

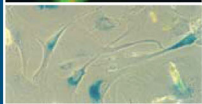
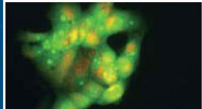
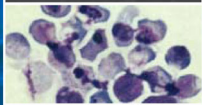
CANCER DRUG DISCOVERY AND DEVELOPMENT

Apoptosis, Senescence, and Cancer

SECOND EDITION

Edited by

David A. Gewirtz
Shawn E. Holt
Steven Grant



 HUMANNA PRESS

APOPTOSIS, SENESCENCE, AND CANCER

CANCER DRUG DISCOVERY AND DEVELOPMENT

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APOPTOSIS, SENESCENCE, AND CANCER

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PREFACE

The goals of chemotherapy (and radiotherapy) are to eliminate tumor cell targets by promoting cell death. In recent years, a major focus has been placed on programmed cell death or apoptosis as the primary mechanism of cell killing. However, tumor cells may respond to various forms of treatment in diverse ways, only some of which culminate in cell death and loss of clonogenic survival. In addition to apoptosis, cell death may occur through mitotic catastrophe, autophagy (a subtype of apoptosis), or anoikis. Alternatively, cells may undergo either transient or prolonged growth arrest; in addition, senescence arrest or accelerated senescence is now recognized as a response to various treatments, which may also play a role in preventing cell transformation. Consequently, “permanent” growth arrest, possibly mediated through senescence, could contribute to loss of self-renewal capacity, particularly in solid tumors. The question of why some tumor cells within a population ultimately recover proliferative capacity (whether in cell culture, in xenograft models as a component of tumor growth delay, or in patients in relation to disease recurrence) remains an unresolved question in the fields of experimental chemotherapy and radiotherapy and a critical problem in the clinical treatment of malignancies. The possibility that surviving and recovering cells represent a resistant stem cell population has recently gained credence, although evidence in support of this hypothesis is far from conclusive.

The purpose of this book is to contribute to an understanding of the growth arrest and cell death pathways mediating the response to chemotherapy in tumor cells. The book is divided into six sections. The first reviews the major cell death pathways. The second develops the themes of telomeres, telomerase, and senescence in genetic stability and tumorigenesis. The third provides an in-depth dissection of the critical DNA damage and response signaling pathways. The fourth deals with the fundamental limitations on therapy conferred by drug resistance, as well as current approaches to circumvent or attenuate drug resistance. The fifth and sixth sections provide an analysis of our understanding of the responses to both conventional strategies and newly developed therapies against cancer.

It is our hope that this book will provide basic scientists and clinicians with a deeper and more thorough understanding of the cellular responses of malignant cells to common therapeutic modalities, which may determine the effectiveness of treatment, both in the initial phase of the disease and the latter stages, including recurrence and metastatic disease.

David A. Gewirtz

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INTRODUCTION AND OVERVIEW

The title of this book might suggest that one mode of cell death (*apoptosis*) and one mode of growth arrest (*senescence*) represent the critical elements of tumor cell responses to various forms of cancer therapy. However, a quick glance through the section and chapter headings will readily convey the range of possible responses to both conventional therapies, such as standard cytotoxic drugs and radiation, to more recent therapies, such as monoclonal antibodies and targeting of specific receptor and signaling pathways, to developing modalities, such as photodynamic therapy and approaches targeting the vascular system. With regard to senescence, in addition to the relatively recent realization that senescence is likely to mediate the growth arrest response to many therapeutic treatments and could potentially act as a rational drug response, a considerable section of this book has been devoted to the role of senescence in genomic instability and tumor development. In addition, the reader will find that the theme of genomic instability runs through other sections, such as the consideration of mitotic catastrophe, as well as regulatory functions of ataxia telangiectasia mutated (ATM)/ATM-related (ATR) and DNA-dependent protein kinase. Likewise, the relationship between c-myc and cyclin-dependent kinase inhibitors is considered in terms of tumor progression, as well as their relevance for cancer therapy and the promotion of apoptosis.

CELL DEATH PATHWAYS

For almost two decades, the cell death response to chemotherapy and radiation has focused almost exclusively on *apoptosis*. In view of the importance of apoptotic cell death, two introductory chapters in this section provide a detailed and learned overview of both the intrinsic and the extrinsic pathways of apoptosis. However, there is extensive evidence that apoptosis is not the only mode of cell death or possibly the primary mode of cell death in solid tumors. It is therefore of particular importance to review the approaches utilized to conclude that a particular mode of cell death is relevant to drug or radiation treatment.

It has been well established for many years that at least one other mode of cell death, that of *mitotic catastrophe*, is quite common, particularly in tumors that have been exposed to ionizing radiation. However, the basis for the cell “choosing” or preferring a particular mode of cell death is not understood, even at the most fundamental level. Relatively recently, at least one additional mode of cell death has been recognized, known as *autophagy*. Autophagy is a complex response because, unlike apoptosis or mitotic catastrophe, autophagy can function as a cytoprotective mechanism when initiated under certain modes of cell stress, such as nutrient deprivation. Finally, an area that is appropriately receiving renewed attention is a subspecies of apoptosis that is termed “anoikis,” or cell death subsequent to loss of adhesion to substratum. The ability of tumor cells to resist anoikis may provide the necessary survival advantage that permits a tumor cell to metastasize.

SENESCENCE GROWTH ARREST

Within the same time frame that autophagy has been recognized as a mode of cell death, *stress-induced senescence* (also known as premature or accelerated senescence) has been recognized as a unique mode of growth arrest. The uniqueness and signaling elements of this pathway has resisted facile dissection as the genetic elements that appear to be involved overlap quite closely with those involved in the G1 arrest pathway, specifically induction of p53 (although it is clear that p53-independent senescence also exists), p21^{waf1/cip1}, dephosphorylation of pRb- or Rb-related proteins such as p130 and p107, and suppression of E2F-mediated transcription. Analysis of the molecular elements involved in stress-induced senescence has also been hampered by reliance on a limited number of senescence markers, specifically senescence-associated β -galactosidase staining, cell morphology (flattening, enlargement, and granulation), and *telomere dysfunction*, which is in dramatic contrast to the multiple signaling and regulatory elements events that have been identified for death-related pathways. Of particular interest, the specific therapeutic targeting of telomeres to induce senescence may provide an under-explored, if not novel, targeted approach for cancer therapy.

DRUGS, MECHANISMS, AND RESISTANCE

Although a great deal of information has been published on the mechanism of action of different classes of antitumor drugs, the chapters in this book attempt to focus, in part, on the importance of different modes of cell death. We have attempted to cover major classical chemotherapeutic agents including the *antimetabolites*, *platinum-based* compounds and *alkylating* agents, *topoisomerase I* and *topoisomerase II* inhibitors, and *microtubule* poisons. In addition to these classical and conventional therapies, relatively new approaches such as *antibody* therapy, *tyrosine kinase*, and *epithelial growth factor receptor* (EGFR) inhibitors are described in detail in this book. Many “cutting edge” therapies are at various stages of development, including *antiangiogenic* agents and *photodynamic* therapy. Finally, the possibility of *TRAIL* as a therapeutic target has been considered, in particular because of its potential to be a highly selective target that is limited to tumor cells.

It is likely that any reader of this book will already be familiar with the difficulties encountered in cancer therapy, many of which are associated with various forms of resistance, including intrinsic mechanisms or those that develop in response to the treatment challenge. To address this issue, specific signaling pathways are considered both in the context of conferring resistance and for converting these pathways into potential targets for drug development and sensitization to existing therapies.

DNA DAMAGE RESPONSE

Although not all cancer therapies involve DNA damage, many of the traditional and conventional treatments do promote cellular stress, either directly through damage to DNA or indirectly through interference with the function of alternative targets. It was therefore considered to be of particular relevance to address specific components of the DNA damage response and signaling pathways, focusing on *ATM/ATR*, *H2AX/53BP1*, and *DNA PK*.

UNANSWERED QUESTIONS

What we still do not understand, despite our best efforts, is precisely how tumor cells “decide” on the nature of their response to these treatment modalities, especially related to *recovery* and/or *resistance*. It is generally thought that less severe “lesions” result in a transient growth arrest, and once such lesions are repaired (or the cell determines that the lesion is not “life-threatening”), growth will resume. Although without solid experimental evidence, it is possible to speculate that disease recurrence at the site of the primary tumor could be related to recovery after transient growth arrest; that is, one can consider this transient growth arrest to be analogous to tumor cell dormancy. A closely related question, one which we have not attempted to address in this book, is the nature of the signaling response that is required for proliferative recovery in “dormant” tumors. Finally, the basis for the therapeutic selectivity of many if not most of current conventional or more novel treatment modalities is still far from being fully elucidated.

The editors express their deep appreciation to all the contributors to this text, scientists, researchers, and clinicians who somehow managed to take the time and effort to provide the benefits of their expertise in specific fields of research to contribute chapters to this book. We also credit the editors at Humana Press for their endless patience with the process of developing this book.

We would like to dedicate this book to all those who have suffered and continue to suffer from the ravages of cancer in the hope that the information gathered in this text might provide some small element of guidance in the efforts of our scientific colleagues to defeat this disease.

David A. Gewirtz
Shawn E. Holt
Steven Grant

I

APOPTOSIS AND ALTERNATIVE MODES OF CELL DEATH

1

The Intrinsic Pathway of Apoptosis

Scott H. Kaufmann, MD, PhD

SUMMARY

Virtually all anticancer drugs induce apoptosis in susceptible cell types. This biochemically distinct form of cell death reflects, in large part, activation of caspases, a distinct family of intracellular cysteine proteases. At least two separable processes, one starting with ligation of specific cell surface receptors (so-called death receptors) and the other involving release of cytochrome c from mitochondria, result in transduction of various signals into caspase activity. The mitochondrial or intrinsic pathway appears to play a predominant role in the apoptotic response to anticancer drugs. Bcl-2 family members regulate this pathway by modulating the release of key proapoptotic polypeptides, including cytochrome c and second mitochondrial activator of caspases (Smac)/direct inhibitor of apoptosis (IAP)-binding protein with low pI (DIABLO), from mitochondria. Several Bcl-2 family members that facilitate mitochondrial permeabilization are transcriptional targets of the p53 tumor suppressor gene, providing a partial explanation for the ability of DNA-damaging agents to induce apoptosis. Other proapoptotic Bcl-2 family members are released from cytoskeletal sites upon treatment with paclitaxel or loss of adherence. The antiapoptotic protein XIAP (X chromosome-linked IAP) binds procaspase 9 and prevents its activation. The gene encoding XIAP is activated by nuclear factor- κ B, contributing to the antiapoptotic effects of this transcription factor. In this chapter, the various components of the intrinsic pathway are reviewed, alterations in this pathway in various cancers are described, and evidence that some of these same antiapoptotic alterations might contribute to anticancer drug resistance under certain circumstances is discussed.

Key Words: Apoptosis; caspases; carcinogenesis; Bcl-2; IAP; drug resistance.

1. ANTICANCER DRUGS CAN INDUCE APOPTOSIS

Studies performed over the past 15 years have demonstrated that virtually all the agents currently utilized to treat cancer can induce apoptosis (1), a morphologically and biochemically distinct cell death process (2), in susceptible cells. Morphologically, this process is characterized by plasma membrane blebbing, cell shrinkage, and chromatin condensation followed by disassembly of the cell into multiple membrane-enclosed fragments, which are then engulfed by neighboring cells or professional phagocytes (2).

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These morphological changes are thought to reflect, at least in part, the action of caspases, unique intracellular proteases that digest polypeptides required for cellular integrity and survival (3–5).

Additional analyses have suggested that apoptosis also occurs after administration of antileukemic therapy in the clinical setting (6–10) and after treatment of solid tumors in certain animal models (2,11,12). These observations, which suggest that proximal effects of diverse anticancer drugs can lead to activation of a common final death pathway, have prompted many investigators to speculate that alterations in the apoptotic machinery or its regulation might lead to a form of broad-spectrum drug resistance (13–17). It is important to emphasize, however, that cells can also suffer other fates, including senescence (see Chaps. 11 and 19) and so-called mitotic catastrophe (see Chap. 4), after exposure to chemotherapeutic agents (18–20). These alternative outcomes need to be considered in determining the overall response of tumor cells to antineoplastic agents (20).

This chapter describes apoptosis as an endpoint of anticancer drug exposure. In the sections that follow, current understanding of the apoptotic machinery, particularly the intrinsic pathway of apoptosis, is summarized. In addition, cancer-associated alterations of this pathway are described, and evidence suggesting that these alterations might contribute to drug resistance is discussed.

2. CASPASE ACTIVATION: SHARPENING THE SCALPEL

Although early studies suggested that the vast majority of cellular polypeptides remain intact as cells undergo apoptosis, more than 500 polypeptides are now known to be selectively degraded during this death process (4,5). Many proteases, including calpains, cathepsins, and the proteasome, have been implicated in these proteolytic events (21,22), but the vast majority of cleavages are thought to be mediated by caspases (4,5,23), a family of intracellular cysteine proteases that cleave next to aspartate residues.

Of the 12 known human caspases, seven have established (caspases 3, 6, 8, 9, and 10) or postulated (caspases 2 and 7) roles in apoptosis. Each of these is synthesized as an inactive zymogen that contains a prodomain as well as sequences that give rise to a large subunit and a small subunit.

Recent studies have suggested that the process of caspase activation differs for initiator caspases, which transduce various signals into protease activity, and effector caspases, which are responsible for the bulk of proteolytic cleavages within apoptotic cells. In particular, the initiator caspase of the intrinsic pathway, procaspase 9, appears to be a monomer (24) with misaligned catalytic cysteine and histidine residues and a catalytic pocket that cannot bind substrates (25). Procaspase 9 dimerization *in vitro* results in conformational changes that open the substrate-binding pocket and align the catalytic residues at one of the two active sites in the dimer (25). Notably, these changes can occur without any proteolytic cleavage of procaspase 9 (25–27). Other initiator caspases such as procaspases 8 and 10 also appear to exist as monomers that can be activated by dimerization *in vitro* (24,28).

In contrast to initiator caspases, the zymogens of effector caspases are dimers under physiological conditions (24,29). Activation of these caspases involves proteolytic cleavage at several aspartate residues, including one between the large and

small subunits and another between the prodomain and the large subunit (4,23,30). X-ray crystallography of procaspase 7 and the mature enzyme has revealed that these proteolytic cleavages are accompanied by release of the two prodomains and by conformational changes that produce an enzyme with two catalytically competent active sites, each containing residues from one large and one small subunit (29,30). Caspases 3 and 6 are likely activated in a similar fashion.

3. CLEAVAGE BY EFFECTOR PROTEASES: SURGICAL EXCISION OF CRITICAL CELLULAR COMPONENTS

Once activated, caspase 3 selectively cleaves several hundred substrates (4,5), and caspase 6, which is activated by caspase 3 (31), cleaves a smaller group of polypeptides (4,32). These cleavages contribute to many of the changes observed during apoptosis. For example, caspase 3 cleaves the actin filament severing enzyme gelsolin to a constitutively active fragment that has been implicated in cytoplasmic blebbing, one of the morphological hallmarks of apoptosis (33,34). Caspase 3 also cleaves intermediate filament proteins, including lamin B, the nuclear/mitotic apparatus protein NuMA, and cytokeratins (4,5), whereas caspase 6 appears to be uniquely capable of cleaving lamin A in nuclei that contain this particular intermediate filament protein (32,35). To the extent that these polypeptides are normally responsible for the maintenance of cell shape, the destabilizing caspase-mediated cleavages of these intermediate filaments likewise contribute to apoptotic morphological changes.

Caspase-induced cleavages also participate in the biochemical changes observed in apoptotic cells. For example, caspase 3 cleaves the nuclease inhibitor ICAD (inhibitor of caspase-activated deoxyribonuclease), allowing subsequent internucleosomal cleavage of DNA (36) by the constitutively expressed nuclear enzyme CAD (37–39). In addition, caspase 3 cleaves many protein kinases, removing their inhibitory domains and increasing their activity (4). Although the manner in which this unregulated kinase activity contributes to the apoptotic phenotype remains largely unknown, the constitutively active fragment of protein kinase C δ (40) activates a phospholipid scramblase that catalyzes transfer of phosphatidylserine from the inner leaflet of the plasma membrane, where it is normally located, to the outer leaflet (41). This loss of phospholipid asymmetry, another hallmark of apoptotic cells (42), provides a signal recognized by the adaptor molecule MFG-E8 (43,44) and/or a phosphatidylserine receptor present on the surfaces of macrophages (45–47), thereby facilitating phagocytosis of the dying cell. Caspase-mediated degradation of additional polypeptides inhibits many processes that are required for continued cell survival, including DNA repair pathways and components of the protein translation machinery (4,5). Collectively, these caspase-mediated cleavages interfere with cellular homeostasis and appear to reinforce the “decision to die.”

4. CYTOCHROME C: A CRITICAL MOLECULE FOR LIFE AND DEATH

Within the cell, two distinct but interacting pathways can lead to the activation of caspases 3 and 6. One involves transduction of receptor-mediated events into proteolytic activity of caspase 8 and/or caspase 10. This pathway is reviewed in Chapter 2. The other involves transduction of various signals into a change in permeability of the

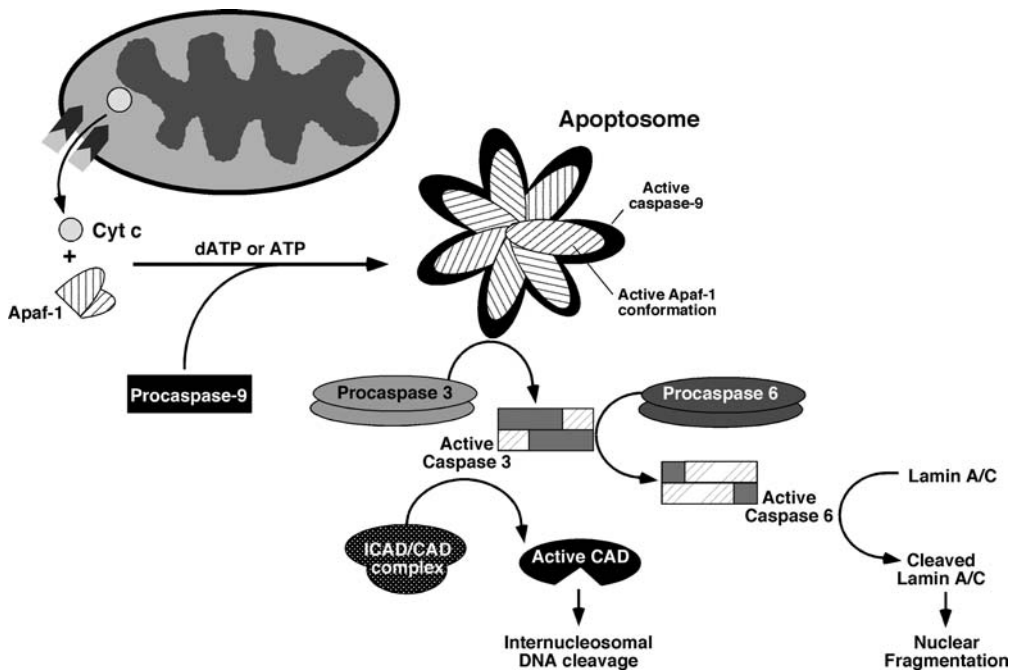


Fig. 1. The mitochondrial pathway. Upon release from the mitochondrial intermembrane space, cytochrome c binds to and induces an ATP dATP-dependent (48) conformational change in Apaf-1, thereby facilitating apoptosome formation. Activated caspase 9 then proteolytically activates caspase 3, which cleaves a myriad of substrates, including the nuclease inhibitor ICAD (inhibitor of caspase-activated deoxyribonuclease) and other procaspases, to produce apoptotic biochemical and morphological changes as described in Section 3.

outer mitochondrial membrane, which then leads to activation of caspase 9. This signal transduction cascade, which is termed the intrinsic (or mitochondrial) pathway (Fig. 1), is the subject of the remainder of this chapter.

A critical step in this pathway is the selective release of polypeptides from the mitochondrial intermembrane space into the cytoplasm (49,50). The most widely studied of these released polypeptides is cytochrome c, a component of the electron transport chain that is loosely bound to the outer leaflet of the inner mitochondrial membrane. Release of the cytochrome c from mitochondria within any particular cell during apoptosis appears to be rapid and quantitative (51). Upon its appearance in the cytoplasm, cytochrome c binds the scaffolding molecule Apaf-1 (apoptotic protease-activating factor-1) (52), which then undergoes a nucleoside triphosphate-dependent conformational change and binds procaspase 9 (53). The resulting $M_r \sim 700,00$ complex termed the “apoptosome” contains multiple Apaf-1 and procaspase 9 molecules (54–57). In a manner that is not completely understood at the molecular level (58), binding to this complex activates procaspase 9, which then proteolytically activates caspase 3 (31,53) to initiate cellular disassembly as described in Section 3.

The role of cytochrome c in caspase activation has been extensively studied. To activate caspase 9 *in vitro*, cytochrome c must contain the heme prosthetic group (59,60), although either oxidized or reduced cytochrome c will suffice (61). Once

the apoptosome forms, cytochrome *c* appears to become dispensable. The absence of detectable cytochrome *c* in the active apoptosome (26,56,57) and the ability of Apaf-1 truncation mutants to activate procaspase 9 in the absence of cytochrome *c* (31) suggest that cytochrome *c* is only required to induce the Apaf-1 conformational change that leads to apoptosome formation.

The biochemical basis for the efflux of cytochrome *c* from mitochondria has been the subject of considerable speculation (reviewed in 49,62–65). Some models have suggested that cytochrome *c* release reflects opening of a permeability transition pore composed of resident mitochondrial membrane proteins such as the voltage-dependent anion channel (VDAC) and adenine nucleoside translocator (ANT) acting in concert with the mitochondrial matrix protein cyclophilin D. Results of murine gene-targeting studies, however, have failed to confirm an essential role for VDAC (66), ANT (67,68), or cyclophilin D (69–71) in apoptosis triggered during development or by exogenous stimuli. Accordingly, more recent studies have focused on the role of Bcl-2 family members in the release of mitochondrial proteins to the cytoplasm.

5. BCL-2 FAMILY MEMBERS AND THE INTEGRATION OF CELLULAR STRESS

The biology and biochemistry of Bcl-2 family proteins have been extensively reviewed elsewhere (72–74) and are only briefly described here. *Bcl-2*, the founding gene in this family, was identified because it is juxtaposed to the immunoglobulin heavy chain promoter in indolent B-cell lymphomas containing the t(14;18) chromosomal translocation. Subsequent analysis demonstrated that Bcl-2 overexpression inhibits cell death (75,76). Since these early observations, approximately 20 related mammalian polypeptides have been identified. On the basis of functional and structural criteria, these polypeptides can be divided into three groups (Fig. 2), the antiapoptotic group I family members and the proapoptotic group II and group III family members. Group I family members, which include Bcl-2, Bcl- x_L , Bcl-w, Mcl-1, A1/Bfl1, Boo/Divia,

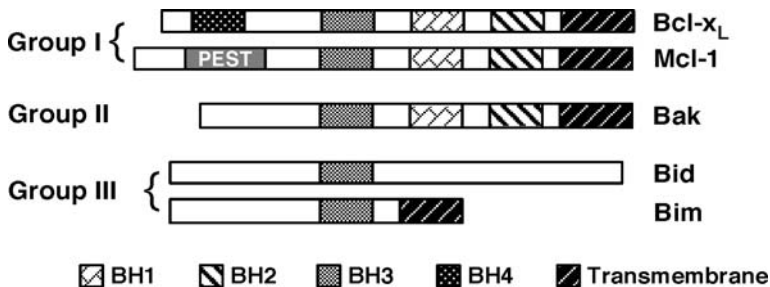


Fig. 2. Schematic representation of selected Bcl-2 family members. Group I polypeptides are antiapoptotic and include Bcl-2, Bcl- x_L , Bcl-w, Mcl-1, A1, Boo/Divia, Nrf3, and Bcl-B. Among these, Mcl-1 is unique in lacking a BH4 (Bcl-2 homology 4) domain and containing a proline/glutamate/serine/threonine-rich (PEST) sequence, which is often seen in short-lived polypeptides. Group II family members are proapoptotic and include Bax, Bak, and Bok/Mtd. Among this group, Bax and Bak are generally expressed in somatic cells. Group III family members, which share limited sequence homology only in their 15-amino acid BH3 domains, include Bid, Bad, Bik, Bim, Blk, Bmf, Hrk, Bnip3, Nix, Noxa, PUMA, and Bcl-G.

Nrf3, and Bcl-B (72–74), generally contain four short, conserved BH (Bcl-2 homology) domains, BH1–BH4, and most contain a C-terminal transmembrane domain that targets them to the cytoplasmic surfaces of various intracellular membranes, including the outer mitochondrial membrane and the endoplasmic reticulum. Group II family members, which include Bax, Bak, and Bok/Mtd (72–74), lack the N-terminal BH4 domain but contain the other BH domains. Finally, group III family members, which include Bid, Bad, Bik, Bim, Blk, Bmf, Hrk, Bnip3, Nix, Noxa, PUMA, and Bcl-G, are a more heterogeneous collection of polypeptides that share limited sequence homology only in their 15-amino acid BH3 domains (73,77).

Current understanding, which is summarized in Fig. 3, suggests that group II Bcl-2 family members, especially Bax and Bak, directly mediate cytochrome c

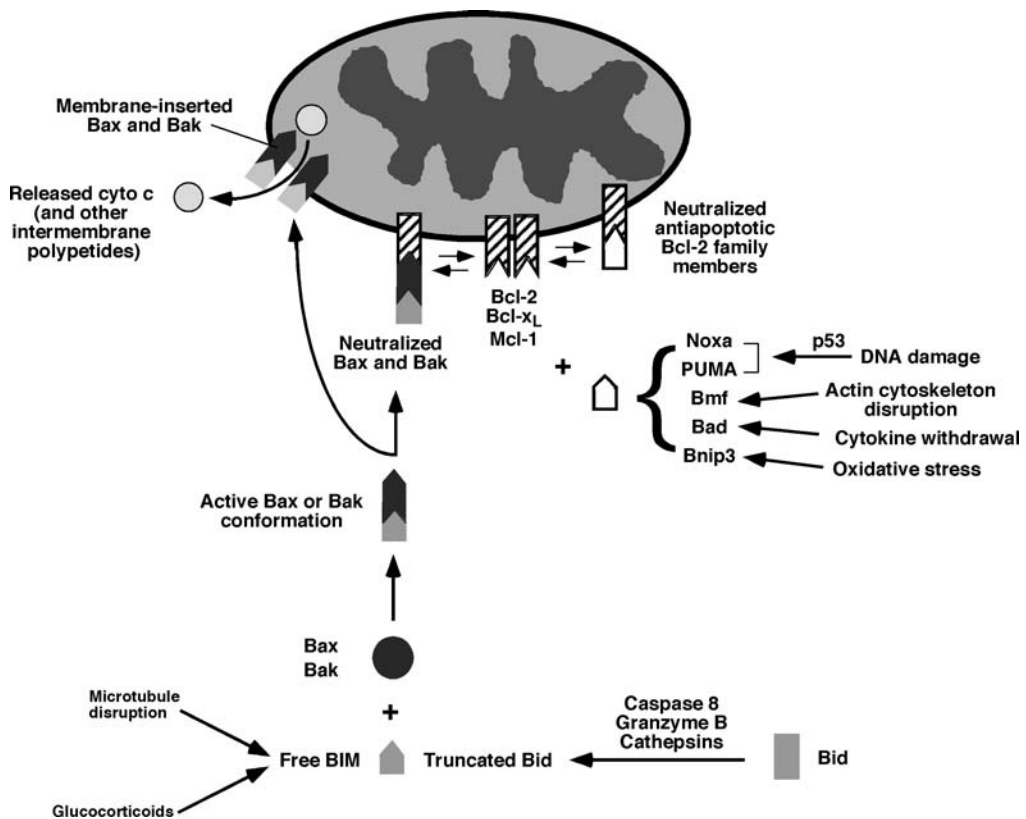


Fig. 3. Regulation of the mitochondrial pathway by Bcl-2 family members. As indicated in the text, insertion of Bax and/or Bak into the outer mitochondrial membrane appears to be directly responsible for the release of cytochrome c and other polypeptides of the mitochondrial intermembrane space. One group of BH3-only polypeptides, typified by Bim and truncated Bid, is currently thought to directly induce the conformational change implicated in Bax- and/or Bak-induced mitochondrial outer membrane permeabilization. Another group of BH3-only polypeptides, including Puma, Bmf, and Bad, is thought to influence Bax and/or Bak membrane insertion less directly, that is, by binding and neutralizing antiapoptotic Bcl-2 family members. Recent data also indicate that certain group III family members bind preferentially to different group I family members (79), providing a potential explanation for the ability of various antiapoptotic Bcl-2 family members to protect differentially against different types of cellular stress.

release (63,73,74). Bax is found in the cytoplasm or loosely bound to the mitochondrial surface in non-apoptotic cells (78) but is inserted in the outer mitochondrial membrane after exposure of cells to apoptotic stimuli (80). This membrane insertion appears to involve changes in the conformation and oligomerization state of Bax (81–83). During the process of apoptosis, Bak, which normally resides in the outer mitochondrial membrane, also undergoes a conformational change that results in its deeper insertion in the outer mitochondrial membrane (84–86). Importantly, deletion of either *BAX* or *BAK* modestly diminishes the ability of various stimuli to induce mitochondrial cytochrome c release and subsequent development of apoptotic changes in fibroblasts, whereas deletion of both genes has a much more profound effect (85,87).

Further support for a role of these polypeptides in outer mitochondrial membrane permeabilization comes from a series of observations made under cell-free conditions. After activation (see Section 6 below), Bax induces cytochrome c release from isolated mitochondria (88,89) or lipid vesicles prepared from mitochondrial membranes (89). The latter observation again calls into question earlier claims that resident mitochondrial membrane proteins are required for cytochrome c release and instead suggests that group II Bcl-2 family members are sufficient by themselves to breach the outer mitochondrial membrane.

Although it is clear that Bax and Bak play important roles in cytochrome c release, several points still require further study. Although it is possible that Bax and/or Bak oligomerize to form a pore (90), the details of this pore are unclear (91). In addition, it is unclear whether other mitochondrial proteins are released by the same process as that of cytochrome c. It has been observed, for example, that cytochrome c release during apoptosis in fibroblasts requires expression of either Bax or Bak, whereas second mitochondrial activator of caspases (Smac) release (see Section 9 below) requires expression of both (92). The mechanistic basis for this observation remains to be determined.

6. GROUP III FAMILY MEMBERS: ARMING THE ASSASSIN

Insertion of Bax and Bak into the mitochondrial outer membrane appears to be facilitated by group III Bcl-2 family members, the so-called BH3-only members of this family. As recently reported by Kuwana et al. (93) and summarized in Fig. 3, it currently appears that BH3-only polypeptides can do this in one of two ways.

Some of the BH3-only polypeptides are thought to interact directly with group II polypeptides to induce their insertion into the mitochondrial outer membrane. Evidence in support of this model includes the detection of a direct interaction of some of these polypeptides (notably Puma and a truncated form of Bid) with Bax (94), the demonstration that truncated Bid is capable of inducing Bax-mediated permeabilization of the mitochondrial outer membrane or vesicles prepared from mitochondrial membrane lipids (89), and the demonstration that the effects of truncated Bid on Bax-mediated permeabilization can be reproduced by 24- or 25-mer peptides containing the BH3 domains of Bid or Bim (93).

By contrast, peptides corresponding to the BH3 domains of other group III Bcl-2 family members are unable to directly induce Bax-mediated permeabilization of lipid vesicles *in vitro* (93). Nonetheless, all these BH3 domain peptides are able to facilitate membrane permeabilization in a cell-free system containing lipid vesicles, truncated Bid, Bax, and the antiapoptotic family member Bcl-x_L. These observations suggest that

the other BH3-only family members act by neutralizing group I Bcl-2 family members as described in greater detail in the next section.

Why are there so many different group III polypeptides? It appears as if these polypeptides monitor and respond to different types of cellular stress. Bid is a constitutively expressed cytoplasmic polypeptide that is cleaved by caspase-8 (95,96), granzyme B (97), or lysosomal cathepsins (98) to release a fragment with enhanced ability to activate Bax. Other constitutively expressed BH3-only polypeptides are activated in other ways: Bad by dephosphorylation and release from cytoplasmic 14-3-3 proteins upon cytokine withdrawal (99); Bim by detachment from microtubules during paclitaxel treatment (100,101); and Bmf by release from actin filaments when cell-substrate attachment is disrupted (102). By contrast, Noxa and Puma are synthesized in a p53-dependent manner in response to DNA damage (103,104), providing a potential explanation for the ability of DNA damage within nuclei to signal mitochondrial cytochrome c release (105–107). It also appears as if Puma is also synthesized in a p53-independent manner in response to other agents such as glucocorticoids (106).

7. GROUP I POLYPEPTIDES: KEEPING THE KILLERS IN CHECK

The effects of these group III Bcl-2 family members are inhibited by group I family members, including Bcl-2, Bcl-x_L, and Mcl-1. Over the years, several different explanations for the antiapoptotic effects of these polypeptides have been proposed. First, it was suggested that group I polypeptides inhibit apoptosis by binding and neutralizing group II polypeptides, especially Bax (80,108,109) or Bak (110). The identification of Bcl-x_L mutants that fail to bind Bax or Bak but still inhibit apoptosis (111) cast doubt on this model, at least as a universal mechanism of apoptosis inhibition. Second, it was proposed that Bcl-2 prevents apoptosis by increasing the antioxidant capacity of cells (112). Observations that place apoptosis-associated increases in reactive oxygen species downstream of caspase 3 activation (113,114) cast doubt on this model. Third, it was suggested that Bcl-2 prevents apoptosis by inhibiting the release of calcium from endoplasmic reticulum stores (115–119). Unfortunately, not all proapoptotic stimuli that are inhibited by Bcl-2 induce calcium release from intracellular stores. Finally, it has been hypothesized that group I polypeptides inhibit apoptosis by binding group III polypeptides, thereby preventing the release of cytochrome c (and other polypeptides) from mitochondria (120,121). This latter hypothesis has received the most attention.

Recent analysis of the binding between BH3 domains and group I polypeptides has demonstrated unanticipated specificity of these interactions (79). Whereas the group I polypeptides Bcl-x_L and Bcl-w bind all tested BH3 peptides except Noxa with nanomolar affinity, other group I family members such as Mcl-1 and A1 bind Noxa, Puma, and Bim with much higher affinity than the remaining BH3 domain peptides. Coupled with the realization that different BH3-only polypeptides monitor different types of stress as described in the preceding section, these results suggest that different group I polypeptides should protect preferentially against certain types of proapoptotic stimuli. This has been documented to a certain extent (110,122) but requires further study.

As might be expected for polypeptides that are the final arbiters of cytochrome c release by various stresses, expression and activity of the group I Bcl-2 family members are highly regulated. Genes encoding Bcl-2, Bcl-x_L, and Mcl-1 are activated

by mitogen-activated protein (MAP) kinase signaling in some cell types (123,124), cytokine-mediated activation of STAT (signal transducer and activator of transcription) factors in others (125,126), and the phosphatidylinositol-3 kinase/Akt pathway, possibly acting through the transcription factor nuclear factor (NF)- κ B, in others (127–130). In addition, the activity of group I polypeptides is regulated post-translationally by phosphorylation. Bcl-2 is activated by protein kinase C α -mediated or extracellular signal-regulated kinase (ERK)-mediated phosphorylation on Ser⁷⁰ (131,132) and other sites (133). Mcl-1 is likewise phosphorylated and stabilized in an ERK-dependent manner (134–136) as well as an ERK-independent manner (130,137). These observations provide at least a partial explanation for the antiapoptotic effects of MAP kinase activation (138,139). Further study will undoubtedly identify additional ways in which signal transduction pathways impinge on the antiapoptotic functions of Bcl-2 family members (e.g., 140,141), adding further complexity to the regulation of apoptosis in various cells. When the observed tissue-specific differences in expression of the antiapoptotic Bcl-2 family members (142,143) are also considered, it starts to become clear how the ability of various stimuli to induce cytochrome c release can vary widely from one cell type to another.

Although the model depicted in Fig. 3 provides an explanation for much of the available experimental data, it is important to realize that several issues require further investigation. For example, because extremely high concentrations of isolated BH3 domains rather than physiological concentrations of native polypeptides were utilized to detect the interactions *in vitro*, it remains to be demonstrated that all the proposed interactions occur *in situ*. Previous studies reporting inability to detect direct binding of Bim to Bax or Bak despite extensive efforts (102,144) highlight the importance of this issue. In addition, it is unclear what drives Bax or Bak into the mitochondrial membrane in the presence of BH3-only polypeptides such as Puma, Noxa, Bad, and Bmf, which are unable to directly activate Bax under cell-free conditions. One possibility is that there is another stimulus for group II polypeptide activation. It has been observed, for example, that activation of Jun N-terminal kinase, which commonly occurs during apoptosis, is accompanied by the release of Bax from cytoplasmic 14-3-3 proteins and subsequent localization to mitochondria (145). A second and related possibility is that group II polypeptides have an intrinsic propensity to insert in the outer mitochondrial membrane unless neutralized by group I polypeptides. Consistent with this possibility, it has been reported that Bak is sequestered by Mcl-1 and Bcl-x_L but becomes active when released from both (110,146).

8. CASPASE INHIBITION BY XIAP: CLOSING THE BARN DOOR AFTER THE COW HAS ESCAPED

Whereas Bcl-2 family members regulate apoptosis upstream of apoptosome formation, the mitochondrial pathway is regulated downstream of apoptosome formation by members of the inhibitor of apoptosis (IAP) family (147,148). These polypeptides, which are characterized by the presence of one or more zinc finger-like baculovirus inhibitor repeat (BIR) domains (Fig. 4), were originally identified in viruses because of their ability to inhibit apoptosis and enhance viral replication in infected cells. It is now clear that there are at least two subgroups of BIR-containing polypeptides (150). One, which contains survivin and its homologues in yeast and worms,

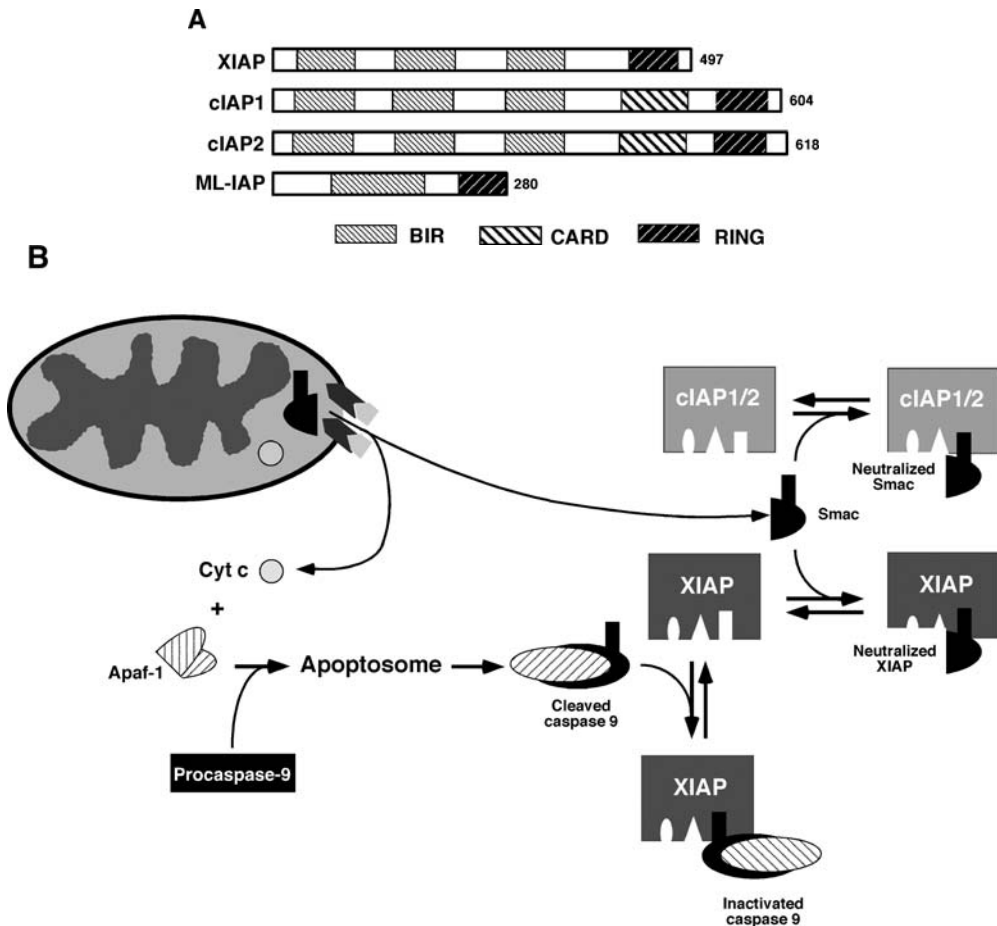


Fig. 4. Regulation of the mitochondrial pathway by inhibitor of apoptosis (IAP) proteins. **(A)** Schematic representation of several antiapoptotic baculovirus inhibitor repeat (BIR) proteins. Shaded areas depict BIR domains, caspase recruitment domains (CARDs), and RING fingers. Numbers at right indicate the number of amino acids in each polypeptide. Melanoma-associated IAP (ML-IAP) is also known as livin. **(B)** Current understanding of X chromosome-linked IAP (XIAP) and cIAP1/2 function. The third BIR domain of XIAP is known to bind procaspase 9 and prevent its activation (not depicted—see 149) as well as inhibit partially cleaved caspase 9. Smac, which is released from mitochondria, competes for this BIR domain, freeing caspase 9. Conversely, cIAP1, cIAP2, or ML-IAP can bind and sequester Smac, liberating XIAP and facilitating caspase 9 inactivation. Because Smac can also bind to XIAP BIR2, which inhibits caspases 3 and 7, cIAP/Smac interactions likely regulate caspases 3 and 7 in a similar fashion.

is required for successful function of the mitotic spindle (151,152). The other, which includes X chromosome-linked IAP (XIAP), cellular IAP1 (cIAP1), cIAP2, and melanoma-associated IAP (ML-IAP), regulates apoptosis (147). In addition to one or more BIR domains, these antiapoptotic IAP proteins commonly contain a RING finger that is thought to transfer ubiquitin to bound caspases and other polypeptides, thereby marking the binding partners for proteasome-mediated degradation (153–156).

The mechanism of caspase inhibition by XIAP has been extensively studied (147,157). The third BIR of XIAP has multiple effects on caspase 9. First, it binds to procaspase 9

and inhibits the dimerization that is required for activation (149,158). Second, BIR3 binds to a cleavage intermediate that is transiently generated when procaspase 9 is clipped between the large and small subunits (158). In addition to these effects on caspase 9, XIAP also inhibits caspases 3 and 7. Structural studies suggest that the second BIR of XIAP binds the surfaces of these target caspases, orienting the molecules so that a short peptide immediately upstream from BIR2 can block access to the active sites of caspases 3 and 7 (147,157,159). These observations suggest that XIAP should be able to inhibit the intrinsic pathway at multiple steps.

On the basis of these observed effects, it has been suggested that XIAP acts as a cellular buffer for small amounts of caspases that are inadvertently activated (160). Consistent with this view, XIAP expression is regulated by NF- κ B (4), a transcription factor that enhances survival (161). Nonetheless, confirming this postulated function of XIAP in intact cells or organisms has been problematic. Although XIAP overexpression inhibits apoptosis induced by various stimuli (162–164), XIAP retains its antiapoptotic activity even when the binding sites for caspases 3 and 9 have both been mutated (165), raising the possibility that some other critical antiapoptotic function of the polypeptide has been missed. Equally disconcerting for current models, targeted disruption of the mouse *XIAP* gene fails to yield a phenotype suggestive of increased apoptosis (166). Conversely, tissue-specific XIAP overexpression *in vivo* inhibits not only thymocyte apoptosis but also thymocyte maturation (167), raising the possibility that XIAP might serve multiple functions that are not yet completely understood.

9. ADDITIONAL PROAPOPTOTIC MITOCHONDRIAL PEPTIDES: THE EXECUTIONER'S ARSENAL

In addition to cytochrome *c*, dozens of other polypeptides are released from mitochondria during apoptosis (168). One of these, endonuclease G, was initially reported to produce CAD-independent internucleosomal DNA degradation (169), although subsequent observations have called this conclusion into question (170,171). Apoptosis-inducing factor (AIF), a mitochondrial oxidoreductase (172) that is released from the intermembrane space during apoptosis (173), was originally said to be critical for apoptotic changes induced by certain oxidative stimuli (174), but more recent studies have cast doubt on the suggestion that AIF plays any role in apoptosis (175). Perhaps more informative from the standpoint of mitochondrial pathway regulation are Smac/direct IAP-binding protein with low pI (DIABLO) and HtRA2, two polypeptides that reportedly modulate the function of XIAP, cIAP1, and cIAP2 (176).

Smac/DIABLO was purified based on its ability to facilitate cytochrome *c*-induced caspase activation under cell-free conditions (177) and to bind XIAP in cells undergoing apoptosis (176). Although some studies have suggested that Smac/DIABLO is easier (178) or harder (179) to release from mitochondria than cytochrome *c*, others have found that these two polypeptides are released in parallel (180). Once in the cytoplasm, Smac/DIABLO binds to XIAP (176–180), disrupting its binding to caspases (158,180). X-ray crystallography and mutational analysis have demonstrated that the tetrapeptide alanine-valine-proline-isoleucine (AVPI), a sequence exposed at the N-terminus of mature Smac when the mitochondrial targeting sequence is proteolytically removed (176,177,181), plays a critical role in the function of Smac/DIABLO by binding to shallow grooves on the surface of XIAP (182,183).

The recognition that Smac acts by inhibiting XIAP has provided new insight into the action of cIAP1, cIAP2, and ML-IAP (Fig. 4). Earlier studies demonstrated that these polypeptides inhibit apoptosis in intact cells and under cell-free conditions (147,184). Curiously, however, these polypeptides are poor inhibitors of caspases 3 and 9 ($K_i > 10 \mu\text{M}$) compared with XIAP ($K_i < 1 \text{ nM}$ for caspase 9 and 10 nM for caspase 3) (185). Recent experiments have demonstrated, however, that these other IAP proteins avidly bind Smac, thereby freeing XIAP from Smac inhibition and allowing caspase inhibition (186–188). Thus, the regulation of proteases in the intrinsic pathway involves interactions between an inhibitor (XIAP), regulators of the inhibitor (e.g., Smac/DIABLO), and binding partners of the regulator (e.g., cIAP1, cIAP2, ML-IAP).

Despite the elegant biochemical studies implicating Smac in the regulation of XIAP, disruption of the *Smac/DIABLO* gene yields no developmental phenotype (189), making it difficult to confirm that modulation of apoptosis is the sole (or even major) function of this polypeptide. The existence of additional polypeptides with a similar function could provide a potential explanation for the lack of an apoptotic phenotype in *Smac* knockout mice. Consistent with this possibility, four groups have reported that the mitochondrial protein HtRA2/Omi, a member of a family of polypeptides that act as chaperones at physiological temperature and as proteases after heat-induced activation (190), also binds to and inhibits XIAP (191–194). Like Smac/DIABLO, HtRA2/Omi relies on an N-terminal targeting sequence for mitochondrial uptake and is then cleaved to yield a mature polypeptide with the N-terminal sequence AVPS. Although it has been suggested that the intrinsic serine protease activity of HtRA2 might contribute to its proapoptotic effects in certain contexts (191,194), possibly by degrading IAP proteins (195), a missense mutation of the HtRA2 active site serine (196) or targeted *HtRA2* gene deletion (197) results in mice with a neurodegenerative phenotype rather than impaired apoptosis, hinting that the major physiological function of HtRA2 is proteolytic clearance of misfolded mitochondrial polypeptides rather than regulation of XIAP.

In addition to Smac/DIABLO and HtRA2/Omi, mammalian cells contain at least three additional polypeptides that could bind XIAP when released to the cytoplasm (176,198). What is unclear at this point, however, is whether any of these XIAP-binding polypeptides are bona fide apoptotic regulators under physiological conditions (199). Although a variety of studies have established that the IAP-binding polypeptides hid, grim, reaper, and sickle, all of which start with N-terminal AVPI-like sequences, act as tissue-specific IAP antagonists in *Drosophila* (199), the importance of this mechanism of caspase regulation remains to be unambiguously demonstrated in mammalian systems.

10. ACTIVATION OF THE APOPTOTIC MACHINERY BY CHEMOTHERAPEUTIC AGENTS

Because apoptosis can be detected in circulating leukemia cells after institution of antileukemic therapy (6–10) and in murine solid tumors after effective antineoplastic treatment *in vivo* (2,11,12), there has been considerable interest in identifying the apoptotic pathway(s) activated by various antineoplastic agents. Many agents, including doxorubicin, etoposide and teniposide, methotrexate, cisplatin, and bleomycin, induce

the synthesis of FasL (200,201) by activating the transcription factors NF- κ B and AP-1 (202,203). In addition, Fas receptor expression is upregulated in a p53-dependent fashion by DNA-damaging agents (204). Although these observations have been used to implicate the death receptor pathway (Chap. 2) in drug-induced killing, several observations suggest that Fas/FasL interactions might not be required for killing by most agents. First, cells with defects in Fas expression or Fas-mediated signaling remain susceptible to a variety of drugs (205–210). Second, blocking anti-Fas antibodies inhibit the cytotoxic effects of agonistic anti-Fas antibodies or upregulated Fas but not the previously mentioned anticancer drugs (205–207,211). Third, inhibition of the death receptor pathway by crmA, an inhibitor of caspases 8 and 10 (212), fails to inhibit the induction of apoptosis by these drugs (205,209).

Although these observations suggest that signaling through the Fas/FasL pathway is not absolutely required for anticancer drug-induced apoptosis, the possibility that induction of Fas and FasL contributes to apoptosis, particularly after prolonged exposure to low doses of DNA-damaging agents (135,213), has not been ruled out. This possibility is discussed in greater detail in Chap. 2. In addition, whereas most drug-induced apoptosis occurs independent of Fas/FasL signaling, a small group of drugs do in fact appear to trigger apoptosis by activating death receptor pathways. These include 5-fluorouracil (214), 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (215), proteasome inhibitors (216), *trans*-retinoic acid (217), CI-1040 (135), and fenretinide (218) as well as decapeptide (219) and other histone deacetylase inhibitors (220). Interestingly, in instances where it has been critically examined, this reliance on the death receptor pathway appears to vary from one cell line to another (135,218,221). In the case of colon cancer cells treated with fluoropyrimidines, the presence of wild-type p53 reportedly determines whether drug-induced apoptosis proceeds through the Fas pathway (222). The critical determinant for other drugs is currently unknown but is almost certainly not p53.

For the majority of anticancer drugs, previous studies not only ruled out an essential role for the Fas/FasL pathway in drug-induced apoptosis (see first paragraph of this Section) but also directly implicated the mitochondrial pathway in this process. Observations that are important in this regard include the demonstration that cytochrome *c* release accompanies induction of apoptosis by a variety of agents (113,120,223,224), the determination that Bax translocates to mitochondria in response to various drugs independent of (i.e., upstream of) caspase activity (83,225), and the demonstration that combined deletion of *BAX* and *BAK* inhibits drug-induced apoptosis (87,226). As might be expected, if the mitochondrial pathway plays a predominant role, dominant-negative caspase-9 constructs (227,228) and antiapoptotic Bcl-2 family members (210,229) inhibit drug-induced apoptosis. The demonstration that *Caspase9* or *Apaf-1* gene deletion delays the induction of apoptosis by staurosporine, dexamethasone, etoposide, and ionizing radiation in mouse fibroblasts or thymocytes, whereas *Caspase8* deletion does not, provides additional support for the view that most agents activate the mitochondrial pathway (1). For some drugs in some cell types, the signaling immediately upstream of Bax and/or Bak activation appears to involve changes in expression or localization of BH3-only family members (see Section 6), but in other cells and for other drugs, the changes that lead to Bax and/or Bak activation still require clarification.

11. LIFE ON THE EDGE: ALTERATIONS IN THE APOPTOTIC MACHINERY IN CANCER CELLS

Changes that disable the apoptotic machinery are now considered one of the hallmarks of neoplastic transformation (230–234). How inhibition of apoptosis contributes to neoplastic transformation is perhaps best understood for the oncoprotein *myc*, a transcription factor that is upregulated in proliferating cells but cannot induce neoplastic transformation unless an antiapoptotic Bcl-2 family member is also overexpressed (75,235,236). When cells are cultured under favorable conditions, *myc* overexpression drives proliferation, but when cells encounter unfavorable growth conditions, *myc* overexpression causes apoptosis (237,238). This *myc*-induced apoptosis appears to result from several distinct proapoptotic changes, including upregulation of p19^{ARF}, which binds Mdm2 and allows upregulation of p53-dependent apoptotic mediators (239), upregulation of Bim (240), and downregulation of Bcl-2 and Bcl-x_L (241). These proapoptotic changes must be overcome if cells are to be successfully transformed by *myc*. According to current understanding, other proliferation-inducing oncogenes likewise require the cooperation of antiapoptotic changes (233).

Because transforming oncogenes activate the intrinsic apoptotic pathway when cells encounter unfavorable growth conditions (242,243), it is perhaps not surprising that the intrinsic pathway is inhibited in a number of different ways in various cancers (233–245). Antiapoptotic Bcl-2 family members are overexpressed in some cancers (15,246,247). In others, constitutive activation of the mitogen-activated kinase pathway (139) induces activation (Bcl-2) or stabilization (Mcl-1) of group I Bcl-2 family members (see Section 7). In leukemia cell lines (248) and a substantial portion of mismatch repair-deficient colon and gastric cancers (249), the *BAX* gene is mutated, although the heterozygous nature of these mutations in clinical cancer (249) stands in contrast to results obtained in animal models (250) and by itself fails to completely account for any apoptotic defect. In other tumors, changes that upregulate the Akt pathway (251), including autocrine or paracrine activation of receptor tyrosine kinases, *PI3KINASE* gene mutation (252) or amplification (253), *PTEN* deletion, or *AKT2* gene amplification (254), inhibit mitochondrial cytochrome c release (255).

The most common change that impinges on the mitochondrial pathway is mutation or deletion of the tumor suppressor p53, a transcription factor that is widely reported to induce apoptosis upon forced overexpression (256,257). Although many intrinsic pathway components, including Bax, Apaf-1, Bid, and caspase-6, have been identified as p53 transcriptional targets (256–258), gene-targeting studies have identified the BH3-only polypeptides Puma and, to a lesser extent, Noxa, as the most important contributors to p53-induced apoptosis (105–107). It currently appears that Puma plays a predominant role in p53-induced apoptosis after certain DNA-damaging stimuli (106), but further study is required to determine whether similar results are obtained after all types of DNA damage and in all cell types.

Despite the frequency of p53 mutations in clinical cancer, there is reason to question whether these lesions arise to counterbalance the proapoptotic effects of oncogenes early in carcinogenesis. Current evidence suggests that p53 mutations play an important role *in vivo* to counter the proapoptotic effects of *myc* overexpression in Burkitt's lymphoma-like B-cell neoplasms in mice (239). In epithelial neoplasms, however, p53 mutations are a late occurrence (259,260), raising the possibility that other antiapoptotic

changes like those involving the PI3 kinase/Akt pathway (251,261), which often occur in atypia and carcinoma in situ, might play a more important role early in tumorigenesis.

12. ARE ALTERATIONS IN THE APOPTOTIC MACHINERY DETERMINANTS OF DRUG SENSITIVITY?

The demonstration that anticancer drugs can kill cells by inducing apoptosis, coupled with the observation that cancer cells have alterations that inhibit apoptotic pathways, has led many authors to hypothesize that alterations in apoptotic pathways might contribute to drug resistance (e.g., 13,15,17,262). Two aspects of this hypothesis are important to emphasize. First, what is being postulated is resistance of cells relative to what their sensitivity would be if apoptotic pathways were not altered, not absolute resistance to therapy. Other changes in cancer cells, e.g., their aberrant entry into the cell cycle and loss of cell cycle checkpoints, enhance sensitivity to anticancer drugs and undoubtedly contribute to the (admittedly limited) therapeutic index of these agents in some settings. Second, the hypothesis that alterations in apoptotic pathways contribute to drug resistance can only be true to the extent that apoptosis plays a role in cellular responses to various chemotherapeutic agents. As indicated elsewhere in this monograph, cells can also suffer other fates after drug treatment, and these other fates are not necessarily modulated by changes in the apoptotic machinery.

Clinical studies examining the correlation between expression of various components of the apoptotic machinery and treatment outcome have been extensively reviewed (15,263,264). These studies, while potentially important for identifying predictive markers that might alter treatment and for confirming activation of various pathways that might serve as drug targets in future trials, do not establish causality. Accordingly, the following comments will focus on controlled studies that have evaluated the relationship between alterations in components of the mitochondrial pathway and drug sensitivity in model systems. These studies have focused primarily on the effects of p53 alterations and the changes in Bcl-2 family members and IAPs.

p53 alterations appear to affect sensitivity to both ionizing radiation and drugs. Studies in tissue culture have led to the conclusion that p53 deficiency can render both E1A/ras-transformed fibroblasts (265) and HCT116 colon cancer cells (266) resistant to the induction of apoptosis by various anticancer drugs *in vitro*. When the same cell lines are grown as xenografts, responses are diminished in the absence of p53. Consistent with these results, studies have also established that ionizing radiation or high-dose 5-fluorouracil induces less apoptosis in epithelial cells of the small and large intestines of p53^{+/+} mice compared with that of p53^{+/+} littermates (267,268). Collectively, these results suggest that changes in p53 status can affect drug sensitivity in at least some cell types.

Increased expression of antiapoptotic Bcl-2 family members can also affect drug sensitivity. Virtually, all published studies agree that Bcl-2 or Bcl-x_L overexpression delays the appearance of apoptotic changes after treatment with a variety of agents (247, 269–271). In addition, assays that compare the ability of isogenic cells to regrow after drug treatment *in vitro* or *in vivo* have established that forced overexpression of Bcl-2 or Bcl-x_L can enhance the ability of cell populations to recover after a variety of drug treatments (247,271,272). Nonetheless, the role of group I Bcl-2 family polypeptides

in anticancer drug resistance is far from settled. Whether conclusions based on limited studies conducted predominantly in lymphohematopoietic cells can be generalized to all cell types and all therapeutic agents remains a matter of some controversy (20). Moreover, the effects of other antiapoptotic Bcl-2 family members remain to be studied in a similar fashion.

At the present time, the effects of alterations in proapoptotic Bcl-2 family members also require further study. It has been reported that *BAX* deletion inhibits drug-induced apoptosis in fibroblasts (273) and HCT116 cells *in vitro* (226,274,275). It has also been reported that *BAX* deletion renders mouse oocytes resistant to polycyclic aromatic hydrocarbon-induced apoptosis *in vivo* (276). Nonetheless, the effects of *BAX* deletion on anticancer drug sensitivity remain to be established *in vivo*. Moreover, it is important to emphasize that *BAX*^{-/-} cells represent an extreme situation that might not occur in human cancers *in vivo*. Instead, mismatch repair-deficient tumors appear to have inactivating mutations in a single *BAX* allele (249), and it is unclear whether the resulting cells or cell lines completely lack Bax protein. If Bax is incompletely downregulated in human tumors, then models in which Bax is downregulated might be more pertinent to clinical anticancer drug resistance than those containing *BAX* deletions. Similar comments apply to Bak.

Information regarding the role of BH3-only protein alterations in drug resistance is likewise incomplete. In perhaps the best-studied example, *Bim* gene deletion not only renders murine thymocytes selectively resistant to paclitaxel-induced apoptosis *in vitro* (100) but also confers resistance to transformed baby mouse kidney xenografts treated with paclitaxel *in vivo* (277). It remains to be determined whether quantitative Bim alterations that fall short of complete deletion also affect drug sensitivity. Likewise, it remains to be determined whether *Puma* deletion, which renders fibroblasts, thymocytes, or HCT116 cells resistant to a number of treatments, including doxorubicin, etoposide, and ionizing radiation *in vitro* (105–107,278), affects long-term responses to anticancer treatments in tumor cells *in vivo*.

The effects of altered IAP levels also require further study. Examination of isogenic cell lines *in vitro* has established that cIAP1, cIAP2, and XIAP diminish apoptosis triggered by a number of agents, including camptothecin and menadione (162). A more recent study also demonstrated that an XIAP antisense oligonucleotide sensitized NIH-H60 non-small-cell lung cancer cells to etoposide, doxorubicin, and paclitaxel *in vitro* and to vinorelbine *in vivo* (279). Antisense oligonucleotides, however, are notorious for producing off-target effects. Further studies using additional model systems *in vitro* and *in vivo* are required to determine whether enhanced expression of cIAP1, cIAP2, and/or XIAP enhances the ability of tumor cell populations to survive drug treatment.

Moving further downstream, silencing of *APAF-1* has been reported in a large fraction of melanoma cell lines and clinical samples (280). Consistent with prior observations in *Apaf-1*^{-/-} murine fibroblasts and thymocytes (281), the *Apaf-1*-deficient melanoma cells were reportedly resistant to doxorubicin-induced apoptosis (280). Although the authors concluded that they had identified a major mechanism of drug resistance in melanoma, subsequent observations have called this conclusion into question. In particular, others have not been able to reproduce the frequent *Apaf-1* downregulation in melanoma cell lines (282–284) or clinical samples (284).

In short, alterations of p53 and certain Bcl-2 family members are clearly capable of altering long-term survival after drug treatment in some model systems *in vitro* and *in vivo*. There is currently less evidence that overexpression of XIAP or downregulation

of Apaf-1, caspase-9, or the effector caspases modulates long-term sensitivity of cells to anticancer drugs, particularly *in vivo*. On the contrary, recent evidence suggests that changes in the intrinsic pathway beyond the mitochondrial permeabilization step (Figs. 1 and 3) affect the rate of cell death but not the ultimate extent of cell death after proapoptotic stimuli (285). These observations invite that speculation that proapoptotic Bcl-2 family members, once activated, might be able to kill cells through the release of cytochrome c and other caspase-independent processes even if cells are limited in their ability to activate caspases. The inability of broad-spectrum caspase inhibitors to enhance long-term survival of camptothecin-treated cells despite inhibition of apoptosis (286) is consistent with this notion. Collectively, these results do not negate the importance of the mitochondrial pathway in determining the response to anticancer drugs but instead suggest that future studies are likely to be most productive if they focus on upstream components of this pathway.

13. CONCLUSIONS

Previous studies have demonstrated that anticancer treatments can induce apoptosis in susceptible cell types *in vitro* and *in vivo*. Although a few agents require an intact death receptor pathway to trigger apoptosis, many agents appear to utilize the mitochondrial pathway. Despite recent improvements in our understanding of this pathway, fundamental issues currently remain unsettled. These include determination of the precise mechanism by which proapoptotic Bcl-2 family members induce outer mitochondrial membrane permeabilization as well as conclusive demonstration that other proapoptotic mitochondrial proteins such as Smac and HtRA2 play a critical role in apoptosis *in vivo*. Because the intrinsic pathway is extensively targeted for inhibition during the development of cancer, it has been speculated that changes in this pathway also contribute to drug resistance. With the exception of p53, Bcl-2, Bcl-x_L, Bim, and possibly XIAP, however, careful *in vivo* studies to establish this point remain to be performed.

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NOTE

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REFERENCES

1. Kaufmann SH, Earnshaw WC. Induction of apoptosis by cancer chemotherapy. *Exp Cell Res* 2000; 256:42–9.
2. Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980; 68:251–306.
3. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol* 1999; 15:269–90.

4. Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates and functions during apoptosis. *Annu Rev Biochem* 1999; 68:383–424.
5. Fischer U, Janicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ* 2003; 10(1):76–100.
6. Li X, Gong J, Feldman E, Seiter K, Traganos F, Darzynkiewicz Z. Apoptotic cell death during treatment of leukemias. *Leuk Lymphoma* 1994; 13(Suppl 1):65–70.
7. Seiter K, Feldman EJ, Halicka HD, et al. Phase I clinical and laboratory evaluation of topotecan and cytarabine in patients with acute leukemia. *J Clin Oncol* 1997; 15(1):44–51.
8. Stahnke K, Fulda S, Friesen C, Staub G, Debatin K-M. Activation of apoptosis pathways in peripheral blood lymphocytes by *in vivo* chemotherapy. *Blood* 2001; 98:3066–73.
9. Byrd JC, Kitada S, Flinn IW, et al. The mechanism of tumor cell clearance by rituximab *in vivo* in patients with B-cell chronic lymphocytic leukemia: evidence of caspase activation and apoptosis induction. *Blood* 2002; 99:1038–43.
10. Stahnke K, Eckhoff S, Mohr A, Meyer LH, Debatin K-M. Apoptosis induction in peripheral leukemia cells by remission induction treatment *in vivo*: selective depletion and apoptosis in a CD34+ subpopulation of leukemia cells. *Leukemia* 2003; 17(11):2130–9.
11. Martin DS, Stolfi RL, Colofiore JR, Nord LD, Sternberg S. Biochemical modulation of tumor cell energy *in vivo*: II. A lower dose of adriamycin is required and a greater antitumor activity is induced when cellular energy is depressed. *Cancer Invest* 1994; 12(3):296–307.
12. Meyn RE, Stephens LC, Hunter NR, Milas L. Apoptosis in murine tumors treated with chemotherapy agents. *Anticancer Drugs* 1995; 6(3):443–50.
13. Green DR, Bissonnette RP, Cotter TG. Apoptosis and cancer. *Imp Adv Oncol* 1994; 1994:37–52.
14. Fisher DE. Apoptosis in cancer therapy: crossing the threshold. *Cell* 1994; 78(4):539–42.
15. Reed JC. Dysregulation of apoptosis in cancer. *J Clin Oncol* 1999; 17(9):2941–53.
16. Makin G, Hickman JA. Apoptosis and cancer chemotherapy. *Cell Tissue Res* 2000; 301:143–52.
17. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis. A link between cancer genetics and chemotherapy. *Cell* 2002; 108(2):153–64.
18. Schmitt CA, Fridman JS, Yang M, et al. A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. *Cell* 2002; 109:335–46.
19. Roninson IB. Tumor cell senescence in cancer treatment. *Cancer Res* 2003; 63:2705–15.
20. Brown JM, Attardi LD. The role of apoptosis in cancer development and treatment response. *Nat Rev Cancer* 2005; 5:231–7.
21. Patel T, Gores GJ, Kaufmann SH. The role of proteases during apoptosis. *FASEB J* 1996; 10(5):587–97.
22. Jaattela M, Tschopp J. Caspase-independent cell death in T lymphocytes. *Nat Immunol* 2003; 4(5):416–23.
23. Creagh EM, Martin SJ. Caspases: cellular demolition experts. *Biochem Soc Trans* 2001; 29:696–702.
24. Boatright KM, Renatus M, Scott FL, et al. A unified model for apical caspase activation. *Mol Cell* 2003; 11(2):529–41.
25. Renatus M, Stennicke HR, Scott FL, Liddington RC, Salvesen GS. Dimer formation drives the activation of the cell death protease caspase 9. *Proc Natl Acad Sci USA* 2001; 98:14250–5.
26. Rodriguez J, Lazebnik Y. Caspase-9 and APAF-1 form an active holoenzyme. *Genes Dev* 1999; 13:3179–84.
27. Stennicke HR, Deveraux QL, Humke EW, Reed JC, Dixit VM, Salvesen GS. Caspase-9 can be activated without proteolytic processing. *J Biol Chem* 1999; 274(13):8359–62.
28. Chang DW, Xing Z, Capacio VL, Peter ME, Yang X. Interdimer processing mechanism of procaspase-8 activation. *EMBO J* 2003; 22(16):4132–42.
29. Chai J, Wu Q, Shiozaki E, Srinivasula SM, Alnemri ES, Shi Y. Crystal structure of a procaspase-7 zymogen: mechanisms of activation and substrate binding. *Cell* 2001; 107:399–407.
30. Yan N, Shi Y. Mechanisms of apoptosis through structural biology. *Ann Rev Cell Dev Biol* 2005; 21:35–56.
31. Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES. Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol Cell* 1998; 1:949–57.
32. Ruchaud S, Korfali N, Villa P, et al. Caspase-6 gene knockout reveals a role for Lamin A cleavage in apoptotic chromatin condensation. *EMBO J* 2002; 21:1967–77.
33. Kothakota S, Azuma T, Reinhard C, et al. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* 1997; 278(5336):294–8.

34. Kamada S, Kusano H, Fujita H, et al. A cloning method for caspase substrates that uses the yeast two-hybrid system: cloning of the antiapoptotic gene gelsolin. *Proc Natl Acad Sci USA* 1998; 95:8532–7.
35. Rao L, Perez D, White E. Lamin proteolysis facilitates nuclear events during apoptosis. *J Cell Biol* 1996; 135:1441–55.
36. Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 1980; 284(5756):555–6.
37. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 1998; 391:43–50.
38. Samejima K, Earnshaw WC. Differential localization of ICAD-L and ICAD-S in cells due to removal of a C-terminal NLS from ICAD-L by alternative splicing. *Exp Cell Res* 2000; 255(2): 314–20.
39. Nagata S. Apoptotic DNA fragmentation. *Exp Cell Res* 2000; 256:12–8.
40. Emoto Y, Manome Y, Meinhardt G, et al. Proteolytic activation of protein kinase C δ by an ICE-like protease in apoptotic cells. *EMBO J* 1995; 14(24):6148–56.
41. Frasch SC, Henson PM, Kailey JM, et al. Regulation of phospholipid scramblase activity during apoptosis and cell activation by protein kinase Cdelta. *J Biol Chem* 2000; 275:23065–73.
42. Martin SJ, Reutelingsperger CP, McGahon AJ, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 1995; 182(5):1545–56.
43. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. *Nature* 2002; 417:182–7.
44. Hanayama R, Tanaka M, Miyasaka K, et al. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science* 2004; 304(5674):1147–50.
45. Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM. A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* 2000; 405:85–90.
46. Hoffmann PR, deCathelineau AM, Ogden CA, et al. Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. *J Cell Biol* 2001; 155:649–59.
47. Lauber K, Blumenthal SG, Waibel M, Wesselborg S. Clearance of apoptotic cells: getting rid of the corpses. *Mol Cell* 2004; 14(3):277–87.
48. Mesner PW Jr, Bible KC, Martins LM, et al. Characterization of caspase processing and activation of HL-60 cell cytosol under cell-free conditions: nucleotide requirement and inhibitor profile. *J Biol Chem* 1999; 274(32):22635–45.
49. Wang X. The expanding role of mitochondria in apoptosis. *Genes Dev* 2001; 15(22):2922–33.
50. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science* 2004; 305 (5684):626–9.
51. Goldstein JC, Waterhouse NJ, Juin P, Evan GI, Green DR. The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nat Cell Biol* 2000; 2(3):156–62.
52. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 1997; 90(3):405–13.
53. Li P, Nijhawan D, Budihardjo I, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; 91(4):479–89.
54. Zou H, Li Y, Liu X, Wang X. An APAF-1-cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* 1999; 274(17):11549–56.
55. Cain K, Bratton SB, Langlais C, et al. Apaf-1 oligomerizes into biologically active approximately 700-kDa and inactive approximately 1.4-MDa apoptosome complexes. *J Biol Chem* 2000; 275(9): 6067–70.
56. Acehan D, Jiang X, Morgan DG, Heuser JE, Wang X, Akey CW. Three-dimensional structure of the apoptosome. Implications for assembly, procaspase-9 binding, and activation. *Mol Cell* 2002; 9(2):423–32.
57. Hill MM, Adrain C, Duriez PJ, Creagh EM, Martin SJ. Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. *EMBO J* 2004; 23(10):2134–45.
58. Shi Y. Caspase activation: revisiting the induced proximity model. *Cell* 2004; 117:855–8.
59. Liu X, Kim CN, Yang J, Jemerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome C. *Cell* 1996; 86(1):147–57.
60. Kluck RM, Martin SJ, Hoffman BM, Zhou JS, Green DR, Newmeyer DD. Cytochrome C activation of CPP32-like proteolysis plays a critical role in a xenopus cell-free apoptotic system. *EMBO J* 1997; 16(15):4639–49.

61. Hampton MB, Zhivotovsky B, Slater AF, Burgess DH, Orrenious S. Importance of the redox state of cytochrome C during caspase activation in cytosolic extracts. *Biochem J* 1998; 329:95–9.
62. Zamzami N, Kroemer G. The mitochondrion in apoptosis: how Pandora's box opens. *Nat Rev Mol Cell Biol* 2001; 2(1):67–71.
63. Martinou JC, Green DR. Breaking the mitochondrial barrier. *Nat Rev Mol Cell Biol* 2001; 2:63–7.
64. Newmeyer DD, Ferguson-Miller S. Mitochondria: releasing power for life and unleashing the machineries of death. *Cell* 2003; 112:481–90.
65. Jiang X, Wang X. Cytochrome C-mediated apoptosis. *Annu Rev Biochem* 2004; 73:87–106.
66. Anflous K, Armstrong DD, Craigen WJ. Altered mitochondrial sensitivity for ADP and maintenance of creatine-stimulated respiration in oxidative striated muscles from VDAC1-deficient mice. *J Biol Chem* 2001; 276:1954–60.
67. Graham BH, Waymire KG, Cottrell B, Trounce IA, MacGregor GR, Wallace DC. A mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoform of the adenine nucleotide translocator. *Nat Genet* 1997; 16:226–34.
68. Kokoszka JE, Waymire KG, Levy SE, et al. The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature* 2004; 427:461–5.
69. Baines CP, Kaiser RA, Purcell NH, et al. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* 2005; 434:658–62.
70. Nakagawa T, Shimizu S, Watanabe T, et al. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* 2005; 434:652–8.
71. Basso E, Fante L, Fowlkes J, Petronilli V, Forte MA, Bernardi P. Properties of the permeability transition pore in mitochondria devoid of cyclophilin D. *J Biol Chem* 2005; 280(19):18558–61. Epub ahead of print.
72. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 1999; 13:1899–911.
73. Cory S, Huang DC, Adams JM. The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* 2003; 22(53):8590–607.
74. Coultas L, Strasser A. The role of the Bcl-2 protein family in cancer. *Semin Cancer Biol* 2003; 13(2):115–23.
75. Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 1998; 335(6189):440–2.
76. Hockenbery DM, Nuñez G, Millman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990; 348:334–6.
77. Puthalakath H, Strasser A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. *Cell Death Differ* 2002; 9:505–12.
78. Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ. Movements of bax from cytosol to mitochondria during apoptosis. *J Cell Biol* 1997; 139:1281–92.
79. Chen L, Willis SN, Wei A, et al. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* 2005; 17(3):393–403.
80. Antonsson B, Montessuit S, Sanchez B, Martinou JC. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. *J Biol Chem* 2001; 276(15):11615–23.
81. Desagher S, Osen-Sand A, Nichols A, et al. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome C release during apoptosis. *J Cell Biol* 1999; 144(5):891–901.
82. Eskes R, Desagher S, Antonsson B, Martinou JC. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol Cell Biol* 2000; 20:929–35.
83. Makin GWJ, Corfe BM, Griffiths GJ, Thistlethwaite A, Hickman JA, Dive C. Damage-induced Bax N-terminal change, translocation to mitochondria and formation of Bax dimers/complexes occur regardless of cell fate. *EMBO J* 2001; 20:6306–15.
84. Griffiths GJ, Dubrez L, Morgan CP, et al. Cell damage-induced conformational changes of the pro-apoptotic protein Bak *in vivo* precede the onset of apoptosis. *J Cell Biol* 1999; 144(5):903–14.
85. Wei MC, Zong WX, Cheng EH, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 2001; 292(5517):727–30.
86. Cheng EH, Wei MC, Weiler S, et al. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell* 2001; 8(3):705–11.
87. Zong W-X, Lindsten T, Ross AJ, MacGregor GR, Thompson CB. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev* 2001; 15:1481–6.

88. Jurgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC. Bax directly induces release of cytochrome C from isolated mitochondria. *Proc Natl Acad Sci USA* 1998; 95(9):4997–5002.
89. Kuwana T, Mackey MR, Perkins G, et al. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* 2002; 111(3):331–42.
90. Antonsson B, Conti F, Ciavatta A, et al. Inhibition of bax channel-forming activity by Bcl-2. *Science* 1997; 277:370–72.
91. Epand RF, Martinou JC, Montessuit S, Epand RM, Yip CM. Direct evidence for membrane pore formation by the apoptotic protein Bax. *Biochem Biophys Res Commun* 2002; 298(5):744–9.
92. Kandasamy K, Srinivasula SM, Alnemri ES, et al. Involvement of proapoptotic molecules Bax and Bak in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial disruption and apoptosis: differential regulation of cytochrome c and Smac/DIABLO release. *Cancer Res* 2003; 63(7):1712–21.
93. Kuwana T, Bouchier-Hayes L, Chipuk JE, et al. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* 2005; 17:525–35.
94. Cartron P-F, Gallenne T, Bougras G, et al. The first helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA. *Mol Cell* 2004; 16:807–18.
95. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998; 94:491–501.
96. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome C release from mitochondria in response to activation of cell surface death receptors. *Cell* 1998; 94(4):481–90.
97. Barry M, Heibein JA, Pinkoski MJ, et al. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. *Mol Cell Biol* 2000; 20:3781–94.
98. Stoka V, Turk B, Schendel SL, et al. Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. *J Biol Chem* 2001; 276:3149–57.
99. Gajewski TF, Thompson CG. Apoptosis meets signal transduction: elimination of a BAD influence. *Cell* 1996; 87(4):589–92.
100. Puthalakath H, Huang DCS, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* 1999; 3:287–96.
101. Bouillet P, Metcalf D, Huang DC, et al. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 1999; 286(5445):1735–8.
102. Puthalakath H, Villunger A, O'Reilly LA, et al. Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science* 2001; 293(5536):1829–32.
103. Yu JJ, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol Cell* 2001; 7:673–82.
104. Oda E, Ohki R, Murasawa H, et al. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 2000; 288:1053–8.
105. Yu J, Wang Z, Kinzler KW, Vogelstein B, Zhang L. PUMA mediates the apoptotic response to p53 in colorectal cancer cells. *Proc Natl Acad Sci USA* 2003; 100(4):1931–6.
106. Villunger A, Michalak EM, Coultas L, et al. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 2003; 302(5647):1036–8.
107. Jeffers JR, Parganas E, Lee Y, et al. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell* 2003; 4(4):321–8.
108. Oltvai ZN, Korsmeyer SJ. Checkpoints of dueling dimers foil death wishes. *Cell* 1994; 79(2):189–92.
109. Perez D, White E. TNF-alpha signals apoptosis through a Bid-dependent conformational change in Bax that is inhibited by E1B 19K. *Mol Cell* 2000; 6(1):53–63.
110. Cuconati A, Mukherjee C, Perez D, White E. DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. *Genes Dev* 2003; 17:2922–32.
111. Cheng EH, Levine B, Boise LH, Thompson CB, Hardwick JM. Bax-independent inhibition of apoptosis by Bcl-XL. *Nature* 1996; 379:554–6.

112. Hockenbery DM, Oltvai ZN, Yin X-M, Millman CL, Korsmeyer SJ. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 1993; 75:241–52.
113. Bossy-Wetzel E, Newmeyer DD, Green DR. Mitochondrial cytochrome C release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J* 1998; 17(1):37–49.
114. Ricci JE, Munoz-Pinedo C, Fitzgerald P, et al. Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain. *Cell* 2004; 117(6):773–86.
115. Baffy G, Miyashita T, Williamson JR, Reed JC. Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production. *J Biol Chem* 1993; 268:6511–9.
116. Lam M, DUBYAK G, Chen L, Nunez G, Miesfeld RL, Distelhorst CW. Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca²⁺ fluxes. *Proc Natl Acad Sci USA* 1994; 91(14):6569–73.
117. Scorrano L, Oakes SA, Opferman JT, et al. BAX and BAK regulation of endoplasmic reticulum Ca²⁺: a control point for apoptosis. *Science* 2003; 300(5616):135–9.
118. Bassik MC, Scorrano L, Oakes SA, Pozzan T, Korsmeyer SJ. Phosphorylation of BCL-2 regulates ER Ca(2+) homeostasis and apoptosis. *EMBO J* 2004; 23:1207–16.
119. Distelhorst CW, Shore GC. Bcl-2 and calcium: controversy beneath the surface. *Oncogene* 2004; 23:2875–80.
120. Yang J, Liu X, Bhalla K, et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 1997; 275:1129–32.
121. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997; 275(5303):1132–6.
122. Vrana JA, Bieszczyk CK, Cleaveland ES, et al. An MCL1-overexpressing Burkitt lymphoma subline exhibits enhanced survival on exposure to serum deprivation, topoisomerase inhibitors, or staurosporine but remains sensitive to 1-beta-D-arabinofuranosylcytosine. *Cancer Res* 2002; 62(3): 892–900.
123. Boucher MJ, Morisset J, Vachon PH, Reed JC, Laine J, Rivard N. MEK/ERK signaling pathway regulates the expression of Bcl-2, Bcl-X(L), and Mcl-1 and promotes survival of human pancreatic cancer cells. *J Cell Biochem* 2000; 79(3):355–69.
124. Milella M, Kornblau SM, Estrov Z, et al. Therapeutic targeting of the MEK/MAPK signal transduction module in acute myeloid leukemia. *J Clin Invest* 2001; 108(6):851–9.
125. Catlett-Falcone R, Landowski TH, Oshiro MM, et al. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity* 1999; 10:105–15.
126. Epling-Burnette PK, Liu JH, Catlett-Falcone R, et al. Inhibition of STAT3 signaling leads to apoptosis of leukemic large granular lymphocytes and decreased Mcl-1 expression. *J Clin Invest* 2001; 107(3):351–62.
127. Wang JM, Chao JR, Chen WS, Kuo ML, Yen JJ, Yang-Yen H-F. The antiapoptotic gene mcl-1 is up-regulated by the phosphatidylinositol 3-kinase/Akt signaling pathway through a transcription factor complex containing CREB. *Mol Cell Biol* 1999; 19:6195–206.
128. Bernal A, Pastore RD, Asgary Z, et al. Survival of leukemic B cells promoted by engagement of the antigen receptor. *Blood* 2001; 98:3050–7.
129. Henson ES, Gibson EM, Villanueva J, Bristow NA, Haney N, Gibson SB. Increased expression of Mcl-1 is responsible for the blockage of TRAIL-induced apoptosis mediated by EGF/ErbB1 signaling pathway. *J Cell Biochem* 2003; 89(6):1177–92.
130. Kobayashi S, Werneburg NW, Bronk SF, Kaufmann SH, Gores GJ. Interleukin-6 contributes to myeloid cell leukemia-1 upregulation (Mcl-1) and TRAIL resistance via an Akt signaling pathway in cholangiocarcinoma cells. *Gastroenterology* 2005; 128:2054–65.
131. Ruvolo PP, Deng X, Carr BK, May WS. A functional role for mitochondrial protein kinase C alpha in Bcl2 phosphorylation and suppression of apoptosis. *J Biol Chem* 1998; 273:25436–42.
132. Deng X, Ruvolo P, Carr B, May WS Jr. Survival function of ERK1/2 as IL-3-activated, staurosporine-resistant Bcl2 kinases. *Proc Natl Acad Sci USA* 2000; 97(4):1578–83.
133. Deng X, Gao F, Flagg T, May WS Jr. Mono- and multisite phosphorylation enhances Bcl2's antiapoptotic function and inhibition of cell cycle entry functions. *Proc Natl Acad Sci USA* 2004; 101(1):153–8.

134. Domina AM, Smith JH, Craig RW. Myeloid cell leukemia 1 is phosphorylated through two distinct pathways, one associated with extracellular signal-regulated kinase activation and the other with G2/M accumulation or protein phosphatase 1/2A inhibition. *J Biol Chem* 2000; 275:21688–94.
135. Meng X, Chandra J, Loegering D, et al. Central role of FADD in apoptosis induction by the mitogen activated protein kinase kinase inhibitor CI1040 (PD184352) in acute lymphocytic leukemia cell lines *in vitro*. *J Biol Chem* 2003; 278:47236–339.
136. Domina AM, Vrana JA, Gregory MA, Hann SR, Craig RW. MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol. *Oncogene* 2004; 23(31):5301–15.
137. Yu C, Bruzek LM, Meng XW, et al. The role of Mcl-1 down-regulation in the pro-apoptotic activity of the raf kinase inhibitor BAY 43-9006. *Oncogene* 2005; 24:6861–9.
138. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 1995; 270(5240):1326–31.
139. Dent P, Grant S. Pharmacologic interruption of the mitogen-activated extracellular-regulated kinase/mitogen-activated protein kinase signal transduction pathway: potential role of promoting cytotoxic drug action. *Clin Cancer Res* 2001; 7:775–83.
140. Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ. Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. *J Biol Chem* 2003; 278:18811–6.
141. Luciano F, Jacquet A, Colosetti P, et al. Phosphorylation of Bim-El by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. *Oncogene* 2003; 22:6785–93.
142. Krajewski S, Krajewska M, Shabaik A, et al. Immunohistochemical analysis of *in vivo* patterns of Bcl-X expression. *Cancer Res* 1994; 54:5501–7.
143. Krajewski S, Bodrug S, Krajewska M, et al. Immunohistochemical analysis of Mcl-1 protein in human tissues. *Am J Pathol* 1995; 146(6):1309–19.
144. Huang DCS, Strasser A. BH3-only proteins-essential initiators of apoptotic cell death. *Cell* 2000; 103:839–42.
145. Tsuruta F, Sunayama J, Mori Y, et al. JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins. *EMBO J* 2004; 23(8):1889–99.
146. Willis SN, Chen L, Dewson G, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* 2005; 19(11):1294–305.
147. Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. *Nat Rev Cell Mol Biol* 2002; 3:401–10.
148. Vaux DL, Silke J. IAPs, RINGs and ubiquitylation. *Nat Rev Mol Cell Biol* 2005; 6:287–97.
149. Shiozaki EN, Chai J, Rigotti DJ, et al. Mechanism of XIAP-mediated inhibition of caspase-9. *Mol Cell* 2003; 11(2):519–27.
150. Silke J, Vaux DL. Two kinds of BIR-containing protein – inhibitors of apoptosis, or required for mitosis. *J Cell Sci* 2001; 114:1821–7.
151. Uren AG, Wong L, Pakusch M, et al. Survivin and the inner centromere protein INCENP show similar cell-cycle localization and gene knockout phenotype. *Curr Biol* 2000; 10(21):1319–28.
152. Li F. Survivin study: what is the next wave. *J Cell Physiol* 2003; 197(1):8–29.
153. Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* 2000; 288:874–7.
154. Huang H-K, Joazeiro CAP, Bonfoco E, Kamada S, Levenson JD, Hunter T. The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes *in vitro* monoubiquitination of caspase 3 and 7. *J Biol Chem* 2000; 275(35):26661–4.
155. Suzuki Y, Nakabayashi Y, Takahashi R. Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. *Proc Natl Acad Sci USA* 2001; 98(15):8662–7.
156. MacFarlane M, Merrison W, Bratton SB, Cohen GM. Proteasome-mediated degradation of Smac during apoptosis: XIAP promotes Smac ubiquitination *in vitro*. *J Biol Chem* 2002; 277:36611–6.
157. Goyal L. Cell death inhibition: keeping caspases in check. *Cell* 2001; 104(6):805–8.
158. Srinivasula SM, Hegde R, Saleh A, et al. A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature* 2001; 410:112–6.
159. Scott FL, Denault JB, Riedl SJ, Shin H, Renatus M, Salvesen GS. XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. *EMBO J* 2005; 24(3):645–55.

160. Miller LK. An exegesis of IAPs: salvation and surprises from BIR motifs. *Trends Cell Biol* 1999; 9(8):323–8.
161. Baldwin AS. Control of oncogenesis and cancer therapy resistance by the transcription factor NF- κ B. *J Clin Invest* 2001; 107:241–6.
162. Liston P, Roy N, Tamai K, et al. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* 1996; 379:349–53.
163. Duckett CS, Nava VE, Gedrich RW, et al. A conserved family of cellular genes related to the baculovirus IAP gene and encoding apoptosis inhibitors. *EMBO J* 1996; 15:2685–94.
164. Uren AG, Pakusch M, Hawkins CJ, Puls KL, Vaux DL. Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. *Proc Natl Acad Sci USA* 1996; 93:4974–8.
165. Silke J, Hawkins CJ, Ekert PG, et al. The anti-apoptotic activity of XIAP is retained upon mutation of both the caspase 3- and caspase 9-interacting sites. *J Cell Biol* 2002; 157:115–24.
166. Harlin H, Reffey SB, Duckett CS, Lindsten T, Thompson CB. Characterization of XIAP-deficient mice. *Mol Cell Biol* 2001; 21(10):3604–8.
167. Conte D, Liston P, Wong JW, Wright KE, Korneluk RG. Thymocyte-targeted overexpression of XIAP transgene disrupts T lymphoid apoptosis and maturation. *Proc Natl Acad Sci USA* 2001; 98(9):5049–54.
168. Ekert PG, Vaux DL. The mitochondrial death squad - hardened killers or innocent bystanders. *Curr Opin Cell Biol* 2005; 17:626–30.
169. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* 2001; 412:95–9.
170. Samejima K, Tone S, Earnshaw WC. CAD/DFF40 nuclease is dispensable for high molecular weight DNA cleavage and stage I chromatin condensation in apoptosis. *J Biol Chem* 2001; 276:45427–32.
171. Irvine RA, Adachi N, Shibata DK, et al. Generation and characterization of endonuclease G null mice. *Mol Cell Biol* 2005; 25(1):294–302.
172. Miramar MD, Costantini P, Ravagnan L, et al. NADH oxidase activity of mitochondrial apoptosis-inducing factor. *J Biol Chem* 2001; 276:16391–8.
173. Susin SA, Lorenzo HK, Zamzami N, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 1999; 397(6718):441–6.
174. Joza N, Susin SA, Daugas E, et al. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature* 2001; 410:549–54.
175. Klein JA, Longo-Guess CM, Rossmann MP, et al. The harlequin mouse mutation downregulates apoptosis-inducing factor. *Nature* 2002; 419:367–74.
176. Verhagen AM, Ekert PG, Pakusch M, et al. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000; 102(1):45–53.
177. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome C-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000; 102(1):33–42.
178. Chauhan D, Hideshima T, Rosen S, Reed JC, Kharbanda S, Anderson KC. Apaf-1/Cytochrome c-independent and Smac-dependent induction of apoptosis in multiple myeloma (MM) cells. *J Biol Chem* 2001; 276:24453–6.
179. Adrain C, Creagh EM, Martin SJ. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. *EMBO J* 2001; 20(23):6627–36.
180. Ekert PG, Silke J, Hawkins CJ, Verhagen AM, Vaux DL. DIABLO promotes apoptosis by removing MIHA/XIAP from processed caspase 9. *J Cell Biol* 2001; 152:483–90.
181. Burri L, Strahm Y, Jawkins CJ, et al. Mature DIABLO/Smac is produced by the IMP protease complex on the mitochondrial inner membrane. *Mol Biol Cell* 2005; 16:2926–33.
182. Chai J, Du C, Kyin S, Wang X, Shi Y. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature* 2000; 406(6798):855–62.
183. Liu Z, Sun C, Olejniczak ET, et al. Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature* 2000; 408(6815):1004–8.
184. Deveraux QL, Reed JC. IAP family proteins – suppressors of apoptosis. *Genes Dev* 1999; 13:239–52.
185. Deveraux QL, Roy N, Stennicke HR, et al. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J* 1998; 17(8):2215–23.

186. Wilkinson JC, Wilkinson AS, Scott FL, Csomos RA, Salvesen GS, Duckett CS. Neutralization of Smac/Diablo by inhibitors of apoptosis (IAPs). A caspase-independent mechanism for apoptotic inhibition. *J Biol Chem* 2004; 279(49):51082–90.
187. Hwain S, Renatus M, Eckelman BP, Nunes VA, Sampaio CAM, Salvesen GS. The BIR domain of IAP-like protein 2 is conformationally unstable: implications for caspase inhibition. *Biochem J* 2005; 385:1–10.
188. Vucic D, Franklin MC, Wallweber HJA, et al. Engineering ML-IAP to produce an extraordinarily potent caspase 9 inhibitor: implications for Smac-dependent anti-apoptotic activity of ML-IAP. *Biochem J* 2005; 385:11–20.
189. Okada H, Suh WK, Jin J, et al. Generation and characterization of Smac/DIABLO-deficient mice. *Mol Cell Biol* 2002; 22:3509–17.
190. Clausen T, Southan C, Ehrmann M. The HtrA family of proteases: implications for protein composition and cell fate. *Mol Cell* 2002; 10:443–55.
191. Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell* 2001; 8(3): 613–21.
192. Hegde R, Srinivasula SM, Zhang Z, et al. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. *J Biol Chem* 2002; 277:432–8.
193. Martins LM, Iaccarino I, Tenev T, et al. The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. *J Biol Chem* 2002; 277:439–44.
194. Verhagen AM, Silke J, Ekert PG, et al. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J Biol Chem* 2002; 277(1): 445–54.
195. Srinivasula SM, Gupta S, Datta P, et al. Inhibitor of apoptosis proteins are substrates for the mitochondrial serine protease Omi/Htr A2. *J Biol Chem* 2003; 278:31469–72.
196. Jones JM, Datta P, Srinivasula SM, et al. Loss of Omi mitochondrial protease activity causes the neuromuscular disorder of mnd2 mutant mice. *Nature* 2003; 425:721–7.
197. Martins LM, Morrison A, Klupsch K, et al. Neuroprotective role of the Reaper-related serine protease HtrA2/Omi revealed by targeted deletion in mice. *Mol Cell Biol* 2004; 24(22):9848–62.
198. Hegde R, Srinivasula SM, Datta P, et al. The polypeptide chain-releasing factor GSPT1/eRF3 is proteolytically processed into an IAP-binding protein. *J Biol Chem* 2003; 278(40):38699–706.
199. Vaux DL, Silke J. Mammalian mitochondrial IAP binding proteins. *Biochem Biophys Res Commun* 2003; 304:499–504.
200. Friesen C, Herr I, Krammer PH, Debatin K-M. Involvement of the CD95 (APO-1/Fas) receptor/ligand system in drug-induced apoptosis in leukemia cells. *Nat Med* 1996; 2:574–7.
201. Muller M, Strand S, Hug H, et al. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. *J Clin Invest* 1997; 99(3):403–13.
202. Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A, Green DR. DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in t lymphocytes via the activation of NF- κ B and AP-1. *Mol Cell* 1998; 1(4):543–51.
203. Eichhorst ST, Muller M, Li-Weber M, Schulze-Bergkamen H, Angel P, Krammer PH. A novel AP-1 element in the CD95 ligand promoter is required for induction of apoptosis in hepatocellular carcinoma cells upon treatment with anticancer drugs. *Mol Cell Biol* 2000; 20(20):7826–37.
204. Müller M, Strand S, Hug H, et al. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1-Fas) receptor/ligand system and involves activation of wild-type p53. *J Clin Invest* 1997; 99:403–13.
205. Villunger A, Egle A, Kos M, et al. Drug-induced apoptosis is associated with enhanced Fas (Apo-1/CD95) ligand expression but occurs independently of Fas (Apo-1/CD95) signaling in human T-acute lymphatic leukemia cells. *Cancer Res* 1997; 57(16):3331–4.
206. Eischen CM, Kottke TJ, Martins LM, et al. Comparison of apoptosis in wild-type and Fas-resistant cells: chemotherapy-induced apoptosis is not dependent on Fas/Fas ligand interactions. *Blood* 1997; 90(3):935–43.
207. Tolomeo M, Dusonchet L, Meli M, et al. The CD95/CD95 ligand system is not the major effector in anticancer drug-mediated apoptosis. *Cell Death Differ* 1998; 5:735–42.

208. Landowski TH, Shain KH, Oshiro MM, Buyuksal I, Painter JS, Dalton WS. Myeloma cells selected for resistance to CD95-mediated apoptosis are not cross-resistant to cytotoxic drugs: evidence for independent mechanisms of caspase activation. *Blood* 1999; 4(1):265–74.
209. Glaser T, Wagenknecht B, Groscurth P, Krammer PH, Weller M. Death ligand/receptor-independent caspase activation mediates drug-induced cytotoxic cell death in malignant glioma cells. *Oncogene* 1999; 18:5044–53.
210. Newton K, Strasser A. Ionizing radiation and chemotherapeutic drugs induce apoptosis in lymphocytes in the absence of Fas or FADD/MORT1 signaling. implications for cancer therapy. *J Exp Med* 2000; 191(1):195–200.
211. Richardson DS, Allen PD, Kelsey SM, Newland AC. Inhibition of FAS/FAS-ligand does not block chemotherapy-induced apoptosis in drug sensitive and resistant cells. *Adv Exp Med Biol* 1999; 457:259–66.
212. Zhou Q, Salvesen GS. Viral caspase inhibitors CrmA and p35. *Methods Enzymol* 2000; 322:143–54.
213. Fulda S, Meyer E, Friesen C, Susin SA, Kroemer G, Debatin K-M. Cell type specific involvement of death receptor and mitochondrial pathways in drug-induced apoptosis. *Oncogene* 2001; 20:1063–75.
214. Houghton JA, Harwood FG, Gibson AA, Tillman DM. The Fas signaling pathway is functional in colon carcinoma cells and induces apoptosis. *Clin Cancer Res* 1997; 3:2205–9.
215. Ito Y, Pandey P, Place A, et al. The novel triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid induces apoptosis of human myeloid leukemia cells by a caspase-8-dependent mechanism. *Cell Growth Differ* 2000; 11(5):261–7.
216. Tani E, Kitagawa H, Ikemoto H, Matsumoto T. Proteasome inhibitors induce Fas-mediated apoptosis by c-Myc accumulation and subsequent induction of FasL message in human glioma cells. *FEBS Lett* 2001; 504:53–8.
217. Altucci L, Rossin A, Raffelsberger W, Reitmair A, Chomienne C, Gronemeyer H. Retinoic acid-induced apoptosis in leukemia cells is mediated by paracrine action of tumor-selective death ligand TRAIL. *Nat Med* 2001; 7(6):680–6.
218. Kalli KR, Devine KE, Cabot MC, et al. Heterogeneous role of caspase-8 in fenretinide-induced apoptosis in epithelial ovarian carcinoma cell lines. *Mol Pharmacol* 2003; 64(6):1434–43.
219. Aron JL, Parthun MR, Marcucci G, et al. Depsipeptide (FR901228) induces histone acetylation and inhibition of histone deacetylase in chronic lymphocytic leukemia cells concurrent with activation of caspase 8-mediated apoptosis and down-regulation of c-FLIP protein. *Blood* 2003; 102(2):652–8.
220. Nebbioso A, Clarke N, Voltz E, et al. Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells. *Nat Med* 2005; 11(1):77–84.
221. Petak I, Tillman DM, Harwood FG, Mihalik R, Houghton JA. Fas-dependent and -independent mechanisms of cell death following DNA damage in human colon carcinoma cells. *Cancer Res* 2000; 60:2643–50.
222. Petak I, Tillman DM, Houghton JA. p53 dependence of Fas induction and acute apoptosis in response to 5-fluorouracil-leucovorin in human colon carcinoma cell lines. *Clin Cancer Res* 2000; 6:4432–41.
223. Amarante-Mendes GP, Kim CN, Liu L, et al. Bcr-Abl exerts its antiapoptotic effect against diverse apoptotic stimuli through blockage of mitochondrial release of cytochrome c and activation of caspase-3. *Blood* 1998; 91(5):1700–5.
224. Kottke TJ, Blajeski AL, Martins LM, et al. Comparison of paclitaxel-, 5-Fluoro-2'-deoxyuridine- and epidermal growth factor-induced apoptosis: evidence for EGF-induced anoikis. *J Biol Chem* 1999; 274:15927–36.
225. Jia L, Patwari Y, Srinivasula SM, et al. Bax translocation is crucial for the sensitivity of leukaemic cells to etoposide-induced apoptosis. *Oncogene* 2001; 0:4817–26.
226. Zhang L, Yu J, Park BH, Kinzler KW, Vogelstein B. Role of BAX in the apoptotic response to anticancer agents. *Science* 2000; 290:989–92.
227. Perkins CL, Fang G, Kim CN, Bhalla KN. The role of Apaf-1, Caspase-9, and BID proteins in etoposide- or paclitaxel-induced mitochondrial events during apoptosis. *Cancer Res* 2000; 60(6):1645–53.
228. Svingen PA, Tefferi A, Kottke TJ, et al. Effects of the Bcr/abl kinase Inhibitors AG957 and NSC 680410 on chronic myelogenous leukemia cells *in vitro*. *Clin Cancer Res* 2000; 6:237–49.
229. Yang E, Korsmeyer SJ. Molecular thanatopsis: a discourse on the Bcl2 family and cell death. *Blood* 1996; 88(2):386–401.
230. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; 100(1):57–70.
231. Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature* 2001; 411:342–8.
232. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell* 2004; 116(2):205–19.
233. Lowe SW, Cepro E, Evan G. Intrinsic tumour suppression. *Nature* 2004;432:307–15.

234. Gerl R, Vaux DL. Apoptosis in the development and treatment of cancer. *Carcinogenesis* 2005; 26:263–70.
235. Strasser A, Harris AW, Bath ML, Cory S. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. *Nature* 1990; 348(6299):331–3.
236. Pelengaris S, Khan M, Evan GI. Suppression of Myc-induced apoptosis in beta cells exposes multiple oncogenic properties of Myc and triggers carcinogenic progression. *Cell* 2002; 109:321–34.
237. Askew DS, Ashmun RA, Simmons BC, Cleveland JL. Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest, accelerates apoptosis. *Oncogene* 1991; 6:1915–22.
238. Evan GI, Wyllie AH, Gilbert CS, et al. Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 1992; 69(1): 119–28.
239. Zindy F, Eischen CM, Randle DH, et al. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev* 1998; 12:2424–33.
240. Egle A, Harris AW, Bouillet P, Cory S. Bim is a suppressor of Myc-induced mouse B cell leukemia. *Proc Natl Acad Sci USA* 2004; 101:6164–9.
241. Eischen CM, Woo D, Roussel MF, Cleveland JL. Apoptosis triggered by myc-induced suppression of Bcl-X(L) or Bcl-2 is bypassed during lymphomagenesis. *Mol Cell Biol* 2001; 21:5063–70.
242. Soengas MS, Alarcon RM, Yoshida H, et al. Apaf-1 and Caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science* 1999; 284(5411):156–9.
243. Schmitt CA, Fridman JS, Yang M, Baranov E, Hoffman RM, Lowe SW. Dissecting p53 tumor suppressor functions *in vivo*. *Cancer Cell* 2002; 1(3):289–98.
244. Green DR, Evan GI. A matter of life and death. *Cancer Cell* 2002; 1:19–30.
245. Kaufmann SH, Vaux DL. Alterations in the apoptotic machinery and their potential role in anticancer drug resistance. *Oncogene* 2003; 22:7414–30.
246. Schmitt CA, Ct, Lowe SW. Genetic analysis of chemoresistance in primary murine lymphomas. *Nat Med* 2000; 6:1029–35.
247. Schmitt CA, Lowe SW. Bcl-2 mediates chemoresistance in matched pairs of primary E μ -myc lymphomas *in vivo*. *Blood Cells Mol Dis* 2001; 27:206–16.
248. Meijerink JPP, Mensink EJBM, Wang K, et al. Hematopoietic malignancies demonstrate loss-of-function mutations of BAX. *Blood* 1998; 91:2991–7.
249. Rampino N, Yamamoto H, Ionov Y, et al. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* 1997; 275(5302):967–9.
250. Degenhardt K, Chen G, Linsten T, White E. BAX and BAK mediate p53-independent suppression of tumorigenesis. *Cancer Cell* 2002; 2:193–203.
251. Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002; 2:489–501.
252. Samuels Y, Wang Z, Bardelli A, et al. High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004; 304(5670):554.
253. Shayesteh L, Lu Y, Kuo WL, et al. PIK3CA is implicated as an oncogene in ovarian cancer. *Nat Genet* 1999; 21(1):99–102.
254. Kaufmann SH, Gores GJ. Apoptosis in cancer: cause and cure. *Bioessays* 2000; 22:1007–17.
255. Kennedy SG, Kandel ES, Cross TK, Hay N. Akt/protein kinase B inhibits cell death by preventing the release of cytochrome C from mitochondria. *Mol Cell Biol* 1999; 19(8):5800–10.
256. Fridman JS, Lowe SW. Control of apoptosis by p53. *Oncogene* 2003; 22:9030–40.
257. Michalak EM, Villunger A, Erlacher M, Strasser A. Death squads enlisted by the tumour suppressor p53. *Biochem Biophys Res Commun* 2005; 331:786–98.
258. Vousden KH, Lu X. Live or let die: the cell's response to p53. *Nat Rev Cancer* 2002; 2(8):594–604.
259. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; 61:759–67.
260. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 Mutations in Human Cancers. *Science* 1991; 253(5015):49–53.
261. Di Cristofano A, Pandolfi PP. The multiple roles of PTEN in tumor suppression. *Cell* 2000; 100: 387–90.
262. Hannun YA. Apoptosis and the dilemma of cancer chemotherapy. *Blood* 1997; 89(6):1845–53.
263. Mow BF, Blajeski AL, Chandra J, Kaufmann SH. Apoptosis and the response to anticancer therapy. *Curr Opin Oncol* 2001; 13(6):453–62.
264. Belka C, Budach W. Anti-apoptotic Bcl-2 proteins: structure, function and relevance for radiation biology. *Int J Radiat Biol* 2002; 78:643–58.

265. Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 1993; 74:957–67.
266. Bunz F, Hwang PM, Torrance C, et al. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J Clin Invest* 1999; 104(3):263–9.
267. Merritt AJ, Allen TD, Potten CS, Hickman JA. Apoptosis in small intestinal epithelial from p53-null mice: evidence for a delayed, p53-independent G2/M-associated cell death after gamma-irradiation. *Oncogene* 1997; 14:2759–66.
268. Pritchard DM, Potten CS, Hickman JA. The relationships between p53-dependent apoptosis, inhibition of proliferation, and 5-fluorouracil-induced histopathology in murine intestinal epithelia. *Cancer Res* 1998; 58:5453–65.
269. Miyashita T, Reed JC. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood* 1993; 81(1):151–7.
270. Yin DX, Schimke RT. Bcl-2 Expression delays drug-induced apoptosis but does not increase clonogenic survival after drug treatment in HeLa cells. *Cancer Res* 1995; 55:4922–8.
271. Simonian PL, Grillot DA, Nunez G. Bcl-2 and Bcl-XL can differentially block chemotherapy-induced cell death. *Blood* 1997;90:1208–16.
272. Liu R, Page C, Beidler DR, Wicha MS, Nunez G. Overexpression of Bcl-xL promotes chemotherapy resistance of mammary tumors in a syngeneic mouse model. *Am J Pathol* 1999; 155:1861–7.
273. McCurrach ME, Conner TM, Knudson CM, Korsmeyer SJ, Lower SW. Bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. *Proc Natl Acad Sci USA* 1997; 94:2345–9.
274. Theodorakis P, Lomonosova E, Chinnadurai G. Critical requirement of BAX for manifestation of apoptosis induced by multiple stimuli in human epithelial cancer cells. *Cancer Res* 2002; 62:3373–6.
275. Yamaguchi H, Bhalla K, Wang HG. Bax plays a pivotal role in thapsigargin-induced apoptosis of human colon cancer HCT116 cells by controlling Smac/Diablo and Omi/HtrA2 release from mitochondria. *Cancer Res* 2003; 3(7):1483–9.
276. Matikainen T, Perez GI, Jurisicova A, et al. Aromatic hydrocarbon receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. *Nat Genet* 2001; 28(4):355–60.
277. Tan T-T, Degenhardt K, Nelson DA, et al. Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. *Cancer Cell* 2005; 7:227–38.
278. Yamaguchi H, Chen J, Bhalla K, Wang HG. Regulation of Bax activation and apoptotic response to microtubule-damaging agents by p53 transcription-dependent and -independent pathways. *J Biol Chem* 2004; 279(38):39431–7.
279. Hu Y, Cherton-Horvat G, Dragowska V, et al. Antisense oligonucleotides targeting XIAP induce apoptosis and enhance chemotherapeutic activity against human lung cancer cells *in vitro* and *in vivo*. *Clin Cancer Res* 2003; 9(7):2826–36.
280. Soengas MS, Capodici P, Polsky D, et al. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. *Nature* 2001; 409(6817):207–11.
281. Yoshida H, Kong Y-Y, Yoshida R, et al. Apaf1 Is required for mitochondrial pathways of apoptosis and brain development. *Cell* 1998; 94:739–50.
282. Svingen PA, Rodriguez J, Mesner PW Jr, et al. Components of the cell death machine and drug sensitivity of the NCI cell line panel. *Clin Cancer Res* 2004; 10:6807–20.
283. Peltenburg L, Meersma D, de Bruin E, Smit N, Schrier P, Medema JP. Expression and function of the apoptosis effector Apaf-1 in melanoma. *Cell Death Differ* 2005; 12:678–9.
284. Allen JD, Zhang XD, Scott CL, Boyle GM, Hersey P, Strasser A. Is Apaf-1 expression frequently abrogated in melanoma. *Cell Death Differ* 2005; 12:680–1.
285. Ekert PG, Read SH, Silke J, et al. Apaf-1 and caspase-9 accelerate apoptosis, but do not determine whether factor-deprived or drug-treated cells die. *J Cell Biol* 2004; 165:835–42.
286. Sane AT, Bertrand R. Caspase inhibition in camptothecin-treated U-937 cells is coupled with a shift from apoptosis to transient G1 arrest followed by necrotic cell death. *Cancer Res* 1999; 59:3565–9.
287. Willis SN, Adams JM. Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol* 2005; 17:617–25.
288. Youle RJ. Cell Biology. Cellular demolition and the rules of engagement. *Science* 2007; 315:776–7.
289. Riedl SJ, Salvesen, GF. The apoptosome: signalling platform of cell death. *Nature Rev Mol Cell Biol* 2007; 8:405–13.

2

The Extrinsic Pathway of Apoptosis

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SUMMARY

Defects in the extrinsic pathway are linked to several disease states, including cancer. Pharmacologic manipulation of the extrinsic pathway holds exciting promise for cancer treatment. This review will discuss the current understanding of the molecular signaling events that originate from extracellular sources to initiate apoptosis, how the pathway is activated by conventional chemotherapeutic agents, and novel opportunities to exploit the extrinsic pathway for cancer treatments.

Key Words: Apoptosis; TNF; Fas; TRAIL; NF- κ B; chemotherapy; death receptor; decoy receptor; extrinsic pathway; autoimmunity.

1. OVERVIEW OF SIGNALING EVENTS

1.1. Introduction

Apoptosis is essential to development and maintaining a healthy life for multicellular organisms. It is a rapid, catastrophic process that is precisely regulated in both its initiation and its execution. Although the phenomena had been described for almost a century, in 1972, Kerr, Wyllie, and Currie (1) first coined the term “apoptosis” to differentiate naturally occurring developmental cell death from the necrosis that results from acute tissue injury. They also noted that apoptosis was responsible for maintaining tissue homeostasis by mediating the equilibrium between cell proliferation and cell death in a particular tissue. Morphologic characteristics of apoptosis include cell membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation. Under normal circumstances, cells undergoing apoptosis are recognized by macrophages or neighboring cells that consume the cells’ fractionated carcasses. There are two distinct molecular signaling pathways that lead to apoptotic cell death: (i) the extrinsic, or extracellularly activated, pathway and (ii) the intrinsic, or mitochondria-mediated, pathway. Both pathways activate a cascade of proteolytic enzymes called caspases that mediate the rapid dismantling of cellular organelles and architecture. Caspases are a family of proteins containing a nucleophilic cysteine

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residue that participates in the cleavage of aspartic acid-containing motifs (2). There are two groups of caspases, the initiator (or apical) caspases and the effector (or executioner) caspases. Initiator caspases are capable of autocatalytic activation whereas effector caspases need activation by initiating caspase cleavage. Initiator caspases mediate the primary signaling events that result in extrinsic apoptosis activation.

Dr. P. Bruns, a German physician, first hinted at the existence of the extrinsic pathway leading to apoptosis in a paper he published in 1868 (3). He reported that acute bacterial infection caused tumor regression in a subset of his patients. More than a century later, tumor necrosis factor (TNF) and lymphotoxin were isolated and found to be effective at killing cultured tumor cells (4,5). After these factors were cloned (6,7), it became evident they were highly homologous. The advent of automated DNA sequence analysis made possible the identification of previously unknown messenger RNA (mRNA) transcripts and brought with it the discovery of an entire family of homologous TNF proteins (8,9).

The TNF family acts by binding the extracellular domains of receptor proteins: there are 19 ligands in the TNF family that can bind one or more of 29 receptors belonging to the TNF receptor (TNFR) family (8,9). Members of the TNF family are primarily produced as type II transmembrane proteins, arranged in stable homotrimers. The structures of the receptors are diverse, and recent observations from crystallization analyses indicate many similarities, but also many differences, among them (10). For the purpose of this review, we will focus only on the TNFR family members that can activate caspases and elicit death signals and will pay particular attention to signaling in the context of cancer therapy.

Each member of the TNF family binds to one or more receptors in the TNFR family, and some receptors bind one or more ligands. TNFRs contain one to four copies of a conserved cysteine-rich domain that follows a hydrophobic amino terminus and precedes the transmembrane domain. Ligand binding elicits a multitude of responses (including apoptosis, proliferation, and inflammation) and the given response depends upon the adapter proteins the bound receptor recruits. TNFRs signal through two classes of adapter proteins, TNFR-associated factors (TRAFs) and “death domain” (DD)-containing proteins. The subset of TNFRs that can activate apoptosis also possesses the DDs (11). The DD is a conserved stretch of approximately 80 amino acids found to be essential for transducing the apoptotic signal. The death receptors that appear to play important roles in mediating apoptosis are those that bind TNF (TNF- α , TNFSF2), FasL (also known as CD95L, TNFSF6), or TNFR apoptosis-inducing ligand (TRAIL; also known as Apo2L, TNFSF10). There are four other DD-containing TNFR members, but there is little evidence directly coupling these receptors with caspase activation. They are the receptor for nerve growth factor (NGFR), the ectodermal dysplasia receptor (EDAR), DR3, and DR6. This chapter will focus only on the receptors for TNF, FasL, and TRAIL because of their major role in human disease and significant potential for therapeutic interventions.

1.2. Molecular Signaling Events

The death-inducing TNFRs recruit initiating caspases and in doing so can activate a cascade of caspase cleavage that rapidly lead to cell death (Fig. 1). The generalized sequence of events is as follows: after binding ligand, the receptors undergo conformational changes that result in recruitment of an assembly of proteins, termed the

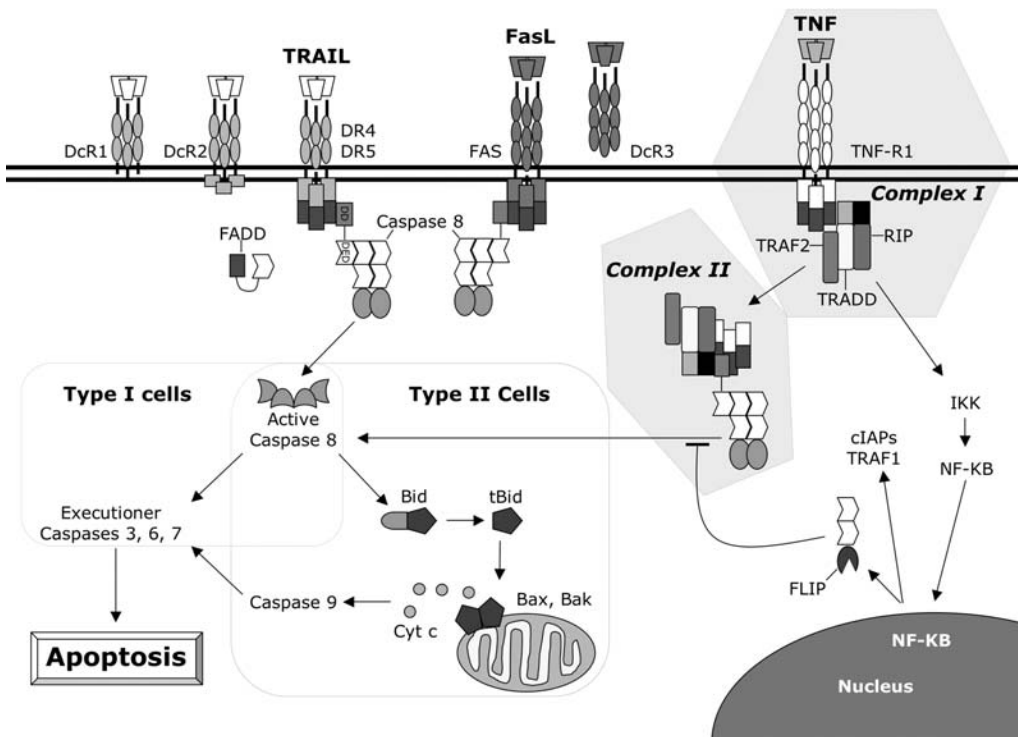


Fig. 1. The extrinsic pathway of apoptosis. Soluble tumor necrosis factor (TNF) family ligands TNF receptor (TNFR) apoptosis-inducing ligand (TRAIL), FasL, and TNF form trimers that recognize and bind their cognate death receptors. I) FasL and TRAIL: after binding ligand, DR4, DR5, and Fas undergo conformational changes resulting in assembly of the death-inducing signaling complex (DISC). Decoy receptor 1 (DcR1), DcR2, and DcR3 bind ligand with high affinity but do not induce apoptosis. DR4, DR5, and Fas then recruit Fas-associated death domain (FADD) through complementary death domains (DDs). FADD can recruit caspase 8 through their complementary death-effector domains (DEDs). Recruitment of caspases 8 to the DISC leads to its autoproteolytic cleavage, releasing two subunits that form active enzyme. In type I cells, caspase 8 cleaves and sufficiently activates effector caspases 3, 6, and 7 to fully engage the apoptotic response. In type II cells, activated caspase 8 cleaves Bid, which stimulates Bax and Bak to release factors from the mitochondria, including cytochrome c, thus activating the intrinsic pathway of apoptosis and augmenting active caspase 8. II) TNF: TNF binds TNF-R1 and recruits TNFR-associated DD (TRADD) through its DD and a complex of proteins containing receptor-interacting protein (RIP) and TNFR-associated factor 2 (TRAF2) (Complex I). Complex I can activate inhibitor of nuclear factor (NF)- κ B (I κ B)-kinase complex, thereby freeing NF- κ B for entry into the nucleus and rapid transcription of anti-apoptotic genes, including FLICE inhibitory protein (FLIP) and cIAP1/2. Complex I then dissociates from TNF-R1 where it binds FADD and caspase 8 (Complex II). If in sufficient abundance, FLIP can block Complex II's caspase 8 from self-activation. Otherwise, complex II triggers a caspase 8-driven apoptotic response.

death-inducing signaling complex (DISC). The DISC was first described in FasL–Fas apoptotic signaling (12). TRAIL binding to its death-inducing receptors acts in a manner similar to FasL, whereas TNF-mediated signaling is more complex and will be discussed in Section 1.3 further detail. The ligand-bound Fas or TRAIL death receptors recruit a DD-containing adapter protein, Fas-associated DD (FADD) (13).

FADD contains a second important death receptor-signaling motif, the death-effector domain (DED), and is the only protein in either the human or the mouse genome that contains both a DD and a DED. Bound FADD recruits initiator caspases 8 and 10 through complimentary DED domains (14,15). Recruitment of caspases 8 and 10 to the DISC leads to their autoproteolytic cleavage and release of two caspase subunits that form a mature active enzyme (16,17). If in sufficient abundance, activated caspase 8/10 cleaves and activates effector caspases 3 and 7, thereby fully engaging the caspase cascade. In some cells, named type I cells, activation of these effector caspases by activated caspase 8/10 alone is sufficient to induce apoptosis (18). In type II cells, activated caspase 8/10 stimulates the release of factors from the mitochondria, including cytochrome c, Smac/DIABLO, and Omi/Htr2A, thereby engaging the intrinsic pathway of apoptosis (see Chap. 1 for detailed review).

Why cells behave in a type I or a type II manner is not well understood. Gene expression analysis comparing type I and type II cells using Fas activation has been performed (19). The expression analysis of type I cell lines showed a preponderance of mesenchymal-like genes, whereas the type II cell lines preferentially express epithelium-like markers. A chemical screen for growth inhibition of these cells revealed that actin-binding compounds selectively inhibited growth of type I cells and tubulin-interacting compounds inhibited growth of type II cells. The functional significance of this observation may become useful in chemotherapeutic treatment selection for cancers with these types of gene expression profiles.

Caspase 8/10 connects the intrinsic and extrinsic pathways by cleaving Bid, a BH3-only member of the Bcl-2 family, which can mediate destabilization of the outer mitochondrial membrane by interacting with other Bcl-2 family members (20,21). To date, Bid is the only known physiologic mediator that connects the extrinsic pathway with release of apoptotic factors from mitochondria. However, a recent report showed the tumor suppressor protein RASSF1A associated with activated death receptors to contribute to Bax activation (22). The mechanism of action proposed is that RASSF1A binds to modulator of apoptosis (MAP)-1, a BH3-like protein, which can associate with Bax resulting in Bax translocation to the mitochondria. RASSF1A or MAP-1 siRNA-mediated knockdown diminished TRAIL-induced apoptosis, but this effect was shown only in the presence of cyclohexamide. RASSF1A could immunoprecipitate with TNF-R1 after a relatively long TNF treatment (2–3 h) while in the presence of cyclohexamide. Therefore, the significance of RASSF1A/MAP-1 modulation of Bax translocation in the context of a delayed death ligand response requiring protein synthesis inhibition is not clear. Nevertheless, it is interesting that a signaling pathway not involving Bid and connecting death receptors to the mitochondria has been discovered.

In humans, the *CASPASE 8* gene is found on chromosome 2q33–q34 in tandem with two other highly homologous proteins, *CASPASE 10* and *FLICE* inhibitory protein (FLIP). Caspase 10 contains both a caspase domain and a DED. Caspase 10 is also recruited to the DISC, and whether it can functionally substitute for caspase 8 is controversial (23–25). Studies of the role of FLIP recruitment to the DISC have revealed both activating and inhibitory functions depending on expression level. Although many FLIP isoforms are expressed in cells, only two are present at the protein level, a 55-kDa variant (FLIP_L) and a 26-kDa form (FLIP_S) (26). FLIP_S contains DEDs but lacks the caspase domain and acts as a direct inhibitor of caspase 8 cleavage. FLIP_L contains tandem DEDs but lacks critical residues in its caspase domain including

the catalytic cysteine, suggesting it to be a classical dominant-negative inhibitor. However, there are reports of FLIP_L acting as an inducer of caspase 8 autoproteolytic activation (27,28). These results were recently challenged by a study using siRNA to selectively knockdown each FLIP isoform (29). Separate knockdown of either FLIP_L or FLIP_S enhanced DISC formation and caspase 8 activation, suggesting the endogenous role of FLIP as primarily inhibitory.

1.3. TNF Pathway

TNF is a pro-inflammatory cytokine produced by a wide range of immune cells, including monocytes, macrophages, T cells, B cells, and natural killer (NK) cells (8,9). Large amounts of soluble TNF are released in response to lipopolysaccharide (LPS) and other bacterial products. High concentrations of TNF induce septic shock, and prolonged exposure to low concentrations of TNF can result in cachexia, a wasting syndrome. TNF is involved in the progression of many human diseases, including autoimmune diseases (Crohn's disease, rheumatoid arthritis), neurodegeneration, and cancer (30).

There are two receptors for TNF, TNF-R1 and TNF-R2 (31,32). TNF-R1 is expressed ubiquitously and has a DD, whereas TNF-R2 has no DD and is found mainly in cells of the immune system and endothelium. TNF-R1 principally regulates the immune system by activating pro-survival signaling. TNF-R1 elicits an anti-apoptotic action by activating nuclear factor (NF)- κ B, AP-1, and other transcription factor pathways. This explains why TNF-induced apoptosis using *in vitro* systems often requires the inhibition of RNA or protein synthesis. TNF-binding TNF-R1 does not bind FADD directly to activate caspase 8/10 cleavage, in contrast to Fas and the TRAIL death receptors. Instead, TNF-R1 binds to the DD-containing adapter protein TNFR-associated DD (TRADD) (33). The DD of TRADD binds other DD-containing proteins, including FADD and receptor-interacting protein (RIP). TRADD can also recruit one of two TRAF proteins (TNFR-associated factor), TRAF2 and TRAF5 (34,35). RIP is essential for TNF-induced NF- κ B activation (36). In unstimulated cells, NF- κ B is held in the cytoplasm by the inhibitor of NF- κ B, I κ B. TNF activates NF- κ B by initiating ubiquitin-mediated degradation of I κ B. Phosphorylation of I κ B dissociates it from NF- κ B, releasing it for entry into the nucleus and initiating transcription of a large number of mostly anti-apoptotic, pro-survival genes. These include cellular-inhibitors of apoptosis (c-IAP1 and c-IAP2), FLIP, Bfl-1/A1, A20, Mn super oxide dismutase (MnSOD), and others (for a review see 37). I κ B is phosphorylated by the I κ B kinase (IKK) complex. IKK activity can be purified as a complex containing two kinase subunits, IKK α (IKK1) and IKK β (IKK2), and a regulatory subunit, NF- κ B essential modifier (NEMO; IKK γ). IKK β is necessary and sufficient for phosphorylation of I κ B α and I κ B β . Studies with TRAF2- and RIP-deficient murine embryo fibroblasts (MEFs) showed that both molecules are independently recruited to TNF-R1 (38). It also appears that TRAF2 is sufficient to recruit the IKK complex to TNF-R1, but RIP is necessary for the activation of the IKKs (38). After phosphorylation by IKKs, I κ B proteins are ubiquitinated by members of the Skp1, Cullin, and F box proteins (SCF) family of ubiquitin ligases. The liberated NF- κ B dimers translocate to the nucleus where they bind DNA. Activated NF- κ B is then down-regulated by multiple pathways, including a negative feedback loop where newly synthesized I κ B α binds to nuclear NF- κ B and exports it to the cytosol. TNF can also activate other transcription

factors through c-Jun NH₂-terminal kinase (JNK) and p38/mitogen-activated protein kinase (MAPK). TRAF2 stimulates JNK through the MAPK kinase MKK7, promoting phosphorylation of c-Jun thereby increasing AP-1 activity.

This pathway of NF- κ B activation is referred to as the classical, or canonical, pathway but is one of two major pathways that activate NF- κ B. The non-canonical, or alternative, pathway results in the specific activation of two of the five NF- κ B subunits, p52 and RelB. The other subunits are p50, RelA, and RelC, and these form heterodimers that are transcriptionally active. Unlike the classical pathway, the alternative pathway is based on IKK α homodimers that prefer the precursor of p52, p100 (39). IKK α binds RelB and sequesters it in the cytoplasm; activation of IKK α results in the degradation of the carboxy-terminus of RelB and nuclear translocation of p52/RelB dimers. The alternative pathway is activated mainly by cytokines involved in development and maintenance of secondary lymphoid organs (40). Another pathway of NF- κ B activation is independent of IKK, and receptor signaling, and is instead based on activation of casein kinase 2 (CK2). CK2 activation can induce I κ B α degradation through its phosphorylation (41). This pathway only has a minor role in physiologic NF- κ B activation.

TNF can also activate caspase-mediated apoptosis, but it appears that the NF- κ B pathway must be disabled for this to occur (42). *In vivo* TNF-induced apoptosis has a minor role in comparison with its overwhelming function in regulating inflammation. TNF-mediated caspase activation occurs when TRADD binds FADD through a DD interaction. Recent evidence shows that FADD only associates with the TNF-R1 complex after it has been internalized by endocytosis (43,44). FADD-bound TRADD recruits caspase-8 and self-activates if not inhibited by NF- κ B-induced anti-apoptotic proteins. The NF- κ B targets, c-FLIP, TRAF1, and c-IAP1, have all been found to co-immunoprecipitate with a TNF-R1/TRADD/FADD cytosolic complex. These anti-apoptotic factors could help to dampen an apoptotic response. Therefore, apoptotic signaling through TNF-R1 includes an NF- κ B-mediated rescue response that results in cell death if newly synthesized survival signals fail to be activated.

1.4. Fas Ligand

Fas plays a major role in the regulation of apoptosis of immune cells and has been implicated in immune system diseases and cancer (45,46). Fas–FasL interactions are important for regulating the immune system in several ways: Fas is involved in cytotoxic T-cell-mediated killing, destruction of inflammatory and immune cells in immune-privileged sites, and deletion of self-reacting B cells and activated T cells at the end of an immune response. Dysregulation of Fas or FasL expression is associated with several disease states. Elevated serum levels of FasL have been seen in patients with NK-cell large granular lymphocyte leukemia, systemic lupus erythematosus, rheumatoid arthritis, Sjogren's syndrome, lymphohistiocytosis, myocarditis, and acute graft-versus-host disease (45,46). Some tumors have been reported to express FasL, which may be a mechanism they developed to evade attacking lymphocytes (47).

Fas-mediated cell death was identified indirectly by the generation of monoclonal antibodies that recognized cell surface antigens on a malignant human lymphoblast cell line (48). Once cloned, Fas was found to map to the chromosomal location of a mouse lymphoproliferative disorder known as *lpr* (49). A point mutation near the extracellular carboxyl domain of FasL gives rise to the *gld* phenotype (50). Both *lpr* and *gld* mice fail

to delete excess lymphocytes and display a lymphoproliferative phenotype including lymphadenopathy and splenomegaly. One other receptor, decoy receptor 3 (DcR3), binds to FasL. DcR3 lacks an apparent transmembrane sequence and appears to be secreted. DcR3 was found genetically amplified in several human cancers including lung and colon carcinomas and is overexpressed in several adenocarcinomas, glioma cell lines, and glioblastomas (51–53). There is preliminary data suggesting that serum DcR3 level might be a useful predictive marker for cancer diagnosis (54).

1.5. TRAIL

TRAIL was identified *in silico* using TNF sequence homology searches of the human genome database of expressed sequence tags (55,56). TRAIL is unique among the TNF superfamily, and most other cytokines, because it can bind five different receptors (57,58). Two of these receptors contain DD and are pro-apoptotic. They are type 1 transmembrane receptors and referred to herein as DR4 (death receptor 4/TRAIL R1) and DR5 (death receptor 5/KILLER/TRICK2/TRAIL R2). The three other TRAIL receptors lack DDs, including one that binds TRAIL very weakly at physiologic temperatures (osteoprotegerin/OPG). The remaining two receptors are also called “decoys” because they bind TRAIL with high affinity but cannot transduce the death signal. Decoy receptor 1 (DcR1/TRID/TRAIL-R3) lacks an intracellular domain (it is attached to the plasma membrane by a glycoposphatidylinositol anchor), and decoy receptor 2 (DcR2/TRUNDD/TRAIL R4) has a truncated DD in its cytoplasmic tail.

If bound to either DR4 or DR5, TRAIL and FasL exert their actions in an analogous manner. Binding of TRAIL triggers DISC formation, caspase 8/10 activation, and rapid apoptosis in sensitive cells. Similarly to Fas, apoptosis triggered by TRAIL can engage the mitochondrial pathway in type II cells or independently of the mitochondria in type I cells (59). The apoptotic signaling pathway downstream of FasL and TRAIL acts through FADD and appears to be very similar. However, one significant difference between TRAIL and FasL is their potential as chemotherapeutic agents. When administered systemically in mice, FasL induces a rapid cytotoxic effect in hepatocytes whereas TRAIL appears relatively non-toxic (60,61). This observation, plus TRAIL’s dramatic ability to kill cancer cells while leaving normal cells unharmed, opened up an exciting new opportunity for development of a “silver bullet” for cancer therapy. TRAIL and DR4- and DR5-activating antibodies recently have entered into clinical trials.

Several mechanisms have been proposed to explain why some cancer cells are highly sensitive to TRAIL-induced death. An attractive hypothesis is differential expression of the decoy and death receptors. However, the decoy receptors do not consistently appear highly expressed in normal cells or to be absent in cancer cells (62,63). Mutant death receptors or defective receptor processing has been observed in TRAIL-resistant cancer cells (64,65). Additional intracellular factors leading to TRAIL resistance affect the caspase 8/c-FLIP ratio, such as loss of caspase 8 and caspase 10 because of mutations or gene methylation (66), caspase-associated ring protein (CARP)-dependent degradation of caspase 8 (67), or high c-FLIP expression levels (62,63). Up-regulation of FLIP was detected in many tumors (68–70), and expression of FLIP in transgenic mice results in escape from T-cell immune surveillance and subsequent tumor growth (71,72). Further downstream in the TRAIL apoptotic pathway, Bax mutations or increased expression of IAP family members, in particular XIAP and survivin, can also cause resistance.

Recently, the human oncogene c-Myc was identified as a bio-marker for TRAIL sensitivity (73). In this study, a panel of human tumor cell lines was examined, and a direct linear correlation was observed between TRAIL sensitivity and high c-Myc expression. Myc was found to bind the FLIP promoter and repress FLIP transcription (73). Furthermore, Myc was isolated from an siRNA screen to identify modulators of TRAIL sensitivity (74). A separate study showed Myc up-regulation of DR5 expression (75), but how Myc regulates DR5 is not clear. Other oncogenic proteins can sensitize cells to TRAIL, including E1A (76) and oncogenic Ras (77,78), but Ras-mediated TRAIL sensitization has not been observed consistently (75).

2. ROLE OF THE EXTRINSIC PATHWAY IN DISEASE

The TNF family plays important functions in innate and adaptive immunity and directly activates pathways leading to cell survival, proliferation, differentiation, and death. Dysregulation of the TNF family members that can elicit apoptosis results in diseases of the immune system, neurodegenerative disorders, and cancer. Two known genetic diseases that are associated with defects in the extrinsic pathway include Fas-linked autoimmune lymphoproliferative syndrome (ALPS) and TNF-R1-associated periodic syndrome (TRAPS) (8). Fas-mediated apoptosis is required for normal lymphocyte homeostasis and peripheral immune tolerance (79). In Fas-deficient *lpr* mice and in patients with heterozygous dominant-interfering defective Fas alleles (encoding defective Fas proteins that complex with normal Fas), abnormal accumulation of lymphocytes often results in systemic autoimmunity (79,80). Afflicted individuals develop pathogenic autoantibodies—frequently against hematopoietic cells—that cause hemolytic anemia, thrombocytopenia, or neutropenia (81). Mouse knockout studies show the TRAIL pathway may also be involved in autoimmune disease (82). In TRAPS, heterozygous dominant alleles of defective TNF-R1 appear to enhance the pro-inflammatory effects of TNF. This may be due in part to a decrease in TNF-R1 shedding (83).

TNF and TNFR families are being targeted for therapies against a wide range of human diseases such as atherosclerosis, osteoporosis, autoimmune disorders, allograft rejection, and cancer. For example, pharmaceuticals to inhibit TNF have been developed to control previously recalcitrant inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease (84,85). Specific TNF antagonists include the TNF antibody infliximab (Remicade) and the TNFR-immunoglobulin G (IgG) fusion protein etanercept (Enbrel) (86).

What are the physiological functions of apoptosis-inducing TNF family members? The use of inhibitory antibodies and mouse knockout models has provided great insight into this question (Table 1).

2.1. Lessons from Knockout Animals

2.1.1. TNF AS A TUMOR PROMOTER

Mouse TNF and TNF-R1 knockout studies show that TNF plays an essential role in protecting against infection by pathogenic organisms. There is also growing evidence that TNF signaling is involved in fostering tumor growth. Expression studies show abnormally high concentrations of TNF in tumors. Studies of various hematopoietic and solid tumor types found an association between TNF expression, poor survival, and

Table 1
Major extrinsic pathway proteins and their official names according to the HUGO Gene Nomenclature Committee (HGNC) are shown. Knock-out mice generated for these proteins are described.

<i>Molecular pathway molecules</i>	<i>Official nomenclature (HGNC)</i>	<i>Human chromosome</i>	<i>Mouse knockout phenotype</i>
Death ligand TNF (TNF- α , Cachectin)	TNFSF2	6p21.3	Viable, highly susceptible to challenge with an infectious agent and resistant to lipopolysaccharide (LPS)-induced death following D-galactosamine treatment (188)
FasL (CD95)	TNFSF6	1q23	Viable but early death (50% at 4 months); FasL ^(-/-) mice exhibit splenomegaly and lymphadenopathy associated with lymphocytic infiltration into multiple organs and autoimmune disease (189); <i>Gld</i> mice carry mutations in FasL and suffer from autoimmune disease (50)
TRAIL (Apo2L)	TNFSF10	3q26	Viable; susceptible to induced and spontaneous tumorigenesis (87)
Death receptor TNF-R1	TNFRSF1A	12p13.2	Viable, resistant to LPS-induced death following D-galactosamine (190)
Fas (CD95, Apo1)	TNFRSF6	10q24.1	No Fas ^(-/-) mouse published; mice carrying the lymphoproliferation (<i>lpr</i>) mutation have defects in the Fas antigen gene. The <i>lpr</i> mice develop lymphadenopathy and suffer from a systemic lupus erythematosus-like autoimmune disease, indicating an important role for Fas antigen in the negative selection of autoreactive T cells in the thymus (191)
DR4 (TRAILR1, APO2) DR5 (TRAILR2, KILLER, TRICK2)	TNFRSF10A TNFRSF10B	8p21 8p22–p21	Viable, but has an enlarged thymus, defective apoptotic response to ionizing radiation (88)
Adapter proteins FADD (MORT)	FADD	11q13.3	Embryonic lethal (d11.5); mice show signs of cardiac failure and abdominal hemorrhage (89)

(Continued)

Table 1
(Continued)

<i>Molecular pathway molecules</i>	<i>Official nomenclature (HGNC)</i>	<i>Human chromosome</i>	<i>Mouse knockout phenotype</i>
TRADD	TRADD	16q22	
Caspase 8 (FLICE, MACH, MCH5)	CASP8	2q33–q34	Embryonic lethal (d13.5); embryos exhibit impaired heart muscle development and accumulation of erythrocytes (90)
Caspase 10 (MCH4)	CASP10	2q33–q34	
FLIP (I-FLICE, CASPER, FLAME1, CASH, CLARP, MRIT)	CFLAR	2q33–q34	Embryonic lethal (d10.5); exhibit impaired heart development (192)
RIP (RIP1)	RIPK1	6p25.2	Viable, but fails to thrive; displays extensive apoptosis in both the lymphoid and adipose tissues and dies at 1–3 days of age (193)
TRAF2 (TRAP)	TRAF2	9q34	Traf2 ^{-/-} mice appear normal at birth but die prematurely; atrophy of the thymus and spleen and depletion of B-cell precursors are observed (35)
TRAF5	TRAF5	1q32	CD27- and CD40-mediated lymphocyte activation is substantially impaired in traf5 ^{-/-} lymphocytes (194)

resistance to therapy (91–94). TNFR1^{-/-} mice show reduced levels of metastatic lung disease following intravenous tumor cell injection (95) and reduced liver metastases following intrasplenic tumor cell injection (96). TNF itself has been proposed as a tumor promoter. Using a standard mouse model of two-step chemical carcinogenesis, it was found that TNF^{-/-} mice developed 10 times fewer skin tumors than wild-type mice (97). Similar results indicating a role for TNF in tumor promotion were seen by using a model of hepatic carcinogenesis in TNF-R1^{-/-} mice and was unaffected in TNF-R2^{-/-} mice (98).

Recent work by Karin and colleagues showed that TNF mediates an inflammatory response by LPS, causing metastatic growth to the lung of intravenously injected colon adenocarcinoma cells (99). Inhibiting NF-κB signaling in the colon cells prevented their metastasis and resulted in their apoptosis. TRAIL expression by mouse lung tissue following LPS administration mediated the death of NF-κB-deficient cancer cells. DR5 was also found up-regulated in tumors following LPS treatment but only in cells that were NF-κB deficient. These results shed light on the interplay between TNF and

TRAIL signaling and show that if NF- κ B is blocked, TNF-mediated growth can be converted into TRAIL-mediated death.

2.1.2. FASL AND NEGATIVE SELECTION

Higher organisms have developed several mechanisms to eliminate unwanted cells rapidly, and Fas is an important mediator of this process. FasL expression at sites of immune privilege identified an important role for FasL in the interaction between non-lymphoid tissues and the immune system. The concept of “tumor counterattack” has been proposed to explain the observation that Fas is down-regulated and FasL up-regulated in the same tumor cells (100). According to this concept, “immune cells are unable to destroy tumor cells since they are attacked by the FasL expressing cells.” In light of this observation, significant evidence has accumulated that indicates Fas may play a major role in immune suppression of tumorigenesis. FasL expression has been reported on numerous tumors of varying origin, including colon, gastric, lung, and astrocytoma (47). Disease progression is associated with increasing levels of FasL (101, 102), and FasL expression has also been found higher in some metastatic tumors than in primary ones (103,104). Animal studies have corroborated the FasL counterattack theory. For example, subcutaneous injection of FasL-expressing murine melanoma cells into Fas-deficient *lpr* mutant mice resulted in delayed tumor growth compared with that in wild-type mice (105). However, conflicting results cast doubt on the Fas counterattack theory, instead suggesting that FasL can also play a growth stimulatory role (47,106). These observations, plus those suggesting that FasL may have stimulatory effects, including induced motility of tumor cells (107) and growth signaling (108) suggest that the initial theory of tumor counterattack may be oversimplified and that FasL does more than trigger apoptosis.

2.1.3. TRAIL AND TUMOR SURVEILLANCE

Recent evidence from mouse knockout studies, plus TRAIL’s ability to activate apoptosis in various cancer cells, led to the hypothesis that a principal function of TRAIL is to kill transformed cells. Unlike FasL, TRAIL is not expressed at detectable levels on the surfaces of T cells, NKT cells, B cells, dendritic cells, monocytes, or most NK cells (109). The one exception is mouse liver NK cells, which constitutively express TRAIL in an interferon (IFN)-dependent manner (110,111). Cell surface TRAIL expression increases through several pathways. Antigen-dependent activation of CD4⁺ and CD8⁺ T cells from peripheral blood induces expression of TRAIL mRNA, as does stimulation of macrophages with IFN- γ . The expression of TRAIL on liver NK cells and their anti-metastatic potential depend on the presence of IFN- γ and interleukin (IL)-12, as these effects were not observed in mice deficient in IFN- γ (87,111). Tumor cells from cancer patients activate macrophages to produce TRAIL, and these macrophages can release molecules that induce increased expression of DR4 and DR5 on tumor cells (112).

Studies with TRAIL knockout mice confirm a role for TRAIL in anti-tumor immune surveillance by NK cells, specifically in host defense against tumor initiation and metastasis (87,111). TRAIL^{-/-} mice were more susceptible to experimental and spontaneous tumor metastasis and were also more likely to form tumors following exposure to the chemical carcinogen methylcholanthrene (87). There are two knockout mice for the receptor for TRAIL. Mice have only one receptor for TRAIL, mouse KILLER,

and it shares its highest homology with human DR5 (113). Two knockout models for the TRAIL receptor have been generated and both are viable (88,114). Both develop normally, but one mouse strain has an enlarged thymus (88). This study showed that DR5 has a limited role during embryogenesis and early stages of development, but plays an organ-specific role in response to DNA-damage. When exposed to ionizing radiation, certain DR5^{-/-} tissues show reduced apoptosis, including the thymus and spleen. Mice wild-type for DR5 show a selective increase in DR5 expression following IR in the thymus and spleen (115), suggesting a significant connection between IR-induced DR5 and resulting apoptosis in these tissues.

Genetic defects in TRAIL signaling have not been strongly correlated with disease states in humans. However, deletions and mutations of DR4 and DR5 have been observed in some human tumors (63,65,116–118).

An intriguing study has implicated TRAIL in mammary tissue lumen formation and suggests that dysregulation of TRAIL signaling may be a hallmark of early breast cancer lesions (119). Using an *in vitro* cell culture model of 3D acinar-like structures using immortalized mammary epithelial cells, TRAIL was found to partially mediate both the apoptotic and the autophagic cell death associated with lumen formation. Autophagy is a cellular process where a multi-membrane vacuole containing cytoplasmic contents fuses with the lysosome. This results in degradation of the vacuole contents by lysosomal enzymes into recyclable macromolecules (120). Recent evidence indicates that autophagy can mediate a form of programmed cell death, where there is an accumulation of vacuoles resulting in massive organelle degradation. How TRAIL is activated during mammary acinar morphogenesis is not known, but this is the first report connecting autophagy with TRAIL function (119). Little is known about how and to what degree the extrinsic pathway signaling affects autophagy, but there are reports that TNF signaling can induce autophagy and possibly contribute to TNF-induced apoptosis (121,122).

3. ROLE OF EXTRINSIC PATHWAY IN CHEMOTHERAPY AND RADIOSENSITIVITY

Chemotherapy and radiation, when used successfully, act to inhibit tumor growth. Ionizing radiation and DNA-damaging chemotherapeutics can elicit an apoptotic response that is principally mediated through activation of the p53 tumor suppressor protein. p53 is the most commonly mutated protein found in human cancers and is a potent transcriptional activator of genes that play principal roles in cell-cycle arrest and apoptosis (123). Recent evidence suggests that p53 also influences apoptosis by directly interacting with members of the Bcl-2 family (124). Members of the Bcl-2 family that p53 can activate transcriptionally include Bax, Puma, Noxa, Bnip3L, Bak, and Bid. p53 also directly contributes to activation of the extrinsic pathway. Death receptors for both TRAIL and FasL have been identified as p53 target genes (113,125).

KILLER/DR5 was originally discovered as a DNA-damage-inducible p53 target gene (113) and is transcriptionally activated by p53 (126). Certain tissues, including the spleen, small intestine, and thymus, show large increases in DR5 expression following ionizing radiation that is dependent on transcriptionally active p53 (88,115,127). DR4 may also be regulated by p53 in a limited number of cell lines (128). Several studies have found a p53-dependent increase in Fas or FasL, which contributes to

mediating the apoptotic response after conventional chemotherapy (125,129–131), but Fas is not essential for mediating p53's effects. Lymphocytes from *lpr* mice, or those expressing DN-FADD, are equally sensitive to chemotherapy and ionizing radiation; p53 deficiency or constitutive expression of Bcl-2 markedly increased the resistance of lymphocytes to gamma radiation or anticancer drugs, but lymphocytes were still sensitive to killing by FasL (132). Furthermore, apoptosis induced by chemotherapeutic drugs is not altered in embryonic fibroblasts from FADD and caspase 8 knockout mice (89,90), indicating only a partial role for the death receptor pathway in response to chemotherapeutic agents. Nevertheless, partial resistance of DR5-null tissues to ionizing radiation implicates the extrinsic pathway in DNA-damage-induced apoptosis.

4. EXPLOITING THE EXTRINSIC PATHWAY FOR CHEMOTHERAPY-INDUCED KILLING

Ever since the discovery of TNF, great attention has been focused on the TNF ligands as mediators of cancer cell death (133–135). Through the efforts of many scientists over the course of decades, clear pictures are emerging of the basic mechanisms of extrinsic pathway-signaling events. Understanding these events has led to exciting advances in using extrinsic pathway signaling for cancer therapy.

4.1. TRAIL

Despite the ability of TNF and FasL to induce apoptosis in cancer cells, severe toxic side effects preclude both ligands from use in systemic anticancer therapy. Systemic administration of TNF caused an inflammatory response resembling septic shock in humans (136). FasL or agonistic anti-Fas antibody caused lethal liver injuries in preclinical models (137). By contrast, recombinant human TRAIL showed no toxicity when systemically administered in rodents and non-human primates (60,61,138). Recombinant human TRAIL has apoptosis-inducing capacity in various tumor cells in culture and in tumor implants in severe combined immune deficiency (SCID) mice (139). Recombinant TRAIL (Genentech/Amgen) and activating DR4 and DR5 antibodies (Human Genome Sciences/Cambridge Antibody Technology) are currently under clinical investigation. Getting TRAIL into clinic trials was delayed by observations that certain preparations of recombinant human TRAIL had selective toxicity toward normal human hepatocytes (140). Because TRAIL was toxic to cultured human hepatocytes, and not to mouse or non-human primates, it resulted in the careful analysis of different TRAIL preparations. It became apparent that TRAIL protein fused with non-physiological amino acid tags or with preparations of native TRAIL using different stabilizing chemicals resulted in multimerized, highly potent versions of TRAIL (141–143). Therefore, the potential toxicity of these TRAIL versions toward normal cells can be avoided if native TRAIL is properly prepared, or if activating monoclonal antibodies specific to DR4 or DR5 are used (139,144,145). Another approach to minimizing off-target TRAIL toxicity is the combination of TRAIL with an inhibitor of caspase 9, which can protect normal cells but is ineffective in protection tumor cells possessing a type I signaling mechanism (143,146).

Like most normal cells, many cancer cells are resistant to TRAIL-induced apoptosis. However, many conventional and novel chemotherapies can act synergistically when combined with TRAIL. Chemotherapy or irradiation sensitized resistant cells to TRAIL

in vitro and *in vivo* (63,147–150). Many cytotoxic chemotherapeutic agents result in DNA damage or other cellular stress that causes stabilization of the p53 tumor suppressor protein. p53 transcriptionally activates DR5 and other pro-apoptotic proteins that enhance the TRAIL signal (113). Therefore, combining TRAIL with such agents should prove to be a useful therapeutic strategy in tumors harboring functional p53. However, tumor progression and resistance to chemotherapies occur because tumors select for cells defective in p53 signaling. An exciting facet of death receptor signaling is that it can occur in the absence of functional p53. Inhibitors of histone deacetylases (HDACs) can induce apoptosis in cancer cells and are currently in clinical trials. One action of HDACs is the increased expression of TRAIL, DR5, Fas, and FasL in leukemic cells, resulting in selective apoptosis of these cells (151,152). HDACs enhance synthesis of several proteins involved in TRAIL signaling including DR5 and when combined with TRAIL show the ability to sensitize TRAIL-resistant cells (153,154). Both glucocorticoids and IFN- γ also increase DR5 expression, which may enhance TRAIL activity (155). These are some of the many strategies being approached to combine TRAIL with novel agents that target proteins in both the extrinsic and intrinsic pathways, thereby increasing their sensitivity to the killing potential of TRAIL. Promising compounds identified that have been combined with TRAIL are discussed below.

4.2. TNF

Recombinant TNF was approved for isolated limb perfusion therapy against sarcomas in Europe in 1998 (156). TNF combined with chemotherapeutic agents such as melphalan shows specificity toward destruction of tumor vasculature and is very effective when used for localized treatment of sarcomas and melanomas (157,158). TNF plus melphalan is awaiting approval following phase III clinical trials for use in the US.

4.3. NF- κ B

Substantial evidence indicates that NF- κ B regulates oncogenesis and tumor progression. Many anticancer agents induce NF- κ B nuclear translocation and activation of their target genes, which impinge on cellular resistance to anticancer agents. TNF is up-regulated by some chemotherapeutic agents, thus activating NF- κ B. FasL and TRAIL also can activate NF- κ B signaling (107,159). Several strategies have been investigated to block pro-survival death receptor signals so that extrinsic apoptotic signals can dominate.

NF- κ B inhibitors have been identified that enhance the cytotoxic effects of many conventional chemotherapies and novel anticancer agents (160). Inhibiting the proteasome is an approach taken to block NF- κ B activation through degradation of I κ B proteins. The proteasome inhibitor Bortezomib (Velcade; PS-341) has recently been approved for treating multiple myeloma and is in clinical trials testing effectiveness against several other cancer types (161,162). Bortezomib also has been effectively combined with many conventional chemotherapeutic agents and radiation (for a recent review see 163). Proteasome inhibition results in stabilization of several critical regulators of apoptosis, including p53, Bid, and Bax; therefore, the effectiveness of Bortezomib may depend only partially on inhibiting NF- κ B.

Strategies have been developed to inhibit NF- κ B directly. Two compounds were identified, BAY 11-7082 and BAY 11-7085, that block I κ B α phosphorylation and prevent its degradation (164). Bay 11-7082 was used to enhance mitochondria dysfunction induced by UCN-01, a cell-cycle checkpoint-abrogating agent (165). A recent study showed that rituximab (Rituxin), the anti-CD20 antibody approved for treatment of non-Hodgkin's lymphoma, can inhibit IKK activity and block constitutive NF- κ B signaling (166). Non-steroidal anti-inflammatory drugs (NSAIDs), including Cox-2 inhibitors, have been identified as inducing regression of adenomatous polyps of the colon, and NF- κ B has been implicated in mediating NSAID action. Aspirin and sulindac have both been shown to inhibit IKK activity and may prove useful in targeting NF- κ B (167,168). Many other IKK inhibitors have been identified, but further studies are necessary to determine whether they will be clinically useful (160,169).

4.4. FLIP

Whether a tumor cell is sensitive to death ligand-induced apoptosis depends on both receptor cell surface expression and an intact downstream-signaling pathway. FLIP is an important regulator of the death signal, and a compound was recently discovered that reduces FLIP expression. The synthetic oleanane triterpenoid 2-cyano-3, 12-dioxooleana-1,9-dien-28-oic acid (CDDO) was reported to have potent differentiating, anti-proliferative, and anti-inflammatory properties and reduce tumor growth *in vivo* (170,171). CDDO was initially constructed to mimic naturally occurring inhibitors of nitric oxide production induced by IFN- γ . The mechanism of action of CDDO and its imidazol derivative (CDDO-Im) are not fully understood, but CDDO was found to induce apoptosis involving caspase 8 cleavage (172). Later, it was observed that CDDO activates a pathway resulting in FLIP degradation (173,174). CDDO or CDDO-Im can cause apoptosis and cell death in a number of different human cancers, but it has shown potent synergy when used in combination with TNF or TRAIL (175–177). *In vivo* studies using nude mice bearing human breast cancer MDA-MB-435 xenografts showed CDDO-induced tumor growth arrest by using daily treatments for 25 days (178). A later study did not recapitulate tumor growth arrest using CDDO-Im in nude mice bearing MDA-MB-468 breast cancer xenografts but restricted tumor growth when combined with TRAIL (179).

4.5. Recruiting the Intrinsic Pathway to Sensitize Cells to the Extrinsic Pathway

Because the extrinsic pathway is linked to intrinsic apoptotic signaling, combining extrinsic and intrinsic pathway activators should elicit a “double whammy.” For example, TRAIL has been combined with several agents identified or specifically designed to target intrinsic signaling.

The Bcl-2 family is the major mediator of outer mitochondrial membrane permeabilization resulting in the release of pro-apoptotic factors, such as cytochrome c, Smac/DIABLO, and Omi/Htr2A (180). Overexpression of anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-XL, A1, or Mcl-1, is frequently observed in many tumor types and contributes to chemotherapeutic resistance. Several strategies are under investigation to target these anti-apoptotic proteins. These include (i) interfering oligonucleotides to down-regulate expression; (ii) use of BH3-only peptides or controlled Bax expression

to abrogate protection; and (iii) small molecules that can inhibit protective interactions. The only agent of these categories that is currently in clinical trials are nuclease-resistant antisense oligonucleotides targeting Bcl-2 mRNA (G3139). G3139 (Genasense) is in phase II and III clinical trials treating a wide variety of adult and childhood tumors (181). However, G3139 was not approved for treatment of melanoma because results from phase III trials showed it did not extend survival (182). There are no reports investigating G3139 in combination with TRAIL pre-clinically, but it was shown to sensitize Fas- and IFN- γ -resistant renal cancer cell line to IFN- γ combined with an Fas-activating antibody (183). Adenoviruses that express Bax and TRAIL under control of the promoter for the human telomerase protein subunit (hTERT) were used to treat nude mice bearing ovarian tumor xenografts (184). Though preliminary, these strategies (or agents) diminished tumor growth while maintaining relatively low toxicity.

Anti-apoptotic Bcl-2 family members are held in check by Bcl-2 family proteins that contain only the BH3 member of the four Bcl-2 homology domains, the so-called BH3-only proteins. A novel approach was taken to generate stabilized BH3 peptides termed SAHBs (stabilized α -helix of Bcl-2 domains) (185). These peptides proved to be protease-resistant and cell-permeable molecules that bound with high affinity to multidomain BCL-2 member pockets. A SAHB of the BH3 domain from BID was effective in inhibiting growth of human leukemia xenografts *in vivo* in short-term assays. A small molecule BH3 mimetic, ABT-737, shows promise in the treatment of Bcl-2- or Bcl-XL-overexpressing tumors (186). ABT-737 was identified using a structure-based combinatorial chemical approach to target Bcl-XL and binds Bcl-XL, Bcl-2, and Bcl-w with high affinity (186). ABT-737 synergized with paclitaxel and the activated BH3-only protein Myr-Bid to cause apoptotic cell death. Because overexpression of Bcl-2 and Bcl-XL is the key to many cancers' resistance to apoptotic stimuli, Bid SAHBs or ABT-737 will very likely synergize with other chemotherapeutic agents, including TRAIL and other extrinsic pathway activators. Whether Mcl-1 overexpression will mediate resistance of tumors remains to be determined in clinical trials.

Other classes of apoptotic targeting agents have also been combined with TRAIL resulting in significant tumor regression. A small molecule SMAC mimic potentiates TNF- and TRAIL-induced death (187). Treatment of glioblastoma cells with a combination of TRAIL and the SMAC mimic resulted in apoptosis of tumor cells, but normal cells were not harmed. Data from animal studies have not been published yet.

5. CONCLUDING REMARKS

Despite being investigated for decades, the TNF and the TNFR family of proteins continue to provide important insights into human health and disease. Here, we focused on the current understanding of their role in activating the extrinsic pathway of apoptosis, how this affects oncogenesis, and how this knowledge can be used for targeted chemotherapeutic design. Several promising cancer-killing agents that engage the extrinsic signaling pathway are in clinical trials, and several more appear promising in preclinical studies. The next stage of clinical research must include rational combination of chemotherapeutic agents that both activate apoptotic signaling pathways and block pro-survival mechanisms, while minimizing off-target toxicities. A major challenge to be overcome is determining whether a patient will respond to agents that activate the extrinsic or intrinsic pathways of apoptosis prior to their treatment.

REFERENCES

1. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; 26(4):239–57.
2. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science* 1998; 281(5381):1312–6.
3. Bruns P. Die Heilwirkung des Erysipels auf Geschwulste. *Beitr Klin Chir* 1868; 3:443–6.
4. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci USA* 1975; 72(9):3666–70.
5. Granger GA, Shacks SJ, Williams TW, Kolb WP. Lymphocyte *in vitro* cytotoxicity: specific release of lymphotoxin-like materials from tuberculin-sensitive lymphoid cells. *Nature* 1969; 221(5186):1155–7.
6. Pennica D, Nedwin GE, Hayflick JS, et al. Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature* 1984; 312(5996):724–9.
7. Gray PW, Aggarwal BB, Benton CV, et al. Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. *Nature* 1984; 312(5996):721–4.
8. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 2001; 104(4):487–501.
9. Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 2003; 3(9):745–56.
10. Zhang G. Tumor necrosis factor family ligand-receptor binding. *Curr Opin Struct Biol* 2004; 14(2):154–60.
11. Tartaglia LA, Ayres TM, Wong GH, Goeddel DV. A novel domain within the 55 kd TNF receptor signals cell death. *Cell* 1993; 74(5):845–53.
12. Kischkel FC, Hellbardt S, Behrmann I, et al. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J* 1995; 14(22):5579–88.
13. Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 1995; 81(4):505–12.
14. Boldin MP, Goncharov TM, Goltsev YV, Wallach D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* 1996; 85(6):803–15.
15. Muzio M, Chinnaiyan AM, Kischkel FC, et al. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* 1996; 85(6):817–27.
16. Donepudi M, Mac Sweeney A, Briand C, Grutter MG. Insights into the regulatory mechanism for caspase-8 activation. *Mol Cell* 2003; 11(2):543–9.
17. Boatright KM, Renshaw M, Scott FL, et al. A unified model for apical caspase activation. *Mol Cell* 2003; 11(2):529–41.
18. Scaffidi C, Fulda S, Srinivasan A, et al. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 1998; 17(6):1675–87.
19. Algeciras-Schimmich A, Pietras EM, Barnhart BC, et al. Two CD95 tumor classes with different sensitivities to antitumor drugs. *Proc Natl Acad Sci USA* 2003; 100(20):11445–50.
20. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998; 94(4):491–501.
21. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 1998; 94(4):481–90.
22. Baksh S, Tommasi S, Fenton S, et al. The tumor suppressor RASSF1A and MAP-1 link death receptor signaling to Bax conformational change and cell death. *Mol Cell* 2005; 18(6):637–50.
23. Sprick MR, Rieser E, Stahl H, Grosse-Wilde A, Weigand MA, Walczak H. Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. *EMBO J* 2002; 21(17):4520–30.
24. Kischkel FC, Lawrence DA, Tinel A, et al. Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. *J Biol Chem* 2001; 276(49):46639–46.
25. Milhas D, Cu villier O, Therville N, et al. Caspase-10 triggers bid cleavage and caspase cascade activation in FasL-induced apoptosis. *J Biol Chem* 2005; 280(20):19836–42.

26. Shu HB, Halpin DR, Goeddel DV. Casper is a FADD- and caspase-related inducer of apoptosis. *Immunity* 1997; 6(6):751–63.
27. Micheau O, Thome M, Schneider P, et al. The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. *J Biol Chem* 2002; 277(47):45162–71.
28. Chang DW, Xing Z, Pan Y, et al. c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. *EMBO J* 2002; 21(14):3704–14.
29. Sharp DA, Lawrence DA, Ashkenazi A. Selective knockdown of the long variant of cellular FLICE inhibitory protein augments death receptor-mediated caspase-8 activation and apoptosis. *J Biol Chem* 2005; 280(19):19401–9.
30. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. *Cell Death Differ* 2003; 10(1):45–65.
31. Hohmann HP, Remy R, Brockhaus M, van Loon AP. Two different cell types have different major receptors for human tumor necrosis factor (TNF alpha). *J Biol Chem* 1989; 264(25):14927–34.
32. Stauber GB, Aiyer RA, Aggarwal BB. Human tumor necrosis factor-alpha receptor. Purification by immunoaffinity chromatography and initial characterization. *J Biol Chem* 1988; 263(35):19098–104.
33. Hsu H, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell* 1995; 81(4):495–504.
34. Hsu H, Shu HB, Pan MG, Goeddel DV. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* 1996;84(2):299–308.
35. Yeh WC, Shahinian A, Speiser D, et al. Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity* 1997; 7(5):715–25.
36. Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity* 1996; 4(4):387–96.
37. Kucharczak J, Simmons MJ, Fan Y, Gelinas C. To be, or not to be: NF-kappaB is the answer—role of Rel/NF-kappaB in the regulation of apoptosis. *Oncogene* 2003; 22(56):8961–82.
38. Devin A, Cook A, Lin Y, Rodriguez Y, Kelliher M, Liu Z. The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. *Immunity* 2000; 12(4):419–29.
39. Senftleben U, Cao Y, Xiao G, et al. Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science* 2001; 293(5534):1495–9.
40. Luo JL, Kamata H, Karin M. IKK/NF-kappaB signaling: balancing life and death—a new approach to cancer therapy. *J Clin Invest* 2005; 115(10):2625–32.
41. Kato T Jr, Delhase M, Hoffmann A, Karin M. CK2 Is a C-Terminal IkappaB Kinase Responsible for NF-kappaB Activation during the UV Response. *Mol Cell* 2003; 12(4):829–39.
42. Wang CY, Mayo MW, Baldwin AS Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science* 1996; 274(5288):784–7.
43. Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* 2003; 114(2):181–90.
44. Schneider-Brachert W, Tchikov V, Neumeyer J, et al. Compartmentalization of TNF receptor 1 signaling: internalized TNF receptorsomes as death signaling vesicles. *Immunity* 2004; 21(3):415–28.
45. Nagata S. Fas ligand-induced apoptosis. *Annu Rev Genet* 1999; 33:29–55.
46. Peter ME, Krammer PH. The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ* 2003; 10(1):26–35.
47. Houston A, O’Connell J. The Fas signalling pathway and its role in the pathogenesis of cancer. *Curr Opin Pharmacol* 2004; 4(4):321–6.
48. Trauth BC, Klas C, Peters AM, et al. Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science* 1989; 245(4915):301–5.
49. Itoh N, Yonehara S, Ishii A, et al. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* 1991; 66(2):233–43.
50. Takahashi T, Tanaka M, Brannan CI, et al. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* 1994; 76(6):969–76.
51. Pitti RM, Marsters SA, Lawrence DA, et al. Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* 1998; 396(6712):699–703.
52. Bai C, Connolly B, Metzker ML, et al. Overexpression of M68/DcR3 in human gastrointestinal tract tumors independent of gene amplification and its location in a four-gene cluster. *Proc Natl Acad Sci USA* 2000; 97(3):1230–5.

53. Roth W, Isenmann S, Naumann U, et al. Locoregional Apo2L/TRAIL eradicates intracranial human malignant glioma xenografts in athymic mice in the absence of neurotoxicity. *Biochem Biophys Res Commun* 1999; 265(2):479–83.
54. Wu Y, Han B, Sheng H, et al. Clinical significance of detecting elevated serum DcR3/TR6/M68 in malignant tumor patients. *Int J Cancer* 2003; 105(5):724–32.
55. Wiley SR, Schooley K, Smolak PJ, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 1995; 3(6):673–82.
56. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* 1996; 271(22):12687–90.
57. LeBlanc HN, Ashkenazi A. Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ* 2003; 10(1):66–75.
58. Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene* 2003; 22(53):8628–33.
59. Ozoren N, El-Deiry WS. Defining characteristics of Types I and II apoptotic cells in response to TRAIL. *Neoplasia* 2002; 4(6):551–7.
60. Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. *Nat Med* 1999; 5(2):157–63.
61. Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999; 104(2):155–62.
62. Zhang XD, Franco A, Myers K, Gray C, Nguyen T, Hersey P. Relation of TNF-related apoptosis-inducing ligand (TRAIL) receptor and FLICE-inhibitory protein expression to TRAIL-induced apoptosis of melanoma. *Cancer Res* 1999; 59(11):2747–53.
63. Kim K, Fisher MJ, Xu S-Q, El-Deiry WS. Molecular determinants of response to TRAIL in killing of normal and cancer cells. *Clin Can Res* 2000; 6:335–46.
64. Jin Z, McDonald ER, 3rd, Dicker DT, El-Deiry WS. Deficient tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor transport to the cell surface in human colon cancer cells selected for resistance to TRAIL-induced apoptosis. *J Biol Chem* 2004; 279(34):35829–39.
65. Ozoren N, Fisher MJ, Kim K, et al. Homozygous deletion of the death receptor DR4 gene in a nasopharyngeal cancer cell line is associated with TRAIL resistance. *Int J Oncol* 2000; 16(5):917–25.
66. Grotzer MA, Eggert A, Zuzak TJ, et al. Resistance to TRAIL-induced apoptosis in primitive neuroectodermal brain tumor cells correlates with a loss of caspase-8 expression. *Oncogene* 2000; 19(40):4604–10.
67. McDonald ER, 3rd, El-Deiry WS. Suppression of caspase-8- and -10-associated RING proteins results in sensitization to death ligands and inhibition of tumor cell growth. *Proc Natl Acad Sci USA* 2004; 101(16):6170–5.
68. Thomas RK, Kallenborn A, Wickenhauser C, et al. Constitutive expression of c-FLIP in Hodgkin and Reed-Sternberg cells. *Am J Pathol* 2002; 160(4):1521–8.
69. Bullani RR, Huard B, Viard-Leveugle I, et al. Selective expression of FLIP in malignant melanocytic skin lesions. *J Invest Dermatol* 2001; 117(2):360–4.
70. Irmeler M, Thome M, Hahne M, et al. Inhibition of death receptor signals by cellular FLIP. *Nature* 1997; 388(6638):190–5.
71. Djerbi M, Screpanti V, Catrina AI, Bogen B, Biberfeld P, Grandien A. The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. *J Exp Med* 1999; 190(7):1025–32.
72. Medema JP, de Jong J, van Hall T, Melief CJ, Offringa R. Immune escape of tumors *in vivo* by expression of cellular FLICE-inhibitory protein. *J Exp Med* 1999; 190(7):1033–8.
73. Ricci MS, Jin Z, Dews M, et al. Direct repression of FLIP expression by c-myc is a major determinant of TRAIL sensitivity. *Mol Cell Biol* 2004; 24(19):8541–55.
74. Aza-Blanc P, Cooper CL, Wagner K, Batalov S, Deveraux QL, Cooke MP. Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. *Mol Cell* 2003; 12(3):627–37.
75. Wang Y, Engels IH, Knee DA, Nasoff M, Deveraux QL, Quon KC. Synthetic lethal targeting of MYC by activation of the DR5 death receptor pathway. *Cancer Cell* 2004; 5(5):501–12.

76. Routes JM, Ryan S, Clase A, et al. Adenovirus E1A oncogene expression in tumor cells enhances killing by TNF-related apoptosis-inducing ligand (TRAIL). *J Immunol* 2000; 165(8):4522–7.
77. Drosopoulos KG, Roberts ML, Cermak L, et al. Transformation by oncogenic RAS sensitizes human colon cells to TRAIL-induced apoptosis by up-regulating death receptor 4 and death receptor 5 through a MEK-dependent pathway. *J Biol Chem* 2005; 280(24):22856–67.
78. Nesterov A, Nikrad M, Johnson T, Kraft AS. Oncogenic Ras sensitizes normal human cells to tumor necrosis factor- α -related apoptosis-inducing ligand-induced apoptosis. *Cancer Res* 2004; 64(11):3922–7.
79. Siegel RM, Chan FK, Chun HJ, Lenardo MJ. The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nat Immunol* 2000; 1(6):469–74.
80. Lenardo M, Chan KM, Hornung F, et al. Mature T lymphocyte apoptosis—immune regulation in a dynamic and unpredictable antigenic environment. *Annu Rev Immunol* 1999; 17:221–53.
81. Rieux-Laucat F, Le Deist F, Hivroz C, et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* 1995;268(5215):1347–9.
82. Lamhamedi-Cherradi SE, Zheng SJ, Maguschak KA, Peschon J, Chen YH. Defective thymocyte apoptosis and accelerated autoimmune diseases in TRAIL-/- mice. *Nat Immunol* 2003; 4(3):255–60.
83. Galon J, Aksentijevich I, McDermott MF, O’Shea JJ, Kastner DL. TNFRSF1A mutations and autoinflammatory syndromes. *Curr Opin Immunol* 2000; 12(4):479–86.
84. Maini RN, Taylor PC. Anti-cytokine therapy for rheumatoid arthritis. *Annu Rev Med* 2000; 51:207–29.
85. Papadakis KA, Targan SR. The role of chemokines and chemokine receptors in mucosal inflammation. *Inflamm Bowel Dis* 2000; 6(4):303–13.
86. Feldmann M. Development of anti-TNF therapy for rheumatoid arthritis. *Nat Rev Immunol* 2002; 2(5):364–71.
87. Cretney E, Takeda K, Yagita H, Glaccum M, Peschon JJ, Smyth MJ. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. *J Immunol* 2002; 168(3):1356–61.
88. Finnberg N, Gruber JJ, Fei P, et al. DR5 knockout mice are compromised in radiation-induced apoptosis. *Mol Cell Biol* 2005; 25(5):2000–13.
89. Yeh WC, Pompa JL, McCurrach ME, et al. FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. *Science* 1998; 279(5358):1954–8.
90. Varfolomeev EE, Schuchmann M, Luria V, et al. Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity* 1998; 9(2):267–76.
91. Naylor MS, Stamp GW, Foulkes WD, Eccles D, Balkwill FR. Tumor necrosis factor and its receptors in human ovarian cancer. Potential role in disease progression. *J Clin Invest* 1993; 91(5):2194–206.
92. Warzocha K, Salles G, Bienvenu J, et al. Prognostic significance of TNF alpha and its p55 soluble receptor in malignant lymphomas. *Leukemia* 1997; 11(Suppl 3):441–3.
93. Foa R, Massaia M, Cardona S, et al. Production of tumor necrosis factor- α by B-cell chronic lymphocytic leukemia cells: a possible regulatory role of TNF in the progression of the disease. *Blood* 1990; 76(2):393–400.
94. Herrmann F, Helfrich SG, Lindemann A, Schleiermacher E, Huber C, Mertelsmann R. Elevated circulating levels of tumor necrosis factor predict unresponsiveness to treatment with interferon alfa-2b in chronic myelogenous leukemia. *J Clin Oncol* 1992; 10(4):631–4.
95. Tomita Y, Yang X, Ishida Y, et al. Spontaneous regression of lung metastasis in the absence of tumor necrosis factor receptor p55. *Int J Cancer* 2004; 112(6):927–33.
96. Kitakata H, Nemoto-Sasaki Y, Takahashi Y, Kondo T, Mai M, Mukaida N. Essential roles of tumor necrosis factor receptor p55 in liver metastasis of intrasplenic administration of colon 26 cells. *Cancer Res* 2002; 62(22):6682–7.
97. Suganuma M, Okabe S, Marino MW, Sakai A, Sueoka E, Fujiki H. Essential role of tumor necrosis factor alpha (TNF- α) in tumor promotion as revealed by TNF- α -deficient mice. *Cancer Res* 1999; 59(18):4516–8.
98. Knight B, Yeoh GC, Husk KL, et al. Impaired preneoplastic changes and liver tumor formation in tumor necrosis factor receptor type 1 knockout mice. *J Exp Med* 2000; 192(12):1809–18.
99. Luo JL, Maeda S, Hsu LC, Yagita H, Karin M. Inhibition of NF- κ B in cancer cells converts inflammation-induced tumor growth mediated by TNF α to TRAIL-mediated tumor regression. *Cancer Cell* 2004; 6(3):297–305.

100. Strand S, Hofmann WJ, Hug H, et al. Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells—a mechanism of immune evasion. *Nat Med* 1996; 2(12):1361–6.
101. Chopin D, Barei-Moniri R, Maille P, et al. Human urinary bladder transitional cell carcinomas acquire the functional Fas ligand during tumor progression. *Am J Pathol* 2003; 162(4):1139–49.
102. Belluco C, Esposito G, Bertorelle R, et al. Fas ligand is up-regulated during the colorectal adenoma-carcinoma sequence. *Eur J Surg Oncol* 2002; 28(2):120–5.
103. Kase H, Aoki Y, Tanaka K. Fas ligand expression in cervical adenocarcinoma: relevance to lymph node metastasis and tumor progression. *Gynecol Oncol* 2003; 90(1):70–4.
104. Mann B, Gratchev A, Bohm C, et al. FasL is more frequently expressed in liver metastases of colorectal cancer than in matched primary carcinomas. *Br J Cancer* 1999; 79(7-8):1262–9.
105. Hahne M, Rimoldi D, Schroter M, et al. Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. *Science* 1996; 274(5291):1363–6.
106. Igney FH, Krammer PH. Tumor counterattack: fact or fiction. *Cancer Immunol Immunother* 2005; 54(11):1127–36.
107. Barnhart BC, Legembre P, Pietras E, Bubici C, Franzoso G, Peter ME. CD95 ligand induces motility and invasiveness of apoptosis-resistant tumor cells. *EMBO J* 2004; 23(15):3175–85.
108. Desbarats J, Birge RB, Mimouni-Rongy M, Weinstein DE, Palermo JS, Newell MK. Fas engagement induces neurite growth through ERK activation and p35 upregulation. *Nat Cell Biol* 2003; 5(2): 118–25.
109. Smyth MJ, Takeda K, Hayakawa Y, Peschon JJ, van den Brink MR, Yagita H. Nature's TRAIL—on a path to cancer immunotherapy. *Immunity* 2003; 18(1):1–6.
110. Smyth MJ, Cretney E, Takeda K, et al. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. *J Exp Med* 2001; 193(6):661–70.
111. Takeda K, Hayakawa Y, Smyth MJ, et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nat Med* 2001; 7(1): 94–100.
112. Herbeuval JP, Lambert C, Sabido O, et al. Macrophages from cancer patients: analysis of TRAIL, TRAIL receptors, and colon tumor cell apoptosis. *J Natl Cancer Inst* 2003; 95(8):611–21.
113. Wu GS, Burns TF, McDonald ER, 3rd. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet* 1997; 17(2):141–5.
114. Diehl GE, Yue HH, Hsieh K, et al. TRAIL-R as a negative regulator of innate immune cell responses. *Immunity* 2004; 21(6):877–89.
115. Fei P, Bernhard EJ, El-Deiry WS. Tissue-specific induction of p53 targets *in vivo*. *Cancer Res* 2002; 62(24):7316–27.
116. Shin MS, Kim HS, Lee SH, et al. Mutations of tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and receptor 2 (TRAIL-R2) genes in metastatic breast cancers. *Cancer Res* 2001; 61(13):4942–6.
117. Lee SH, Shin MS, Kim HS, et al. Alterations of the DR5/TRAIL receptor 2 gene in non-small cell lung cancers. *Cancer Res* 1999; 59(22):5683–6.
118. Lee SH, Shin MS, Kim HS, et al. Somatic mutations of TRAIL-receptor 1 and TRAIL-receptor 2 genes in non-Hodgkin's lymphoma. *Oncogene* 2001; 20(3):399–403.
119. Mills KR, Reginato M, Debnath J, Queenan B, Brugge JS. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is required for induction of autophagy during lumen formation *in vitro*. *Proc Natl Acad Sci USA* 2004; 101(10):3438–43.
120. Shintani T, Klionsky DJ. Autophagy in health and disease: a double-edged sword. *Science* 2004; 306(5698):990–5.
121. Prins J, Ledgerwood E, Ameloot P, et al. Tumour necrosis factor induced autophagy and mitochondrial morphological abnormalities are mediated by TNFR-I and/or TNFR-II and do not invariably lead to cell death. *Biochem Soc Trans* 1998; 26(4):S314.
122. Jia L, Dourmashkin RR, Allen PD, Gray AB, Newland AC, Kelsey SM. Inhibition of autophagy abrogates tumour necrosis factor alpha induced apoptosis in human T-lymphoblastic leukaemic cells. *Br J Haematol* 1997; 98(3):673–85.
123. El-Deiry WS. The role of p53 in chemosensitivity and radiosensitivity. *Oncogene* 2003; 22(47): 7486–95.
124. Schuler M, Green DR. Transcription, apoptosis and p53: catch-22. *Trends Genet* 2005; 21(3):182–7.

125. Muller M, Wilder S, Bannasch D, et al. p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. *J Exp Med* 1998; 188(11):2033–45.
126. Takimoto R, El-Deiry WS. Wild-type p53 transactivates the KILLER/DR5 gene through an intronic sequence-specific DNA-binding site. *Oncogene* 2000; 19(14):1735–43.
127. Burns T, Bernhard E, El-Deiry W. Tissue specific expression of p53 target genes suggests a key role for KILLER/DR5 in p53-dependent apoptosis *in vivo*. *Oncogene* 2001; 20:4601–12.
128. Liu X, Yue P, Khuri FR, Sun SY. p53 upregulates death receptor 4 expression through an intronic p53 binding site. *Cancer Res* 2004; 64(15):5078–83.
129. Friesen C, Herr I, Krammer PH, Debatin KM. Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. *Nat Med* 1996; 2(5):574–7.
130. Fulda S, Sieverts H, Friesen C, Herr I, Debatin KM. The CD95 (APO-1/Fas) system mediates drug-induced apoptosis in neuroblastoma cells. *Cancer Res* 1997; 57(17):3823–9.
131. Muller M, Strand S, Hug H, et al. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. *J Clin Invest* 1997; 99(3):403–13.
132. Newton K, Strasser A. Ionizing radiation and chemotherapeutic drugs induce apoptosis in lymphocytes in the absence of Fas or FADD/MORT1 signaling. Implications for cancer therapy. *J Exp Med* 2000; 191(1):195–200.
133. Debatin KM, Krammer PH. Death receptors in chemotherapy and cancer. *Oncogene* 2004; 23(16):2950–66.
134. Kelley SK, Ashkenazi A. Targeting death receptors in cancer with Apo2L/TRAIL. *Curr Opin Pharmacol* 2004; 4(4):333–9.
135. Wajant H, Gerspach J, Pfizenmaier K. Tumor therapeutics by design: targeting and activation of death receptors. *Cytokine Growth Factor Rev* 2005; 16(1):55–76.
136. Hersh EM, Metch BS, Muggia FM, et al. Phase II studies of recombinant human tumor necrosis factor alpha in patients with malignant disease: a summary of the Southwest Oncology Group experience. *J Immunother* 1991; 10(6):426–31.
137. Ogasawara J, Watanabe-Fukunaga R, Adachi M, et al. Lethal effect of the anti-Fas antibody in mice. *Nature* 1993; 364(6440):806–9.
138. Kelley SK, Harris LA, Xie D, et al. Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of *in vivo* efficacy, pharmacokinetics, and safety. *J Pharmacol Exp Ther* 2001; 299(1):31–8.
139. Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* 2002; 2(6):420–30.
140. Jo M, Kim TH, Seol DW, et al. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. *Nat Med* 2000; 6(5):564–7.
141. Leverkus M, Neumann M, Mengling T, et al. Regulation of tumor necrosis factor-related apoptosis-inducing ligand sensitivity in primary and transformed human keratinocytes. *Cancer Res* 2000; 60(3):553–9.
142. Lawrence D, Shahrokh Z, Marsters S, et al. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. *Nat Med* 2001; 7(4):383–5.
143. Kim SH, Kim K, Kwagh JG, et al. Death induction by recombinant native TRAIL and its prevention by a caspase 9 inhibitor in primary human esophageal epithelial cells. *J Biol Chem* 2004; 279(38):40044–52.
144. Ichikawa K, Liu W, Zhao L, et al. Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. *Nat Med* 2001; 7(8):954–60.
145. Chuntharapai A, Dodge K, Grimmer K, et al. Isotype-dependent inhibition of tumor growth *in vivo* by monoclonal antibodies to death receptor 4. *J Immunol* 2001; 166(8):4891–8.
146. Ozoren N, Kim K, Burns TF, Dicker DT, Mosconi AD, El-Deiry WS. The caspase 9 inhibitor Z-LEHD-FMK protects human liver cells while permitting death of cancer cells exposed to tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Res* 2000; 60(22):6259–65.
147. Wang S, El-Deiry WS. Requirement of p53 targets in chemosensitization of colonic carcinoma to death ligand therapy. *Proc Natl Acad Sci USA* 2003; 100(25):15095–100.
148. Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. *Cancer Res* 1999; 59(3):734–41.
149. Nagane M, Pan GH, Weddle JJ, Dixit VM, Cavenee WK, Huang HJS. Increased death receptor 5 expression by chemotherapeutic agents in human gliomas causes synergistic cytotoxicity with

- tumor necrosis factor-related apoptosis-inducing ligand *in vitro* and *in vivo*. *Cancer Res* 2000; 60(4): 847–53.
150. Chinnaiyan AM, Prasad U, Shankar S, et al. Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. *Proc Natl Acad Sci USA* 2000; 97(4):1754–9.
 151. Insinga A, Monestiroli S, Ronzoni S, et al. Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. *Nat Med* 2005; 11(1):71–6.
 152. Nebbioso A, Clarke N, Voltz E, et al. Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells. *Nat Med* 2005; 11(1):77–84.
 153. Inoue H, Shiraki K, Ohmori S, et al. Histone deacetylase inhibitors sensitize human colonic adenocarcinoma cell lines to TNF-related apoptosis inducing ligand-mediated apoptosis. *Int J Mol Med* 2002; 9(5):521–5.
 154. Shetty S, Graham BA, Brown JG, et al. Transcription factor NF-kappaB differentially regulates death receptor 5 expression involving histone deacetylase 1. *Mol Cell Biol* 2005; 25(13):5404–16.
 155. Meng RD, El-Deiry WS. p53-independent upregulation of KILLER/DR5 TRAIL receptor expression by glucocorticoids and interferon-gamma. *Exp Cell Res* 2001; 262(2):154–69.
 156. Eggermont AM, ten Hagen TL. Tumor necrosis factor-based isolated limb perfusion for soft tissue sarcoma and melanoma: ten years of successful antivasculature therapy. *Curr Oncol Rep* 2003; 5(2): 79–80.
 157. Lienard D, Ewalenko P, Delmotte JJ, Renard N, Lejeune FJ. High-dose recombinant tumor necrosis factor alpha in combination with interferon gamma and melphalan in isolation perfusion of the limbs for melanoma and sarcoma. *J Clin Oncol* 1992; 10(1):52–60.
 158. Renard N, Lienard D, Lespagnard L, Eggermont A, Heimann R, Lejeune F. Early endothelium activation and polymorphonuclear cell invasion precede specific necrosis of human melanoma and sarcoma treated by intravascular high-dose tumour necrosis factor alpha (rTNF alpha). *Int J Cancer* 1994; 57(5):656–63.
 159. Wajant H. TRAIL and NFkappaB signaling—a complex relationship. *Vitam Horm* 2004; 67:101–32.
 160. Nakanishi C, Toi M. Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. *Nat Rev Cancer* 2005; 5(4):297–309.
 161. Richardson PG, Sonneveld P, Schuster MW, et al. Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. *N Engl J Med* 2005; 352(24):2487–98.
 162. Rajkumar SV, Richardson PG, Hideshima T, Anderson KC. Proteasome inhibition as a novel therapeutic target in human cancer. *J Clin Oncol* 2005; 23(3):630–9.
 163. Boccadoro M, Morgan G, Cavenagh J. Preclinical evaluation of the proteasome inhibitor bortezomib in cancer therapy. *Cancer Cell Int* 2005; 5(1):18.
 164. Pierce JW, Schoenleber R, Jesmok G, et al. Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects *in vivo*. *J Biol Chem* 1997; 272(34):21096–103.
 165. Dai Y, Rahmani M, Dent P, Grant S. Blockade of histone deacetylase inhibitor-induced RelA/p65 acetylation and NF-kappaB activation potentiates apoptosis in leukemia cells through a process mediated by oxidative damage, XIAP downregulation, and c-Jun N-terminal kinase 1 activation. *Mol Cell Biol* 2005; 25(13):5429–44.
 166. Jazirehi AR, Huerta-Yepez S, Cheng G, Bonavida B. Rituximab (chimeric anti-CD20 monoclonal antibody) inhibits the constitutive nuclear factor-{kappa}B signaling pathway in non-Hodgkin's lymphoma B-cell lines: role in sensitization to chemotherapeutic drug-induced apoptosis. *Cancer Res* 2005; 65(1):264–76.
 167. Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature* 1998; 396(6706):77–80.
 168. Yamamoto Y, Yin MJ, Lin KM, Gaynor RB. Sulindac inhibits activation of the NF-kappaB pathway. *J Biol Chem* 1999; 274(38):27307–14.
 169. Reed JC, Pellecchia M. Apoptosis-based therapies for hematologic malignancies. *Blood* 2005; 106(2):408–18.
 170. Suh N, Wang Y, Honda T, et al. A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxolean-1,9-dien-28-oic acid, with potent differentiating, antiproliferative, and anti-inflammatory activity. *Cancer Res* 1999; 59(2):336–41.
 171. Place AE, Suh N, Williams CR, et al. The novel synthetic triterpenoid, CDDO-imidazolide, inhibits inflammatory response and tumor growth *in vivo*. *Clin Cancer Res* 2003; 9(7):2798–806.

172. Ito Y, Pandey P, Place A, et al. The novel triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid induces apoptosis of human myeloid leukemia cells by a caspase-8-dependent mechanism. *Cell Growth Differ* 2000; 11(5):261–7.
173. Kim Y, Suh N, Sporn M, Reed JC. An inducible pathway for degradation of FLIP protein sensitizes tumor cells to TRAIL-induced apoptosis. *J Biol Chem* 2002; 277(25):22320–9.
174. Pedersen IM, Kitada S, Schimmer A, et al. The triterpenoid CDDO induces apoptosis in refractory CLL B cells. *Blood* 2002; 100(8):2965–72.
175. Stadheim TA, Suh N, Ganju N, Sporn MB, Eastman A. The novel triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) potently enhances apoptosis induced by tumor necrosis factor in human leukemia cells. *J Biol Chem* 2002; 277(19):16448–55.
176. Suh WS, Kim YS, Schimmer AD, et al. Synthetic triterpenoids activate a pathway for apoptosis in AML cells involving downregulation of FLIP and sensitization to TRAIL. *Leukemia* 2003; 17(11):2122–9.
177. Zou W, Liu X, Yue P, et al. c-Jun NH2-terminal kinase-mediated up-regulation of death receptor 5 contributes to induction of apoptosis by the novel synthetic triterpenoid methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate in human lung cancer cells. *Cancer Res* 2004; 64(20):7570–8.
178. Lapillonne H, Konopleva M, Tsao T, et al. Activation of peroxisome proliferator-activated receptor gamma by a novel synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces growth arrest and apoptosis in breast cancer cells. *Cancer Res* 2003; 63(18):5926–39.
179. Hyer ML, Croxton R, Krajewska M, et al. Synthetic triterpenoids cooperate with tumor necrosis factor-related apoptosis-inducing ligand to induce apoptosis of breast cancer cells. *Cancer Res* 2005; 65(11):4799–808.
180. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell* 2004; 116(2):205–19.
181. Dias N, Stein CA. Potential roles of antisense oligonucleotides in cancer therapy. The example of Bcl-2 antisense oligonucleotides. *Eur J Pharm Biopharm* 2002; 54(3):263–9.
182. Fischer U, Schulze-Osthoff K. New approaches and therapeutics targeting apoptosis in disease. *Pharmacol Rev* 2005; 57(2):187–215.
183. Kelly JD, Dai J, Eschwege P, et al. Downregulation of Bcl-2 sensitises interferon-resistant renal cancer cells to Fas. *Br J Cancer* 2004; 91(1):164–70.
184. Huang X, Lin T, Gu J, et al. Combined TRAIL and Bax gene therapy prolonged survival in mice with ovarian cancer xenograft. *Gene Ther* 2002; 9(20):1379–86.
185. Walensky LD, Kung AL, Escher I, et al. Activation of apoptosis in vivo by a hydrocarbon-stapled BH3 helix. *Science* 2004; 305(5689):1466–70.
186. Oltersdorf T, Elmore SW, Shoemaker AR, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 2005; 435(7042):1869–78.
187. Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harran PG. A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. *Science* 2004; 305(5689):1471–4.
188. Marino MW, Dunn A, Grail D, et al. Characterization of tumor necrosis factor-deficient mice. *Proc Natl Acad Sci USA* 1997; 94(15):8093–8.
189. Karray S, Kress C, Cuvellier S, et al. Complete loss of Fas ligand gene causes massive lymphoproliferation and early death, indicating a residual activity of gld allele. *J Immunol* 2004; 172(4):2118–25.
190. Rothe J, Lesslauer W, Lotscher H, et al. Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*. *Nature* 1993; 364(6440):798–802.
191. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 1992; 356(6367):314–7.
192. Yeh WC, Itie A, Elia AJ, et al. Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. *Immunity* 2000; 12(6):633–42.
193. Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P. The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. *Immunity* 1998; 8(3):297–303.
194. Nakano H, Sakon S, Koseki H, et al. Targeted disruption of Traf5 gene causes defects in CD40- and CD27-mediated lymphocyte activation. *Proc Natl Acad Sci USA* 1999; 96(17):9803–8.

3

Evaluating the Importance of Apoptosis and Other Determinants of Cell Death and Survival

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SUMMARY

Apoptosis, the cell's intrinsic death program, has emerged as a key regulator in physiological growth control and regulation of tissue homeostasis. Research into the underlying mechanistic basis and its importance in disease and treatment has progressed tremendously. However, it is important to note that apoptosis is only one form of cell death and that we should not overlook the importance of other death mechanisms. The particular death pathway that is the most important determinant for cancer therapy is not necessarily that which has the fastest kinetics, as is the bias in many laboratories, but rather that which displays the most sensitive dose–response relationship. In this chapter, we argue that in many situations apoptosis is not the most important determinant of treatment response. Furthermore, we provide a summary of the different forms of cell death and discuss their contribution to cancer development and treatment response.

Key Words: Apoptosis; clonogenic survival; senescence; cancer therapy.

1. INTRODUCTION

The realization that cell death often occurs as part of an active, programmed process has significantly altered our understanding of both cancer development and cancer treatment. Cell death is now often considered as a “choice” made by a cell that has become irreparably damaged or that has become potentially dangerous because of the activation of a growth-promoting oncogene. Apoptosis, a well-described form of programmed cell death characterized by the activation of caspases (1), is often used synonymously with cell death itself. This is understandable, as this form of cell death results in rapid and normally complete destruction and removal of the cell. However,

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when we consider cell death in the context of both the development and the treatment of cancer, it is perhaps more useful to expand the definition to include other processes that lead to the inactivation of the proliferative or “clonogenic” capacity of the cell. These other processes include senescence, mitotic catastrophe, autophagy, and necrosis (2).

It has become clear that apoptosis represents a powerful and important cellular defense against cancer development. Correspondingly, loss of apoptotic sensitivity is considered one of the six hallmarks of cancer (3). The propensity of individual cells to undergo apoptosis is markedly influenced both positively and negatively by a variety of different genes, many of which become mutated and/or deregulated in human cancers (2). Furthermore, apoptosis can be highly influenced by the local microenvironment of a tumor, which is characterized by extreme heterogeneity in oxygenation and nutrient supply (4). Consequently, the cellular apoptotic sensitivity can vary remarkably among different tumors and even within the same tumor. As anticancer agents, including the most commonly used chemotherapy agents and radiation, are capable of activating apoptosis, it has become widely assumed that apoptotic sensitivity is also an important contributor to overall treatment sensitivity (5,6). This has led to the search for novel agents capable of inducing apoptosis in human cancers and also for mechanisms of increasing the apoptotic sensitivity of tumor cells. However, as already eluded to above, apoptosis represents only one form of cell death that can contribute to the inactivation of tumor cells. The importance of other non-apoptotic pathways are frequently overlooked with respect to their role in preventing cancer and, even more so, in determining treatment sensitivity. In fact, several critical analyses of the literature have concluded that apoptosis and the genes controlling it, such as p53, are not major contributors to treatment response (7–19). How then has the widely held view, that apoptosis is a major determinant of treatment sensitivity, become so persuasive? In part, this may be due to the difficult or in some cases impossible task of separating treatment response from patient prognosis. Apoptosis, and the genes controlling it, may alter several other tumor parameters that are important for patient prognosis but unrelated to cellular treatment sensitivity. These parameters include the rate of tumor growth, invasiveness, and metastasis. A second and perhaps even more important reason results from the fact that many laboratory experiments fail to consider all the potentially relevant forms of cell death (2,8,20,21). Each process of cell death is activated with different kinetics and with a different dose-response following treatment. The most important form of cell death is that which displays the most sensitive dose–response relationship and not necessarily that which occurs with the fastest kinetics. Many laboratory assays are biased to detect apoptosis or other forms of rapid cell death. This can lead to dramatic misinterpretations of cellular sensitivity to treatment.

2. FORMS OF CELL DEATH

Understanding and evaluating the contribution of different forms of cell death to cancer development and treatment requires consideration of their individual mechanisms of action and their genetic determinants. For the purposes here, we discuss each of these briefly.

2.1. Apoptosis

The most well-defined form of cell death is apoptosis, an active, complex, and highly regulated process initiated within the cell in response to either an intracellular or

an extracellular cue. Apoptosis is an essential and normal part of many physiological processes including embryonic development, the immune system, and maintenance of tissue homeostasis (22). Consequently, alterations in the control of apoptosis lead to several human disorders including cancer and autoimmune disease (lower apoptosis) or neurodegenerative disease or immunodeficiency (higher apoptosis). Apoptotic cell death is characterized by the sequential activation of several different cysteine proteases known as caspases (23). These proteins are initially expressed in an inactive procaspase form (24) and are additionally kept in check by a family of inhibitor of apoptosis (IAP) proteins (25). Apoptosis begins following the activation of an “initiator” caspase such as caspase 8 or 9. These caspases subsequently activate other effector caspases, which then cleave a large set of cellular proteins leading to the ultimate destruction of the cell. Caspase 8 activation is termed the “extrinsic” pathway because it is normally activated on the binding of a ligand and subsequent activation of a death receptor present in the cellular membrane (26). Caspase 9 activation is part of the “intrinsic” pathway that is activated within the cell in response to various forms of cell damage or cell stress. Conditions within the tumor microenvironment such as hypoxia (4) and nutrient deprivation (27) can activate apoptosis through this intrinsic pathway. DNA damage caused by chemotherapy agents and radiation also can lead to caspase 9-dependent apoptosis (28–30).

What then determines whether a particular cell will initiate apoptosis in response to cellular stress? Another way to pose this question is to ask what determines the dose–response relationship for the induction of apoptosis to a particular form of damage or cell stress. Critical in this relationship is the balance of pro- and anti-apoptotic proteins, chief among which are the family of BH3-containing proteins such as BCL2 and BAX (31,32). These proteins control permeabilization of the outer membrane of the mitochondria and the subsequent release of cytochrome c (33). Cytochrome c release leads to formation of the apoptosome and caspase activation. Thus, activation of apoptosis in response to cancer treatment depends highly on the specific balance of these pro and anti-apoptotic proteins. This balance is known to vary widely among both different cell types in the body and different tumors. For example, activation of apoptosis through the intrinsic pathway can be highly influenced by p53, which is itself activated in response to DNA damage and hypoxia in human tumors. The ability of p53 to induce apoptosis is due in large part to its transactivation of pro-apoptotic proteins such as BAX and PUMA. Many tumors show loss of p53 function and are thus unable to initiate apoptosis through this pathway. However, in those tumors that retain p53 function, its ability to induce apoptosis is still highly dependent on the relative level of other pro- and anti-apoptotic proteins in the cell (34). Consequently, some cells may require significantly higher levels of stress or damage than others to tip this balance in favor of apoptosis. This is also true of normal tissues. Although p53 is activated in response to DNA damage in most normal cell types, apoptosis is much more easily activated in certain cell types, such as those of lymphoid origin (35). Many other normal cell types almost never undergo apoptosis even after very high amounts of cell damage. Thus, the importance of p53 as an inducer of apoptosis is context dependent. This example applies to most regulators of apoptosis and leads to the conclusion that evaluation of the dose–response relationship is critical for evaluating the relative importance of apoptosis in response to damage. However, it is important to remember that apoptosis may occur independently of p53 as well.

2.2. Senescence

Senescence is a term that reflects the permanent inactivation of proliferation that, like apoptosis, is initiated by a genetic program. Cells undergoing senescence remain metabolically active yet do not actively cycle and thus for cancer will not contribute to further growth of the tumor. Senescence is also a normal part of physiology that increases as organisms age. Senescence was first visualized in cultured primary cells that exhibit an initial period of exponential growth, followed by a permanent arrest termed replicative senescence (36). Replicative senescence correlates with the gradual shortening of telomeres during the exponential growth period. In addition to this replicative form of senescence, “premature” senescence can also be elicited by various cellular stresses such as those caused by oncogene activation or by DNA damage (37,38). In both situations, the cells enter a permanent cell-cycle arrest characterized morphologically by a flattened cytoplasm and increased granularity or biochemically by stimulating senescence-associated β -galactosidase expression (39). Senescence-inducing stresses typically do not induce shortening of the telomeres but instead are controlled by a number of gene products that are only beginning to become understood. The best understood part of senescence induction involves the activation of cell-cycle inhibitor proteins. Senescence activation following DNA damage is influenced by p53, and its target gene CDKN1A, also known as p21 (40). This is thought to initiate a cell-cycle checkpoint that leads eventually to permanent arrest, perhaps through widespread gene silencing (41). Also critical in the induction of senescence is the *p16* gene from the INK4A locus and the retinoblastoma (RB) gene product (42,43). In cells that retain p53 function, p16 has been shown in some cases to be necessary for the induction of senescence after DNA damage (44). However, this may not be universally true as other studies suggest that the maintenance of the senescent state and not its induction requires p16 (45,46). Like apoptosis, avoidance of senescence is thought to play a role during tumor development. However, the considerable overlap between the genes that control apoptosis and those that control senescence make the situation difficult to assess. Nonetheless, recent evidence from several model systems indicates that tumors do indeed downregulate senescence through a variety of mechanisms (47–50).

2.3. Mitotic Catastrophe (Chromosome Aberrations)

Mitotic catastrophe is a term that has evolved to encompass the type of cell death that results from or following aberrant mitosis. This is often morphologically associated with the accumulation of multinucleated, giant cells containing uncondensed chromosomes. This process is thought to occur when cells proceed through mitosis in an inappropriate manner. For example, mitotic catastrophe frequently occurs if cells enter mitosis with unrepaired or misrepaired DNA damage. Death because of mitotic catastrophe contrasts with that described for apoptosis and senescence in that it is more of a trigger for cell death rather than a genetic program for cell death. Consequently, mitotic catastrophe resulting in cell fusion, polyploidy, or failure to perform cytokinesis may subsequently lead to cell death by apoptosis, senescence, or necrosis (51). The important distinction is that cell death is caused by the mitotic catastrophe rather than the initial cell damage itself.

Several checkpoints in G2 and throughout mitosis exist to prevent mitotic catastrophe. These include two genetically distinct G2 checkpoints that are activated by

DNA damage and block mitosis by preventing the activation of the mitotic kinase CDK1. Cells that show deregulated expression of CDK1 enter into mitosis prematurely and die through mitotic catastrophe (52). An “early” G2 checkpoint is regulated by activation of the DNA damage sensing protein ataxia telangiectasia mutated (ATM) (53). This checkpoint is activated within minutes of DNA damage and prevents cells that are in G2 phase at the time of damage induction from entering mitosis. Inhibition of CDK1 occurs following ATM- and CHK2-dependent phosphorylation of the CDK1 inhibitor CDC25C. A “late” G2 checkpoint is controlled in a similar way through initial activation of the ATM- and Rad3-related (ATR) protein and subsequent phosphorylation of CHK1. This late G2 checkpoint prevents cells that arrive in G2 phase with remaining DNA damage from entering mitosis. In addition, p53 can contribute to G2 arrest by upregulation of its targets p21 and 14-3-3 σ (54). Bypass of this checkpoint permits premature entry into mitosis even if the DNA has not been fully replicated or repaired, leading to mitotic catastrophe.

Several mitotic checkpoints are also thought to exist to ensure proper spindle assembly and attachment prior to cytokinesis. The spindle checkpoint is regulated by a number of different kinases, including the aurora kinases (A, B, and C), polo kinases (PLK1, 2, and 3), as well as the BUB1 and BUBR1 spindle checkpoint kinases. Deregulation of these kinases has been shown to lead to mitotic catastrophe, although it is still unclear precisely how this occurs (55).

Many of the abovementioned genes and the pathways that control them are modified during cancer. For example, transmission of the signals from ATM and ATR to CHK1 and CHK2 is dependent on the tumor suppressor genes *BRCA1* and/or *NBS* (56). Consequently, the propensity to undergo mitotic catastrophe can vary significantly among different cell types that may have lost these tumor suppressor gene functions. For example, breast cancer cells with aberrant *BRCA1* proteins would be expected to exhibit increased sensitivity to mitotic catastrophe. Moreover, some chemotherapeutic drugs such as paclitaxel induce this form of cell death by directly disrupting the mitotic spindles or damaging microtubules (57).

2.4. Autophagy

Autophagy is a process in which cells digest their own cytoplasm to generate small macromolecules that are essential for cell survival. Autophagy is induced in response to growth factor removal and is thought to sustain overall survival during times of low nutrient environment (58). The molecular basis of autophagy, and its relationship to cell survival mechanisms, is an active area of current research. DNA-dependent protein kinase (DNA-PK) was recently demonstrated to play a critical role in ionizing radiation autophagy (59). The mammalian target of rapamycin (mTOR) pathway, which is a general sensor of nutrient status, appears to be critically required to regulate autophagy (60). Intracellular structures called phagophores engulf parts of the cytoplasm, forming cytoplasm-filled vacuoles. These are subsequently delivered to the lysosomes for degradation. Cell death can result in a distinct manner in cells that undergo excessive autophagy. This form of cell death is distinct from apoptosis or necrosis and is thus unique to the autophagic process (61,62). The precise function for death by autophagy in mammals is not fully understood (63,64) but has been implicated in several pathologies including cancer (65). Indeed, the involvement of the

mTOR pathway, which is activated by PI3 kinase signaling, suggests that autophagy may often become differentially regulated during cancer.

2.5. *Necrosis and Others*

Necrosis is considered to be inappropriate or accidental death that usually occurs under extremely unfavorable conditions that are incompatible with a critical normal physiologic process. Examples of conditions that can activate necrosis include extreme pH, energy loss, and ion imbalance (66). Necrosis has been typically thought of as an uncontrollable, an irreversible, and a chaotic form of cell death. More recently, however, studies have suggested that necrosis is also a regulated process that can be modulated (67). It is characterized by cellular swelling, membrane deformation, and organelle break down and is usually the consequence of such conditions as infection, inflammation, or ischemia (22). However, whether active signaling pathways for this form of death exist is uncertain.

3. INVOLVEMENT OF CELL DEATH DURING TUMOR DEVELOPMENT AND TREATMENT

From this brief overview, it is already clear that various mechanisms of cell death can contribute to cancer development and treatment response in largely varying degrees. The loss or downregulation of cell death pathways clearly occurs during cancer development, not only in the case of apoptosis but in other mechanisms of cell death. In the case of cancer development, it is easy to see how even subtle changes in cell death can contribute to tumor growth and development. A carefully regulated balance between cell proliferation and cell death is absolutely required for maintenance of tissue homeostasis. This equilibrium needs to be only modestly affected to allow proliferation to exceed cell death and consequently to the development of a tumor. Selection against apoptosis may be especially important, as many oncogenes not only promote proliferation but also sensitize the cell to death by apoptosis (68). Consequently for the cell to realize the true potential of an oncogene, it must also counter-balance this increased apoptotic sensitivity.

However, it is less clear whether the changes in sensitivity to cell death that occur during carcinogenesis will also significantly alter the response of these same tumors to treatment. There are certainly compelling experiments that have clearly demonstrated that apoptosis can influence tumor response. This has been perhaps most elegantly studied in the E μ -myc lymphoma model developed by Lowe and colleagues (69). Tumors arising because of the presence of myc activation in the B-cell lineages are highly influenced by secondary pathways that affect apoptosis. For example, when these mice are crossed with p53 +/– heterozygous mice, all the lymphomas that develop demonstrate loss of heterozygosity for p53 (34). Furthermore, loss of apoptosis through knockout of p53, overexpression of BCL-2, or loss of INK4A/ARF dramatically accelerates tumor onset and tumor growth. This model clearly demonstrates that apoptosis acts as a barrier to cancer development. This model has also provided evidence that apoptosis is important for cancer therapy. When mice-harboring lymphomas were treated with chemotherapy agents, the tumors with loss of p53, INK4A/ARF, or overexpression of BCL2 also responded much more poorly than those expressing only

myc (34). These data clearly demonstrate that in this model, the propensity to undergo apoptosis influences tumor response.

Despite this compelling data, we cannot necessarily extrapolate this result to human tumors, especially those of non-lymphoid origin. The model described above displays an extreme sensitivity to apoptosis because of two distinct factors. First, the cells of lymphoid origin generally show a much higher propensity to die by apoptosis—in other words, the balance between pro- and anti-apoptotic proteins is already tipped in favor of apoptosis in these cells (35,70). Second, overexpression of myc also results in an increased sensitivity toward apoptosis (71). Consequently, the control tumors in this case are extremely sensitive to apoptosis. This is perhaps best exemplified by the fact that a single dose of cyclophosphamide is sufficient to cause a long-term remission in the majority of mice. In solid human tumors, which develop primarily from epithelial cells, the situation may be substantially different. Indeed, in a comprehensive review of the literature, Brown and Wilson concluded that “there is little or no support that apoptosis, and the genes govern it, determine the response to therapy” (9). In contrast to the results with the lymphoma model, they found that the propensity to undergo apoptosis in human tumors of epithelial origin played no role in predicting treatment sensitivity.

Despite the aforementioned review of clinical data, the concept that selection of cells with resistance to apoptosis during carcinogenesis results in a co-selection of cells that will be resistant to treatment has become a persuasive one in the research community. Why has this concept become so widespread? We argue that this has arisen in part due to the use of inappropriate assays to evaluate treatment responses. It is important to evaluate all potential forms of cell death that may contribute to treatment response (8,20). Furthermore, for each type of cell death, it is critical to consider both the kinetics of cell death and its dose–response relationship with the treatment.

4. CONSIDERATION OF CELL DEATH KINETICS AND DOSE RESPONSES IN RELATION TO SURVIVAL

It is often said that “you only live once,” but it is equally true that “you only die once.” In many cells, several forms of cell death may be possible in response to cancer treatment. The form of cell death that actually inactivates any particular cell is obviously the one that occurs first, that is, that with the fastest kinetics. In cells that retain considerable apoptotic sensitivity, the most rapid form of cell death is often apoptosis, even though it may not be the most sensitive. This is particularly true when treating cells with high doses of a DNA-damaging agent. However, this does not imply that other mechanisms of cell death would not have occurred if given the opportunity (Fig. 1). The important consideration in such a case is whether the probability that the cell survived would be changed if that rapid form of cell death was eliminated. Differences in the rate of cell death have complicated our understanding of the importance of apoptosis for two distinct reasons. The first is because many assays are biased to detect rapid changes in cell number. Rapid forms of cell death are thus more easily detected by these types of assays (discussed in Section 5). The second is because the most rapid form of cell death can mask other forms of death. Consequently, if multiple forms of cell death are activated in the same cell, the one

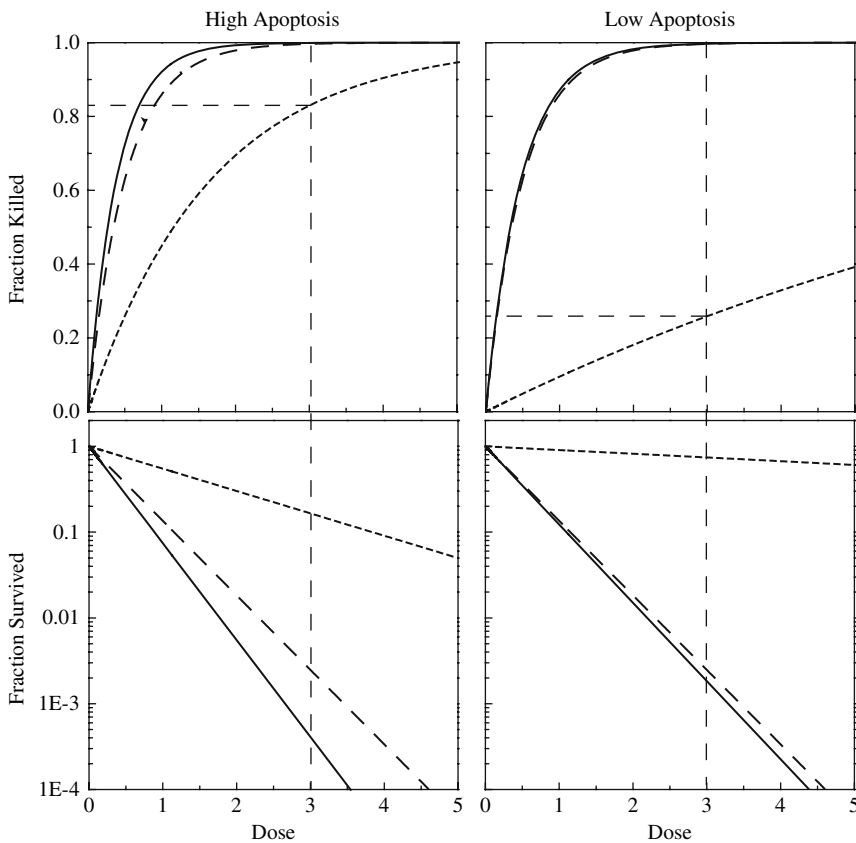


Fig. 1. Importance of dose–response relationships for different modes of cell death. Shown are hypothetical dose–response curves for death (upper frames) and survival (lower frames) in response to an anti-cancer agent. The dose–response relationships for overall (solid), apoptosis only (short dash), and non-apoptosis mechanisms (long dash) are shown separately. Two important concepts are illustrated here. First, a significant change in the sensitivity to one mode of cell death, in this case apoptosis, does not necessarily impact significantly on overall survival. The two examples have substantially different sensitivities to death by apoptosis, but much smaller differences in overall survival. The mode of cell death that is the most sensitive (in this case non-apoptotic death) is the one most important for determining the overall response in both cases. Second, even though apoptosis may not be an important determinant of the overall level of cell kill, the vast majority of cells may still die through this process. In the examples above, doses that are sufficient to kill more than three logs of cells induce apoptosis rates of more than 80% in the apoptosis sensitive cells and more than 25% in the resistant cells. However, in both cases, this is of little consequence to overall survival. Thus, simply because cells die by apoptosis does not imply that apoptosis is an important determinant of outcome.

that is manifested first will be the only one observed. The most rapid form of death will not necessarily be the most important (sensitive).

Interestingly, studies with the E μ -myc model of lymphoma also provide one of the best examples of how disabling one cell death pathway can reveal another that is equally as sensitive (72). In this model, the development of lymphoma (time to onset/aggressiveness) is very strongly influenced by loss of apoptotic sensitivity. Loss

of the INK4A/ARF locus reduces apoptosis and accelerates tumor development in this model. This locus encodes two tumor suppressors: ARF, which is responsible for activating p53 in response to oncogene activation, and INK4A, which encodes the p16 CDK inhibitor. Thus, the loss in apoptotic sensitivity following deletion of the entire INK4A/ARF locus is due entirely to ARF. Indeed, when the E μ -myc transgenic mice were crossed with a INK4A (*p16*) knockout mouse, no reduction in apoptosis or acceleration of tumor development was observed. As expected, when p14ARF knockout mice were crossed with the E μ -myc mice, lymphomas demonstrated significantly reduced apoptosis and rapid tumor onset. Loss of p14ARF in this case appeared equivalent to loss of p53 or overexpression of BCL2. However, the tumors that arose in the E μ -myc p14ARF mice, despite showing low levels of apoptosis, were found to be just as chemosensitive as those tumors arising in the control mice. It was convincingly demonstrated that instead of dying by apoptosis, the tumor cells from the p14ARF knockouts entered premature senescence. Thus, in these mice, loss of apoptosis simply revealed an equally sensitive form of cell death that took longer to manifest—premature senescence. It is likely that this principle is also present for other forms of cell death in a variety of other human tumors. This concept may explain in part the poor correlation between apoptotic sensitivity and treatment response that is found in the literature (7,9,11,12,15–19).

The example shown above also illustrates the importance of the dose–response relationship between apoptosis and the treatment agent. Modifying the dose–response relationship for a particular form of cell death will only affect overall treatment sensitivity if this is the most sensitive form of cell death (Fig. 1). There are many examples in the literature using genetically matched cell lines in which dramatic changes in apoptotic sensitivity do not lead to any change in overall treatment sensitivity as assessed by clonogenic survival. For example, we have shown that HCT116 cells can be dramatically sensitized to undergo apoptosis in response to a variety of DNA-damaging agents following knockout of the *p21* gene (18). However, when treatment sensitivity is assessed by a clonogenic assay to these same agents, no differences are found between the matched cell lines. Clearly in this case, induction of cell death by alternative modes of cell death occurred at doses far lower than those required to induce apoptosis—even in the cell line that exhibited increased apoptosis because of loss of the *p21* gene.

5. LABORATORY EVALUATION OF TREATMENT RESPONSES AND THEIR INTERPRETATIONS

As mentioned in Section 4, one of the principle reasons for the widely held view that apoptosis plays an important role in treatment sensitivity arises from the use *in vitro* and *in vivo* assays that are biased or inappropriate for assessing overall treatment sensitivity. Outlined below is a brief description of some of these assays and their strengths and limitations with respect to evaluation of treatment response.

5.1. *In Vitro*

A number of *in vitro* assays are used to assess treatment sensitivity, including those based primarily on the cell growth. The MTT assay, which measures the activity of a mitochondrial enzyme, or assays based on cellular protein or DNA content are often

considered measures of viability and surrogates for treatment sensitivity. However, these assays are chiefly based on measurements of cell number. These assays are normally executed several days after exposure of cells to a damaging agent and as such are influenced not only by cell death but also by transient changes in the rate of cell growth. As many of the same genes that influence cell death also influence cell proliferation, especially in the case of apoptosis, it is difficult or impossible to interpret overall treatment sensitivity from these types of assays. A good example is data from the NCI60 cell line database suggesting that p53 is a strong predictor of treatment sensitivity (73). In this study, treatment sensitivity was evaluated by a short-term (2-day) growth assay. As p53 elicits temporary cell-cycle blocks in response to DNA damage, it is not surprising that this assay would produce such a result. Regardless of how it is performed, any assay that is performed at such short times after treatment will by definition ignore any forms of cell death that occur after longer times. Indeed, critical analysis of the role of p53 in response to radiation (74) and other treatment stresses has shown that it is not a reliable predictor of response.

As an alternative, several *in vitro* assays are based on the detection of specific modes of cell death including apoptosis. The least effective assays do not evaluate treatment at the cellular level but instead measure an average or population response. These assays include those that measure changes in the average level of caspase activity or the amount of DNA fragmentation. These assays are useful to demonstrate that apoptosis is occurring but are relatively non-quantitative. Somewhat better are cell-based assays for apoptosis that evaluate the known features of apoptosis such as DNA condensation or exposure of phosphatidyl serine at the cell surface. Other modes of cell death can similarly be identified using assays based on the known morphological and/or biochemical features of that particular form of cell death. For example, senescence is often detected by an increase in cellular β -galactosidase activity.

There are two fundamental limitations of all these death-based assays that limit their usefulness as measures of treatment sensitivity. The first is that they give a picture of the response only at the point in time—the time at which the cell population is evaluated. Given the fact that cells in a population can die by several different processes that each operate with different kinetics that may further vary in different cell types, one can never be sure that the cells that remain viable at the time of assessment will not subsequently die by some form of cell death. This can be partially solved by making continuous measurements as a function of time. However, this introduces further complication as surviving cells begin to proliferate and dilute the dead cells. Furthermore, as the end stages of apoptosis can result in complete cell destruction, the apoptotic cells are also eliminated from assessment as time progresses. This is particularly a problem when attempting to assess cell death by these assays *in vivo*. The second major problem is that it is difficult or perhaps even impossible to simultaneously assess all possible modes of cell death. Consequently, these death-based assays can never give a full picture of treatment response.

The solution to the problem of identifying all forms of cell death was solved in 1956 by Puck and Markus, who developed an assay based on the ability of a single cell to grow into a colony. This “clonogenic assay” has formed the basis of *in vitro* cellular response studies in tumors and also some normal tissues (75). The clonogenic assay tests for the ability of a cell to recover from treatment in such a way that it can proliferate again and form a clone of substantial size (normally evaluated 10–20 days after

treatment). It thus can measure the ability of a cell to survive from all possible (known and unknown) forms of cell death. This assay is analogous to the well-accepted and well-proven assays of treatment sensitivity in other organisms such as yeast and bacteria.

Clonogenic survival shows a typical log-linear relationship with treatment dose implying that the probability of cell kill increases in a roughly linear way with dose (Fig. 1). In other words, each incremental increase in dose kills the same percentage of remaining cells. This relationship is well supported by *in vivo* dose responses of tumors containing large numbers of cells, which correspondingly require a very low percentage of cell survival for cure. The relationship between treatment dose and clonogenic survival established *in vitro* has successfully predicted the doses required to cure transplanted tumors *in vivo* even when rates of apoptosis do not correlate (18,76,77). For example, given the fact that a 1 cm³ tumor contains more than 10⁹ cells, treatment requires killing more than 99.9999999% of the cells to have a chance at being effective. The log-linear relationship of cell survival and treatment dose allows one to predict the curative doses required to reach these levels.

This numerical example also exemplifies the importance of comparing dose-response relationships for the various death processes when evaluating their relative importance. For example, if a particular treatment dose results in 0.1% survival (99.9% death) as assessed by a clonogenic assay, but causes only 35% apoptosis, one can conclude that apoptosis is not an important contributor to cell death. The fact that 99.9% of cells are killed implies that cell death is equally effective in the 65% of cells that do not die by apoptosis. One can easily imagine in this case that substantial increases or decreases in apoptosis induction would not affect clonogenic survival. The literature is full of examples in which modest levels of cell death are assessed using treatment doses that will inactivate several logs of cell kill if assessed by the clonogenic assay. Obviously, the relevance of such studies is highly questionable.

The clonogenic assay is not without problems. The assay often involves plating of dilute concentrations of cells under conditions that are significantly different from those found *in vivo*. Furthermore, the *in vitro* conditions ignore the unique microenvironmental parameters of a tumor that can be important contributors to treatment sensitivity such as oxygen and cell-to-cell contact. Several investigators have also pointed out that the long-term culture of cells *in vitro* can result in selection of resistant clones that are not reflective of the original cells *in vivo*. In some instances, it is thus desirable to use primary cells derived from tumors *in vivo*. In this case, it is important that these primary cells can also tolerate *in vitro* culture conditions without substantial death even without treatment. This can be assessed by the plating efficiency—a measure of the number of cells that retain clonogenic capacity in the absence of treatment. When this value is very low (less than 5%), the predictive power of the clonogenic assay comes into question. However, such a low-plating efficiency is indicative of the fact that the cells do not tolerate *in vitro* culture well and thus place doubts on any *in vitro* assays that may be used with these cells. Thus, we feel strongly that the clonogenic assay is far and away the best *in vitro* tool for assessing treatment response.

5.2. *In Vivo*

There is clearly a need to evaluate cellular treatment sensitivity within the context of the normal environment, a so-called *in vivo* assay. However, even in this context, several common assays can be highly biased or inappropriate for evaluating treatment

response. In particular, assays that are based primarily on evaluating or comparing tumor size (or presence) at fixed times after treatment are difficult to interpret and heavily biased toward tumors that display rapid forms of cell death like apoptosis. For example, investigators may treat either spontaneous or transplanted tumors and then evaluate the size of the tumor at one or more times after treatment. This type of experiment is heavily influenced by the intrinsic tumor growth rate, which is itself influenced by cell loss that occurs through death mechanisms such as apoptosis. A tumor that suffers a high loss factor will grow much more slowly than a similar tumor with a lower cell loss factor. To illustrate this problem, we can consider two different tumors that behave identically with the exception that one has a much slower growth rate because of a higher rate of spontaneous apoptosis (Fig. 2). If we treat each of these two tumors with a dose that produces the same amount of overall cell kill (the same level of survival), it will take substantially longer for the slow-growing tumor to reappear. This may cause misinterpretation of the treatment sensitivity as the “time to relapse” was significantly longer in the slow-growing (apoptotically sensitive) tumor, even though treatment sensitivity and curability are identical. In fact, at any time after treatment, the slow-growing tumor will appear smaller than the other tumor leading one to this mistaken conclusion.

Misinterpretations of treatment sensitivity can also result from differences in the kinetics of cell death following treatment. Again, let us consider a hypothetical situation in which two tumors have equal growth rates and equal overall responses to treatment (Fig. 2). However, in this case, cell death in one of the tumors occurs principally through a rapid apoptotic mechanism and in the other through a much slower mechanism based on mitotic catastrophe. If one follows the size of the tumors after treatment, the apoptotic tumor will regress rapidly because of the induction of apoptosis. The other tumor, although it will eventually show an equivalent loss in cell number, will not regress quickly and thus “appear” to respond more poorly. Again, assessment of tumor size at a fixed time after treatment will not yield the correct information on treatment sensitivity. Owing to both of the factors illustrated in these examples, comparison of tumor size at fixed times after treatment is nearly impossible to relate to overall treatment sensitivity.

A much better way to evaluate the treatment sensitivity of tumors is to measure the growth *delay* that results from treatment. The growth delay is a measure of the difference in times for treated and untreated tumors to reach a certain size (e.g., three times the starting volume). For this value to be meaningful, it must be evaluated at a sufficiently long time after treatment such that all forms of cell death have had an opportunity to have taken place. At this time, the tumor should be growing at a rate that is equivalent again to the untreated tumor (Fig. 2). The difference in time required for the control and treated tumors to reach equivalent sizes directly reflects the percentage of cells that survived the treatment. As seen in Fig. 2, this value is completely independent of the rate of tumor regression after treatment. To remove the problems associated with comparing tumors that have different intrinsic growth rates (our first example above), one must use the specific growth delay (78). This is simply equal to the growth delay expressed as a fraction of the growth rate (e.g., time to reach three times starting volume) of the untreated tumor.

Although measurements of growth delay correct for the two errors outlined in our example, they do suffer from other potential problems. Tumor growth rate can be

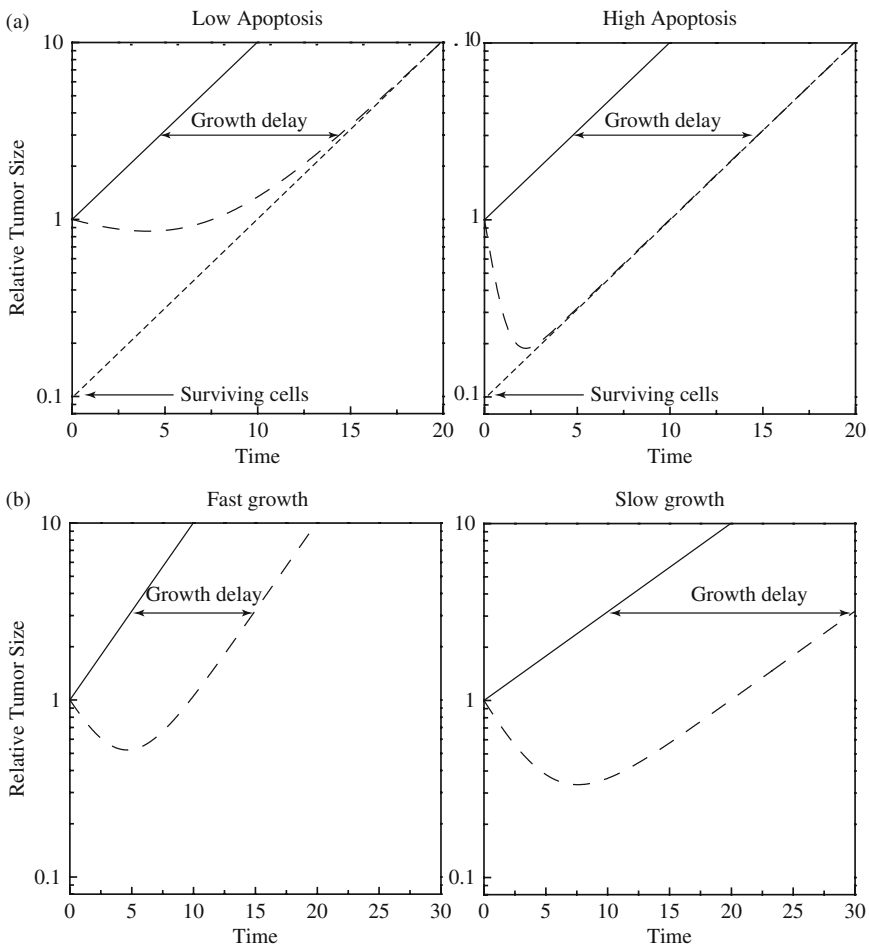


Fig. 2. Interpretation of tumor responses *in vivo*. These illustrations highlight two distinct ways that apoptosis can result in misinterpretation of *in vivo* treatment responses. (A) Plotted are the hypothetical responses of two tumors with the same growth rates and same overall response to treatment. In the left panel, the tumor cells die primarily through non-apoptotic pathways. In the right panel, cells die primarily through apoptotic pathways. Comparison of the size of the tumors after treatment leads one to the incorrect conclusion that the apoptosis-prone tumor is more sensitive. In this case, the tumor cells die quickly leading to a large tumor regression. However, in both cases, the same numbers of cells die, and after a sufficiently long period of time (when the size of the tumor is determined solely by the survivors), the two tumors are identical. The correct evaluation of response is made by comparing the growth delay at a point where the tumor has restored its normal growth rate. (B) Plotted are the hypothetical responses of two tumors that again have an identical overall treatment sensitivity (the same survival) but very different rates of growth. In this case, the slow growth of the tumor in the left panel could be attributed to high spontaneous rates of apoptosis. Despite the fact that the same numbers of cells survive, the slow-growing tumor has a much longer growth delay. This difference arises not because of a difference in sensitivity but rather to the trivial explanation that the tumor simply grows slower and thus takes a longer time to reach its evaluation size. The correction to make in this case is to use specific growth delay (see text).

affected by treatment in ways that are unrelated to the overall amount of cell death of the tumor cells. For example, damage to tumor vasculature may slow tumor growth in a way that is unrelated to killing the tumor cells (79). This extra growth delay may be incorrectly interpreted as increased cell death even though no additional tumor cells may be killed.

The gold standard of all assays of treatment sensitivity is thus those that evaluates tumor cure. This is evaluated by a so-called TCD50 assay, in which the dose required to cure 50% of the tumors is calculated. In these experiments, cure typically follows a sigmoidal function of treatment dose. Although this is the best of all assays, there has been a steady decrease in its use. This is likely due to the fact that a large number of mice must be used to accurately define the dose–response relationship. Furthermore, this assay takes a comparatively long time, typically requiring many months to be sure that cure has been achieved. The TCD50 assay is somewhat analogous to the *in vitro* clonogenic assay, as it ultimately measures the probability that a single cell can survive and reform the tumor. For example, at doses that will cure 50% of treated tumors, the average number of surviving cells per tumor is just slightly above 1. The assay is unaffected by rates of tumor growth or rates of cell death. When performed correctly, this assay provides the ultimate measure of overall treatment sensitivity.

The TCD50 assay also allows one to consider the possible existence of tumor stem cells. This is an old concept that has received renewed interest and implies that only a small fraction of tumor cells are truly clonogenic and capable of unlimited proliferation (80,81). This concept may also explain the poor “plating efficiency” that has been observed when primary tumor cells are cultured *in vitro*. The bulk of the tumor cells thus represent somewhat more differentiated cells that lack this clonogenic capacity. Obviously in such a case, it becomes important to evaluate the response of the tumor stem cells and not the response of the bulk of the tumor. As the TCD50 is influenced only by those cells that have the ability to both survive and reform the tumor, it accurately reflects the treatment sensitivity of the relevant cells in the tumor. The behavior of the non-stem cells in this case is irrelevant.

6. CONCLUSIONS

Our understanding of the molecular basis and regulation of the apoptotic pathway and its importance in cancer has, somewhat paradoxically, clouded our ability to understand the important molecular determinants of treatment response. Experiments demonstrating that apoptosis could indeed influence treatment sensitivity have contrasted with other studies showing that dramatic changes in apoptosis can occur without affecting the overall treatment response. Thus, the question of whether apoptosis, or the genes controlling it, is important for cancer therapy remains controversial.

We have argued that conflicting conclusions over the importance of apoptosis results both from studies with model systems that cannot be extrapolated to most human tumors and from the use of assays that may be heavily biased toward detection of specific modes of cell death. Several elegant genetic models have convincingly demonstrated that it is possible to create transformed cells that become, and retain, extreme sensitivity to apoptosis. In these cases, apoptosis becomes the most sensitive form of cell death in response to treatment and is thus a dominant determinant of overall response. However, in real human cancers, apoptotic sensitivity is dramatically

lower and correspondingly also less important (or even unimportant) for treatment response. The important point is that all the different forms of cell death outlined in Section 2 are potentially important in tumors of various types. Proper evaluation of treatment response thus requires careful analysis of each of these forms of cell death prior to making any conclusions. This is most accurately assessed *in vitro* by use of the clonogenic assay that is capable of integrating all forms of cell death. Similarly, *in vivo* assays of tumor response must also be conducted in ways that allow evaluation of all forms of cell death. Short-term assays that are influenced by tumor growth rates or the rates of regression should be avoided. Proper selection and interpretation of these assays is necessary for future experiments aimed at evaluating the importance of apoptosis and other determinants of cell death and survival.

The finding that apoptosis can, but rarely does, contribute to tumor response has important implications on how to move forward with research into better treatments for cancer. Especially important is to not make the mistake to generalize this finding to all forms of cell death. Just because apoptosis, and the genes controlling it, may not be important for treatment sensitivity in many tumors does not mean that the genotype of cancer cells is an unimportant factor during treatment. Cells that die as a result of non-apoptotic mechanisms may still be highly influenced by cancer-associated mutations. Particularly important in many tumors of epithelial origin are the molecular determinants of mitotic catastrophe. These include the more than 130 known DNA repair proteins that play crucial roles in determining treatment sensitivity (82). The importance of the DNA damage response and in particular DNA repair on treatment sensitivity has been demonstrated repeatedly for a vast number of DNA-damaging agents (83). Loss of critical proteins that participate in the DNA damage response, for example, the proteins required for the repair of double-strand breaks by ionizing radiation, almost universally cause sensitivity to DNA-damaging agents (84). This finding also supports the argument that activation of apoptosis and/or other forms of cell death in response to *initial* DNA damage is rarely important as a determinant of treatment sensitivity. DNA repair, which may require several days for completion, remains a critical determinant of cell survival. In these cases, cell death results only in the cells that are incapable of repair or that undergo misrepair. These cells tend to die at comparatively long times after treatment and importantly after mitotic catastrophe (74). Continued research aimed at improving our understanding of this and other forms of cell death will undoubtedly lead to new approaches for improving cancer treatment.

REFERENCES

1. Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem* 1999;68:383–424.
2. Okada H, Mak TW. Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat Rev Cancer* 2004;4(8):592–603.
3. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100(1):57–70.
4. Graeber TG, Osmanian C, Jacks T, et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 1996;379(6560):88–91.
5. Kerr JF, Winterford CM, Harmon BV. Apoptosis. Its significance in cancer and cancer therapy. *Cancer* 1994;73(8):2013–26.
6. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 2002;108(2):153–64.

7. Aldridge DR, Arends MJ, Radford IR. Increasing the susceptibility of the rat 208F fibroblast cell line to radiation-induced apoptosis does not alter its clonogenic survival dose-response. *Br J Cancer* 1995;71(3):571–7.
8. Brown JM, Attardi LD. The role of apoptosis in cancer development and treatment response. *Nat Rev Cancer* 2005;5(3):231–7.
9. Brown JM, Wilson G. Apoptosis genes and resistance to cancer therapy: what does the experimental and clinical data tell us? *Cancer Biol Ther* 2003;2(5):477–90.
10. Ekert PG, Read SH, Silke J, et al. Apaf-1 and caspase-9 accelerate apoptosis, but do not determine whether factor-deprived or drug-treated cells die. *J Cell Biol* 2004;165(6):835–42.
11. Han JW, Dionne CA, Kedersha NL, Goldmacher VS. p53 status affects the rate of the onset but not the overall extent of doxorubicin-induced cell death in rat-1 fibroblasts constitutively expressing c-Myc. *Cancer Res* 1997;57(1):176–82.
12. Lock RB, Stribinskiene L. Dual modes of death induced by etoposide in human epithelial tumor cells allow Bcl-2 to inhibit apoptosis without affecting clonogenic survival. *Cancer Res* 1996;56(17):4006–12.
13. Merritt AJ, Allen TD, Potten CS, Hickman JA. Apoptosis in small intestinal epithelial from p53-null mice: evidence for a delayed, p53-independent G2/M-associated cell death after gamma-irradiation. *Oncogene* 1997;14(23):2759–66.
14. Ruth AC, Roninson IB. Effects of the multidrug transporter P-glycoprotein on cellular responses to ionizing radiation. *Cancer Res* 2000;60(10):2576–8.
15. Tannock IF, Lee C. Evidence against apoptosis as a major mechanism for reproductive cell death following treatment of cell lines with anti-cancer drugs. *Br J Cancer* 2001;84(1):100–5.
16. Westphal CH, Rowan S, Schmaltz C, Elson A, Fisher DE, Leder P. atm and p53 cooperate in apoptosis and suppression of tumorigenesis, but not in resistance to acute radiation toxicity. *Nat Genet* 1997;16(4):397–401.
17. Wouters BG, Denko NC, Giaccia AJ, Brown JM. A p53 and apoptotic independent role for p21waf1 in tumour response to radiation therapy. *Oncogene* 1999;18(47):6540–5.
18. Wouters BG, Giaccia AJ, Denko NC, Brown JM. Loss of p21Waf1/Cip1 sensitizes tumors to radiation by an apoptosis-independent mechanism. *Cancer Res* 1997;57(21):4703–6.
19. Yin DX, Schimke RT. BCL-2 expression delays drug-induced apoptosis but does not increase clonogenic survival after drug treatment in HeLa cells. *Cancer Res* 1995;55(21):4922–8.
20. Brown JM, Wouters BG. Apoptosis, p53, and tumor cell sensitivity to anticancer agents. *Cancer Res* 1999;59(7):1391–9.
21. Abend M. Reasons to reconsider the significance of apoptosis for cancer therapy. *Int J Radiat Biol* 2003;79(12):927–41.
22. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972;26(4):239–57.
23. Alnemri ES, Livingston DJ, Nicholson DW, et al. Human ICE/CED-3 protease nomenclature. *Cell* 1996;87(2):171.
24. Salvesen GS, Dixit VM. Caspases: intracellular signaling by proteolysis. *Cell* 1997;91(4):443–6.
25. Holcik M, Gibson H, Korneluk RG. XIAP: apoptotic brake and promising therapeutic target. *Apoptosis* 2001;6(4):253–61.
26. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol* 1999;15:269–90.
27. Fridman JS, Lowe SW. Control of apoptosis by p53. *Oncogene* 2003;22(56):9030–40.
28. Shinoura N, Sakurai S, Asai A, Kirino T, Hamada H. Over-expression of APAF-1 and caspase-9 augments radiation-induced apoptosis in U-373MG glioma cells. *Int J Cancer* 2001;93(2):252–61.
29. Taneja N, Tjalkens R, Philbert MA, Rehemtulla A. Irradiation of mitochondria initiates apoptosis in a cell free system. *Oncogene* 2001;20(2):167–77.
30. Soengas MS, Alarcon RM, Yoshida H, et al. Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science* 1999;284(5411):156–9.
31. Bouillet P, Strasser A. BH3-only proteins - evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. *J Cell Sci* 2002;115(Pt 8):1567–74.
32. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2002;2(9):647–56.

33. Kluck RM, Esposti MD, Perkins G, et al. The pro-apoptotic proteins, Bid and Bax, cause a limited permeabilization of the mitochondrial outer membrane that is enhanced by cytosol. *J Cell Biol* 1999;147(4):809–22.
34. Schmitt CA, McCurrach ME, de Stanchina E, Wallace-Brodeur RR, Lowe SW. INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. *Genes Dev* 1999;13(20):2670–7.
35. Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 1993;362(6423):847–9.
36. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res* 1961;25:585–621.
37. MacLaren A, Black EJ, Clark W, Gillespie DA. c-Jun-deficient cells undergo premature senescence as a result of spontaneous DNA damage accumulation. *Mol Cell Biol* 2004;24(20):9006–18.
38. Meng A, Wang Y, Van Zant G, Zhou D. Ionizing radiation and busulfan induce premature senescence in murine bone marrow hematopoietic cells. *Cancer Res* 2003;63(17):5414–9.
39. Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc Natl Acad Sci USA* 1995;92(20):9363–7.
40. Stein GH, Drullinger LF, Soulard A, Dulic V. Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. *Mol Cell Biol* 1999;19(3):2109–17.
41. Narita M, Nunez S, Heard E, et al. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* 2003;113(6):703–16.
42. Kamiyo T, Zindy F, Roussel MF, et al. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* 1997;91(5):649–59.
43. Sage J, Miller AL, Perez-Mancera PA, Wysocki JM, Jacks T. Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature* 2003;424(6945):223–8.
44. Brookes S, Rowe J, Ruas M, et al. INK4a-deficient human diploid fibroblasts are resistant to RAS-induced senescence. *EMBO J* 2002;21(12):2936–45.
45. Chang BD, Broude EV, Dokmanovic M, et al. A senescence-like phenotype distinguishes tumor cells that undergo terminal proliferation arrest after exposure to anticancer agents. *Cancer Res* 1999;59(15):3761–7.
46. Elmore LW, Di X, Dumur C, Holt SE, Gewirtz DA. Evasion of a single-step, chemotherapy-induced senescence in breast cancer cells: implications for treatment response. *Clin Cancer Res* 2005;11(7):2637–43.
47. Braig M, Lee S, Loddenkemper C, et al. Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* 2005;436(7051):660–5.
48. Chen Z, Trotman LC, Shaffer D, et al. Crucial role of p53-dependent cellular senescence in suppression of tumor-deficient tumorigenesis. *Nature* 2005;436(7051):725–30.
49. Collado M, Gil J, Efeyan A, et al. Tumour biology: senescence in premalignant tumours. *Nature* 2005;436(7051):642.
50. Michaloglou C, Vredeveld LC, Soengas MS, et al. BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* 2005;436(7051):720–4.
51. Chu K, Teele N, Dewey MW, Albright N, Dewey WC. Computerized video time lapse study of cell cycle delay and arrest, mitotic catastrophe, apoptosis and clonogenic survival in irradiated 14-3-3sigma and CDKN1A (p21) knockout cell lines. *Radiat Res* 2004;162(3):270–86.
52. Russell P, Nurse P. cdc25+ functions as an inducer in the mitotic control of fission yeast. *Cell* 1986;45(1):145–53.
53. Xu B, Kim ST, Lim DS, Kastan MB. Two molecularly distinct G(2)/M checkpoints are induced by ionizing irradiation. *Mol Cell Biol* 2002;22(4):1049–59.
54. Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B. 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* 1999;401(6753):616–20.
55. Nitta M, Kobayashi O, Honda S, et al. Spindle checkpoint function is required for mitotic catastrophe induced by DNA-damaging agents. *Oncogene* 2004;23(39):6548–58.
56. Yarden RI, Pardo-Reoyo S, Sgagias M, Cowan KH, Brody LC. BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage. *Nat Genet* 2002;30(3):285–9.
57. Jordan MA, Wendell K, Gardiner S, Derry WB, Copp H, Wilson L. Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. *Cancer Res* 1996;56(4):816–25.

58. Huang WP, Klionsky DJ. Autophagy in yeast: a review of the molecular machinery. *Cell Struct Funct* 2002;27(6):409–20.
59. Daido S, Yamamoto A, Fujiwara K, Sawaya R, Kondo S, Kondo Y. Inhibition of the DNA-dependent protein kinase catalytic subunit radiosensitizes malignant glioma cells by inducing autophagy. *Cancer Res* 2005;65(10):4368–75.
60. Noda T, Ohsumi Y. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J Biol Chem* 1998;273(7):3963–6.
61. Bursch W, Hocheegger K, Torok L, Marian B, Ellinger A, Hermann RS. Autophagic and apoptotic types of programmed cell death exhibit different fates of cytoskeletal filaments. *J Cell Sci* 2000;113 (Pt 7):1189–98.
62. Schwartz LM, Smith SW, Jones ME, Osborne BA. Do all programmed cell deaths occur via apoptosis? *Proc Natl Acad Sci USA* 1993;90(3):980–4.
63. Isahara K, Ohsawa Y, Kanamori S, et al. Regulation of a novel pathway for cell death by lysosomal aspartic and cysteine proteinases. *Neuroscience* 1999;91(1):233–49.
64. Xue L, Fletcher GC, Tolkovsky AM. Autophagy is activated by apoptotic signalling in sympathetic neurons: an alternative mechanism of death execution. *Mol Cell Neurosci* 1999;14(3):180–98.
65. Liang XH, Jackson S, Seaman M, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 1999;402(6762):672–6.
66. Nicotera P, Melino G. Regulation of the apoptosis-necrosis switch. *Oncogene* 2004;23(16):2757–65.
67. Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB. Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes Dev* 2004;18(11):1272–82.
68. Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature* 2001;411(6835):342–8.
69. Schmitt CA, Rosenthal CT, Lowe SW. Genetic analysis of chemoresistance in primary murine lymphomas. *Nat Med* 2000;6(9):1029–35.
70. Aldridge DR, Radford IR. Explaining differences in sensitivity to killing by ionizing radiation between human lymphoid cell lines. *Cancer Res* 1998;58(13):2817–24.
71. Bearss DJ, Subler MA, Hundley JE, Troyer DA, Salinas RA, Windle JJ. Genetic determinants of response to chemotherapy in transgenic mouse mammary and salivary tumors. *Oncogene* 2000;19(8):1114–22.
72. Schmitt CA, Fridman JS, Yang M, et al. A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. *Cell* 2002;109(3):335–46.
73. Weinstein JN, Myers TG, O'Connor PM, et al. An information-intensive approach to the molecular pharmacology of cancer. *Science* 1997;275(5298):343–9.
74. Gudkov AV, Komarova EA. The role of p53 in determining sensitivity to radiotherapy. *Nat Rev Cancer* 2003;3(2):117–29.
75. Puck TT, Marcus PI. Action of x-rays on mammalian cells. *J Exp Med* 1956;103(5):653–66.
76. Bunz F, Hwang PM, Torrance C, et al. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J Clin Invest* 1999;104(3):263–9.
77. Waldman T, Zhang Y, Dillehay L, et al. Cell-cycle arrest versus cell death in cancer therapy. *Nat Med* 1997;3(9):1034–6.
78. Begg A. Principles and practice of the tumor growth delay assay. In: Kallman R, ed. *Rodent Tumor Models in Experimental Cancer Therapy*. New York: Pergamon Press; 1987:114.
79. Zips D, Hessel F, Krause M, et al. Impact of adjuvant inhibition of vascular endothelial growth factor receptor tyrosine kinases on tumor growth delay and local tumor control after fractionated irradiation in human squamous cell carcinomas in nude mice. *Int J Radiat Oncol Biol Phys* 2005;61(3):908–14.
80. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003;100(7):3983–8.
81. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature* 2004;432(7015):396–401.
82. Wood RD, Mitchell M, Sgouros J, Lindahl T. Human DNA repair genes. *Science* 2001;291(5507):1284–9.
83. Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. *Nature* 2001;411 (6835):366–74.
84. Collis SJ, DeWeese TL, Jeggo PA, Parker AR. The life and death of DNA-PK. *Oncogene* 2005;24(6):949–61.

4

Mitotic Catastrophe

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SUMMARY

Mitotic catastrophe (MC) is the result of premature or inappropriate entry of cells into mitosis, usually occurring because of chemical or physical stresses. MC is characterized by changes in nuclear morphology and the eventual appearance of polyploid cell progeny in affected cell populations, is markedly enhanced in cells lacking p53 function, and is the result of overaccumulation of cyclin B1 in cells delayed late in the cell cycle by the inducing agent. Thus, MC is considered to be the predominant mechanism underlying mitotic-linked cell death. Along with characteristic features associated with MC, a delayed DNA damage phenotype has been noted in these populations, suggesting a potential role for MC in mutagenesis and the acquisition of genomic instability. Although generally lethal, some cells can survive MC through mechanisms that are incompletely understood. Cytological features associated with meiotic cell division have been noted in polyploid cell populations produced through MC, a finding that might be particularly relevant in the understanding of tumor progression and that might provide a novel mechanism for the generation of quasi-diploid progeny from MC-induced polyploid cell populations. This review summarizes the literature pertaining to MC and describes current lines of research in this interesting research area.

Key Words: Mitotic catastrophe; cell cycle regulation; cyclin B1; endopolyploid cells; mitosis; meiosis; carcinogenesis; tumor progression; delayed DNA damage; SPCC.

1. MOLECULAR MECHANISMS UNDERLYING MITOTIC CATASTROPHE

1.1. Mitotic Catastrophe is the Result of Premature Entry into Mitosis Following Abrogation of G2/M Checkpoint Function

Exposure of some cell types to a broad class of agents can lead to a loss of regulation of cell division, such that cells enter into a premature mitosis, an event that culminates in a phenomenon called mitotic catastrophe (MC). MC is characterized by the aberrant

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nuclear morphology observed following premature mitotic entry (1) and often results in the generation of aneuploid and polyploid cell progeny. Ultrastructural studies of HeLa cells (2,3) during long-duration hyperthermia characterized a general disorganization of cellular organelles that occurs in cells undergoing spontaneous premature chromosome condensation (SPCC). Cells undergoing SPCC can either fail to achieve cytokinesis (early-SPCC) or divide and fuse shortly thereafter (late-SPCC) (4). The early and late classifications arose based on two different observations: in the case of Chinese hamster ovary (CHO) and HeLa cells heated for up to 24 h at 41.5°C, cells arrest in mid-S phase and SPCC figures observed in cell preparations have the characteristic appearance of cells in S phase undergoing chromosome condensation due to mitotic factors (5), thus producing early-SPCC. Following radiation and other agents that block primarily in late S and G2 phases, the condensed chromosome morphology is more fibrillar, looking identical to the morphology obtained when a G2 phase cell is induced to prematurely enter mitosis (5); thus, these cells are termed late-SPCC. The difficulty in distinguishing SPCC from normal mitoses in some cell lines, especially late-SPCC, has led to the use of nuclear fragmentation as an endpoint indicative of MC; studies have shown that these two quantities correlate well with SPCC (4,6).

Stress-induced SPCC and subsequent MC are found following activation of cyclin B1/cdc2 kinase complex occurring while cells are delayed in S or G2 phases (4,7), indicating that stress-induced MC is the result of abrogation of cell cycle regulatory pathways, in particular the G2 checkpoint (8–10). MC has been found to occur in cells exposed to a variety of treatments, including adriamycin (11), 5-fluorouracil (12), etoposide (13), temozolomide (14), topotecan (15), camptothecin (16), combretastatin (17), paclitaxel (13,18), phytoestrogens (19), overexpression of c-H Ras (20), prolonged mild heat shock (17,21,22), UV radiation (23), methyl-methanesulfonate (23), ionizing radiation (4,24–26), and hyperthermia combined with radiation (27). Specific effects on mitotic spindle formation and mitotic microtubule stability, mediated by cyclin B1/cdc2 kinase activity, have been identified as playing an important role in MC (28,29). Furthermore, there is evidence in support of the notion that cell cycle delays play a critical role in the development of SPCC and MC. All the aforementioned studies reported that delays late in the cell cycle were observed prior to MC. In SPCC and MC induced by long-duration mild heat shock of HeLa cells, blockage in G1 during the heat treatment using either cycloheximide or caffeine (both agents known to induce a G1 block in HeLa cells) results in a reduction in S delay, SPCC, and cytotoxicity (30). Inhibition of cell cycle progression following radiation exposure leads to protective effects on cell survival, which has prompted some authors to consider that repair of potentially lethal damage can occur during the arrest interval (31). It has been proposed that one of the cellular functions of mutations in the tumor-suppressor gene p53 is to promote MC as a mechanism for removing damaged cells from populations following genotoxic stress (32). The mechanisms underlying this function of p53 involve its activity as a modulator of G2 checkpoint mechanisms.

1.2. p53 is an Important Repressor of MC

All living organisms have evolved mechanisms to regulate the timing of cell division within the context of the completion of DNA replication. Events associated with mitosis, such as nuclear envelope breakdown, chromosome condensation, and assembly of the mitotic apparatus are mediated through the specific phosphorylation of cellular proteins

by large multi-protein complexes containing, in addition to other proteins, the cdc2 and cyclin B1 gene products. The activity of this complex is stimulated through the action of the cdc25C phosphatase; inhibition is mediated by wee1 and other inhibitory kinases; in both cases, changes in the phosphorylation state of key residues of cdc2 result in changes in cyclin-dependent kinase activity. Cdc25C activity is inhibited in the presence of unreplicated DNA, whereas inhibitory kinases such as wee1 are constitutively active. Cyclin B1 biosynthesis also contributes to the regulation of mitotic entry, as cyclin B1 levels are cell cycle regulated, with the gene being expressed only in late S and G2 phases in human cells (33); proteasome-mediated degradation of cyclin B1 begins in anaphase, resulting in undetectable levels of the protein by the time cells enter the next S phase (34). These pathways act in concert to ensure that mitosis does not commence prior to the completion of DNA replication. Other mechanisms function to inhibit entry into mitosis if DNA is damaged, resulting in a G2 block; there are many pathways involved here, some are p53-dependent, and others are not. The end result is to reduce the activity of cyclin B1/cdc2-containing complexes, and the mechanisms that have been elucidated demonstrate the incredible diversity at play in biological organisms. Chk1, chk2, atm, and atr gene products all contribute to the activation of p53 in response to genotoxic stress, whereas p53-mediated inhibition of cyclin B1/cdc2 activity can occur through expression of the cdk inhibitor p21, direct effects on cyclin B1 or cdc2 biosynthesis, inhibition of cdc25C nuclear transport through chk1/chk2- and 14-3-3- σ -dependent pathways (35), or destabilization of cyclin B1/cdc2 complexes by gadd45 (36). One study (37) has also implicated chk2 in the phosphorylation-mediated inactivation of cdc25A and radiation-induced inhibition of DNA synthesis that is generally thought to be responsible for S phase delays following irradiation (38).

In the absence of checkpoint activation, mitosis can occur very rapidly, provided the right activating pathways predominate over the inactivating pathways (Fig. 1). When cells are damaged or otherwise perturbed from the usual sequence of cell cycle events, many of these regulatory pathways serve to check further progress through the cell cycle, until either a sensed deficiency (e.g., incomplete DNA replication) is ameliorated or some other imbalance is fixed. This balance between inhibitory and stimulatory pathways relies on the abundance of these same regulators, as it has been demonstrated that overexpression of cyclin B1 in G2-arrested cells leads to MC in genetic manipulation studies (8), independent of the activity of the inhibitory pathways. So, under ordinary conditions, these checkpoints serve to ensure that orderly progress through the cell cycle occurs. Under extraordinary conditions, however, these regulatory pathways can become abrogated, resulting in a deviation from the expected cellular behavior. MC is just one of these deviations.

1.3. MC Has Been Associated with Overexpression of Cyclin B1 in p53-Deficient Cells

Previous studies of heat- and radiation-induced MC in HeLa cells (4,7,27) have demonstrated that a common feature of the effect of the two modalities is the high accumulation of cyclin B1 that occurs prior to the appearance of MC. Further studies indicate that this phenomenon is not limited to HeLa cells, being also observed in hamster and mouse cell lines, as long as p53 is non-functional (24,39), and in human glioblastoma U87MG cells transduced with a dominant-negative p53 adenovirus

Alterations in cell cycle control pathways that lead to mitotic catastrophe

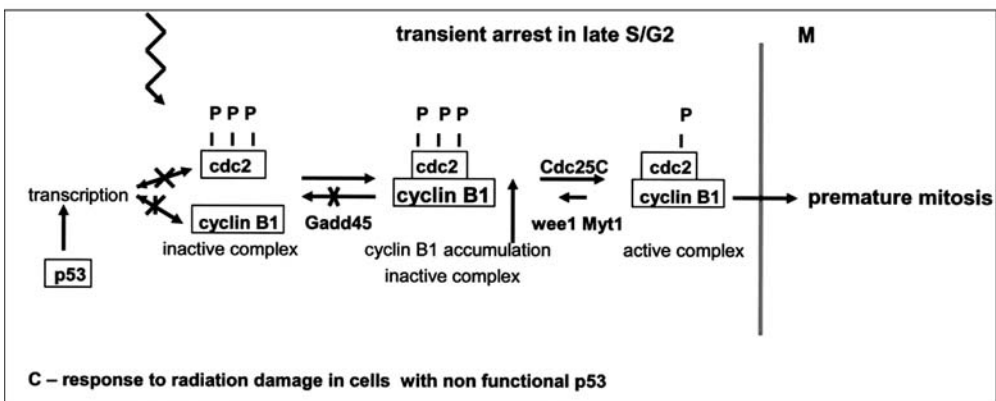
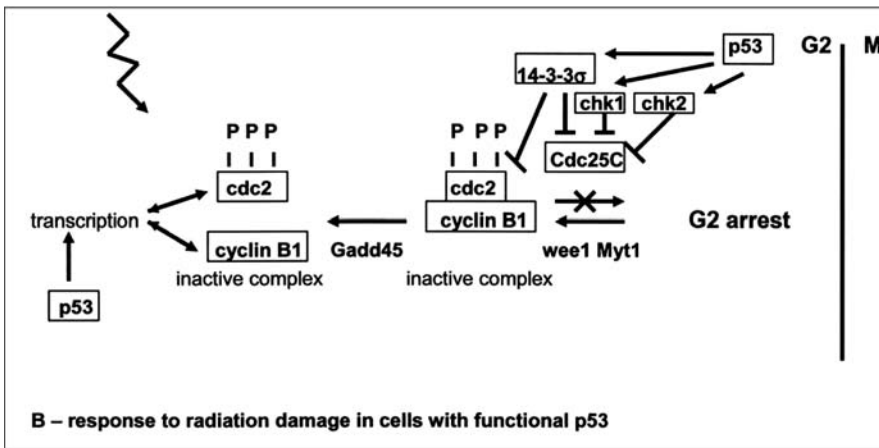
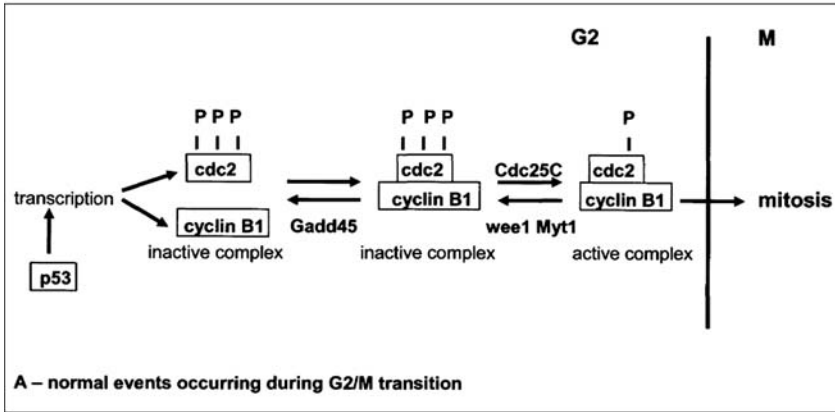


Fig. 1. A complex set of pathways acts to regulate entry of cells into mitosis, where active cyclin B1/cdc2 kinase complexes initiate nuclear envelope breakdown, chromosome condensation, assembly of the mitotic spindle, and other events required for cell division. Control of nuclear levels of activated cyclin B1/cdc2 complexes is accomplished through pathways related to biosynthesis, post-transcriptional modification, and intracellular transport of the component proteins in these complexes. In the nucleus, cyclin B1/cdc2 is normally maintained in an inactive state by the tyrosine kinases

construct that rendered the cells, otherwise wild-type p53, impaired in their p53 function (40). Furthermore, experiments have shown that in some cell lines, this elevation of cyclin B1 levels persists for several cell generations in the treated population. Although studies have demonstrated that p53 deficiency can lead to the disruption of many cell cycle regulatory pathways (9,41), other reports have demonstrated that effects of p53 on cyclin B1 gene expression alone can contribute significantly to MC (42). p53 acts to regulate cellular levels of cyclin B1, through a transcriptional repression mechanism that is not completely understood (43). Nevertheless, studies have shown that inhibition of p53-dependent regulation of cyclin B1 transcription results in MC in G2-arrested cells (14,42), and overexpression of cyclin B1 can overcome the G2 block (44). Other studies (45) reported that MC is independent of p21 (Waf-1) and 14-3-3- σ , but p21 can lead to G1 arrest in tetraploid cells produced during MC. Another study (46) demonstrated that p53-mediated transcriptional regulation of cyclin B1 requires a short region at the C-terminal regulatory domain of p53. Thus, it appears that, in addition to those p53-dependent pathways related to cyclin B1/cdc2 activation, at least one pathway has evolved that functions to simply repress cyclin B1 synthesis, and abrogation of this pathway can lead to MC and other subsequent effects on cell cycle regulation. Control of cyclin B1 levels during a G2 arrest is an important function, as elevation of cyclin B1 can reduce the level of control of other cell cycle regulators simply by mass action, if their levels are not increased accordingly (47). Nevertheless, the generality of this phenomenon as a toxicological mechanism underlying radiation-induced MC has not been established in a variety of human tumor cell lines. Radiation is an important treatment modality for a variety of human cancers. According to the American Cancer Society, in 2003, over a million persons were diagnosed with cancer in the USA. Radiation therapy will be an important clinical treatment for a significant fraction of these patients. However, an important complication associated with radiation therapy is the appearance of tumors containing radioresistant cells (48). Although mechanisms underlying this phenomenon are unknown, it has been suggested that radiation-induced chromosomal instability



Fig. 1. *wee1* and *myt1*: *wee1* inactivates *cdc2* kinase through an inhibitory tyrosine phosphorylation, whereas *myt1* inhibits *cdc2* through phosphorylation at threonine 14 and tyrosine 15 residues in a cyclin-dependent manner. Dephosphorylation of *cdc2* at threonine 14 and tyrosine 15 in late G2 by *cdc25C* promotes entry into mitosis. DNA damage activates the DNA-PK/ATM/ATR kinases triggering two parallel cascades of events that inactivate the cyclin B1/*cdc2* complex. In the first cascade, the *chk* kinases phosphorylate and inactivate *cdc25C*, thus preventing activation of *cdc2*. In the second cascade, p53 induces a set of genes that either inactivate or inhibit nuclear translocation of cyclin B1/*cdc2* complex: 14-3-3- σ binds to the phosphorylated cyclin B1/*cdc2* complex and prevents its transport into the nucleus (14-3-3- σ also acts to sequester *cdc25C* in the cytoplasm, thus preventing cyclin B1/*cdc2* complex activation in the nucleus); Gadd45 binds to and dissociates the cyclin B1/*cdc2* complex; and p21Cip1 inhibits *cdc2* kinase activity. In the absence of functional P53, those events usually leading to a decrease in nuclear cyclin B1/*cdc2* activity, along with the loss of P53-mediated transcriptional regulation of cyclin B1 and *cdc2* gene expression, results in an abnormally high accumulation of cyclin B1/*cdc2* complexes in the nucleus. Once there, we hypothesize that the negative regulatory effects of *wee1* and *myt1* are effectively diluted by the high levels of cyclin B1/*cdc2* present, resulting in premature mitotic entry and subsequent mitotic catastrophe. Panels: simplified cascade of events occurring at the border of G2/M in (A) an untreated cell; (B) an irradiated cell having functional P53; (C) an irradiated cell having non-functional P53.

may contribute to induced radioresistance (49). Moreover, as most human tumors have defects in p53 or p53-related pathways (32), they are resistant to treatments aimed at inducing death through apoptotic pathways. Thus, it is conceivable to infer that if MC is a major mode of radiation-induced cell death in cell lacking p53 function, clinical interventions designed to enhance its production in tumor cells might lead to a therapeutic gain. The elucidation of the mechanisms underlying radiation-induced MC might therefore have a major impact on the clinical effectiveness of radiation therapy.

2. STRESS-INDUCED MC

2.1. Cell Cycle Delays

Results have been reported (1,6,7) on the effects of MC induced by long-duration 41.5°C hyperthermia treatment in HeLa cells, and a role for increases in cyclin B1/cdc2 activity occurring during delays in S phase in the appearance of MC has been identified (7). Experimental conditions that inhibited the ability of cells to undergo the heat-induced block in S phase eliminated the induction of heat-induced MC and were protective toward cell survival (30). It was also reported that MC is a phenomenon distinct from apoptosis in HeLa cells (2). Reports on the effects of ionizing radiation in the induction of MC have also been published, in which the extent of cell cycle delays have been characterized following 0, 5, 10, and 20 Gy of X-ray exposure. These data (4) demonstrated that radiation-induced MC shares many features with heat-induced MC. Radiation-induced cell cycle delays occurred primarily in late S and G2 phases, as opposed to the mid-S delays observed at 41.5°C, and the morphology of chromatin in cells undergoing premature mitosis was different from that observed in the heat experiments, where heat produced primarily early-SPCC and radiation exposure led to the appearance of late-SPCC. Although the details of the phase-location of cell cycle delays and the morphology of SPCC were different for the two treatments, both heat and radiation exposure resulted in significant MC. For both stresses, cyclin B1 levels increased prior to the appearance of MC; for radiation, this increase in cyclin B1 was dose-dependent up to 10 Gy. The increased levels of cyclin B1 protein correlated well with increases in cyclin B1-dependent kinase activity; both increases appeared prior to MC in the treated populations. Although the yield of MC was not dose-dependent in these experiments, the extent of MC was (measured as the number of fragments per fragmented cell). In these experiments, cyclin B1 levels also apparently peaked again in later cell generations after the induction of MC, suggesting that cyclin B1 gene expression might be persistently altered in the treated populations. The observed increase in cyclin B1 levels was thought to occur because expression of this gene in human cells is known to occur only in late S/G2 phases (33); simple arrest of cells at this part of the cell cycle might allow for a longer time for transcription and accumulation of cyclin B1 (7). Although such an explanation might seem simplistic, recent reports have shown that transcriptional regulation of cyclin B1 is mediated by p53 during a radiation-induced G2 block and is apparently not related to effects on mRNA or protein stability (43). As HeLa cells are deficient in p53 function because of human papilloma virus (HPV) infection, these developments prompted investigations on the effects of p53 status on radiation-induced MC.

Evaluation of other cell lines for the induction of MC by irradiation began in the authors' laboratory; a thorough analysis is still ongoing using the Large-Scale Digital

Cell Analysis System (LSDCAS) (50,51). LSDCAS holds a promise of being quite useful in these studies, as the analysis of many cell lines using cytological assays is complicated by low plating efficiencies, and it is only the proliferative cells in the culture that are at risk of radiation-induced MC. LSDCAS ameliorates this situation by allowing for the analysis of proliferative cells, even in the presence of a high fraction of a non-proliferative cell population. LSDCAS has advantages over other time-lapse systems currently in use (52), as this system allows for the monitoring of thousands of microscope fields for up to a month following irradiation. A summary of the findings related to radiation-induced MC within the context of p53 status by using the cytological approach is presented in Table 1. Although it was found that all cell lines were delayed late in the cell cycle, it was only those cell lines with non-functional p53 that exhibited high levels of cyclin B1 during these delays, and the subsequent yield of MC was much higher in these cell lines. These results suggest that lack of p53 function is critical to the induction of radiation-induced MC.

Table 1
Summary of the Incidence of Radiation-Induced Mitotic Catastrophe (MC) in Various Cell Lines

<i>Cell line</i>	<i>Origin</i>	<i>p53 status</i>	<i>Induction of MC by irradiation</i>	<i>Delays late in the cell cycle?</i>	<i>Overexpression of cyclin B1?</i>
HeLa S3	Cervical carcinoma	Non-functional (HPV)	+++	Yes	+++
HeLa Clone 3	Derived from HeLa S3	Non-functional (HPV)	+++	Yes	+++
V79	Chinese hamster lung	Mutant	+++	Yes	+++
GM10115	CHO hybrid w/human chromosome 4	Mutant	+++	Yes	++
115	Chromosomally unstable variant of GM10115 (obtained from W.F. Morgan)	Mutant	+++	Yes	+++
CS9	Chromosomally unstable variant of GM10115 (obtained from W.F. Morgan)	Mutant	+++	Yes	+++

(Continued)

Table 1
(Continued)

<i>Cell line</i>	<i>Origin</i>	<i>p53 status</i>	<i>Induction of MC by irradiation</i>	<i>Delays late in the cell cycle?</i>	<i>Overexpression of cyclin B1?</i>
LS12	Chromosomally unstable variant of GM10115 (obtained from W.F. Morgan)	Mutant	+++	Yes	+++
138	Chromosomally unstable variant of GM10115 (obtained from W.F. Morgan)	Mutant	+++	Yes	+++
U87-MG	Human glioblastoma	Wildtype	+	Yes	—
GM00037F	Primary human fibroblasts		+	Yes	—
MEF-12(1)	Mouse embryo fibroblasts	Wildtype	+	Yes	+
MEF-10(1)	Derived from MEF-12(1)	Mutant	+++	Yes	+++

2.2. LET Effects on the Induction of MC

Experiments studying the induction of MC in V79 Chinese hamster cells by low-energy protons (24) demonstrated that high Linear Energy Transfer (LET) protons are much more effective in causing MC than X-rays. In particular, doses as low as 0.5 Gy produce detectable MC in these cells, whereas the limit of detection following X-ray exposure is about 2 Gy using cytological assays. Radiation-induced MC shows an Relative Biological Effectiveness (RBE) effect that appears to be similar to that observed for survival, indicating the importance of MC as a mechanism of radiation-induced cell killing (24). Other studies (53,54) have shown that the cells are delayed for longer intervals late in the cell cycle following high-LET proton irradiation, when compared with delays caused by X-irradiation, in accord with earlier studies using alpha particles (55). Thus, the longer duration of cell cycle delays induced by high-LET protons is thought to contribute to the increased yield of radiation-induced MC in these studies.

3. DELAYED DNA DAMAGE ASSOCIATED WITH MC

3.1. p53-Deficient Cells Undergoing MC Later Present a Novel Form of Delayed DNA Damage

It is generally thought that abrogation of G2 checkpoint function in irradiated populations is a lethal event; yet, the mechanisms underlying these processes are poorly understood. Beginning some 24 h following irradiation, DNA degradation has been observed in irradiated HeLa cells (56). It has been reported that a novel form

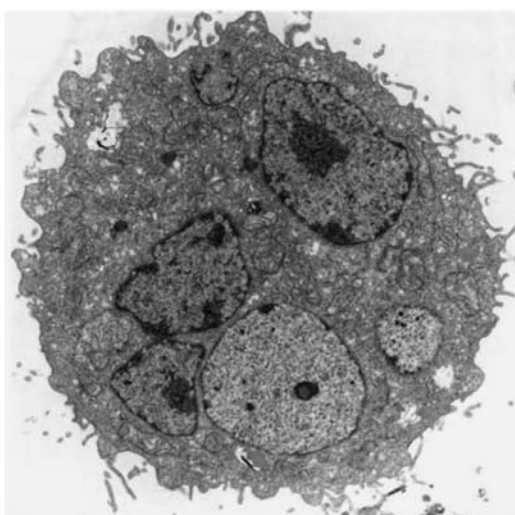
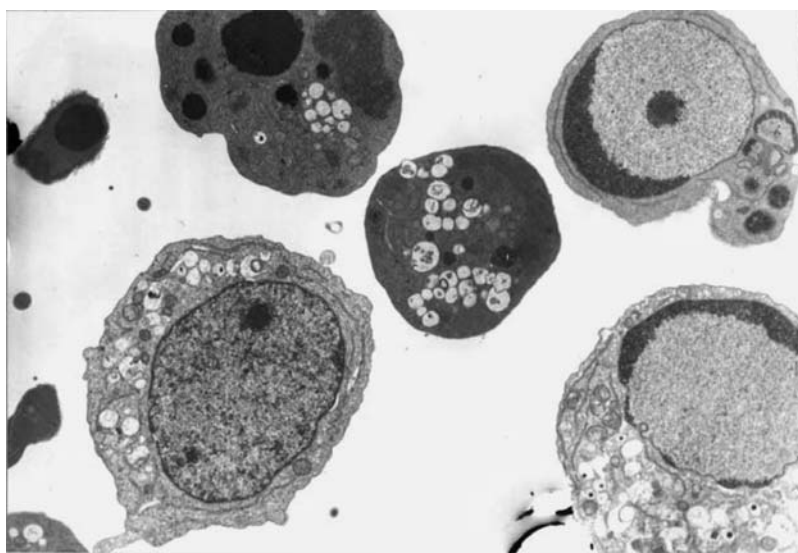


Fig. 2. Morphological comparison of cells undergoing apoptosis (top panel) and mitotic catastrophe (bottom panel). Glutaraldehyde-fixed cells were embedded in resin, sectioned, and stained with osmium tetroxide, as described in Swanson et al. (2). Top panel: HL-60 cells undergoing etoposide-induced apoptosis fixed at 4 h post-etoposide treatment ($68 \mu\text{M}$) ($\times 2950$). Apoptotic cells can be seen presenting blebbing of the membrane, extensive vacuolization, and condensation and marginalization of chromatin. Bottom panel: HeLaS3 cells irradiated with 10 Gy X-irradiation were fixed at 24 h post-irradiation ($\times 3800$). Multiple nuclei can be seen in this giant cell; each nuclear fragment is enveloped in an intact nuclear membrane, a characteristic feature of mitotic catastrophe as reported in Swanson et al. (2).

of DNA damage is found in HeLa cells following radiation-induced MC (57). This damage produces “non-sticky” free 3'-OH groups in cellular DNA (a chemical form of DNA damage not directly produced by ionizing radiation) and is thus recognized by the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay, yet apoptosis does not occur under these conditions (17,22,26,58,59). These results were preceded by a study using transmission electron microscopy that demonstrated in cells undergoing heat-induced MC that the reformation of the nuclear membrane occurred within chromatin fragments (2), consistent with observation that delayed DNA damage is also observed following heat-induced MC (unpublished data from the authors' laboratory), and that morphological changes associated with apoptosis and other modes of cell death were not associated with MC (Fig. 2 and Table 2). Reformation of the nuclear membrane itself is unlikely to cause chromatin disaggregation; rather, it has been suggested that cytoplasmic nucleases partially digest incompletely condensed chromatin during SPCC, thus producing the observed damage (57,60). Alternatively, aberrant re-condensation of chromatin in mitosis-arrested cells through degradation of cyclin B1, mediated by proteasome activities that usually occur in late

Table 2
Modes of Cell Death

<i>Event</i>	<i>Definition</i>	<i>Morphology</i>	<i>Assays</i>
Apoptosis	Programmed cell death. A distinct mode of cell destruction. Represents a major regulatory mechanism in eliminating abundant and unwanted cells during embryonic development, growth, differentiation, and normal cell turnover	Rapid cell shrinkage; condensed and marginalized chromatin; convolution or “blebbing” of the plasma membrane	Agarose gel electrophoresis; electron microscopy; TUNEL; FCM; WB; IP; IF
Anoikis	Detachment-induced apoptosis occurring when cells lose contact with the extracellular matrix	Same features as for apoptosis, except that it occurs in rounded-up cells detached from the extracellular matrix	Agarose gel electrophoresis; electron microscopy; TUNEL; FCM; WB; IP; IF
MC	Post-mitotic event driven by abrogation of the G2/M checkpoint in cells that have non-functional p53 and are exposed to various damaging agents. Often results in the generation of aneuploid and polyploid cell progeny	Giant cells; spontaneous premature chromosome condensation (SPCC); nuclear fragmentation	Light microscopy; Electron microscopy; TUNEL; FCM; WB; IP; IF

anaphase, may contribute to this effect (61). Regardless of the mechanism(s) underlying this late-appearing DNA damage, cells that are able to survive this process might possess a high level of gene mutations. In fact, it has been demonstrated that exposure of cells to various non-genotoxic stresses (including heat exposure) can lead to persistent genetic instability in surviving cells (62). In another study using LSDCAS (50), 50% of the colonies derived from cells surviving a 5 Gy radiation exposure were found to be abnormal with continuous production of MC for many cell generations following irradiation; moreover, the incidence of MC in some surviving clones did not diminish during colony formation.

4. PROMOTING MC MAY ADVANTAGEOUSLY INCREASE CLINICAL OUTCOME

Some types of human glioblastomas have functional p53 but fail to undergo apoptosis because of radiation treatment rendering the prognosis extremely unfavorable. Somatic mutations in phosphatase and tensin homologue on chromosome 10 gene (PTEN), a phosphatase tumor suppressor protein (63) that dephosphorylates phosphatidylinositol-3-phosphate (PIP3), a lipid second messenger that is produced by activated phosphatidylinositol-3-kinase (PI3K) (64–66), occur in a vast number of tumors and most markedly in glioblastomas; these mutations represent one of the culprit in the gliomas' resistance to radiation treatment. In general, PTEN mutants retain protein tyrosine phosphatase activity but are not able to dephosphorylate PIP3 (66,67); thus, PTEN-deficient tumors have elevated levels of PIP3. Activation of PI3K through mitogens or growth factors, both in normal and in cancer cells, starts a cascade of events that includes activation of Akt/protein kinase B, a serine-threonine kinase that phosphorylates and inhibits pro-apoptotic BAD (68), caspase 9 (69), and members of the fork head family of transcription factors (70), and increases in nuclear factor- κ B activity, thus facilitating the induction of genes that suppress apoptosis (71–73). Moreover, PI3K/Akt favors translocation of Mdm2 from the cytoplasm onto the nucleus where it negatively regulates the pro-apoptotic function of p53 (74). Another protein, survivin, has also been shown to play a role as suppressor of apoptosis in gliomas. Survivin is a 16.5-kDa protein member of the inhibitor of apoptosis (IAP) family; it is expressed in G2/M phases of the cell cycle (75–77) and is associated with gliomas progression from low to high grade (78–82). It has been shown that survivin enhances survival of tumor cells through suppression of apoptosis through inhibition of the caspase cascade pathway (83–85). It has also been reported (86) that radiation increases levels of phospho-survivin, a more stable survivin isoform than its unphosphorylated counterpart (87), and that radiation causes survivin to compartmentalize in the nucleus, a phenomenon that has been associated with enhanced double-strand DNA repair capability of exposed cells (86). Thus, survivin appears to be implicated in radiation resistance through the activation of more than one pathway in human glioblastomas. A preferential upregulation of genes involved in the DNA repair/replication pathways following radiation exposure of U87MG cells has also been reported (88,89). In both normal and neoplastic astrocytic cells, DNA repair, not programmed cell death, is responsible for p53 induction in response to cytotoxic agents (90). Thus, it appears that upregulation of DNA repair is a fundamental property of astrocytes exposed to clastogenic agents. The result of this capability is that these cells are resistant to the action of cytotoxic agents.

Recent studies (40) have shown that transduction of a dominant-negative p53 adenovirus construct in p53 wild-type U87MG cells leads to the induction of MC through overaccumulation of cyclin B1 and abrogation of the G2/M checkpoint. These results suggest that although mutant adenovirus transduction methodology cannot be used as anticancer therapy because of the inherent damage to the surrounding normal cells, interventions aimed at facilitating abrogation of the G2/M check point and at inducing MC may represent a novel mechanism to augment radiation efficacy in wild-type p53 cells that are resistant to apoptosis.

5. MC AND TUMOR PROGRESSION

5.1. Endopolyploid Cell Formation and Reductive Cell Division in Populations that Have Undergone MC

Illidge and collaborators (91) have reported polyploid giant cell formation (what the authors of this review would refer to as MC) after irradiation of human lymphoblastoid Namalwa cells. The polyploid cell population escapes death and is capable of releasing reproductive descendants as it enters an endocyclic path in which the polyploid cells divide and give rise to diploid or tetraploid cells during a restitution period. Data from the same group (92) also showed the presence of chromosome double loops in the giant cells by the second week post-irradiation. These formations are referred to as polyploid “bouquets.” Cells bearing these features return to an interphase state and subsequently separate into several secondary nuclei within the giant cell. These sub-nuclei eventually resume DNA synthesis and sequester cytoplasm around their genetic material giving rise to secondary cells that are able to continue mitotic propagation. Using the same cell line, Ivanov and collaborators (93) have also reported that a proportion of lymphoblastoid Namalwa cells exposed to severe genotoxic treatment undergo MC and endoreduplication giving rise to endopolyploid cells that resemble cells in meiotic prophase. Homologous DNA recombination repair occurs in these cells, and they replicate their DNA for more than a few rounds of endomitotic cycles producing mitotic descendants. Thus, endopolyploid cells, originated from MC cells, present a survival advantage over the remaining proportion of cell population that can die through apoptosis.

During megakaryocyte differentiation, the immature megakaryocyte (promegakaryoblast) undergoes a series of endocycles increasing its ploidy to a 2X DNA content (94). Megakaryocytes of 8–16N DNA content express cyclin B1 and cyclin B1-dependent H1 kinase activity with the cells retaining the ability to enter subsequent endomitotic cycles. It has been reported (95) that chromosome segregation did not require cyclin B1 degradation, whereas inhibiting the inactivation of cyclin B1/cdc2 complexes stopped chromosome decondensation and prevented migration of telophase chromosomes (96). Studies performed using a non-destructible form of cyclin B1 in prometaphase kidney cells of normal rats have shown that the principal effect of inactivation of cyclinB1/cdc2 complexes is on the spindle dynamics that regulate chromosome migration and cytokinesis (97). Thus, de-regulated degradation of cyclin B1 could partly explain the processes occurring during endomitosis. Interestingly, overaccumulation of cyclin B1 protein and activation of cyclin B1/cdc2 complexes were also described by the authors of this review (4,7,27,57) in irradiated and mild-heated HeLa cells undergoing MC.

These findings point out that MC and endomitosis might share common pathways of activation.

Apart from the megakaryocyte in which polyploidization is a phenomenon occurring during normal differentiation, the other reported findings pertain to cells that lack p53 function. These results contribute to the notion that cancer cells that escape cell death after genotoxic treatment and undergo MC and endoreduplication are not reproductively dead but retain proliferative potential.

Moreover, work carried out by our group (98) shows that irradiated HeLa cells that have undergone MC and have been cultured for up to 20 days post-irradiation present similar morphological features as those present in meiotic prophase and described in ref. 21. Meiosis or reduction division occurs in germ cells, and it results in haploid cells with new combination of genes in the daughter cells because of chromosome crossover; mitosis occurs in somatic cells, and it involves the division of one diploid mother cell into two identical diploid daughter cells. Although meiosis is considered a rare event in normal somatic cells, this might not be the case in cancer cells where a highly unstable genome is present (99). In particular, we report that following irradiation, HeLa cells undergo MC and form endopolyploid cells. By the end of the first week post-irradiation, the majority of the cells that underwent MC die. At 7–9 days post-irradiation and later, the polyploid cells that have escaped death stop DNA synthesis and initiate a de-polyploidization process. A portion of the endopolyploid cells is then able to segregate its nuclei and to produce viable descendants. DNA image cytometry reveals that when mitoses are resumed, the cells' DNA content is reduced from 8N to quasi-diploid in anaphases by subsequent reduction divisions that appear to occur omitting S phase passage. These data imply that endopolyploid tumor cells conserve their original individual genomic integrity and can re-initiate mitosis through reduction division (98). Thus, the ability of a cancer cell exposed to genotoxic agents to first overcome death and then to put in place an array of pathways that imply the occurrence of MC, polyploidization and endoreduplication, reduction division, and restitution to mitotic division needs to be looked at very closely as these pathways are likely to be responsible for the acquisition of resistance to treatment and to confer a higher chance for long-term survival and might, therefore, be important mechanisms in tumor progression *in vivo*.

5.2. Cells that Undergo MC and Escape Death Later are Thought to Divide Through Neosis

Sundaram and collaborators (100), using irradiated mouse embryo fibroblasts and human tumor cell lines, have reported what they define as a novel type of cell division in cancer cells called neosis. After radiation treatment, cells undergo MC and while the majority of them die, a small proportion of cells divide through neosis. Neosis is characterized by karyokinesis through nuclear budding followed by asymmetric, intracellular cytokinesis from which several small mononucleated cells originate. These small mononucleated cells have extended mitotic life span and were termed by the authors Raju cells. Raju cells are thought to be progenitors of transformed cells. In the human metastatic neuroblastoma HTB11 cell line, neosis was preceded by an absence of mitotic activity for about 8 weeks; the onset of mitotic crisis was accompanied by the formation of large polyploid cells (MC cells) followed by neosis and production of small Raju cells with an extended mitotic life span (100).

The results reported above are intriguing and interesting, and mirror preliminary results of work in progress in the laboratories of the authors of this review. We are analyzing late MC events in irradiated HeLa cells, and we find that by day 18 post-irradiation, the HeLa cell population that has undergone MC and has escaped death consists of polyploid cells, multi-nucleated cells, small mononucleated cells, and endomitotic giant cells that are in the process of extruding small mononucleated cells that resemble the Raju cells described by Sundaram and collaborators (100). Thus, a captivating parallel seems to exist between the phenomenon described by Sundaram and collaborators (100) and that noticed by us. Whatever the name used to designate delayed division of MC or giant cells and their progeny, it will be important to determine how this progeny of apparently normal mononucleated cells arising from reduction division contributes to tumor progression. We have begun extensive analysis of genotoxically treated human cancer cells using LSDCAS. Experiments performed using LSDCAS will allow for the quantitative determination of the ultimate fate of irradiated cells that undergo MC and escape death. Understanding the mechanisms underlying the appearance of clonogenic cells from polyploid cell populations might lend further light on the mechanisms involved in cancer resistance to genotoxic treatment.

6. CONCLUDING REMARKS

MC is a common mechanism underlying mitotic-linked cell death, and it is the result of premature entry into mitosis occurring during delays late in the cell cycle. A predominant effect in cells lacking p53 function, MC has been observed after a variety of treatments with cytotoxic agents and generally results in the production of polyploid cell populations. The mechanism underlying the premature entry into mitosis, which immediately precedes MC, involves the overaccumulation of cyclin B1, which is thought to overcome mitosis inhibitory pathways through mass action. Although most cells die because of MC, there is evidence that a small fraction of cells can survive this event. The mechanism underlying the restoration of clonogenicity in populations that have undergone MC is still an active research area, but some reports in the literature suggest a potential role for meiotic cell division mechanisms in the generation of quasi-diploid cell fractions from polyploid parental populations. Understanding the specific mechanisms underlying the temporary change from a pro-mitotic to a pro-meiotic division regimen might lend important insights into phenomena occurring during human tumor progression.

REFERENCES

1. Mackey MA, Morgan WF, Dewey WC. Nuclear fragmentation and premature chromosome condensation induced by heat shock in S-phase Chinese hamster ovary cells. *Cancer Res* 1988; 48: 6478–6483.
2. Swanson PE, Carroll SB, Zhang XF, Mackey MA. Spontaneous premature chromosome condensation micronucleus formation, and non-apoptotic cell death in heated HeLa S3 cells. *Am J Pathol* 1995; 146: 963–971.
3. Swanson PE, Zhang XF, Mackey MA. Non-apoptotic cell death in heated HeLa S3 cells—Ultrastructural observations. *Lab Invest* 1995; 72: A142–A142.
4. Ianzini F, Mackey MA. Spontaneous premature chromosome condensation and mitotic catastrophe following irradiation of HeLa S3 cells. *Int J Radiat Biol* 1997; 72: 409–421.
5. Rao PN, Johnson RT. Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. *Nature* 1970; 225: 159–164.

6. Mackey MA, Anolik SL, Roti Roti JL. Cellular mechanisms associated with the lack of chronic thermotolerance expression in HeLa S3 cells. *Cancer Res* 1992; 52: 1101–1106.
7. Mackey MA, Zhang XF, Hunt C, Sullivan S, Blum J, Laszlo A, Roti Roti JL. Uncoupling of M-phase kinase activation from the completion of S phase by heat shock. *Cancer Res* 1996; 56: 1770–1774.
8. Heald R, McLoughlin M, McKeon F. Human wee1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated cdc2 kinase. *Cell* 1993; 74: 463–474.
9. Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B. 14-3-3 σ is required to prevent mitotic catastrophe after DNA damage. *Nature* 1999; 401: 616–620.
10. Canman CE. Replication checkpoint: preventing mitotic catastrophe. *Curr Biol* 2001; 11: R121–124.
11. Flatt PM, Tang LJ, Scatena CD, Szak ST, Pietenpol JA. p53 Regulation of G2 checkpoint is retinoblastoma protein dependent. *Molec Cell Biol* 2000; 20: 4210–4223.
12. Yoshikawa R, Kusunoki M, Yanagi H, Noda M, Furuyama JI, Yamamura T, Hashimoto-Tamaoki T. Dual antitumor effects of 5-fluorouracil on the cell cycle in colorectal carcinoma cells: a novel target mechanism concept for pharmacokinetic modulating chemotherapy. *Cancer Res* 2001; 61: 1029–1037.
13. Lock RB, Stribinskiene L. Dual modes of death induced by etoposide in human epithelial tumor cells allow Bcl-2 to inhibit apoptosis without affecting clonogenic survival. *Cancer Res* 1996; 56: 4006–4012.
14. Hirose Y, Berger MS, Pieper RO. Abrogation of the Chk1-mediated G(2) checkpoint pathway potentiates temozolomide-induced toxicity in a p53-independent manner in human glioblastoma cells. *Cancer Res* 2001; 61: 5843–5849.
15. Jackson JR, Gilmartin A, Imburgia C, Winkler JD, Marshall LA, Roshak A. An indolocarbazole inhibitor of human checkpoint kinase (Chk1) abrogates cell cycle arrest caused by DNA damage. *Cancer Res* 2000; 60: 566–572.
16. Bruschi GC, de Souza CC, Fagundes MR, Dani MA, Goldman MH, Morris NR, Liu L, Goldman GH. Sensitivity to camptothecin in *Aspergillus nidulans* identifies a novel gene, scaA+, related to the cellular DNA damage response. *Mol Genet Genomics* 2001; 265: 264–275.
17. Nabha SM, Wall NR, Mohammad RM, Pettit GR, Al-Katib AM. Effects of combretastatin A-4 prodrug against a panel of malignant human B-lymphoid cell lines. *Anticancer Drugs* 2000; 11: 385–392.
18. Huang TS, Shu CH, Chao Y, Chen SN, Chen LL. Activation of MAD 2 checkpoint and persistence of cyclin B1/CDC 2 activity associate with paclitaxel-induced apoptosis in human nasopharyngeal carcinoma cells. *Apoptosis* 2000; 5: 235–241.
19. Balabhadrapathruni S, Thomas TJ, Yurkow EJ, Amenta PS, Thomas T. Effects of genistein and structurally related phytoestrogens on cell cycle kinetics and apoptosis in MDA-MB-468 human breast cancer cells. *Oncol Reports* 2000; 7: 3–12.
20. Miranda EI, Santana C, Rojas E, Hernandez S, Ostrosky-Wegman P, Garcia-Carranca A. Induced mitotic death of HeLa cells by abnormal expression of c-H-ras. *Mutat Res* 1996; 349: 173–182.
21. Erenpreisa JE, Ivanov A, Dekena G, Vitina A, Krampe R, Freivalds T, Selivanova G, Roach HI. Arrest in metaphase and anatomy of mitotic catastrophe: mild heat shock in two human osteosarcoma cell lines. *Cell Biol Int* 2000; 24: 61–70.
22. Nakahata K, Miyakoda M, Suzuki K, Kodama S, Watanabe M. Heat shock induces centrosomal dysfunction, and causes non-apoptotic mitotic catastrophe in human tumour cells. *Int J Hyperthermia* 2002; 18: 332–343.
23. Lackinger D, Kaina B. Primary mouse fibroblasts deficient for c-Fos, p53 or for both proteins are hypersensitive to UV light and alkylating agent-induced chromosomal breakage and apoptosis. *Mutat Res-Fund Mol* 2000; M 457: 113–123.
24. Ianzini F, Cherubini R, Mackey MA. Mitotic catastrophe induced by exposure of V79 Chinese hamster cells to low energy protons. *Int J Radiat Biol* 1999; 75: 717–723.
25. Ruth AC, Roninson IB. Effects of the multidrug transporter P-glycoprotein on cellular responses to ionizing radiation. *Cancer Res* 2000; 60: 2576–2578.
26. Redpath JL, Bengtsson U, DeSimone J, Lao X, Wang X, Stanbridge EJ. Sticky anaphase aberrations after G2-phase arrest of gamma-irradiated human skin fibroblasts: TP53 independence of formation and TP53 dependence of consequences. *Radiat Res* 2003; 159: 57–71.
27. Mackey MA, Ianzini F. Enhancement of radiation-induced mitotic catastrophe by moderate hyperthermia. *Int J Radiat Biol* 2000; 76: 273–280.

28. Sato N, Mizumoto K, Nakamura M, Tanaka M. Radiation-induced centrosome overduplication and multiple mitotic spindles in human tumor cells. *Exp Cell Res* 2000; 255: 321–326.
29. Holmfeldt P, Larsson N, Segerman B, Howell B, Morabito J, Cassimeris L, Gullberg M. The catastrophe-promoting activity of ectopic Op18/stathmin is required for disruption of mitotic spindles but not interphase microtubules. *Mol Biol Cell* 2001; 12: 73–83.
30. Mackey MA. *In vitro* effects and biological potential of long duration, moderate hyperthermia. In: H. Seegenschmiedt, ed. *Medical Radiology*, Springer Verlag: Heidelberg, 1993;21–29.
31. Phillips RA, Tolmach LJ. Repair of potentially lethal damage in X-irradiated HeLa cells. *Radiat Res* 1966; 29: 413–432.
32. Vogelstein B, Kinzler KW. Achilles' heel of cancer. *Nature* 2001; 412: 865–866.
33. Pines J, Hunter T. Isolation of a human cyclin cDNA: Evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. *Cell* 1989; 58: 833–846.
34. Pines J, Hunter T. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. *J Cell Biol* 1991; 115: 1–17.
35. Taylor WR, Stark GR. Regulation of the G2/M transition by p53. *Oncogene* 2001; 20: 1803–1815.
36. Zhan Q, Antonore MJ, Wang XW, Carrier F, Smith MI, Harris CC, Fornace AJ. Association of cdc2 and inhibition of cdc2/cyclin B1 kinase activity by the p53-regulated protein gadd45. *Oncogene* 1999; 18: 2892–2900.
37. Falck J, Mailand N, Syljuasen RG, Bartek J, Lukas J. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature* 2001; 410: 842–847.
38. Murnane JP. Cell cycle regulation in response to DNA damage in mammalian cells: A historical perspective. *Cancer Metastasis Rev* 1995; 14: 17–29.
39. Ianzini F, Bertoldo A, Kosmacek EA, Phillips SL, Mackey MA. Lack of p53 function promotes radiation-induced mitotic catastrophe in mouse embryonic fibroblast cells. *Cancer Cell Int* 2006; 6: 11. doi:10.1186/1475-2867-6-11
40. Ianzini FF, Domann FE, Kosmacek EA, Phillips SL, Mackey MA. Human glioblastoma U87MG cells transduced with a dominant negative p53 adenovirus construct undergo radiation-induced mitotic catastrophe. *Rad Res*, in press.
41. Passalaris TM, Benanti JA, Gewin L, Kiyono T, Galloway DA. The G2 checkpoint is maintained by redundant pathways. *Molec Cell Biol* 1999; 19: 5872–5881.
42. Innocente SAQ, Abrahamson JLA, Cogswell JP, Lee JM. p53 regulates a G2 checkpoint through cyclin B1. *Proc Natl Acad Sci USA* 1999; 96: 2147–2152.
43. Taylor WR, Agarwal ML, Agarwal A, Stacey DW, Stark GR. p53 inhibits entry into mitosis when DNA synthesis is blocked. *Oncogene* 1999; 18: 283–95.
44. Park M, Chae HD, Yun J, Jung M, Kim Y-S, Kim S-H, Moon HH, Shin DY. Constitutive activation of cyclin B1-associated cdc2 kinase overrides p53-mediated G2/M arrest. *Cancer Res* 2000; 60: 542–545.
45. Andreassen PR, Lacroix FB, Lohez OD, Margolis RL. Neither p21(WAF1) nor 14-3-3sigma prevents G(2) progression to mitotic catastrophe in human colon carcinoma cells after DNA damage, but p21(WAF1) induces stable G(1) arrest in resulting tetraploid cells. *Cancer Res* 2001; 61: 7660–7668.
46. Nakamura S, Yoshihito G, Roth JA, Mukhopadhyay T. C-terminus of p53 is required for G2 arrest. *Oncogene* 2002; 21: 2102–2107.
47. Murray AW. Creative blocks: cell cycle checkpoints and feedback controls. *Nature* 1992; 359: 599–604.
48. Weichselbaum R, Dahlberg W, Little JB, Ervin TJ, Miller D, Hellman S, Rheinwald JG. Cellular X-ray repair parameters of early passage squamous cell carcinoma lines derived from patients with known responses to radiotherapy. *Br J Cancer* 1984; 49: 595–601.
49. Limoli CL, Corcoran JJ, Jordan R, Morgan WF, Schwartz JL. A role for chromosomal instability in the development of and selection for radioresistant cell variants. *Br J Cancer* 2001; 84: 489–92.
50. Ianzini F, Mackey MA. Development of the large-scale digital cell analysis system. *Radiat Prot Dosimetry* 2002; 99: 289–293.
51. Davis PJ, Kosmacek EA, Sun Y, Ianzini F, Mackey MA. The large scale digital cell analysis system: open source, freely available software for live cell imaging. *J Microsc*, in press.
52. Forrester HB, Albright N, Ling CC, Dewey WC. Computerized video time-lapse analysis of apoptosis of REC:Myc cells X-irradiated in different phases of the cell cycle. *Radiat Res* 2000; 154: 625–639.

53. Ianzini F, Cherubini R, Mackey MA. Mitotic catastrophe and cell-cycle delays in V79 cells exposed to high-LET proton beams. *Istituto Nazionale Di Fisica Nucleare-LNL-Annual Report 1999 2000*; 160/00: 119–120.
54. Ianzini F, Cherubini R, Mackey MA. 2001. Effects on cell-cycle progression in V79 cells irradiated with 35 keV/ μm proton beams. *Istituto Nazionale Di Fisica Nucleare-LNL-Annual Report 2000 2001*; 178(2001): 73–74.
55. Lucke-Huhle C, Comper W, Hieber L, Pech M. Comparative study of G2 delay and survival after ^{241}Am Americium- α and ^{60}Co Cobalt- γ irradiation. *Radiat Environ Biophys* 1982; 20: 171–185.
56. Hawkins RB, Tolmach LJ, Griffiths TD. DNA synthesis in generation 1 in X-irradiated HeLa cells. *Radiat Res* 1981; 85: 85–98.
57. Ianzini F, Mackey MA. Delayed DNA damage associated with mitotic catastrophe following X-irradiation of HeLa S3 cells. *Mutagenesis* 1998; 13: 337–344.
58. Nabha SM, Mohammad RM, Dandashi MH, Coupaye-Gerard B, Aboukameel A, Pettit GR, Al-Katib AM. Combrestatin-A4 prodrug induced mitotic catastrophe in chronic lymphocytic leukemia cell line independent of caspase activation and Poly(ADP-ribose) polymerase cleavage. *Clin Cancer Res* 2002; 8: 2735–41.
59. Roninson IB, Broude EV, Hange B-D. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist Updat* 2001; 4: 303–313.
60. Ianzini F, Mackey MA. Delayed induction of DNA strand breaks following mitotic catastrophe occurring as a result of radiation G2 checkpoint abrogation. *Radiat Res* 1997; 148: 520.
61. Lee EA, Keutmann MK, Dowling M, Harris E, Chan G, Kao GD. Inactivation of the mitotic checkpoint targets human cancer cells to killing by microtubule-disrupting drugs. *Mol Cancer Ther* 2004; 3: 724–731.
62. Li CY, Little JB, Hu K, Zhang W, Zhang L, Dewhirst MW, Huang Q. Persistent genetic instability in cancer cells induced by non-DNA-damaging stress exposures. *Cancer Res* 2001; 61: 428–432.
63. Maehama T, Dixon JE. PTEN: a tumour suppressor that functions as a phospholipid phosphatase. *Trends Cell Biol* 1999; 9: 125–128.
64. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 1998; 95: 29–39.
65. Maehama TT, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 1998; 273: 13375–13338.
66. Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA, Wigler MH, Downes CP, Tonk NK. The lipid phosphatase activity of PTEN is critical for its tumor suppressor function. *Proc Natl Acad Sci USA* 1998; 95: 13513–13518.
67. Furnari FB, Huang HJ, Cavenee WK. The phosphoinositol phosphatase activity of PTEN mediates a serum-sensitive G1 growth arrest in glioma cells. *Cancer Res* 1998; 58: 5002–5008.
68. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 1997; 91: 231–241.
69. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. Regulation of cell death protease caspase-9 by phosphorylation. *Science* 1998; 282: 1318–1321.
70. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating, inhibiting a Forkhead transcription factor. *Cell* 1999; 96: 857–868.
71. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF- κ B activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 1999; 401: 82–85.
72. Kane LP, Shapiro VS, Stokoe D, Weiss A. Induction of NF- κ B by the Akt/PKB kinase. *Curr Biol* 1999; 9: 601–604.
73. Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS. NF- κ B antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* 1998; 281: 1680–1683.
74. Mayo LD, Donner DB. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc Natl Acad Sci USA* 2001; 98: 11598–11603.
75. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* 1997; 3: 917–921.

76. Ambrosini G, Adida C, Sirugo G, Altieri DC. Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. *J Biol Chem* 1998; 273: 11177–11182.
77. Kobayashi K, Hatano M, Otaki M, Ogasawara T, Tokuhisa T. Expression of a murine homologue of the inhibitor of apoptosis protein is related to cell proliferation. *Proc Natl Acad Sci USA* 1999; 96: 1457–1462.
78. Tan EC, Leung T, Manser E, Lim L. The human active breakpoint cluster region-related gene encodes a brain protein with homology to guanine nucleotide exchange proteins and GTPase-activating proteins. *J Biol Chem* 1993; 268: 27291–27298.
79. Chakravarti A, Noll E, Black PM, Finkelstein DF, Finkelstein DM, Dyson NJ, Loeffler JS. Quantitatively determined survivin expression levels are of prognostic value in human gliomas. *J Clin Oncol* 2002; 20: 1063–1068.
80. Sasaki T, Lopes MB, Hankins GR, Helm GA. Expression of survivin, an inhibitor of apoptosis protein, in tumors of the nervous system. *Acta Neuropathol* 2002; 104: 105–109.
81. Kajiwara Y, Yamasaki F, Hama S, Yahara Y, Yoshioka H, Sugiyama K, Arita K, Kurisu K. Expression of survivin in astrocytic tumors: correlation with malignant grade and prognosis. *Cancer* 2003; 97: 1077–1083.
82. Katoh M, Wilmotte R, Belkouch MC, de Tribolet N, Pizzolato G, Dietrich PY. Survivin in brain tumors: an attractive target for immunotherapy. *J Neurooncol* 2003; 64: 71–76.
83. Conway EM, Pollefeyt S, Cornelissen J, DeBaere I, Steiner-Mosonyi M, Ong K, Baens M, Collen D, Schuh AC. Three differentially expressed survivin cDNA variants encode proteins with distinct antiapoptotic functions. *Blood* 2000; 95: 1435–1442.
84. Wright ME, Han DK, Hockenbery DM. Caspase-3 and inhibitor of apoptosis protein(s) interactions in *Saccharomyces cerevisiae* and mammalian cells. *FEBS Lett* 2000; 481: 13–18.
85. Shin S, Sung BJ, Cho YS, Kim HJ, Ha NC, Hwang JI, Chung CW, Jung YK, Oh BH. An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. *Biochemistry* 2001; 40: 1117–1123.
86. Chakravarti A, Zhai GG, Zhang M, Malhotra R, Latham DE, Delaney MA, Robe P, Nestler U, Song Q, Loeffler J. Survivin enhances radiation resistance in primary human glioblastoma cells via caspase-independent mechanisms. *Oncogene* 2004; 23: 7494–7506.
87. Altieri DC. Survivin, versatile modulation of cell division and apoptosis in cancer. *Oncogene* 2003; 22: 8581–8589.
88. Otomo T, Hishii M, Arai H, Sato K, Sasai K. Microarray analysis of temporal gene responses to ionizing radiation in two glioblastoma cell lines: up-regulation of DNA repair genes. *J Radiat Res* 2004; 45: 53–60.
89. Wei Xu G, Nutt CL, Zlatescu MC, Keeney M, Chin-Yee I, Cairncross JG. Inactivation of p53 Sensitizes U87MG Glioma Cells to 1, 3-bis(2-Chloroethyl)-1-nitrosourea. *Cancer Res* 2001; 61: 4155–4159.
90. Nutt CL, Chambers AF, Cairncross JG. Wild-type p53 renders mouse astrocytes resistant to 1, 3-Bis(2-chloroethyl)-1-nitrosourea despite the absent of a p53-dependent cell cycle arrest. *Cancer Res* 1996; 56: 2748–2751.
91. Illidge TM, Cragg MS, Fringes B, Olive P, Erenpreisa JA. Polyploid giant cells provide a survival mechanism for p53 mutant cells after DNA damage. *Cell Biol Int* 2000; 24: 621–633.
92. Erenpreisa JA, Cragg MS, Fringes B, Sharakhov I, Illidge TM. Release of mitotic descendants by giant cells from irradiated Burkitt's lymphoma cell line. *Cell Biol Int* 2000; 24: 635–648.
93. Ivanov A, Cragg MS, Erenpreisa J, Emzins D, Lukman H, Illidge TM. Endopolyploid cells produced after severe genotoxic damage have the potential to repair DNA double strand breaks. *J Cell Sci* 2003; 116: 4095–4106.
94. Vitrat N, Cohen-Solal K, Pique C, Le Couedic JP, Norol F, Larsen AK, Katz A, Vainchenker W, Debili N. Endomitosis of human megakaryocytes are due to abortive mitosis. *Blood* 1998; 91: 3711–3723.
95. Holloway SL, Glotzer M, King RW, Murray AW. Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. *Cell* 1993; 73: 1393–1402.
96. Murray AW, Desai AB, Salmon ED. Real time observation of anaphase *in vitro*. *Proc Natl Acad Sci USA* 1996; 93: 12327–12328.
97. Wheatley SP, Hinchcliffe EH, Glotzer M, Hyman AA, Sluder G, Wang Y. CDK1 inactivation regulates anaphase spindle dynamics and cytokinesis *in vivo*. *J Cell Biol* 1997; 138: 385–393.

98. Erenpreisa J, Kalejs M, Ivanov A, Illidge TM, Ianzini F, Kosmacek EA, Mackey MA, Dalmane A, Cragg MS. Genomes segregation in polyploid tumor cells following mitotic catastrophe. *Cell Biol Int* 2005; 29: 1005–1011.
99. Storchova Z, Pellman D. From polyploidy to aneuploidy, genome instability and cancer. *Nat Rev Mol Cell Biol* 2004; 5: 45–54.
100. Sundaram M, Guernsey DL, Rajaraman MM, Rajaraman R. Neosis: a novel type of cell division in cancer. *Cancer Biol Ther* 2004; 3: 207–218.

5

Autophagy and Autophagic Cell Death

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SUMMARY

Macroautophagy or autophagy is a degradative pathway terminating in the lysosomal compartment after the formation of a cytoplasmic vacuole that engulfs macromolecules and organelles. During the last decade, progress made in our understanding of the molecular controls of autophagy has uncovered the importance of tumor suppressor molecules in the stimulation of autophagy. Downexpression of autophagy is an early event during tumorigenesis. However, the relation between autophagy and tumor progression seems to be more complex because cancer cells are able to trigger autophagy in response to various situations including changes in their extracellular environment and cancer treatments. The role of autophagy in cancer cells balances between two apparently opposite outcomes. Autophagy as a stress response mechanism can protect cancer cells from various insults. But autophagy can eliminate cancer cells by triggering autophagic cell death. These two aspects of autophagy will be discussed in this review.

Key Words: Autophagy; apoptosis; cell death; cancer; macroautophagy; lysosome; signal transduction.

1. INTRODUCTION

Autophagy is a lysosomal degradative mechanism occurring in different modes (chaperone-mediated autophagy, microautophagy, and macroautophagy) (1). This chapter is dedicated to macroautophagy (hereafter referred as to autophagy). Autophagy is a general and evolutionarily conserved vacuolar catabolic pathway terminating in the lysosomal compartment (2–4). It contributes to the quality control of cytoplasmic components by recycling macromolecules (autophagy is responsible for the turnover of long-lived proteins) and removing organelles when damaged or in excess (peroxisomes, mitochondria). From a cell biology standpoint, autophagy is characterized by the formation of a multimembrane-bound autophagosome that engulfs portions of the

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cytoplasm. The delimiting membrane of the autophagosome is derived from an 'isolation' membrane or phagophore of unknown origin (5). In mammalian cells, most autophagosomes can fuse directly with lysosomes or merge with endocytic compartments (6,7) to form an amphisome where the sequestered material is denatured because of the acidic environment. The final step is the fusion of amphisomes with the lysosomal compartment where the sequestered material is degraded.

The discovery of autophagy by de Duve and Wattiaux (8) was contemporary with that of lysosomes. The physiological importance of autophagy in maintaining cell homeostasis in organs such as liver and in cultured cells rapidly emerged (9,10). At the same time, the term autophagic cell death or type II programmed cell death (PCD II) was introduced to describe a cell death different from apoptosis or type I PCD (PCD I) (11,12). From these data, it appeared that autophagy is a cell response to stress, which under certain circumstances can lead to cell death. However, the precise role of autophagy as a cell death mechanism remains to be explored (13).

Identification of its molecular machinery and signaling pathways has shed some light on the importance of autophagy in physiological processes and diseases (1,2,14). Among the autophagy-related (*ATG*) genes, discovered in yeast and almost integrally conserved in all eukaryotic phyla, which control the formation of the autophagosome (15), *beclin 1* (the mammalian ortholog of the yeast *ATG6*) is a tumor suppressor gene that contributes to a complex with the class III phosphatidylinositol-3-kinase (PI3K) to the formation of the autophagosome (16,17). Other tumor suppressor gene products [p53, PTEN, TSC1/TSC2, death-associated protein kinase (DAP kinase)] are involved in the control of autophagy (18). Interestingly, autophagy is also stimulated in cancer cells by ceramide (19,20), a tumor suppressor lipid (21).

The aim of this chapter is to discuss the role of autophagy in cancer cell survival and death. Readers interested in a complete review of autophagy are referred to the different chapters of the book *Autophagy* edited by Daniel Klionsky (22). In addition, several recent reviews were dedicated to the role of autophagy during cell death (13,23–25) and tumor progression (26–28).

2. MOLECULAR CONTROL OF AUTOPHAGY

In this section, we will briefly summarize the molecular machinery involved in the formation and maturation of autophagic vacuoles and signaling pathways that modulate autophagy. Readers who want a more complete view of the role of Atg and signaling of autophagy can consult several recent reviews on these topics (29–32).

2.1. *ATG Complexes Implicated in the Formation of Autophagosome*

The discovery of *APG* (autophagy, 33) and *AUT* (autophagocytosis, 34) genes that regulate autophagy in the yeast *Saccharomyces cerevisiae* has contributed to our understanding of the molecular control of autophagy. Many *APG* and *AUT* genes are identical to *CVT* genes involved in the cytoplasm-to-vacuole (the yeast lysosome) targeting of some enzymes (aminopeptidase I, vacuolar α -mannosidase) under nutrient-rich conditions (35), whereas autophagy directs them to the vacuole under starvation conditions. Recently, a unique nomenclature *ATG* for these genes has been introduced (15) and will be used hereafter in this review.

2.1.1. CONJUGATION SYSTEMS

The formation of the autophagosome is dependent on two conjugation systems reminiscent of the ubiquitination of proteins in the proteasomal pathway that function in a coordinated manner (29,30,32,36). The first conjugation system involves four Atg proteins 5, 7, 10, and 12. The final complex is formed by Atg5–Atg12 non-covalently associated with the coiled-coil protein Atg16. Membrane-associated multimerization of Atg16 and Atg5–Atg12 conjugate is required for autophagy. In this conjugation system, Atg7 and Atg10 play the role of the E1 and E2 enzymes in the ubiquitin pathway, respectively. This conjugation system is conserved in mammalian cells where it initiates the formation of the sequestering membrane before its retrieval to cytosol (37). The second conjugation system is characterized by the conjugation of a protein Atg8 with a phospholipid, phosphatidylethanolamine. This system is dependent on the activity of Atg7 and Atg3, which have functions similar to those of the E1 and E2 enzymes in the ubiquitin pathway, respectively. Atg4, which is a protease belonging to the cysteine protein family of caspases, is required for the processing of Atg8 before the conjugation reactions and for its release from the phospholipid anchor (38). Several Atg8 mammalian homologs GATE-16 Golgi-associated ATPase enhancer of 16KDa, MAP1-LC3 Microtubule associated protein 1 light chain 3, GABARAP γ -aminobutyric acid A receptor-associated protein (GATE-16, MAP1-LC3, GABARAP) can be engaged in Atg3-dependent conjugating reactions (36,39). One of these homologs, MAP1-LC3, is engaged in the formation of the autophagosome after a processing similar to that of yeast Atg8 (40). As the lipid-modified form of Atg8 (MAP-LC3) remains associated with the autophagosome's outer and inner membrane, these proteins are useful to monitor autophagy either morphologically or biochemically because of the change in electrophoretic mobility due to the lipid modification of the protein during autophagy (41,42). The role of other Atg proteins in biogenesis of the autophagosome has been discussed in recent reviews (32,43).

2.1.2. CLASS III PI3K

The first evidence for the implication of this enzyme stemmed from the discovery that 3-methyladenine (3-MA) is able to block the formation of autophagosomes (44). After this seminal observation, it was demonstrated that wortmannin and LY294002, two PI3K inhibitors, interfere with the formation of autophagosomes in rat hepatocytes (45). Further studies have shown that 3-MA inhibits autophagy by interfering with the activity of class III PI3K (46).

Class III PI3K is associated with the membrane-anchored p150 adapter, which tethers the enzyme to cytoplasmic membranes (47). In *S. cerevisiae*, the class III PI3K (Vps34) and its membrane adaptor Vps15 have been shown to control autophagy (48). Vps34 and Vps15 exist in two complexes that control different membrane transport processes. Complex I, which also contains Atg14 and Atg6(Vps30), controls autophagy, whereas complex II, which is composed of Vps38 and Atg6(Vps30) in addition to Vps34/Vps15, governs the vesicular transport to the vacuole (yeast lysosome). In mammalian cells, class III PI3K forms a complex with Beclin 1 (the ortholog of the yeast Atg6) (17).

In addition to a role in membrane transport processes (reviewed in 49), class III PI3K controls autophagy by its interaction with Beclin 1 (50,51). Moreover, this interaction is essential for tumor repressor function of Beclin 1 but does not interfere with the class III PI3K-dependent vacuolar protein sorting (50).

3. REGULATION AND SIGNALING OF AUTOPHAGY

3.1. Overview

As a great variety of stimuli are able to modulate autophagy, it is not surprising that numerous signaling pathways have been shown to control this process: tyrosine kinase receptors, protein kinase A, casein kinase II, MAP kinases, and calcium have been shown to interfere with autophagy (31,52). However, the mechanisms by which these signaling pathways contribute to the control of autophagy are still largely unknown and require the identification of downstream targets. This variety probably reflects the diversity of stimuli able to modulate autophagy and also the cell-type specificity needed to integrate these stimuli. It can be envisioned that stimuli are *in fine* transduced to a common target upstream of the molecular machinery engaged in the formation of the autophagosome. The kinase TOR (target of rapamycin) occupies this privileged position in autophagic signaling in all eukaryotic cells (53,54). The class I PI3K-signaling pathway, which acts upstream of mammalian Target of Rapamycin (mTOR), plays a major role in the control of autophagy in metazoans (46,55,56).

3.2. Class I PI3K/mTOR Pathway

Class I PI3K enzymes phosphorylate PtdIns4P and PtdIns(4,5)P₂ to produce PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃, which, through Pleckstrin homology (PH) domains, recruit protein kinase B (Akt/PKB) and its activator phosphoinositide-dependent kinase-1 (PDK1) (57,58) at the plasma membrane. The PI3K/PKB-signaling pathway modulates the function of numerous substrates involved in cell survival, cell cycle progression, and cellular growth, which are frequently altered in human cancers (59,60). The plasma membrane-associated class I PI3 kinase transduces a negative signal for the biogenesis of autophagic vacuoles in HT-29 cells (46,61). Through its lipid phosphatase activity, the tumor suppressor PTEN, which is mutated in various cancers, dephosphorylates the phospholipid produced by class I PI3 kinase (60,62,63). Overexpression of wild-type PTEN, but not of a phosphoinositide phosphatase-deficient mutant of PTEN, counteracts the down-regulation of autophagy in HT-29 cells after activation of PI3 kinase (61). PTEN positively regulates autophagy by inhibition of the PI3K/PKB-signaling pathway in human intestinal cancer cells. In addition, a dominant-negative mutant of PKB increases autophagy and a constitutively active form of PKB decreases autophagy (61), suggesting a critical role of the PKB oncogene in the negative regulation of autophagy in HT-29 cells. The expression of a mutant of Ras (Val12Y40C) that specifically activates the class I PI3K-signaling pathway decreases autophagy in transformed fibroblasts (64). These different data allow us to speculate that genetic disruption of autophagy control can stimulate oncogenesis.

Further experiments on colon and breast cancer cells showed that the lipid tumor suppressor ceramide stimulates autophagy by down-regulation of the PI3 kinase/PKB pathway (19). Interestingly, programmed autophagy is induced by ecdysone in the fat body during *Drosophila* development through the inhibition of the class I PI3K pathway (65). Another study in *Drosophila* shows the importance of the class I PI3K and its downstream effector the kinase TOR in regulating starvation-induced autophagy (55). However, TOR can sense starvation independently of the class I PI3K pathway (reviewed in 53). In fact, the first evidence for a role of mTOR in the control of autophagy was obtained in rat hepatocytes (66), where the inhibitory effect of amino

acids on autophagy could be relieved by rapamycin, an inhibitor of TOR. Accordingly, the inhibition of TOR in yeast, where the class I PI3K pathway is absent, stimulates autophagy (67). The role of events downstream of TOR controlling autophagy has been discussed in recent reviews (31,68).

4. AUTOPHAGY IN CANCER CELLS

Autophagy may suppress tumor development by causing cell killing that would limit cell population size or otherwise by maintaining a low DNA mutation that would prevent oncogenesis (69). Although autophagy has been found to occur in various types of tumor cells (70), cancer cells often display reduced autophagic capacity (16,27,28,71). A reduced rate of autophagic activity has also been reported in chemically transformed rat pancreatic cells in which autophagic capacity increases at precancerous stages and then diminishes during adenoma to carcinoma transition (72). This suggests that autophagic activity is decreased during malignant progression.

4.1. Autophagy in Tumor Development

The link between autophagy and tumor development is also supported by the fact that some tumor suppressor proteins and oncoproteins are involved in the control of autophagic activity in cells (reviewed in 27,28,31). Beclin 1, PTEN, PKB/class I PI3-kinase pathway, DAPK, Ras, c-Myc, p53, and Bcl-2 are examples of these categories of proteins (Fig. 1). The implication of *beclin 1* in tumor progression was suggested by the observation that this gene is monoallelically deleted in a high percentage of human cancers and tumor cell lines (16). Stable transfection of *beclin 1* into MCF-7 human breast carcinoma cells reduced their tumorigenicity in nude mice and slowed their proliferation rate (16). Furthermore, *beclin 1*-haploinsufficient mice displayed an increased incidence of spontaneous tumors (73,74), and the cell-proliferative capacity was markedly increased in some of their tissues.

The increased expression of Akt/PKB and the loss of function of PTEN protein, an inhibitor of the PI3K/PKB pathway, are frequently observed in cancer cells. As discussed in Section 3, the PTEN tumor suppressor can induce autophagy in human

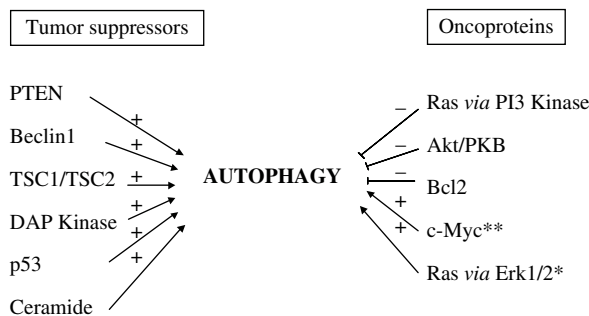


Fig. 1. Control of autophagy by tumor suppressors and oncoproteins. Autophagy is stimulated by tumor suppressors and inhibited by oncoproteins. **The proto-oncogene c-Myc has been shown to induce autophagy independently of its oncogenic property (75). *The stimulation of autophagy through the ras-dependent ERK1/2-signaling pathway has been shown in human colon cancer cells (76,77).

colon cancer cell lines whereas the activation of Akt/PKB has an inhibitory effect on autophagy (61). These findings suggest that PTEN and the Akt/PKB pathway may participate in tumor progression by interfering with autophagy. Furthermore, Akt/PKB-induced inhibition of autophagy is mediated through mTOR, which is regarded as a “gate keeper” of the autophagy pathway (see Section 3). This inhibition requires the expression of two tumor suppressors, TSC1 and TSC2 (88,89). The role of mTOR in oncogenesis is supported by the fact that the well-known inhibitor of mTOR, rapamycin, displays antitumor effects in several tumor models (80).

DAP kinase and its homolog Drp-1, which have tumor suppressor properties and control apoptotic membrane blebbing, have been shown to stimulate autophagy in response to amino acid starvation, tamoxifen, and interferon (INF)- γ (81).

Recent observations indicate that p53, a known regulator of cell growth and proliferation, is also involved in the modulation of mTOR activity and autophagy (82).

4.2. Autophagy in Established Tumors

Cancer cells are able to induce autophagy in response to stress conditions by adapting their metabolism to survive. This might occur when tumors are poorly vascularized and cancer cells have to survive in low oxygen and reduced nutrient conditions (83–85). In agreement with this assumption, it has been reported that endostatin, an inhibitor of angiogenesis, induces autophagic cell death in endothelial cells (86). Although these

Table 1
Autophagy as a Response to Cancer Therapy

<i>Effectors</i>	<i>Cells</i>	<i>References</i>
Epidermal Growth Factor Receptor EGFR inhibitors	Glioma	(87)
Platelet derived growth factor Anti-PDGF	Glioma	(88)
Interferon (INF)- γ	Cervix cancer cells	(81)
Tumor necrosis factor (TNF)- α	Leukemic cells	(101)
Peroxisome proliferator activated receptor PPAR- γ activation	Neuroblastoma	(89)
Radiation	Breast, colon, prostate	(99)
Arsenic trioxide	Glioma	(90)
Celecoxib	Colon cancer cells	Unpublished results
Lipoxygenase inhibitors	Pancreatic cancer cells	(91)
Resveratrol	Ovarian cancer cells	(92)
Tamoxifen	Breast cancer cells	(97)
Temozolomide	Glioma	(93)
Etoposide	Murine pluripotent hematopoietic stem cells	(111)
Rapamycin	Breast cancer cells	(94)
	Glioma	(95)
Histone deacetylase inhibitors	Cervix cancer cells	(96)

studies point out the role of autophagy in solid tumor development, the relationship between angiogenesis and autophagy needs to be investigated further.

4.3. Autophagy and Cancer Therapy

Although many anticancer drugs have shown to induce apoptosis in cancer cells, autophagy is also frequently observed in response to these treatments (26) (Table 1). Anticancer drug-induced autophagy can lead either to cell death or alternatively to protection against cell death induced by these treatments (Table 1). For example, tamoxifen, a well-known anticancer drug, induces autophagic cell death in human mammary carcinoma MCF-7 cells (97,98). By contrast, autophagy is a cell survival mechanism in response to certain drugs and treatments such as low radiation, sulindac, and tumor necrosis factor (TNF)- α (99–101). However, the mechanisms by which anticancer drugs promote autophagy remain to be fully elucidated.

5. AUTOPHAGIC CELL DEATH

Although apoptosis (PCD I) is well characterized, less is known about the autophagic cell death (PCD II) that can also occur in cells (24,25). Apoptotic cells display nuclear fragmentation, membrane blebbing, externalization of phosphatidylserine at the outer leaflet of the plasma membrane, and activation of caspases. The latter proteases mediate cell death by cleaving several cellular components that are eliminated by phagocytosis. Programmed autophagic cell death is characterized by the appearance of autophagic vacuoles. This self-digestion mechanism of cells causes a reduction of cell mass in tissues and may especially take place in situations in which the number of dead cell bodies exceeds phagocytosis capacity. It is worth noting that the induction of autophagic vacuoles in cells may reflect autophagy-induced cell death or alternatively autophagy-induced cell survival, but the molecular distinction between these two types of autophagy is currently not defined. In addition, the distinction between apoptosis and autophagic cell death is not so clear-cut because autophagic vacuoles can be observed in dying cells with apoptotic features (27,102).

5.1. Autophagy and Apoptosis

Numerous reports reveal that apoptosis and autophagy pathways are linked (reviewed in 25,27,103,104). This interpretation is based on results showing that (i) autophagy and apoptosis share some common molecular signaling pathways, (ii) several inducers of apoptosis can promote stimulation of autophagy, and (iii) in some instances, specific inhibition of each cell death pathway (apoptosis or autophagy) can switch cells to the other form of death.

It has been reported that several actors of both death receptor- and mitochondria-mediated apoptosis are also involved in the regulation of autophagy. For example, activation of a receptor-interacting protein (RIP) and c-Jun N-terminal kinase (JNK) pathway induced autophagic cell death in L929 cells in response to inhibition of caspase activation (105). TRAIL, a well-known ligand of death receptor-mediated apoptosis, induces autophagy in a model of the formation of lumens in mammary acini (106). Stimulation of autophagy is observed in human T-lymphoblastic cells in response to TNF α , another ligand of the death receptor pathway (101). Recently, the interaction of

Atg5 with the death domain of Fas-associated death domain protein (FADD) has been shown to play a crucial role in INF γ -induced cell death independently of detectable activation of caspase 8 (107). Moreover, ceramide, a well-known mediator of death receptor-induced apoptosis, is also an actor of autophagy activation and autophagic cell death (19,20). The kinase Akt/PKB, which is involved in the insulin-like growth factor (IGF) receptor-signaling pathway, exerts negative control in both apoptosis and autophagy (28,59). In addition to its pro-apoptotic activity, DAP kinase can mediate autophagic vacuolization in a caspase-independent manner (27). Inhibition of caspase-8 activity, which is known to be mainly stimulated by death receptor signaling, has been shown to induce autophagic cell death in L929 cells associated with the up-regulation of the *atg7* and *beclin 1* genes (105).

Mitochondria are another common mediator of apoptosis and autophagy (104,108–110). Their removal by autophagy in response to etoposide has been observed (98). In addition, Tolkovsky and co-workers (112) reported that the blockade of apoptosis leads to selective elimination of mitochondria in neuronal cells in response to several apoptotic inducers including the withdrawal of nerve growth factor (NGF). This effect is prevented by 3-MA treatment, indicating that the loss of mitochondria was caused by their sequestration by autophagosomes. Similarly, Kirkland et al. (113) demonstrated that autophagy and lipid peroxidation are required in the reduction of mitochondrial mass in NGF-deprived sympathetic neurons. It has been shown that depolarized mitochondria can be selectively sequestered by autophagosomes in primary hepatocytes (114). This observation led to the suggestion that autophagy may protect against apoptosis by elimination of damaged mitochondria that release pro-apoptotic signaling molecules such as cytochrome c, Apaf, and reactive oxygen species. It has been shown in yeast that the autophagic degradation of mitochondria induced by rapamycin or nitrogen starvation requires the protein Uth1p mainly localized in the outer mitochondrial membrane of these cells (115). The lack of Uth1p was found to induce resistance to both Bax expression and rapamycin, a well-known inducer of autophagy, suggesting it plays role in a death pathway related to autophagy. Another link between autophagy and mitochondria is the interaction of Beclin 1 with the antiapoptotic Bcl-2 protein (116). Recently, it has been shown that this interaction negatively regulates autophagy during cell starvation (117). These findings are consistent with previous observations showing that overexpression of Bcl-2 prevents autophagy (110), whereas its down-regulation stimulates this process (118). The BH3-like domain protein BNIP3 and the BH3-interacting protein Hspin1 stimulate autophagy in cells (20,119,120). As the mechanisms involved in the recognition of mitochondria by the autophagic machinery are unknown at present, the literature data suggest that the protein Uth1p in yeast, the BNIP3 protein in mammalian cells, or lipid oxidation of the mitochondrial membrane are possibly involved in this process.

The existence of a double switch between apoptosis and autophagy has been demonstrated by experiments involving the blockage of one or another death pathway. There are examples in the literature in which the inhibition of caspase activation can lead to autophagic cell death (105,112). Reciprocally, it has been shown that impaired autophagy can trigger apoptotic cell killing (121).

5.2. Autophagic Cell Death

Autophagy related to cell death can occur in response to stress conditions, such as anticancer treatments, as summarized in Table 1. Tamoxifen is one anticancer drug whose induction of autophagic cell death is well characterized in MCF-7 cells (97,98). Tamoxifen-induced autophagy is associated with increased expression of Beclin 1 (19). Both these responses are mediated by ceramide, which is considered a tumor suppressor lipid (21). The association of autophagic vacuoles with cell death is also observed in various cell types during development and aging and also in several diseases, notably neurological disorders (13,122–124). In *Drosophila* development, the involution of the salivary glands requires apoptosis associated with autophagic features (125). Neuronal development is associated with both apoptosis (126) and autophagy-related cell death (127).

The link between autophagy and cell death was first demonstrated by experiments showing that 3-MA, a pharmacological inhibitor of autophagy activity, inhibits autophagy and cell death in MCF-7 cells (97). More recently, the role of autophagy in cell death has been shown by RNA interference silencing of the *Atg* genes in different cellular models (105). In mouse L929 fibroblastic cells, reduction of the expression of the *atg7* and *beclin 1* genes inhibits autophagic cell death induced by Z-VAD-fmk, a caspase inhibitor with broad specificity. Similarly, in cells lacking apoptotic machinery (*Bax*^{-/-}*Bak*^{-/-} cells), etoposide treatment causes non-apoptotic cell death through *atg*-dependent mechanism (128). In *Dictyostelium discoideum*, disruption of the *atg1* autophagy gene impairs autophagy vacuolization but cells still die by a non-vacuolar mechanism (129), supporting the notion that distinct cell death mechanisms may co-exist in a given cell.

6. CONCLUSION

The importance of autophagy during tumor progression has become increasingly clear in recent years (27,28,130). The expression of several tumor suppressors including tumor suppressor gene products and tumor suppressor lipids is required to stimulate autophagy. Accordingly, a defect in autophagy is observed in many cancer cells and is probably an early event during tumor progression. However, the mechanism by which some tumor suppressor molecules control autophagy remains to be clarified. Uncovering the mechanism by which *beclin 1*, the only identified tumor suppressor gene product among *atg*, regulates autophagy is an important step toward the understanding of its tumor suppressor capacity (76,77). Suppression of autophagy probably contributes to the proliferation of cancer cells and to the increase in tumor mass through the inactivation (complete or partial) of tumor suppressor gene products that restrain the activation of mTOR.

Although it is generally agreed that autophagy is low in cancer cells, the presence of autophagic vacuoles is observed, *in situ*, in many tumors (70) and autophagy is stimulated by chemotherapeutic treatments and radiation in a large panel of cancer cells (28). These observations suggest that gene amplification and alternative control of autophagy are effective in cancer cells. Alternatively, one cannot exclude that in the tumor mass, cancer cells with unaltered autophagic potential are selected by stresses such as limitation in nutrient and oxygen supply and the use of non-lethal doses of anticancer treatment. In this scenario, autophagy would be a cell survival mechanism

that contributes to the adaptation of cancer to adverse conditions. Alternatively, autophagy is a cell death mechanism (PCD II), but in many cases signs of both apoptosis and autophagy can be observed in cancer cells (27,102). In this latter case, the temporal intervention of autophagy is important to consider. When autophagy prevails before the point of no return of apoptosis (mitochondrial depolarization), it can be assumed that autophagy would counteract or delay cell death. However, when autophagy is triggered after mitochondrial depolarization, in cells doomed to die, it can contribute to cell dismantlement. This last function is probably a heightened version of the autophagic removal of faulty mitochondria observed in non-apoptotic cells (109). In this context, investigation of the molecular relation between autophagy and mitochondria would better define the role of autophagy during apoptosis but also in mitochondrial diseases and aging. As previously mentioned, cancer cells can trigger autophagic cell death in response to various cancer treatments. However, the mechanisms by which these treatments stimulate autophagy are largely unknown. Elucidation of these molecular controls would be of value in establishing whether autophagic cell death is simply an intensified version of autophagy as classically defined (responsive to amino acids, controlled by mTOR) or a mechanism with alternative controls. Knowing more about the key mechanisms that regulate autophagy may provide some clues about the consequences of manipulating autophagy in cancer cells.

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REFERENCES

1. Cuervo AM. Autophagy: in sickness and in health. *Trends Cell Biol* 2004; 14:70–77.
2. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science* 2000; 290:1717–1721.
3. Klionsky DJ, Ohsumi Y. Vacuolar import of proteins and organelles from the cytoplasm. *Annu Rev Cell Dev Biol* 1999; 15:1–32.
4. Seglen PO, Bohley P. Autophagy and other vacuolar protein degradation mechanisms. *Experientia* 1992; 48:158–172.
5. Fengsrud M, Roos N, Berg T, Liou W, Slot JW, Seglen PO. Ultrastructural and immunocytochemical characterization of autophagic vacuoles in isolated hepatocytes: effects of vinblastine and asparagine on vacuole distributions. *Exp Cell Res* 1995; 221:504–519.
6. Stromhaug PE, Seglen PO. Evidence for acidity of prelysosomal autophagic/endocytic vacuoles (amphisomes). *Biochem J* 1993; 291:115–121.
7. Liou W, Geuze HJ, Geelen MJ, Slot JW. The autophagic and endocytic pathways converge at the nascent autophagic vacuoles. *J Cell Biol* 1997; 136:61–70.
8. de Duve C, Wattiaux R. Functions of lysosomes. *Annu Rev Physiol* 1966; 28:435–492.
9. Mortimore GE, Schworer CM. Induction of autophagy by amino-acid deprivation in perfused rat liver. *Nature* 1977; 270:174–176.
10. Mortimore GE, Kadowaki M. Autophagy: its mechanism and regulation. In: Ciechanover AJ, Schwartz AL, eds. *Cellular proteolytic systems*. New York, NY: Wiley-Liss, 1994; 65–87.
11. Schweichel JU, Merker HJ. The morphology of various types of cell death in prenatal tissues. *Teratology* 1973; 7:253–266.
12. Clarke PG. Developmental cell death: morphological diversity and multiple mechanisms. *Anat Embryol* 1990; 181:195–213.

13. Levine B, Yuan J. Autophagy in cell death: an innocent convict. *J Clin Invest* 2005; 115:2679–2688.
14. Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell* 2004; 6:463–477.
15. Klionsky DJ, Cregg JM, Dunn WA Jr, Emr SD, Sakai Y, Sandoval IV, Sibirny A, Subramani S, Thumm M, Veenhuis M, Ohsumi Y. A unified nomenclature for yeast autophagy-related genes. *Dev Cell* 2003; 5:539–545.
16. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 1999; 402:672–676.
17. Kihara A, Kabeya Y, Ohsumi Y, Yoshimori T. Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. *EMBO Rep* 2001; 2:330–335.
18. Botti J, Djavaheri-Mergny M, Pilatte Y, Codogno P. Autophagy signaling and the cogwheels of cancer. *Autophagy* 2006; 2:67–73.
19. Scarlatti F, Bauvy C, Ventruti A, Sala G, Cluzeaud F, Vandewalle A, Ghidoni R, Codogno P. Ceramide-mediated macroautophagy involves inhibition of protein kinase B and up-regulation of Beclin 1. *J Biol Chem* 2004; 279:18384–18391.
20. Daido S, Kanzawa T, Yamamoto A, Takeuchi H, Kondo Y, Kondo S. Pivotal role of the cell death factor BNIP3 in ceramide-induced autophagic cell death in malignant glioma cells. *Cancer Res* 2004; 64:4286–4293.
21. Hannun YA, Linares CM. Sphingolipid breakdown products: anti-proliferative and tumor-suppressor lipids. *Biochim Biophys Acta* 1993; 1154:223–236.
22. Klionsky DJ. *Autophagy*. Georgetown, TX: Landes Bioscience, 2004.
23. Kroemer G, Jäättelä M. Lysosomes and autophagy in cell death control. *Nat Rev Cancer* 2005; 5:886–897.
24. Bursch W. Multiple cell death programs: Charon's lifts to Hades. *FEMS Yeast Res* 2004; 5:101–110.
25. Lockshin RA, Zakeri Z. Apoptosis, autophagy, and more. *Int J Biochem Cell Biol* 2004; 36:2405–2419.
26. Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. *Nat Rev Cancer* 2005; 5:726–734.
27. Gozuacik D, Kimchi A. Autophagy as a cell death and tumor suppressor mechanism. *Oncogene* 2004; 23:2891–2906.
28. Ogier-Denis E, Codogno P. Autophagy: a barrier or an adaptive response to cancer. *Biochim Biophys Acta* 2003; 1603:113–128.
29. Ohsumi Y. Molecular dissection of autophagy: two ubiquitin-like systems. *Nat Rev Mol Cell Biol* 2001; 2:211–216.
30. Mizushima N, Ohsumi Y, Yoshimori T. Autophagosome formation in mammalian cells. *Cell Struct Funct* 2002; 27:421–429.
31. Meijer AJ, Codogno P. Regulation and role of autophagy in mammalian cells. *Int J Biochem Cell Biol* 2004; 36:2445–2462.
32. Nair U, Klionsky DJ. Molecular mechanisms and regulation of specific and nonspecific autophagy pathways in yeast. *J Biol Chem* 2005; 280:41785–41788.
33. Tsukada M, Ohsumi Y. Isolation and characterization of Autophagy-Defective mutants of *Saccharomyces-Cerevisiae*. *FEBS Lett* 1993; 333:169–174.
34. Thumm M, Egner R, Koch B, Schlumpberger M, Straub M, Veenhuis M, Wolf DH. Isolation of autophagocytosis mutants of *Saccharomyces cerevisiae*. *FEBS Lett* 1994; 349:275–280.
35. Kim J, Klionsky DJ. Autophagy, cytoplasm-to-vacuole targeting pathway, and pexophagy in yeast and mammalian cells. *Annu Rev Biochem* 2000; 69:303–342.
36. Tanida I, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. *Int J Biochem Cell Biol* 2004; 36:2503–2518.
37. Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, Tokuhisa T, Ohsumi Y, Yoshimori T. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J Cell Biol* 2001; 152:657–668.
38. Marino G, Lopez-Otin C. Autophagy: molecular mechanisms, physiological functions and relevance in human pathology. *Cell Mol Life Sci* 2004; 61:1439–1454.
39. Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, Yoshimori T. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. *J Cell Sci* 2004; 117:2805–2812.

40. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 2000; 19:5720–5728.
41. Yoshimori T. Autophagy: a regulated bulk degradation process inside cells. *Biochem Biophys Res Commun* 2004; 313:453–458.
42. Mizushima N. Methods for monitoring autophagy. *Int J Biochem Cell Biol* 2004; 36:2491–2502.
43. Thumm M. Structure and function of the yeast vacuole and its role in autophagy. *Microsc Res Tech* 2000; 51:563–572.
44. Seglen PO, Gordon PB. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proc Natl Acad Sci USA* 1982; 79:1889–1892.
45. Blommaert EF, Krause U, Schellens JP, Vreeling-Sindelarova H, Meijer AJ. The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. *Eur J Biochem* 1997; 243:240–246.
46. Petiot A, Ogier-Denis E, Blommaert EF, Meijer AJ, Codogno P. Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. *J Biol Chem* 2000; 275:992–998.
47. Panaretou C, Domin J, Cockcroft S, Waterfield MD. Characterization of p150, an adaptor protein for the human phosphatidylinositol (PtdIns) 3-kinase. *J Biol Chem* 1997; 272:2477–2485.
48. Kihara A, Noda T, Ishihara N, Ohsumi Y. Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in *Saccharomyces cerevisiae*. *J Cell Biol* 2001; 152:519–530.
49. Gilmool DJ, Simonsen A, Stenmark H. Cellular functions of phosphatidylinositol 3-phosphate and FYVE domain proteins. *Biochem J* 2001; 355:249–258.
50. Furaya N, Yu J, Byfield M, Pattingre S, Levine B. The evolutionarily conserved domain of Beclin1 is required for Vps34 binding and tumor function. *Autophagy* 2005; 1:41–47.
51. Zeng X, Overmeyer JH, Maltese WA. Functional specificity of the mammalian Beclin-Vps34 PI 3-kinase complex in macroautophagy versus endocytosis and lysosomal enzyme trafficking. *J Cell Sci* 2006; 119:259–270.
52. Møller MT, Samari HR, Holden L, Seglen PO. Regulation of mammalian autophagy by protein phosphorylation. In: Klionsky DJ, ed. *Autophagy*. Georgetown, TX: Landes Bioscience 2004; 49–59.
53. van Sluijters DA, Dubbelhuis PF, Blommaert EFC, Meijer AJ. Amino-acid-dependent signal transduction. *Biochem J* 2000; 351:545–550.
54. Codogno P, Meijer AJ. Autophagy and signaling: their role in cell survival and cell death. *Cell Death Differ* 2005; 12:1509–1518.
55. Scott RC, Schuldiner O, Neufeld TP. Role and regulation of starvation-induced autophagy in the *Drosophila* fat body. *Dev Cell* 2004; 7:167–178.
56. Melendez A, Taloczy Z, Seaman M, Eskelinen EL, Hall DH, Levine B. Autophagy genes are essential for dauer development and life-span extension in *C. elegans*. *Science* 2003; 301:1387–1391.
57. Brazil DP, Hemmings BA. Ten years of protein kinase B signalling: a hard Akt to follow. *Trends Biochem Sci* 2001; 26:657–664.
58. Katso R, Okkenhaug K, Ahmadi K, White S, Timms J, Waterfield MD. Cellular function of phosphoinositide 3-kinases: implications for development, immunity, homeostasis, and cancer. *Annu Rev Cell Dev Biol* 2001; 17:615–675.
59. Franke TF, Hornik CP, Segev L, Shostak GA, Sugimoto C. PI3K/Akt and apoptosis: size matters. *Oncogene* 2003; 22:8983–8998.
60. Parsons R. Human cancer, PTEN and the PI-3 kinase pathway. *Semin Cell Dev Biol* 2004; 15:171–176.
61. Arico S, Petiot A, Bauvy C, Dubbelhuis PF, Meijer AJ, Codogno P, Ogier-Denis E. The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *J Biol Chem* 2001; 276:35243–35246.
62. Di Cristofano A, Pandolfi PP. The multiple roles of PTEN in tumor suppression. *Cell* 2000; 100:387–390.
63. Maehama T, Taylor GS, Dixon JE. PTEN and myotubularin: novel phosphoinositide phosphatases. *Annu Rev Biochem* 2001; 70:247–279.

64. Furuta S, Hidaka E, Ogata A, Yokota S, Kamata T. Ras is involved in the negative control of autophagy through the class I PI3-kinase. *Oncogene* 2004; 23:3898–3904.
65. Rusten TE, Lindmo K, Juhasz G, Sass M, Seglen PO, Brech A, Stenmark H. Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway. *Dev Cell* 2004; 7:179–192.
66. Blommaert EF, Luiken JJ, Blommaert PJ, van Woerkom GM, Meijer AJ. Phosphorylation of ribosomal protein S6 is inhibitory for autophagy in isolated rat hepatocytes. *J Biol Chem* 1995; 270:2320–2326.
67. Noda T, Ohsumi Y. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J Biol Chem* 1998; 273:3963–3966.
68. Stromhaug PE, Klionsky DJ. Approaching the molecular mechanism of autophagy. *Traffic* 2001; 2:524–531.
69. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; 100:57–70.
70. Alva AS, Gultekin SH, Baehrecke EH. Autophagy in human tumors: cell survival or death. *Cell Death Differ* 2004; 11:1046–1048.
71. Kisen GO, Tessitore L, Costelli P, Gordon PB, Schwarze PE, Baccino FM, Seglen PO. Reduced autophagic activity in primary rat hepatocellular carcinoma and ascites hepatoma cells. *Carcinogenesis* 1993; 14:2501–2505.
72. Rez G, Toth S, Palfia Z. Cellular autophagic capacity is highly increased in azaserine-induced premalignant atypical acinar nodule cells. *Carcinogenesis* 1999; 20:1893–1898.
73. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* 2003; 112:1809–1820.
74. Yue Z, Jin S, Yang C, Levine AJ. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci USA* 2003; 100:15077–15082.
75. Tsuneoka M, Umata T, Kimura H, Koda Y, Nakajima M, Kosai K, Takahashi T, Takahashi Y, Yamamoto A. c-Myc induces autophagy in rat 3Y1 fibroblast cells. *Cell Struct Funct* 2003; 28:195–204.
76. Ellington AA, Berhow MA, Singletary KW. Inhibition of Akt signaling and enhanced ERK1/2 activity are involved in induction of macroautophagy by triterpenoid B-group soyasaponins in colon cancer cells. *Carcinogenesis* 2006; 27(2):298–306.
77. Pattingre S, Bauvy C, Codogno P. Amino acids interfere with the ERK1/2-dependent control of macroautophagy by controlling the activation of Raf-1 in human colon cancer HT-29 cells. *J Biol Chem* 2003; 278:16667–16674.
78. Stein RC, Waterfield MD. PI3-kinase inhibition: a target for drug development? *Mol Med Today* 2000; 6:347–357.
79. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* 2002; 4:648–657.
80. Hidalgo M, Rowinsky EK. The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. *Oncogene* 2000; 19:6680–6686.
81. Inbal B, Bialik S, Sabanay I, Shani G, Kimchi A. DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. *J Cell Biol* 2002; 157:455–468.
82. Feng Z, Zhang H, Levine AJ, Jin S. The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci USA* 2005; 102:8204–8209.
83. Hockel S, Schlenger K, Vaupel P, Hockel M. Association between host tissue vascularity and the prognostically relevant tumor vascularity in human cervical cancer. *Int J Oncol* 2001; 19:827–832.
84. David H, Ellermann J, Uerlings I. Primary phase of hepatocytic autophagocytosis under ischaemic conditions. *Exp Toxicol Pathol* 1992; 44:74–80.
85. Shankar A, Loizidou M, Taylor I. The vascularity of colorectal liver metastases. *Eur J Surg Oncol* 1996; 22:389–396.
86. Chau YP, Lin SY, Chen JH, Tai MH. Endostatin induces autophagic cell death in EAhy926 human endothelial cells. *Histol Histopathol* 2003; 18:715–726.
87. Steinbach JP, Eisenmann C, Klumpp A, Weller M. Co-inhibition of epidermal growth factor receptor and type 1 insulin-like growth factor receptor synergistically sensitizes human malignant glioma cells to CD95L-induced apoptosis. *Biochem Biophys Res Commun* 2004; 321:524–530.

88. Takeuchi H, Kanzawa T, Kondo Y, Kondo S. Inhibition of platelet-derived growth factor signalling induces autophagy in malignant glioma cells. *Br J Cancer* 2004; 90:1069–1075.
89. Emmans VC, Rodway HA, Hunt AN, Lillycrop KA. Regulation of cellular processes by PPARgamma ligands in neuroblastoma cells is modulated by the level of retinoblastoma protein expression. *Biochem Soc Trans* 2004; 32:840–842.
90. Kanzawa T, Kondo Y, Ito H, Kondo S, Germano I. Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. *Cancer Res* 2003; 63:2103–2108.
91. Anderson KM, Seed T, Vos M, Mulshine J, Meng J, Alrefai W, Ou D, Harris JE. 5-Lipoxygenase inhibitors reduce PC-3 cell proliferation and initiate nonnecrotic cell death. *Prostate* 1998; 37:161–173.
92. Opirari AW Jr, Tan L, Boitano AE, Sorenson DR, Aurora A, Liu JR. Resveratrol-induced autophagocytosis in ovarian cancer cells. *Cancer Res* 2004; 64:696–703.
93. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death Differ* 2004; 11:448–457.
94. Paglin S, Lee NY, Nakar C, Fitzgerald M, Plotkin J, Deuel B, Hackett N, McMahill M, Sphicas E, Lampen N, Yahalom J. Rapamycin-sensitive pathway regulates mitochondrial membrane potential, autophagy, and survival in irradiated MCF-7 cells. *Cancer Res* 2005; 65:11061–11070.
95. Takeuchi H, Kondo Y, Fujiwara K, Kanzawa T, Aoki H, Mills GB, Kondo S. Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors. *Cancer Res* 2005; 65:3336–3346.
96. Shao Y, Gao Z, Marks PA, Jiang X. Apoptotic and autophagic cell death induced by histone deacetylase inhibitors. *Proc Natl Acad Sci USA* 2004; 101:18030–18035.
97. Bursch W, Ellinger A, Kienzl H, Torok L, Pandey S, Sikorska M, Walker R, Hermann RS. Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy. *Carcinogenesis* 1996; 17:1595–1607.
98. Bursch W, Hohegger K, Torok L, Marian B, Ellinger A, Hermann RS. Autophagic and apoptotic types of programmed cell death exhibit different fates of cytoskeletal filaments. *J Cell Sci* 2000; 113:1189–1198.
99. Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D, Yahalom J. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* 2001; 61:439–444.
100. Bauvy C, Gane P, Arico S, Codogno P, Ogier-Denis E. Autophagy delays sulindac sulfide-induced apoptosis in the human intestinal colon cancer cell line HT-29. *Exp Cell Res* 2001; 268:139–149.
101. Jia L, Dourmashkin RR, Allen PD, Gray AB, Newland AC, Kelsey SM. Inhibition of autophagy abrogates tumour necrosis factor alpha induced apoptosis in human T-lymphoblastic leukaemic cells. *Br J Haematol* 1997; 98:673–685.
102. Bursch W. The autophagosomal-lysosomal compartment in programmed cell death. *Cell Death Differ* 2001; 8:569–581.
103. Jäättelä M. Multiple cell death pathways as regulators of tumour initiation and progression. *Oncogene* 2004; 23:2746–2756.
104. Piacentini M, Evangelisti C, Mastroberardino PG, Nardacci R, Kroemer G. Does prothymosin-alpha act as molecular switch between apoptosis and autophagy. *Cell Death Differ* 2003; 10:937–939.
105. Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science* 2004; 304:1500–1502.
106. Mills KR, Reginato M, Debnath J, Queenan B, Brugge JS. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is required for induction of autophagy during lumen formation *in vitro*. *Proc Natl Acad Sci USA* 2004; 101:3438–3443.
107. Pyo JO, Jang MH, Kwon YK, Lee HJ, Jun JI, Woo HN, Cho DH, Choi B, Lee H, Kim JH, Mizushima N, Oshumi Y, Jung YK. Essential roles of Atg5 and FADD in autophagic cell death: dissection of autophagic cell death into vacuole formation and cell death. *J Biol Chem* 2005; 280:20722–20729.
108. Camougrand N, Kissova I, Velours G, Manon S. Uth1p: a yeast mitochondrial protein at the crossroads of stress, degradation and cell death. *FEMS Yeast Res* 2004; 5:133–140.
109. Lemasters JJ, Qian T, He L, Kim JS, Elmore SP, Cascio WE, Brenner DA. Role of mitochondrial inner membrane permeabilization in necrotic cell death, apoptosis, and autophagy. *Antioxid Redox Signal* 2002; 4:769–781.

110. Tolkovsky AM, Xue L, Fletcher GC, Borutaite V. Mitochondrial disappearance from cells: a clue to the role of autophagy in programmed cell death and disease. *Biochimie* 2002; 84:233–240.
111. Reipert S, Berry J, Hughes MF, Hickman JA, Allen TD. Changes of mitochondrial mass in the hemopoietic stem cell line FDCP-mix after treatment with etoposide: a correlative study by multi-parameter flow cytometry and confocal and electron microscopy. *Exp Cell Res* 1995; 221:281–288.
112. Xue L, Fletcher GC, Tolkovsky AM. Mitochondria are selectively eliminated from eukaryotic cells after blockade of caspases during apoptosis. *Curr Biol* 2001; 11:361–365.
113. Kirkland RA, Adibhatla RM, Hatcher JF, Franklin JL. Loss of cardiolipin and mitochondria during programmed neuronal death: evidence of a role for lipid peroxidation and autophagy. *Neuroscience* 2002; 115:587–602.
114. Elmore SP, Qian T, Grissom SF, Lemasters JJ. The mitochondrial permeability transition initiates autophagy in rat hepatocytes. *FASEB J* 2001; 15:2286–2287.
115. Kissova I, Deffieu M, Manon S, Camougrand N. Uth1p is involved in the autophagic degradation of mitochondria. *J Biol Chem* 2004; 279:39068–39074.
116. Liang XH, Kleeman LK, Jiang HH, Gordon G, Goldman JE, Berry G, Herman B, Levine B. Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. *J Virol* 1998; 72:8586–8596.
117. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 2005; 122:927–939.
118. Saeki K, Yuo A, Okuma E, Yazaki Y, Susin SA, Kroemer G, Takaku F. Bcl-2 down-regulation causes autophagy in a caspase-independent manner in human leukemic HL60 cells. *Cell Death Differ* 2000; 7:1263–1269.
119. Yanagisawa H, Miyashita T, Nakano Y, Yamamoto D. HSpin1, a transmembrane protein interacting with Bcl-2/Bcl-xL, induces a caspase-independent autophagic cell death. *Cell Death Differ* 2003; 10:798–807.
120. Vande Velde C, Cizeau J, Dubik D, Alimonti J, Brown T, Israels S, Hakem R, Greenberg AH. BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. *Mol Cell Biol* 2000; 20:5454–5468.
121. Boya P, González-Polo R-A, Casares N, Perfettini JL, Larochette N, Métivier D, Meley D, Souquere S, Yoshimori T, Pierron G, Codogno P, Kroemer G. Inhibition of macroautophagy triggers apoptosis. *Mol Cell Biol* 2005; 25:1025–1040.
122. Baehrecke EH. Autophagic programmed cell death in *Drosophila*. *Cell Death Differ* 2003; 10:940–945.
123. Larsen KE, Sulzer D. Autophagy in neurons: a review. *Histol Histopathol* 2002; 17:897–908.
124. Yuan J, Lipinski M, Degtrev A. Diversity in the mechanisms of neuronal cell death. *Neuron* 2003; 40:401–413.
125. Martin DN, Baehrecke EH. Caspases function in autophagic programmed cell death in *Drosophila*. *Development* 2004; 131:275–284.
126. Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME. Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* 1997; 275:661–665.
127. Xue LZ, Fletcher GC, Tolkovsky AM. Autophagy is activated by apoptotic signalling in sympathetic neurons: an alternative mechanism of death execution. *Mol Cell Neurosci* 1999; 14:180–198.
128. Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB, Tsujimoto Y. Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. *Nat Cell Biol* 2004; 6:1221–1228.
129. Kosta A, Roisin-Bouffay C, Luciani MF, Otto GP, Kessin RH, Golstein P. Autophagy gene disruption reveals a non-vacuolar cell death pathway in *Dictyostelium*. *J Biol Chem* 2004; 279:48404–48409.
130. Edinger AL, Thompson CB. Defective autophagy leads to cancer. *Cancer Cell* 2003; 4:422–424.

6

Regulation and Function of Detachment-Induced Cell Death (Anoikis) in Cancer Progression and Metastasis

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SUMMARY

Epithelial cells and possibly all normal cell types must receive adhesion-dependent signals from their microenvironments to survive. The cell death that is triggered when adherent cells detach has been termed “anoikis,” and the appreciation that tumor metastasis involves the suppression of anoikis has prompted aggressive investigation into the biochemical and molecular mechanisms that control the process. Much of this work has focused on integrins and the integrin-mediated signaling events that mediate attachment-induced normal cell survival and the molecular mechanisms that are activated in metastatic tumor cells that uncouple these responses. However, it also appears that unique cell death pathway(s) can be activated in metastatic tumor cells when conventional caspase-dependent apoptotic pathways are disrupted, some of which resemble those activated in cells undergoing autophagy (auto-digestion). Here, we will review what is known about the signaling pathways that control attachment-mediated survival in normal cells and the molecular alterations that appear to disrupt these pathways in metastatic cells. We will also review the evidence that alternative cell death mechanism(s) can be activated in metastatic tumors when apoptosis is disrupted.

Key Words: Integrin; AKT; caspase; Bim; autophagy.

1. SOCIAL CONTROLS ON CELL SURVIVAL AND CELL DEATH

Over a century ago, Paget first proposed the “seed and soil” hypothesis, whereby tumor metastasis depends not only on specific tumor cell properties (the “seed”) but also on appropriate characteristics found in the host microenvironment (the “soil”) (1,2). Work performed by cancer metastasis researchers over the past three decades

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has confirmed Paget's hypothesis and identified some of the specific host factors involved (2,3). Indeed, one important early observation demonstrated that tumor cells are remarkably heterogeneous with respect to their abilities to survive in the circulation and colonize particular host microenvironments, such that the vast majority of cells die en route (2).

Martin Raff (4) first proposed the idea that cell death (apoptosis) is the default pathway in all mammalian cells beyond the blastomere stage of development. In a pioneering opinion piece on the subject, he proposed that this default cell death was suppressed in normal cells through signals generated by their specific microenvironments, involving cytokines, hormones, and adhesion molecules (4). He also argued that migration from a normal cell's tissue of origin would strip it of these survival signals, resulting in apoptosis. He suggested that this response would serve as a safeguard to the inappropriate cellular migration that occurs during tumor metastasis, the process that results in the vast majority of the mortality observed in patients with solid tumors.

These observations prompted Frisch and Francis (5) and other investigators to directly characterize the effects of detachment on cell death in populations of cells that normally require attachment for their expansion (epithelial and endothelial cells and fibroblasts). These studies demonstrated that detachment results in rapid induction of apoptosis, and Frisch and Francis coined the term "anoikis" to describe the process (5). Anoikis is studied *in vitro* by plating cells on supports that do not allow for cellular attachment (bacterial plates and plates coated with poly-L-lysine) or by rotating them in continuous suspension culture. It is also likely that the classical approach for isolating tumorigenic and metastatic cells by growing them in soft agar also selects for cells that resist anoikis. Whether any of these conditions accurately model the pressures metastatic tumor cells are subjected to *in vivo* remains an unanswered question. Furthermore, it is not clear how long a tumor cell must resist anoikis in transit between the primary tumor and the distant site, and it is possible that the *in vitro* conditions exaggerate the stress exerted upon tumor cells *in vivo*.

2. ADHESION-DEPENDENT SURVIVAL SIGNALING

Integrins play a central role in mediating adhesion between adherent cells and the extracellular matrix, and the signal transduction pathways they activate to suppress apoptosis in attached cells have been the focus of most of the investigation in the field overall (6–9). Although several different protein kinases are independently activated by integrins, the work performed to date indicates that they converge on the phosphatidylinositol-3' (PI-3) kinase/AKT (AKR mouse thymoma transforming oncogene) pathway to promote survival (7,10,11). In addition, many of the genetic alterations that accumulate in metastatic tumors, and in particular the mutational inactivation of the lipid phosphatase on chromosome 10 (PTEN) (12) and activation of the Ras pathway (3,11,13), also collaborate to promote AKT activation and metastasis and to suppress anoikis.

2.1. Role of AKT

AKT activation is mediated by the generation of 3'-polyphosphoinositides, which bind to AKT's pleckstrin homology (PH) domain and recruit it to the inner surface of the plasma membrane (14), and by phosphorylation of AKT at T308 and S473 (10,15).

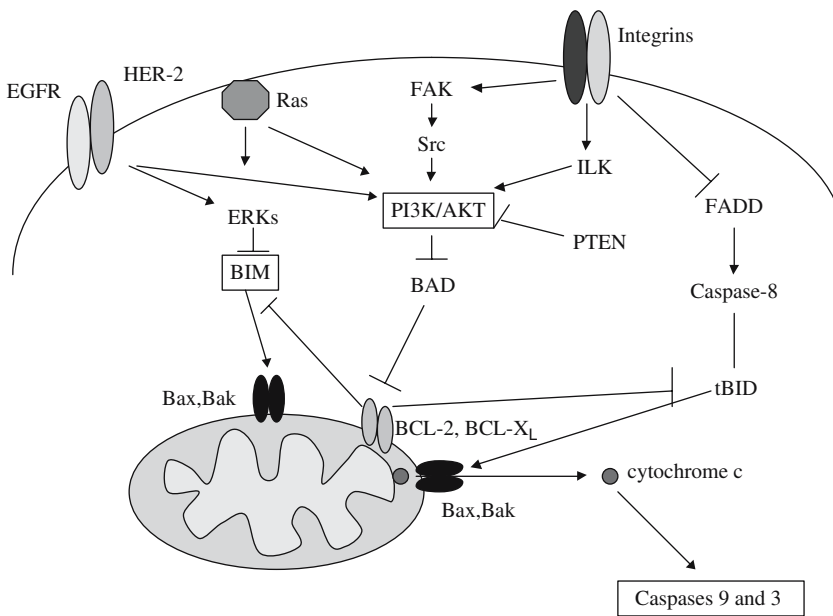


Fig. 1. Control of anoikis in apoptosis-proficient cells. Integrins and members of the erbB family of growth factor receptors (EGFR, HER-2), as well as mutant forms of Ras, drive activation of the ERK and AKT pathways, leading to phosphorylation of BH3-only members of the BCL-2 family (Bim, Bad) and suppression of cytochrome c release. Thus, detachment from the matrix disengages integrin signaling and unleashes these proteins to activate Bax and Bak, resulting in cytochrome c release and cell death. Under these circumstances, effector caspases (caspases 3, 6, and 7) carry out the proteolysis that is required for DNA fragmentation.

Integrin-linked kinase (ILK) is a serine-threonine, PH domain-containing kinase that binds to the cytoplasmic tails of $\beta 1$ -integrins (16,17) and functions to suppress anoikis (18) (Fig. 1). Several studies have demonstrated that ILK promotes phosphorylation of AKT on S473, and siRNA-mediated knockdown of ILK blocks integrin-mediated AKT activation and survival signaling (19–22). Whether ILK directly phosphorylates S473 or indirectly promotes phosphorylation by activating another AKT kinase remains unclear. Nonetheless, there is consensus that ILK-mediated activation of AKT plays an important (if not exclusive) role in adhesion-mediated cell survival (Fig. 1).

2.2. Role of Focal Adhesion Kinase

The focal adhesion kinase (FAK) protein tyrosine kinase is a second integrin-associated signal transduction intermediate that plays a central role in integrin-mediated survival (8,23) (Fig. 1). Dominant active forms of FAK block anoikis (23,24), whereas inhibition of FAK by various approaches induces cell rounding and detachment and promotes cell death (24,25). The molecular mechanisms involved in FAK's effects are still under investigation but appear to involve crosstalk with Src (26,27) (Fig. 1), and parallel studies have established that active Src can also block detachment-induced cell death (26,28). Furthermore, FAK also regulates the activity of the death receptor-associated protein serine/threonine kinase receptor-interacting protein (RIP) (29,30),

which regulates survival by promoting activation of the transcription factor, nuclear factor (NF)- κ B (31–34). FAK is cleaved by caspases, resulting in inactivation of its survival-promoting activity and reinforcing cell death signaling (35–38). Direct contacts between FAK and its cytoskeletal interacting proteins (vinculin, talin, paxillin, and the actin cytoskeleton) also appear to be crucial for survival signaling, presumably because they control the activation of the BH3-only members of the BCL-2 family that appear to control commitment to cell death (see Section 3.3).

2.3. Role of PTEN

The lipid PTEN is a third important signaling intermediate that controls AKT activation and anoikis (39,40) (Fig. 1). The protein was originally cloned at a frequent site of loss of heterozygosity on chromosome 10q in advanced solid tumors (41–43), and early analysis of its sequence suggested that it might be a protein phosphatase (41). However, subsequent studies demonstrated that PTEN is more active as a lipid phosphatase that removes phosphate groups from the 3' position of polyphosphoinositides (12,42). Thus, PTEN is a direct antagonist of PI-3 kinase and downstream targets that contain PH domains (e.g., ILK and AKT). Loss of wild-type PTEN is a relatively common event not only in glioblastomas but also in a variety of other solid malignancies, leading to elevated basal AKT activation (12). Reintroduction of wild-type PTEN into cells that lack it tends not to have a dramatic effect on spontaneous apoptosis but does dramatically sensitize cells to anoikis (39,40).

2.4. Role of Ras

Ras pathway activation leading to activation of the extracellular signal-regulated kinases (ERKs) can also suppress anoikis (44–46) (Fig. 1), but it is not clear that the ERKs are as directly regulated by adhesion-dependent signaling as the PI-3 kinase/AKT pathway is. Rather, ERK activation appears to be more commonly driven by either mutations in the Ras proteins themselves (particularly K-ras in lung and pancreatic cancers) or constitutive growth factor receptor [erbB family of growth factor receptor (EGFR), human epidermal growth factor receptor-2 (HER-2)] signaling (Fig. 1). Interestingly, detachment results in downregulation of EGFR expression in MCF-10a cells, and it is possible that this effect is required for subsequent cell death (44).

3. MOLECULAR MECHANISMS UNDERLYING DETACHMENT-INDUCED APOPTOSIS

The observation that loss of matrix attachment results in apoptosis was first reported by Frisch and Francis in 1994 (5). Since then, studies aimed at understanding the molecular mechanisms underlying the phenomenon coupled with advances in the field of apoptosis overall have helped to elucidate several of the key players and to create hypotheses for how they are regulated. Overall, there is good consensus that AKT and the ERKs play important roles in suppressing anoikis and that the cytoskeleton-associated, proapoptotic, BH3-only BCL-2 family members, BCL-2-interacting mediator of cell death (Bim) and Bcl-2-modifying factor (Bmf), are critical for commitment to cell death, at least in certain cell types (Fig. 1). However, parallel studies have implicated AKT and Bim more broadly in apoptosis regulation, raising

the question of whether they are especially important for this specialized pathway of cell death. Furthermore, we are finding that metastatic tumors that possess defects in the regulation of caspase activation (overexpression of BCL-2, inactivation of caspase-3) can still die just as efficiently as non-metastatic cells that do not possess these defects, and death in these cells is still associated with “classical” oligonucleosomal DNA fragmentation (so-called DNA laddering) (47). We will therefore begin with a discussion of the consensus that exists regarding the roles of caspases and BCL-2 family proteins in anoikis and conclude with a section describing the “unorthodox” anoikis that is often observed in metastatic tumors.

3.1. *Anoikis: Extrinsic or Intrinsic?*

Historically, apoptotic responses have been described as “extrinsic” or “intrinsic” based on whether initiator caspase activation is driven by cell surface (death) receptors or by Bax/Bak-mediated release of cytochrome c from mitochondria (48,49). Thus, extrinsic pathway stimuli initiate apoptosis through formation of a “death-inducing signaling complex” (DISC) and Fas-associated death domain (FADD)-dependent aggregation of procaspase-8, whereas intrinsic pathway stimuli initiate apoptosis through formation of an “apoptosome” and Apaf-1-dependent aggregation of procaspase-9. Complicating things is the observation that activated caspase-8 can trigger the intrinsic pathway by cleaving the BH3 protein, Bid, which translocates to mitochondria to induce cytochrome c release (50,51). Thus, extrinsic pathways have been further subclassified as “type I” or “type II” (52) based on whether induction of apoptosis requires this so-called mitochondrial amplification loop (as it does in type II cells).

Abundant evidence implicates caspase-8 as an important contributor to cell death during anoikis (53–60) (Fig. 1), although precisely how it is activated remains the subject of some controversy. Several groups have demonstrated that caspase-8 is activated early in cells undergoing anoikis in a manner that is insensitive to inhibitors of caspase-9 or caspase-3 (53,54,61). Furthermore, dominant-negative forms of FADD and other molecular strategies to inhibit FADD-dependent caspase-8 activation are strong inhibitors of anoikis-associated cell death, and proteins that modulate the FADD-dependent activation of caspase-8 [death-associated protein 3 (DAP3), Fas-associated death domain-like interleukin-1beta converting enzyme (FLICE) inhibitory protein (FLIP)] also regulate anoikis (54). Beyond these observations, there is less consensus with respect to how FADD and caspase-8 are activated. One study concluded that detachment induces increased Fas and FasL expression in endothelial cells and that FasL was required for cell death (55). However, another group disputed these conclusions and suggested instead that FADD-dependent activation of caspase-8 occurs independently of death ligand-induced aggregation of death receptors (7). Other complicating issues include the suggestion that Bid is not processed to tBid in cells undergoing anoikis but rather the full-length protein translocates to mitochondria during the response (62). Furthermore, caspase-8 activation is at least partially blocked by BCL-2 and BCL-X_L (54), proteins that are thought to act primarily at the level of mitochondrial cytochrome c release (49) and downstream of FADD-dependent activation of caspase-8. Other work argued that caspase-8 activation occurs downstream of cytochrome c release in human intestinal epithelial cells (63), and evidence has been advanced that Bax translocates to mitochondria within 15 min in detached primary breast epithelial

cells and subsequently forms large aggregates within 1 h that are associated with loss of cytochrome c and mitochondrial membrane potential, suggesting that the intrinsic pathway might be activated directly (64). Whether an orthodox or unorthodox extrinsic pathway or an intrinsic pathway predominates could also be cell-type dependent.

3.2. *BCL-2 Family Proteins in Apoptosis*

Members of the BCL-2 family of cell death regulators play evolutionarily conserved roles in promoting or inhibiting apoptosis in eukaryotes (49,65,66). The BCL-2 family can be divided into three structure-based subcategories. The founding member of the family (BCL-2) and its anti-apoptotic structural cousins (BCL-X_L, MCL-1, A1) contain four so-called BCL-2 homology (BH) domains, and they inhibit apoptosis (at least in part) by directly binding to and neutralizing the proapoptotic effects of other members of the family (Fig. 1). The proapoptotic, multidomain Bax, Bak, and Bok proteins contain only three of the four BH domains found in their anti-apoptotic relatives, and they can aggregate to form pores in the outer mitochondrial membrane, thereby facilitating cytochrome c release and downstream caspase activation (Fig. 1). The third subcategory consists of a group of proapoptotic proteins that only share their BH3 domains with the other members of the family. The BH3-only proteins appear to function at the interface of upstream signaling events and commitment to cell death (65,66) (Fig. 1). Thus, the mechanisms that control the activation or inactivation of the BH3-only proteins have become the subject of intensive investigation.

The best worked out of these mechanisms involves the activation of the BH3-only protein Bid by activated caspases 8 or 10 (50,51) (Fig. 1). Full-length Bid is a largely inert protein that localizes to the cytosol in resting viable cells. Active caspase-8 promotes the N-terminal cleavage of Bid, forming an active form of the protein (tBid) that translocates to mitochondria to promote cytochrome c release and possibly other ultrastructural events (67) that facilitate this release. Bid-deficient cells fail to display cytochrome c release in response to death receptors and other caspase-8 activators (68,69), indicating that Bid and/or tBid plays a non-redundant role in this pathway. There is also evidence that Bim can be regulated by proteolytic cleavage (70), but additional investigation is required to establish the general relevance of these observations.

Phosphorylation-mediated inhibition of BH3 protein function represents a second major mechanism for the control of these proteins by upstream signaling pathways. The best-characterized example of this mechanism is found in the case of Bad (71,72), which is phosphorylated on S132 by AKT (73), on S112 by cAMP-dependent protein kinase (PKA) (74), and on S132 by p70S6K (75) (Fig. 1). Phosphorylation of Bad promotes its association with 14-3-3 proteins, thereby sequestering it away from the anti-apoptotic proteins it neutralizes (BCL-2 and BCL-X_L) (71). Not surprisingly, Bad has been implicated in the cell death that is induced by cytokine withdrawal (72), presumably because growth factors suppress Bad activation through their effects on AKT activation. The BH3-only protein Bim also appears to be regulated by phosphorylation (45,46,76,77) (Fig. 1). However, as we will discuss below (Section 3.3), most of the available evidence implicates the ERKs rather than AKT in the suppression of Bim function. Bim phosphorylation appears to promote its ubiquitylation and degradation through the proteasome.

There is general consensus that apoptosis-associated cytochrome c release is dependent on the multi-domain proapoptotic BCL-2 proteins (i.e., Bax and Bak) (78),

but precisely how they are activated remains unclear. Letai and co-workers (79) used isolated mitochondria to obtain evidence that a subset of the BH3-only members directly bind to and activate Bax and Bak to form oligomeric pores in the outer mitochondrial membrane, and this result was subsequently confirmed and extended by Kuwana et al. (80) in reconstituted lipid bilayers (Fig. 1). However, it has also been argued that the effects of the BH3-only proteins are really limited to disrupting interactions between the pro- and anti-apoptotic multi-domain family members (65), as convincing evidence for direct binding of the BH3-only proteins to either Bax or Bak has been difficult to obtain. Thus, it is possible that the functional differences noted in the previous studies (79) had more to do with differences in the spectrum of BH3-binding partners rather than to differential effects on Bax or Bak activation (65).

3.3. *BCL-2 Family Proteins in Anoikis*

Bmf is a BH3-only member of the BCL-2 family that was isolated in a yeast two-hybrid screen for MCL-1-interacting partners (81). It interacts with the myosin V actin motor in resting cells by binding to dynein light chain 2 (DLC2). Yeast two-hybrid studies indicated that Bmf interacts with all the major anti-apoptotic BCL-2 family members interrogated but does not interact stably with Bax or Bak (81). The protein is expressed at high levels in hematopoietic cells as well as in epithelial cells of the kidney, liver, and pancreas. Transient overexpression of the protein in Jurkat cells induced apoptosis, and stable combined overexpression of Bmf and BCL-2 sensitized hematopoietic cells to growth factor withdrawal-induced apoptosis relative to cells expressing BCL-2 alone (81). These observations support the idea that Bmf plays a role in monitoring the integrity of the actin cytoskeleton and could orchestrate the cell death that occurs following disruption of integrin signaling. Consistent with this idea, Bmf was released from DLC2 and formed complexes in human MCF-7 breast adenocarcinoma cells cultured on poly-Hema plates (81). Whether Bmf is actually required for detachment-induced cell death remains to be determined, for example, by silencing its expression with siRNA.

The BH3-only protein Bim also interacts with the cytoskeleton in resting cells through its interaction with cytoplasmic DLC1 (82). However, this interaction appears to promote the interaction of Bim with microtubular dynein motor complexes through DLC1-mediated binding to dynein intermediate chain. Thus, studies with Bim-deficient thymocytes established that the protein plays a central role in cell death induced by the microtubule-stabilizing drug, paclitaxel (83), whereas paclitaxel appears to have little to no effect on mobilization of Bmf (81). Conversely, in the original study that described the involvement of Bmf in anoikis, detachment did not induce release of Bim from microtubule dynein motor complexes in MCF-7 cells under conditions that promoted release of Bmf (81).

Nonetheless, several more recent studies strongly suggest that Bim does play a critical role in anoikis (Fig. 1). Detachment leads to a rapid and sustained increase in Bim protein levels, and a Bim-specific siRNA construct inhibited anoikis-associated cell death (44). Bim stabilization is associated with a decrease in EGFR and ERK signaling (44) (Fig. 1), and in fact, upstream ERK antagonists (MEK inhibitors) promote sensitivity to detachment-induced cell death in human MDA-MB-231 breast adenocarcinoma cells (76). Overall, it appears that the Ras–Raf–ERK pathway controls cellular Bim expression by phosphorylation of S69, leading to ubiquitylation of the

protein and its degradation via the proteasome (76). Interestingly, cells arrested at the G₁/S transition may also display resistance to anoikis (45). This mechanism also paradoxically appears to involve ERK activity, but in this case ERK activation is apparently not controlled by upstream Ras–Raf–MEK activation but rather by inhibition of ERK phosphatase activity (45).

The BH3-only protein Bid has also been implicated in anoikis, but its involvement may be somewhat unorthodox. Typically, as discussed above (Section 3.2), Bid is activated by caspase-8-mediated cleavage, which generates a truncated form of the protein (tBid) that possesses an enhanced ability to translocate to mitochondria to activate Bax or Bak (Fig. 1). Thus, Bid is a central player in cell death that is driven by caspase-8 activation, most notably in response to death receptor engagement. However, it has been suggested that full-length Bid translocates to mitochondria in cells undergoing anoikis, where it may be capable of activating Bax and/or Bak independently of cleavage (62). Further investigation is required to establish the prevalence of these effects and to define the biochemical mechanisms involved. For example, it is conceivable that unique posttranslational modification(s) induced during anoikis are required for the response. It is also interesting to note that full-length Bid has recently been implicated in DNA repair (84), arguing that, as is true of FADD (85) and caspase-8 (86), its spectrum of physiological activities extends beyond the regulation of cell death.

3.4. Caspase-Independent Mechanisms in Anoikis

Given their evolutionarily conserved roles in cell death, most investigators equate caspase dependence with apoptosis (87). Similarly, most of the investigations into the molecular mechanisms that regulate anoikis have focused on pathways that directly or indirectly regulate caspase activation (Fig. 1). However, the bulk of this work has been conducted with non-transformed cells (HUVEC endothelial cells, MDCK cells, human MCF10a breast epithelial cells), and it is not clear that anoikis becomes a barrier to cancer progression before solid tumors become metastatic. Indeed, growth in suspension does lead to induction of apoptosis in many of the commonly studied human solid tumor cell lines, although perhaps not surprisingly the kinetics of these responses may be somewhat delayed (>8 h) relative to those observed in the non-transformed cells. Because solid tumors acquire defects in the control of apoptosis as they progress, the relative resistance of frank carcinomas with respect to what is observed in normal epithelial cell lines is to be expected.

In an effort to directly assess the importance of anoikis resistance in tumor metastasis, we have compared isogenic non-metastatic and metastatic variants of various human solid tumor cell lines with respect to their abilities to undergo anoikis *in vitro*. Our work has focused on variants of two commonly studied human prostate cancer cell lines (LNCaP and PC-3) that were selected for enhanced spontaneous metastasis through orthotopic “recycling” in nude mice (47), although we have performed experiments with human colon cancer cell lines as well (28,88). Others have performed similar studies with human oral squamous cell carcinoma lines (89). We previously reported that LNCaP cells selected for increased metastatic potential (LNCaP-LN3) were resistant to thapsigargin- and doxorubicin-induced apoptosis (90) and displayed androgen-independent growth *in vitro* and *in vivo* (91). Apoptosis resistance appeared to be driven by increased expression of BCL-2 (90), consistent with the observation that androgen-independent primary tumors also display elevated BCL-2 levels.

In subsequent experiments, we measured apoptosis-associated DNA fragmentation in the non-metastatic and metastatic LNCaP subclones produced when the cells were cultured in suspension by two different strategies (rotation or plating on agarose) (47). We expected that the elevated BCL-2 expression and global apoptosis resistance displayed by the metastatic subclones would translate into increased resistance to anoikis. Unexpectedly, rates of DNA fragmentation were indistinguishable in the non-metastatic and metastatic cells. In subsequent experiments, we were also surprised to find that even enforced stable expression of BCL-2 failed to inhibit anoikis-associated DNA fragmentation in the non-metastatic or metastatic cells (47).

Further characterization of the biochemical mechanisms that mediated DNA fragmentation in the cell lines did reveal important differences between the non-metastatic and metastatic cells (V.Bondar, manuscript submitted). In the former, suspension culture induced cytochrome c release from mitochondria and caspase-3 activation, and DNA fragmentation was partially blocked by the pan-caspase inhibitor zVADfmk. However, in the metastatic cells or the BCL-2 transfectants, anoikis did not trigger significant cytochrome c release or caspase-3-like activity, and zVADfmk had no effect on “DNA laddering.” Similar results were obtained in human MCF-7 breast adenocarcinoma cells that lack caspase-3 because of mutation. In these apoptosis-resistant cells, a chemical inhibitor of cathepsin B (CA0-074Me) attenuated DNA fragmentation (Fig. 2), and we are currently exploring the possibility that the response is mediated via lysosomal proteases and/or an alternative physiological cell death pathway known as “autophagy” that, like apoptosis, may also be relevant to tumor progression and metastasis (92) (Fig. 2).

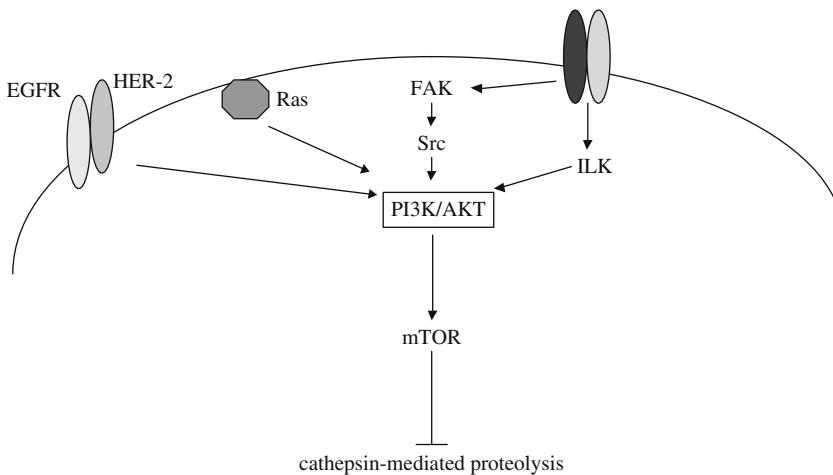


Fig. 2. Hypothetical model: cathepsins mediate anoikis in apoptosis-resistant cells. Many metastatic tumors acquire defects in the control of apoptosis that prevent cytochrome c release and/or effector caspase activation. However, these cells can still undergo a form of anoikis-associated cell death that involves classical “DNA laddering.” We speculate that in these cells, lysosomal proteases (cathepsins) carry out the proteolytic events that are required for cell death, because a specific chemical inhibitor of cathepsin B can inhibit DNA fragmentation under these circumstances. Cell death is still associated with decreased AKT phosphorylation, but in our model, this allows for cathepsin mobilization, perhaps by relieving molecular target of rapamycin (mTOR)-mediated inhibition of autophagy.

Although our data create an interesting story, their physiological significance remains unclear. Because the LNCaP cell line was isolated from a prostate cancer lymph node metastasis, the parental cell line had already acquired molecular defects that rendered it metastasis-competent in the patient, and the further increase in metastatic potential generated by the orthotopic “recycling” might be a laboratory phenomenon. Furthermore, there is no way of knowing how long a tumor subclone must resist anoikis to allow it to seed a distant organ site: if the process takes place in less than an hour, then all the solid tumor cell lines studied by our group and others already possess enough resistance to survive the process. Finally, suspension culture often stimulates these cell lines to form aggregates, and previous work has shown that three-dimensional culture provides survival support that involves Ras pathway activation.

Nonetheless, independent studies have also implicated caspase-independent mechanisms in anoikis. An interesting example of this can be found in the recent implication of the Bit-1 protein in anoikis in endothelial cells (93). Bit-1 apparently localizes to the mitochondria in resting cells but is released during anoikis. It forms a complex with the Groucho family member and transcriptional coregulator, AES (amino terminal enhancer of split), which in turn interacts with the groucho/transducin-like enhancer of split (TLE) transcriptional repressors. Thus, AES binding to TLE proteins releases their transcriptional repression activities (94,95). Bit-1 in turn appears to be an inhibitor of AES, displacing the protein from preexisting AES–TLE complexes to facilitate transcriptional repression. Bit-1 also possesses peptidyl-tRNA hydrolase activity, and it is possible that this activity or other functions of the protein participate in cell death. It is still not clear how Bit-1 promotes caspase-independent cell death, because the targets identified to date (BCL-2, NF- κ B) are classical inhibitors of caspase activation and apoptosis.

So, how are we to reconcile the caspase-dependent and caspase-independent mechanisms in anoikis? Perhaps the simplest hypothesis is that the caspase-dependent pathways are the default ones in normal cells and that the caspase-independent pathways only become functionally relevant when the caspase-dependent pathways are disabled. Whether there is one caspase-independent pathway or multiple pathways remains to be determined, and the potential relevance of other emerging physiological cell death mechanisms (i.e., autophagy) needs to be evaluated. It is of interest that defects in the control of autophagy may accumulate during breast cancer progression, and it is possible that these defects act in concert with disruption of caspase activation to promote anoikis resistance. There are clearly plenty of unanswered questions in this interesting and highly relevant subspecialty within the field of apoptosis research.

REFERENCES

1. Paget S. The distribution of secondary growths in cancer of the breast. *Lancet* 1889; 1:571–3.
2. Fidler IJ. Critical determinants of metastasis. *Semin Cancer Biol* 2002; 12:89–96.
3. Kerbel RS. Significance of tumor-host interactions in cancer growth and metastases. *Cancer Metastasis Rev* 1995; 14:259–62.
4. Raff MC. Social controls on cell survival and cell death. *Nature* 1992; 356:397–400.
5. Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* 1994; 124:619–26.
6. Frisch SM, Ruoslahti E. Integrins and anoikis. *Curr Opin Cell Biol* 1997; 9:701–6.
7. Frisch SM, Sreaton RA. Anoikis mechanisms. *Curr Opin Cell Biol* 2001; 13:555–62.
8. Ruoslahti E. Fibronectin and its integrin receptors in cancer. *Adv Cancer Res* 1999; 76:1–20.

9. Liu W, Ahmad SA, Reinmuth N, et al. Endothelial cell survival and apoptosis in the tumor vasculature. *Apoptosis* 2000; 5:323–8.
10. Franke TF, Kaplan DR, Cantley LC. PI3K: downstream AKTion blocks apoptosis. *Cell* 1997; 88:435–7.
11. Khwaja A, Rodriguez-Viciana P, Wennstrom S, Warne PH, Downward J. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J* 1997; 16:2783–93.
12. Sansal I, Sellers WR. The biology and clinical relevance of the PTEN tumor suppressor pathway. *J Clin Oncol* 2004; 22:2954–63.
13. Bernstein SC, Weinberg RA. Expression of the metastatic phenotype in cells transfected with human metastatic tumor DNA. *Proc Natl Acad Sci USA* 1985; 82:1726–30.
14. Thompson JE, Thompson CB. Putting the rap on Akt. *J Clin Oncol* 2004; 22:4217–26.
15. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. *Nature* 2001; 411:355–65.
16. Dedhar S, Williams B, Hannigan G. Integrin-linked kinase (ILK: a regulator of integrin and growth-factor signalling. *Trends Cell Biol* 1999; 9:319–23.
17. Wu C. PINCH, N(i)ck and the ILK: network wiring at cell-matrix adhesions. *Trends Cell Biol* 2005; 15:460–6.
18. Attwell S, Roskelley C, Dedhar S. The integrin-linked kinase (ILK) suppresses anoikis. *Oncogene* 2000; 19:3811–5.
19. Troussard AA, Mawji NM, Ong C, Mui A, St-Arnaud R, Dedhar S. Conditional knock-out of integrin-linked kinase demonstrates an essential role in protein kinase B/Akt activation. *J Biol Chem* 2003; 278:22374–8.
20. Fukuda T, Chen K, Shi X, Wu C. PINCH-1 is an obligate partner of integrin-linked kinase (ILK) functioning in cell shape modulation, motility, and survival. *J Biol Chem* 2003; 278:51324–33.
21. Duxbury MS, Ito H, Benoit E, Waseem T, Ashley SW, Whang EE. RNA interference demonstrates a novel role for integrin-linked kinase as a determinant of pancreatic adenocarcinoma cell gemcitabine chemoresistance. *Clin Cancer Res* 2005; 11:3433–8.
22. Nho RS, Xia H, Kahm J, Kleidon J, Diebold D, Henke CA. Role of integrin-linked kinase in regulating phosphorylation of Akt and fibroblast survival in type I collagen matrices through a beta1 integrin viability signaling pathway. *J Biol Chem* 2005; 280:26630–9.
23. Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY. Control of adhesion-dependent cell survival by focal adhesion kinase. *J Cell Biol* 1996; 134:793–9.
24. Xia H, Nho RS, Kahm J, Kleidon J, Henke CA. Focal adhesion kinase is upstream of phosphatidylinositol 3-kinase/Akt in regulating fibroblast survival in response to contraction of type I collagen matrices via a beta 1 integrin viability signaling pathway. *J Biol Chem* 2004; 279:33024–34.
25. Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. Focal adhesion kinase gene silencing promotes anoikis and suppresses metastasis of human pancreatic adenocarcinoma cells. *Surgery* 2004; 135:555–62.
26. Hisano C, Tanaka R, Fujishima H, et al. Suppression of anoikis by v-Src but not by activated c-H-ras in human gallbladder epithelial cells. *Cell Biol Int* 2003; 27:415–21.
27. Wei L, Yang Y, Zhang X, Yu Q. Altered regulation of Src upon cell detachment protects human lung adenocarcinoma cells from anoikis. *Oncogene* 2004; 23:9052–61.
28. Windham TC, Parikh NU, Siwak DR, et al. Src activation regulates anoikis in human colon tumor cell lines. *Oncogene* 2002; 21:7797–807.
29. Stanger BZ, Leder P, Lee TH, Kim E, Seed B. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell* 1995; 81:513–23.
30. Kurenova E, Xu LH, Yang X, et al. Focal adhesion kinase suppresses apoptosis by binding to the death domain of receptor-interacting protein. *Mol Cell Biol* 2004; 24:4361–71.
31. Liu ZG, Hsu H, Goeddel DV, Karin M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. *Cell* 1996; 87:565–76.
32. Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity* 1996; 4:387–96.
33. Ting AT, Pimentel-Muinos FX, Seed B. RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. *EMBO J* 1996; 15:6189–96.
34. Funakoshi-Tago M, Sonoda Y, Tanaka S, et al. Tumor necrosis factor-induced nuclear factor kappaB activation is impaired in focal adhesion kinase-deficient fibroblasts. *J Biol Chem* 2003; 278:29359–65.

35. Gervais FG, Thornberry NA, Ruffolo SC, Nicholson DW, Roy S. Caspases cleave focal adhesion kinase during apoptosis to generate a FRNK-like polypeptide. *J Biol Chem* 1998; 273:17102–8.
36. Widmann C, Gibson S, Johnson GL. Caspase-dependent cleavage of signaling proteins during apoptosis. A turn-off mechanism for anti-apoptotic signals. *J Biol Chem* 1998; 273:7141–7.
37. Levkau B, Herren B, Koyama H, Ross R, Raines EW. Caspase-mediated cleavage of focal adhesion kinase pp125FAK and disassembly of focal adhesions in human endothelial cell apoptosis. *J Exp Med* 1998; 187:579–86.
38. Wen LP, Fahrni JA, Troie S, Guan JL, Orth K, Rosen GD. Cleavage of focal adhesion kinase by caspases during apoptosis. *J Biol Chem* 1997; 272:26056–61.
39. Lu Y, Lin YZ, LaPushin R, et al. The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells. *Oncogene* 1999; 18:7034–45.
40. Davies MA, Lu Y, Sano T, et al. Adenoviral transgene expression of MMAC/PTEN in human glioma cells inhibits Akt activation and induces anoikis. *Cancer Res* 1998; 58:5285–90.
41. Li J, Yen C, Liaw D, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 1997; 275:1943–7.
42. Myers MP, Stolarow JP, Eng C, et al. P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. *Proc Natl Acad Sci USA* 1997; 94:9052–7.
43. Steck PA, Pershouse MA, Jasser SA, et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* 1997; 15:356–62.
44. Reginato MJ, Mills KR, Paulus JK, et al. Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. *Nat Cell Biol* 2003; 5:733–40.
45. Collins NL, Reginato MJ, Paulus JK, Sgroi DC, Labaer J, Brugge JS. G1/S cell cycle arrest provides anoikis resistance through Erk-mediated Bim suppression. *Mol Cell Biol* 2005; 25:5282–91.
46. Marani M, Hancock D, Lopes R, Tenev T, Downward J, Lemoine NR. Role of Bim in the survival pathway induced by Raf in epithelial cells. *Oncogene* 2004; 23:2431–41.
47. Bondar VM, McConkey DJ. Anoikis is regulated by BCL-2-independent pathways in human prostate carcinoma cells. *Prostate* 2002; 51:42–9.
48. Strasser A, O'Connor L, Dixit VM. Apoptosis signaling. *Annu Rev Biochem* 2000; 69:217–45.
49. Hengartner MO. The biochemistry of apoptosis. *Nature* 2000; 407:770–6.
50. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998; 94:491–501.
51. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 1998; 94:481–90.
52. Scaffidi C, Fulda S, Srinivasan A, et al. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 1998; 17:1675–87.
53. Frisch SM. Evidence for a function of death-receptor-related, death-domain-containing proteins in anoikis. *Curr Biol* 1999; 9:1047–9.
54. Rytomaa M, Martins LM, Downward J. Involvement of FADD and caspase-8 signalling in detachment-induced apoptosis. *Curr Biol* 1999; 9:1043–6.
55. Aoudjit F, Vuori K. Matrix attachment regulates Fas-induced apoptosis in endothelial cells: a role for c-flip and implications for anoikis. *J Cell Biol* 2001; 152:633–43.
56. Stupack DG, Puente XS, Boutsaboualoy S, Storgard CM, Cheresh DA. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *J Cell Biol* 2001; 155:459–70.
57. Skurk C, Maatz H, Kim HS, et al. The Akt-regulated forkhead transcription factor FOXO3a controls endothelial cell viability through modulation of the caspase-8 inhibitor FLIP. *J Biol Chem* 2004; 279:1513–25.
58. Miyazaki T, Shen M, Fujikura D, et al. Functional role of death-associated protein 3 (DAP3) in anoikis. *J Biol Chem* 2004; 279:44667–72.
59. Marconi A, Atzei P, Panza C, et al. FLICE/caspase-8 activation triggers anoikis induced by beta1-integrin blockade in human keratinocytes. *J Cell Sci* 2004; 117:5815–23.
60. Maubant S, Saint-Dizier D, Boutillon M, et al. Blockade of $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins by RGD mimetics induces anoikis and not integrin-mediated death in human endothelial cells. *Blood* 2006; 108(9):3035–44.
61. Bozzo C, Sabbatini M, Tiberio R, Piffanelli V, Santoro C, Cannas M. Activation of caspase-8 triggers anoikis in human neuroblastoma cells. *Neurosci Res* 2006; 29(6):347–50.

62. Valentijn AJ, Gilmore AP. Translocation of full-length Bid to mitochondria during anoikis. *J Biol Chem* 2004; 279:32848–57.
63. Grossmann J, Walther K, Artinger M, Kiessling S, Scholmerich J. Apoptotic signaling during initiation of detachment-induced apoptosis (“anoikis”) of primary human intestinal epithelial cells. *Cell Growth Differ* 2001; 12:147–55.
64. Wang P, Valentijn AJ, Gilmore AP, Streuli CH. Early events in the anoikis program occur in the absence of caspase activation. *J Biol Chem* 2003; 278:19917–25.
65. Willis SN, Adams JM. Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol* 2005; 17:617–25.
66. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell* 2004; 116:205–19.
67. Scorrano L, Ashiya M, Buttle K, et al. A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. *Dev Cell* 2002; 2:55–67.
68. Gross A, Yin XM, Wang K, et al. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J Biol Chem* 1999; 274:1156–63.
69. Yin XM, Wang K, Gross A, et al. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature* 1999; 400:886–91.
70. Chen D, Zhou Q. Caspase cleavage of BimEL triggers a positive feedback amplification of apoptotic signaling. *Proc Natl Acad Sci USA* 2004; 101:1235–40.
71. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* 1996; 87:619–28.
72. Datta SR, Ranger AM, Lin MZ, et al. Survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis. *Dev Cell* 2002; 3:631–43.
73. Datta SR, Dudek H, Tao X, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 1997; 91:231–41.
74. Harada H, Becknell B, Wilm M, et al. Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. *Mol Cell* 1999; 3:413–22.
75. Harada H, Andersen JS, Mann M, Terada N, Korsmeyer SJ. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. *Proc Natl Acad Sci USA* 2001; 98:9666–70.
76. Fukazawa H, Noguchi K, Masumi A, Murakami Y, Uehara Y. BimEL is an important determinant for induction of anoikis sensitivity by mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitors. *Mol Cancer Ther* 2004; 3:1281–8.
77. Wang P, Gilmore AP, Streuli CH. Bim is an apoptosis sensor that responds to loss of survival signals delivered by epidermal growth factor but not those provided by integrins. *J Biol Chem* 2004; 279:41280–5.
78. Wei MC, Zong WX, Cheng EH, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 2001; 292:727–30.
79. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* 2002; 2:183–92.
80. Kuwana T, Bouchier-Hayes L, Chipuk JE, et al. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* 2005; 17:525–35.
81. Puthalakath H, Villunger A, O’Reilly LA, et al. Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science* 2001; 293:1829–32.
82. Puthalakath H, Huang DC, O’Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* 1999; 3:287–96.
83. Bouillet P, Metcalf D, Huang DC, et al. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 1999; 286:1735–8.
84. Zinkel SS, Hurov KE, Ong C, Abtahi FM, Gross A, Korsmeyer SJ. A role for proapoptotic BID in the DNA-damage response. *Cell* 2005; 122:579–91.
85. Chen G, Bhojani MS, Heaford AC, et al. Phosphorylated FADD induces NF-kappaB, perturbs cell cycle, and is associated with poor outcome in lung adenocarcinomas. *Proc Natl Acad Sci USA* 2005; 102:12507–12.

86. Su H, Bidere N, Zheng L, et al. Requirement for caspase-8 in NF-kappaB activation by antigen receptor. *Science* 2005; 307:1465–8.
87. Samali A, Zhivotovsky B, Jones D, Nagata S, Orrenius S. Apoptosis: cell death defined by caspase activation. *Cell Death Differ* 1999; 6:495–6.
88. Termuhlen PM, Sweeney-Gotsch BM, Berman RS, et al. Increased apoptosis in metastatic human colonic adenocarcinomas. *Cancer Biol Ther* 2002; 1:58–63.
89. Swan EA, Jasser SA, Holsinger FC, Doan D, Bucana C, Myers JN. Acquisition of anoikis resistance is a critical step in the progression of oral tongue cancer. *Oral Oncol* 2003; 39:648–55.
90. McConkey DJ, Greene G, Pettaway CA. Apoptosis resistance increases with metastatic potential in cells of the human LNCaP prostate carcinoma line. *Cancer Res* 1996; 56:5594–9.
91. Pettaway CA, Pathak S, Greene G, et al. Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. *Clin Cancer Res* 1996; 2:1627–36.
92. Liang XH, Jackson S, Seaman M, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 1999; 402:672–6.
93. Jan Y, Matter M, Pai JT, et al. A mitochondrial protein, Bit1, mediates apoptosis regulated by integrins and Groucho/TLE corepressors. *Cell* 2004; 116:751–62.
94. Ren B, Chee KJ, Kim TH, Maniatis T. PRDI-BF1/Blimp-1 repression is mediated by corepressors of the Groucho family of proteins. *Genes Dev* 1999; 13:125–37.
95. Roose J, Molenaar M, Peterson J, et al. The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* 1998; 395:608–12.

II

TELOMERES AND TELOMERASE, SENESCENCE, GENOMIC INSTABILITY, AND TUMORIGENESIS

7

Structure and Function of the Telomere

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SUMMARY

Telomeres are specialized nucleoprotein structures found at the ends of linear chromosomes that guard against aberrant chromosomal rearrangements and prevent the ends of DNA molecules from being recognized by DNA damage-sensing mechanisms. These structures were initially characterized by Hermann Muller in the 1930s and have subsequently been the subject of intense study. The essential role of the telomere in protecting chromosomes is compromised by the continuous shortening of chromosome ends that accompanies DNA replication. At least two mechanisms have been found that counteract this telomere attrition, and these mechanisms have been implicated in tumorigenesis in that they allow unchecked cellular proliferation. This chapter summarizes our current understanding of the structure and function of the mammalian telomere, its maintenance, and its role in tumor formation.

Key Words: Telomerase; ALT; tumorigenesis; genomic instability; chromosome stability; cancer; aging.

1. INTRODUCTION

Telomeres are found at the ends of DNA molecules and are required for the maintenance of the linear chromosomes present in eukaryotes. These specialized structures protect against chromosome end-to-end fusion events as well as prevent the ends of DNA molecules from being recognized by DNA damage-sensing mechanisms. This essential role is eventually compromised by the progressive shortening of chromosome ends that is caused, at least in part, by DNA replication. Telomere dysfunction typically results in the activation of cellular responses such as programmed cell death (apoptosis) or cell growth arrest (senescence), thereby minimizing the likelihood that an aberrantly growing cell will develop into a tumor. By contrast, telomere stabilization facilitates evasion of such checkpoints by tumor cells and allows uninterrupted proliferation. These observations underline the importance of the telomere in tumor suppression.

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2. HISTORICAL PERSPECTIVE

As early as the 1930s, it was recognized that there was likely a chromosomal “cap” that prevented chromosome ends from fusing with one another. *Drosophila* geneticist, Hermann Muller called this structure a telomere, a term derived from the Greek words meaning “end” (telos) and “part” (meros). In a 1938 lecture entitled “The Remaking of Chromosomes,” Muller proposed that

...the terminal gene must have a special function, that of sealing the ends of the chromosome, so to speak, and that for some reason, a chromosome cannot persist indefinitely without having its ends thusly “sealed.” This gene may accordingly be distinguished by a special term, the “telomere” (1).

Muller’s hypothesis that the ends of a chromosome were somehow functionally distinct from the rest of the molecule was based on X-ray-induced chromosomal breakage experiments that he began in the latter half of the 1920s (and for which he would later win a Nobel prize) (2). In these experiments, Muller analyzed the chromosomal rearrangements obtained after inducing double-stranded breaks by X-ray irradiation. Although he obtained many inversions, translocations, and deletions, none of these involved the extreme chromosomal termini, suggesting that the natural chromosome ends were unable to fuse with the newly broken ends.

At about the same time as Muller’s studies, Barbara McClintock was performing elegant experiments in maize that would ultimately provide a confirmation of Muller’s findings. In these studies, she could generate a dicentric chromosome that would break during mitosis as its centromeres migrated to opposing spindle poles (3). She observed that newly broken ends were highly reactive and would fuse to one another before the next mitosis, producing a new dicentric chromosome. In sharp contrast, she found that normal chromosome ends were highly resistant to such fusion events. These data strongly supported Muller’s contention that there was something special about the naturally occurring chromosome end, or telomere, and that this feature was necessary for chromosomal stability.

Further significant insights into telomere biology would unfortunately have to wait until the 1970s, as they required an important change in scientific thought. During the 1960s, many researchers abandoned the idea of the chromosome as a conglomeration of discrete components, beginning instead to consider the implications of the chromosome as a single uninterrupted DNA molecule (2). This led to a switch in the techniques being applied to the study of telomeres, favoring molecular biological approaches over cytogenetics. It was during this time that several groups began sequencing the chromosomal termini in ciliated protozoa and found that, depending on the organism, the termini were composed of tandem repeats of either hexameric or octameric GC-rich sequences (4–6). In pivotal work, Szostak and Blackburn (7) demonstrated the functional relevance of these sequences when they showed that the terminal repeats from the ciliate *Tetrahymena thermophila* could provide telomere function in the budding yeast, *Saccharomyces cerevisiae*. As part of these studies, they also demonstrated that the chromosome ends of *Saccharomyces* contained sequence repeats not unlike those observed in ciliates. In rapid succession, simple telomeric repeats were identified and characterized in many higher eukaryotes, including humans.

Attrition of telomeric repeats is caused, at least in part, by the incomplete replication of the chromosome. This “end-replication problem” is a consequence of both the unidirectional action and the primer requirements of conventional DNA polymerases (8,9). Consequently, each time a cell replicates its DNA, telomeric sequences are lost. The mechanism by which these repeats are maintained remained elusive for some time. No known polymerases could be implicated in the maintenance of chromosome ends by the addition of telomere-specific repeats. In 1985, however, Greider and Blackburn (10) isolated protein extracts from *Tetrahymena* that possessed just such an activity. The enzyme responsible for this activity was called telomerase and would later be found to be a holoenzyme containing both RNA and protein subunits (see below, Section 6). To assay telomerase activity, Greider and Blackburn (10) used a synthetic single-stranded oligonucleotide as a primer and observed that it could be elongated by both dTTP and ^{32}P -dGTP in an extract-dependent fashion. In addition, the reaction products obtained showed a six-nucleotide periodicity, corresponding to the telomeric repeat found in *Tetrahymena*, 5'(TTGGGG)3'. Although a more sensitive polymerase chain reaction (PCR)-based assay would later be devised (11,12), it was the advent of this initial telomerase assay that facilitated much of the work characterizing both telomeres and telomerase over the past two decades.

3. THE PRIMARY STRUCTURE OF TELOMERIC DNA

In vertebrates, telomeres are typically 10–50 kb in length and are composed of tandem repeats of the hexanucleotide, 5'(TTAGGG)3' (see Fig 1A). The length of the telomere is species specific, being approximately 10 kb in humans and 50 kb in inbred strains of *Mus musculus* (13,14). Furthermore, telomere length is dependent on both the type and the replication history of the relevant tissue (15,16). In the human germline, for example, the array of telomeric repeats is approximately 15 kb in length, whereas it is shorter in most somatic cells. Lengthening of the telomere is normally accomplished by the sequential addition of telomeric repeats to a pre-existing telomere terminus and requires the action of the large, multi-subunit ribonucleoprotein complex known as telomerase.

An interesting feature of the telomere is the strand asymmetry that results from the G-rich nature of the telomeric repeat. The G-rich strand is oriented in the 5' to 3' direction, toward the chromosome end, and in humans, there is a 75–200 nucleotide stretch at the chromosomal terminus where this strand is unpaired (17–19). The presence of the G-rich single-stranded protrusion is correlated with proper telomeric capping (20). Additionally, the G-strand overhang is involved in the generation of secondary structure at the telomere [the telomere loop (t-loop), see below, Section 4], which is thought to further stabilize the chromosome end (21). Although work in human cell culture systems has indicated that telomerase may be involved in the generation of G-strand overhangs (22), other studies have suggested that the enzyme is not specifically required for their maintenance. For example, G-strand overhangs have been shown to be maintained in mouse strains that lack telomerase (23). Thus far, very little is actually known about how these structures are generated and/or maintained.

4. THE SECONDARY STRUCTURE OF TELOMERIC DNA

Telomeres allow ongoing cellular proliferation despite the presence of naturally occurring chromosome ends, which resemble double-stranded DNA breaks. This is accomplished by protecting these ends from detection by the genome surveillance machinery, nucleolytic attack, and recombinogenic processing. Consistent with these roles, telomeres directly sequester the ends of the DNA molecule through the formation of a circular structure called a t-loop (see Fig 1C) (21). The t-loop is formed by the invasion of internal double-stranded telomeric repeats by the single-stranded G-rich protrusion. Moreover, the displacement of the Watson strand by the G-strand overhang results in the formation of another structure, the aptly named displacement loop (d-loop). Both these structures have been directly visualized by electron microscopy (EM) (21). Importantly, t-loops have also been observed in the context of isolated telomeric chromatin, providing further evidence that these structures are present *in vivo* (24).

A less characterized and more controversial telomeric structure is the G-quadruplex. *In vitro* studies have indicated that G-rich telomeric DNA is capable of forming complex four-stranded structures (25). Formation of these structures is mediated by a motif known as a G-quadruplex (sometimes called a G-quartet). Specifically, a G-quadruplex is a relatively stable planar association of four guanines. *In vitro*, G-quadruplexes can stack upon one another, giving rise to four-stranded structures. The carbonyl groups of each guanine can participate in the coordination of a cation, thereby trapping it in between “stacks.” Interestingly, this “cationic trapping” results in further stabilization of the stacked structure. Recent work has provided the first evidence that G-quadruplexes actually exist *in vivo*. In this study, a fluorescent G-quadruplex-binding compound, 3,6-bis(1-methyl-4-vinylpyridinium)carbazole diiodide (BMVC), was used to probe chromosomal DNA purified from human cells (26). A fluorescent emission signal specific for G-quadruplexes was detected at telomeric DNA. Furthermore, BMVC fluorescent signal was found to localize adjacent to chromosomal termini on metaphase chromosome preparations, suggesting that G-quadruplexes may be present at human telomeres.

With regard to the physiological relevance of these observations, a number of ideas have been proposed. It has been suggested that G-quadruplexes might interact with the template RNA of telomerase, possibly playing a role in telomere elongation (27). Alternatively, G-quadruplexes might be involved in the formation of highly stable telomeric



Fig. 1. (TIN2-interacting protein 1/POT1- and TIN2-organizing protein/POT1-interacting protein 1) (aqua rectangle). The functions and interacting partners of these factors are summarized in Table 1. (C) The G-rich overhang is involved in the generation of two telomeric structural elements that are thought to stabilize the chromosome end. The telomere loop (t-loop) is a circular structure formed by the invasion of internal double-stranded telomeric repeats by the single-stranded G-rich protrusion. The displacement of the Watson strand by the G-rich overhang results in the formation of the displacement loop (d-loop). (D) Telomeric proteins are shown associated with the telomere in the context of secondary structural elements such as the t- and d-loops. Shown are many possible associations, including the same grouping of telomeric proteins as depicted in panel B, as well as subsets of this complex that might also exist *in vivo*. Examples of the latter group include complexes of TRF1 and its associated proteins, or TRF2 and its associated proteins. (Note: panels B and D are intended only to demonstrate the association of telomeric proteins with telomeric DNA and are not meant to suggest any specific stoichiometry or order of recruitment).

DNA complexes that provide additional “capping” function at the telomere (28). Several studies have provided evidence that G-quadruplexes may affect the activity of telomerase at chromosome ends. In one study, treatment of cells with the G-quadruplex-interacting compound telomestatin was found to block telomerase activity (29). Interestingly, subsequent work has shown that this compound interacts strongly with the G-rich overhangs found at the end of telomeres and promotes the formation of stable intramolecular G-quadruplex structures (30,31). Taken together, these data suggest that binding of telomestatin to the intramolecular G-quadruplex structures in telomeric DNA abrogates telomerase function through the occlusion of the telomerase holoenzyme. Indeed, there are other telomestatin-like compounds that seem to exert their anti-proliferative effects through interaction with the G-rich portion of the telomere. TMPyP4, a compound that facilitates the formation of intermolecular G-quadruplexes, has an anti-proliferative effect on both telomerase-positive cells and cells that use a telomerase-independent mechanism for telomere maintenance [alternative lengthening of telomeres (ALT), see below, Section 8] (32). In addition, treatment of cells with TMPyP4 results in an increase in the number of chromosome end-to-end fusions observed, whereas no such effect is seen with telomestatin treatment. With respect to the physiological relevance of G-quadruplexes, the above observations are consistent with those of a later study wherein the authors explored the effects of treating cells with guanosine-rich oligodeoxyribonucleotides (GROs) (33). Synthetic GROs based on telomeric DNA sequences formed G-quadruplexes *in vitro* and had profound anti-proliferative effects when added to cultured HeLa cells. Although the current data are insufficient to establish any definitive conclusions, one might imagine that the effect of these G-quadruplex-containing oligodeoxyribonucleotides is elicited by the binding and sequestration of the active cellular pool of telomerase, causing telomere dysfunction and cell morbidity. It is difficult, however, to draw conclusions about how GROs might exert their anti-proliferative effects, as it is not clear from this study that these molecules can even gain access to the nucleus. The data obtained thus far implicate both interaction with the RNA component of telomerase and interactions within and between telomeres as mechanisms by which G-quadruplexes might affect telomerase activity.

5. TELOMERIC PROTEINS AND THE TELOSOME

The chromosomal capping function of telomeres and their ability to evade DNA repair mechanisms are dependent, to a large extent, on the secondary structure of telomeric DNA. This DNA structure, in turn, requires the activity of a number of proteins that localize to the telomere. In mammals, these proteins can be organized into three distinct classes. The first class is that of the telomeric DNA-binding proteins and contains TTAGGG repeat binding factor 1 (TRF1), TRF2, and protection of telomeres 1 (POT1) (34–37). TRF1 and TRF2 bind to duplex telomeric DNA, whereas POT1 interacts with single-stranded DNA (ssDNA), most probably at the d-loop (see Fig 1B, D) (37,38). The specific activities of these factors are summarized in Table 1.

In turn, the telomere-binding proteins interact with a second class of factors and the resulting multi-protein complexes have been implicated in the regulation of telomere metabolism. This includes TRF1-interacting nuclear protein 2 (TIN2), human repressor activator protein 1 (hRAP1), and TIN2-interacting protein 1/POT1- and

Table 1
Protein and RNA Factors Found at the Normal Human Telomere

<i>Factor</i>	<i>Definition</i>	<i>Relevant telomere-interacting partners</i>	<i>Known or proposed function</i>	<i>References</i>
TRF1	TTAGGG repeat binding factor 1	Double-stranded telomeric DNA, NBS1, TIN2, tankyrase1/2	Negatively regulates telomere maintenance by telomerase	(34,35,39,46,49,95)
TRF2	TTAGGG repeat binding factor 2	Double-stranded telomeric DNA, TIN2, POT1, hRAP1, NBS1	Plays an essential role in telomere end capping; negatively regulates telomere length	(36,38–40,52)
POT1	Protection of telomeres 1	Single-stranded telomeric DNA (ssDNA), TRF2, TPP1, PINX1	ssDNA-binding protein that binds to and protects the G-rich strand; negatively regulates telomere length	(37,38,41–43)
TIN2	TRF1-interacting nuclear protein 2	TRF1, TRF2, TPP1	Recruits POT1 to duplex telomeric DNA through TPP1; protects TRF1 from tankyrase-mediated destruction	(39)
TPP1	TIN2-interacting protein 1/POT1- and TIN2-organizing protein/POT1-interacting protein 1	POT1, TIN2	Regulates telomere length by recruiting POT1 to telomeres	(41–43)
hRAP1	Human repressor activator protein 1	TRF2, MRE11/RAD50, Ku	Recruits MRN and Ku to telomeres; negatively regulates telomere length	(40)
PINX1	Pin2/TRF1-interacting factor 1	TRF1, hTERT, hTR(hTER)	Negatively regulates telomerase activity by binding to both hTERT and hTR(hTER)	(45,111)

tankyrase 1	TRF1-interacting, ankyrin-like poly(ADP-ribose) polymerase (PARP)	TRF1	ADP-ribosylates TRF1, leading to dissociation from the telomere and ubiquitin-dependent degradation	(46)
tankyrase 2	TRF1-interacting, ankyrin-like PARP	TRF1	Homolog of tankyrase 1 that can also liberate TRF1 from telomeres	(47–49)
hTERT	Human telomerase reverse transcriptase	hTR(hTER), PINX1, Ku, hEST1A/B	A specialized reverse transcriptase that extends telomeric DNA by addition of the sequence, TTAGGG; may also stabilize the telomere by a mechanism independent of DNA elongation	(45,50,112,79)
hTR (hTER)	Human telomerase RNA	hTERT, PINX1	Template RNA required for TTAGGG addition to telomeres by hTERT	(45,68,69,73,111)
hEST1A/B	Human ever shorter telomeres 1A/B	hTERT	Activates telomerase, possibly by recruitment of hTERT to the telomere	(79,80)
Ku70/86 (Ku)	Heterodimer involved in non-homologous end-joining (NHEJ)	hRAP1, hTERT, DNA-PK	NHEJ proteins that may play a role in telomere processing or the recognition of critically short telomeres as DSBs	(50,51)
MRE11/RAD50/ NBS1 (MRN)	Meiotic recombination 11/radiation sensitive 50/Nijmegen breakage syndrome 1	TRF2, hRAP1	Like Ku, MRN is involved in DNA double-strand break (DSB) repair and may be involved in maintenance of the telomere, possibly through the nuclease function of MRE11	(50,52)

(Continued)

Table 1
(Continued)

<i>Factor</i>	<i>Definition</i>	<i>Relevant telomere-interacting partners</i>	<i>Known or proposed function</i>	<i>References</i>
DNA-PK	DNA-dependent protein kinase	Ku	Suppresses chromosome end-to-end fusions	(50)
WRN	Werner syndrome protein	TRF1, TRF2 (<i>in vitro</i>)	RecQ helicase that also has a 3'-5' exonuclease activity; involved in recombinational repair of stalled replication forks and DSBs; promotes lagging strand synthesis at the telomere, thereby regulating length of the 3' G-rich overhang; dissociates d-loop <i>in vitro</i>	(54-56,113)
BLM	Bloom syndrome protein	TRF1, TRF2 (<i>in vitro</i>)	Like WRN, RecQ helicase that has been implicated in telomere metabolism and may promote telomere elongation by alternative lengthening of telomeres (ALT); lacks the nuclease domain present in Werner syndrome helicase/exonuclease (WRN)	(53,54,56)

TIN2-organizing protein/POT1-interacting protein 1 (TPP1), which together with the telomere-binding proteins make up the so-called shelterin complex (39–44). Additional factors that modulate telomere structure and activity include the Pin2/ TRF1-interacting factor (PINXI) and tankyrases 1 and 2 (45–49). The telomere dysfunction seen upon depletion of TRF1 or TRF2 is likely due to the inability of these factors to localize to and function at the telomere. The effects of these various factors on telomere length, telomere-capping, and overall telomere status are also summarized in Table 1.

The final class of proteins present at the telomere are those that have functions in other cellular processes and include a number of factors involved in the response to DNA damage (50). These include the Ku70/86 heterodimer and the MRE11/RAD50/NBS1 (MRN) complex (50–52). Similar to the telomere-associated factors described above, these complexes do not bind the telomere directly; rather, they require interaction with telomere-specific factors for their efficient localization to the telomere. Other factors, such as the RecQ helicases Werner (WRN) and Bloom (BLM), may be recruited to telomeric sites through association with TRF1 and/or TRF2 where they affect telomere homeostasis (53–56). The functions of these proteins are discussed further in Table 1. Finally, there are a number of proteins that are thought to be involved in sensing telomere dysfunction (57,58). For example, the phosphorylated form of the histone variant H2AX, γ -H2AX, is part of the cellular response to DNA damage and interacts with the MRN complex in DNA damage foci induced by critically short telomeres (57,59). This interaction may contribute to the growth arrest and/or apoptotic responses elicited by non-functional telomeres. Similar to γ -H2AX, the ATM (ataxia telangiectasia-mutated) and ATR (ATM and RAD3-related) kinases, 53BP1 (p53-binding protein 1) and RAD17 (radiation sensitive 17), are DNA damage repair proteins that localize to telomere-associated DNA damage foci and are involved in the activation of telomere-induced senescence and apoptosis (57,58). Interestingly, such factors are not constitutively present at the telomere, rather they are recruited in response to a critically short telomere.

The telomere-binding protein TRF1 plays an important role in telomere length control, whereas TRF2 is required for the end-capping function that prevents chromosome ends from being recognized as double-strand breaks (20,60). There are many proteins, as mentioned above, that associate with each factor and contribute to the overall stability of the telomere. The recent finding that the protein TIN2 interacts with TRF1, TRF2, and POT1 indicates that there is crosstalk between the complexes associated with these factors and provides evidence for the so-called telosome model (42,61–63). Specifically, the telosome is considered to be a high-molecular-weight complex of proteins associated with the telomere (63). As mentioned above, abrogation of any of a number of TRF1- or TRF2-associated factors results in telomere dysfunction. The telosome model suggests that this effect might be the result of an overall decrease in the stability of the telosome, as opposed to the loss of specific functions provided by its components. Moreover, this model hypothesizes that it is the integration or summation of a number of signals from discrete pathways that contribute to the overall stability of the telosome and, consequently, the control of telomere metabolism. This model is consistent with recent work indicating that it is the state of the telomere and not necessarily its length that determines whether cells senesce. In these studies, the telomere-binding protein TRF2 was overexpressed, increasing the rate of telomere erosion without a concomitant acceleration of the onset of senescence (64). The likeliest explanation

for this observation is that in cells overexpressing TRF2, although telomeric DNA is of a shorter length than what would typically activate senescence, the increased levels of TRF2 contribute to the formation of multimeric telomere complexes that are at least as stable as those in cells with normal length telomeres. Therefore, the authors argue, the most relevant checkpoint in telomere-driven senescence would be the state or stability of the telosome and not the length of telomeric DNA. Under physiological conditions, wherein telomeric protein levels are more tightly regulated, telomere length likely determines the quantity of protein bound to the telomere and, therefore, telosome status.

6. TELOMERASE

The human telomerase holoenzyme consists of two essential subunits, a telomere-specific reverse transcriptase (RT) [human telomerase RT (hTERT)] and an RNA template (hTR/hTER), and functions as an interdependent dimer or multimer (65). Whereas these components are necessary and sufficient for telomerase activity *in vitro*, there are a number of accessory proteins that modify the activity and/or assembly of telomerase. Two of these factors are the heat-shock protein 90 (hsp90) and the chaperonin, p23. Both are involved in preserving the folding states of proteins and, consequently, preserving the physiological activities of many enzymes. As both proteins have been shown to bind telomerase directly, it is thought that they enhance telomerase function by facilitating the assembly of the active holoenzyme (66,67). Consistent with this role, an inhibitor of hsp90, geldanamycin, has been shown to reduce telomerase activity *in vitro* (66,68). Furthermore, recent studies have shown that the use of antisense oligonucleotides to downregulate hsp90 and p23 expression results in decreased telomerase activity both *in vitro* and *in vivo* (69). Taken together, these results indicate that although hsp90 and p23 may not be required for telomerase activity, their expression has a stimulatory effect on the hTERT-mediated elongation of chromosomal termini. The same holds true for another protein proposed to be part of the telomerase holoenzyme, the nucleolar protein, dyskerin. Cells with mutant alleles of dyskerin show reduced levels of hTR and, not surprisingly, have similarly affected levels of telomerase activity (70,71). Moreover, antisense RNA-mediated downregulation of dyskerin negatively impacts on telomerase function, as described for hsp90 and p23 (69). What is arguably most intriguing about this particular telomerase holoenzyme component is that *dyskerin* point mutants have been shown to be causative agents of the human skin and bone marrow disorder, dyskeratosis congenital (DKC) (72). Dyskerin is an rRNA pseudouridylase and has been proposed to modulate telomerase levels through direct interaction with the H/ACA domain of hTR (70,71). Studies in mice suggest that DKC disease phenotypes are caused by impaired ribosomal biogenesis, as opposed to telomere shortening (71). This is because *dyskerin* knockout mice immediately display symptoms of DKC, although they suffer detectable telomere attrition only in later generations. Despite such nuances, it is clear that dyskerin does play a role in telomere metabolism, although the molecular details remain unclear.

7. REGULATION OF TELOMERASE ACTIVITY

Accessory subunits of the telomerase multi-protein complex such as hsp90 and p23 are expressed at relatively comparable levels in normal and cancerous tissues (69). Similarly, the telomerase template RNA, hTR, is expressed fairly widely and is even

found in many tissues that do not have detectable telomerase activity. By contrast, levels of the catalytic component of the holoenzyme, hTERT, are much more variable. Transcription of hTERT is either low or completely undetectable in the majority of somatic tissues (11,73,74). Interestingly, there are many renewable somatic tissues that represent notable exceptions to this rule as they have been shown to possess moderate levels of telomerase activity (75). These renewable tissues include the lining of the gut, hair follicles, the proliferating keratinocyte layer of the skin, and cells of the hematopoietic system, the last group being the most extensively characterized due to the ease of obtaining such samples. Whereas transcriptional regulation of hTERT represents an efficient means of modulating telomerase activity, many additional regulatory mechanisms exist that affect the levels of active enzyme.

Alternative splicing of the hTERT transcript is a post-transcriptional control that might also play a role in the regulation of telomerase activity, although the relative contribution of this pathway to overall telomerase regulation has yet to be determined. There are, for example, several splice variants of the hTERT message that lack domains crucial for catalytic activity (76,77). Translation of many of these transcripts produces non-functional proteins, whereas the variant known as hTERT α is a dominant-negative inhibitor of normal telomerase activity. This splice variant lacks a conserved RT motif and may exhibit its transdominant effects through sequestration of both DNA and protein factors needed for proper functioning of the active enzyme.

A third mechanism involved in the regulation of telomerase is the human ever-shorter telomeres 1A/B (hEST1A)-mediated enhancement of telomere elongation. hEST1A was identified as the human homolog of the yeast EST1 protein, which is involved in the activation of the telomerase holoenzyme, presumably by recruiting it to the chromosomal terminus (78). The human homolog likely functions similarly to its yeast counterpart, as it has the ability to bind single-stranded telomeric DNA and its overexpression results in telomere elongation (79,80). Somewhat puzzling, however, is the observation that this enhancement of telomerase function is also accompanied by an impairment of the end-protecting capability of the telomere. That is, hEST1A overexpression results in a dramatic increase in the number of chromosomal end-to-end fusions observed. These data are interesting, however, in that they suggest that longer telomeres do not necessarily provide additional chromosomal protection.

Yet another mechanism that contributes to the regulation of telomerase activity is that of protein phosphorylation. A number of potential phosphorylation sites in the telomerase reverse transcriptase have been identified, and many have been implicated in enzyme activation. In one of these studies, phosphorylation of hTERT by the Akt kinase resulted in the upregulation of telomerase activity (81). This post-translational modification of hTERT may play a role in the subcellular targeting of the protein, thereby affecting assembly of a functional holoenzyme. For example, the nucleolar localization of telomerase has also been implicated in regulation of activity (82,83). In one such study, hTERT was exogenously expressed in primary fibroblasts. The exogenous protein was localized to the nucleolus and returned to the nucleoplasm upon entry into S phase. The nucleolar localization of hTERT was abrogated upon transformation, whereas DNA damage induced a transient sequestration of hTERT into the nucleolus (82). The multi-level regulation of telomerase activity may be useful therapeutically, as it provides a variety of potential targets for inhibition of the enzyme.

8. ALTERNATIVE LENGTHENING OF TELOMERES

Telomere stabilization facilitates tumorigenesis. The majority of human tumors utilize telomerase to offset the critical shortening of telomeres that would otherwise occur under conditions of rapid cell division. A subset of tumors and cell lines are capable of maintaining chromosome ends in the absence of telomerase, however (84,85). Such cells are said to use ALT for telomere maintenance. Although the specific genetic determinants required for ALT are unknown, there are several key differences between ALT-positive and telomerase-positive cells.

In contrast to the fairly homogenous telomeres found in telomerase-positive cells, in most ALT-positive cells telomeres are quite heterogenous, ranging from 4 kb to >25 kb in size (84). However, a single telomerase-negative immortal cell line, AG11395, in which the terminal DNA array is composed of telomeric repeats interspersed with SV40 sequences, has been described (86,87). Moreover, ALT cells contain extrachromosomal DNA molecules that contain telomeric repeats (88,89). These circular molecules are readily detected by both pulsed-field gel electrophoresis (PFGE) and EM and their existence, combined with studies in *Kluyveromyces lactis* (90), suggest that telomeres may be maintained in ALT-positive cells by a mechanism involving rolling-circle replication and homologous recombination (89). ALT-positive cells also differ from telomerase-positive cells in that a subpopulation of cells in an ALT-positive cell line often show co-localization of telomeric DNA, the telomeric-binding proteins TRF1 and TRF2, and the promyelocytic leukemia (PML) nuclear body in a complex called the ALT-associated PML body (APB) (91). The PML nuclear body is a large multiprotein structure that has been implicated in various cellular processes, including growth control, differentiation, and apoptosis (92,93). It is possible that the association of TRF1 and TRF2 with the PML body generates signals that mediate transcriptional changes in response to telomere status. An alternative possibility is that they are sites of telomere elongation through recombination. As discussed above (see Section 5) and in Table 1, a number of factors involved in the DNA damage response have been detected at telomeres. DNA repair factors such as the MRN and Ku70/86 complexes might be involved in recombination-based telomere maintenance at APBs. Finally, these structures may represent the accumulation of byproducts of the telomere elongation reaction. Interestingly, cell synchrony experiments show that the number of APB-positive cells is increased significantly when cultures are enriched for late S or G2/M phase cells (94,95). This observation suggests that the formation of the APB complex, whatever its function, is a cell cycle-dependent event. In the AG11395 cell line, telomeric components do not co-localize with the PML nuclear body, although these cells contain a multiprotein complex wherein recombination factors, telomeric factors, and many other APB components are present (86,87). Whatever their role, APBs are tightly linked with ALT and have served as a diagnostic marker for cells that use this mechanism for telomere maintenance.

Another significant feature of ALT-positive cells is an increased frequency of chromosome end-to-end fusions (96). This condition is evidenced quite clearly by the presence of anaphase chromosome bridges, structures that result from the bi-directional migration of the two halves of a dicentric chromosome. Conveniently, these structures serve as simple visual indicators of the chromosome fusion-breakage cycle that occurs in cells with elevated levels of telomere dysfunction. As mentioned above, it is thought that the mechanism by which telomeres are maintained in ALT-positive human cells involves

recombination between chromosome ends. Such a mechanism is strongly suggested by a number of studies in *S. cerevisiae* examining both the genetic requirements for telomerase-independent telomere maintenance and the structure of the telomere itself (78,97,98). In human cells, rapid changes in the length of the telomere repeat array were observed rather than the gradual changes in size more commonly associated with telomerase activity or the telomere loss that accompanies cell division (99). Subsequently, it was demonstrated that a unique tag embedded in a single telomere will spread to other telomeres in ALT cell lines (100). However, this only occurs when the tag is flanked by telomeric sequences, suggesting that the recombination event occurs within the TTAGGG repeat array. By contrast, characterization of telomeric structure in mouse embryonic stem (ES) cells deficient in telomerase raised the possibility that recombination may occur within subtelomeric repeats (101). The idea that ALT functions in human cells by a recombination-based mechanism is further supported by the finding that the ALT pathway becomes activated upon telomerase inhibition in cells deficient in DNA mismatch repair (MSH6^{-/-}) (102). In addition, it was recently shown that sister chromatid exchange at telomeres is elevated in ALT cells (103,104). Although such studies implicate DNA recombination in the maintenance of telomeres by ALT, the specific molecular details of this mechanism remain undetermined.

9. CELLULAR IMPLICATIONS OF TELOMERE DYSFUNCTION

As mentioned previously, telomeres provide an essential cellular role by facilitating evasion of DNA damage-sensing mechanisms, thereby preventing the chromosomal termini from being recognized as double-stranded breaks. Telomeres achieve this by providing an end-capping function, guarding against potentially deleterious chromosomal rearrangements. Loss of telomere function by either the critical shortening of telomeric DNA or the perturbation of telomeric proteins involved in end-capping ultimately provokes cellular responses such as programmed cell death (apoptosis) or cell cycle arrest (senescence).

In most cells, the rate of telomere elongation, if any, is insufficient to offset the progressive shortening of telomeres that is associated with DNA replication. Recent work has shown that critically short telomeres directly engage DNA damage repair mechanisms (57,58). That is, upstream sensor kinases typically undergo autophosphorylation and initiate a phosphorelay that eventually signals downstream effectors, such as protein kinases and transcription factors, to change the cellular expression profile. Although this induced profile is marked by upregulation of factors necessary to repair DNA damage, there can also be a concomitant growth arrest or an induction of apoptosis. The specific response triggered is largely dependent on the background of the cell (20,105). In mammals, the upstream kinases, ATM and ATR, initiate signaling cascades that act through signal transducers such as γ -H2AX, a histone protein involved in the alteration of chromatin structure at the site of DNA damage (106). Upon telomere attrition, γ -H2AX and a number of other DNA damage response proteins directly engage the chromosomal terminus, as shown by chromatin immunoprecipitation and whole genome-scanning techniques (58). Notably, these studies provided the first direct evidence that DNA damage repair checkpoints recognize dysfunctional telomeres as double-stranded DNA breaks. Intriguingly, these checkpoints are activated in response to both truncated telomeric DNA and dysfunctional telomeres that result

from the depletion or mutation of telomeric-binding proteins such as TRF2 (*105,106*). Such results imply that in the case of critically short telomeres, DNA damage repair mechanisms directly sense DNA ends, which have been generated and/or de-protected because of an insufficiently high concentration of the telomeric proteins that ordinarily cap chromosomal termini. One possibility is that the inability of telomeric proteins to associate with the telomere might de-stabilize t-loop structures, in turn allowing telomeres to be recognized as DNA damage. To date, however, no conclusive evidence has been obtained to address such speculation.

With respect to telomere-induced senescence or apoptosis, mutations arise that abrogate these normal cellular responses and allow continued proliferation despite critically short or otherwise dysfunctional telomeres. Ablation or impairment of any number of signaling components, from the upstream sensor kinases to the downstream effector proteins, can allow a cell to evade these surveillance mechanisms. Indeed, the circumvention of both senescence and apoptosis are crucial steps in cellular immortalization and tumor progression.

10. THE ROLES OF TELOMERASE ACTIVATION IN GENETIC INSTABILITY AND TUMOR PROGRESSION

As discussed previously, the majority of human tumors have overcome telomere crisis by the reactivation of the telomerase holoenzyme. It has been suggested, however, that dysfunctional telomeres actually play a large part in promoting tumorigenesis. In mice, ablation of the RNA component of telomerase results in telomere shortening, increased genomic instability, and a decreased proliferative capacity in high-renewal tissues (*107*). The cellular senescence and apoptosis responsible for this decrease in proliferation are dependent on the action of the tumor-suppressor protein, p53 (*106*). Interestingly, coincident telomere attrition and p53 deficiency result in increased tumor formation. This increased tumorigenesis is likely because dysfunctional telomeres cause a global chromosomal instability that allows for the accumulation of growth-promoting mutations. Although this hypermutational state may facilitate the progression of early tumors, it would be disastrous to more advanced tumors. In addition to those genetic changes that promote cell growth, mutations might also be produced that decrease the rate of proliferation or reduce overall cell viability. In particular, these mutations might even re-activate pathways involved in senescence or apoptosis, re-establishing anti-proliferative processes that were likely bypassed in the earliest stages of tumor formation.

It had been reasoned that the possible accumulation of growth-arresting mutations might be circumvented by the upregulation of telomerase and the consequent stabilization of the genome (*108*). According to this model, tumor cells that were newly telomerase-positive would be selected and amplified as their viability is increased with respect to the remainder of the tumor population. Indeed, this hypothesis has been supported by studies in many human cell culture systems. In one study, telomerase was added exogenously to human mammary epithelial cells and genomic instability assayed by spectral karyotyping (*109*). It was found that the re-introduction of telomerase, the activity of which was confirmed by telomerase holoenzyme assays, produced an extremely stable karyotype compared with that of control cells. These data are consistent with those of another study, wherein the authors established a cell culture

system to model the role of telomere dysfunction on both genomic instability and tumor progression (110). Briefly, the authors used fluorescence in situ hybridization (FISH) to assay genomic instability, also in human mammary epithelial cell cultures, while monitoring telomere lengths and telomerase activity. Following these cells through telomere crisis and immortalization, they observed that the frequency of chromosomal rearrangements, as assayed by comparative genome hybridization, correlated with telomere attrition, becoming more prominent as telomeres shortened. Following telomere crisis, cultures were enriched for cells that had reactivated telomerase. These cells, not surprisingly, showed a marked decrease in chromosomal instability with respect to earlier populations. These findings were then confirmed in breast carcinomas of varying stages, providing further evidence that the genomic instability resulting from telomere attrition promotes cell immortalization and tumorigenesis.

11. CONCLUDING REMARKS

It can be said that our cells walk a “tight-rope” of telomere length, where a fall to one side results in growth arrest or apoptosis and a fall to the other allows unchecked cell growth and, potentially, tumorigenesis. Under the latter conditions, where telomeres are abnormally stable, efficient tumor formation is still not guaranteed, however. Just as with normal cells, there is likely a narrow range of telomere dysfunction that is optimal for tumor cells. Although an initial decrease in telomere length allows for chromosome end-to-end fusions and many other genetic rearrangements that can produce growth-enhancing alterations, continued growth of the tumor requires the subsequent rescue of this telomere dysfunction. Therefore, efficient tumor progression likely involves the initial shortening of telomeres, as well as their eventual stabilization. As it is unlikely that screens for telomeric proteins have been saturated, it is possible that future work will reveal additional factors that might be exploited in an attempt to exogenously manipulate telomeres and perturb tumor progression.

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REFERENCES

1. Muller HJ. The remaking of chromosomes. *Collecting Net* 1938; 8:182–95, 98.
2. Gall JG. Beginning of the end: origins of the telomere concept. In: Blackburn EH, Greider CW, eds. *Telomeres*. Cold Spring Harbor Laboratory Press. *Cold Spring Harbor, NY* 1995:1–10.
3. McClintock B. The stability of broken ends of chromosomes in *Zea mays*. *Genetics* 1941; 26: 234–82.
4. Blackburn EH, Gall JG. A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in *Tetrahymena*. *J Mol Biol* 1978; 120(1):33–53.
5. Oka Y, Shiota S, Nakai S, Nishida Y, Okubo S. Inverted terminal repeat sequence in the macronuclear DNA of *Stylonychia pustulata*. *Gene* 1980; 10(4):301–6.

6. Klobutcher LA, Swanton MT, Donini P, Prescott DM. All gene-sized DNA molecules in four species of hypotrichs have the same terminal sequence and an unusual 3' terminus. *Proc Natl Acad Sci USA* 1981; 78(5):3015–9.
7. Szostak JW, Blackburn EH. Cloning yeast telomeres on linear plasmid vectors. *Cell* 1982; 29(1):245–55.
8. Watson JD. Origin of concatemeric T7 DNA. *Nat New Biol* 1972; 239(94):197–201.
9. Olovnikov AM. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *J Theor Biol* 1973; 41(1):181–90.
10. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell* 1985; 43(2 Pt 1):405–13.
11. Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994; 266:2011–5.
12. Kim NW, Wu F. Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucleic Acids Res* 1997; 25(13):2595–7.
13. Lange T, Shiue L, Myers RM, et al. Structure and variability of human chromosome ends. *Mol Cell Biol* 1990; 10(2):518–27.
14. Kipling D, Cooke HJ. Hypervariable ultra-long telomeres in mice. *Nature* 1990; 347(6291):400–2.
15. Cooke HJ, Smith BA. Variability at the telomeres of the human X/Y pseudoautosomal region. *Cold Spring Harb Symp Quant Biol* 1986; 51(Pt 1):213–9.
16. Lindsey J, McGill NI, Lindsey LA, Green DK, Cooke HJ. *in vivo* loss of telomeric repeats with age in humans. *Mutat Res* 1991; 256(1):45–8.
17. Makarov VL, Hirose Y, Langmore JP. Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. *Cell* 1997; 88(5):657–66.
18. McElligott R, Wellinger RJ. The terminal DNA structure of mammalian chromosomes. *EMBO J* 1997; 16(12):3705–14.
19. Wright WE, Tesmer VM, Huffman KE, Levene SD, Shay JW. Normal human chromosomes have long G-rich telomeric overhangs at one end. *Genes Dev* 1997; 11(21):2801–9.
20. van Steensel B, Smogorzewska A, de Lange T. TRF2 protects human telomeres from end-to-end fusions. *Cell* 1998; 92:401–13.
21. Griffith JD, Comeau L, Rosenfield S, et al. Mammalian telomeres end in a large duplex loop. *Cell* 1999; 97(4):503–14.
22. Masutomi K, Yu EY, Khurts S, et al. Telomerase maintains telomere structure in normal human cells. *Cell* 2003; 114(2):241–53.
23. Hemann MT, Greider CW. G-strand overhangs on telomeres in telomerase-deficient mouse cells. *Nucleic Acids Res* 1999; 27(20):3964–9.
24. Nikitina T, Woodcock CL. Closed chromatin loops at the ends of chromosomes. *J Cell Biol* 2004; 166(2):161–5.
25. Williamson JR, Raghuraman MK, Cech TR. Monovalent cation-induced structure of telomeric DNA: the G-quartet model. *Cell* 1989; 59(5):871–80.
26. Chang CC, Kuo IC, Ling IF, et al. Detection of quadruplex DNA structures in human telomeres by a fluorescent carbazole derivative. *Anal Chem* 2004; 76(15):4490–4.
27. Choi KH, Choi BS. Formation of a hairpin structure by telomere 3' overhang. *Biochim Biophys Acta* 1994; 1217(3):341–4.
28. Frank-Kamenetskii M. DNA structure. The turn of the quadruplex? *Nature* 1989; 342(6251):737.
29. Shin-ya K, Wierzbka K, Matsuo K, et al. Telomestatin, a novel telomerase inhibitor from *Streptomyces anulatus*. *J Am Chem Soc* 2001; 123(6):1262–3.
30. Kim MY, Vankayalapati H, Shin-Ya K, Wierzbka K, Hurley LH. Telomestatin, a potent telomerase inhibitor that interacts quite specifically with the human telomeric intramolecular g-quadruplex. *J Am Chem Soc* 2002; 124(10):2098–9.
31. Gomez D, Paterski R, Lemarteleur T, Shin-Ya K, Mergny JL, Riou JF. Interaction of telomestatin with the telomeric single-strand overhang. *J Biol Chem* 2004; 279(40):41487–94.
32. Kim MY, Gleason-Guzman M, Izbiccka E, Nishioka D, Hurley LH. The different biological effects of telomestatin and TMPyP4 can be attributed to their selectivity for interaction with intramolecular or intermolecular G-quadruplex structures. *Cancer Res* 2003; 63(12):3247–56.
33. Dapic V, Abdomerovic V, Marrington R, et al. Biophysical and biological properties of quadruplex oligodeoxyribonucleotides. *Nucleic Acids Res* 2003; 31(8):2097–107.

34. Chong L, van Steensel B, Broccoli D, et al. A human telomeric protein. *Science* 1995; 270(5242):1663–7.
35. Broccoli D, Chong L, Oelmann S, et al. Comparison of the human and mouse genes encoding the telomeric protein, TRF1: chromosomal localization, expression and conserved protein domains. *Hum Mol Genet* 1997; 6(1):69–76.
36. Bilaud T, Brun C, Ancelin K, Koering CE, Laroche T, Gilson E. Telomeric localization of TRF2, a novel human telobox protein. *Nat Genet* 1997; 17(2):236–9.
37. Baumann P, Cech TR. Pot1, the putative telomere end-binding protein in fission yeast and humans. *Science* 2001; 292(5519):1171–5.
38. Yang Q, Zheng YL, Harris CC. POT1 and TRF2 cooperate to maintain telomeric integrity. *Mol Cell Biol* 2005; 25(3):1070–80.
39. Kim SH, Kaminker P, Campisi J. TIN2, a new regulator of telomere length in human cells. *Nat Genet* 1999; 23(4):405–12.
40. Li B, Oestreich S, de Lange T. Identification of human Rap1: implications for telomere evolution. *Cell* 2000; 101(5):471–83.
41. Ye JZ, Hockemeyer D, Krutchinsky AN, et al. POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex. *Genes Dev* 2004; 18(14):1649–54.
42. Houghtaling BR, Cuttonaro L, Chang W, Smith S. A dynamic molecular link between the telomere length regulator TRF1 and the chromosome end protector TRF2. *Curr Biol* 2004; 14(18):1621–31.
43. Liu D, Safari A, O'Connor MS, et al. PTop interacts with POT1 and regulates its localization to telomeres. *Nat Cell Biol* 2004; 6(7):673–80.
44. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev* 2005; 19(18):2100–10.
45. Zhou XZ, Lu KP. The Pin2/TRF1-interacting protein PinX1 is a potent telomerase inhibitor. *Cell* 2001; 107(3):347–59.
46. Smith S, Giriat I, Schmitt A, de Lange T. Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. *Science* 1998; 282(5393):1484–7.
47. Lyons RJ, Deane R, Lynch DK, et al. Identification of a novel human tankyrase through its interaction with the adaptor protein Grb14. *J Biol Chem* 2001; 276(20):17172–80.
48. Kuimov AN, Kuprash DV, Petrov VN, et al. Cloning and characterization of TNKL, a member of tankyrase gene family. *Genes Immun* 2001; 2(1):52–5.
49. Kaminker PG, Kim SH, Taylor RD, et al. TANK2, a new TRF1-associated poly(ADP-ribose) polymerase, causes rapid induction of cell death upon overexpression. *J Biol Chem* 2001; 276(38):35891–9.
50. Bailey SM, Meyne J, Chen DJ, et al. DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes. *Proc Natl Acad Sci USA* 1999; 96(26):14899–904.
51. Cary RB, Peterson SR, Wang J, Bear DG, Bradbury EM, Chen DJ. DNA looping by Ku and the DNA-dependent protein kinase. *Proc Natl Acad Sci USA* 1997; 94(9):4267–72.
52. Zhu XD, Kuster B, Mann M, Petrini JH, de Lange T. Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres. *Nat Genet* 2000; 25(3):347–52.
53. Lillard-Wetherell K, Machwe A, Langland GT, et al. Association and regulation of the BLM helicase by the telomere proteins TRF1 and TRF2. *Hum Mol Genet* 2004; 13(17):1919–32.
54. Du X, Shen J, Kugan N, et al. Telomere shortening exposes functions for the mouse Werner and Bloom syndrome genes. *Mol Cell Biol* 2004; 24(19):8437–46.
55. Crabbe L, Verdun RE, Haggblom CI, Karlseder J. Defective telomere lagging strand synthesis in cells lacking WRN helicase activity. *Science* 2004; 306(5703):1951–3.
56. Opresko PL, von Kobbe C, Laine JP, Harrigan J, Hickson ID, Bohr VA. Telomere-binding protein TRF2 binds to and stimulates the Werner and Bloom syndrome helicases. *J Biol Chem* 2002; 277(43):41110–9.
57. Takai H, Smogorzewska A, de Lange T. DNA damage foci at dysfunctional telomeres. *Curr Biol* 2003; 13(17):1549–56.
58. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, et al. A DNA damage checkpoint response in telomere-initiated senescence. *Nature* 2003; 426(6963):194–8.
59. Iijima K, Komatsu K, Matsuura S, Tauchi H. The Nijmegen breakage syndrome gene and its role in genome stability. *Chromosoma* 2004; 113(2):53–61.
60. van Steensel B, de Lange T. Control of telomere length by the human telomeric protein TRF1. *Nature* 1997; 385(6618):740–3.

61. Liu D, O'Connor MS, Qin J, Songyang Z. Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins. *J Biol Chem* 2004; 279(49):51338–42.
62. Ye JZ, Donigian JR, van Overbeek M, et al. TIN2 binds TRF1 and TRF2 simultaneously and stabilizes the TRF2 complex on telomeres. *J Biol Chem* 2004; 279(45):47264–71.
63. Blackburn EH. Telomere states and cell fates. *Nature* 2000; 408(6808):53–6.
64. Karlseder J, Smogorzewska A, de Lange T. Senescence induced by altered telomere state, not telomere loss. *Science* 2002; 295(5564):2446–9.
65. Beattie TL, Zhou W, Robinson MO, Harrington L. Functional multimerization of the human telomerase reverse transcriptase. *Mol Cell Biol* 2001; 21(18):6151–60.
66. Holt SE, Aisner DL, Baur J, et al. Functional requirement of p23 and Hsp90 in telomerase complexes. *Genes Dev* 1999; 13(7):817–26.
67. Forsythe HL, Jarvis JL, Turner JW, Elmore LW, Holt SE. Stable association of hsp90 and p23, but not hsp70, with active human telomerase. *J Biol Chem* 2001; 276(19):15571–4.
68. Masutomi K, Kaneko S, Hayashi N, et al. Telomerase activity reconstituted *in vitro* with purified human telomerase reverse transcriptase and human telomerase RNA component. *J Biol Chem* 2000; 275(29):22568–73.
69. Chang JT, Chen YL, Yang HT, Chen CY, Cheng AJ. Differential regulation of telomerase activity by six telomerase subunits. *Eur J Biochem* 2002; 269(14):3442–50.
70. Mitchell JR, Wood E, Collins K. A telomerase component is defective in the human disease dyskeratosis congenita. *Nature* 1999; 402(6761):551–5.
71. Ruggero D, Grisendi S, Piazza F, et al. Dyskeratosis congenita and cancer in mice deficient in ribosomal RNA modification. *Science* 2003; 299(5604):259–62.
72. Heiss NS, Knight SW, Vulliamy TJ, et al. X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions. *Nat Genet* 1998; 19(1):32–8.
73. Avilion AA, Piatyszek MA, Gupta J, Shay JW, Bacchetti S, Greider CW. Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues. *Cancer Res* 1996; 56(3):645–50.
74. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. *Eur J Cancer* 1997; 33(5):787–91.
75. Broccoli D, Young JW, de Lange T. Telomerase activity in normal and malignant hematopoietic cells. *Proc Natl Acad Sci USA* 1995; 92(20):9082–6.
76. Colgin LM, Wilkinson C, Englezou A, Kilian A, Robinson MO, Reddel RR. The hTERTalpha splice variant is a dominant negative inhibitor of telomerase activity. *Neoplasia* 2000; 2(5):426–32.
77. Yi X, White DM, Aisner DL, Baur JA, Wright WE, Shay JW. An alternate splicing variant of the human telomerase catalytic subunit inhibits telomerase activity. *Neoplasia* 2000; 2(5):433–40.
78. Lundblad V, Blackburn EH. An alternative pathway for yeast telomere maintenance rescues est1-senescence. *Cell* 1993; 73:347–60.
79. Snow BE, Erdmann N, Cruickshank J, et al. Functional conservation of the telomerase protein Est1p in humans. *Curr Biol* 2003; 13(8):698–704.
80. Reichenbach P, Hoss M, Azzalin CM, Nabholz M, Bucher P, Lingner J. A human homolog of yeast Est1 associates with telomerase and uncaps chromosome ends when overexpressed. *Curr Biol* 2003; 13(7):568–74.
81. Kang SS, Kwon T, Kwon DY, Do SI. Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J Biol Chem* 1999; 274(19):13085–90.
82. Wong JM, Kusdra L, Collins K. Subnuclear shuttling of human telomerase induced by transformation and DNA damage. *Nat Cell Biol* 2002; 4(9):731–6.
83. Yang Y, Chen Y, Zhang C, Huang H, Weissman SM. Nucleolar localization of hTERT protein is associated with telomerase function. *Exp Cell Res* 2002; 277(2):201–9.
84. Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J* 1995; 14:4240–8.
85. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat Med* 1997; 3:1271–4.
86. Marciniak RA, Cavazos D, Montellano R, Chen Q, Guarente L, Johnson FB. A novel telomere structure in human ALT cell line. *Cancer Res* 2005; 65:2730–7.
87. Fasching CL, Bower K, Reddel RR. Telomerase-independent telomere length maintenance in the absence of ALT-associated PML bodies. *Cancer Res* 2005; 65:2722–9.

88. Ogino H, Nakabayashi K, Suzuki M, et al. Release of telomeric DNA from chromosomes in immortal human cells lacking telomerase activity. *Biochem Biophys Res Commun* 1998; 248:223–7.
89. Cesare AJ, Griffith JD. Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops. *Mol Cell Biol* 2004; 24(22):9948–57.
90. Natarajan S, McEachern MJ. Recombinational telomere elongation promoted by DNA circles. *Mol Cell Biol* 2002; 22(13):4512–21.
91. Yeager T, Neumann A, Englezou A, Huschtscha L, Noble J, Reddel R. Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. *Cancer Res* 1999; 59:4175–9.
92. Bernardi R, Pandolfi PP. Role of PML and the PML-nuclear body in the control of programmed cell death. *Oncogene* 2003; 22(56):9048–57.
93. Salomoni P, Pandolfi PP. The role of PML in tumor suppression. *Cell* 2002; 108(2):165–70.
94. Grobelny JV, Godwin AK, Broccoli D. ALT-associated PML bodies are present in viable cells and are enriched in cells in the G2/M phase of the cell cycle. *J Cell Sci* 2000; 113:4577–85.
95. Wu G, Lee WH, Chen PL. NBS1 and TRF1 colocalize at promyelocytic leukemia bodies during late S/G2 phases in immortalized telomerase-negative cells. Implication of NBS1 in alternative lengthening of telomeres. *J Biol Chem* 2000; 275(39):30618–22.
96. Montgomery E, Argani P, Hicks JL, DeMarzo AM, Meeker AK. Telomere lengths of translocation-associated and nontranslocation-associated sarcomas differ dramatically. *Am J Pathol* 2004; 164(5):1523–9.
97. Teng SC, Zakian VA. Telomere-telomere recombination is an efficient bypass pathway for telomere maintenance in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1999; 19(12):8083–93.
98. Teng SC, Chang J, McCowan B, Zakian VA. Telomerase-independent lengthening of yeast telomeres occurs by an abrupt Rad50p-dependent, Rif-inhibited recombinational process. *Mol Cell* 2000; 6(4):947–52.
99. Murnane JP, Sabatier L, Marder BA, Morgan WF. Telomere dynamics in an immortal human cell line. *EMBO J* 1994; 13(20):4953–62.
100. Dunham MA, Neymann AA, Fasching CL, Reddel RR. Telomere maintenance by recombination in human cells. *Nat Genet* 2000; 26:447–50.
101. Niida H, Shinkai Y, Hande MP, et al. Telomere maintenance in telomerase-deficient mouse embryonic stem cells: characterization of an amplified telomeric DNA. *Mol Cell Biol* 2000; 20(11):4114–27.
102. Bechter OE, Zou Y, Walker W, Wright WE, Shay JW. Telomeric recombination in mismatch repair deficient human colon cancer cells after telomerase inhibition. *Cancer Res* 2004; 64(10):3444–51.
103. Londono-Vallejo JA, Der-Sarkissian H, Cazes L, Bacchetti S, Reddel RR. Alternative lengthening of telomeres is characterized by high rates of telomeric exchange. *Cancer Res* 2004; 64(7):2324–7.
104. Bailey SM, Brenneman MA, Goodwin EH. Frequent recombination in telomeric DNA may extend the proliferative life of telomerase-negative cells. *Nucleic Acids Res* 2004; 32(12):3743–51.
105. Karlseder J, Broccoli D, Dai Y, Hardy S, de Lange T. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science* 1999; 283(5406):1321–5.
106. Chin L, Artandi SE, Shen Q, et al. p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. *Cell* 1999; 97(4):527–38.
107. Lee HW, Blasco MA, Gottlieb GJ, Horner JW, 2nd, Greider CW, DePinho RA. Essential role of mouse telomerase in highly proliferative organs. *Nature* 1998; 392(6676):569–74.
108. de Lange T. Activation of telomerase in a human tumor. *Proc Natl Acad Sci USA* 1994; 91(8):2882–5.
109. Elmore LW, Turner KC, Gollahon LS, Landon MR, Jackson-Cook CK, Holt SE. Telomerase protects cancer-prone human cells from chromosomal instability and spontaneous immortalization. *Cancer Biol Ther* 2002; 1(4):391–7.
110. Chin K, de Solorzano CO, Knowles D, et al. In situ analyses of genome instability in breast cancer. *Nat Genet* 2004; 36(9):984–8.
111. Banik SS, Counter CM. Characterization of interactions between PinX1 and human telomerase subunits hTERT and hTR. *J Biol Chem* 2004; 279(50):51745–8.
112. Counter CM, Meyerson M, Eaton EN, et al. Telomerase activity is restored in human cells by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase. *Oncogene* 1998; 16(9):1217–22.
113. Orren DK, Theodore S, Machwe A. The Werner syndrome helicase/exonuclease (WRN) disrupts and degrades D-loops *in vitro*. *Biochemistry* 2002; 41(46):13483–8.

8

Overview of Senescence

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SUMMARY

Cellular senescence refers to the limited capacity of normal somatic cells to proliferate indefinitely *in vitro*. There are at least two types of senescence that prevent infinite proliferation: replicative senescence and stress-induced senescence (SIS). In replicative senescence, failure of the DNA polymerase to faithfully duplicate the chromosomal ends leads to progressive telomeric DNA sequence loss with each subsequent cell division and signaling for growth arrest. There are, at least, two mechanisms that prevent further telomere shortening and as a consequence bypass replicative senescence: upregulation/reactivation of endogenous telomerase and alternative lengthening for telomeres (ALT) pathway. In SIS, however, several stressors have been found to signal for growth arrest, such as DNA damage, damage to chromatin structure, oxidative stress, and oncogene activation. The ability to induce growth arrest as a consequence of telomere shortening or stress is thus the essential feature distinguishing the mortality of normal cells (presumably to provide a barrier against the accumulation of mutations and the formation of cancer) and the immortality of tumor cells.

Key Words: Cellular senescence; aging; telomeres; telomerase; alternative lengthening for telomeres; end replication problem.

1. CELLULAR SENESCENCE

Cellular senescence has been defined as the limited capacity to proliferate indefinitely *in vitro*. The ability to proliferate indefinitely in tissue culture is one of the many differences between normal human cells and established human cell lines. For nearly 40 years (1), the contrast between the immortality of cancer cells and the senescence of normal cells has been controversial. The major source of skepticism rests on the knowledge that *in vitro* culture conditions represent a departure from the *in vivo* environment and that the aneuploidy and altered growth controls accumulated by tumor cells might simply allow them to divide under conditions inadequate to support the long-term proliferation of normal cells.

Presently, two distinct processes to elicit cellular senescence are recognized: senescence due to cell replication (replicative senescence) and senescence due to other causes

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[stress-induced senescence (SIS)]. Replicative senescence is a specific type of cellular senescence that results from an intrinsic mechanism: loss of telomere sequences with each round of cell division. SIS, on the contrary, results from a number of stressors, such as DNA damage, damage to chromatin structure, oxidative stress, and increased oncogene activity.

Cellular senescence is a viable, metabolic active, and non-dividing *in vitro* state, in which the phenotype of senescent cells involves changes in cell morphology (cells become enlarged and flattened in shape), dramatic changes in chromatin structure, changes in gene expression, and lack of response to mitogenic stimuli (2,3,4). It has been suggested that cellular senescence is a fundamental cellular program involved in aging and is a potential tumor suppressive mechanism to limit the replicative capacity of potential cancer cells. Thus, efforts have lately been directed to develop chemopreventive agents that turn immortal tumor cells back to mortality.

2. REPLICATIVE SENESCENCE

Replicative (from the Latin words *replicare*, meaning to fold back, to repeat, to duplicate) senescence is a specific type of cellular senescence referring to limited number of *in vitro* cell divisions. Hayflick (1,5) postulated that the limited proliferative capacity of somatic cells was pre-programmed. Today, the “Hayflick limit” refers to the “preset” number of doublings that a particular cell type is able to achieve. Currently, the best understood mechanism for replicative senescence involves shortening of the chromosomal ends (telomeres), which results from a problem in completely duplicating the telomeric ends. Consequently, the only known way to retard replicative aging and to exceed the Hayflick limit in intact normal cells is to prevent telomere shortening through either the expression of telomerase or activation of the alternative lengthening of telomeres (ALT) pathway. Cellular immortalization through the ectopic expression of the catalytic component of telomerase [human telomerase reverse transcriptase (hTERT)] (6–10) or through activation of ALT pathway (11–14) has been reported in a number of articles. These results suggest that the number of cell divisions is limited, not by chronological or metabolic age but rather by the process of telomere shortening, which acts as a genetic, mitotic clock.

2.1. DNA Duplication

2.1.1. DNA POLYMERASE CANNOT INITIATE DNA SYNTHESIS DE NOVO

During the process of cell division, genomic DNA must be faithfully replicated. To this end, the cell must engage in the enormous task of copying the 3.2 billion base pairs comprising the human genome into a new DNA strand. To ensure proper duplication of each base pair without errors and in a timely manner, we must meticulously regulate and monitor replication.

DNA replication is a very intricate mechanism, which involves several proteins just in the process of replication initiation (15,16). Prior to the S phase, initiation of replication starts by assembling a multiprotein structure called the pre-replicative complex (pre-RC), which is then converted to a replication fork during the G1–S transition. For replication initiation to continue, origin unwinding, stabilization of single stranded DNA, and loading of DNA polymerases are required. There are essentially three polymerases for replication: DNA pol α , DNA pol δ , and DNA pol ϵ . After the

origin of replication becomes unwound, DNA pol α is recruited to the origin where the enzyme mediates the production of short RNA primers used for the initiation of leading and lagging strand synthesis. Following RNA primer synthesis, DNA pol α is replaced by DNA pol δ and/or DNA pol ϵ . The exchange of DNA polymerases produces an increase in DNA synthesis processivity and adds proofreading exonuclease activity during the process of DNA replication through the S phase. It has been thought that both polymerases perform mutually exclusive essential functions (17,18). Previous reports have shown that DNA pol δ displays greater processivity of DNA synthesis of both leading and lagging strand in the absence of pol ϵ , whereas DNA pol ϵ fails to significantly elongate DNA strands in the absence of DNA pol δ , suggesting that the main DNA polymerase is DNA pol δ (17).

DNA polymerases mediate the synthesis of a strand of DNA by using a complementary DNA strand as template. Sequences of DNA nucleotides are essentially copied by base-pairing into complementary new nucleotide sequences. The DNA replication is semiconservative, because each of the two daughter cells receives from their progenitor a DNA double helix containing one old and one new strand. Furthermore, the DNA replication fork is asymmetrical. One strand is synthesized continuously and is known as the leading strand, whereas the other strand is synthesized discontinuously and is known as the lagging strand. The leading strand requires only the RNA primer at the start of the replication. However, the lagging strand requires constantly synthesized RNA primers to generate the Okazaki fragments throughout the strand. When the DNA polymerase from the previous Okazaki fragment runs into the RNA primer of the downstream Okazaki fragment, DNA polymerase's exonuclease activity removes the ribonucleotides and DNA polymerase replaces them with deoxynucleotides. DNA ligase then joins the 3'-end of the new DNA fragment to the 5'-end of the previous one to complete the process. Thus, the complete duplication of the entire genome requires a complementary DNA strand for annealing an RNA primer and initiation of strand synthesis by DNA polymerase. This poses a problem for DNA sequences at the end of linear chromosomes (explained below).

2.2. The End Replication Problem

2.2.1. DNA POLYMERASE CANNOT FAITHFULLY DUPLICATE THE ENDS OF CHROMOSOMES

After the DNA structure was discovered and the duplication mechanisms during S phase of the cell cycle were elucidated, it was apparent first to Olovnikov (19,20) and later to Watson (21) that in the absence of special mechanisms, replication of linear chromosomes would be incomplete. Because of the requirement for an RNA primer to initiate DNA synthesis, it has been postulated that lack of telomeric end synthesis is due to inability to position RNA primers at the very end of telomeres (Fig. 1).

DNA polymerases, the enzymes responsible for duplicating the DNA strands during the S phase of the cell cycle, can only replicate DNA in the 5'-to-3' direction and cannot initiate synthesis of the lagging DNA chain without the assistance of RNA primer templates. One result of these restrictions is that there is no template downstream of the 3' telomeric end that DNA polymerase can use to synthesize the rest of the lagging strand (Fig. 1). The end replication problem mandates that the 5'-end of the daughter strands will shorten with each round of cell division. Thus, in actively proliferating

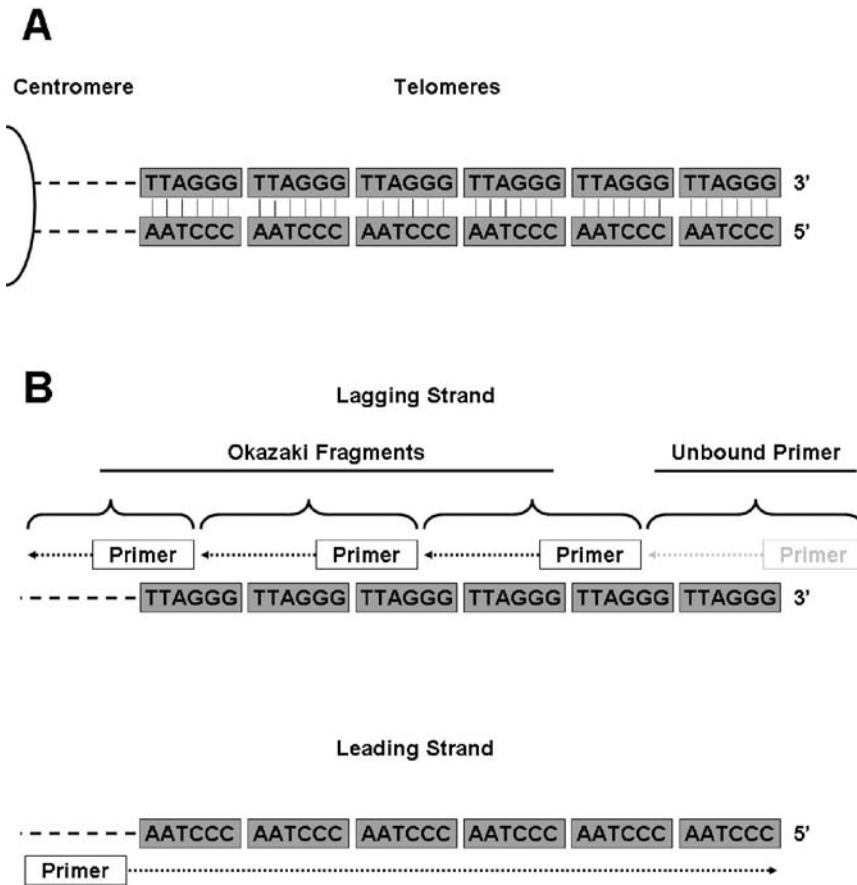


Fig. 1. (A) The chromosomal ends are called telomeres and consist of tandem repeats of the DNA sequence TTAGGG in vertebrates. (B) Upon unwinding of DNA strands, RNA primers are synthesized to initiate DNA replication. The lagging strand is copied by DNA polymerase synthesizing the new strand discontinuously; thus RNA primers are positioned at different intervals along the DNA template. Following elongation of the RNA primers by DNA polymerase, the Okazaki fragments are formed. The lack of DNA sequences further downstream of the 3' telomeric end prevents binding of RNA primers and precludes complete DNA replication, resulting in shorter chromosomal ends at each round of cell division. By contrast, the leading strand is copied by DNA polymerase synthesizing the new strand continuously; thus RNA primers are positioned only at the start of the replication.

cells, chromosomes shorten progressively from both ends (20), and in the absence of any compensating mechanisms, the ends of the chromosomes are not replicated completely, resulting in a net loss of terminal chromosomal DNA with each cell division. It has been proposed that the erosion of telomeres during cell division is the mitotic clock that limits the replicative capacity of normal somatic cells (22,23) and that the reaching of a critical telomere length signals replicative senescence or aging (19,20,24).

The inability to position RNA primers at the very end of telomeres is not the only hypothesis accounting for telomere shortening. Two additional extensively studied

hypotheses are (i) unrepaired oxidative damage to telomeres (25) and (ii) nucleolytic degradation of one or both strands (26).

2.3. *Telomere Theory*

2.3.1. **TELOMERES ARE TANDEM REPEATS AT THE END OF CHROMOSOMES AND PROTECT AGAINST GENOME INSTABILITY**

Telomeres (from the Greek words *telos*, meaning end, and *meros*, meaning part) are the terminal ends of linear chromosomes. For nearly 65 years, it has been known that the chromosomal ends behave differently than the internal free ends. For example, X-ray ionizing radiation can cause internal inversions far more readily than terminal inversions (27,28). Compared with the telomeric free ends of linear chromosomes, the internal free ends of broken chromosomes are more reactive and easily undergo end-to-end fusion reactions with internal free ends from other broken chromosomes. In the absence of a repair system, the high reactivity of internal double-strand breaks could eventually lead to gross karyotypic changes. To prevent the development of abnormal karyotypes, organisms have developed monitoring systems that arrest cell division in response to DNA damage, allowing time for DNA repair before proceeding into mitosis. The *p53* gene product functions as a monitoring system for genomic damage (29,30–33).

Telomeres are composed of tandem repeats of the sequence TTAGGG in vertebrates (34,35) and are thought to prevent the degradation or aberrant recombination of the chromosomal ends (36–38). In addition, telomeres also contain specific proteins, such as the TTAGGG repeat binding factor 1 (TRF1) (39,40), TRF2 (41,42), tankyrase 1 (TANK1) (43,44), tankyrase 2 (TANK2) (45), TRF1-interacting nuclear protein 2 (TIN2) (46), PIN2-interacting protein 1 (PINX1) (47), and protection of telomeres 1 (POT1) (48). Among vertebrates, the size of telomeres is variable (49,50–53) and their precise length can only be estimated. Capping the chromosomal ends to prevent signaling for cell cycle arrest and helping in maintaining chromosomal stability are among the different biological roles that have been described for telomeres (54). They protect chromosomes from degradation and fusion (53), contribute to the architecture of the nucleus (55, 56), and play a role in gene expression through the phenomenon of telomere silencing (57,58).

In the last 10 years, a dual role in chromosomal homeostasis has been described for telomeres: they buffer the loss of terminal sequences resulting from incomplete DNA replication by DNA polymerases due to the end replication problem (19–21), and telomeres provide sites for the *de novo* elongation of the chromosome by telomerase, thus compensating for the DNA sequence loss. Overall, telomeres are special protein/DNA structures essential for chromosomal stability.

2.4. *Telomerase*

2.4.1. **TELOMERASE IS A REVERSE TRANSCRIPTASE THAT ELONGATES THE 3'-END OF TELOMERES**

In most immortal eukaryotic cells, telomerase functions as the compensating mechanism that maintains telomere length. Telomerase is a ribonucleoprotein enzyme

that mediates the synthesis of the telomeric repeat sequence de novo using its RNA (59) component [human telomerase RNA (hTR)] as a template and the telomerase catalytic protein subunit (hTERT) (60–62) for the addition of telomeric repeats to the 3'-ends of the chromosomes. Telomerase activity was first identified in *Tetrahymena* and later in other species, including ciliates, yeasts, mice, and humans (63–67). Because most normal somatic cells in vertebrates express no or very low levels of telomerase activity, it is thought that their telomeres progressively shorten, undergo genomic instability, and cease proliferating once a critical length is reached (24). It has been suggested that upregulation or reactivation of telomerase may result in chromosomal stability leading to continuous cell proliferation (23). Thus, telomerase is thought to partially or completely compensate for the progressive telomere erosion that would occur in its absence.

Although telomerase is not expressed in most normal somatic cells, telomerase activity is found in the vast majority of primary tumors (68,69). This observation suggests that telomerase activity plays a role in preventing senescence in malignant infinitely dividing cells. It also seems reasonable to assume that cells with extreme proliferative requirements, such as those in renewal tissues (e.g., the blood, skin, and intestine), should also be capable of expressing telomerase. In support of this concept, recent reports have demonstrated the presence of telomerase activity in hematopoietic progenitor cells and activated lymphocytes (70–72), the epidermis (73,74), and the intestine (75). Nevertheless, the telomeres of cells from these renewal tissues also shorten with age (76–78), suggesting that the level of expression of telomerase in cells of renewal tissues is sufficient to slow, but not prevent, telomere erosion. This low level of telomerase expression appears to extend the life span of these cells but does not confer immortality.

2.5. ALT Mechanism

2.5.1. TELOMERASE-INDEPENDENT TELOMERE MAINTENANCE MECHANISM

Even though most immortal cell lines and human tumors bypass cellular senescence by compensating for the telomere loss through reactivation of telomerase (79), a telomerase-independent mechanism of telomere elongation has also been described (11,80,81). The ALT pathway has been studied for nearly 12 years (11,80,81). It has been determined, at least in some cases, that elongation of telomeres in immortal cell lines expressing no telomerase activity is mediated by homologous recombination of telomeric DNA sequences (12,13). To date, all the human ALT cell lines analyzed have several characteristics in common: (i) cells lack significant levels of telomerase activity (80), (ii) telomeres are highly heterogenous in length, ranging from <2Kb to >20–80 Kb (80–82), (iii) ALT cells' telomeres show rapid telomeric lengthening or shortening events (13), and (iv) the presence of ALT-associated promyelocytic leukemia (PML) bodies (APB) is detected in ALT cells (83).

3. STRESS-INDUCED SENESCENCE

Another type of cellular senescence is SIS resulting from a number of stressors, such as DNA damage, damage to chromatin structure, oxidative stress, and increased oncogene activity. This variety of cellular senescence suggests that multiple stresses can trigger a common cellular response. Senescence is activated once a cell has

reached a critical level of damage, regardless of the type of damage. Common features with replicative senescence are morphology (enlarged cell shape), senescence-associated β -galactosidase activity, cell cycle regulation, gene expression, and telomere shortening.

3.1. DNA Damage

3.1.1. DIRECT GENOMIC DNA DAMAGE INDUCES SENESCENCE

It has been known for years that direct damaging of DNA, either by irradiation of cells (84) or by treatment with reactive chemicals (85), can induce cells to undergo senescence (86,87), which has been usually interpreted as an arrest to allow time for repair. Prolonged arrest due to ionizing radiation has often been reported to be dependent on p53 (84–88). In mammalian cells, both homologous and non-homologous mechanisms seem to be involved in repairing double-strand breaks (89). Other types of DNA damage can also induce senescence. For example, absence of Ku86 (a protein essential for repair of DNA breaks through non-homologous end joining) results in a high frequency of end-to-end chromosomal fusions despite having long telomeric segments, and induction of senescence (90). Generation of chromosomal fusions has also been reported as a consequence of inactivating the function of the telomere-binding protein TRF2 (91).

In line with this notion that DNA damaging induces senescence (and/or apoptosis), telomere shortening can also be interpreted as loss of DNA material and hence trigger senescence as well. It has been reported that unprotected chromosomes can induce DNA damage checkpoint cascades and ultimately lead to senescence both in mice and humans in a p53-dependent manner (91–94). Thus, initial telomere length setting determines the proliferative capacity of each cell.

3.2. Damage to Chromatin Structure

3.2.1. EPIGENETIC CHANGES IN MICROENVIRONMENT OF HETEROCHROMATIN INDUCE SENESCENCE

Major epigenetic mechanisms include methylation modification of DNA and acetylation/packaging of DNA by histone proteins into chromatin structure. It has been known that formation of heterochromatin-like chromatin structures is implicated in epigenetic silencing (95–98). In fact, functional chromosomal domains have been linked to silencing in yeast (reviewed in 97) and *Drosophila* (reviewed in 96). In addition, heterochromatin-like regions in mammalian cells have also been reported to be underacetylated at selected lysine residues in histone H4 (99). Thus, proper function of histone deacetylase has been assumed to be required for the reformation of these regions during the latter part of each cell cycle (100). Accordingly, histone acetylation has already been related to senescence (101). Interestingly, telomere length in mice has also been reported recently to be directly regulated by histone methylation (102). Therefore, besides increased oxidative stress (103,104) and telomere shortening (20,22,76,105), alteration of epigenetic maintenance mechanisms can also induce cellular senescence.

3.3. Oxidative Stress

3.3.1. STRESS-INDUCED PREMATURE SENESCENCE IS RELATED TO OXIDATIVE STRESS

Premature growth arrest in response to stress has previously been described in both fibroblasts and melanocytes as stress-induced premature senescence (SIPS) (reviewed in 106). SIPS has been demonstrated for a variety of experimental conditions, including exposure to hydrogen peroxide (H_2O_2), tert-butylhydroperoxide (t-BHP), hyperoxia, UV light, and radioactivity. Oxidative stress is the most well understood inducer of SIPS (reviewed in 106). Because growth arrest occurs at G_1 in human dermal fibroblasts post- H_2O_2 treatment, it has been speculated that cyclin-dependent kinase inhibitors are involved. In support of this, mRNA levels of p21^{WAF1} and p16^{INK4a} increased 72 h post- H_2O_2 stress followed by a decrease in hyperphosphorylated Rb which last several weeks (25,107). It was also reported that there is a decrease in hyperphosphorylated Rb 5 days post-chronic t-BHP treatment (108). Thus, p16/Rb pathway seems to function as a stress sensor mechanism to prevent further DNA damage due to exposure to oxidative damage.

3.4. Oncogene Activity

3.4.1. OVEREXPRESSION OF ONCOGENES INDUCES SENESCENCE IN NORMAL CELLS

Activated oncogenes have been known to trigger tumor suppressor mechanisms in primary cells to prevent propagation of potentially transformed cells. For example, *c-myc* has been shown to cause apoptosis (109–112), activated *ras* has been shown to elicit senescence (113,114), and β -catenin has been shown to induce p53-dependent apoptosis (115). Tumorigenesis is an evolutionary process that selects for genetic and epigenetic changes to escape normal anti-proliferative and cell-death-inducing mechanisms and allows clonal expansion of malignantly transformed cells (116). Thus, induction of apoptosis and/or senescence upon increased oncogenic signaling is crucial for eliminating or preventing dissemination of cells with damaged DNA or deregulated proliferation. Several mechanisms to restrain oncogenesis have been proposed, including hypoxia (117), telomere attrition (118), and induced expression of the Arf tumor suppressor (119). However, the mechanism representing the major force guarding against genetic instability and tumorigenesis is actually unknown.

4. CONCLUSION

It is generally believed that unlimited replicative capacity is a required attribute for most malignant tumors to sustain long-term growth. As a result, cellular senescence has been thought as a potential tumor suppressor mechanism preventing cells from replicating indefinitely. However, direct experimental evidence that cellular senescence is a tumor suppressor pathway has remained elusive. Because telomere shortening regulates the onset of cellular senescence, one should undoubtedly conclude that loss of the growth controls without inducing a compensatory mechanism on telomere dynamics would lead to formation of tumors with an extended, but not infinite, life span because of continued telomere shortening. That is, if tumor cells lack a telomere maintenance mechanism, these cells would have a finite life span and consequently would not

produce life-threatening tumors. Clearly, dysfunction of cellular senescence must be among the changes required for perpetuation of malignant tumors.

In the past 15 years, two pathways that bypass cellular senescence have been extensively investigated: a telomerase-dependent (upregulation/reactivation of telomerase) pathway and a telomerase-independent (ALT) pathway. Because both pathways seem to abrogate the same basic mechanism that mediates cellular senescence, e.g., loss of telomeric sequences, targeting telomere maintenance mechanisms has become an attractive anti-neoplastic strategy. Continued progress in the study of cellular senescence, telomeres, and their role in cancer progression should help in the development of novel therapeutic strategies against cancer.

REFERENCES

1. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res* 1961; 25:585–621.
2. Narita M, Nunez S, Heard E, Narita M, Lin AW, Hearn SA, et al. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* 2003; 113(6):703–716.
3. Serrano M, Blasco MA. Putting the stress on senescence. *Curr Opin Cell Biol* 2001; 13(6):748–753.
4. Shelton DN, Chang E, Whittier PS, Choi D, Funk WD. Microarray analysis of replicative senescence. *Curr Biol* 1999; 9(17):939–945.
5. Hayflick L. The limited *in vitro* lifetime of human diploid cell strains. *Exp Cell Res* 1965; 37:614–636.
6. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, et al. Extension of life-span by introduction of telomerase into normal human cells. *Science* 1998; 279(5349):349–352.
7. Morales CP, Gandia KG, Ramirez RD, Wright WE, Shay JW, Spechler SJ. Characterisation of telomerase immortalised normal human oesophageal squamous cells. *Gut* 2003; 52(3):327–333.
8. Ouellette MM, McDaniel LD, Wright WE, Shay JW, Schultz RA. The establishment of telomerase-immortalized cell lines representing human chromosome instability syndromes. *Hum Mol Genet* 2000; 9(3):403–411.
9. Ramirez RD, Morales CP, Herbert BS, Rohde JM, Passons C, Shay JW, et al. Putative telomerase-independent mechanisms of replicative aging reflect inadequate growth conditions. *Genes Dev* 2001; 15(4):398–403.
10. Vaziri H, Benchimol S. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr Biol* 1998; 8(5):279–282.
11. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat Med* 1997; 3(11):1271–1274.
12. Dunham MA, Neumann AA, Fasching CL, Reddel RR. Telomere maintenance by recombination in human cells. *Nat Genet* 2000; 26(4):447–450.
13. Murnane JP, Sabatier L, Marder BA, Morgan WF. Telomere dynamics in an immortal human cell line. *EMBO J* 1994; 13(20):4953–4962.
14. Varley H, Pickett HA, Foxon JL, Reddel RR, Royle NJ. Molecular characterization of inter-telomere and intra-telomere mutations in human ALT cells. *Nat Genet* 2002; 30(3):301–305.
15. Bell SP, Dutta A. DNA replication in eukaryotic cells. *Annu Rev Biochem* 2002; 71:333–374.
16. Mendez J, Stillman B. Perpetuating the double helix: molecular machines at eukaryotic DNA replication origins. *Bioessays* 2003; 25(12):1158–1167.
17. Fukui T, Yamauchi K, Muroya T, Akiyama M, Maki H, Sugino A, et al. Distinct roles of DNA polymerases delta and epsilon at the replication fork in *Xenopus* egg extracts. *Genes Cells* 2004; 9(3):179–191.
18. Waga S, Masuda T, Takisawa H, Sugino A. DNA polymerase epsilon is required for coordinated and efficient chromosomal DNA replication in *Xenopus* egg extracts. *Proc Natl Acad Sci USA* 2001; 98(9):4978–4983.
19. Olovnikov AM. [Principle of marginotomy in template synthesis of polynucleotides]. *Dokl Akad Nauk SSSR* 1971; 201(6):1496–1499.

20. Olovnikov AM. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *J Theor Biol* 1973; 41(1):181–190.
21. Watson JD. Origin of concatemeric T7DNA. *Nat New Biol* 1972; 239(94):197–201.
22. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature* 1990; 345(6274):458–460.
23. Shay JW. Aging and cancer: are telomeres and telomerase the connection. *Mol Med Today* 1995; 1(8):378–384.
24. Allsopp RC, Harley CB. Evidence for a critical telomere length in senescent human fibroblasts. *Exp Cell Res* 1995; 219(1):130–136.
25. Vonzglinicki T, Saretzki G, Docke W, Lotze C. Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts – a model for senescence. *Exp Cell Res* 1995; 220(1):186–193.
26. Makarov VL, Hirose Y, Langmore JP. Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. *Cell* 1997; 88(5):657–666.
27. Muller HJ. The remaking of chromosomes. *Collect Net* 1938; 13:181–198.
28. Muller HJ, Herskowitz IH. Concerning the healing of chromosome ends produced by breakage in *Drosophila melanogaster*. *Am Nat* 1954; 88(840):177–208.
29. Perry ME, Levine AJ. Tumor-suppressor p53 and the cell cycle. *Curr Opin Genet Dev* 1993; 3(1):50–54.
30. Lu X, Lane DP. Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes. *Cell* 1993; 75(4):765–778.
31. Lane Cancer. DP. A death in the life of p53. *Nature* 1993; 362(6423):786–787.
32. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 1992; 356(6366):215–221.
33. Livingstone LR, White A, Sprouse J, Livanos E, Jacks T, Tlsty TD. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell* 1992; 70(6):923–935.
34. Blackburn EH. Structure and function of telomeres. *Nature* 1991; 350(6319):569–573.
35. Blackburn EH. Telomeres. *Trends Biochem Sci* 1991; 16(10):378–381.
36. Blackburn EH. The molecular structure of centromeres and telomeres. *Annu Rev Biochem* 1984; 53:163–194.
37. Zakian VA. Structure and function of telomeres. *Annu Rev Genet* 1989; 23:579–604.
38. Hastie ND, Allshire RC. Human telomeres: fusion and interstitial sites. *Trends Genet* 1989; 5(10):326–331.
39. Chong L, van Steensel B, Broccoli D, Erdjument-Bromage H, Hanish J, Tempst P, et al. A human telomeric protein. *Science* 1995; 270(5242):1663–1667.
40. van Steensel B, de Lange T. Control of telomere length by the human telomeric protein TRF1. *Nature* 1997; 385(6618):740–743.
41. Bilaud T, Brun C, Ancelin K, Koering CE, Laroche T, Gilson E. Telomeric localization of TRF2, a novel human telobox protein. *Nat Genet* 1997; 17(2):236–239.
42. Broccoli D, Smogorzewska A, Chong L, de Lange T. Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. *Nat Genet* 1997; 17(2):231–235.
43. Smith S, Giriat I, Schmitt A, de Lange T. Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. *Science* 1998; 282(5393):1484–1487.
44. Smith S, de Lange T. Tankyrase promotes telomere elongation in human cells. *Curr Biol* 2000; 10(20):1299–1302.
45. Kaminker PG, Kim SH, Taylor RD, Zebarjadian Y, Funk WD, Morin GB, et al. TANK2, a new TRF1-associated poly(ADP-ribose) polymerase, causes rapid induction of cell death upon overexpression. *J Biol Chem* 2001; 276(38):35891–35899.
46. Kim SH, Kaminker P, Campisi J. TIN2, a new regulator of telomere length in human cells. *Nat Genet* 1999; 23(4):405–412.
47. Zhou XZ, Lu KP. The Pin2/TRF1-interacting protein PinX1 is a potent telomerase inhibitor. *Cell* 2001; 107(3):347–359.
48. Baumann P, Cech TR. Pot1, the putative telomere end-binding protein in fission yeast and humans. *Science* 2001; 292(5519):1171–1175.
49. Starling JA, Maule J, Hastie ND, Allshire RC. Extensive telomere repeat arrays in mouse are hypervariable. *Nucleic Acids Res* 1990; 18(23):6881–6888.

50. Lejnine S, Makarov VL, Langmore JP. Conserved nucleoprotein structure at the ends of vertebrate and invertebrate chromosomes. *Proc Natl Acad Sci USA* 1995; 92(6):2393–2397.
51. Kipling D, Cooke HJ. Hypervariable ultra-long telomeres in mice. *Nature* 1990; 347(6291):400–402.
52. Cooke HJ, Smith BA. Variability at the telomeres of the human X/Y pseudoautosomal region. *Cold Spring Harb Symp Quant Biol* 1986; 1(51 Pt):213–219.
53. Biessmann H, Mason JM. Genetics and molecular biology of telomeres. *Adv Genet* 1992; 30:185–249.
54. McClintock B. The stability of broken ends of chromosomes in *Zea mays*. *Genetics* 1941; 26:234–282.
55. de Lange T. Human telomeres are attached to the nuclear matrix. *EMBO J* 1992; 11(2):717–724.
56. Gilson E, Laroche T, Gasser SM. Telomeres and the functional architecture of the nucleus. *Trends Cell Biol* 1993; 3(4):128–134.
57. Baur JA, Zou Y, Shay JW, Wright WE. Telomere position effect in human cells. *Science* 2001; 292(5524):2075–2077.
58. Karpen GH. Position-effect variegation and the new biology of heterochromatin. *Curr Opin Genet Dev* 1994; 4(2):281–291.
59. Feng J, Funk WD, Wang SS, Weinrich SL, Ailion AA, Chiu CP, et al. The RNA component of human telomerase. *Science* 1995; 269(5228):1236–1241.
60. Kilian A, Bowtell DD, Abud HE, Hime GR, Venter DJ, Keese PK, et al. Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum Mol Genet* 1997; 6(12):2011–2019.
61. Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, et al. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell* 1997; 90(4):785–795.
62. Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, et al. Telomerase catalytic subunit homologs from fission yeast and human. *Science* 1997; 277(5328):955–959.
63. Shippen-Lentz D, Blackburn EH. Functional evidence for an RNA template in telomerase. *Science* 1990; 247(4942):546–552.
64. Morin GB. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell* 1989; 59(3):521–529.
65. Lin JJ, Zakian VA. An *in vitro* assay for *Saccharomyces* telomerase requires EST1. *Cell* 1995; 81(7):1127–1135.
66. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell* 1985; 43(2 Pt 1):405–413.
67. Cohn M, Blackburn EH. Telomerase in yeast. *Science* 1995; 269(5222):396–400.
68. Counter CM, Hirte HW, Bacchetti S, Harley CB. Telomerase activity in human ovarian carcinoma. *Proc Natl Acad Sci USA* 1994; 91(8):2900–2904.
69. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994; 266(5193):2011–2015.
70. Hiyama K, Hirai Y, Kyoizumi S, Akiyama M, Hiyama E, Piatyszek MA, et al. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J Immunol* 1995; 155(8):3711–3715.
71. Chiu CP, Dragowska W, Kim NW, Vaziri H, Yui J, Thomas TE, et al. Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow. *Stem Cells* 1996; 14(2):239–248.
72. Broccoli D, Young JW, de Lange T. Telomerase activity in normal and malignant hematopoietic cells. *Proc Natl Acad Sci USA* 1995; 92(20):9082–9086.
73. Taylor RS, Ramirez RD, Ogoshi M, Chaffins M, Piatyszek MA, Shay JW. Detection of telomerase activity in malignant and nonmalignant skin conditions. *J Invest Dermatol* 1996; 106(4):759–765.
74. Harle-Bachor C, Boukamp P. Telomerase activity in the regenerative basal layer of the epidermis in human skin and in immortal and carcinoma-derived skin keratinocytes. *Proc Natl Acad Sci USA* 1996; 93(13):6476–6481.
75. Bachor C, Bachor OA, Boukamp P. Telomerase is active in normal gastrointestinal mucosa and not up-regulated in precancerous lesions. *J Cancer Res Clin Oncol* 1999; 125(8–9):453–460.
76. Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RC. Telomere reduction in human colorectal carcinoma and with ageing. *Nature* 1990; 346(6287):866–868.

77. Vaziri H, Dragowska W, Allsopp RC, Thomas TE, Harley CB, Lansdorp PM. Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age. *Proc Natl Acad Sci USA* 1994; 91(21):9857–9860.
78. Lindsey J, McGill NI, Lindsey LA, Green DK, Cooke HJ. *in vivo* loss of telomeric repeats with age in humans. *Mutat Res* 1991; 256(1):45–48.
79. Cech TR. Beginning to understand the end of the chromosome. *Cell* 2004; 116(2):273–279.
80. Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J* 1995; 14(17):4240–4248.
81. Bryan TM, Reddel RR. Telomere dynamics and telomerase activity in *in vitro* immortalised human cells. *Eur J Cancer* 1997; 33(5):767–773.
82. Reddel RR. Alternative lengthening of telomeres, telomerase, and cancer. *Cancer Lett* 2003; 194(2):155–162.
83. Yeager TR, Neumann AA, Englezou A, Huschtscha LI, Noble JR, Reddel RR. Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. *Cancer Res* 1999; 59(17):4175–4179.
84. Little JB. Delayed initiation of DNA synthesis in irradiated human diploid cells. *Nature* 1968; 218(146):1064–1065.
85. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res* 1991; 51(23 Pt 1):6304–6311.
86. Di Leonardo A, Linke SP, Clarkin K, Wahl GM. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev* 1994; 8(21):2540–2551.
87. Wahl GM, Carr AM. The evolution of diverse biological responses to DNA damage: insights from yeast and p53. *Nat Cell Biol* 2001; 3(12):E277–E286.
88. Weichselbaum RR, Epstein J, Little JB. *In vitro* radiosensitivity of human diploid fibroblasts derived from patients with unusual clinical responses to radiation. *Radiology* 1976; 121(2):479–482.
89. Johnson RD, Jasin M. Double-strand-break-induced homologous recombination in mammalian cells. *Biochem Soc Trans* 2001; 29(Pt 2):196–201.
90. Samper E, Goytisolo FA, Slijepcevic P, van Buul PP, Blasco MA. Mammalian Ku86 protein prevents telomeric fusions independently of the length of TTAGGG repeats and the G-strand overhang. *EMBO Rep* 2000; 1(3):244–252.
91. Karlseder J, Broccoli D, Dai Y, Hardy S, de Lange T. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science* 1999; 283(5406):1321–1325.
92. d’Adda dF, Teo SH, Jackson SP. Functional links between telomeres and proteins of the DNA-damage response. *Genes Dev* 2004; 18(15):1781–1799.
93. Lansdorp PM. Repair of telomeric DNA prior to replicative senescence. *Mech Ageing Dev* 2000; 118(1–2):23–34.
94. Narayan S, Jaiswal AS, Multani AS, Pathak S. DNA damage-induced cell cycle checkpoints involve both p53-dependent and -independent pathways: role of telomere repeat binding factor 2. *Br J Cancer* 2001; 85(6):898–901.
95. Laurenson P, Rine J. Silencers, silencing, and heritable transcriptional states. *Microbiol Rev* 1992; 56(4):543–560.
96. Pirrotta V, Rastelli L. White gene expression, repressive chromatin domains and homeotic gene regulation in *Drosophila*. *Bioessays* 1994; 16(8):549–556.
97. Rivier DH, Rine J. Silencing: the establishment and inheritance of stable, repressed transcription states. *Curr Opin Genet Dev* 1992; 2(2):286–292.
98. Shaffer CD, Wallrath LL, Elgin SC. Regulating genes by packaging domains: bits of heterochromatin in euchromatin. *Trends Genet* 1993; 9(2):35–37.
99. Jeppesen P, Turner BM. The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. *Cell* 1993; 74(2):281–289.
100. Annunziato AT, Seale RL. Histone deacetylation is required for the maturation of newly replicated chromatin. *J Biol Chem* 1983; 258(20):12675–12684.
101. Ogryzko VV, Hirai TH, Russanova VR, Barbie DA, Howard BH. Human fibroblast commitment to a senescence-like state in response to histone deacetylase inhibitors is cell cycle dependent. *Mol Cell Biol* 1996; 16(9):5210–5218.

102. Garcia-Cao M, O'Sullivan R, Peters AH, Jenuwein T, Blasco MA. Epigenetic regulation of telomere length in mammalian cells by the Suv39h1 and Suv39h2 histone methyltransferases. *Nat Genet* 2004; 36(1):94–99.
103. Sohal RS, Allen RG. Oxidative stress as a causal factor in differentiation and aging: a unifying hypothesis. *Exp Gerontol* 1990; 25(6):499–522.
104. Stadtman ER. Protein oxidation and aging. *Science* 1992; 257(5074):1220–1224.
105. Wright WE, Shay JW. Telomere positional effects and the regulation of cellular senescence. *Trends Genet* 1992; 8(6):193–197.
106. Toussaint O, Medrano EE, von Zglinicki T. Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. *Exp Gerontol* 2000; 35(8):927–945.
107. Chen QM, Bartholomew JC, Campisi J, Acosta M, Reagan JD, Ames BN. Molecular analysis of H₂O₂-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication. *Biochem J* 1998; 332:43–50.
108. Dumont P, Burton M, Chen QM, Gonos ES, Fripiat C, Mazarati JB, et al. Induction of replicative senescence biomarkers by sublethal oxidative stresses in normal human fibroblast. *Free Radic Biol Med* 2000; 28(3):361–373.
109. Amati B, Littlewood TD, Evan GI, Land H. The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max. *EMBO J* 1993; 12(13):5083–5087.
110. Bissonnette RP, Echeverri F, Mahboubi A, Green DR. Apoptotic cell death induced by c-myc is inhibited by bcl-2. *Nature* 1992; 359(6395):552–554.
111. Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, et al. Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 1992; 69(1):119–128.
112. Harrington EA, Bennett MR, Fanidi A, Evan GI. c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO J* 1994; 13(14):3286–3295.
113. Bringold F, Serrano M. Tumor suppressors and oncogenes in cellular senescence. *Exp Gerontol* 2000; 35(3):317–329.
114. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 1997; 88(5):593–602.
115. Pauklin S, Kristjuhan A, Maimets T, Jaks V. ARF and ATM/ATR cooperate in p53-mediated apoptosis upon oncogenic stress. *Biochem Biophys Res Commun* 2005; 334(2):386–394.
116. Lowe SW, Cepero E, Evan G. Intrinsic tumour suppression. *Nature* 2004; 432(7015):307–315.
117. Graeber TG, Peterson JF, Tsai M, Monica K, Fornace AJ Jr, Giaccia AJ. Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. *Mol Cell Biol* 1994; 14(9):6264–6277.
118. Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, et al. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J* 1992; 11(5):1921–1929.
119. Zindy F, Williams RT, Baudino TA, Rehg JE, Skapek SX, Cleveland JL, et al. Arf tumor suppressor promoter monitors latent oncogenic signals *in vivo*. *Proc Natl Acad Sci USA* 2003; 100(26):15930–15935.

9

Contributions of Telomerase to Tumorigenesis

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SUMMARY

In eukaryotic cells, the telomere acts as a molecular cap, protecting the chromosome ends and preventing end-to-end fusions. In addition to this end protection function, dysfunctional telomeres limit replicative potential by inducing a non-proliferative state termed replicative senescence. In premalignant cells, telomere loss results in genomic instability, which promotes the formation and selection of pro-tumorigenic mutations. Telomeres are maintained by the reverse transcriptase telomerase, an enzyme whose activity is tightly controlled by limiting the expression of the catalytic subunit, telomerase reverse transcriptase (TERT). The majority of human tumors exhibit abundant telomerase activity, which protects the chromosome ends to prevent additional genomic instability and confers unlimited replicative potential. This unique biochemical activity is vital to tumorigenesis and presents an ideal target for potential pharmacologic and immunologic therapies.

Key Words: Telomerase reverse transcriptase; telomere; tumorigenesis; transformation; cancer.

1. INTRODUCTION

Over the past decade, several lines of investigation have established the critical role of the biology of telomeres and telomerase in cancer development. Early work in the field defined the essential role of telomeres in chromosome end protection. However, later studies demonstrated a correlation between telomere length and replicative lifespan in cultured human fibroblasts. Specifically, telomere attrition occurs with successive cell divisions, suggesting that telomere structure specifies the number of cell divisions permitted in cultured diploid human fibroblasts (1,2). Telomeres are maintained by telomerase, a reverse transcriptase that synthesizes de novo telomeric repeats (3). Most human cancers express abundant telomerase activity, and genetic and cell biological

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studies have confirmed that telomerase expression facilitates cell immortalization, suggesting one function for telomerase activation in cell transformation. In addition to its role in cell immortalization, recent work suggests that telomerase may play additional roles during cancer development and tumor progression. This chapter will review our current understanding of the roles of telomerase and telomere biology in malignant transformation.

2. TELOMERE STRUCTURE

Eukaryotic chromosomes terminate in large arrays composed of tandem repeated guanine-rich sequences termed telomeres. Although the sequence and length of telomeres vary among eukaryotic organisms, in mammals the telomeric repeat is TTAGGG (4,5). The extreme terminus of the telomere is composed of a single stranded overhang of variable length that invades distal telomeric sequences forming a lariat structure, or t-loop, which is thought to protect the chromosome terminus while masking it from cellular DNA repair machinery (6). A number of proteins bind specifically to telomeres and regulate telomere function and structure. In mammals, telomere repeat-binding factors (TRF1 and TRF2) localize to the telomere through their ability to bind to double-stranded telomeric DNA (6). TRF1 has been shown to negatively regulate telomere length whereas TRF2 protects telomere integrity, preventing telomeric end-to-end chromosome fusions (7,8). Protection of telomeres 1 (POT1) binds to the 3' single-stranded telomeric overhang, protecting the chromosome end (9) and regulating access to the telomere (10).

These telomere-binding proteins are a foundation for the formation of large protein complexes at telomeres. Indeed, recent work indicates that TRF1 is linked to TRF2 through interactions with the TRF1 interacting nuclear factor 2 (TIN2) and POT1 and TIN2 organizing protein (PTOP1) (11). These telomere-specific complexes play critical roles in regulating telomere maintenance. In addition, a number of proteins implicated as members of DNA repair complexes are found at the telomere. The homologous recombination repair Mre11 complex, which consists of Mre11, Rad50, and Nbs1, is recruited to the telomere by TRF2 (12). Also present at the telomere is the non-homologous end-joining DNA-dependent protein kinase (DNA-PK) complex, consisting of Ku70 and Ku86 and the catalytic subunit DNA-PKcs (13,14) Through yet undefined mechanisms, these numerous proteins participate in the maintenance of telomeres.

Telomeres are maintained by telomerase, a tetramer composed of two subunits of the telomerase reverse transcriptase (TERT) catalytic subunit and two copies of the telomerase RNA component (TERC) as well as an accessory protein known as Dyskerin (3,15,16). Telomerase adds telomeric repeats to the telomere using a template provided by the RNA subunit *TERC* (17). *TERC* is a member of the H/ACA family of small nucleolar RNAs and binds TERT through interactions involving the pseudoknot/templating domain and two conserved loop structures (18,19). Co-expression of *in vitro* translated TERT and *in vitro* transcribed TERC reconstitutes telomerase activity, providing evidence that these two components form the minimal enzymatic core (20). Although accessory proteins such as TEP1 have been found associated with the telomerase holoenzyme, the functions of this and other proteins remain undefined (21,22). Both p23 and Hsp 90 are associated with telomerase and play a role in the assembly of the telomerase holoenzyme (22).

3. TELOMERES AND CELL MORTALITY

In normal human cells, hTERT expression closely correlates with telomerase activity, suggesting that hTERT is the rate-limiting telomerase subunit (23). High levels of telomerase activity have been identified in cells with greater replicative potential, such as cancer cell lines derived from tumors and immortal cell lines derived from the transformation of human cells with viral oncoproteins (24). By contrast, mortal cell lines express telomerase only transiently and at low levels insufficient to maintain telomere length (25). When such cells are cultured *in vitro*, they are only capable of undergoing a finite number of cellular divisions (26,27). Preliminary studies concluded that the number of cellular divisions a somatic cell is able to undergo correlates with telomere length (1,28), and there is a weak correlation between the age of an individual and the average length of the telomeres in their somatic cells (2). Whether such cells would similarly stop dividing *in vivo* remains undefined; nevertheless, these observations suggest that the telomere serves as a molecular clock that can limit cell lifespan by counting cell divisions.

Human cells that are cultured for extended time *in vitro* cease dividing and enter a metabolically active, but non-proliferative state termed mortality stage 1 (M1) or replicative senescence (27,29). The observation that telomere length shortens with each division (1,28) has led some groups to postulate that cells enter replicative senescence due to production of critically short telomeres (30). Whereas some evidence suggests that a single short telomere may induce the senescence program (31), other studies suggest that telomere state rather than a specific telomere length triggers senescence (32,33). Functional ablation of the retinoblastoma (Rb) and p53 tumor suppressor pathways, by expression of the SV40 large T antigen or human papillomavirus E6 and E7 oncoproteins for example, permits cells to bypass the senescence state induced by each of these stimuli, suggesting that these tumor suppressor pathways play important roles in triggering the senescence program (34).

Cells that bypass replicative senescence through the inactivation of these tumor suppressor pathways continue to proliferate until they reach another barrier, termed mortality stage 2 (M2) or crisis (34). Crisis is characterized by widespread apoptotic cell death, and cells in crisis exhibit short telomeres yet continue to replicate their DNA, suggesting that cells entering M2 have lost the ability to halt cell cycle progression (35). At a low frequency (approximately one in 10^7), cells survive crisis and achieve immortality, resulting in a line of immortal descendants (36). Surviving cells harbor stable telomere lengths and the majority of such cells express detectable levels of telomerase (37).

The notion that telomere length and replicative potential are intimately intertwined is supported by the evidence that exogenous expression of hTERT in post-senescent cells results in immortalization without crisis. In pre-senescent cells such as fibroblasts, endothelial cells, and mesothelial cells, expression of hTERT induces constitutive activation of telomerase and confers telomere length stability and immortality (38–40). However, in other cells including many epithelial cell types, expression of hTERT fails to immortalize without the simultaneous inactivation of the p53 and Rb pathways (41–44). Moreover, several other stimuli, including exposure to genotoxic agents, increased hypoxic stress, and oncogene activation, also induce a state that shares functional and morphologic similarity to replicative senescence (45–47). The senescent state caused by these stimuli cannot be reversed by the expression of hTERT (48), and such cells are senescent despite having normal telomere lengths. These stimuli

likely do not involve alteration of the telomere structure, suggesting that telomere dysfunction is not necessary to induce this state. Indeed, it is not yet clear whether short telomeres suffice to induce replicative senescence, or simply a trait of many of the early characterized senescent cells: whereas some groups have been able to determine the precise number of short telomeres needed to trigger senescence (49), others have demonstrated robust cell viability in cells with critically short telomeres (50). These observations have led to the postulate that there are different types of senescent states, triggered by excessive or accumulated stresses. Nevertheless, it remains unclear as to how telomerase expression and telomere length contribute to each of these types of senescence.

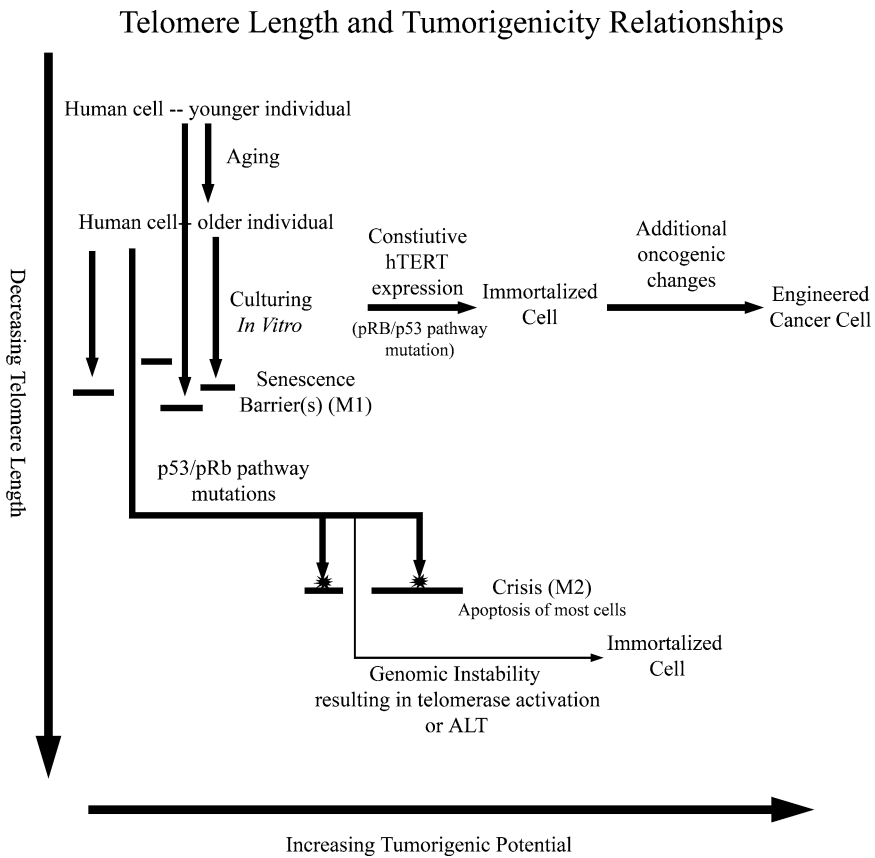


Fig. 1. Relationships between telomere length and tumorigenicity in human cells. Telomere lengths decrease with cell proliferation in cell types that suppress telomerase activity. Culturing of such cells *in vitro* leads to continued shortening followed by senescence (possibly linked to overall telomere length). Cells containing pRb and p53 pathway mutations continue dividing until cells enter crisis, triggered by critically short telomere lengths. Crisis is characterized by genomic instability resulting in apoptosis except in rare cases where telomerase or the alternative lengthening of telomeres (ALT) pathway is activated. The resulting immortal cells are not necessarily tumorigenic but can be transformed with additional oncogenes. Introduction of human telomerase reverse transcriptase (hTERT) (and loss of pRb or p53 pathway function) in pre-senescent cultured cells results in immortalized cells with longer telomeres than post-crisis cells. Additional oncogenic changes can render these cells tumorigenic.

Confounding these observations, there exists a minor yet important class of cells that is immortal but does not express telomerase and is believed to operate under a second pathway for telomere maintenance termed alternative lengthening of telomeres (ALT) (51,52). Such cells are capable of maintaining their telomere lengths without elevated levels of hTERT expression or telomerase activity. There is some evidence suggesting that this pathway may involve telomeric recombination, but overall this alternative mechanism of telomere maintenance remains poorly understood (53). Nevertheless, approximately 10–15% of human cancers display the ALT phenotype, suggesting that ALT may not contribute to transformation as efficiently as telomerase. A visual summary of the preceding observations relating to telomere length and tumorigenicity are included (Fig. 1).

Several lines of evidence have suggested that the structure of the telomere and not its actual length is the more important determinant of the immortalized versus senescent phenotype. A host of telomere-associated proteins binds the complex, high-order structure of the t-loop, among them TRF2 (54). De Lange and colleagues have demonstrated that loss of TRF2 from the telomeres leads to destabilization of the t-loop, chromosomal fusions, and cell death (8). TRF2 and the other proteins associated with the telomere are thought to protect the telomere by preventing its recognition as a double-strand break. However, at present, precisely how telomere-binding proteins and telomerase cap and protect chromosomes remains undefined (55).

4. TELOMERES, DNA DAMAGE, AND REPLICATIVE LIFESPAN

One prediction that follows from these observations is that uncapped or dysfunctional telomeres should be recognized as DNA double-strand breaks. The core histone, H2AX, is phosphorylated in response to chemical and physical agents that damage DNA at sites surrounding DNA breaks (56). These collections of phosphorylated H2AX mark DNA damage foci and serve to recruit other DNA repair factors to the site of damage (57). In senescent cells, these DNA damage foci accumulate over time, suggesting that the senescent state can also be characterized by an ongoing DNA-damage response (58). Conversely, in cells from patients afflicted with ataxia telangiectasia, Bloom's syndrome, or Werner's syndrome, whose DNA repair response is impaired, the onset of senescence is more rapid (59,60). Furthermore, the chronic treatment of normal cells with DNA-damaging agents such as ionizing radiation or hydrogen peroxide also leads to an accelerated onset of senescence in the absence of telomere shortening (61,62). Such observations have raised the question of whether shortened telomeres are triggering the senescent state or are simply correlative with cell division, which itself is correlated with DNA damage. Another possible interpretation of these data lies in observations by several groups that telomeres are either damaged by or particularly sensitive to DNA-damaging agents (63–66). Damage to the telomere without causing simultaneous telomere shortening may promote telomere uncapping, leading to the onset of senescence. Nevertheless, the several reports of senescence-associated DNA-damage foci do not agree on whether such foci form preferentially or only coincidentally at the telomeres (58,67).

Thus, the interplay between DNA damage, the telomere, and senescence remains intriguing yet poorly understood. Indeed, it is possible that this accumulated damage arises primarily at telomeres, thus making telomeres a prime stimulus for initiating

senescence. However, because a wide range of cellular stresses can trigger senescence, it is clear that other mechanisms including changes in chromatin state and oxidative damage may also contribute to this process.

5. THE TELOMERE AND CANCER

The current paradigm for the role of telomere biology in cancer is twofold. In normal cells, the telomere limits replicative potential while transient expression of telomerase maintains telomeres. In the malignant state, telomere loss results in genomic instability, scrambling the genomes of a population of premalignant cells and driving the selection of pro-tumorigenic mutations while expression of telomerase or maintenance of telomeres by the ALT pathway facilitates immortalization.

5.1. Potential Tumor-Suppressing Functions of the Telomere

Although the role that the telomere plays in senescence is not entirely understood, many of the observations mentioned previously are predictive of an *in vivo* cell-growth-limiting function of the telomere. In this model of tumor suppression, most cells are expected to divide a limited number of times in a single individual. The presence of the telomere, a molecular clock that counts these cell divisions, and telomerase, which potentially preserves genomic stability through maintenance of the telomeric cap, serves as a mechanism to limit replication, forcing cells that somehow step outside of their allotted number of divisions into apoptosis. Any cell division in excess of this amount required for most cell types may be the result of a necessary biological process (e.g., regeneration of the digestive tract and immunologic responses), and such cells often exhibit active telomere maintenance through expression of telomerase and potentially other factors. However, excessive cell division is clearly detrimental, and thus, activation of the p53 and Rb pathways in response to telomere shortening or some correlating phenomenon acts to ensure that any cells that have gained the ability to grow in the absence of proliferation signals or that have become insensitive to antiproliferative signals do not become immortal. Furthermore, the phenomenon of telomere uncapping, manifested as crisis *in vitro*, may play an additional role in eliminating cells that have somehow been able to become post-senescent by escaping p53 and Rb activities.

5.2. Tumor-Promoting Functions of the Telomere

In addition, the telomere contributes to cancer progression through an indirect mechanism involving genomic instability. As mentioned previously in Section 3, cells in culture that have undergone a critical number of cell divisions can reach a proliferative barrier termed crisis. This cellular state is characterized by rampant genomic instability resulting from critical telomere shortening. The few surviving cells that are able to stably maintain their telomere lengths through expression of hTERT or the ALT pathway exhibit aneuploidy, a hallmark of many epithelial malignancies (68). This genomic instability may facilitate the accumulation of the additional genetic changes necessary for transformation. An example of this phenomenon is seen in murine embryo fibroblasts (MEFs) derived from *mTerc* and *p53* null mice (69). Mice lacking *mTerc* are viable despite being unable to maintain telomere lengths with each successive

division; however, with each passing generation, telomere lengths become critically short such that late generation mice become increasingly less viable. These late generation *mTerc* null mice with extremely short telomeres are refractory to transformation by pairs of oncogenes compared with early generation mice with longer telomeres, suggesting that limiting the lifespan of murine cells by artificially beginning with short telomeres inhibits transformation. Furthermore, as the senescent state induced by telomere shortening is in part due to the activity of p53, *mTerc*-deficient mice become prone to tumor formation in a p53 heterozygous background. Karyotype analysis of cells derived from the tumors of these mice identified several reciprocal translocations, a rare phenomenon in murine tumors, but reminiscent of structures seen in human cancer (70). These observations implicate critical telomere shortening as a major mechanism that drives genomic instability in human tumorigenesis and demonstrate that whereas short telomeres may be able to limit replicative potential and therefore be antineoplastic, cells that are capable of escaping the chaos generated by loss of telomeric sequences are more prone to transformation.

5.3. Telomerase and Cancer

Although most human cancer cell lines and tumors exhibit constitutively high levels of telomerase activity, most normal human cells express little or low levels of telomerase. These observations suggest that telomerase activation in premalignant cells results in an immortalized state. In support of this hypothesis, overexpression of telomerase facilitates cell immortalization (38), whereas inhibition of telomerase in already immortal cancer cell lines results in loss of telomeric sequences, a loss of proliferative capacity, and the onset of crisis (71–73).

Consistent with the view that telomerase activation plays an important role in facilitating human cell transformation, co-expression of hTERT with cooperating oncogenes such as the rat sarcoma viral oncogene homolog (RAS) and the early region of Simian Virus 40 (SV40) generates cells with a transformed phenotype, capable of forming colonies in semi-solid medium or tumors when injected subcutaneously into immunodeficient mice (74). Such systems have been used to transform a variety of cell types and investigate members of contributing pathways by substitution experiments. As an example, introduction of an oncogenic allele of Harvey Ras (H-RAS), the SV40 large T and small t antigens, and hTERT into human fibroblasts is sufficient to transform these cells; however, this same combination of genes without hTERT fails to permit transformation. In this setting, hTERT contributes to cell immortalization but may also play other, yet undefined roles in cell transformation.

6. ALTERNATIVE FUNCTIONS OF TELOMERASE

Indeed, somewhat unexpectedly, emerging evidence suggests that telomerase has an additional role in tumorigenesis distinct from its role in maintaining telomere structure. For example, ALT phenotype cells harboring the SV40 ER and H-RAS fail to form tumors unless hTERT is co-expressed (75). Moreover, ALT phenotype cells derived from *mTerc* null mice exhibit an attenuated metastatic phenotype compared with cells in which telomerase was restored (76). Consistent with these observations, several groups have noted that hTERT expression and telomerase activity are present in a number of precancerous lesions. The presence of hTERT expression before sufficient

telomere shortening has occurred to make telomerase activity requisite for continued replication suggests that hTERT may contribute to transformation beyond its effects on telomere length. Indeed, mice engineered to overexpress mTERT show an increased ability to form skin or breast tumors, although murine telomeres are already extremely long (77,78). Indeed, analysis of murine tumors has found a small increase in telomerase activity (79,80). As an initial hint to these alternative functions, some reports suggest that hTERT-expressing cells are relatively resistant to apoptosis (48,81,82). Moreover, ectopic expression of mTERT in the mouse epithelium leads to an overgrowth of hair in these mice due to the increased proliferation and mobilization of hair follicle stem cells (83,84). Finally, hTERT appears to play a role in setting chromatin state (85). Taken together, these results suggest that a catalytically active telomerase enzyme may have a function beyond telomere maintenance, although the mechanisms behind these alternative functions have yet to be fully uncovered.

7. TELOMERE-BASED THERAPIES AND THERAPEUTIC CONSIDERATIONS

Given the strength of the connection between malignancy and telomere maintenance, several groups are engaged in the development of pharmacologic inhibitors of telomerase to abrogate telomerase activity in patients. Using several different methodologies, several groups have chosen to target hTERT. Genetic studies demonstrate that disrupting functional telomerase complexes with dominantly interfering mutants of hTERT leads to cell death by apoptosis dependent on telomere length in many but not all cell lines (71,86). In addition, Damm et al. have reported the characterization of BIBR3132, a small molecule inhibitor of hTERT (87) which appears to inhibit telomerase processivity (88). Cancer cell lines, when treated with these molecules, undergo rapid telomere shortening and eventually enter a non-proliferative state similar to senescence. Although effective, such drugs have a reversible mode of action, resulting in telomere lengthening and resumed proliferation once the drug is removed. This observation suggests that therapies involving telomerase inhibitors of this class would likely need to be administered for extended periods of time.

Several other groups have addressed the challenge of targeting telomerase activity by focusing on the telomerase RNA component. Suppression of telomerase RNA subunit by antisense constructs selectively suppresses tumor cell growth (89,90). Expression of hTERC mutants expected to incorporate mutant telomere sequences has been shown to lead to apoptosis of cancer cells, suggesting that the telomere itself may be a potential antitumor target (91,92). Furthermore, ablation of hTERC expression by RNAi has also proven effective alone and in combination with mutant RNA expression to inhibit the growth of transformed cells (93). Another class of telomerase drugs is telomeric mimic sequences, targeted to the hTERC template and expected to form duplexes with hTERC, requesting telomerase away from telomeres (94). Such drugs have shown efficacy in animal models but have limitations similar to those mentioned above.

The constitutive expression of hTERT in the majority of cancer cell lines has provided two additional classes of telomere-specific anticancer therapy involving the telomerase promoter and using telomerase as a target of immunotherapy. Many studies have shown that the fusion of proapoptotic genes such as *bax* or toxic genes such as thymidine kinase to the telomerase promoter can promote selective cell death of cancer

cells when these constructs are expressed (95,96). Moreover, telomerase-expressing cells have been successfully targeted by cytotoxic T lymphocytes that recognize hTERT peptides (97–100).

Despite the success of generating methodologies and drugs to exploit telomerase activity and expression in cancer cells, any successful therapy will need to be mindful of a multitude of factors specific to telomerase. These considerations range from cell types that normally exhibit telomerase activity as well as any alternative functions of telomerase and long-term effects of telomerase inhibition. Given that many tumors have activated telomerase, telomere lengths vary in different tumors. Thus, even given complete pharmacological ablation of telomerase activity, each cell in the tumor may have tens or hundreds of population doubling left before the onset of crisis. One may therefore expect the use of antitelomerase therapy in conjunction with other therapies that typically cause complete but temporary regression of a tumor, as such situations require the selection and excessive proliferation of a resistant clone. Another promising aspect of antitelomerase therapy not addressed in the above example is the phenomenon of oncogene addiction seen often in cancer cell lines in which the removal of a transforming factor leads to immediate cell death and regression of the tumor. Such a phenomenon has been observed in cancer cell lines in which telomerase has been inhibited, leading to cell death in the absence of critically short telomeres, perhaps due to the aforementioned capping (or alternative) activity of telomerase (101,102).

As has been seen with other antineoplastic agents, one can expect clones to arise which are resistant to the antitelomerase therapy employed. Prediction of the mechanism of such resistance may help to design proper therapies involving telomerase. Given the already existent ALT phenotype, one might expect that resistant clones may maintain telomere lengths by this mechanism. However, genetic or antisense inhibition of telomerase has not to date resulted in a resistant clone with the ALT phenotype.

Ultimately, the ability to effectively implement an integrated antineoplastic therapy involving targeting of the telomere or telomerase will depend intimately on the elucidation of the mechanisms by which these biologic factors contribute to replicative potential. Answering such questions may also depend on determining the biological relevance of senescence and whether such a phenotype has an *in vivo* equivalent. It remains to be seen whether telomere length, structure, or some combination of the two is a determining factor of replicative potential, or if telomere uncapping or shortening is simply one (albeit a sometimes critical) destabilizing event in a series of stresses placed upon the cell.

REFERENCES

1. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature* 1990; 345(6274):458–60.
2. Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RC. Telomere reduction in human colorectal carcinoma and with ageing. *Nature* 1990; 346(6287):866–8.
3. Greider CW, Blackburn EH. A telomeric sequence in the RNA of tetrahymena telomerase required for telomere repeat synthesis. *Nature* 1989; 337(6205):331–7.
4. Meyne J, Ratliff RL, Moyzis RK. Conservation of the human telomere sequence (TTAGGG)_n among vertebrates. *Proc Natl Acad Sci USA* 1989; 86(18):7049–53.
5. Moyzis RK, Buckingham JM, Cram LS, et al. A highly conserved repetitive DNA sequence, (TTAGGG)_n, present at the telomeres of human chromosomes. *Proc Natl Acad Sci USA* 1988; 85(18):6622–6.

6. Griffith JD, Comeau L, Rosenfield S, et al. Mammalian telomeres end in a large duplex loop. *Cell* 1999; 97(4):503–14.
7. van Steensel B, de Lange T. Control of telomere length by the human telomeric protein TRF1. *Nature* 1997; 385(6618):740–3.
8. van Steensel B, Smogorzewska A, de Lange T. TRF2 protects human telomeres from end-to-end fusions. *Cell* 1998; 92(3):401–13.
9. Baumann P, Cech TR. Pot1, the putative telomere end-binding protein in fission yeast and humans. *Science* 2001; 292(5519):1171–5.
10. Kelleher C, Kurth I, Lingner J. Human protection of telomeres 1 (POT1) is a negative regulator of telomerase activity *in vitro*. *Mol Cell Biol* 2005; 25(2):808–18.
11. Ye JZ, Donigian JR, van Overbeek M, et al. TIN2 binds TRF1 and TRF2 simultaneously and stabilizes the TRF2 complex on telomeres. *J Biol Chem* 2004; 279(45):47264–71.
12. Zhu XD, Kuster B, Mann M, Petrini JH, de Lange T. Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres. *Nat Genet* 2000; 25(3):347–52.
13. Hsu HL, Gilley D, Blackburn EH, Chen DJ. Ku is associated with the telomere in mammals. *Proc Natl Acad Sci USA* 1999; 96(22):12454–8.
14. Bianchi A, de Lange T. Ku binds telomeric DNA *in vitro*. *J Biol Chem* 1999; 274(30):21223–7.
15. Nakamura TM, Cech TR. Reversing time: origin of telomerase. *Cell* 1998; 92(5):587–90.
16. Meyerson M. Role of telomerase in normal and cancer cells. *J Clin Oncol* 2000; 18(13):2626–34.
17. Feng J, Funk WD, Wang SS, et al. The RNA component of human telomerase. *Science* 1995; 269(5228):1236–41.
18. Mitchell JR, Cheng J, Collins K. A box H/ACA small nucleolar RNA-like domain at the human telomerase RNA 3' end. *Mol Cell Biol* 1999; 19(1):567–76.
19. Mitchell JR, Collins K. Human telomerase activation requires two independent interactions between telomerase RNA and telomerase reverse transcriptase. *Mol Cell* 2000; 6(2):361–71.
20. Weinrich SL, Pruzan R, Ma L, et al. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. *Nat Genet* 1997; 17(4):498–502.
21. Harrington L, McPhail T, Mar V, et al. A mammalian telomerase-associated protein. *Science* 1997; 275(5302):973–7.
22. Holt SE, Aisner DL, Baur J, et al. Functional requirement of p23 and Hsp90 in telomerase complexes. *Genes Dev* 1999; 13(7):817–26.
23. Meyerson M, Counter CM, Eaton EN, et al. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell* 1997; 90(4):785–95.
24. Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994; 266(5193):2011–5.
25. Masutomi K, Yu EY, Khurts S, et al. Telomerase maintains telomere structure in normal human cells. *Cell* 2003; 114(2):241–53.
26. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res* 1961; 25:585–621.
27. Hayflick L. The limited *in vitro* lifetime of human diploid cell strains. *Exp Cell Res* 1965; 37: 614–36.
28. Allsopp RC, Vaziri H, Patterson C, et al. Telomere length predicts replicative capacity of human fibroblasts. *Proc Natl Acad Sci USA* 1992; 89(21):10114–8.
29. Wright WE, Shay JW. The two-stage mechanism controlling cellular senescence and immortalization. *Exp Gerontol* 1992; 27(4):383–9.
30. Harley CB, Kim NW, Prowse KR, et al. Telomerase, cell immortality, and cancer. *Cold Spring Harb Symp Quant Biol* 1994; 59:307–15.
31. Hemann MT, Strong MA, Hao LY, Greider CW. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell* 2001; 107(1):67–77.
32. Karlseder J, Smogorzewska A, de Lange T. Senescence induced by altered telomere state, not telomere loss. *Science* 2002; 295(5564):2446–9.
33. Stewart SA, Ben-Porath I, Carey VJ, O'Connor BF, Hahn WC, Weinberg RA. Erosion of the telomeric single-strand overhang at replicative senescence. *Nat Genet* 2003; 33(4):492–6.
34. Shay JW, Wright WE, Werbin H. Defining the molecular mechanisms of human cell immortalization. *Biochim Biophys Acta* 1991; 1072(1):1–7.
35. Wei W, Sedivy JM. Differentiation between senescence (M1) and crisis (M2) in human fibroblast cultures. *Exp Cell Res* 1999; 253(2):519–22.

36. Shay JW, Van Der Haegen BA, Ying Y, Wright WE. The frequency of immortalization of human fibroblasts and mammary epithelial cells transfected with SV40 large T-antigen. *Exp Cell Res* 1993; 209(1):45–52.
37. Counter CM, Avilion AA, LeFeuvre CE, et al. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J* 1992; 11(5): 1921–9.
38. Bodnar AG, Ouellette M, Frolkis M, et al. Extension of life-span by introduction of telomerase into normal human cells. *Science* 1998; 279(5349):349–52.
39. Vaziri H, Benchimol S. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr Biol* 1998; 8(5):279–82.
40. Yang J, Chang E, Cherry AM, et al. Human endothelial cell life extension by telomerase expression. *J Biol Chem* 1999; 274(37):26141–8.
41. Dickson MA, Hahn WC, Ino Y, et al. Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol Cell Biol* 2000; 20(4):1436–47.
42. Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelutz AJ. Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature* 1998; 396(6706):84–8.
43. Ramirez RD, Morales CP, Herbert BS, et al. Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions. *Genes Dev* 2001; 15(4):398–403.
44. DiRenzo J, Signoretti S, Nakamura N, et al. Growth factor requirements and basal phenotype of an immortalized mammary epithelial cell line. *Cancer Res* 2002; 62(1):89–98.
45. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 1997; 88(5):593–602.
46. McConnell BB, Starborg M, Brookes S, Peters G. Inhibitors of cyclin-dependent kinases induce features of replicative senescence in early passage human diploid fibroblasts. *Curr Biol* 1998; 8(6):351–4.
47. Lundberg AS, Hahn WC, Gupta P, Weinberg RA. Genes involved in senescence and immortalization. *Curr Opin Cell Biol* 2000; 12(6):705–9.
48. Gorbunova V, Seluanov A, Pereira-Smith OM. Expression of human telomerase (hTERT) does not prevent stress-induced senescence in normal human fibroblasts but protects the cells from stress-induced apoptosis and necrosis. *J Biol Chem* 2002; 277(41):38540–9.
49. Zou Y, Sfeir A, Gryaznov SM, Shay JW, Wright WE. Does a sentinel or a subset of short telomeres determine replicative senescence? *Mol Biol Cell* 2004; 15(8):3709–18.
50. Zhu J, Wang H, Bishop JM, Blackburn EH. Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening. *Proc Natl Acad Sci USA* 1999; 96(7):3723–8.
51. Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J* 1995; 14(17):4240–8.
52. Murnane JP, Sabatier L, Marder BA, Morgan WF. Telomere dynamics in an immortal human cell line. *EMBO J* 1994; 13(20):4953–62.
53. Dunham MA, Neumann AA, Fasching CL, Reddel RR. Telomere maintenance by recombination in human cells. *Nat Genet* 2000; 26(4):447–50.
54. Stansel RM, de Lange T, Griffith JD. T-loop assembly *in vitro* involves binding of TRF2 near the 3' telomeric overhang. *EMBO J* 2001; 20(19):5532–40.
55. Chan SW, Blackburn EH. New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin. *Oncogene* 2002; 21(4):553–63.
56. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* 1998; 273(10):5858–68.
57. Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol* 2000; 10(15):886–95.
58. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, et al. A DNA damage checkpoint response in telomere-initiated senescence. *Nature* 2003; 426(6963):194–8.
59. Schulz VP, Zakian VA, Ogburn CE, et al. Accelerated loss of telomeric repeats may not explain accelerated replicative decline of Werner syndrome cells. *Hum Genet* 1996; 97(6):750–4.
60. Shiloh Y, Tabor E, Becker Y. In vitro phenotype of ataxia-telangiectasia (AT) fibroblast strains: clues to the nature of the “AT DNA lesion” and the molecular defect in AT. *Kroc Founder* 1985; 19:111–21.

61. Chen QM, Prowse KR, Tu VC, Purdom S, Linskens MH. Uncoupling the senescent phenotype from telomere shortening in hydrogen peroxide-treated fibroblasts. *Exp Cell Res* 2001; 265(2):294–303.
62. Suzuki K, Mori I, Nakayama Y, Miyakoda M, Kodama S, Watanabe M. Radiation-induced senescence-like growth arrest requires TP53 function but not telomere shortening. *Radiat Res* 2001; 155(1 Pt 2):248–53.
63. Henle ES, Han Z, Tang N, Rai P, Luo Y, Linn S. Sequence-specific DNA cleavage by Fe²⁺-mediated fenton reactions has possible biological implications. *J Biol Chem* 1999; 274(2):962–71.
64. Oikawa S, Kawanishi S. Site-specific DNA damage at GGG sequence by oxidative stress may accelerate telomere shortening. *FEBS Lett* 1999; 453(3):365–8.
65. Oikawa S, Tada-Oikawa S, Kawanishi S. Site-specific DNA damage at the GGG sequence by UVA involves acceleration of telomere shortening. *Biochemistry* 2001; 40(15):4763–8.
66. Petersen S, Saretzki G, von Zglinicki T. Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. *Exp Cell Res* 1998; 239(1):152–60.
67. Sedelnikova OA, Horikawa I, Zimonjic DB, Popescu NC, Bonner WM, Barrett JC. Senescing human cells and ageing mice accumulate DNA lesions with unreparable double-strand breaks. *Nat Cell Biol* 2004; 6(2):168–70.
68. Karlseder J, Broccoli D, Dai Y, Hardy S, de Lange T. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science* 1999; 283(5406):1321–5.
69. Chin L, Artandi SE, Shen Q, et al. p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. *Cell* 1999; 97(4):527–38.
70. Artandi SE, Chang S, Lee SL, et al. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature* 2000; 406(6796):641–5.
71. Hahn WC, Stewart SA, Brooks MW, et al. Inhibition of telomerase limits the growth of human cancer cells. *Nat Med* 1999; 5(10):1164–70.
72. Herbert B, Pitts AE, Baker SI, et al. Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. *Proc Natl Acad Sci USA* 1999; 96(25):14276–81.
73. Zhang X, Mar V, Zhou W, Harrington L, Robinson MO. Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Genes Dev* 1999; 13(18):2388–99.
74. Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW, Weinberg RA. Creation of human tumour cells with defined genetic elements. *Nature* 1999; 400(6743):464–8.
75. Stewart SA, Hahn WC, O'Connor BF, et al. Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. *Proc Natl Acad Sci USA* 2002; 99(20):12606–11.
76. Chang S, Khoo CM, Naylor ML, Maser RS, DePinho RA. Telomere-based crisis: functional differences between telomerase activation and ALT in tumor progression. *Genes Dev* 2003; 17(1):88–100.
77. Gonzalez-Suarez E, Samper E, Ramirez A, et al. Increased epidermal tumors and increased skin wound healing in transgenic mice overexpressing the catalytic subunit of telomerase, mTERT, in basal keratinocytes. *EMBO J* 2001; 20(11):2619–30.
78. Artandi SE, Alson S, Tietze MK, et al. Constitutive telomerase expression promotes mammary carcinomas in aging mice. *Proc Natl Acad Sci USA* 2002; 99(12):8191–6.
79. Blasco MA, Rizen M, Greider CW, Hanahan D. Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. *Nat Genet* 1996; 12(2):200–4.
80. Broccoli D, Godley LA, Donehower LA, Varmus HE, de Lange T. Telomerase activation in mouse mammary tumors: lack of detectable telomere shortening and evidence for regulation of telomerase RNA with cell proliferation. *Mol Cell Biol* 1996; 16(7):3765–72.
81. Dudognon C, Pendino F, Hillion J, Saumet A, Lanotte M, Segal-Bendirdjian E. Death receptor signaling regulatory function for telomerase: hTERT abolishes TRAIL-induced apoptosis, independently of telomere maintenance. *Oncogene* 2004; 23(45):7469–74.
82. Holt SE, Glinsky VV, Ivanova AB, Glinsky GV. Resistance to apoptosis in human cells conferred by telomerase function and telomere stability. *Mol Carcinog* 1999; 25(4):241–8.
83. Flores I, Cayuela ML, Blasco MA. Effects of telomerase and telomere length on epidermal stem cell behavior. *Science* 2005; 309(5738):1253–6.
84. Sarin KY, Cheung P, Gilson D, et al. Conditional telomerase induction causes proliferation of hair follicle stem cells. *Nature* 2005; 436(7053):1048–52.
85. Masutomi K, Possemato R, Wong JM, et al. The telomerase reverse transcriptase regulates chromatin state and DNA damage responses. *Proc Natl Acad Sci USA* 2005; 102(23):8222–7.
86. Zhang R, Wang X, Guo L, Xie H. Growth inhibition of BEL-7404 human hepatoma cells by expression of mutant telomerase reverse transcriptase. *Int J Cancer* 2002; 97(2):173–9.

87. Damm K, Hemmann U, Garin-Chesa P, et al. A highly selective telomerase inhibitor limiting human cancer cell proliferation. *EMBO J* 2001; 20(24):6958–68.
88. Pascolo E, Wenz C, Lingner J, et al. Mechanism of human telomerase inhibition by BIBR1532, a synthetic, non-nucleosidic drug candidate. *J Biol Chem* 2002; 277(18):15566–72.
89. Nosrati M, Li S, Bagheri S, et al. Antitumor activity of systemically delivered ribozymes targeting murine telomerase RNA. *Clin Cancer Res* 2004; 10(15):4983–90.
90. Kondo S, Kondo Y, Li G, Silverman RH, Cowell JK. Targeted therapy of human malignant glioma in a mouse model by 2–5A antisense directed against telomerase RNA. *Oncogene* 1998; 16(25):3323–30.
91. Kim MM, Rivera MA, Botchkina IL, Shalaby R, Thor AD, Blackburn EH. A low threshold level of expression of mutant-template telomerase RNA inhibits human tumor cell proliferation. *Proc Natl Acad Sci USA* 2001; 98(14):7982–7.
92. Guiducci C, Cerone MA, Bacchetti S. Expression of mutant telomerase in immortal telomerase-negative human cells results in cell cycle deregulation, nuclear and chromosomal abnormalities and rapid loss of viability. *Oncogene* 2001; 20(6):714–25.
93. Li S, Rosenberg JE, Donjacour AA, et al. Rapid inhibition of cancer cell growth induced by lentiviral delivery and expression of mutant-template telomerase RNA and anti-telomerase short-interfering RNA. *Cancer Res* 2004; 64(14):4833–40.
94. Zahler AM, Williamson JR, Cech TR, Prescott DM. Inhibition of telomerase by G-quartet DNA structures. *Nature* 1991; 350(6320):718–20.
95. Gu J, Kagawa S, Takakura M, et al. Tumor-specific transgene expression from the human telomerase reverse transcriptase promoter enables targeting of the therapeutic effects of the Bax gene to cancers. *Cancer Res* 2000; 60(19):5359–64.
96. Majumdar AS, Hughes DE, Lichtsteiner SP, Wang Z, Lebkowski JS, Vasserot AP. The telomerase reverse transcriptase promoter drives efficacious tumor suicide gene therapy while preventing hepatotoxicity encountered with constitutive promoters. *Gene Ther* 2001; 8(7):568–78.
97. Nair SK, Heiser A, Boczkowski D, et al. Induction of cytotoxic T cell responses and tumor immunity against unrelated tumors using telomerase reverse transcriptase RNA transfected dendritic cells. *Nat Med* 2000; 6(9):1011–7.
98. Arai J, Yasukawa M, Ohminami H, Kakimoto M, Hasegawa A, Fujita S. Identification of human telomerase reverse transcriptase-derived peptides that induce HLA-A24-restricted antileukemia cytotoxic T lymphocytes. *Blood* 2001; 97(9):2903–7.
99. Vonderheide RH, Schultze JL, Anderson KS, et al. Equivalent induction of telomerase-specific cytotoxic T lymphocytes from tumor-bearing patients and healthy individuals. *Cancer Res* 2001; 61(23):8366–70.
100. Vonderheide RH, Hahn WC, Schultze JL, Nadler LM. The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. *Immunity* 1999; 10(6):673–9.
101. Kraemer K, Fuessel S, Schmidt U, et al. Antisense-mediated hTERT inhibition specifically reduces the growth of human bladder cancer cells. *Clin Cancer Res* 2003; 9(10 Pt 1):3794–800.
102. Saretzki G, Ludwig A, von Zglinicki T, Runnebaum IB. Ribozyme-mediated telomerase inhibition induces immediate cell loss but not telomere shortening in ovarian cancer cells. *Cancer Gene Ther* 2001; 8(10):827–34.

10

The Role of Telomeres in Genomic Instability

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SUMMARY

Genomic instability is now considered to play an important role in promoting the genetic changes necessary for cancer. It has been reported that exposure of cells to ionizing radiation or chemical agents can promote genomic instability, although the mechanisms involved are not understood. Knowledge concerning mechanisms of genomic instability has been derived from studies in yeast and human genetic disease, where mutations in a variety of genes involved in DNA replication, repair, and cell cycle regulation have been found to promote genomic instability. However, the identity of the mechanisms responsible for the genomic instability common to many cancer cells has yet to be determined, although increasing evidence demonstrates that loss of telomere function is important in this process. Telomeres are nucleoprotein structures that serve as caps that protect the ends of chromosomes and are maintained by the enzyme telomerase. Telomeres are maintained in the germ line but shorten with age in most somatic cells due to the lack of sufficient telomerase. This telomere shortening serves as a signal for replicative cell senescence, which protects against the unlimited cell division required for advanced forms of cancer. As a result, malignant cancer cells invariably maintain their telomeres, which is necessary to prevent massive chromosome fusion and cell death. However, telomere loss can occur during “crisis,” in which cells that fail to senesce undergo critical telomere shortening prior to re-establishing the ability to maintain telomeres. In addition, it is now clear that even tumor cells that maintain telomeres continue to show a high rate of telomere loss, suggesting a fundamental defect in telomere maintenance in many cancer cells. Telomere loss results in sister chromatid fusion that initiates prolonged breakage/fusion/bridge (B/F/B) cycles, in which chromosomes repeatedly fuse and break for many cell generations. This prolonged instability results in various chromosomal rearrangements that are commonly associated with cancer, including amplification, deletions, nonreciprocal translocations, and duplications of whole chromosome arms. This instability is not confined to the chromosome that originally lost its telomere but can be transferred to other chromosomes as well. Thus, the loss of a single telomere can result in a large population of cells with a variety of changes in many different chromosomes. Understanding the mechanisms of telomere

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loss and the factors that control chromosome instability resulting from B/F/B cycles is therefore an important goal in cancer research.

Key Words: Amplification; chromosome fusion; deletion; duplication; genomic instability; isochromosome; telomere; translocation.

1. GENOMIC INSTABILITY AND CANCER

Genomic instability has been proposed to play an important role in cancer by accelerating the accumulation of genetic changes responsible for cancer cell evolution (1,2). Genomic instability can occur through a variety of different mechanisms, including a defective DNA damage response, defects in DNA replication, and defects in chromosome segregation. Most cancer cells do not show an increased rate of mutation at the nucleotide level, suggesting that increased misincorporation during replication or a failure to correct mismatched or damaged nucleotides is not a common mechanism for instability in human cancer (3). Instead, most genomic instabilities associated with human cancer involve chromosome instability (CIN), which includes gross chromosome rearrangements and/or alterations in chromosome number. Studies in yeast have identified a large number of genes that can lead to CIN when inactivated, including genes involved in spindle formation, cell cycle checkpoints, recombination pathways, and telomere maintenance (4). In mammals, defects leading to CIN can arise through either inherited mutations associated with genetic diseases or mutations arising in somatic cells. A number of human genetic diseases demonstrate increased CIN and cancer predisposition. Many of the proteins responsible for these diseases, including ATM in ataxia telangiectasia, NBS1 in Nijmegen breakage syndrome, FANC genes in Fanconi anemia, and BRCA1 and BRCA2 in hereditary breast cancer, are critical for cell pathways signaling the presence of DNA damage and are necessary for proper cell cycle regulation and repair (5). However, unlike the well-defined genetics of these inherited syndromes, the identity of the genetic defects leading to CIN in sporadic human tumors is much less well defined. Alterations in the MAD2 protein involved in the mitotic checkpoint (6) and the *hCDC4* gene involved in the G1/S checkpoint (7) have been found to be associated with CIN in a small number of human cancers. Inactivation of the Rb protein can also promote CIN by uncoupling cell cycle progression from mitotic control (8). However, the identity of specific genes responsible for CIN in many tumors has yet to be determined, although increasing evidence now points to loss of telomere function as a major contributor to CIN in human cancer (see Section 5).

2. STRESS-INDUCED GENOMIC INSTABILITY

Genomic instability can also result from exposure to ionizing radiation or chemical agents (9–11). Unlike mutations or chromosomal rearrangements occurring at the time of exposure, these alterations continue to occur for many cell generations. Like the genomic instability associated with genetic disease or cancer, this stress-induced genomic instability can take different forms, ranging from small mutations to gross chromosomal rearrangements. The high percentage of cells in the population that demonstrate increased genomic instability following exposure to ionizing radiation strongly suggests that this stress-induced genomic instability is not due to mutations in one or a few genes. Instead, it has been proposed that stress-induced genomic

instability is due to epigenetic changes leading to alterations in gene expression that result in increased DNA damage or reduced repair. These epigenetic changes could result from long-term activation of signal transduction pathways or prolonged release of cytokines in response to cell damage. In fact, exposure to ionizing radiation has been demonstrated to result in changes in expression of a large number of genes (12), and irradiated cells have been demonstrated to generate factors that can promote instability in unirradiated cells (13,14). In addition, radiation-induced genomic instability has been found to correlate with increased levels of reactive oxygen species that can lead to a higher rate of spontaneous DNA damage (14,15). However, the exact mechanisms by which ionizing radiation can promote long-term genomic instability through *trans*-acting mechanisms have yet to be established.

An alternative mechanism by which radiation or chemicals can induce genomic instability in a high percentage of cells is through *cis*-acting mechanisms involving the formation of unstable chromosomes. One mechanism by which unstable chromosomes could be created is through the juxtaposition of incompatible chromatin structures as a result of the DNA damage-induced chromosome rearrangements. The possibility of unstable chromatin structures resulting from chromosome rearrangements was first proposed from data demonstrating variability in the stability of integration sites of transfected DNA (16). This model proposes that unstable chromosome regions would be more likely to undergo subsequent rearrangements, leading to a cascade of chromosome rearrangements involving multiple chromosomes. In support of this hypothesis, subsequent studies found that chromosome rearrangements involving centromeric heterochromatin showed a high rate of subsequent rearrangements (17,18). Thus, DNA damage occurring near centromeres might be more likely to initiate CIN. Similarly, DNA damage occurring near the ends of chromosomes, called telomeres, might also promote CIN. In fact, there is increasing evidence that the loss of telomeres can lead to CIN and that telomere loss is a mechanism for CIN in cancer (see Section 5).

3. TELOMERE STRUCTURE AND FUNCTION

Telomeres are DNA–protein complexes that contain short repeat sequences added on to the ends of chromosomes by the enzyme telomerase (19,20). Telomeres serve multiple functions, including protecting the ends of chromosomes (21) and preventing chromosome fusion (22). Telomeres are maintained in germ line cells but shorten with age in most somatic cells due to the lack of sufficient telomerase activity to compensate for the loss of DNA from the ends of chromosomes in dividing cells (22). Telomere shortening in somatic cells is a signal for replicative cell senescence (22), which results from the inability of the telomere to form a cap that protects the end of the chromosome (23). Primary human fibroblasts that have lost the ability to senesce continue to show telomere shortening and eventually enter “crisis,” which involves extensive chromosome fusion, aneuploidy, and cell death (24,25). Similarly, human epithelial cells that fail to senesce can enter “agonescence,” which like crisis involves increased chromosome fusion (26,27). Thus, it has been proposed that most somatic cells must first regain the ability to maintain telomeres to undergo the large number of cell divisions required for cancer (24,28). Consistent with this hypothesis, the introduction of telomerase into normal human fibroblasts can result in a greatly extended capacity to divide in culture (29). In addition to telomerase, immortal human cells have

also been found to maintain telomeres through a telomerase-independent mechanism, termed alternative lengthening of telomeres (ALT) (30,31), in which telomeres are maintained through recombination (30,32). Studies found that most tumors demonstrate telomerase activity (33,34), although tumors maintaining telomeres through the ALT mechanism have also been observed (35).

A large number of different proteins have now been identified that are involved in telomere structure and function (36). Two of the initial proteins that were identified included TRF1, which is involved in the regulation of telomere length (37,38), and TRF2, which is involved in the formation of capping structures that prevent chromosome fusion (39). Subsequent studies have identified a myriad of other telomere-associated proteins, many of which have other functions in DNA repair and cell cycle regulation (36). This overlap in proteins involved in the cellular response to DNA double-strand breaks and telomere maintenance points to the requirement for processing and sequestering the ends of chromosomes to prevent telomeres from being recognized as double-strand breaks and to prevent chromosome fusion. This relationship between DNA repair and telomere maintenance is further illustrated by increasing evidence that telomere function influences the cellular response to DNA damage (40–43). Furthermore, mice deficient in telomerase are sensitive to ionizing radiation (42,43) and alkylating agents (44). Because radiosensitivity is not seen in early generations of these mice, it is attributed to the cumulative effects of loss of telomere function due to gradual shortening rather than to the absence of telomerase. A slower rate of repair of double-strand breaks in cells from telomerase-deficient mice suggests that a deficit in repair pathway is involved. Consistent with this conclusion, another study reported that expression of telomerase in human fibroblasts can affect transcription of proteins involved in DNA repair (41). In addition, it has been reported that ionizing radiation increases telomerase activity in some cells, although the mechanism and significance of this observation are not clear (40).

4. MECHANISMS AND CONSEQUENCES OF TELOMERE LOSS

Telomere loss can initiate CIN through chromosome fusion. Although as discussed in section 3, the loss of telomere function can serve as a signal for cell senescence, chromosome fusion is common in cells that are deficient in the senescence pathway. The importance of telomeres in preventing chromosome fusion is demonstrated by the extensive chromosome fusion observed in cells in crisis or agonescence when telomeres become critically short (24–27). Similarly, the loss of telomere function brought about by expression of dominant-negative TRF2 also results in extensive chromosome fusion and cell death (39). The type of fusions resulting from telomere loss depends on which chromosome ends lack telomeres. Loss of telomeres from both ends of the same chromosome can result in the formation of ring chromosome, whereas the loss of telomeres from the ends of two different chromosomes can result in the formation of dicentric chromosomes. Both these types of chromosome aberrations are visible at metaphase and are common in cells in which telomere function has been severely compromised, such as in yeast defective in telomerase (45) or in mammalian cells deficient in TRF2 (39).

Unlike the extensive chromosome fusion observed in TRF2-deficient cells or cells in crisis that have severe telomere dysfunction, the loss of a single telomere is not

necessarily lethal in cells that are deficient in the senescence pathway. Although fusion between two different chromosomes will not occur, following DNA replication, the loss of a single telomere can result in fusion of sister chromatids (46–49). This type of chromosome rearrangement is more difficult to monitor than ring or dicentric chromosomes, because sister chromatid fusions are not detectable by routine cytogenetic analysis. As first described by McClintock (50), sister chromatid fusion can result in prolonged CIN involving breakage/fusion/bridge (B/F/B) cycles. B/F/B cycles occur when the fused sister chromatids form a bridge during anaphase and then break when the sister chromatids are pulled toward the daughter cells during cytokinesis (Fig. 1). This process is then repeated in the next cell cycle, as the chromosome will again be without a telomere in each of the daughter cells. Because of unequal breakage of the fused sister chromatids, repeated B/F/B cycles result in amplification and deletion events on the end of the chromosome. These B/F/B cycles can continue until the chromosome acquires a new telomere and again becomes stable.

There are multiple mechanisms by which a chromosome can lose a telomere. As mentioned above, telomere loss may occur from exposure to exogenous sources of DNA damage such as ionizing radiation or chemical mutagens. The likelihood of the loss of a telomere due to double-strand breaks occurring near the end of a chromosome may be increased by a deficiency in DNA repair in subtelomeric or telomeric regions. Studies

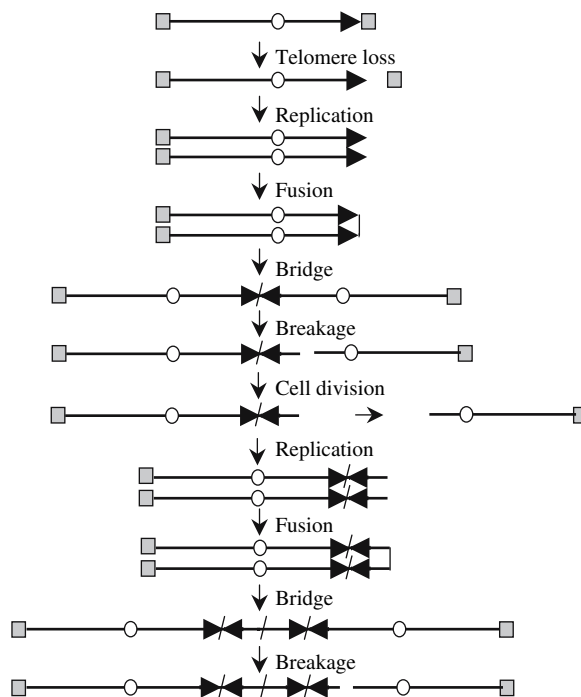


Fig. 1. Chromosome instability resulting from breakage/fusion/bridge (B/F/B) cycles initiated by telomere loss. B/F/B cycles are initiated when sister chromatids fuse following the loss of a telomere. The sister chromatids then form a bridge during anaphase when their centromeres are pulled in opposite directions. Breakage occurs at locations other than the site of fusion, resulting in amplification of sequences in one daughter cell and deletions in the other. The telomeres (squares), centromeres (circles), and orientation of the subtelomeric sequences (horizontal arrows) are shown.

in yeast have shown that regions around telomeres are deficient in nonhomologous end joining (51), a primary mechanism for double-strand break repair in mammalian cells. Studies in human cells have also found a reduced rate of repair of single-strand breaks or ultraviolet light-induced DNA damage within telomeric repeat sequences (52,53). A deficiency in DNA repair in regions near telomeres in mammalian cells would explain why the spectrum of rearrangements resulting from the introduction of double-strand breaks with the *I-SceI* endonuclease near telomeres is much different than that observed at interstitial sites. Double-strand breaks at interstitial sites most often result in small deletions (54,55), whereas double-strand breaks near telomeres most often result in sister chromatid fusions or direct telomere addition at double-strand breaks near telomeres (46–48,56). DNA damage occurring at or near telomeres may therefore be much more likely to result in telomere loss and complex chromosome rearrangements. In fact, a role for radiation-induced telomere loss in cancer has been suggested by studies in radiation-induced breast cancer in BALB/c mice. The increased radiation-induced breast cancer in these mice has been associated with a deficiency in the DNA-PKcs (DNA-dependent protein kinase active site) gene involved in double-strand break repair (57). However, cells deficient in DNA-PKcs also show increased end-to-end chromosome fusion, demonstrating that DNA-PKcs also has a role in telomere maintenance (58–60). This combined deficiency in double-strand break repair and telomere maintenance may together contribute to the predisposition of BALB/c mice to cancer, because the dysfunctional telomeres can recombine with misrepaired double-strand breaks in the cells from these mice following ionizing radiation (61).

Telomere loss can also occur through spontaneous events resulting from alterations in proteins involved in telomere maintenance. This type of telomere loss can involve gradual telomere shortening due a failure to compensate for telomere attrition, or sudden loss of telomere function due to rapid deletion of telomeric repeat sequences or failure of capping structures. Gradual loss of telomeres in cancer cells has been shown to result from a periodic reduction in expression of telomerase (62,63). Cancer cells can also show rapid fluctuations in telomere length due to an inability to properly regulate telomere length (64). These increases and decreases in telomere length are likely to be due to changes in expression of proteins involved in the regulation of telomere length. In fact, defects in a large number of proteins involved in telomere maintenance have been shown to promote chromosome fusion, including Ku and DNA-PK (58–60), the Mre11/Rad50/Nbs1 complex (65–67), and the Werner syndrome helicase (WRN) helicase (68). Breaks at telomeres could also result when replication forks become stalled at telomeres, as has been demonstrated in yeast (69,70). Consistent with this possibility, reduced function of proteins involved in the resolution of stalled replication forks, such as Nbs1 or WRN, can influence telomere loss (67,68). Deletions due to recombination within telomeric repeat sequences can also result in rapid loss of telomeres, as has been observed in cells expressing TRF2 protein with a deletion in the basic amino acid domain at its N terminus (71). Finally, a failure to maintain the capping structure that protects the ends of chromosomes could result from altered function of a number of different proteins known to be involved in cap formation, including telomerase itself (72,73) and TRF2 (39).

5. TELOMERE LOSS AND CANCER

Spontaneous telomere loss is likely to be an important source of CIN in cancer. As mentioned above, loss of telomere function can occur when cells escape senescence and therefore enter crisis or agonescence (24–27). The chromosome fusion due to loss of telomere function at crisis has been proposed to be an important mechanism for the chromosome rearrangements leading to cancer (74). Consistent with this conclusion, *in vivo* studies in humans demonstrated a dramatic increase in CIN during the transition from ductal hyperplasia to ductal carcinoma *in situ* when breast epithelial cells appear to progress through crisis and become immortal through expression of telomerase (75). However, this study also found that more advanced cancers continued to show a high rate of telomere instability even after expression of telomerase. This observation is consistent with studies demonstrating a high rate of telomere instability and anaphase bridges in early passage tumor cells in culture (76) and the high rate of loss of individual telomeres in a human tumor cell line (47,48). Therefore, despite the expression of telomerase, cancer cells commonly have problems maintaining telomeres, suggesting that transformation is associated with a fundamental defect in telomere function. A fundamental change in telomere function in cancer cells is also indicated by the fact that despite the expression of telomerase, most cancer cells have telomeres shorter than those found in normal cells (77,78).

Additional proof of the importance of telomere loss as a mechanism for CIN can be found in the increasing evidence demonstrating that B/F/B cycles are a common mechanism for chromosome rearrangements in human cancer. Despite B/F/B cycles being described in maize more than 50 years ago (50), until recently there has been little evidence for a role of B/F/B cycles in CIN in human cells. Several studies had demonstrated that high-copy gene amplification occurs through B/F/B cycles in hamster cells (79–82). However, highly amplified genes in human cells are found on double-minute chromosomes or as homogeneously staining regions cells (83). More recently, structures consistent with B/F/B cycles have been observed in low-copy gene amplification in some human tumor cell lines (84,85). Although not initially apparent, B/F/B cycles may also be an early step in high-copy gene amplification in human cells. Most clones of a human tumor cell line selected in culture for gene amplification contained double minute (DM) chromosomes; however, one clone showed amplification consistent with B/F/B cycles (86). When placed under more stringent selection conditions, the amplified region in this latter clone converted to DM chromosomes, leading to the conclusion that B/F/B cycles can be an initial step in the formation of DM chromosomes. Consistent with this conclusion, the regions amplified by B/F/B cycles in hamster cells have also been shown to form DM chromosomes (81,87,88). In fact, telomere instability and anaphase bridges have been observed in many early passage tumor cells (76). In addition, in histologic tumor sections, anaphase bridges show a correlation with abnormal karyotypes in sarcomas and pancreatic and colon carcinomas (89). This initiation of gene amplification by B/F/B cycles would explain why inverted repeats are commonly observed in amplified regions in human cancer cells (90).

Cell cycle regulation plays an important role in preventing CIN through B/F/B cycles. Cells involved in B/F/B cycles enter the next cell cycle with a chromosome lacking a telomere, which would signal a cell cycle arrest. Studies on replicative senescence resulting from telomere shortening (91–94) or loss of telomere function due to overexpression of dominant-negative TRF2 (94) demonstrate that the presence

of double-strand break repair foci associated with telomeres is a signal for replicative cell senescence (92). The importance of a similar mechanism in preventing CIN through B/F/B cycles is demonstrated by studies in telomerase-deficient mice. The progressive telomere shortening in telomerase-deficient mice results in only a modest increase in cancer (95) because in addition to promoting chromosome fusion, telomere shortening can also serve as a tumor suppressor mechanism (96). However, mice that are deficient in both telomerase and p53 show a dramatic increase in the frequency of carcinomas that are similar to those seen in humans (97,98). Cytogenetic analysis of these tumors demonstrated chromosome rearrangements consistent with B/F/B cycles, suggesting that telomere loss and B/F/B cycles play an important role in human cancer. Similarly, lymphomas in mice with knockouts in p53 and various proteins involved nonhomologous end joining were also shown to contain chromosome rearrangements consistent with B/F/B cycles (99–102).

6. MECHANISMS OF RESTORATION OF LOST TELOMERES

There are multiple mechanisms by which a chromosome can acquire a new telomere and either prevent or terminate B/F/B cycles. The most straightforward mechanism is through de novo addition of a telomere by telomerase, which has been observed in *Tetrahymena* and yeast (103,104). Direct telomere addition at the end of a broken chromosome has also been observed in mouse embryonic stem (ES) cells and human tumor cells, as well as at the ends of chromosomes terminal deletions associated with various human genetic diseases (105–107). Extracts from HeLa cells have demonstrated that human telomerase is capable of de novo addition of telomeres to DNA ends using only a few base pairs of homology (108). Despite this evidence, there is little data to suggest that de novo addition of telomeres is a common occurrence at double-strand breaks resulting from ionizing radiation. In fact, as mentioned earlier, mammalian cells have been found to demonstrate similar types of chromosome rearrangements in response to ionizing radiation regardless of whether they express telomerase (109,110). Why direct telomere addition to the ends of broken chromosomes should be a rare event in mammalian cells expressing telomerase is not known. One possibility is that the small amount of telomerase in mammalian cells is not capable of competing with the ubiquitous double-strand break repair proteins. Alternatively, de novo addition of telomeres may be actively suppressed. In yeast, de novo telomere addition is inhibited by the PIF1 helicase (Petite integration frequency), which has been proposed to prevent de novo telomere addition from competing with double-strand break repair and generating terminal deletions (111).

In addition to direct telomere addition, telomeres can also be acquired by either break-induced replication (BIR) or translocation of the ends of other chromosomes. BIR has been observed in yeast (45,112) and occurs when the broken end of a chromosome invades a region of homology near another telomere and initiates replication, thereby duplicating the end of that chromosome. Like de novo addition by telomerase, BIR results in the net gain of a telomere and thereby stabilizes the genome. Telomeres can also be acquired through telomere capture, involving the translocation of the ends of other chromosomes, as has been observed in human cancer cells (48,113) and in mouse ES cells (46). However, unlike de novo synthesis or BIR, telomere acquisition through translocation can have important consequences for the stability of the genome,

as translocations can lead to genetic imbalances or transfer of instability to other chromosomes (see below).

7. THE USE OF SELECTABLE MARKER GENES TO MONITOR THE CONSEQUENCES OF TELOMERE LOSS

To more fully understand the consequences of telomere loss for CIN, mouse ES and human tumor cell lines were developed that contain an Herpes simplex virus-thymidine kinase (*HSV-tk*) selectable marker gene located adjacent to a telomere (46–49,56). Using ganciclovir to select for loss of the *HSV-tk* gene, cell clones were isolated that had lost the telomere on this marker chromosome. Because of the presence of an I-*SceI* recognition sequence that is also present at this telomere, double-strand breaks can also be introduced at this location following transfection with expression vectors containing the I-*SceI* gene. I-*SceI* has been used extensively to introduce double-strand breaks in mammalian cells to study recombination and DNA repair processes (54,55,114–117). This approach has the advantage over previous studies in that selecting for telomere loss instead of later downstream events such as amplification provides the method to study the process from the beginning and therefore identify the early events following telomere loss. Several important observations have been made through the analysis of the consequences of telomere loss on individual chromosomes. The first important observation is the high rate of spontaneous loss of the telomeric *HSV-tk* gene in the human tumor cell line relative to the mouse ES cells. In the human tumor cell line, the mutation rate in telomeric *HSV-tk* genes was 10^{-4} events/cell/generation, which was 100-fold higher than the mutation rate at interstitial *HSV-tk* genes in this cell line (48). Most of the mutations involving the telomeric *HSV-tk* gene were due to telomere loss, whereas those at the interstitial *HSV-tk* gene were due to point mutations. By contrast, the rate of spontaneous mutation of the telomeric *HSV-tk* gene in the mouse ES cell line was less than 10^{-6} and was never observed to result from telomere loss (56). Thus, the human tumor cell line has a high rate of spontaneous telomere loss, which is consistent with the telomere instability, anaphase bridges, and CIN that have been observed in human tumor cells both in culture and in vivo (75,76).

A second important observation made from these studies is that the types of events resulting from telomere loss are much different than those observed at double-strand breaks at interstitial sites. Two major types of events were observed following spontaneous or I-*SceI*-induced telomere loss: direct addition of telomeres to the end of the broken chromosome and inverted repeats resulting from sister chromatid fusion (46–49,56). By contrast, mutations resulting from I-*SceI*-induced double-strand breaks at interstitial sites primarily involve small deletions (54,55). Although other types of events have also been observed, including large deletions and translocations, direct telomere addition or inverted repeats have not been reported. This observation suggests that double-strand breaks occurring near telomeres are processed differently than those occurring at other locations. In fact, studies in yeast have demonstrated that double-strand breaks generated with the I-*SceI* endonuclease are repaired by nonhomologous end joining much more efficiently at interstitial sites than near telomeres (51).

A third important observation derived from these studies is the prolonged period that B/F/B cycles can continue once initiated in human tumor cells (46–49). This is a critical observation, as the prolonged B/F/B cycles result in a large clonal expansion

of cells containing an unstable chromosome without a telomere. Unlike the mouse ES cells where B/F/B cycles lasted only a few generations (46), chromosomes that had lost a telomere in the human tumor cell line were still involved in B/F/B cycles in 20% of the cells in the population even after more than 20 generations (47). The presence of B/F/B cycles was evident from the absence of a telomere and the presence of anaphase bridges specific to the marker chromosome that had lost its telomere. Because of unequal breakage of the fused sister chromatids at anaphase, these prolonged B/F/B cycles resulted in extensive amplification of DNA on the end of the marker chromosome even without selection. The use of cosmid probes containing DNA from different locations on the chromosome demonstrated that the fused sister chromatids most often broke within 1 Mb of the site of fusion. Thus, the region near the site of fusion is prone to breakage, possibly due to the presence of the large inverted repeat that can serve as a hotspot for recombination (118–120). However, breaks also occurred at a lower frequency at other locations, as demonstrated by the presence of large duplications on the end of the chromosome in some cells. Studies in hamster cells have shown that the breakage at other sites tends to occur at fragile sites, and is favored when there is selection for amplification of specific genes (82,85). Because each amplification/duplication is accompanied by a corresponding deletion on the other sister chromatid, B/F/B cycles are highly efficient in generating large deletions, consistent with the presence of both amplified DNA and deletions in tumors from telomerase-deficient mice (121). Studies in yeast have found that much of the instability resulting from telomere loss is due to deletions resulting from degradation and not B/F/B cycles (122). However, the extent of degradation in mouse ES cells following telomere loss was relatively small, consistent with the small deletions observed at I-SceI-induced double-strand breaks at interstitial sites in mammalian cells (54,55). By contrast, the deletions resulting from B/F/B cycles can be much larger, some consisting of large portions of the chromosome.

A fourth important observation made in the course of these studies is that although chromosomes involved in B/F/B cycles can be stabilized through the acquisition of a telomere, this does not commonly occur by direct telomere addition by telomerase. An extensive analysis of a tumor cell subclone in which the marker chromosome was actively involved in B/F/B cycles demonstrated that most of the acquired telomeres originated by translocation or duplication of existing telomeres (49). Only approximately 16% of the telomeres that were acquired during B/F/B cycles did not contain detectable translocations or duplications. In fact, direct telomere addition may be completely absent, because it is possible that these chromosomes also have duplications or translocations that are too small to be detected. This low level of direct telomere addition is consistent with studies demonstrating that the expression of telomerase has little effect on the cellular response to ionizing radiation (109,110). Why direct telomere addition should be a rare event during B/F/B cycles even in human tumor cells that express telomerase suggests that direct telomere addition is actively suppressed, as proposed in yeast (111). In this regard, it is interesting to note that translocations were also a common event for telomere acquisition during B/F/B cycles in mouse ES cells (46), even though direct telomere addition was by far the most common event at double-strand breaks occurring near telomeres (56). One possible explanation for this difference is that direct telomere addition involving de novo synthesis of telomeres by telomerase preferentially occurs near existing telomeres.

A fifth important observation from these studies is that the mechanism by which telomeres are acquired during B/F/B cycles has important ramifications for the stability of the genome as a whole. Multiple mechanisms of telomere acquisition were observed, all of which resulted in the stabilization of the marker chromosome (Fig. 2). One mechanism for telomere acquisition that was observed in about 8% of the cells involved small duplications of the other end of the marker chromosome (49). The mechanism for these small duplications is not known but may occur through BIR, as BIR was found to be involved in a similar duplication of the other end of a chromosome that had lost a telomere in yeast (112). This mechanism of telomere acquisition stabilizes both the chromosome undergoing B/F/B cycles and the genome as a whole, because it involves the net gain of a telomere. Another mechanism for telomere acquisition involved translocations resulting from large duplications of the arms of other chromosomes,

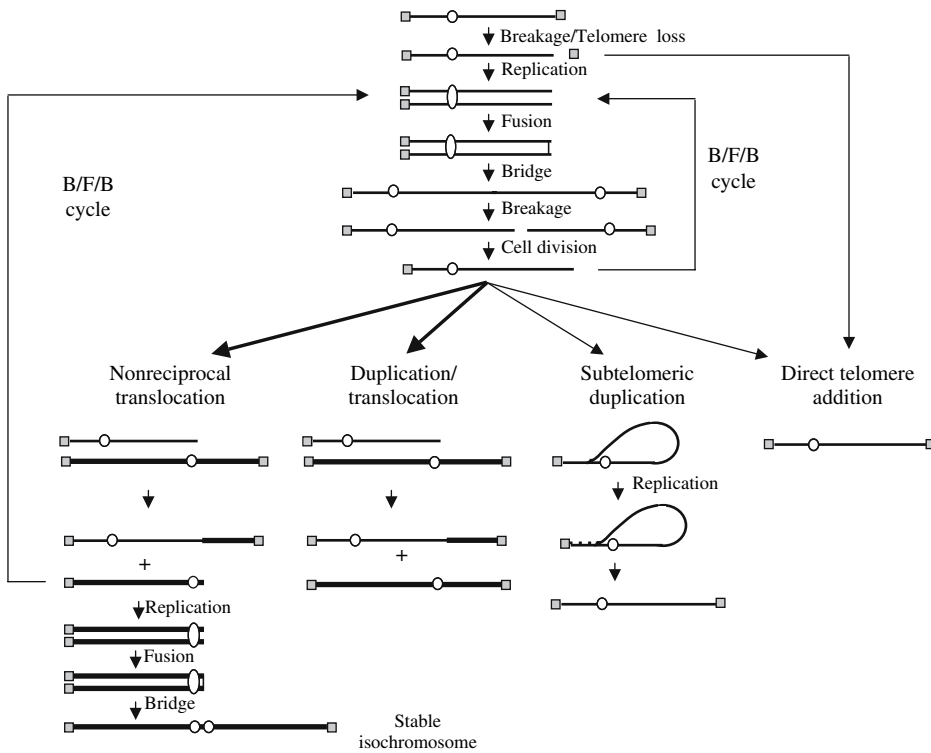


Fig. 2. Mechanisms of telomere acquisition during breakage/fusion/bridge (B/F/B) cycles and their consequences for stability of the genome. The loss of a telomere, either as a result of a double-strand break or failure to properly maintain telomeres, results in either direct addition of telomeres or sister chromatid fusion and B/F/B cycles. B/F/B cycles continue until the chromosome acquires a new telomere by (i) nonreciprocal translocation (NRT), (ii) translocation involving duplications of large portions of other chromosomes, (iii) small duplications of a telomeric region on the other end of the same chromosome, or (iv) direct addition of a telomere, possibly involving de novo synthesis by telomerase. The chromosome donating the NRT can enter the B/F/B cycle due to the loss of its telomere, or when breakage occurs near the centromere, the fused sister chromatids can form a stable dicentric isochromosome. Telomere acquisition by NRT can therefore transfer instability from one chromosome to another, while duplications and direct addition of telomeres result in stabilization of the genome, although they can contribute to the clonal karyotypic evolution of the cancer cell.

which accounted for one-fourth of the acquired telomeres (49). However, unlike the small duplications of the other end of the marker chromosome, these large duplications of chromosome arms had a significant impact on the genome as a whole, because they resulted in large gene imbalances. Finally, telomeres were also acquired through nonreciprocal translocation (NRT), which involved the transfer of large portions of the arms of other chromosomes. Like large duplications, NRTs also accounted for one-fourth of the telomeres acquired during B/F/B cycles (49). However, these NRTs were unique in that they resulted in the loss of the telomere from the donor chromosome. As a result, the chromosomes donating these NRTs became unstable and underwent subsequent rearrangements, including NRTs, duplications, and isochromosome formation. Thus, in addition to gene amplification and deletions, telomere loss is an efficient mechanism for generating chromosome duplications and NRTs, both of which are commonly associated with human cancer (113,123). The mechanism by which these translocations occur is not known. However, the presence of duplications at the sites of translocations on chromosomes that have undergone B/F/B cycles suggests that it may be an active process that is initiated when the chromosome end without a telomere invades another chromosome and initiates DNA replication, similar to BIR (124). Combined with the clonal expansion of the cell containing a chromosome without a telomere due to the prolonged B/F/B cycles, these results demonstrate that the loss of a single telomere can rapidly result in a population of cells with extensive karyotypic heterogeneity involving multiple chromosomes. Telomere loss is therefore likely to be a major mechanism for CIN in cancer cells, not only during crisis but also during later stages of tumor cell progression. Understanding the mechanisms of telomere loss and the factors that control CIN associated with B/F/B cycles is therefore an important goal in cancer research.

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REFERENCES

1. Tlsty TD. Genomic instability and its role in neoplasia. *Curr Topics Microbiol Immunol* 1997; 221:37–46.
2. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature* 1998; 396:643–9.
3. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med* 2004; 10(8):789–99.
4. Kolodner RD, Putnam CD, Myung K. Maintenance of genome stability in *saccharomyces cerevisiae*. *Science* 2002; 297:552–7.
5. Surralles J, Jackson SP, Jasin M, Kastan MB, West SC, Joenje H. Molecular cross-talk among chromosome fragility syndromes. *Genes Dev* 2004; 18:1359–70.
6. Michel LS, Liberal V, Chatterjee A, et al. MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells. *Nature* 2001;409(6818):355–9.
7. Rajagopalan H, Lengauer C. Aneuploidy and cancer. *Nature* 2004; 432(7015):338–41.
8. Hernando E, Nahle Z, Juan G, et al. Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. *Nature* 2004; 430(7001):797–802.
9. Little JB. Genomic instability and radiation. *J Radiol Prot* 2003; 23:173–81.

10. Murnane JP. Role of induced genetic instability in the mutagenic effects of chemicals and radiation. *Mutat Res* 1995; 367:11–23.
11. Morgan WF, Day JP, Kaplan MI, McGhee EM, Limoli CL. Genomic instability induced by ionizing radiation. *Radiat Res* 1996; 146:247–58.
12. Amundson SA, Fornace AJ Jr. Gene expression profiles for monitoring radiation exposure. *Radiat Prot Dosimetry* 2001; 97(1):11–6.
13. Hall EJ, Hei TK. Genomic instability and bystander effects induced by high-LET radiation. *Oncogene* 2003; 22(45):7034–42.
14. Azzam EI, de Toledo SM, Little JB. Oxidative metabolism, gap junctions and the ionizing radiation-induced bystander effect. *Oncogene* 2003; 22(45):7050–7.
15. Limoli CL, Giedzinski E, Morgan WF, Swarts SG, Jones GD, Hyun W. Persistent oxidative stress in chromosomally unstable cells. *Cancer Res* 2003; 63(12):3107–11.
16. Murnane JP. The role of recombinational hotspots in genome instability in mammalian cells. *Bioessays* 1990; 12:577–81.
17. Smith LE, Parks KK, Hasegawa LS, Eastmond DA, Grosovsky AJ. Targeted breakage of paracentromeric heterochromatin induces chromosomal instability. *Mutagenesis* 1998; 13(5):435–43.
18. Pongsaensook P, Ritter LE, Parks KK, Grosovsky AJ. Cis-acting transmission of genomic instability. *Mutat Res* 2004; 568(1):49–68.
19. McEachern MJ, Krauskopf A, Blackburn EH. Telomeres and their control. *Ann Rev Genet* 2000; 34:331–58.
20. Blackburn EH. Switching and signaling at the telomere. *Cell* 2001; 106:661–73.
21. Blackburn EH, Greider CW. *Telomeres*. Plainview, NY: Cold Spring Harbor Laboratory Press, 1995.
22. de Lange T. Telomere dynamics and genome instability in human cancer. In: Blackburn EH, Greider CW, eds. *Telomeres*. Plainview, NY: Cold Spring Harbor Press; 1995:265–93.
23. Karlseder J, Smogorzewska A, de Lange T. Senescence induced by altered telomere state, not telomere loss. *Science* 2002; 295:2446–9.
24. Counter CM, Avilion AA, LeFeuvre CE, et al. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J* 1992; 11:1921–9.
25. Ducray C, Pommier J-P, Martins L, Boussin F, Sabatier L. Telomere dynamics, end-to-end fusions and telomerase activation during the human fibroblast immortalization process. *Oncogene* 1999; 18:4211–23.
26. Romanov SR, Kozakiewicz BK, Hoist CR, Stampfer MR, Haupt LM, Tlsty TD. Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. *Nature* 2001; 409:633–7.
27. Yaswen P, Stampfer MR. Molecular changes accompanying senescence and immortalization of cultured human mammary epithelial cells. *Int J Biochem Cell Biol* 2002; 34:1382–94.
28. Harley CB. Telomeres and aging. In: Blackburn EH, Greider CW, eds. *Telomeres*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1995:247–63.
29. Bodnar AG, Ouellette M, Frolkis M, et al. Extension of life-span by introduction of telomerase into normal human cells. *Science* 1998; 279:349–52.
30. Murnane JP, Sabatier L, Marder BA, Morgan WF. Telomere dynamics in an immortal human cell line. *EMBO J* 1994; 13:4953–62.
31. Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J* 1995; 14:4240–8.
32. Dunham MA, Neumann AA, Fasching CL, Reddel RR. Telomere maintenance by recombination in human cells. *Nat Genet* 2000; 26:447–50.
33. Shay JW, Wright WE. Telomerase activity in human cancer. *Curr Opin Oncol* 1996; 8:66–71.
34. Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994; 266:2011–5.
35. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat Med* 1997; 3:1271–4.
36. Smogorzewska A, de Lange T. Regulation of telomerase by telomeric proteins. *Annu Rev Biochem* 2004; 73:177–208.
37. Karlseder J, Kachatrian L, Takai H, et al. Targeted deletion reveals an essential function for the telomere length regulator Trf1. *Mol Cell Biol* 2003; 23(18):6533–41.

38. van Steensel B, de Lange T. Control of telomere length by the human telomeric protein TRF1. *Nature* 1997; 385:740–3.
39. van Steensel B, Smogorzewska A, de Lange T. TRF2 protects human telomeres from end-to-end fusions. *Cell* 1998; 92:401–13.
40. Bouffler SD, Blasco MA, Cox R, Smith PJ. Telomeric sequences, radiation sensitivity and genomic instability. *Int J Radiat Biol* 2001; 77(10):995–1005.
41. Sharma GG, Gupta A, Wang H, et al. hTERT associates with human telomeres and enhances genomic stability and DNA repair. *Oncogene* 2003; 22(1):131–46.
42. Wong KK, Chang S, Weiler SR, et al. Telomere dysfunction impairs DNA repair and enhances sensitivity to ionizing radiation. *Nat Genet* 2000; 26(1):85–8.
43. Goytisolo FA, Samper E, Martin-Caballero J, et al. Short telomeres result in organismal hypersensitivity to ionizing radiation in mammals. *J Exp Med* 2000; 192(11):1625–36.
44. Gonzalez-Suarez E, Goytisolo FA, Flores JM, Blasco MA. Telomere dysfunction results in enhanced organismal sensitivity to the alkylating agent N-methyl-N-nitrosourea. *Cancer Res* 2003; 63(21):7047–50.
45. Hackett JA, Feldser DM, Greider CW. Telomere dysfunction increases mutation rates and genomic instability. *Cell* 2001; 106:275–86.
46. Lo AWI, Sprung CN, Fouladi B, et al. Chromosome instability as a result of double-strand breaks near telomeres in mouse embryonic stem cells. *Mol Cell Biol* 2002; 22:4836–50.
47. Lo AWI, Sabatier L, Fouladi B, Pottier G, Ricoul M, Murnane JP. DNA amplification by breakage/fusion/bridge cycles initiated by spontaneous telomere loss in a human cancer cell line. *Neoplasia* 2002; 6:531–8.
48. Fouladi B, Miller D, Sabatier L, Murnane JP. The relationship between spontaneous telomere loss and chromosome instability in a human tumor cell line. *Neoplasia* 2000; 2:540–54.
49. Sabatier L, Ricoul M, Pottier G, Murnane JP. The loss of a single telomere can result in genomic instability involving multiple chromosomes in a human tumor cell line. *Mol Cancer Res* 2005; 3:139–50.
50. McClintock B. The stability of broken ends of chromosomes in *Zea mays*. *Genetics* 1941; 41: 234–82.
51. Ricchetti M, Dujon B, Fairhead C. Distance from the chromosome end determines the efficiency of double-strand break repair in subtelomeres of haploid yeast. *J Mol Biol* 2003; 328:847–62.
52. Petersen S, Saretzki G, von Zglinicki T. Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. *Exp Cell Res* 1998; 239:152–60.
53. Kruk PA, Rampino NJ, Bohr VA. DNA damage and repair in telomeres: relation to aging. *Proc Natl Acad Sci USA* 1995; 92:258–62.
54. Lin Y, Waldman AS. Capture of DNA sequences at double-strand breaks in mammalian cells. *Genetics* 2001; 158:1665–74.
55. Sargent RG, Brenneman MA, Wilson JH. Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. *Mol Cell Biol* 1997; 17(1):267–77.
56. Sprung CN, Reynolds GE, Jasin M, Murnane JP. Chromosome healing in mouse embryonic stem cells. *Proc Natl Acad Sci USA* 1999; 96:6781–6.
57. Yu Y, Okayasu R, Weil MM, et al. Elevated breast cancer risk in irradiated BALB/c mice associates with unique functional polymorphism of the Prkdc (DNA-dependent protein kinase catalytic subunit) gene. *Cancer Res* 2001; 61(5):1820–4.
58. Bailey SM, Meyne J, Chen DJ, et al. DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes. *Proc Natl Acad Sci USA* 1999; 96:14899–904.
59. Gilley D, Tanaka H, Hande MP, et al. DNA-PKcs is critical for telomere capping. *Proc Natl Acad Sci USA* 2001; 98:15084–8.
60. Espejel S, Franco S, Rodriguez-Perales S, Bouffler SD, Cigudosa JC, Blasco MA. Mammalian Ku86 mediates chromosomal fusions and apoptosis caused by critically short telomeres. *EMBO J* 2002; 21:2207–19.
61. Bailey SM, Cornforth MN, Ullrich RL, Goodwin EH. Dysfunctional mammalian telomeres join with DNA double-strand breaks. *DNA Repair (Amst)* 2004; 3(4):349–57.
62. Schwartz JL, Jordan R, Liber H, Murnane JP, Evans HH. TP53-dependent chromosome instability is associated with transient reductions in telomere length in immortal telomerase-positive cell lines. *Genes Chromosomes Cancer* 2001; 30:236–44.

63. Bryan TM, Englezou A, Dunham MA, Reddel RR. Telomere length dynamics in telomerase-positive immortal human cell populations. *Exp Cell Res* 1998; 239:370–8.
64. Sprung CN, Afshar G, Chavez EA, Lansdorp P, Sabatier L, Murnane JP. Telomere instability in a human cancer cell line. *Mutat Res* 1999; 429:209–23.
65. Zhu X-D, Kuster B, Mann M, Petrini JHJ, de Lange T. Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres. *Nat Genet* 2000; 25:347–52.
66. Ranganathan V, Heine WF, Ciccone DN, et al. Rescue of a telomere length defect in Nijmegen breakage syndrome cells requires NBS and the telomerase catalytic subunit. *Curr Biol* 2001; 11:962–6.
67. Bai Y, Murnane JP. Telomere instability in a human tumor cell line expressing the NBS1 gene with mutations at sites phosphorylated by the ATM protein. *Mol Cancer Res* 2003; 1:1058–69.
68. Bai Y, Murnane JP. Telomere instability in a human tumor cell line expressing a dominant-negative WRN protein. *Hum Genet* 2003; 113:337–47.
69. Ivessa AS, Zhou J-Q, Schulz VP, Monson EK, Zakian VA. *Saccharomyces Rrm3p*, a 5' to 3' DNA helicase that promotes replication fork progression through telomeric and subtelomeric DNA. *Genes Dev* 2002; 16:1383–96.
70. Ivessa AS, Lenzmeier BA, Bessler JB, Goudsouzian LK, Schnakenberg SL, Zakian VA. The *Saccharomyces cerevisiae* helicase Rrm3p facilitates replication past nonhistone protein-DNA complexes. *Mol Cell* 2003; 12:1525–36.
71. Wang RC, Smogorzewska A, de Lange T. Homologous recombination generates T-loop-sized deletions at human telomeres. *Cell* 2004; 119(3):355–68.
72. Zhu J, Wang H, Bishop JM, Blackburn EH. Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening. *Proc Natl Acad Sci USA* 1999; 96:3723–8.
73. Masutomi K, Yu EY, Khurts S, et al. Telomerase maintains telomere structure in normal human cells. *Cell* 2003; 114:241–53.
74. DePinho RA, Polyak K. Cancer chromosomes in crisis. *Nat Genet* 2004; 36(9):932–4.
75. Chin K, de Solorzano CO, Knowles D, et al. In situ analysis of genome instability in breast cancer. *Nat Genet* 2004; 16:984–8.
76. Gisselsson D, Jonson T, Petersen A, et al. Telomere dysfunction triggers extensive DNA fragmentation and evolution of complex chromosome abnormalities in human malignant tumors. *Proc Natl Acad Sci USA* 2001; 98:12683–8.
77. Meeker AK, Hicks JL, Iaculuzio-Donahue CA, et al. Telomere length abnormalities occur early in the initiation of epithelial carcinogenesis. *Clin Cancer Res* 2004; 10:3317–26.
78. Meeker AK, Hicks JL, Gabrielson E, Strauss WM, De Marzo AM, Argani P. Telomere shortening occurs in subsets of normal breast epithelium as well as in situ and invasive carcinoma. *Am J Pathol* 2004; 164:925–35.
79. Ma C, Martin S, Trask B, Hamlin JL. Sister chromatid fusion initiates amplification of the dihydrofolate reductase gene in Chinese hamster cells. *Genes Dev* 1993; 7:605–20.
80. Smith KA, Gorman PA, Stark MB, Groves RP, Stark GR. Distinctive chromosomal structures are formed very early in the amplification of CAD genes in Syrian hamster cells. *Cell* 1990; 63:1219–27.
81. Toledo F, Buttin G, Debatisse M. The origin of chromosome rearrangements at early stages of AMPD2 gene amplification in Chinese hamster cells. *Curr Biol* 1993; 3:255–64.
82. Coquelle A, Pipiras E, Toledo F, Buttin G, Debatisse M. Expression of fragile sites triggers intrachromosomal mammalian gene amplification and sets boundaries to early amplification. *Cell* 1997; 89:215–25.
83. Hahn PJ. Molecular biology of double-minute chromosomes. *Bioessays* 1993; 15:477–84.
84. Shuster MI, Han L, Le Beau MM, et al. A consistent pattern of RIN1 rearrangements in oral squamous cell carcinoma cell lines supports a breakage-fusion-bridge cycle model for 11q23 amplification. *Genes Chromosomes Cancer* 2000; 28:153–63.
85. Hellman A, Ziotorynski E, Scherer SW, et al. A role for common fragile site induction in amplification of human oncogenes. *Cancer Cell* 2002; 1:89–97.
86. Singer MJ, Mesner LD, Friedman CL, Trask BJ, Hamlin JL. Amplification of the human dihydrofolate reductase gene via double minutes is initiated by chromosome breaks. *Proc Natl Acad Sci USA* 2000; 97:7921–6.
87. Coquelle A, Rozier L, Dutrillaux B, Debatisse M. Induction of multiple double-strand breaks within an hsr by meganuclease I-SceI expression of fragile site activation leads to formation of double

- minutes and other chromosomal rearrangements. *Oncogene* 2002; 21:7671–9.
88. Coquelle A, Toledo F, Stern S, Bieth A, Debatisse M. A new role for hypoxia in tumor progression: induction of fragile site triggering genomic rearrangements and formation of complex DMs and HSRs. *Mol Cell* 1998; 2:249–65.
 89. Montgomery E, Wilentz RE, Argani P. Analysis of anaphase figures in routine histologic sections distinguishes chromosomally unstable from chromosomally stable malignancies. *Cancer Biol Ther* 2003; 2:248–52.
 90. Ford M, Fried M. Large inverted duplications are associated with gene amplification. *Cell* 1986; 45:425–30.
 91. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, et al. A DNA damage checkpoint response in telomere-initiated senescence. *Nature* 2003; 426(6963):194–8.
 92. Herbig U, Jobling WA, Chen BP, Chen DJ, Sedivy JM. Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). *Mol Cell* 2004; 14(4):501–13.
 93. Sedelnikova OA, Horikawa I, Zimonjic DB, Popescu NC, Bonner WM, Barrett JC. Senescing human cells and ageing mice accumulate DNA lesions with unreparable double-strand breaks. *Nat Cell Biol* 2004; 6(2):168–70.
 94. Takai H, Smogorzewska A, de Lange T. DNA damage foci at dysfunctional telomeres. *Curr Biol* 2003; 13(17):1549–56.
 95. Rudolph KL, Chang S, Lee H-W, et al. Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell* 1999;96:701–12.
 96. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature* 1990; 345:458–60.
 97. Rudolph KL, Millard M, Bosenberg MW, DePinho RA. Telomere dysfunction and evolution of intestinal carcinoma in mice and humans. *Nat Genet* 2001; 28:155–9.
 98. Artandi SE, Chang S, Lee S-L, et al. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature* 2000; 406:641–5.
 99. Ferguson DO, Sekiguchi JM, Chang S, et al. The nonhomologous end-joining pathway of DNA repair is required for genomic stability and the suppression of translocations. *Proc Natl Acad Sci USA* 2000; 97:6630–3.
 100. Ferguson DO, Alt FW. DNA double strand break repair and chromosomal translocation: lessons from animal models. *Oncogene* 2001; 20:5572–9.
 101. Gao Y, Ferguson DO, Xie W, et al. Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic instability and development. *Nature* 2000; 404:897–900.
 102. Difilippantonio MJ, Zhu J, Chen HT, et al. DNA repair protein Ku80 suppresses chromosomal aberrations and malignant transformation. *Nature* 2000; 404:510–4.
 103. Yu G, Blackburn EH. Developmentally programmed healing of chromosomes by telomerase in *Tetrahymena*. *Cell* 1991; 67:823–32.
 104. Diede SJ, Gottschling DE. Telomerase-mediated telomere addition in vivo requires DNA primase and DNA polymerase α and δ . *Cell* 1999; 99:723–33.
 105. Varley H, Di S SW, Royle NJ. Characterization of terminal deletions at 7q32 and 22q13. 3 healed by de novo telomere addition. *Am J Hum Genet* 2000; 67:610–22.
 106. Wong AC, Ning Y, Flint J, et al. Molecular characterization of a 130-kb terminal microdeletion at 22q in a child with mild mental retardation. *Am J Hum Genet* 1997; 60(1):113–20.
 107. Flint J, Thomas K, Micklem G, et al. The relationship between chromosome structure and function at a human telomeric region. *Nat Genet* 1997; 15(3):252–7.
 108. Morin GB. Recognition of a chromosome truncation site associated with α -thalassaemia by human telomerase. *Nature* 1991; 353:454–6.
 109. Kampinga HH, Van Waarde-Verhagen MA, Van Assen-Bolt AJ, et al. Reconstitution of active telomerase in primary human foreskin fibroblasts: effects on proliferative characteristics and response to ionizing radiation. *Int J Radiat Biol* 2004; 80(5):377–88.
 110. Latre L, Genesca A, Martin M, et al. Repair of DNA broken ends is similar in embryonic fibroblasts with and without telomerase. *Radiat Res* 2004; 162(2):136–42.
 111. Zhou J-Q, Monson EK, Teng S-C, Schultz VP, Zakian VA. Pif1p helicase, a catalytic inhibitor of telomerase in yeast. *Science* 2000; 289:771–4.
 112. Bosco G, Haber JE. Chromosome break-induced replication leads to nonreciprocal translocations and telomere capture. *Genetics* 1998; 150:1037–47.

113. Meltzer PS, Guan X-Y, Trent JM. Telomere capture stabilizes chromosome breakage. *Nat Genet* 1993; 4:252–5.
114. Richardson C, Moynahan ME, Jasin M. Double-strand break repair by interchromosomal recombination: suppression of chromosomal translocations. *Genes Dev* 1998; 12:3831–42.
115. Johnson RD, Jasin M. Sister chromatid gene conversion is a prominent double-strand break repair pathway in mammalian cells. *EMBO J* 2000; 19:3398–407.
116. Richardson C, Jasin M. Frequent chromosomal translocations induced by DNA double-strand breaks. *Nature* 2000; 405:697–700.
117. Rouet P, Smih F, Jasin M. Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Mol Cell Biol* 1994; 14(12):8096–106.
118. Akgun E, Zahn J, Baumes S, et al. Palindrome resolution and recombination in the mammalian germ line. *Mol Cell Biol* 1997; 17:5559–70.
119. Waldman AS, Tran H, Goldsmith EC, Resnick MA. Long inverted repeats are an at-risk motif for recombination in mammalian cells. *Genetics* 1999; 153:1873–83.
120. Gebow D, Miselis N, Liber H. Homologous and nonhomologous recombination resulting in deletion: effects of p53 status, microhomology, and repetitive DNA length and orientation. *Mol Cell Biol* 2000; 20:4028–35.
121. O'Hagan RC, Chang S, Maser RS, et al. Telomere dysfunction provokes regional amplification and deletion in cancer genomes. *Cancer Cell* 2002; 2(2):149–55.
122. Hackett JA, Greider CW. End resection initiates genomic instability in the absence of telomerase. *Mol Cell Biol* 2003; 23:8450–61.
123. Ingvarsson S. Molecular genetics of breast cancer progression. *Semin Cancer Biol* 1999; 9:277–88.
124. Difilippantonio MJ, Petersen S, Chen HT, et al. Evidence for replicative repair of DNA double-strand breaks leading to oncogenic translocation and gene amplification. *J Exp Med* 2002; 196:469–80.

III

DNA DAMAGE RESPONSE, SIGNALING PATHWAYS, AND TUMORIGENESIS

11

Overview of the DNA Damage Checkpoint

ATM and ATR

Aude Dupré, PhD, and Jean Gautier, PhD

SUMMARY

Maintenance of genome stability in response to DNA damage is essential for cell survival. DNA damage occurs following external insults such as ultraviolet light and ionizing radiations or as a consequence of DNA replication or physiological DNA rearrangements. Cells respond to DNA damage by the coordinated induction of cell-cycle arrest and DNA repair or trigger apoptosis if the damage is too extensive. The DNA damage response is induced by two prominent intermediates: double-strand breaks and single-strand DNA. These pathways are well conserved from yeast to mammals and comprise a network of proteins acting together to monitor, sense, and repair DNA damage. In this review, we will focus on the central role played by two protein kinases, Ataxia-Telangiectasia mutated (ATM) and ATM related (ATR), during S-phase checkpoints and how these proteins are regulated by other components of the DNA damage response such as BRCA1, the Mre11-Rad50-Nbs1 complex, Claspin, and ATR-interacting protein (ATRIP). All these proteins, which are involved in sensing the damage (ATM, Mre11, Nbs1), in mediating (BRCA1) or transducing (Chk2) the signal, are essential for maintaining genome stability. Notably, mutations in genes encoding these proteins lead to increased risk for cancer.

Key Words: ATM; ATR; checkpoints; replication; double-strand break DNA; single-stranded DNA.

1. INTRODUCTION

Cells are continuously subjected to DNA damage that arises as a consequence of external insults such as ultraviolet (UV) light, ionizing radiation (IR), oxidative stress, and DNA-damaging agents or as a consequence of DNA replication, meiotic and V(D)J recombination. DNA damage may lead to mutations as well as chromosomal rearrangement or chromosomal loss. To maintain genome stability, cells have evolved a protective mechanism that we refer as the DNA damage response. Critical components

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of the DNA damage response are checkpoint pathways. These pathways regulate cell-cycle arrest, which allows DNA repair and cell survival, or apoptosis if the damage is too extensive (Fig. 1). Much progress has been made in our understanding of these pathways that are rapidly activated following damage. Cells lacking a functional DNA damage response display genome instability, faulty DNA replication, or aberrant chromosome segregation, resulting in an acceleration of the mutation phenotype (1).

The DNA damage response can be functionally separated into three steps using different sets of proteins that act in concert to transduce the signal from the lesion to the physiological output. Proteins of the first group are “sensor” proteins, which recognize aberrant DNA structures or subsequent chromosome alterations and initiate signaling (Fig. 2). Once the damage is detected, the signal is relayed to “transducers,” typically protein kinases, which convey and amplify the DNA damage signal to downstream effectors. These effectors are then modified and activated to evoke the physiological response (Fig. 2). Because some of these transducers are also involved in the assembly of DNA–repair complexes at the site of damage, DNA repair and checkpoint responses are concomitant and functionally linked. Finally, a class of proteins, called “mediators” or “adaptors,” may promote functional interactions between sensors and effectors.

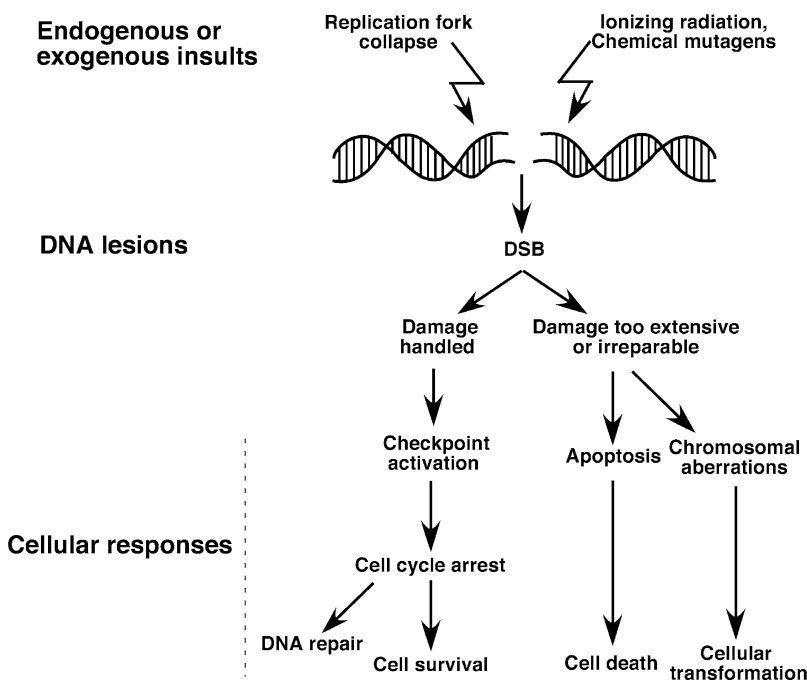


Fig. 1. Schematic representation of cellular responses to double-strand breaks (DSBs). DSBs are induced in response to external insults such as ionizing radiations (IR) and chemical mutagens or occur during normal cellular physiology, that is, when the replication fork collapses. The cellular response to DSBs leads to the activation of cell-cycle checkpoints that allow the coordination of cell-cycle arrest with DNA repair, ensuring cell survival and genome integrity. If DNA damage is too extensive or irreparable, cell can also induce apoptosis. Failure to detect or to repair DSBs can result in mutations, deletions, and rearrangements, which may cause cellular transformation and genomic instability.

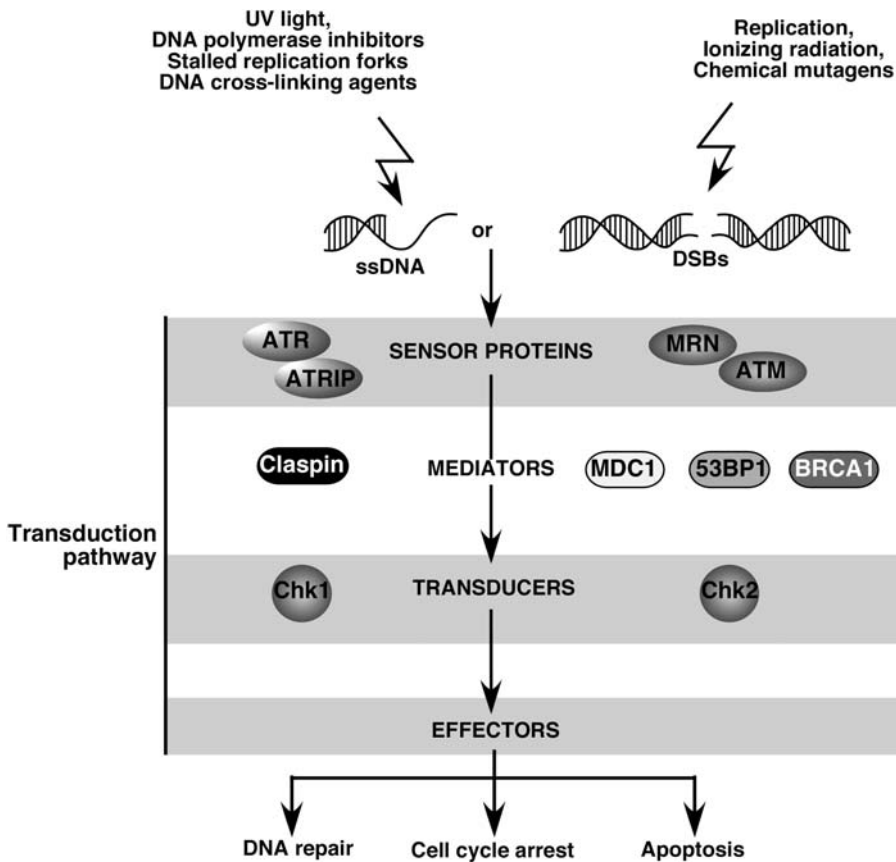


Fig. 2. Overview of the molecular components of the DNA damage response. The cellular response to single-strand DNA (ssDNA) or double-strand breaks (DSBs) can be divided into three different steps that induce cell-cycle arrest, DNA repair, or apoptosis. ssDNA or DSBs are detected by “sensor” proteins, ATR-ATRIP or ATM-MRN respectively. ATM and ATR are then activated and phosphorylate transducers such as Chk1 or Chk2, with the help of “mediator” proteins. Transducers are in turn activated and regulate effectors responsible for cell-cycle arrest at the G1/S and G2/M transitions or during the S phase.

1.1. DNA Lesions and Aberrant DNA Structures

DNA damage includes covalent modifications and non-covalent abnormal structures such as mismatches, loops, and bubbles arising from a string of mismatches (2). Covalent changes are induced by several intrinsic and extrinsic insults and include (i) structures generated at the replication fork: Holliday junctions, stalled or collapsed replication forks; (ii) base damages such as thymine glycols, oxidized or fragmented bases in response to reactive oxygen species, IR, and UV light; (iii) backbone damages including abasic sites, single- and double-strand DNA breaks; (iv) DNA interstrand crosslinks formed by crosslinking agents such as cisplatin, mitomycin C (MMC), and psoralen. It is currently thought that not all aberrant structures are recognized as such and directly trigger a response. Instead, they are converted into a limited number of signaling intermediates. This enables cells to share a limited number of pathways in response to a large number of problems. The most prominent intermediates are

double-strand breaks (DSBs) and single-stranded DNA (ssDNA) that are generated in response to IR, UV light, and radiomimetic agents. During DNA replication, nicked DNA produces DSBs, whereas treatments with hydroxyurea (HU), aphidicholin, or UV create ssDNA. Finally, DSBs could be converted into ssDNA by processing and signaling from these two DNA structures therefore overlaps functionally.

1.2. Cell-Cycle Checkpoint

DNA damage checkpoints are signal transduction pathways that induce cell-cycle arrest or delay in response to DNA damage. Cell-cycle progression is tightly coordinated with repair during the DNA damage response. Here, we will focus on the Ataxia-Telangiectasia mutated (ATM)- and ATM related (ATR)-dependent checkpoints that regulate S-phase progression (Fig. 3). These checkpoints regulate early steps of DNA replication: the assembly or the activation of the pre-Replicative Complex (pre-RC). These checkpoints can be classified in two groups whether it requires or not DNA replication and fork progression.

Checkpoints that are independent of active DNA replication operate during G1 or S phases and are induced by DSBs or ssDNA. During G1, the G1/S checkpoint recognizes the presence of damaged DNA and delays the subsequent S phase by inhibiting origin firing. Similarly, the intra-S-phase checkpoint blocks DNA replication in S phase. Following damage sensing outside of replication forks, ATM and ATR are activated, leading to the phosphorylation and activation of Chk2 and Chk1, respectively. Ultimately, these pathways downregulate Cdk2 and Cdc7 protein kinase activities, which are both required for Cdc45 loading and subsequent replication origin activation (see also Chapter 3.2.1.2.).

During S phase, two DNA replication-dependent checkpoints are active. The first one is called the “Replication” checkpoint and is initiated when progression of replication forks is stalled in response to genotoxic stresses such as deoxynucleotides depletion, chemical inhibition of DNA polymerases, or as a consequence of aberrant structures or DNA damage (Fig. 3). This checkpoint inhibits unfired origins through the activity of ATM and Chk1. In addition, ATM and ATR are involved in the recovery of stalled replication forks. The second checkpoint is the S/M checkpoint that monitors completion of DNA replication before entry into mitosis (Fig. 3). The downstream target of this checkpoint is the inactivation of Cyclin B–cdk1 complexes, thereby preventing mitosis entry. Once activated, Chk2 phosphorylates and inhibits Cdc25 phosphatase activity, leading to the inactivation of Cyclin B–Cdk1 complexes. Other ATM substrates such as Nbs1 or BRCA1 have been involved in the S/M checkpoint (Fig. 3).

Finally, some recent data also support the idea that the signaling pathways regulating these checkpoints are functional even in the absence of DNA damage and help to coordinate progression through the cell cycle (3).

1.3. Genome Instability Syndromes

Several hereditary cancer-prone syndromes exhibit defects in the DNA damage response (Table 1). These disorders include ATM, Ataxia-telangiectasia (A-T)-like disorder (ATLD) (Mre11), Nijmegen Breakage Syndrome (NBS), Werner syndrome (WRN), Fanconi anemia (FA), and familial forms of breast and cervical cancers with

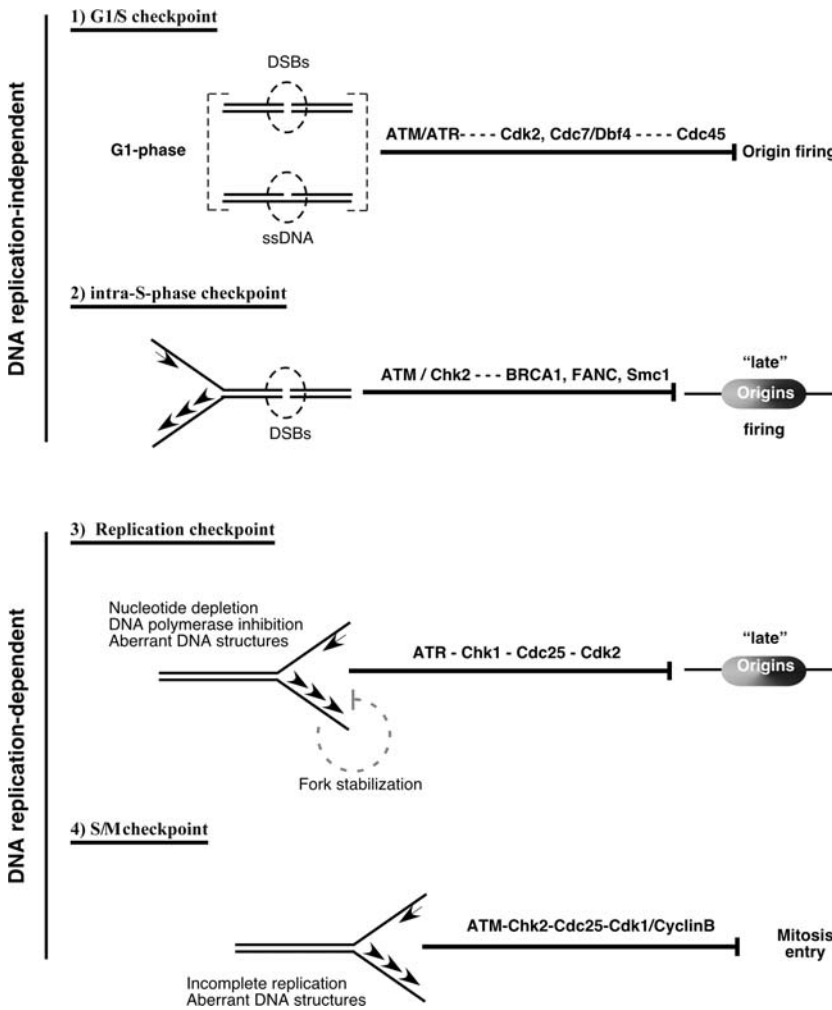


Fig. 3. Checkpoints that influence S phase. Checkpoints that regulate S phase can be divided in two groups whether it requires or not DNA replication and fork progression. The first group is independent of replication forks and includes the G1/S and the intra-S-phase checkpoints triggered by the presence of DSBs or ssDNA. **(A)** The G1/S inhibits S-phase entry by preventing the loading of Cdc45 onto replication origins and origin firing. This mechanism is mediated by the inhibition of two major S-phase kinases, Cdk2 and Cdc7, in response to ATM and ATR activation. **(B)** The intra-S-phase checkpoint blocks DNA replication in S-phase by inhibiting the firing of late replication origins. In response to DSBs, ATM is activated and phosphorylates proteins involved in the DNA damage response including Chk2, the Mre11-Rad50-Nbs1 complex (MRN), BRCA1, Fanconi Anemia (FANC) proteins, and the SMC protein 1 (Smc1). Abrogation of these phosphorylation events following exposure to IR results in a radio-resistant DNA synthesis (RDS). The second group of checkpoints influencing S phase depends on DNA replication. It contains **(C)** the “replication” checkpoint that is induced in response to stalled replication forks, nucleotides depletion or caused by aberrant DNA structures. This checkpoint inhibits unfired origins by activating the ATR/Chk1 pathway, which inactivates the activity of the Cdk2 kinase. **(D)** The S/M checkpoint is activated when DNA replication is incomplete or in the presence of aberrant DNA structures. This checkpoint is mediated by the activation of ATM and Chk2 pathway, which in turn inactivates by phosphorylation the activity of Cdc25 phosphatase. The major M-phase kinase, Cdk1, is inactivated, and mitosis entry is prevented.

Table 1
Clinical presentation associated Genome Instability syndromes

	<i>A-T</i>	<i>NBS</i>	<i>ATLD</i>	<i>FA</i>	<i>WRN</i>	<i>BRCA</i>
Clinical symptoms						
Telangiectasia	+	–	–	–		
Ataxia	+	–	+	–		
Microcephaly	–	+	–	+		
Immunodeficiency	+	+	–	ND		
Malignancy	+	+	ND	+		
Cellular phenotypes						
Radiation sensitivity	+	+	+	+	+	+
Chromosomal instability	+	+	+	+	+	+
RDS phenotype	+	+	+	ND	ND	+

ND, not described.

mutations in the *BRCA1* or *BRCA2* genes. In addition, a splicing mutation in the *atr* gene is responsible for Seckel syndrome.

1.3.1. ATAXIA-TELANGIECTASIA: ATM

ATM protein is the product of the gene that is mutated, lost, or inactivated, in the human autosomal recessive genetic disorder A-T (4,5). Ataxia or the inability to coordinate and control motor movement is a result of cerebellar degeneration. A-T is also characterized by immunodeficiency, genome instability (a markedly increased rate of chromosome breakage in cell culture), thymic and gonadal atrophy, and sensitivity to IR and DSB-inducing agents (6,7). A-T patients commonly develop cancer during their second or third decade of life, particularly Hodgkin's and non-Hodgkin's lymphomas as well as various lymphoid leukemias (8). Some patients with A-T harbor mutations that cause frame-shifting, premature termination of translation, or exon-skipping during mRNA processing (9,10). As these mutations result in the truncation and complete destabilization of ATM, they behave like null mutations. Less severe forms of the disease also occur because of missense mutations in the ATM gene (9).

1.3.2. SECKEL SYNDROME: ATR

Seckel Syndrome is an extremely rare recessive inherited disorder characterized by intrauterine growth retardation, dwarfism, microcephaly, varying degrees of mental retardation, and/or unusual characteristic facial features (11). The gene responsible for this disease has been mapped to chromosome 3 and encodes the ATR protein (12). The mutation identified to date results in a splicing defect leading to reduce levels of wild-type ATR protein.

1.3.3. OTHER CHROMOSOME INSTABILITY SYNDROMES

Two rare inherited diseases have phenotypes similar to A-T. ATLD is a very rare disorder caused by hypomorphic mutations in the *Mre11* gene, which lead to C-terminal truncations or missense mutations. ATLD patients show a phenotype very similar to that of A-T with regard to cerebellar ataxia. In contrast to A-T, these patients do not

develop telangiectasia, and the progression of the disease is very slow. None of the ATLD patients being described to date have developed cancer, and mice carrying a mutation analogous to the ATLD mutation do not develop lymphomas (13). NBS is a recessive autosomal syndrome caused by hypomorphic mutations in the gene encoding the Nbs1 protein. Immunodeficiency, small head and growth failure, mental deficiency, genome instability, and an increased risk of malignancy characterize NBS patients.

Several other chromosomal instability and cancer predisposition syndromes have been linked to ATM and ATR signaling pathways. Bloom syndrome (BLM) is a very rare disorder caused by mutation in the *blm* gene. The BLM protein is a member of the RecQ helicase family (14). Importantly, Bloom patients are predisposed to various cancers such as lymphomas, carcinomas, and leukemias. Cells from Bloom patients are characterized by spontaneous sister-chromatid exchanges, chromosomal breakages, and rearrangements. WNS is caused by mutations in the WRN protein, another RecQ homolog. The most characteristic manifestation of WNS is premature aging. Cells from WNS patients exhibit chromosomal translocations and breakage and are hypersensitive to camptothecin, an inhibitor of DNA topoisomerases I, and some DNA crosslinking agents. FA is a rare recessive disorder that is characterized by bone-marrow depletion leading to aplastic anemia. Many patients eventually develop acute myelogenous leukemia (AML) and carcinomas at a very early age. Cells from FA patients show chromosomal alterations and are sensitive to DNA-crosslinking agents such as MMC but not to IR (15,16). This disease is heterogeneous with mutations affecting at least 12 different genes. Finally, two major genes have been identified that are associated with familial breast and ovarian cancers: *BRCA1* and *BRCA2* (breast cancer susceptibility genes). *BRCA1* mutations account for approximately 40% of inherited cases of breast cancer. Bi-allelic inactivation of *BRCA1* is also observed in sporadic cases of breast cancer. Inherited mutations affect both germline alleles. *BRCA1* and *BRCA2*-deficient cells exhibit hypersensitivity to IR and chromosomal abnormalities.

All of these cancer-prone syndromes were originally thought to be unrelated and to lead to cancer predisposition by affecting distinct pathways. However, recent advances in the understanding of the molecular connections between the DNA damage response and the maintenance of genome stability have unraveled an increasing number of connections between all these pathways affected in these diseases. ATM and ATR protein kinases are critical for the maintenance of genome stability. In the following sections, we attempt to highlight the biochemical and functional connections between these protein kinases and other proteins that are inactivated in disease affecting the maintenance of genome stability.

2. BIOCHEMISTRY OF ATM AND ATR

2.1. ATM and ATR Genes

The gene mutated in A-T (ATM) is localized at the q22–23 locus of chromosome 11 (17). The 150 kb gene encodes a 13 kb transcript with 66 exons (18). ATM is ubiquitously expressed at all stages of development and its level does not fluctuate during the cell cycle or in response to DNA damage (19–22). In most cells, ATM is localized in the nucleus with a small fraction associated with cytoplasmic endosomes or peroxisomes (22–24). In highly differentiated cells, such as purkinje cells of the

cerebellum, ATM is exclusively cytoplasmic, but the mechanisms controlling the subcellular distribution of ATM have not been yet determined (24).

The *ATR* (ATM and Rad3 related) gene was identified by homology with Rad3 and Mec1p in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, respectively, and mTor in human cells (25,26). This gene maps to the 3q22–24 locus of chromosome 3 and is highly expressed in testis, skeletal muscle, and pancreas (25). Unlike ATM, disruption of the *ATR* gene in mice is embryonic lethal, suggesting that ATR plays an essential role in early development or in cell growth (27,28).

2.2. Structural Properties

ATM and ATR genes encode for two related serine/threonine protein kinases of 3056 and 2644 amino acids, respectively (Fig. 4). These proteins are conserved in all eukaryotes and belong to the phosphoinositide 3-kinase-like kinase (PIKK) protein family based on sequence homology in the C-terminal catalytic domain (29). Other mammalian members of this family include DNA-dependent protein kinase (DNA-PK), mTor, and SMG1. No lipid kinase activity has been described for these proteins. ATM and ATR contain also two FAT (FRAP/ATM/TRRAP) domains, commonly found in FRAP proteins: one directly N-terminal to the PI3K domain and the other localized at the C-termini of both proteins called FAT-C (30). The N-terminal portion domain of both proteins has no similarity with other proteins and is composed of large HEAT repeats that form superhelical scaffolding matrices to interact with other macromolecules (31). ATM contains also a Proline-rich motif (PXPXPPX₂P) that could be involved in specific protein–protein interactions (29). ATM and ATR phosphorylate serines or threonines followed by a glutamine, that is, (S/T)-Q motif (32–34). This (S/T)-Q motif is present in all known ATM and ATR substrates including p53, Chk2, BLM, BRCA1, FANCD2, Nbs1, Mre11, TRF1, Smc1, Histone H2AX, and c-Abl.

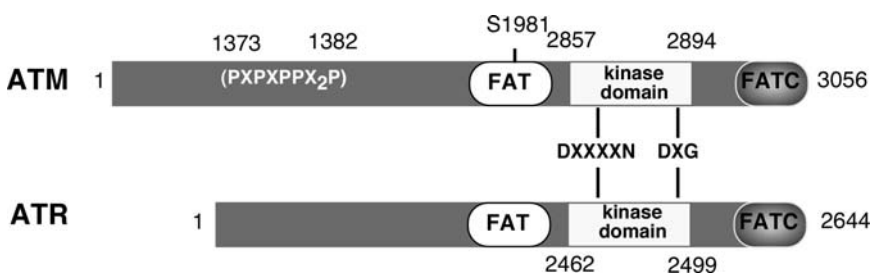


Fig. 4. Structural organization of ATM and ATR. ATM and ATR belong to the phosphoinositide kinase-like kinase (PIKK) family of protein kinases. ATM and ATR share three conserved motifs within their C-terminal sections: the FAT (FRAP/ATM/TRAAP) domain, the FAT-C (FAT-C terminal) domain, and the kinase domain that contains conserved catalytic residues DXNXXN and DXG. The catalytic domain of ATM has about 30% amino acid identity with the PI3K and is the signature motif for members of the PIKK proteins. ATM and ATR are serine/threonine protein kinase activity and no lipid kinase activity has been described for those proteins. Serine 1981 is within the FAT domain of ATM. This residue is important for ATM autophosphorylation and activation in response to IR (see text). The N-terminal region of ATM has no significant amino acid similarity to other proteins. This region is composed of HEAT repeats and contains a proline-rich motif, both probably involved in protein interactions.

2.3. Mechanisms of ATM and ATR Activation

As mentioned earlier, two prominent types of DNA damage intermediates are DSBs and ssDNA coated with replication protein A (ssDNA-RPA). Although ATM- and ATR-dependent responses partially overlap, ATM is central to the DSB-induced response, whereas ATR is primarily involved in responding to ssDNA.

2.3.1. ACTIVATION OF ATM

2.3.1.1. ATM Autophosphorylation. ATM activity is stimulated 2–3-fold following IR or neocarzinostatin (NCS) treatments (35,36). Following IR, ^{32}P is incorporated into ATM supporting a mechanism for ATM activation by autophosphorylation (37,38) (Fig. 5). In undamaged cells, ATM exists as an inactive protein dimer; the catalytic domain of one ATM molecule is blocked by the FAT domain of the other ATM molecule. Following DNA damage, each ATM molecule within a dimer transphosphorylates the other one at serine 1981 within the FAT domain, leading to the dissociation of the dimer and to the generation of active monomers (Fig. 5). ATM activation could be triggered by chromatin alterations as well as by direct interactions between ATM and the broken DNA ends (37). It is important to emphasize that, even though ATM autophosphorylation is a critical process for its activation, additional phosphorylation steps and/or mechanisms are likely to be required to reach full ATM activation. ATM activity is then assayed by phosphorylation of ATM substrates such as Smc1, Chk2, or H2AX that is one of the earliest ATM substrates phosphorylated in response to IR.

2.3.1.2. Co-Factors Required for ATM Activation: MRN Complex. The MRN complex is composed of three proteins: Mre11, Rad50, and Nbs1 (Xrs2 in yeast). Mre11 contains four N-terminal phosphoesterase motifs, required for its 3'–5' exonuclease and endonuclease activities and two DNA-binding domains (39) (Fig. 6). Rad50 belongs to the structural maintenance chromosome (SMC) protein family and contains a Walker A and B motifs brought together by an intramolecular coiled-coil formation, resulting in an active ATPase domain. The central region, also called “hook,” can chelate Zn^{2+} and thus allows intermolecular interactions between Rad50 molecules (39). Nbs1 is composed of two N-terminal domains: Forkhead-associated (FHA) and BRCT (BRCA1-C terminal) phosphopeptide-binding motifs, predominantly found in proteins participating in the DNA damage response and acting to recruit and activate downstream protein kinases (40). The C-terminal region of Nbs1 contains the Mre11-binding domain, the ATM-binding site and three NLS (41–44). In response to DNA damage, Nbs1 and Mre11 are phosphorylated at “SQ” sites (serine 343 and 278 for Nbs1, whereas the “SQ” sites for Mre11 have not been yet identified) leading to the hypothesis that MRN is an effector of the DNA damage response, positioned downstream of ATM (45,46). However, more recent work established that MRN also acts upstream of ATM, as a direct activator (47).

In yeast, Mre11 is one of the first proteins recruited to the sites of DSBs in response to IR (48,49). ATLD and NBS cells are partially deficient for ATM-dependent phosphorylation of p53, Chk2, Smc1, and FANCD2 following irradiation, indicating that full ATM activation requires a functional MRN complex (49–56). *In vitro*, addition of baculovirus-expressed MRN complex to purified ATM recruits ATM to damaged DNA and dissociates ATM into monomers (57). MRN also stimulates

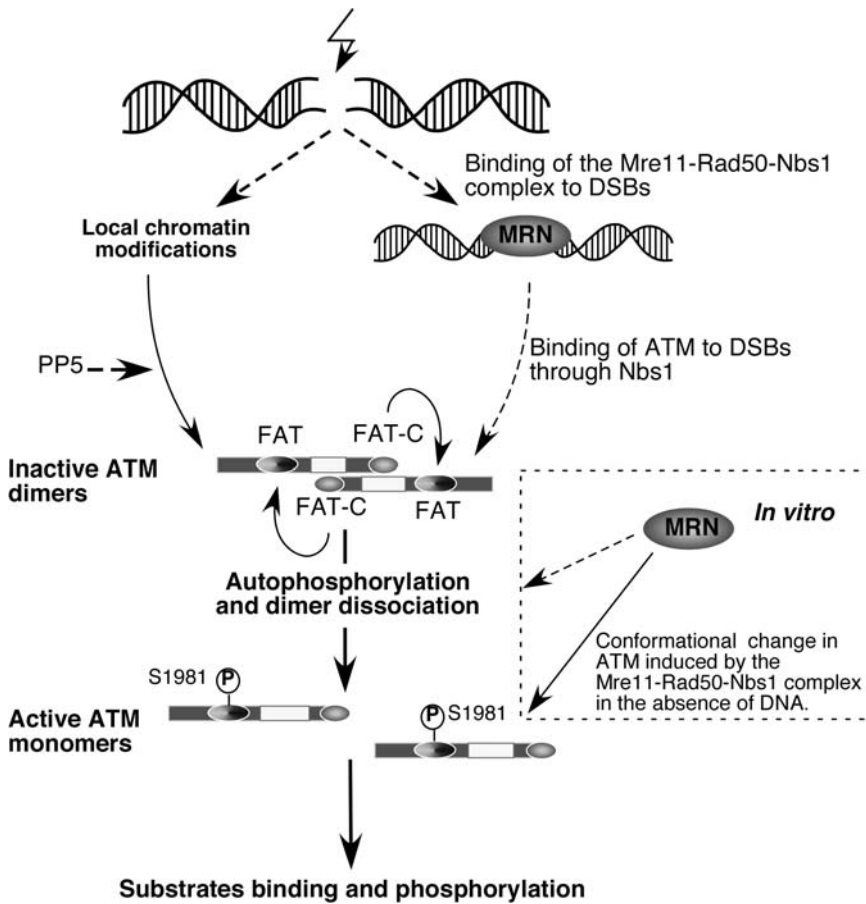


Fig. 5. ATM activation. In untreated cells, ATM is present as inactive dimers. The catalytic domain of both ATM monomers is then blocked by the FAT domain of the other ATM molecule. In the presence of double-strand breaks (DSBs) or following changes in chromatin structure, ATM becomes autophosphorylated on serine 1981 and dissociates into active monomers. In mammalian cells, the Mre11-Rad50-Nbs1 (MRN) complex acts as a direct activator of ATM by promoting ATM's recruitment to damaged DNA in response to IR. *In vitro*, MRN is required for the monomerization of ATM in response to DNA broken molecules. Surprisingly, MRN also increases ATM affinity for its substrates in the absence of DNA, suggesting that MRN may induce a conformational change in ATM increasing its substrate's affinity.

ATM-dependent phosphorylations of p53 and Chk2 in the absence of free DNA ends (58), arguing that MRN may induce a conformational change in ATM, increasing its affinity for substrates (Fig. 5). ATM autophosphorylation was shown to be impaired in cells expressing adenoviral proteins that degrade MRN further supporting the hypothesis that MRN is a direct ATM activator (56). Finally, biochemical evidence supporting this idea came from experiments using *Xenopus* cell-free extracts. Addition of linear double-strand DNA fragments to these extracts activates ATM in an MRN-dependent manner (43,59,60). Active Mre11 and ATM are recovered in a high molecular weight protein/DNA complex associated with DSB-containing DNA. This result establishes that the MRN complex is required for ATM activation and that the

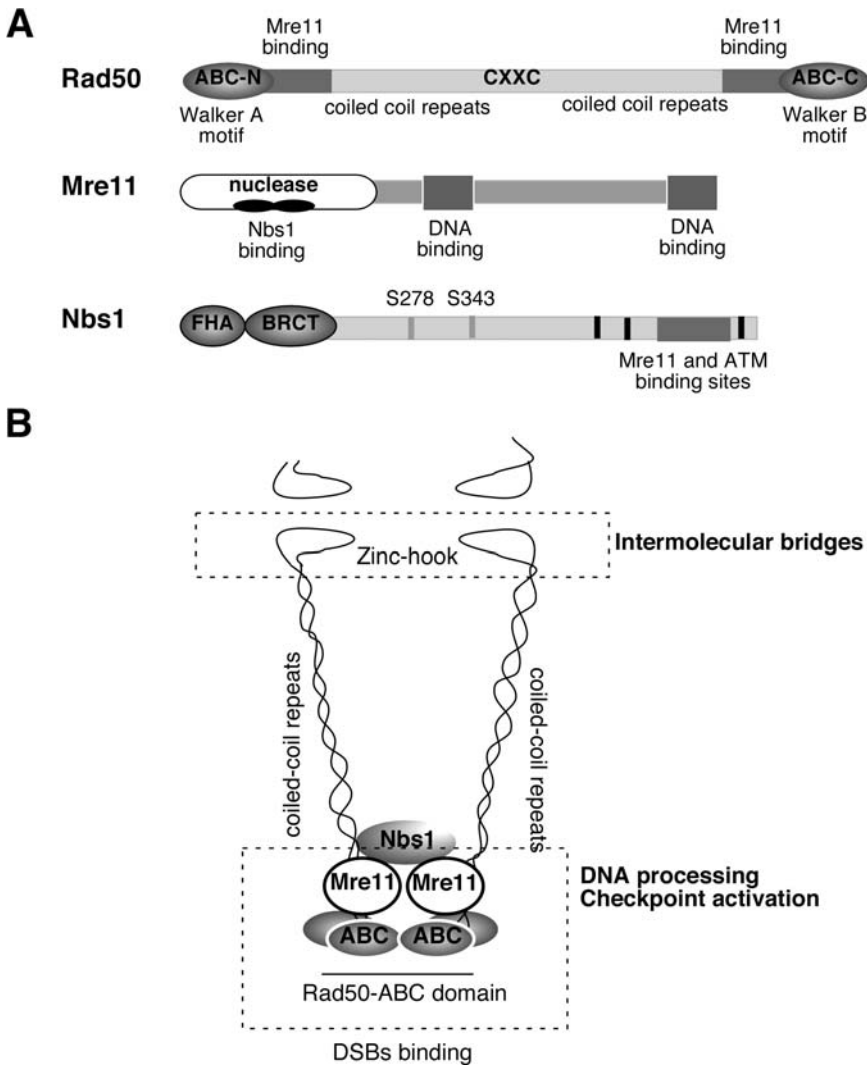


Fig. 6. Structure and spatial organization of the Mre11-Rad50-Nbs1 (MRN) complex. MRN is composed of three different proteins: Mre11, Rad 50, and Nbs1. **(A)** Domain organization of Rad50, Mre11, and Nbs1. Rad50 belongs to the SMC proteins and contains two N- and C- terminus Walker motif (A and B) that binds to ATP through ABC domains. Rad50 has two Mre11-binding domains and two coiled-coil repeats separated by a sequence containing two cysteine residues also called the “Hook” domain. Mre11 is a nuclease containing DNA-binding sites. Finally, Nbs1 is composed of two N-terminus phosphopeptide-binding domains: a Forkhead-associated (FHA) and a BRCA1 C-terminus (BRCT) domains. The C-terminal region of Nbs1 contains an Mre11- and an ATM-binding sites that mediate the recruitment of ATM to damaged DNA and ATM activation. The central region of Nbs1 contains several serine/glutamine motifs phosphorylated by ATM and ATR. In particular, the serine residues 278 and 343 are phosphorylated by ATM in response to IR, and these phosphorylation events are essential for the intra-S-phase checkpoint and the telomere maintenance. **(B)** Spatial organization of the MRN complex. The MRN complex binds to DNA ends through Mre11 and the Walker domains of Rad50. Intramolecular coiled-coil interactions within Rad50 bring together its Walker A and B domains generating an active ATPase. In addition, chelation of Zn^{2+} through the CXXC (hook) sequence at the base of the coiled-coil repeats of Rad50 allows intermolecular cohesion between MRN complexes. The stoichiometry of the complex is thought to be 2Mre11/2Rad50/1Nbs1.

association of MRN with the DNA is required to transduce ATM-dependent pathways in response to DNA damage (59).

Taken together, these results support a model, in which MRN complex binds directly to DSBs, promoting or facilitating the processing of DNA ends. ATM is recruited to DNA by MRN and activated. ATM activation involves ATM autophosphorylation and its monomerization. ATM is then released from the DNA lesion and acts to phosphorylate/activate other “soluble” substrates, probably to amplify and propagate the checkpoint response (49,61). It remains to be determined whether other proteins participate in DNA–ATM–MRN interactions *in vivo* and to clarify how ATM activation is triggered by its binding to DSBs and/or by the MRN complex.

2.3.1.3. Other Proteins Involved in ATM Activation. Several studies have implicated “mediator” proteins in ATM-dependent pathways. Among them are MDC1 (for mediator of DNA damage checkpoint protein, also called NFBP1) (62), 53BP1 (63), BRCA1, and protein phosphatases.

MDC1 contains an FHA and a BRCT domain and is found in a high molecular weight complex containing ATM, BRCA1, MRN, FANCD2, Smc1, BARD1, and 53BP1 (64). A number of studies have suggested that MDC1 is important for the MRN complex-dependent activation of ATM at high doses of IR. First, MDC1 is phosphorylated in an ATM- and Nbs1-dependent manner (64). MDC1 forms foci in a manner that is dependent of the phosphorylated form of H2AX, also called γ H2AX. Finally, MDC1 is required for the accumulation of active ATM, MRN, and BRCA1 into IR-induced foci (61,64–66). This suggests that MDC1 could act as a signal amplifier of the ATM pathway by controlling the stable recruitment of the MRN complex and other checkpoint/DNA-repair proteins in the vicinity of DSBs. In addition, MDC1 may also have a role upstream of ATM, because ATM autophosphorylation as well as Chk1 and Chk2 phosphorylation is deficient in MDC1-downregulated cells in response to DNA damage (64).

53BP1 is a nuclear protein that interacts with p53 through two C-terminal BRCT domains (67–71). In response to IR, etoposide, and NCS, 53BP1 forms discrete foci and colocalizes with γ H2AX and MRN at DSBs sites (72–77). The suppression of 53BP1 expression by siRNA decreases the phosphorylation of various ATM substrates and knockout mice for 53BP1 and ATM have similar phenotypes including growth retardation, immunodeficiencies, and radiation sensitivity (78). Three different mechanisms for the requirement of 53BP1 in ATM activation have been proposed (78). 53BP1 could either facilitate the ability of ATM to phosphorylate its substrates or could be a direct activator of ATM. Because 53BP1 also interacts with ATM in irradiated cells, this could reflect the 53BP1-dependent recruitment of ATM to DSBs (79). In turn, ATM phosphorylates 53BP1 in response to DNA damage but the functional significance of this event is not known (73–75,80,81).

Another interesting candidate to mediate ATM activation is the BRCA1 protein that is also a substrate for ATM, ATR, and Chk2 (82). BRCA1 is a nuclear protein of 1863 amino acids composed of a N-terminal RING domain involved in protein–protein interactions, such as BARD1 protein, and two C-terminal BRCT domains. BRCA1 is required for the phosphorylation of several ATM substrates in response to IR (83,84). Although BRCA1 does not directly activate ATM following immunoprecipitation, BRCA1 has been detected in a high molecular weight complex called

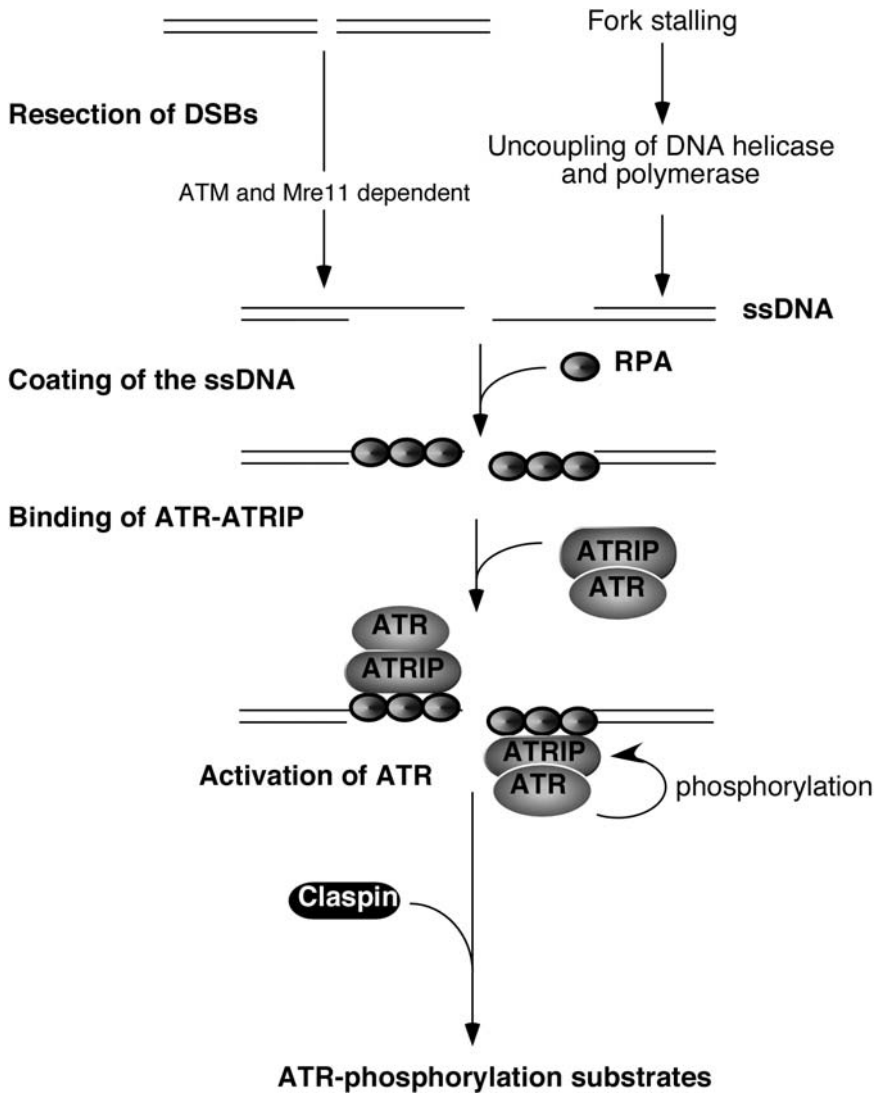


Fig. 7. ATR activation. ATR is directly activated in response to bulky DNA lesions, DNA polymerase inhibitors, stalled replication forks, or hypoxia, which all generate ssDNA as a result of uncoupling between the MCM DNA helicase and the stalled DNA polymerases. In human cells, ATR exists in a stable complex with ATRIP protein, a potential regulatory partner. In the presence of ssDNA, the RPA binds to ssDNA, which induces the recruitment of ATR to DNA damages site through ATRIP–RPA interactions. Following IR treatment, ATR is also activated. This process depends on ATM and the MRN complex that are both required for the processing of the DSB, thus generating ssDNA. ATR is then recruited to ssDNA.

BRCA1-associated genome surveillance complex (BASC), which contains ATM, MRN, and BLM (85). BRCA1 could act as a scaffolding protein, increasing the accessibility of substrates to ATM (83).

Finally, protein phosphatases PP5 and PP2A have been shown to participate in ATM activation (86,87). PP5 interacts with ATM after treatment with NCS. Downregulation

of PP5 by siRNA or by overexpressing a catalytically inactive mutant reduces ATM autophosphorylation and ATM-dependent phosphorylation of p53 in response to NCS resulting in an S-phase checkpoint defect (86). This suggests that PP5 might remove inhibitory phosphorylation on ATM in response to IR. In undamaged cells, ATM interacts constitutively with PP2A and treatment with okadaic acid, an inhibitor of PP2A, induces ATM autophosphorylation. Because ATM and PP2A interactions are suppressed by IR through a mechanism involving phosphorylation, PP2A could modulate ATM activation by catalyzing the dephosphorylation of ATM on serine 1981 or by promoting the dissociation of ATM into active monomers (87).

2.3.2. ATR ACTIVATION

2.3.2.1. ssDNA-RPA Intermediates and ATRIP. ATR is a nuclear protein kinase that can directly bind to DNA *in vitro* (88). In *Xenopus* cell-free extracts, ATR activity is increased following DNA binding (89). ATR is activated primarily in response to bulky DNA lesions, DNA polymerase inhibitors, stalled replication forks, hypoxia, and also in response to DSBs (90–92). Similar to ATM, ATR forms nuclear foci in response to IR (93). Unlike ATM, ATR activation requires processing and generation of ssDNA from DSBs (37,49,94,95) (Fig. 7). ssDNA is rapidly coated by RPA, thus creating a platform for the recruitment of ATR to the damage sites (96–99). ATR's recruitment to ssDNA/RPA intermediates requires the C-terminus end of ATRIP a homolog of yeast Rad26/DDC2 (42,97) (Fig. 7). ATRIP and ATR interact *in vivo* and colocalize to DNA damage sites or stalled DNA replication forks. Although ATRIP is phosphorylated by ATR, this event is not necessary for ATR's recruitment and the initial ATR-dependent response (100,101). ATRIP or ATR protein levels are co-regulated, and downregulation of either protein causes a defect in the DNA damage S-phase checkpoint, suggesting that ATR–ATRIP interactions are essential for their stability and function (97). Once ATR is recruited to ssDNA-RPA, ATR interacts with and phosphorylates its substrates, such as BRCA1 and H2AX.

2.3.2.2. ATR Activation in Response to IR. During G1, S, and G2 phases, ATR is activated following exposure to IR and recruited to damaged DNA (102–104). This process depends on ATM and the MRN complex, which are both required for the processing of DSBs, thus generating a DNA structure able to recruit ATR through RPA and ATRIPs (102,104).

3. FUNCTIONS OF ATM AND ATR

3.1. Roles as Damage Sensors

A DNA damage sensor must physically interact with DNA before activation of the DNA damage response, and this association should be independent of activating modifications of the sensor protein. Mutations in this sensor should affect both DNA–protein interactions and the cellular responses to DNA damage. ATM fulfills in part these criteria. ATM is one of the first proteins detected at DSBs sites in response to IR (48). Purified ATM binds preferentially DNA ends rather than ssDNA or double-strand DNA, an event that can be correlated with an increase of ATM activity (34,105,106). In *Xenopus* extracts, active ATM is preferentially precipitated with DSB-containing DNA and not with circular DNA (59,60). Furthermore, ATM acts at DSB sites to modify transducers of the DSB-signaling pathways such as Nbs1 and Chk2, thus

supporting the hypothesis that ATM could be a DNA damage sensor (49). Finally, active ATM is found in two fractions following the generation of DSBs, a chromatin-bound fraction, possibly signaling locally, and a soluble nuclear fraction, free to move throughout the nucleus, that could propagate the damage response (107). However, ATM activation, as monitored by autophosphorylation, can take place in the absence of DSBs presumably as a sole consequence of changes in chromatin structure. Therefore, DSB recognition might not be essential for ATM-dependent signaling. In summary, the exact requirement for ATM–DNA interactions and their role in ATM activation needs to be further investigated, especially in light of recent data showing DNA-independent ATM activation (58).

ATR is able to bind chromatin even in the absence of external genotoxic stress and associates with ssDNA cellulose *in vitro* (95,96). However, in contrast to ATM, ATR is not able to directly interact with damaged DNA by itself and requires RPA and ATRIP to generate a signal from ssDNA (95,108) (see also Section 2.3.2.1.). Therefore, there is no strong evidence implicating ATR as a DNA damage sensor.

3.2. Signaling to Cell-Cycle Arrest

3.2.1. G1/S CHECKPOINT

DNA replication is initiated at origins of replication, defined by the stepwise assembly of a pre-RC containing the origin recognition complex (ORC), Cdc6, Cdt1, and mini-chromosome maintenance proteins (MCMs) (Fig. 8). The activity of the S-phase kinases Cdk2 and Cdc7 is then required sequentially to activate origins, to promote the loading of Cdc45 onto chromatin and for subsequent DNA unwinding by MCMs and elongation (109,110). Cellular exposure to IR or radiomimetic agents before S-phase triggers a G1/S checkpoint that prevents origin firing and subsequent DNA replication until the damage is repaired. Several pathways have been described that lead to the inhibition of two essential protein kinases for DNA replication initiation, Cdc7 and Cdk2. An important characteristic of these checkpoint pathways is that they do not require an active replication fork to function.

3.2.1.1. p53-Dependent Mechanisms. The dominant checkpoint response to DNA damage during the G1 phase is the ATM/ATR-Chk2/Chk2-p53/MDM2-p21 pathway which triggers G1 arrest (2). In the absence of stress, p53 protein is unstable and a poor activator of transcription. Following DNA damage, p53 is stabilized and activates transcriptional targets involved in the DNA damage response. The activation of p53 is mediated by ATM- and ATR-dependent phosphorylations that are reinforced by Chk1/Chk2, leading to the acetylation, sumoylation, stabilization and nuclear translocation of p53 (2). A critical transcriptional target of p53 for cell-cycle arrest is p21Cip1/Waf1, which inhibits Cdk2 kinase activity and thereby causes G1 arrest. This process inhibits not only the initiation of DNA replication but also prevents the inactivation of the E2F pathway required for a sustained G1 arrest (111).

3.2.1.2. p53-Independent Mechanisms. The p53-dependent response to DNA damage is slow, as it requires transcription and protein synthesis following p53 stabilization. However, cells have developed rapid mechanisms to respond to DNA damage that rely only on post-translational protein modifications. Two distinct, p53-independent checkpoint pathways coexist in G1 and are activated by ATM and ATR, respectively (Fig. 8).

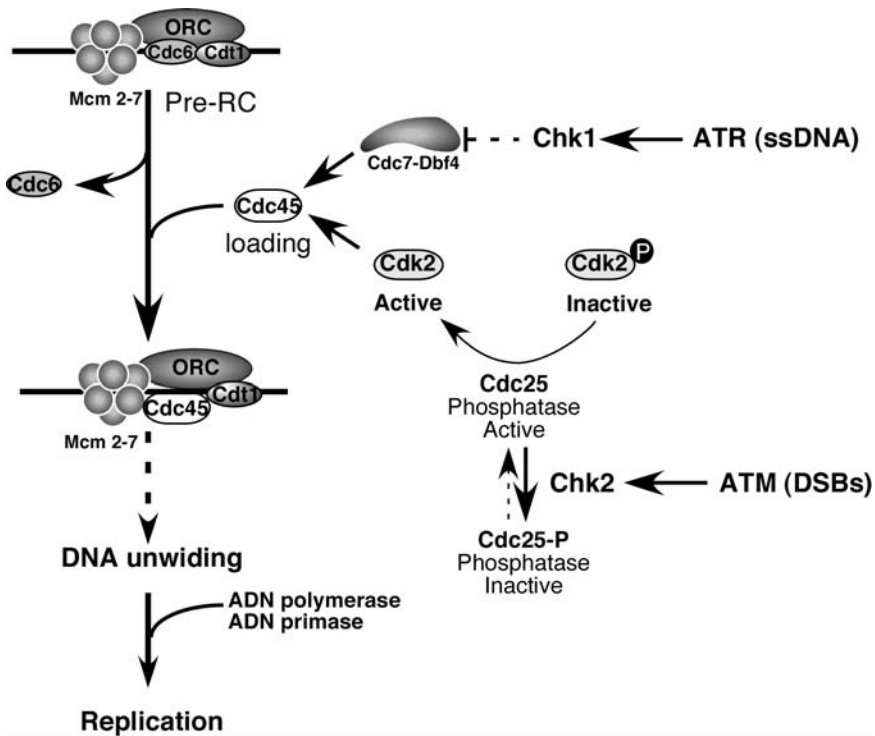


Fig. 8. pre-Replicative Complex (pre-RC) activation. The pre-RC is formed through the stepwise assembly of the origin recognition complex (ORC) and the Cdc6 and Cdt1-dependent loading of MCM helicase. At the onset of S phase, the pre-RC is activated by the action of Cdk2 and Cdc7. Cdk2 and Cdc7 then triggers the loading of the protein Cdc45 onto origins, leading to the activation of MCM proteins and initiation replication. These kinases are the downstream effectors of checkpoint signaling pathways. In the presence of DSBs, ATM is activated and phosphorylates Chk2. In turn, Chk2 is activated and phosphorylates the Cdc25 protein phosphatase leading to its degradation. Cdk2 is then inactivated, which prevents Cdc45 loading onto chromatin and the initiation of DNA replication. In the presence of ssDNA, ATR and Chk1 are activated which inhibits the Cdc7 kinase, probably by dissociating its interaction with Dbf4. Cdc45 loading onto origins is then prevented.

Addition of DSB-containing DNA to *Xenopus* extracts triggers an ATM-dependent checkpoint independently of transcription and protein synthesis (112). This signaling pathway ultimately results in phosphorylation of tyrosine 15 of Cdk2, thus inhibiting its activity. Cdc25A carries out this inhibitory phosphorylation. Inhibition of Cdk2 activity prevents the loading of Cdc45 onto chromatin and blocks subsequent origin activation. A similar pathway was also identified in mammalian cells (113,114).

A second checkpoint pathway that inhibits initiation of DNA replication has been described in *Xenopus* cell-free extracts. This checkpoint is triggered by ssDNA-RPA intermediates generated in G1. These aberrant DNA structures can be generated experimentally by treatment with exonuclease III or by addition of etoposide, an inhibitor of DNA topoisomerase II (99). The signaling is dependent on ATR and RPA. Activation of ATR ultimately results in the inhibition of Cdc7/Dbf4 protein kinase activity. Cdc7 is a conserved serine/threonine kinase, which is required for origin firing with Dbf4, its regulatory subunit (115). The essential targets of Cdc7 have not yet been

unambiguously identified. However, MCM2, a member of the MCM family and a component of the putative replicative helicase, is a potential substrate (116–119). In conjunction with Cdk2, Cdc7 is also required for the loading of Cdc45 onto the chromatin (115). In response to ssDNA/RPA intermediates, Cdc7 activity is downregulated, possibly by dissociation of the Dbf4 subunit, which leads to the inhibition of Cdc45 chromatin loading and a failure to activate the pre-RC (99). This signaling appears to be conserved in mammalian cells as some checkpoint-defective lymphoma cell lines exhibit ATR downregulation (120,121).

3.2.2. S-PHASE CHECKPOINTS

In contrast with the G1/S checkpoints, these pathways usually require active replication to be elicited and are under the control of ATR. ATR and its direct target Chk1 are activated following UV, IR, and HU treatments. ATR phosphorylates Chk1 on serine residues 317 and 345 (122–124). In turn, Chk1 phosphorylates Cdc25A on serine 123 that is then targeted for ubiquitin-dependent degradation, leading to the inhibition of Cdk2-Cyclin E complexes (125). This pathway requires an active replication fork and leads to the inhibition of late-firing origins (126,127). In addition, ATR and Chk1 have been involved in the maintenance of genome integrity during DNA replication and stabilize stalled replication forks (128–130).

3.2.2.1. DNA Damage and Replication Fork Progression. Whether signaling in response to DNA damage can directly influence fork progression is still controversial. However, recent studies show that ATM and ATR can directly phosphorylate the MCM protein complex, suggesting that these protein kinases could regulate the activity of the replicative helicase following DNA damage (116,131,132).

3.2.2.2. Other Regulators of the S-Phase Checkpoint.

Claspin/Mrc1: *Xenopus* claspin or the yeast homolog MRC1 (mediator of replication checkpoint) mediates ATR function during DNA replication. Claspin/Mrc1 interacts with DNA structures that are generated by active replication forks and contributes to ATR-dependent activation of Chk1 (108,133–136). Claspin depletion abolishes ATR-dependent Chk1 phosphorylation and abrogates the cell-cycle arrest triggered by DNA replication inhibition, causing a radio-resistant synthesis (RDS) phenotype (133). In human cells, the phosphorylated form of claspin associates with Chk1 in response to replicative stresses or DNA damage. Downregulation of claspin by SiRNA decreases Chk1 activation in response to HU (137,138). The protein kinase responsible for claspin phosphorylation in response to DNA damage has not been identified, but ATM and ATR are good candidates. Claspin has 12 SQ/TQ motifs in its sequence, coimmunoprecipitates with ATR, and its phosphorylation is caffeine sensitive (137–139). Moreover, phosphorylation of serine 864 is abolished following ATR depletion in *Xenopus* egg extracts (140). Similarly, in yeast, Mrc1 is hyperphosphorylated in response to replication blocks, and this phosphorylation is dependent on the ATR homolog Mec1 (135).

MRN Complex: ATLD and NBS cells with hypomorphic mutations in Mre11 and Nbs1, respectively, are defective in S-phase checkpoint signaling, demonstrating the role of the MRN complex in checkpoint response. ATM phosphorylates Nbs1 on several

sites in response to IR, including serine 343. This phosphorylation is required for proper S-phase checkpoint and takes place at DSBs sites, suggesting that Nbs1 is recruited to DNA damage sites (49,61,141–143). Although Nbs1 retention to DSBs is dependent on γ H2AX, its recruitment to damage sites involves its FHA and BRCT domain (144,145). This result suggests that Nbs1 could act to recruit MRN at DSBs sites.

A second branch of the intra-S-phase checkpoint, reflecting the impact of the ATM-dependent phosphorylations of Nbs1 and Smc1, is thought to be important for the transient arrest of DNA replication in response to IR. Smc1 is phosphorylated on serine 957 and 966 in an ATM- and Nbs1-dependent manner (51,146). Cells derived from knockin mice in which Smc1 cannot be phosphorylated or surexpression of a non-phosphorylatable mutant of Smc1 suppresses the intra-S-phase checkpoint (51,146,147). The phosphorylation of Smc1 is also observed in response to UV or HU treatment, is independent of ATM under these conditions, and thus, is probably under the control of ATR (148). However, a better understanding of the ATM-Nbs1-SMC1 pathway, which also depends on BRCA1 and FANCD2, needs to be developed, specially because the hypersensitivity to radiation/damage in cells lacking Nbs1 or Smc1 seems to be due to the inability of ATM to phosphorylate Smc1 (62,146,147,149).

BRCA1: BRCA1-deficient cells are defective in the S-phase checkpoint and display a RDS phenotype (150). BRCA1 is hyperphosphorylated following exposure to DNA damaging agents. In response to IR, ATM phosphorylates a cluster of serines 1387, 1423, and 1524 that stabilize its interactions with BRCA1 (150,151). ATM-dependent phosphorylations of BRCA1 are essential for the DNA damage response because non-phosphorylatable mutants of BRCA1 do not rescue the radiosensitivity of BRCA1-deficient cancer cells (152).

BRCA1 is also regulated by ATR. Both proteins coimmunoprecipitate and colocalize to nuclear foci in S-phase and after cellular exposure to IR or UV (122). ATR phosphorylates BRCA1 at serines 1143, 1280, 1387, 1423, 1457, and threonine 1394 (122). In response to IR, BRCA1 is phosphorylated by ATM, followed by ATR to maintain these events, suggesting a model in which ATM and ATR functions overlap in response to IR (153). BRCA1 could help integrating the DNA damage response by regulating ATR-dependent Chk1 activation (84).

FANC Proteins: Eleven complementation groups have been described for FA based on somatic cell fusion studies: A, B, C, D1, or BRCA2, D2, E, F, G, I, J, and L, at least eight FA genes have been cloned (154,155). FA proteins cooperate in a common DNA damage response pathway. FANCA, E, C, F, G, and L are constitutively assembled in a nuclear core complex that functions as a monoubiquitin ligase in which the catalytic subunit is FANCL (156–159). The core complex is required for FANCD2 monoubiquitination on Lysine 561 and chromatin association during S phase and following DNA damage (160). FA cells, which lack ubiquitinated FANCD2, are deficient in the assembly of IR-inducible BRCA2 foci (161). FA core complex is also recruited to the chromatin during S phase (162).

BRCA1 could also influence FANCD2 monoubiquitination. BRCA1/BARD1 heterodimers display ubiquitin ligase activity (163,164). However, FANCD2 is still ubiquitinated in cells lacking BRCA1 but is not translocated to Rad51 foci (160,165). Moreover, FANCD2 monoubiquitination is reduced but not suppressed in cells

expressing a truncated form of BRCA1, suggesting that BRCA1/BARD1 regulates the localization and/or the stability of modified FANCD2.

FA proteins regulate the intra-S-phase checkpoint. FA patients who are deficient for BRCA2 (FANCD1) or FANCD2 have a more severe clinical phenotype than patients from other FA subtypes (166,167). Cells from these patients exhibit a RDS phenotype (168).

ATM phosphorylates FANCD2 on serine 222 following exposure to IR. This phosphorylation is required for the activation of the intra-S phase checkpoint and depends on Nbs1 protein but not on FANCD2 monoubiquitination (168). FANCD2 can also be phosphorylated in an ATM-independent manner, and recent studies have indicated that ATR is required for the normal cellular response to DNA crosslinking agents and UV light. FA cells are hypersensitive to MMC and Cisplatin, and cells deficient for ATR are also defective for MMC induced-FANCD2 monoubiquitination (12,96). The mechanism by which ATR regulates FANCD2 monoubiquitination is not known, but ATR could phosphorylate components of the core complex or FANCD2 directly.

3.2.3. REGULATION OF THE S/M CHECKPOINT BY ATM AND ATR

The G2/M checkpoint prevents cells from entering mitosis when DNA is damaged during G2, when cells enter G2 with damaged DNA or if DNA has not been fully replicated during S-phase. The critical target of this checkpoint is the activity of the Cdk1-Cyclin B kinase. Cell-cycle arrest is initiated through ATM-Chk2 pathway and maintenance of the arrest depends on ATR-Chk1 (84,169–172). A-T cells are defective in IR-induced G2 arrest and enter mitosis despite the presence of damage (173). Following IR, ATM phosphorylates and activates Chk2 that is required for G2 arrest (174–177). Treatment of cells with the ATM inhibitor caffeine prevents the phosphorylation and the subsequent activation of Chk2 (178). Once activated, Chk2 phosphorylates and inhibits Cdc25C activity leading to the accumulation of inactive Cdk1-Cyclin B1 and failure to enter mitosis (179–181). Phosphorylated Cdc25C binds 14-3-3, which sequesters Cdc25C in the cytoplasm and facilitates its ubiquitin-dependent degradation (2,125,182). However, cells lacking Cdc25C have a normal G2 checkpoint, possibly because of redundancy and Cdc25A replacing Cdc25C function. Disruption of Chk1-Cdc25A pathway with the chemical Chk1 inhibitor UCN-01 or by antisense oligonucleotides also abrogates the G2 checkpoint induced by IR (181,183). Other ATM and ATR targets have also been involved in the G2/M checkpoint in addition to Chk proteins. Phosphorylation and activation of Chk2 are defective in NBS cells and correlate with the failure to arrest before mitosis (53). Whereas wild-type Nbs1 protein can rescue the G2 arrest, Nbs1 lacking the C-terminus or a form of Nbs1 unable to be phosphorylated by ATM cannot rescue the checkpoint defect. This implies that phosphorylation of Nbs1 by ATM is important for both Chk2 activation and cell-cycle arrest upon DNA damage (53). Mouse embryonic fibroblasts deficient for BRCA1 are genetically unstable and defective in G2 checkpoint (150). Cells lacking BRCA1 fail to activate Chk1. Expression of BRCA1 rescues Chk1 activation by regulating the expression of Wee1 and 14-3-3 proteins (84). This suggests that BRCA1 may regulate the G2 checkpoint through Chk1. The G2 checkpoint is also abrogated by caffeine, and substitution of serine 1423, an ATM target, to alanine in BRCA1 also abrogates

the G2 checkpoint, suggesting that phosphorylation of BRCA1 by ATM may regulate the BRCA1-Chk1 pathway (84,150,184,185).

3.3. Chromatin Remodeling

Histones are subjected to many translational modifications such as acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation (186). Chromatin is involved in transcription, replication, DNA repair, and recombination and any event impairing the stability of its structure may compromise genome integrity. Recent data link chromatin to the DNA damage response. Changes in chromatin structure can trigger a DNA damage response, and in turn, histones are targets of the DNA damage response.

As mentioned previously, chromatin structure is important for ATM activation, and DNA intercalating agents such as chloroquine or inhibitors of histone deacetylases are able to induce ATM autophosphorylation independently of DSBs (37).

Chromatin assembly has been linked with the DNA damage response. Histone regulator A (HIRA) is a chromatin assembly factor acting independently of DNA synthesis. Overexpression of HIRA induces an S-phase arrest, a process that is dependent on ATM and ATR (187). The chromatin assembly factor CAF1 is required for chromatin assembly coupled to DNA synthesis during replication and repair (188). Disruption of CAF1 in human cells using dominant negative mutants or siRNA triggers cell-cycle arrest in S phase and activates ATR (187,189). Under these conditions, DSBs accumulate, indicating that loss of histone chaperones leads to checkpoint activation and inhibition of DNA replication.

Post-translational modifications of histones play a role in the DNA damage response. Mutations of the N-terminal lysine residues of histone H3 suppress its acetylation and increase the sensibility to DSB-inducing agents (190). Similarly, mutation of the N-terminal lysine residues in Histone H4 induces the activation of a Rad9-dependent checkpoint (191). Furthermore, H2AX inactivation in mouse leads to IR radiosensitivity and genome instability and increases tumor susceptibility (145,192,193). Serine 139 of H2AX is rapidly phosphorylated in response to DSBs. In mammals, although γ H2AX is dispensable for the initial recognition of the break, it forms nuclear foci that serve as a hallmark for DSBs and allows the retention of many factors involved in DSBs signaling (145,194). Phosphorylation of H2AX requires ATM in response to IR and ATR in response to UV or DNA replication block (144,195–197). The exact function of H2AX phosphorylation is unclear, but it could induce chromatin changes and facilitate DNA repair through the recruitment of chromatin remodeling factors as it has already been described in yeast (198).

3.4. Telomere Maintenance

Telomeres are the ends of linear chromosomes and comprise protein complexes that prevent the recognition of the chromosome end as damage. When telomeres shorten or lose these protective factors, chromosome fusions and rearrangements occur leading to cell-cycle arrest, senescence, and/or apoptosis (199). Interestingly, many proteins involved in DNA repair and checkpoint pathways are activated in response to telomere dysfunction.

In addition to the telomere-specific binding proteins TRF1 and TRF2, DNA-PK, ATM, and MRN complex are found at telomeres. Together, these proteins promote the formation of a “T-loop” in the DNA, which prevents checkpoint, repair, and recombination activities (199).

The connection between DNA damage and telomere checkpoint has been essentially revealed by using a dominant negative form of TRF2 (TRF2^{ΔB/ΔM}), which inactivates endogenous TRF2. TRF2 is a small protein that, once bound to telomeric sequences, recruits all the proteins implicated in the maintenance of telomere function (199). TRF2^{ΔB/ΔM} expression induces uncapping of the telomere, generation of fused chromosomes, autophosphorylation of ATM, and its association with telomeres (200). This leads to the activation of an ATM-dependent response, inducing cell-cycle arrest and apoptosis (201). Among the proteins that are phosphorylated in response to ATM activation are H2AX, Smc1, Chk1 and Chk2, and p53, and these proteins accumulate in telomeric foci (202–206). A-T cells have an impaired response to telomere uncapping (200,207). However, an ATM-independent pathway has also been described involving the upregulation of p53 and possibly DNA-PK or ATR in the absence of ATM (200,207,208). Interestingly, recent experiments have suggested that TRF2 could play a role at DSBs in addition to telomeres (209).

However, one important question remains to determine how the activation of ATM is prevented on natural chromosome ends. It was recently shown that overexpression of TRF2 abrogates cell-cycle arrest, decreases the phosphorylation of ATM substrates such as Nbs1, and suppresses the upregulation of p53 in response to IR. This suggests that TRF2 could act to protect telomeres from ATM activation. TRF2 directly interacts with unphosphorylated ATM in proximity of serine 1981 and could block ATM autophosphorylation or prevents ATM to interact with other DNA damage proteins (210).

3.5. DNA Repair

The interface between DNA repair and cell-cycle arrest will not be covered in this review and recent reviews can be found. Many of the proteins regulating the cell-cycle response to DNA damage also participate directly or indirectly in regulating DNA repair. DSBs are repaired by two major pathways: non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ is the prominent pathway used in G1, whereas HR is used in S and G2 phases when homologous templates are available. Although ATM and ATR seem to have minor roles in repair, targets of ATM and ATR kinases are essential for repair. This is the case of BRCA1, BRCA2, MRN complex, and FANC proteins, which coordinate DNA repair and cell-cycle arrest during S and G2 phases. Moreover, CDKs have been recently implicated in the regulation of HR (211).

4. CONCLUSIONS

Much progress has been made during the past few years in our understanding of DNA damage response mechanisms. Since the discovery of ATM, many questions remain to be answered. What is the “exact” signal for ATM activation? Does ATM bind directly to the DSB or is it recruited through MRN or another high molecular weight complex? Are there additional molecular mechanisms required for its full activation? However, detection of DNA damage may not be such a simple process and may require more than just one or two proteins to fulfill this role. Supporting this idea is the finding

of “foci” or repair “centers” at damaged DNA sites, where many proteins involved in DNA repair and maintenance aggregate. It is more likely that interactions of these proteins, combined with some unidentified factors might function as DNA damage sensors.

REFERENCES

1. Loeb LA. Mutator phenotype may be required for multistage carcinogenesis. *Cancer Res* 1991;51(12):3075–9.
2. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem* 2004;73:39–85.
3. Shechter D, Costanzo V, Gautier J. ATR and ATM regulate the timing of DNA replication origin firing. *Nat Cell Biol* 2004;6(7):648–55.
4. Savitsky K, Bar-Shira A, Gilad S, et al. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* 1995;268(5218):1749–53.
5. Savitsky K, Sfez S, Tagle DA, et al. The complete sequence of the coding region of the ATM gene reveals similarity to cell cycle regulators in different species. *Hum Mol Genet* 1995;4(11):2025–32.
6. Crawford TO. Ataxia telangiectasia. *Semin Pediatr Neurol* 1998;5(4):287–94.
7. Becker-Catania SG, Gatti RA. Ataxia-telangiectasia. *Adv Exp Med Biol* 2001;495:191–8.
8. Olsen JH, Hahnemann JM, Borresen-Dale AL, et al. Cancer in patients with ataxia-telangiectasia and in their relatives in the nordic countries. *J Natl Cancer Inst* 2001;93(2):121–7.
9. Shiloh Y. Ataxia-telangiectasia and the Nijmegen breakage syndrome: related disorders but genes apart. *Annu Rev Genet* 1997;31:635–62.
10. Concannon P, Gatti RA. Diversity of ATM gene mutations detected in patients with ataxia-telangiectasia. *Hum Mutat* 1997;10(2):100–7.
11. Goodship J, Gill H, Carter J, Jackson A, Splitt M, Wright M. Autozygosity mapping of a seckel syndrome locus to chromosome 3q22.1-q24. *Am J Hum Genet* 2000;67(2):498–503.
12. O’Driscoll M, Jeggo PA. Clinical impact of ATR checkpoint signalling failure in humans. *Cell Cycle* 2003;2(3):194–5.
13. Theunissen JW, Kaplan MI, Hunt PA, et al. Checkpoint failure and chromosomal instability without lymphomagenesis in Mre11(ATLD1/ATLD1) mice. *Mol Cell* 2003;12(6):1511–23.
14. Mohaghegh P, Hickson ID. The Bloom’s syndrome helicase: keeping cancer at bay. *Biologist (London)* 2003;50(1):29–33.
15. Wang X, D’Andrea AD. The interplay of Fanconi anemia proteins in the DNA damage response. *DNA Repair (Amst)* 2004;3(8–9):1063–9.
16. Tischkowitz M, Dokal I. Fanconi anaemia and leukaemia – clinical and molecular aspects. *Br J Haematol* 2004;126(2):176–91.
17. Gatti RA, Berkel I, Boder E, et al. Localization of an ataxia-telangiectasia gene to chromosome 11q22–23. *Nature* 1988;336(6199):577–80.
18. Uziel T, Savitsky K, Platzer M, et al. Genomic Organization of the ATM gene. *Genomics* 1996;33(2):317–20.
19. Lakin ND, Weber P, Stankovic T, Rottinghaus ST, Taylor AM, Jackson SP. Analysis of the ATM protein in wild-type and ataxia telangiectasia cells. *Oncogene* 1996;13(12):2707–16.
20. Gately DP, Hittle JC, Chan GK, Yen TJ. Characterization of ATM expression, localization, and associated DNA-dependent protein kinase activity. *Mol Biol Cell* 1998;9(9):2361–74.
21. Chan DW, Gately DP, Urban S, et al. Lack of correlation between ATM protein expression and tumour cell radiosensitivity. *Int J Radiat Biol* 1998;74(2):217–24.
22. Lim DS, Kirsch DG, Canman CE, et al. ATM binds to beta-adaptin in cytoplasmic vesicles. *Proc Natl Acad Sci USA* 1998;95(17):10146–51.
23. Watters D, Kedar P, Spring K, et al. Localization of a portion of extranuclear ATM to peroxisomes. *J Biol Chem* 1999;274(48):34277–82.
24. Barlow C, Ribaut-Barassin C, Zwingman TA, et al. ATM is a cytoplasmic protein in mouse brain required to prevent lysosomal accumulation. *Proc Natl Acad Sci USA* 2000;97(2):871–6.
25. Cimprich KA, Shin TB, Keith CT, Schreiber SL. cDNA cloning and gene mapping of a candidate human cell cycle checkpoint protein. *Proc Natl Acad Sci USA* 1996;93(7):2850–5.

26. Bentley NJ, Holtzman DA, Flaggs G, et al. The *Schizosaccharomyces pombe* rad3 checkpoint gene. *EMBO J* 1996;15(23):6641–51.
27. de Klein A, Muijtjens M, van Os R, et al. Targeted disruption of the cell-cycle checkpoint gene ATR leads to early embryonic lethality in mice. *Curr Biol* 2000;10(8):479–82.
28. Brown EJ, Baltimore D. ATR disruption leads to chromosomal fragmentation and early embryonic lethality. *Genes Dev* 2000;14(4):397–402.
29. Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev* 2001;15(17):2177–96.
30. Bosotti R, Isacchi A, Sonhammer EL. FAT: a novel domain in PIK-related kinases. *Trends Biochem Sci* 2000;25(5):225–7.
31. Perry J, Kleckner N. The ATRs, ATMs, and TORs are giant HEAT repeat proteins. *Cell* 2003;112(2):151–5.
32. Kim ST, Lim DS, Canman CE, Kastan MB. Substrate specificities and identification of putative substrates of ATM kinase family members. *J Biol Chem* 1999;274(53):37538–43.
33. O'Neill T, Dwyer AJ, Ziv Y, et al. Utilization of oriented peptide libraries to identify substrate motifs selected by ATM. *J Biol Chem* 2000;275(30):22719–27.
34. Smith GC, Cary RB, Lakin ND, et al. Purification and DNA binding properties of the ataxia-telangiectasia gene product ATM. *Proc Natl Acad Sci USA* 1999;96(20):11134–9.
35. Banin S, Moyal L, Shieh S, et al. Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* 1998;281(5383):1674–7.
36. Canman CE, Lim DS, Cimprich KA, et al. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science* 1998;281(5383):1677–9.
37. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 2003;421(6922):499–506.
38. Kozlov S, Gueven N, Keating K, Ramsay J, Lavin MF. ATP activates ataxia-telangiectasia mutated (ATM) *in vitro*. Importance of autophosphorylation. *J Biol Chem* 2003;278(11):9309–17.
39. D'Amours D, Jackson SP. The Mre11 complex: at the crossroads of dna repair and checkpoint signalling. *Nat Rev Mol Cell Biol* 2002;3(5):317–27.
40. Zhang Y, Zhou J, Lim CU. The role of NBS1 in DNA double strand break repair, telomere stability, and cell cycle checkpoint control. *Cell Res* 2006;16(1):45–54.
41. Tauchi H, Kobayashi J, Morishima K, et al. The forkhead-associated domain of NBS1 is essential for nuclear foci formation after irradiation but not essential for hRAD50-hMRE11[middle dot]NBS1 complex DNA repair activity. *J Biol Chem* 2001;276(1):12–5.
42. Falck J, Coates J, Jackson SP. Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. *Nature* 2005;434(7033):605–11.
43. You Z, Chahwan C, Bailis J, Hunter T, Russell P. ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1. *Mol Cell Biol* 2005;25(13):5363–79.
44. Cersaletti K, Wright J, Concannon P. Active role for nibrin in the kinetics of atm activation. *Mol Cell Biol* 2006;26(5):1691–9.
45. Kobayashi J, Antocchia A, Tauchi H, Matsuura S, Komatsu K. NBS1 and its functional role in the DNA damage response. *DNA Repair (Amst)* 2004;3(8–9):855–61.
46. Petrini JH. The Mre11 complex and ATM: collaborating to navigate S phase. *Curr Opin Cell Biol* 2000;12(3):293–6.
47. Paull TT, Lee JH. The Mre11/Rad50/Nbs1 complex and its role as a DNA double-strand break sensor for ATM. *Cell Cycle* 2005;4(6):737–40.
48. Lisby M, Barlow JH, Burgess RC, Rothstein R. Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins. *Cell* 2004;118(6):699–713.
49. Lukas C, Falck J, Bartkova J, Bartek J, Lukas J. Distinct spatiotemporal dynamics of mammalian checkpoint regulators induced by DNA damage. *Nat Cell Biol* 2003;5(3):255–60.
50. Nakanishi K, Taniguchi T, Ranganathan V, et al. Interaction of FANCD2 and NBS1 in the DNA damage response. *Nat Cell Biol* 2002;4(12):913–20.
51. Yazdi PT, Wang Y, Zhao S, Patel N, Lee EY, Qin J. SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. *Genes Dev* 2002;16(5):571–82.
52. Lee JH, Xu B, Lee CH, et al. Distinct functions of Nijmegen breakage syndrome in ataxia telangiectasia mutated-dependent responses to DNA damage. *Mol Cancer Res* 2003;1(9):674–81.
53. Buscemi G, Savio C, Zannini L, et al. Chk2 activation dependence on Nbs1 after DNA damage. *Mol Cell Biol* 2001;21(15):5214–22.

54. Girard PM, Riballo E, Begg AC, Waugh A, Jeggo PA. Nbs1 promotes ATM dependent phosphorylation events including those required for G1/S arrest. *Oncogene* 2002;21(27):4191–9.
55. Uziel T, Lerenthal Y, Moyal L, Andegeko Y, Mittelman L, Shiloh Y. Requirement of the MRN complex for ATM activation by DNA damage. *EMBO J* 2003;22(20):5612–21.
56. Carson CT, Schwartz RA, Stracker TH, Lilley CE, Lee DV, Weitzman MD. The Mre11 complex is required for ATM activation and the G2/M checkpoint. *EMBO J* 2003;22(24):6610–20.
57. Lee JH, Paull TT. ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science* 2005;308(5721):551–4.
58. Lee JH, Paull TT. Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex. *Science* 2004;304(5667):93–6.
59. Costanzo V, Paull T, Gottesman M, Gautier J. Mre11 assembles linear DNA fragments into DNA damage signaling complexes. *PLoS Biol* 2004;2(5):E110.
60. Costanzo V, Robertson K, Ying CY, et al. Reconstitution of an ATM-dependent checkpoint that inhibits chromosomal DNA replication following DNA damage. *Mol Cell* 2000;6(3):649–59.
61. Lukas C, Melander F, Stucki M, et al. Mdc1 couples DNA double-strand break recognition by Nbs1 with its H2AX-dependent chromatin retention. *EMBO J* 2004;23(13):2674–83.
62. Goldberg M, Stucki M, Falck J, et al. MDC1 is required for the intra-S-phase DNA damage checkpoint. *Nature* 2003;421(6926):952–6.
63. Mochan TA, Venere M, DiTullio RA Jr, Halazonetis TD. 53BP1 and NFB1/MDC1-Nbs1 function in parallel interacting pathways activating ataxia-telangiectasia mutated (ATM) in response to DNA damage. *Cancer Res* 2003;63(24):8586–91.
64. Stucki M, Jackson SP. MDC1/NFB1: a key regulator of the DNA damage response in higher eukaryotes. *DNA Repair (Amst)* 2004;3(8–9):953–7.
65. Lee AC, Fernandez-Capetillo O, Pisupati V, Jackson SP, Nussenzweig A. Specific association of mouse MDC1/NFB1 with NBS1 at sites of DNA-damage. *Cell Cycle* 2005;4(1):177–82.
66. Lou Z, Minter-Dykhouse K, Franco S, et al. MDC1 maintains genomic stability by participating in the amplification of ATM-dependent DNA damage signals. *Mol Cell* 2006;21(2):187–200.
67. Iwabuchi K, Bartel PL, Li B, Marraccino R, Fields S. Two cellular proteins that bind to wild-type but not mutant p53. *Proc Natl Acad Sci USA* 1994;91(13):6098–102.
68. Iwabuchi K, Li B, Massa HF, Trask BJ, Date T, Fields S. Stimulation of p53-mediated transcriptional activation by the p53-binding proteins, 53BP1 and 53BP2. *J Biol Chem* 1998;273(40):26061–8.
69. Joo WS, Jeffrey PD, Cantor SB, Finnin MS, Livingston DM, Pavletich NP. Structure of the 53BP1 BRCT region bound to p53 and its comparison to the Brcal BRCT structure. *Genes Dev* 2002;16(5):583–93.
70. Derbyshire DJ, Basu BP, Date T, Iwabuchi K, Doherty AJ. Purification, crystallization and preliminary X-ray analysis of the BRCT domains of human 53BP1 bound to the p53 tumour suppressor. *Acta Crystallogr D Biol Crystallogr* 2002;58(Pt 10 Pt 2):1826–9.
71. Derbyshire DJ, Basu BP, Serpell LC, et al. Crystal structure of human 53BP1 BRCT domains bound to p53 tumour suppressor. *EMBO J* 2002;21(14):3863–72.
72. Schultz LB, Chehab NH, Malikzay A, Halazonetis TD. p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. *J Cell Biol* 2000;151(7):1381–90.
73. Xia Z, Morales JC, Dunphy WG, Carpenter PB. Negative cell cycle regulation and DNA damage-inducible phosphorylation of the BRCT protein 53BP1. *J Biol Chem* 2001;276(4):2708–18.
74. Anderson L, Henderson C, Adachi Y. Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. *Mol Cell Biol* 2001;21(5):1719–29.
75. Rappold I, Iwabuchi K, Date T, Chen J. Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. *J Cell Biol* 2001;153(3):613–20.
76. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* 1998;273(10):5858–68.
77. Maser RS, Monsen KJ, Nelms BE, Petrini JH. hMre11 and hRad50 nuclear foci are induced during the normal cellular response to DNA double-strand breaks. *Mol Cell Biol* 1997;17(10):6087–96.
78. Zgheib O, Huyen Y, DiTullio RA Jr, et al. ATM signaling and 53BP1. *Radiother Oncol* 2005;76(2):119–22.
79. DiTullio RA Jr, Mochan TA, Venere M, et al. 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. *Nat Cell Biol* 2002;4(12):998–1002.

80. Vialard JE, Gilbert CS, Green CM, Lowndes NF. The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage. *EMBO J* 1998;17(19):5679–88.
81. Emili A. MEC1-dependent phosphorylation of Rad9p in response to DNA damage. *Mol Cell* 1998;2(2):183–9.
82. Zhang J, Powell SN. The role of the BRCA1 tumor suppressor in DNA double-strand break repair. *Mol Cancer Res* 2005;3(10):531–9.
83. Foray N, Marot D, Gabriel A, et al. A subset of ATM- and ATR-dependent phosphorylation events requires the BRCA1 protein. *EMBO J* 2003;22(11):2860–71.
84. Yarden RI, Pardo-Reoyo S, Sgagias M, Cowan KH, Brody LC. BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage. *Nat Genet* 2002;30(3):285–9.
85. Wang Y, Cortez D, Yazdi P, Neff N, Elledge SJ, Qin J. BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes Dev* 2000;14(8):927–39.
86. Ali A, Zhang J, Bao S, et al. Requirement of protein phosphatase 5 in DNA-damage-induced ATM activation. *Genes Dev* 2004;18(3):249–54.
87. Goodarzi AA, Jonnalagadda JC, Douglas P, et al. Autophosphorylation of ataxia-telangiectasia mutated is regulated by protein phosphatase 2A. *EMBO J* 2004;23(22):4451–61.
88. Unsal-Kacmaz K, Makhov AM, Griffith JD, Sancar A. Preferential binding of ATR protein to UV-damaged DNA. *Proc Natl Acad Sci USA* 2002;99(10):6673–8.
89. Guo Z, Kumagai A, Wang SX, Dunphy WG. Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in *Xenopus* egg extracts. *Genes Dev* 2000;14(21):2745–56.
90. Shiloh Y. ATM and ATR: networking cellular responses to DNA damage. *Curr Opin Genet Dev* 2001;11(1):71–7.
91. Hammond EM, Dorie MJ, Giaccia AJ. Inhibition of ATR leads to increased sensitivity to hypoxia/reoxygenation. *Cancer Res* 2004;64(18):6556–62.
92. Heffernan TP, Simpson DA, Frank AR, et al. An ATR- and Chk1-dependent S checkpoint inhibits replicon initiation following UVC-induced DNA damage. *Mol Cell Biol* 2002;22(24):8552–61.
93. Cliby WA, Roberts CJ, Cimprich KA, et al. Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. *EMBO J* 1998;17(1):159–69.
94. Unsal-Kacmaz K, Sancar A. Quaternary structure of ATR and effects of ATRIP and replication protein A on its DNA binding and kinase activities. *Mol Cell Biol* 2004;24(3):1292–300.
95. Bomgardner RD, Yean D, Yee MC, Cimprich KA. A novel protein activity mediates DNA binding of an ATR-ATRIP complex. *J Biol Chem* 2004;279(14):13346–53.
96. Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* 2003;300(5625):1542–8.
97. Cortez D, Guntuku S, Qin J, Elledge SJ. ATR and ATRIP: partners in checkpoint signaling. *Science* 2001;294(5547):1713–6.
98. You Z, Kong L, Newport J. The role of single-stranded DNA and polymerase alpha in establishing the ATR, Hus1 DNA replication checkpoint. *J Biol Chem* 2002;277(30):27088–93.
99. Costanzo V, Gautier J. Single-strand DNA gaps trigger an ATR- and Cdc7-dependent checkpoint. *Cell Cycle* 2003;2(1):17.
100. Itakura E, Takai KK, Umeda K, et al. Amino-terminal domain of ATRIP contributes to intranuclear relocation of the ATR-ATRIP complex following DNA damage. *FEBS Lett* 2004;577(1–2):289–93.
101. Itakura E, Umeda K, Sekoguchi E, Takata H, Ohsumi M, Matsuura A. ATR-dependent phosphorylation of ATRIP in response to genotoxic stress. *Biochem Biophys Res Commun* 2004;323(4):1197–202.
102. Jazayeri A, Falck J, Lukas C, et al. ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat Cell Biol* 2006;8(1):37–45.
103. Adams KE, Medhurst AL, Dart DA, Lakin ND. Recruitment of ATR to sites of ionising radiation-induced DNA damage requires ATM and components of the MRN protein complex. *Oncogene* 2006;25(28):3894–904.
104. Myers JS, Cortez D. Rapid activation of ATR by ionizing radiation requires ATM and Mre11. *J Biol Chem* 2006;281(14):9346–50.

105. Chan DW, Son SC, Block W, et al. Purification and characterization of ATM from human placenta. A manganese-dependent, wortmannin-sensitive serine/threonine protein kinase. *J Biol Chem* 2000;275(11):7803–10.
106. Suzuki K, Kodama S, Watanabe M. Recruitment of ATM protein to double strand DNA irradiated with ionizing radiation. *J Biol Chem* 1999;274(36):25571–5.
107. Andegeko Y, Moyal L, Mittelman L, Tsarfaty I, Shiloh Y, Rotman G. Nuclear retention of ATM at sites of DNA double strand breaks. *J Biol Chem* 2001;276(41):38224–30.
108. Kumagai A, Kim SM, Dunphy WG. Claspin and the activated form of ATR-ATRIP collaborate in the activation of Chk1. *J Biol Chem* 2004;279(48):49599–608.
109. Bell SP, Dutta A. DNA replication in eukaryotic cells. *Annu Rev Biochem* 2002;71:333–74.
110. Petersen P, Chou DM, You Z, Hunter T, Walter JC, Walter G. Protein phosphatase 2A antagonizes ATM and ATR in a Cdk2- and Cdc7-independent DNA damage checkpoint. *Mol Cell Biol* 2006;26(5):1997–2011.
111. Massague J. G1 cell-cycle control and cancer. *Nature* 2004;432(7015):298–306.
112. Costanzo V, Robertson K, Bibikova M, et al. Mre11 protein complex prevents double-strand break accumulation during chromosomal DNA replication. *Mol Cell* 2001;8(1):137–47.
113. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. *Nature* 2004;432(7015):316–23.
114. Falck J, Petrini JH, Williams BR, Lukas J, Bartek J. The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways. *Nat Genet* 2002;30(3):290–4.
115. Kim JM, Yamada M, Masai H. Functions of mammalian Cdc7 kinase in initiation/monitoring of DNA replication and development. *Mutat Res* 2003;532(1–2):29–40.
116. Yoo HY, Shevchenko A, Shevchenko A, Dunphy WG. Mcm2 is a direct substrate of ATM and ATR during DNA damage and DNA replication checkpoint responses. *J Biol Chem* 2004;279(51):53353–64.
117. Lei M, Kawasaki Y, Young MR, Kihara M, Sugino A, Tye BK. Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. *Genes Dev* 1997;11(24):3365–74.
118. Zou L, Stillman B. Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. *Mol Cell Biol* 2000;20(9):3086–96.
119. Sato N, Arai K, Masai H. Human and *Xenopus* cDNAs encoding budding yeast Cdc7-related kinases: *in vitro* phosphorylation of MCM subunits by a putative human homologue of Cdc7. *EMBO J* 1997;16(14):4340–51.
120. Jones GG, Reaper PM, Pettitt AR, Sherrington PD. The ATR-p53 pathway is suppressed in noncycling normal and malignant lymphocytes. *Oncogene* 2004;23(10):1911–21.
121. Dierov J, Dierova R, Carroll M. BCR/ABL translocates to the nucleus and disrupts an ATR-dependent intra-S phase checkpoint. *Cancer Cell* 2004;5(3):275–85.
122. Tibbetts RS, Cortez D, Brumbaugh KM, et al. Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. *Genes Dev* 2000;14(23):2989–3002.
123. Zhao H, Piwnicka-Worms H. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. *Mol Cell Biol* 2001;21(13):4129–39.
124. Liu Q, Guntuku S, Cui XS, et al. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev* 2000;14(12):1448–59.
125. Busino L, Chiesa M, Draetta GF, Donzelli M. Cdc25A phosphatase: combinatorial phosphorylation, ubiquitylation and proteolysis. *Oncogene* 2004;23(11):2050–6.
126. Hekmat-Nejad M, You Z, Yee MC, Newport JW, Cimprich KA. *Xenopus* ATR is a replication-dependent chromatin-binding protein required for the DNA replication checkpoint. *Curr Biol* 2000;10(24):1565–73.
127. Lupardus PJ, Byun T, Yee MC, Hekmat-Nejad M, Cimprich KA. A requirement for replication in activation of the ATR-dependent DNA damage checkpoint. *Genes Dev* 2002;16(18):2327–32.
128. Shechter D, Costanzo V, Gautier J. Regulation of DNA replication by ATR: signaling in response to DNA intermediates. *DNA Repair (Amst)* 2004;3(8–9):901–8.
129. Lopes M, Cotta-Ramusino C, Pelliccioli A, et al. The DNA replication checkpoint response stabilizes stalled replication forks. *Nature* 2001;412(6846):557–61.
130. Lucca C, Vanoli F, Cotta-Ramusino C, et al. Checkpoint-mediated control of replisome-fork association and signalling in response to replication pausing. *Oncogene* 2004;23(6):1206–13.
131. Cortez D, Glick G, Elledge SJ. Minichromosome maintenance proteins are direct targets of the ATM and ATR checkpoint kinases. *Proc Natl Acad Sci USA* 2004;101(27):10078–83.

132. Shechter D, Gautier J. MCM proteins and checkpoint kinases get together at the fork. *Proc Natl Acad Sci USA* 2004;101(30):10845–6.
133. Kumagai A, Dunphy WG. Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in *Xenopus* egg extracts. *Mol Cell* 2000;6(4):839–49.
134. Alcasabas AA, Osborn AJ, Bachant J, et al. Mrc1 transduces signals of DNA replication stress to activate Rad53. *Nat Cell Biol* 2001;3(11):958–65.
135. Osborn AJ, Elledge SJ. Mrc1 is a replication fork component whose phosphorylation in response to DNA replication stress activates Rad53. *Genes Dev* 2003;17(14):1755–67.
136. Zhao H, Tanaka K, Nogochi E, Nogochi C, Russell P. Replication checkpoint protein Mrc1 is regulated by Rad3 and Tel1 in fission yeast. *Mol Cell Biol* 2003;23(22):8395–403.
137. Chini CC, Chen J. Human claspin is required for replication checkpoint control. *J Biol Chem* 2003;278(32):30057–62.
138. Chini CC, Chen J. Claspin, a regulator of Chk1 in DNA replication stress pathway. *DNA Repair (Amst)* 2004;3(8–9):1033–7.
139. Jeong SY, Kumagai A, Lee J, Dunphy WG. Phosphorylated claspin interacts with a phosphate-binding site in the kinase domain of Chk1 during ATR-mediated activation. *J Biol Chem* 2003;278(47):46782–8.
140. Kumagai A, Dunphy WG. Repeated phosphopeptide motifs in Claspin mediate the regulated binding of Chk1. *Nat Cell Biol* 2003;5(2):161–5.
141. Lim DS, Kim ST, Xu B, et al. ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. *Nature* 2000;404(6778):613–7.
142. Wu X, Ranganathan V, Weisman DS, et al. ATM phosphorylation of Nijmegen breakage syndrome protein is required in a DNA damage response. *Nature* 2000;405(6785):477–82.
143. Zhao S, Weng YC, Yuan SS, et al. Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. *Nature* 2000;405(6785):473–7.
144. Fernandez-Capetillo O, Celeste A, Nussenzweig A. Focusing on foci: H2AX and the recruitment of DNA-damage response factors. *Cell Cycle* 2003;2(5):426–7.
145. Celeste A, Fernandez-Capetillo O, Kruhlak MJ, et al. Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. *Nat Cell Biol* 2003;5(7):675–9.
146. Kim JS, Krasieva TB, LaMorte V, Taylor AM, Yokomori K. Specific recruitment of human cohesin to laser-induced DNA damage. *J Biol Chem* 2002;277(47):45149–53.
147. Kitagawa R, Bakkenist CJ, McKinnon PJ, Kastan MB. Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-BRCA1 pathway. *Genes Dev* 2004;18(12):1423–38.
148. Pichierrri P, Rosselli F. The DNA crosslink-induced S-phase checkpoint depends on ATR-CHK1 and ATR-NBS1-FANCD2 pathways. *EMBO J* 2004;23(5):1178–87.
149. Stewart GS, Wang B, Bignell CR, Taylor AM, Elledge SJ. MDC1 is a mediator of the mammalian DNA damage checkpoint. *Nature* 2003;421(6926):961–6.
150. Xu B, Kim S, Kastan MB. Involvement of Brca1 in S-phase and G(2)-phase checkpoints after ionizing irradiation. *Mol Cell Biol* 2001;21(10):3445–50.
151. Gatei M, Scott SP, Filippovitch I, et al. Role for ATM in DNA damage-induced phosphorylation of BRCA1. *Cancer Res* 2000;60(12):3299–304.
152. Cortez D, Wang Y, Qin J, Elledge SJ. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. *Science* 1999;286(5442):1162–6.
153. Gatei M, Zhou BB, Hobson K, Scott S, Young D, Khanna KK. Ataxia telangiectasia mutated (ATM) kinase and ATM and Rad3 related kinase mediate phosphorylation of Brca1 at distinct and overlapping sites. *In vivo* assessment using phospho-specific antibodies. *J Biol Chem* 2001;276(20):17276–80.
154. Joenje H, Oostra AB, Wijker M, et al. Evidence for at least eight Fanconi anemia genes. *Am J Hum Genet* 1997;61(4):940–4.
155. Levitus M, Rooimans MA, Steltenpool J, et al. Heterogeneity in Fanconi anemia: evidence for 2 new genetic subtypes. *Blood* 2004;103(7):2498–503.
156. Garcia-Higuera I, Kuang Y, Naf D, Wasik J, D'Andrea AD. Fanconi anemia proteins FANCA, FANCC, and FANCG/XRCC9 interact in a functional nuclear complex. *Mol Cell Biol* 1999;19(7):4866–73.
157. de Winter JP, van der Weel L, de Groot J, et al. The Fanconi anemia protein FANCF forms a nuclear complex with FANCA, FANCC and FANCG. *Hum Mol Genet* 2000;9(18):2665–74.
158. Meetei AR, Sechi S, Wallisch M, et al. A multiprotein nuclear complex connects Fanconi anemia and Bloom syndrome. *Mol Cell Biol* 2003;23(10):3417–26.

159. Meetei AR, Levitus M, Xue Y, et al. X-linked inheritance of Fanconi anemia complementation group B. *Nat Genet* 2004;36(11):1219–24.
160. Garcia-Higuera I, Taniguchi T, Ganesan S, et al. Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol Cell* 2001;7(2):249–62.
161. Wang X, Andreassen PR, D'Andrea AD. Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. *Mol Cell Biol* 2004;24(13):5850–62.
162. Kupfer G, Naf D, Garcia-Higuera I, et al. A patient-derived mutant form of the Fanconi anemia protein, FANCA, is defective in nuclear accumulation. *Exp Hematol* 1999;27(4):587–93.
163. Hashizume R, Fukuda M, Maeda I, et al. The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. *J Biol Chem* 2001;276(18):14537–40.
164. Brzovic PS, Rajagopal P, Hoyt DW, King MC, Klevit RE. Structure of a BRCA1-BARD1 heterodimeric RING-RING complex. *Nat Struct Biol* 2001;8(10):833–7.
165. Folias A, Matkovic M, Bruun D, et al. BRCA1 interacts directly with the Fanconi anemia protein FANCA. *Hum Mol Genet* 2002;11(21):2591–7.
166. Hirsch B, Shimamura A, Moreau L, et al. Association of biallelic BRCA2/FANCD1 mutations with spontaneous chromosomal instability and solid tumors of childhood. *Blood* 2004;103(7):2554–9.
167. Howlett NG, Taniguchi T, Olson S, et al. Biallelic inactivation of BRCA2 in Fanconi anemia. *Science* 2002;297(5581):606–9.
168. Taniguchi T, Garcia-Higuera I, Xu B, et al. Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. *Cell* 2002;109(4):459–72.
169. Brown EJ, Baltimore D. Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. *Genes Dev* 2003;17(5):615–28.
170. Xu X, Tsvetkov LM, Stern DF. Chk2 activation and phosphorylation-dependent oligomerization. *Mol Cell Biol* 2002;22(12):4419–32.
171. Rhind N, Russell P. Roles of the mitotic inhibitors Wee1 and Mik1 in the G(2) DNA damage and replication checkpoints. *Mol Cell Biol* 2001;21(5):1499–508.
172. Walworth NC. DNA damage: Chk1 and Cdc25, more than meets the eye. *Curr Opin Genet Dev* 2001;11(1):78–82.
173. Beamish H, Williams R, Chen P, et al. Rapamycin resistance in ataxia-telangiectasia. *Oncogene* 1996;13(5):963–70.
174. Chaturvedi P, Eng WK, Zhu Y, et al. Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway. *Oncogene* 1999;18(28):4047–54.
175. Matsuoka S, Rotman G, Ogawa A, Shiloh Y, Tamai K, Elledge SJ. Ataxia telangiectasia-mutated phosphorylates Chk2 *in vivo* and *in vitro*. *Proc Natl Acad Sci USA* 2000;97(19):10389–94.
176. Melchionna R, Chen XB, Blasina A, McGowan CH. Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. *Nat Cell Biol* 2000;2(10):762–5.
177. Hirao A, Kong YY, Matsuoka S, et al. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science* 2000;287(5459):1824–7.
178. Zhou BB, Chaturvedi P, Spring K, et al. Caffeine abolishes the mammalian G(2)/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity. *J Biol Chem* 2000;275(14):10342–8.
179. Matsuoka S, Huang M, Elledge SJ. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science* 1998;282(5395):1893–7.
180. Blasina A, Price BD, Turenne GA, McGowan CH. Caffeine inhibits the checkpoint kinase ATM. *Curr Biol* 1999;9(19):1135–8.
181. Lopez-Girona A, Tanaka K, Chen XB, Baber BA, McGowan CH, Russell P. Serine-345 is required for Rad3-dependent phosphorylation and function of checkpoint kinase Chk1 in fission yeast. *Proc Natl Acad Sci USA* 2001;98(20):11289–94.
182. Wilker E, Yaffe MB. 14-3-3 Proteins—a focus on cancer and human disease. *J Mol Cell Cardiol* 2004;37(3):633–42.
183. Luo Y, Rockow-Magnone SK, Kroeger PE, et al. Blocking Chk1 expression induces apoptosis and abrogates the G2 checkpoint mechanism. *Neoplasia* 2001;3(5):411–9.
184. Xu B, Kim ST, Lim DS, Kastan MB. Two molecularly distinct G(2)/M checkpoints are induced by ionizing irradiation. *Mol Cell Biol* 2002;22(4):1049–59.
185. Xu B, O'Donnell AH, Kim ST, Kastan MB. Phosphorylation of serine 1387 in Brcal is specifically required for the Atm-mediated S-phase checkpoint after ionizing irradiation. *Cancer Res* 2002;62(16):4588–91.

186. Turner BM. Histone acetylation and an epigenetic code. *Bioessays* 2000;22(9):836–45.
187. Ye X, Franco AA, Santos H, Nelson DM, Kaufman PD, Adams PD. Defective S phase chromatin assembly causes DNA damage, activation of the S phase checkpoint, and S phase arrest. *Mol Cell* 2003;11(2):341–51.
188. Gaillard PH, Martini EM, Kaufman PD, Stillman B, Moustacchi E, Almouzni G. Chromatin assembly coupled to DNA repair: a new role for chromatin assembly factor I. *Cell* 1996;86(6): 887–96.
189. Hoek M, Stillman B. Chromatin assembly factor 1 is essential and couples chromatin assembly to DNA replication *in vivo*. *Proc Natl Acad Sci USA* 2003;100(21):12183–8.
190. Qin S, Parthun MR. Histone H3 and the histone acetyltransferase Hat1p contribute to DNA double-strand break repair. *Mol Cell Biol* 2002;22(23):8353–65.
191. Megee PC, Morgan BA, Smith MM. Histone H4 and the maintenance of genome integrity. *Genes Dev* 1995;9(14):1716–27.
192. Bassing CH, Alt FW. H2AX may function as an anchor to hold broken chromosomal DNA ends in close proximity. *Cell Cycle* 2004;3(2):149–53.
193. Celeste A, Difilippantonio S, Difilippantonio MJ, et al. H2AX haploinsufficiency modifies genomic stability and tumor susceptibility. *Cell* 2003;114(3):371–83.
194. Rogakou EP, Boon C, Redon C, Bonner WM. Megabase chromatin domains involved in DNA double-strand breaks *in vivo*. *J Cell Biol* 1999;146(5):905–16.
195. Downs JA, Lowndes NF, Jackson SP. A role for *Saccharomyces cerevisiae* histone H2A in DNA repair. *Nature* 2000;408(6815):1001–4.
196. Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol* 2000;10(15):886–95.
197. Fernandez-Capetillo O, Chen HT, Celeste A, et al. DNA damage-induced G2-M checkpoint activation by histone H2AX and 53BP1. *Nat Cell Biol* 2002;4(12):993–7.
198. Widlak P, Pietrowska M, Lanuszewska J. The role of chromatin proteins in DNA damage recognition and repair Mini-review. *Histochem Cell Biol* 2006;125(1–2):119–26.
199. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev* 2005;19(18):2100–10.
200. Takai H, Smogorzewska A, de Lange T. DNA damage foci at dysfunctional telomeres. *Curr Biol* 2003;13(17):1549–56.
201. Brunori M, Luciano P, Gilson E, Geli V. The telomerase cycle: normal and pathological aspects. *J Mol Med* 2005;83(4):244–57.
202. Karlseder J, Broccoli D, Dai Y, Hardy S, de Lange T. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science* 1999;283(5406):1321–5.
203. Karlseder J, Smogorzewska A, de Lange T. Senescence induced by altered telomere state, not telomere loss. *Science* 2002;295(5564):2446–9.
204. Smogorzewska A, de Lange T. Different telomere damage signaling pathways in human and mouse cells. *EMBO J* 2002;21(16):4338–48.
205. Smogorzewska A, Karlseder J, Holtgreve-Grez H, Jauch A, de Lange T. DNA ligase IV-dependent NHEJ of deprotected mammalian telomeres in G1 and G2. *Curr Biol* 2002;12(19):1635–44.
206. d’Adda di Fagagna F, Reaper PM, Clay-Farrace L, et al. A DNA damage checkpoint response in telomere-initiated senescence. *Nature* 2003;426(6963):194–8.
207. Karlseder J. Telomere repeat binding factors: keeping the ends in check. *Cancer Lett* 2003;194(2):189–97.
208. Wong KK, Chang S, Weiler SR, et al. Telomere dysfunction impairs DNA repair and enhances sensitivity to ionizing radiation. *Nat Genet* 2000;26(1):85–8.
209. Bradshaw PS, Stavropoulos DJ, Meyn MS. Human telomeric protein TRF2 associates with genomic double-strand breaks as an early response to DNA damage. *Nat Genet* 2005;37(2):193–7.
210. Karlseder J, Hoke K, Mirzoeva OK, et al. The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response. *PLoS Biol* 2004;2(8):E240.
211. Ira G, Pelliccioli A, Balijja A, et al. DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. *Nature* 2004;431(7011):1011–7.

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Interactions Between Myc- and Cyclin-Dependent Kinase Inhibitors in Cancer

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SUMMARY

Deregulated cell growth and the inhibition of apoptosis are hallmarks of cancer. The *MYC* family of oncogenes are pivotal players in tumorigenesis and are altered in most tumor types. *c-Myc* is the founding member of a family of structurally related basic helix-loop-helix-leucine zipper (bHLH-Zip) proteins that function as sequence-specific transcription factors and are aberrantly expressed in most cancers. *c-Myc* is a key regulator of cell proliferation and differentiation, and its expression is both necessary and sufficient to drive quiescent cells into S phase. Following mitogenic stimulation, *c-Myc* is rapidly induced; remains elevated throughout the cell cycle and, through dimerization with its bHLH-Zip partner Max, regulates the transcription of genes essential for cell growth and division. Conversely, *c-Myc* expression is rapidly suppressed by growth inhibitory signals such as transforming growth factor β . However, these controls are lost in cancers by translocations, amplifications, and alterations in regulatory signaling pathways, resulting in abnormally high levels of *MYC* oncoproteins. The precise roles that *Myc* oncoproteins provide to provoke tumorigenesis are not fully resolved but do include the regulation of target genes that control cell division, differentiation, cell size, and angiogenesis. These target genes include members of the cyclin dependent kinase inhibitors (encompassing the Ink4 family and the Cip/Kip family of inhibitors), responsible for inhibiting the activity of cyclin/cyclin-dependent kinase complexes that regulate cell cycle traverse. The regulation of these inhibitors by *Myc* clearly represents an important target in cancer prevention and therapeutics.

Key Words: *Myc*, CDK, CDK inhibitors, Cyclins, Ink4, Cip/Kip.

1. INTRODUCTION

The genomic integrity of multicellular organisms is ensured by various components of the DNA repair machinery and/or by the decision of cells to undergo apoptosis or senescence. Collectively, these programs prevent cells that have accumulated DNA

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mutations from developing into cancer cells. Once thought to simply provide a proliferative advantage to the malignant cell, the activation of oncogenes is now recognized to also provide new avenues for cancer therapeutics. Foremost among these potential targets are the Myc family of oncogenes (c-Myc, N-Myc, and L-Myc) that function as master regulatory transcription factors overexpressed in approximately 70% of all human cancers (1–4). The array of functions executed by Myc oncoproteins underscore their importance in tumor cell biology, as their enhanced expression drives unrestricted cell proliferation and increases in cell mass and ribosome biogenesis (5–10), inhibits terminal differentiation programs (11,12), and provokes tumor angiogenesis (13,14). Paradoxically, in normal cells, Myc overexpression triggers the apoptotic program (15,16), which acts as a defense mechanism against transformation, and Myc also promotes genomic instability (17). Collectively, these events safeguard cancer cells from progressing toward overt malignancy. Furthermore, Myc functions appear necessary to maintain the tumorigenic state, as even brief inactivation of Myc causes rapid tumor regression because of the induction of apoptosis (17,18). Thus, Myc is widely viewed as an important target in both cancer chemoprevention and cancer therapeutics.

On the flip side of cell growth control are a series of dedicated inhibitors that function to hold the cell cycle in check by inhibiting the activity of cyclin/cyclin-dependent kinase (Cdk) complexes (cyclin D/Cdk4;Cdk6, cyclin E/Cdk2, cyclin A/Cdk2, and cyclin B/Cdk1) that normally phosphorylate key substrates to allow entry and progression through the cell cycle. Cdk inhibitors fall broadly into those that specifically target cyclin-D/Cdk4;Cdk6 complexes, the Ink4 family of inhibitors (p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, and p19^{Ink4d}), and those that broadly inhibit all Cdks, the Cip/Kip family (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}). Notably, at least two of the Inks, p18^{Ink4c} and p16^{Ink4a}, as well as p27^{Kip1} play essential roles as guardians against tumorigenesis. For example, gene targeting has established that mice lacking p16^{Ink4a}, p18^{Ink4c}, or p27^{Kip1} are tumor-prone, especially when exposed to mutagens (19–23). Furthermore, deletion or silencing of the *INK4A* locus and the suppression of *INK4B*, *INK4C*, and *KIP2* expression are common features of many human malignancies (24–28). In addition, the suppression or loss of heterozygosity (LOH) of *KIP1* is a well-established predictor of poor prognosis in many human cancers (29–32). Finally, there are now obvious connections between Myc and many of the Cdk inhibitors in tumorigenesis. Herein, we describe these interactions and their relevance for tumorigenesis and cancer therapy.

2. MYC'S TRANSCRIPTION FUNCTIONS

Initial studies by Bishop and Varmus and colleagues demonstrated that v-Myc was the oncogene of the avian myelocytomatosis (MC29) retrovirus. Subsequent work then revealed that v-Myc's cellular homolog c-Myc, as well as the other Myc family members N-Myc and L-Myc, is activated by chromosomal translocations or amplifications in many human cancers (including breast and prostate, lymphoma, leukemia, colon and cervical carcinoma, melanoma, glioblastoma, and small-cell lung carcinoma). Furthermore, *Myc* genes are often overexpressed in cancers having mutations in other signaling pathways and in those having mutations in tumor suppressors such as p53, which negatively regulate Myc expression (33).

Myc oncoproteins function as transcription factors that repress or activate the expression of targets necessary for cell-cycle entry and traverse and for cell growth. First, their N-terminus harbor domains conserved among Myc family proteins, Myc boxes I and II (MBI and MBII), that regulate Myc's interactions with transcriptional co-activators and co-repressors. Indeed, MBII plays an essential role in transactivation and transrepression of the numerous Myc target genes (34–36). The N-terminus also contains a domain that controls the rates of Myc protein turnover, which have been linked to changes in Myc transcriptional activity (37–39) and appear largely mediated by the F-box protein Fbw7 (40–42). The C-terminal domain of Myc harbors a basic helix-loop-helix leucine zipper (B-HLH-Zip) motif, which mediates specific DNA binding and dimerization with another B-HLH-Zip protein coined Max. Myc–Max heterodimers activate transcription by binding to E-box elements (CACGTG), and activation involves the recruitment of multiple co-activators such as TRRAP and BAF53 (43,44) and their associated histone acetyltransferases [GCN5, CREB-binding protein (CBP), and p300] (45,46) and ATPase/helicases (such as TIP48 and TIP49) (47,48). Furthermore, the central region of Myc harbors another conserved domain termed MBIII, which contributes to Myc's transrepression and transformation functions (49), and yet another domain juxtaposed to Myc's basic DNA-binding motif has been suggested to be a target for monoubiquitylation and to be important for Myc's transactivation functions (39).

In addition to the Myc–Max interaction, Max can also form homodimers or can heterodimerize with several related B-HLH-Zip proteins termed Mad1, Mxi1 (Mads), Mad3, Mad4, Mnt, and Mga (3). However, Mad–Max and Mnt–Max heterodimers function to repress transcription by recruiting histone deacetylases through their ability to interact with the transcriptional co-repressor Sin3 (50–52). Interestingly, Myc–Max dimers can also repress transcription when bound to promoters regulated by the Miz1 transcription factor (53–55). Repression in this scenario occurs either through disruption of the interaction between Miz1 and p300 or by recruiting the DNA methyltransferase DNMT3a (56). Nonetheless, the ultimate balance between transcriptional activation and repression is largely determined by the absolute levels of Myc versus Mad or Mnt proteins, all of which have relatively short half-lives (57).

Myc-induced tumorigenesis requires its transcription functions. A number of genes induced by Myc have been suggested to be important for its biological effects (3,39,58–61), yet important targets also include those that are directly repressed by Myc (55,62–64), and the classification of a true Myc targets is rather controversial, particularly because several putative Myc targets are not regulated by Myc *in vivo* (65,66). Indeed, as much of 15% of the genome has been suggested to be regulated by Myc (see <http://www.myc-cancer-gene.org>), and among these are key targets that play direct roles in tumorigenesis, particularly the Ink and Cip/Kip Cdk inhibitors.

3. MYC AND GROWTH CONTROL

Under normal physiological conditions, the expression of *Myc* genes is tightly regulated by signaling pathways emanating from the engagement of growth factors with their receptors and adhesion complexes. Indeed, resting, quiescent cells express very little Myc RNA or protein, whereas high levels of *Myc* are induced, as an

immediate-early response gene, in cells stimulated by mitogens, and in proliferating cells, Myc protein levels are maintained at a constant level throughout the cell cycle. However, Myc transcription is quickly repressed by growth-inhibitory signals [e.g., by transforming growth factor-beta (TGF - β)] or following the withdrawal of mitogens, and Myc RNAs and proteins all have very short half-lives, allowing for rapid reductions in pools of Myc. This response is important, as silencing of Myc expression is necessary for withdrawal from the cell cycle and for terminal differentiation, and especially as unchecked Myc expression provokes additional genetic alterations events that lead to cancer.

The crucial roles of Myc in growth control were underscored by the creation of the *c-Myc* and *N-Myc* knockout mice, which die at mid-gestation with obvious defects in their growth and development (67). Most importantly, the analysis of conditional Myc knockouts revealed that acute loss of Myc leads to an immediate arrest in G1 in most (but not all) cell types (68–70). Furthermore, Myc overexpression, or even microinjection, is sufficient to drive quiescent cells into cycle and into S phase (5,71–73). Thus, Myc is both necessary and sufficient for cell proliferation.

So how then does Myc regulate cell-cycle entry and traverse? Obvious targets would be expected to include those that are necessary for cell-cycle progression. Indeed, Myc induces the transcription of some of the D-type cyclins (63), cyclin E, and cyclin A (74), and it also induces the transcription of Cdk4 (75). Cyclin D-Cdk4 and cyclin E-Cdk2 complexes phosphorylate many key targets required for S-phase entry, in particular the retinoblastoma protein (pRb) (reviewed in ref. 4). Furthermore, at least in some cell contexts, D-type cyclins and Cdk4 appear to play important roles in Myc-induced transformation (76,77).

Proteins that inhibit cell-cycle traverse are also important targets of Myc. For example, it is well established that Myc represses the expression of Cdk inhibitors p21^{Cip1}, p27^{Kip1}, and p15^{Ink4b} and that their repression contributes to Myc's ability to drive cell-cycle progression. Transcriptional induction of *Cip1* and *Ink4b* requires the functions of the transactivator Miz-1, and Myc's interactions with Miz-1 disables in transactivation functions, by for example recruiting transcriptional co-repressors to the *Cip1* and *Ink4b* promoters (39,55,64,78). Suppression of p27^{Kip1} expression by Myc is more complex, as Myc represses *Kip1* transcription, but also more profoundly provokes p27^{Kip1} degradation (61,63,79,80). Thus, Myc coordinately regulates components of the cell-cycle machinery to drive cell-cycle traverse.

4. INK4 AND CIP/KIP CDK INHIBITORS IN CELL-CYCLE CONTROL

The four Ink4 family members p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, and p19^{Ink4d}, all specifically bind to Cdk4 and Cdk6 and prevent their interactions with the D-type cyclins (81). This effectively cancels the activity of cyclin D-Cdk4/6 complexes in phosphorylating and inactivating pRb, and as a net result, overexpression of any of the Ink4 inhibitors leads to arrest in the G1 phase of the cell cycle in a pRb-dependent manner (82). All four Ink4 family proteins are similar in their structure and harbor several ankyrin repeats that direct their binding and inhibition of Cdk4/6 (reviewed in ref. 83). Interestingly, although they all essentially perform the same function, each Ink4 has distinct patterns of expression during mouse development and differentiation, with p18^{Ink4c} and p19^{Ink4d} playing key roles in regulating cell-cycle exit during development and differentiation, whereas p16^{Ink4a} and p15^{Ink4b} are rather expressed in response to stress- or

growth-inhibitory signals such as those provoked by “culture shock” or directed by TGF- β . In particular, p16^{Ink4a} is induced as cells undergo senescence (84). Remarkably, the *INK4a* locus also encodes an alternative reading frame (ARF) product termed p19^{Arf} (p14^{Arf} in humans), whose role is to rather interact with and inhibit the functions of Mdm2, an oncogene that harnesses the activity of the tumor suppressor p53, by ubiquitylating p53 and targeting it for destruction by the proteasome (85). Thus, in the face of sustained expression of Arf, such as occurs in response to hyperproliferative signals provoked by oncogenes, p53 protein levels rapidly accumulate, and it induces the transcription of downstream targets that induce cell-cycle arrest or apoptosis (86).

Given their roles, one would predict rather dire consequences of inactivating *Ink4* genes on cell-cycle control in general and on development in particular. Indeed, although their functional redundancy likely dampens the effects of deleting the *Inks4s* individually, there are notable phenotypes with regard to growth control associated with the knockout of the *Ink4* genes in mice.

Initial targeting studies of the *Ink4a* locus deleted *Ink4a* exon 2, which contains coding sequences of both Ink4a and Arf, and although these mice develop normally, they are highly prone to tumors (100% penetrance), suggesting that Ink4a was a bona fide tumor suppressor (19). However, the creation of the selective knockout of *Arf* revealed that many of the tumor suppressor properties harbored in this locus were specifically contributed by Arf, as these mice are prone to develop a number of malignancies, and *Arf* null cells are immortal in culture and can be transformed by activated Ras alone (87). Subsequently, the creation of knockin mice that harbor disabling and selective mutations in *Ink4a* demonstrated that these mice do display thymic hyperplasia, that *Ink4a*-deficient T cells are hyper-responsive to mitogens, and that these mice also display an increased incidence of late-onset spontaneous malignancies that are exacerbated by mutagens (21,22). In addition, these studies firmly established the role of p16^{Ink4a} in constraining cellular proliferation, as although p16^{Ink4a} null mouse embryonic fibroblasts (MEFs) possessed normal growth characteristics, they remained susceptible to Ras-induced senescence (88). Furthermore, p16^{Ink4a} null MEFs and lymphocytes exhibit increased rates of immortalization (89). Collectively then, these studies established a tumor suppressor role for p16^{Ink4a}, and as predicted given its cyclin-D/Cdk4;Cdk6 target, these effects were specifically attributed to targeting of the pRb pathway (90).

Mice lacking p15^{Ink4b} are also viable but exhibit extramedullary hematopoiesis and lymphoid hyperplasia in the spleen and lymph nodes (91). Similar to loss of p16^{Ink4a}, the lymphocytes derived from p15^{Ink4b} null mice also demonstrate increased rates in proliferation in response to mitogens (91). Furthermore, although MEFs lacking p15^{Ink4b} show normal contact inhibition and undergo senescence, they display accelerated rates of proliferation in response to serum and show increased plating efficiencies compared with wild-type MEFs. Finally, loss of p15^{Ink4b} also increases susceptibility to transformation by Ras and Myc (91), properties again consistent with functions of a tumor suppressor.

Targeted deletion of p18^{Ink4c} also revealed no effect on normal mouse development, although there is evidence of enhanced epithelial cell growth and increased rates of B- and T-cell proliferation following mitogenic stimulation (20,91,92). As these mice age, there is, however, a rather profound organomegaly and gigantism (20), and Ink4c was found to be essential for the generation of functional plasma cells (93). Furthermore, the

use of the small interfering RNA (siRNA) to knockdown $p18^{Ink4c}$ expression indicated that $p18^{Ink4c}$ functions as a negative regulator of cell growth (94). Finally, formal proof that effects of *Ink4c* loss were indeed due to targeting of cyclin-D/Cdk4 complexes came from the work of Xiong et al. who generated mutant mice deficient in both $p18^{Ink4c}$ and *Cdk4* and showed that loss of *Cdk4* reversed many of the phenotypes associated with loss of $p18^{Ink4c}$ (95).

Mice lacking $p19^{Ink4d}$ are also viable with few developmental defects. However, they do develop testicular hyperplasia that is associated with increased apoptosis of germ cells and male infertility (96,97). Interestingly, $p19^{Ink4d}$ mice also exhibit progressive hearing loss, which is due to the disruption of the maintenance of the postmitotic state of sensory hair cells in the auditory epithelium (98).

Unlike the Ink family that targets CDK4/6, the Cip/Kip family that includes $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ binds to both cyclin-D/Cdk4;Cdk6 and cyclin-E/Cdk2 and cyclin-A/Cdk2 complexes. However, they only functionally block the activity of Cdk2 complexes, whereas their role in modifying cyclin-D-Cdk4;Cdk6 complexes is rather a positive one, as they prevent binding of the Ink4 inhibitors, and they also appear to play an important role in the assembly of functional cyclin-D/Cdk4;Cdk6 complexes (83,99), although this requirement is not absolute and can be overcome by other mitogen-activated events (100).

As a downstream target of the tumor suppressor p53, a key role of $p21^{Cip1}$ following DNA damage is to induce cell-cycle arrest, allowing the cell time to repair its damaged genome and to prevent apoptosis (101–103). Although commonly associated with a growth-inhibitory role, $p21^{Cip1}$ can also function in cell proliferation (104,105). This dichotomy may reflect different roles in distinct subcellular locales, where cytosolic $p21^{Cip1}$ functions in cell-cycle progression and nuclear $p21^{Cip1}$ functions to inhibit Cdk complexes to result in cell-cycle arrest (105,106). $p21^{Cip1}$ also plays important roles in cellular senescence, as targeted deletion of *CIP1* in normal human diploid fibroblasts bypasses senescence (107,108). $p21^{Cip1}$ -deficient cells and primary MEFs also fail to undergo cell-cycle arrest when exposed to various DNA damaging agents (109,110), and as a consequence, this promotes sensitivity to apoptosis following treatment with chemotherapeutics (111–113). Many of these studies however used systems that also lacked functional p53, and many aspects of p53 function appear intact following loss of $p21^{Cip1}$ as, for example, $p21^{Cip1}$ -deficient thymocytes undergo rapid apoptosis following damage (110). Furthermore, the outcome of $p21^{Cip1}$ loss clearly depends on cell context, as keratinocytes derived from $p21^{Cip1}$ -deficient mice exhibit increased proliferative potential (114).

$p27^{Kip1}$ mediates growth arrest induced by various signals, including those directed by TGF- β and contact inhibition (115). The targeted deletion of $p27^{Kip1}$ in mice revealed many important phenotypes relating to growth control, notably an increase in overall size because of extra cell divisions and organ hypertrophy (116–118). Furthermore, $p27^{Kip1}$ loss leads to selective effects on augmenting the proliferative rates of hematopoietic cells and leads to pituitary adenomas (117). Furthermore, $p27^{Kip1+/-}$ mice are tumor prone following exposure to mutagens or γ -irradiation, without showing loss of the wild-type allele; therefore, $p27^{Kip1}$ functions as a haploinsufficient tumor suppressor (119). Finally, $p27^{Kip1}$ -deficient females are sterile, which is likely due to defects in cell-cycle exit during maturation of the ovarian corpora lutea (120).

Of all Cip/Kip family members, inactivation of $p57^{Kip2}$ in the mouse results in the most profound phenotype (121–123). These mice die prematurely soon after birth and exhibit many developmental defects similar to the humans suffering from Beckwith–Wiedemann syndrome, including abnormal proliferation in the limbs, lens, and intestine, cleft palate, organomegaly, and even defects in placental development. Deletion of both $p57^{Kip2}$ and $p27^{Kip1}$ has underscored their importance in the cell cycle, as these doubly deficient mice display defects cell-cycle exit and differentiation of lens fiber cells (122). Furthermore, loss of $p27^{Kip1}$ increases the proliferative rate in the labyrinth zone of $p57^{Kip2}$ null placentas (122). Thus, clearly the Kip inhibitors are key regulators of cell-cycle control.

5. LOSS OF INK4/CDK INHIBITORS IN CANCER

The inactivation of the *Ink4a/Arf* locus is the second most common alteration, behind those that disable p53, in cancer (reviewed in ref. 90), and this can occur through various means: (i) homozygous deletion, generally involving the entire *INK4a/Arf* locus, but only affecting approximately 10% of human tumors (24,124,125); (ii) intra-genic mutations, representing both point and missense mutations, a rather infrequent event (5% of human tumors) that occurs in the second exon common for p16^{Ink4a} and Arf, usually affecting the amino acid sequence of both proteins (24); and (iii) epigenetic silencing by methylation, which is by far the most common mechanism. In this scenario, DNA methylation of cytosine residues present in the CpG residues in the Ink4a promoter leads to gene inactivation (126).

There is widespread evidence for inactivation of *INK4A* particularly in human leukemia and lymphoma. First, a hallmark of T-acute lymphocytic leukemia (T-ALL) is biallelic deletion of these genes (127–129). Second, although deletions of *INK4A* are relatively rare in acute myeloid leukemia (AML) (130), they are frequently deleted in chronic myeloid leukemia (CML) (131), non-Hodgkin's lymphoma (132), primary central nervous system lymphomas (133,134), and relapsed ALL (135–137), and this is associated with poor outcome (136,137). In addition, translocations that inactivate these genes in B-ALL have also been reported (138). Methylation of *INK4A* is also relatively frequent in multiple myeloma, although the relevance of this is currently unclear (139). *INK4A* epigenetic silencing is also seen in non-hematological malignancies, where hypermethylation is a common event in solid tumor types such as colorectal, lung, pancreas, and breast (140) and also in bladder and cervical tumors and in melanoma and glioma (141,142). Despite its major role as a guardian against transformation in cancer in mice compared with p16^{Ink4a}, the role of human p14^{ARF} is somewhat less clear. Indeed, promoter methylation and inactivating missense mutations are rare, although some have been reported in colon cancer (143,144). Furthermore, most cases of deletion of *ARF* usually occur along with concomitant loss of other Ink4 family members (90).

Much of our insight and understanding of the role of the *INK4a/Arf* locus in cancer comes from the use of mouse models. As noted above, targeted deletions of p16^{Ink4a} and especially *Arf* render mice prone to a wide spectrum of spontaneous tumors, including soft-tissue sarcoma, osteosarcoma, melanoma, and histiocytic lymphoma, and lymphoma development is accelerated by carcinogens such as dimethylbenzoic acid (DMBA) (19,21,22,87). Furthermore, malignant melanomas in p16^{Ink4a}/*Arf* doubly

deficient mice that are very similar to those seen in the human malignancy can be induced by activated *Ras* (145). Furthermore, in mice, *Arf* clearly behaves as a classical tumor suppressor, where one sees loss of the wild-type allele in *Arf*^{+/-} mice when spontaneous tumors arise in these mice or when they are crossed to other tumor-prone mice, for example, the E μ -*Myc* transgenic mice, where the onset of lymphomagenesis is dramatically accelerated (146).

Although *INK4A* is one of the most frequently inactivated genes in human cancer, there is now evidence that methylation of *INK4B* may also be important in cancer. The *INK4B* promoter region is hypermethylated in at least 80% of human AML, in about 40% of ALL cases (147,148), and in 60% of chronic myelomonocytic leukemia (CMML) cases (149). Furthermore, *INK4B* is inactivated by deletion in primary cutaneous B-cell lymphomas (150) and up to 30% of ALL (24,151,152). Although inactivation of *INK4B* seems to be a frequent event in various hematological malignancies, it is also seen in other cancer types, including ovarian (153,154), pulmonary squamous cell carcinomas (155), pituitary adenomas (156), neurofibromatosis type 1-related malignancies (157), and bladder cancer (158). Interestingly, *Ink4b* knockout mice are also susceptible to tumor formation; they develop a lympho-proliferative disease, extramedullary hematopoiesis, and a low incidence of angiosarcomas (91).

The loss of p18^{Ink4c} in mice results in organomegaly and widespread hyperplasia and increased incidence of tumorigenesis with advanced age or in the presence of carcinogens (20,23). *Ink4c*-deficient mice display many forms of neoplasia, perhaps resulting from enhanced epithelial cell growth, including testicular tumors, pheochromocytomas, angiosarcomas, lymphomas, and renal cell carcinomas (91). Moreover, leukemogenesis induced by an aberrant *abl*-related gene (*ARG*) kinase involves the suppression of p18^{Ink4c} expression, and blocking *ARG* kinase activity with the drug ST1571 (an inhibitor of the Bcr-Abl tyrosine kinase) induces cell-cycle arrest through up-regulation of p18^{Ink4c} (159). Furthermore, treatment of tumor cell lines with the histone deacetylase inhibitor Trichostatin A activates p18^{Ink4c} (as well as p19^{Ink4d}) (160), and protein kinase C (PKC), known to be increased in many human tumors, may promote human cancer cell growth through suppression of p18^{Ink4c} (94). Recent work utilizing a mouse model of medulloblastoma has also suggested that levels of p18^{Ink4c} contribute to the development of this tumor type (161). Taken together, these data suggest that p18^{Ink4c} functions as a tumor suppressor and that its down-regulation is an important regulator in many human malignancies (23).

Unlike other *Ink4* family members, *p19^{Ink4d}* null mice do not develop spontaneous tumors, and carcinogen treatments do not increase the incidence of tumor development, when compared with their normal littermates (96). Indeed, there is a very little evidence to support a role for p19^{Ink4d} in human cancer. However, elevated expression seen in hematopoietic progenitors and leukemic blast cells may contribute to the premature differentiation block in AML (162), and interestingly, p19^{Ink4d} was found to be the only *Ink4* family member whose expression is induced by UV light (at least in neuroblastoma cells). Furthermore, ectopic expression of p19^{Ink4d} suppresses UV-induced apoptosis and enhances the DNA repair response (163), suggesting a novel role for this *Ink4* family member in the maintenance of DNA integrity, and perhaps cancer prevention.

Given the central function of p21^{Cip1} in cell-cycle regulation, it is perhaps surprising that it plays only modest roles in cancer (164). High levels of the protein are predicted to contribute to chemoresistance in various tumor types (165,166), and hypermethylation

of the gene contributes to its transcriptional silencing in hematopoietic malignancies (167). Polymorphisms that appear to repress *CIP1* expression have also been described in breast cancer (168,169), oral (170), and esophageal cancers (171). In addition, cytosolic localization of p21^{Cip1} has been associated with poor prognosis in breast cancer (172). p21^{Cip1} knockout mice also do develop tumors, including sarcomas and lymphomas, albeit at an advanced age and with rather low penetrance (173). Furthermore, loss of p21^{Cip1} increases the susceptibility to chemically induced skin carcinoma (174) and accelerates tumor onset when treated with carcinogens (175).

p27^{Kip1} is expressed at higher levels in quiescent cells and in those undergoing differentiation. Reduced p27^{Kip1} levels is an observed phenotype in many tumor types, perhaps because of abnormal proteolytic degradation (115), and may contribute to tumor development by increasing proliferative rates. Many cancers exhibit low p27^{Kip1} protein levels including colon, breast, prostate, lung, and ovary to name but a few (reviewed in ref. 176). However, homozygous loss or silencing of the *KIP1* locus is a rare event in human tumors; therefore, the levels of p27^{Kip1} serve as a prognostic marker for disease progression (177). Furthermore, there are only modest effects with regard to malignancy associated with loss of p27^{Kip1} in the mouse; this is perhaps because of the hyperplasia phenotype associated with targeted deletion of p27^{Kip1} where the mice succumb to pituitary adenomas with age (178). p27^{Kip1} deficiency does however cooperate with both oncogenic stimuli (179–181) and most recently with a knockin mutation of Cdk4 (Cdk4 R24C) (182) in enhancing tumor formation.

p57^{Kip2} is located on chromosome 11p15, a region frequently deleted in various human cancers, including lung cancers and Wilms' tumors (183). Interestingly, *KIP2* undergoes imprinting such that the paternally inherited allele is transcriptionally repressed and methylated (184), and inactivation of *KIP2* thus includes selective loss of the maternal allele (185–187). As noted above, p57^{Kip2} knockout mice display phenotypes akin to those seen in patients with Beckwith–Wiedemann syndrome, a hereditary disorder that displays predisposition to cancer, and a subset of these patients harbor missense mutations in *KIP2*. Loss of function and/or expression of *KIP2* is also involved in the proliferation of malignant sporadic adrenal cortical tumors (188), bladder cancer (189), and pancreatic carcinoma (190).

6. REGULATORY CIRCUITS BETWEEN MYC AND CKIS

Deregulated Myc expression prevents cell-cycle withdrawal in response to various antiproliferative signals by activating and repressing transcription of its target genes. Importantly, some of these targets include members of the Cdk inhibitor family, including p21^{Cip1}, p27^{Kip1}, and p15^{Ink4b}. p15^{Ink4b} was initially discovered to be involved in TGF- β growth arrest (191). Prior to this, it was known that TGF- β could down-regulate Myc (192) and that enforced expression of Myc could overcome TGF- β -induced cell-cycle arrest (193). Subsequent works both by the Massague and Eilers groups have now shed some light on the mechanism involved, with a central player being the transcriptional activator Miz1 (53,54). Miz1 was previously identified as a partner of Myc (194,195), and it binds to the initiator elements (Inr) that are present near the CAP sites of several genes that are repressed by Myc (e.g., p21^{Cip1}, see below) and activates their transcription. Ectopic expression of Miz1 was shown to inhibit the proliferation of rodent fibroblasts and to induce cell-cycle arrest. However,

co-expression of Myc restores the proliferation of Miz1-expressing cells. Miz1 regulates transcription of the *Ink4b* by binding to the initiator element of the p15^{Ink4b} promoter. The Myc–Max heterodimer can form a complex with Miz1 at the initiator and inhibit its transcriptional activation. Therefore, abrogation of Miz1 function and suppression of p15^{Ink4b} are critical functions of Myc (53,54).

To date, there have been no direct associations of Myc with p19^{Ink4d} and p18^{Ink4c}, although it has been suggested that the growth arrest of human myeloid cell lines following retinoic acid treatment induces a down-regulation of Myc paralleled by an up-regulation of p18^{Ink4c} by an as yet unknown mechanism (196). Clearly in most cell types, Myc overexpression does not lead to changes in the expression of these two Cdk inhibitors (L. Nilsson and J. Cleveland, unpublished data).

How Myc represses p21^{Cip1} expression has been somewhat controversial (recently reviewed in ref. 197). Several lines of evidence suggest that p21^{Cip1} can also be repressed by binding Miz1 (64,198). Other groups, however, have suggested that repression is Miz1-independent and instead relies on the inhibition of Miz1 transcriptional activity (199). To add further complexity to this issue, yet another group has suggested that p21^{Cip1} repression involves the recruitment of the DNA methyltransferase corepressor Dnmt3a, with Myc and Dnmt3a forming a ternary complex with Miz1 to corepress p21^{Cip1} (156). This then forces one to consider several alternative mechanisms by which Myc represses *p21^{Cip1}* transcription.

Myc represses the transcription of *p27^{Kip1}*, but how this occurs has not been resolved. What is clear, however, is that turnover of p27^{Kip1} levels is profoundly affected by Myc and that this appears to occur through Myc's ability to induce the expression of components of the SCF^{Skp2} complex that targets p27^{Kip1} for degradation by the proteasome (200,201). Specifically, Myc induces the expression of Cullin-1

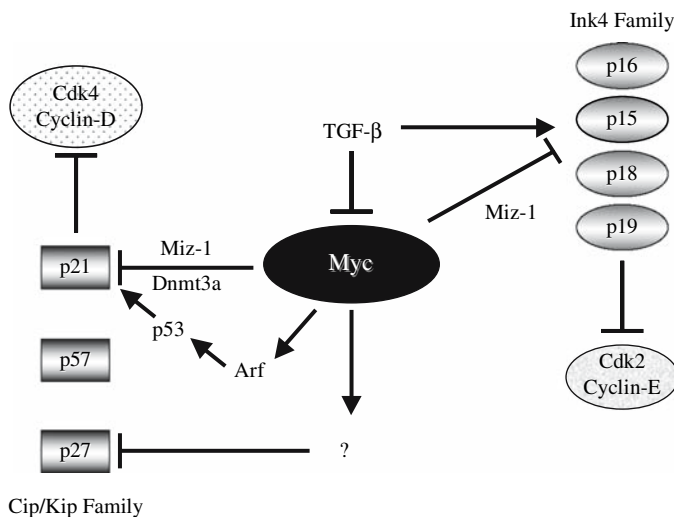


Fig. 1. Regulation of cdk inhibitors by Myc. The Ink4 and Cip/KIP family inhibit CDK4/Cyclin D and CDK2/Cyclin E complexes, respectively. Myc targets p21^{Cip1} repression possibly through binding Miz1 or Dnmt3a. p21^{Cip1} is activated by p53, which is induced by Myc through Arf. The question mark indicates an unknown that Myc targets to repress p27^{Kip1}. TGFβ down-regulates Myc, in turn p15^{Ink4b} regulates TGFβ, Myc may also target p15^{Ink4b} repression through Miz1.

and Cks1 (200) (Keller UB et al., unpublished data), thus linking effects of Myc overexpression in many malignancies to Cks1 overexpression (202–207) and p27^{Kip1} repression (202,205,208–210). Importantly, genetic tests have established that the Myc-to-p27^{Kip1} pathway is a very important regulator of Myc-induced tumorigenesis, as the *Kip1* deficiency markedly accelerates lymphoma development in E μ -Myc transgenic mice (181), whereas lymphoma onset and aggressiveness are impaired in mice lacking *E2f1* or *Cks1*, which are collectively required for Myc-mediated repression of p27^{Kip1} (80) (Keller UB et al., unpublished data). By contrast, p21^{Cip1} does not appear to play limiting role in Myc-driven tumorigenesis, as loss of *Cip1* has essentially no effect on lymphoma development in E μ -Myc mice (181).

Myc is clearly linked and directly regulates the expression of at least three Cdk inhibitors (Fig. 1), and its ability to repress the expression of at least p27^{Kip1} is rate limiting for tumor development. Thus, the Myc-to-p27^{Kip1} pathway is certainly an important target in cancer prevention and therapeutics. However, other Cdk inhibitors also need to be considered as therapeutics targets, particularly given the important role of p16^{Ink4a} in inducing the senescence program (90). Perhaps the most effective approach would be one that targets both, which would eliminate escape routes for the cancer cell.

REFERENCES

1. Ingvarsson S, Sundaresan S, Jin P, et al. Chromosome localization and expression pattern of Lmyc and Bmyc in murine embryonal carcinoma cells. *Oncogene* 1988;3(6):679–85.
2. Sugiyama A, Kume A, Nemoto K, et al. Isolation and characterization of s-myc, a member of the rat myc gene family. *Proc Natl Acad Sci USA* 1989;86(23):9144–8.
3. Nilsson JA, Cleveland JL. Myc pathways provoking cell suicide and cancer. *Oncogene* 2003;22(56):9007–21.
4. Hurlin PJ, Dezfouli S. Functions of myc:max in the control of cell proliferation and tumorigenesis. *Int Rev Cytol* 2004;238:183–226.
5. Eilers M, Schirm S, Bishop JM. The MYC protein activates transcription of the alpha-prothymosin gene. *EMBO J* 1991;10(1):133–41.
6. Iritani BM, Eisenman RN. c-Myc enhances protein synthesis and cell size during B lymphocyte development. *Proc Natl Acad Sci USA* 1999;96(23):13180–5.
7. Johnston LA, Prober DA, Edgar BA, Eisenman RN, Gallant P. Drosophila myc regulates cellular growth during development. *Cell* 1999;98(6):779–90.
8. Gomez-Roman N, Grandori C, Eisenman RN, White RJ. Direct activation of RNA polymerase III transcription by c-Myc. *Nature* 2003;421(6920):290–4.
9. Grandori C, Gomez-Roman N, Felton-Edkins ZA, et al. c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. *Nat Cell Biol* 2005;7(3):311–8.
10. Arabi A, Wu S, Ridderstrale K, et al. c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. *Nat Cell Biol* 2005;7(3):303–10.
11. Freytag SO, Geddes TJ. Reciprocal regulation of adipogenesis by Myc and C/EBP alpha. *Science* 1992;256(5055):379–82.
12. La Rocca SA, Crouch DH, Gillespie DA. c-Myc inhibits myogenic differentiation and myoD expression by a mechanism which can be dissociated from cell transformation. *Oncogene* 1994;9(12):3499–508.
13. Pelengaris S, Khan M, Evan GI. Suppression of Myc-induced apoptosis in beta cells exposes multiple oncogenic properties of Myc and triggers carcinogenic progression. *Cell* 2002;109(3):321–34.
14. Baudino TA, McKay C, Pendeville-Samain H, et al. c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. *Genes Dev* 2002;16(19):2530–43.

15. Askew DS, Ashmun RA, Simmons BC, Cleveland JL. Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* 1991;6(10):1915–22.
16. Evan GI, Wyllie AH, Gilbert CS, et al. Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 1992;69(1):119–28.
17. Felsher DW, Bishop JM. Reversible tumorigenesis by MYC in hematopoietic lineages. *Mol Cell* 1999;4(2):199–207.
18. Pelengaris S, Littlewood T, Khan M, Elia G, Evan G. Reversible activation of c-Myc in skin: induction of a complex neoplastic phenotype by a single oncogenic lesion. *Mol Cell* 1999;3(5):565–77.
19. Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D, DePinho RA. Role of the INK4a locus in tumor suppression and cell mortality. *Cell* 1996;85(1):27–37.
20. Franklin DS, Godfrey VL, Lee H, et al. CDK inhibitors p18(INK4c) and p27(Kip1) mediate two separate pathways to collaboratively suppress pituitary tumorigenesis. *Genes Dev* 1998;12(18): 2899–911.
21. Sharpless NE, Bardeesy N, Lee KH, et al. Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature* 2001;413(6851):86–91.
22. Krimpenfort P, Quon KC, Mooi WJ, Loonstra A, Berns A. Loss of p16Ink4a confers susceptibility to metastatic melanoma in mice. *Nature* 2001;413(6851):83–6.
23. Bai F, Pei XH, Godfrey VL, Xiong Y. Haploinsufficiency of p18(INK4c) sensitizes mice to carcinogen-induced tumorigenesis. *Mol Cell Biol* 2003;23(4):1269–77.
24. Ruas M, Peters G. The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* 1998;1378(2):F115–77.
25. Shay JW, Roninson IB. Hallmarks of senescence in carcinogenesis and cancer therapy. *Oncogene* 2004;23(16):2919–33.
26. Helmrich A, Lee S, O'Brien P, et al. Recurrent chromosomal aberrations in INK4a/ARF defective primary lymphomas predict drug responses *in vivo*. *Oncogene* 2005;24(26):4174–82.
27. Lee MH, Yang HY. Negative regulators of cyclin-dependent kinases and their roles in cancers. *Cell Mol Life Sci* 2001;58(12–13):1907–22.
28. Macaluso M, Montanari M, Cinti C, Giordano A. Modulation of cell cycle components by epigenetic and genetic events. *Semin Oncol* 2005;32(5):452–7.
29. Adachi H, Igawa M, Shiina H, Urakami S, Shigeno K, Hino O. Human bladder tumors with 2-hit mutations of tumor suppressor gene TSC1 and decreased expression of p27. *J Urol* 2003;170 (2 Pt 1):601–4.
30. Woenckhaus C, Fenic I, Giebel J, et al. Loss of heterozygosity at 12p13 and loss of p27KIP1 protein expression contribute to melanoma progression. *Virchows Arch* 2004;445(5):491–7.
31. Rickert CH. Prognosis-related molecular markers in pediatric central nervous system tumors. *J Neuropathol Exp Neurol* 2004;63(12):1211–24.
32. Mawrin C, Kirches E, Schneider-Stock R, et al. Alterations of cell cycle regulators in gliomatosis cerebri. *J Neurooncol* 2005;72(2):115–22.
33. Moberg KH, Tyndall WA, Hall DJ. Wild-type murine p53 represses transcription from the murine c-myc promoter in a human glial cell line. *J Cell Biochem* 1992;49(2):208–15.
34. Stone J, de Lange T, Ramsay G, et al. Definition of regions in human c-myc that are involved in transformation and nuclear localization. *Mol Cell Biol* 1987;7(5):1697–709.
35. Li LH, Nerlov C, Prendergast G, MacGregor D, Ziff EB. c-Myc represses transcription *in vivo* by a novel mechanism dependent on the initiator element and Myc box II. *EMBO J* 1994;13(17):4070–9.
36. Nesbit CE, Tersak JM, Grove LE, Drzal A, Choi H, Prochownik EV. Genetic dissection of c-myc apoptotic pathways. *Oncogene* 2000;19(28):3200–12.
37. Kim SY, Herbst A, Tworkowski KA, Salghetti SE, Tansey WP. Skp2 regulates Myc protein stability and activity. *Mol Cell* 2003;11(5):1177–88.
38. von der Lehr N, Johansson S, Wu S, et al. The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. *Mol Cell* 2003;11(5):1189–200.
39. Adhikary S, Marinoni F, Hock A, et al. The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation. *Cell* 2005;123(3):409–21.
40. Yada M, Hatakeyama S, Kamura T, et al. Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. *EMBO J* 2004;23(10):2116–25.

41. Welcker M, Orian A, Jin J, et al. The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. *Proc Natl Acad Sci USA* 2004;101(24): 9085–90.
42. Welcker M, Orian A, Grim JA, Eisenman RN, Clurman BE. A nucleolar isoform of the Fbw7 ubiquitin ligase regulates c-Myc and cell size. *Curr Biol* 2004;14(20):1852–7.
43. McMahon SB, Van Buskirk HA, Dugan KA, Copeland TD, Cole MD. The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. *Cell* 1998;94(3):363–74.
44. Park J, Wood MA, Cole MD. BAF53 forms distinct nuclear complexes and functions as a critical c-Myc-interacting nuclear cofactor for oncogenic transformation. *Mol Cell Biol* 2002;22(5):1307–16.
45. Vervoorts J, Luscher-Firzlaff JM, Rottmann S, et al. Stimulation of c-MYC transcriptional activity and acetylation by recruitment of the cofactor CBP. *EMBO Rep* 2003;4(5):484–90.
46. McMahon SB, Wood MA, Cole MD. The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc. *Mol Cell Biol* 2000;20(2):556–62.
47. Wood MA, McMahon SB, Cole MD. An ATPase/helicase complex is an essential cofactor for oncogenic transformation by c-Myc. *Mol Cell* 2000;5(2):321–30.
48. Dugan KA, Wood MA, Cole MD. TIP49, but not TRRAP, modulates c-Myc and E2F1 dependent apoptosis. *Oncogene* 2002;21(38):5835–43.
49. Herbst A, Hemann MT, Tworkowski KA, Salghetti SE, Lowe SW, Tansey WP. A conserved element in Myc that negatively regulates its proapoptotic activity. *EMBO Rep* 2005;6(2):177–83.
50. Hurlin PJ, Queva C, Eisenman RN. Mnt, a novel Max-interacting protein is coexpressed with Myc in proliferating cells and mediates repression at Myc binding sites. *Genes Dev* 1997;11(1):44–58.
51. Ayer DE, Eisenman RN. A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation. *Genes Dev* 1993;7(11):2110–9.
52. Nomura T, Khan MM, Kaul SC, et al. Ski is a component of the histone deacetylase complex required for transcriptional repression by Mad and thyroid hormone receptor. *Genes Dev* 1999;13(4):412–23.
53. Staller P, Peukert K, Kiermaier A, et al. Repression of p15INK4b expression by Myc through association with Miz-1. *Nat Cell Biol* 2001;3(4):392–9.
54. Seoane J, Pouponnot C, Staller P, Schader M, Eilers M, Massague J. TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. *Nat Cell Biol* 2001;3(4):400–8.
55. Seoane J, Le HV, Massague J. Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* 2002;419(6908):729–34.
56. Brenner C, Deplus R, Didelot C, et al. Myc represses transcription through recruitment of DNA methyltransferase corepressor. *EMBO J* 2005;24(2):336–46.
57. Ayer DE, Kretzner L, Eisenman RN. Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. *Cell* 1993;72(2):211–22.
58. Oster SK, Ho CS, Soucie EL, Penn LZ. The myc oncogene: MarvelouslyY Complex. *Adv Cancer Res* 2002;84:81–154.
59. Gartel AL, Shchors K. Mechanisms of c-myc-mediated transcriptional repression of growth arrest genes. *Exp Cell Res* 2003;283(1):17–21.
60. Prochownik EV. c-Myc as a therapeutic target in cancer. *Exp Rev Anticancer Ther* 2004;4(2):289–302.
61. Nilsson JA, Keller UB, Baudino TA, et al. Targeting ornithine decarboxylase in Myc-induced lymphomagenesis prevents tumor formation. *Cancer Cell* 2005;7(5):433–44.
62. Perez-Roger I, Solomon DL, Sewing A, Land H. Myc activation of cyclin E/Cdk2 kinase involves induction of cyclin E gene transcription and inhibition of p27(Kip1) binding to newly formed complexes. *Oncogene* 1997;14(20):2373–81.
63. Bouchard C, Thieke K, Maier A, et al. Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. *EMBO J* 1999;18(19):5321–33.
64. Herold S, Wanzel M, Beuger V, et al. Negative regulation of the mammalian UV response by Myc through association with Miz-1. *Mol Cell* 2002;10(3):509–21.
65. Nilsson JA, Nilsson LM, Keller U, Yokota Y, Boyd K, Cleveland JL. Id2 is dispensable for myc-induced lymphomagenesis. *Cancer Res* 2004;64(20):7296–301.
66. Murphy DJ, Swigart LB, Israel MA, Evan GI. Id2 is dispensable for Myc-induced epidermal neoplasia. *Mol Cell Biol* 2004;24(5):2083–90.

67. Davis AC, Wims M, Spotts GD, Hann SR, Bradley A. A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. *Genes Dev* 1993;7(4):671–82.
68. de Alboran IM, O'Hagan RC, Gartner F, et al. Analysis of C-MYC function in normal cells via conditional gene-targeted mutation. *Immunity* 2001;14(1):45–55.
69. Trumpp A, Refaeli Y, Oskarsson T, et al. c-Myc regulates mammalian body size by controlling cell number but not cell size. *Nature* 2001;414(6865):768–73.
70. Arvanitis C, Felsner DW. Conditionally MYC: insights from novel transgenic models. *Cancer Lett* 2005;226(2):95–9.
71. Cavalieri F, Goldfarb M. N-myc proto-oncogene expression can induce DNA replication in Balb/c 3T3 fibroblasts. *Oncogene* 1988;2(3):289–91.
72. Cavalieri F, Goldfarb M. Growth factor-deprived BALB/c 3T3 murine fibroblasts can enter the S phase after induction of c-myc gene expression. *Mol Cell Biol* 1987;7(10):3554–60.
73. Heikkila R, Schwab G, Wickstrom E, et al. A c-myc antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G0 to G1. *Nature* 1987;328(6129):445–9.
74. Jansen-Durr P, Meichle A, Steiner P, et al. Differential modulation of cyclin gene expression by MYC. *Proc Natl Acad Sci USA* 1993;90(8):3685–9.
75. Hermeking H, Rago C, Schuhmacher M, et al. Identification of CDK4 as a target of c-MYC. *Proc Natl Acad Sci USA* 2000;97(5):2229–34.
76. Kozar K, Ciemerych MA, Rebel VI, et al. Mouse development and cell proliferation in the absence of D-cyclins. *Cell* 2004;118(4):477–91.
77. Miliiani de Marval PL, Macias E, Rounbehler R, et al. Lack of cyclin-dependent kinase 4 inhibits c-myc tumorigenic activities in epithelial tissues. *Mol Cell Biol* 2004;24(17):7538–47.
78. Gebhardt A, Frye M, Herold S, et al. Myc regulates keratinocyte adhesion and differentiation via complex formation with Miz1. *J Cell Biol* 2006;172(1):139–49.
79. Yang W, Shen J, Wu M, et al. Repression of transcription of the p27(Kip1) cyclin-dependent kinase inhibitor gene by c-Myc. *Oncogene* 2001;20(14):1688–702.
80. Baudino TA, Maclean KH, Brennan J, et al. Myc-mediated proliferation and lymphomagenesis, but not apoptosis, are compromised by E2f1 loss. *Mol Cell* 2003;11(4):905–14.
81. Russo AA, Tong L, Lee JO, Jeffrey PD, Pavletich NP. Structural basis for inhibition of the cyclin-dependent kinase Cdk6 by the tumour suppressor p16INK4a. *Nature* 1998;395(6699):237–43.
82. Lukas J, Aagaard L, Strauss M, Bartek J. Oncogenic aberrations of p16INK4/CDKN2 and cyclin D1 cooperate to deregulate G1 control. *Cancer Res* 1995;55(21):4818–23.
83. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 1999;13(12):1501–12.
84. Ciemerych MA, Sicinski P. Cell cycle in mouse development. *Oncogene* 2005;24(17):2877–98.
85. Quelle DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* 1995;83(6):993–1000.
86. Zindy F, Eischen CM, Randle DH, et al. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev* 1998;12(15):2424–33.
87. Kamijo T, Zindy F, Roussel MF, et al. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* 1997;91(5):649–59.
88. Sharpless NE, Ramsey MR, Balasubramanian P, Castrillon DH, DePinho RA. The differential impact of p16(INK4a) or p19(ARF) deficiency on cell growth and tumorigenesis. *Oncogene* 2004;23(2): 379–85.
89. Carnero A, Hudson JD, Price CM, Beach DH. p16INK4A and p19ARF act in overlapping pathways in cellular immortalization. *Nat Cell Biol* 2000;2(3):148–55.
90. Sharpless NE. INK4a/ARF: a multifunctional tumor suppressor locus. *Mutat Res* 2005;576(1–2):22–38.
91. Latres E, Malumbres M, Sotillo R, et al. Limited overlapping roles of P15(INK4b) and P18(INK4c) cell cycle inhibitors in proliferation and tumorigenesis. *EMBO J* 2000;19(13):3496–506.
92. Kovalev GI, Franklin DS, Coffield VM, Xiong Y, Su L. An important role of CDK inhibitor p18(INK4c) in modulating antigen receptor-mediated T cell proliferation. *J Immunol* 2001;167(6):3285–92.
93. Tourigny MR, Ursini-Siegel J, Lee H, et al. CDK inhibitor p18(INK4c) is required for the generation of functional plasma cells. *Immunity* 2002;17(2):179–89.

94. Matsuzaki Y, Takaoka Y, Hitomi T, Nishino H, Sakai T. Activation of protein kinase C promotes human cancer cell growth through downregulation of p18(INK4c). *Oncogene* 2004;23(31):5409–14.
95. Pei XH, Bai F, Tsutsui T, Kiyokawa H, Xiong Y. Genetic evidence for functional dependency of p18Ink4c on Cdk4. *Mol Cell Biol* 2004;24(15):6653–64.
96. Zindy F, van Deursen J, Grosveld G, Sherr CJ, Roussel MF. INK4d-deficient mice are fertile despite testicular atrophy. *Mol Cell Biol* 2000;20(1):372–8.
97. Zindy F, den Besten W, Chen B, et al. Control of spermatogenesis in mice by the cyclin D-dependent kinase inhibitors p18(Ink4c) and p19(Ink4d). *Mol Cell Biol* 2001;21(9):3244–55.
98. Chen P, Zindy F, Abdala C, et al. Progressive hearing loss in mice lacking the cyclin-dependent kinase inhibitor Ink4d. *Nat Cell Biol* 2003;5(5):422–6.
99. Cheng M, Olivier P, Diehl JA, et al. The p21(Cip1) and p27(Kip1) CDK ‘inhibitors’ are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J* 1999;18(6):1571–83.
100. Sugimoto M, Martin N, Wilks DP, et al. Activation of cyclin D1-kinase in murine fibroblasts lacking both p21(Cip1) and p27(Kip1). *Oncogene* 2002;21(53):8067–74.
101. Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE, Givol D. Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res* 1994;54(13):3391–5.
102. el-Deiry WS, Harper JW, O’Connor PM, et al. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res* 1994;54(5):1169–74.
103. Di Leonardo A, Linke SP, Clarkin K, Wahl GM. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev* 1994;8(21): 2540–51.
104. Dupont J, Karas M, LeRoith D. The cyclin-dependent kinase inhibitor p21CIP/WAF1 is a positive regulator of insulin-like growth factor I-induced cell proliferation in MCF-7 human breast cancer cells. *J Biol Chem* 2003;278(39):37256–64.
105. Dong Y, Chi SL, Borowsky AD, Fan Y, Weiss RH. Cytosolic p21Waf1/Cip1 increases cell cycle transit in vascular smooth muscle cells. *Cell Signal* 2004;16(2):263–9.
106. Zhou BP, Liao Y, Xia W, Spohn B, Lee MH, Hung MC. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat Cell Biol* 2001;3(3):245–52.
107. Brown JP, Wei W, Sedivy JM. Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. *Science* 1997;277(5327):831–4.
108. Wells SI, Francis DA, Karpova AY, Dowhanick JJ, Benson JD, Howley PM. Papillomavirus E2 induces senescence in HPV-positive cells via pRB- and p21(CIP)-dependent pathways. *EMBO J* 2000;19(21):5762–71.
109. Waldman T, Kinzler KW, Vogelstein B. p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res* 1995;55(22):5187–90.
110. Deng C, Zhang P, Harper JW, Elledge SJ, Leder P. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* 1995;82(4):675–84.
111. Bunz F, Hwang PM, Torrance C, et al. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J Clin Invest* 1999;104(3):263–9.
112. Kondo S, Barna BP, Kondo Y, et al. WAF1/CIP1 increases the susceptibility of p53 non-functional malignant glioma cells to cisplatin-induced apoptosis. *Oncogene* 1996;13(6):1279–85.
113. Lincet H, Poulain L, Remy JS, et al. The p21(cip1/waf1) cyclin-dependent kinase inhibitor enhances the cytotoxic effect of cisplatin in human ovarian carcinoma cells. *Cancer Lett* 2000;161(1):17–26.
114. Missero C, Di Cunto F, Kiyokawa H, Koff A, Dotto GP. The absence of p21Cip1/WAF1 alters keratinocyte growth and differentiation and promotes ras-tumor progression. *Genes Dev* 1996;10(23):3065–75.
115. Bloom J, Pagano M. Deregulated degradation of the cdk inhibitor p27 and malignant transformation. *Semin Cancer Biol* 2003;13(1):41–7.
116. Nakayama K, Ishida N, Shirane M, et al. Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 1996;85(5):707–20.
117. Fero ML, Rivkin M, Tasch M, et al. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell* 1996;85(5):733–44.
118. Kiyokawa H, Kineman RD, Manova-Todorova KO, et al. Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell* 1996;85(5):721–32.
119. Fero ML, Randel E, Gurley KE, Roberts JM, Kemp CJ. The murine gene p27Kip1 is haplo-insufficient for tumour suppression. *Nature* 1998;396(6707):177–80.

120. Tong W, Kiyokawa H, Soos TJ, et al. The absence of p27Kip1, an inhibitor of G1 cyclin-dependent kinases, uncouples differentiation and growth arrest during the granulosa->luteal transition. *Cell Growth Differ* 1998;9(9):787-94.
121. Yan Y, Frisen J, Lee MH, Massague J, Barbacid M. Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev* 1997;11(8):973-83.
122. Zhang P, Wong C, DePinho RA, Harper JW, Elledge SJ. Cooperation between the Cdk inhibitors p27(KIP1) and p57(KIP2) in the control of tissue growth and development. *Genes Dev* 1998;12(20):3162-7.
123. Zhang P, Liegeois NJ, Wong C, et al. Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. *Nature* 1997;387(6629):151-8.
124. Gruis NA, van der Velden PA, Sandkuijl LA, et al. Homozygotes for CDKN2 (p16) germline mutation in Dutch familial melanoma kindreds. *Nat Genet* 1995;10(3):351-3.
125. Pavel S, Smit NP, van der Meulen H, et al. Homozygous germline mutation of CDKN2A/p16 and glucose-6-phosphate dehydrogenase deficiency in a multiple melanoma case. *Melanoma Res* 2003;13(2):171-8.
126. Esteller M, Fraga MF, Paz MF, et al. Cancer epigenetics and methylation. *Science* 2002;297(5588):1807-8; discussion 1807-8.
127. Cayuela JM, Hebert J, Sigaux F. Homozygous MTS1 (p16INK4A) deletion in primary tumor cells of 163 leukemic patients. *Blood* 1995;85(3):854.
128. Cayuela JM, Madani A, Sanhes L, Stern MH, Sigaux F. Multiple tumor-suppressor gene 1 inactivation is the most frequent genetic alteration in T-cell acute lymphoblastic leukemia. *Blood* 1996;87(6):2180-6.
129. Cayuela JM, Gardie B, Sigaux F. Disruption of the multiple tumor suppressor gene MTS1/p16(INK4a)/CDKN2 by illegitimate V(D)J recombinase activity in T-cell acute lymphoblastic leukemias. *Blood* 1997;90(9):3720-6.
130. Faderl S, Kantarjian HM, Estey E, et al. The prognostic significance of p16(INK4a)/p14(ARF) locus deletion and MDM-2 protein expression in adult acute myelogenous leukemia. *Cancer* 2000;89(9):1976-82.
131. Hernandez-Boluda JC, Cervantes F, Colomer D, et al. Genomic p16 abnormalities in the progression of chronic myeloid leukemia into blast crisis: a sequential study in 42 patients. *Exp Hematol* 2003;31(3):204-10.
132. Pinyol M, Hernandez L, Martinez A, et al. INK4a/ARF locus alterations in human non-Hodgkin's lymphomas mainly occur in tumors with wild-type p53 gene. *Am J Pathol* 2000;156(6):1987-96.
133. Nakamura M, Sakaki T, Hashimoto H, et al. Frequent alterations of the p14(ARF) and p16(INK4a) genes in primary central nervous system lymphomas. *Cancer Res* 2001;61(17):6335-9.
134. Hayashi Y, Iwato M, Arakawa Y, et al. Homozygous deletion of INK4a/ARF genes and overexpression of bcl-2 in relation with poor prognosis in immunocompetent patients with primary central nervous system lymphoma of the diffuse large B-cell type. *J Neurooncol* 2001;55(1):51-8.
135. Maloney KW, McGavran L, Odom LF, Hunger SP. Acquisition of p16(INK4A) and p15(INK4B) gene abnormalities between initial diagnosis and relapse in children with acute lymphoblastic leukemia. *Blood* 1999;93(7):2380-5.
136. Carter TL, Watt PM, Kumar R, et al. Hemizygous p16(INK4A) deletion in pediatric acute lymphoblastic leukemia predicts independent risk of relapse. *Blood* 2001;97(2):572-4.
137. Carter TL, Reaman GH, Kees UR. INK4A/ARF deletions are acquired at relapse in childhood acute lymphoblastic leukaemia: a paired study on 25 patients using real-time polymerase chain reaction. *Br J Haematol* 2001;113(2):323-8.
138. Duro D, Bernard O, Della Valle V, Leblanc T, Berger R, Larsen CJ. Inactivation of the P16INK4/MTS1 gene by a chromosome translocation t(9;14)(p21-22;q11) in an acute lymphoblastic leukemia of B-cell type. *Cancer Res* 1996;56(4):848-54.
139. Ribas C, Colleoni GW, Felix RS, et al. p16 gene methylation lacks correlation with angiogenesis and prognosis in multiple myeloma. *Cancer Lett* 2005;222(2):247-54.
140. Esteller M, Cordon-Cardo C, Corn PG, et al. p14ARF silencing by promoter hypermethylation mediates abnormal intracellular localization of MDM2. *Cancer Res* 2001;61(7):2816-21.
141. Kusy S, Larsen CJ, Roche J. p14ARF, p15INK4b and p16INK4a methylation status in chronic myelogenous leukemia. *Leuk Lymphoma* 2004;45(10):1989-94.

142. Kawamoto K, Enokida H, Gotanda T, et al. p16(INK4a) and p14(ARF) methylation as a potential biomarker for human bladder cancer. *Biochem Biophys Res Commun* 2006;339(3):790–6.
143. Esteller M. Epigenetic lesions causing genetic lesions in human cancer: promoter hypermethylation of DNA repair genes. *Eur J Cancer* 2000;36(18):2294–300.
144. Sato M, Horio Y, Sekido Y, Minna JD, Shimokata K, Hasegawa Y. The expression of DNA methyltransferases and methyl-CpG-binding proteins is not associated with the methylation status of p14(ARF), p16(INK4a) and RASSF1A in human lung cancer cell lines. *Oncogene* 2002;21(31): 4822–9.
145. Chin L, Tam A, Pomerantz J, et al. Essential role for oncogenic Ras in tumour maintenance. *Nature* 1999;400(6743):468–72.
146. Eischen CM, Weber JD, Roussel MF, Sherr CJ, Cleveland JL. Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. *Genes Dev* 1999;13(20):2658–69.
147. Tsellou E, Troungos C, Moschovi M, et al. Hypermethylation of CpG islands in the promoter region of the p15INK4B gene in childhood acute leukaemia. *Eur J Cancer* 2005;41(4):584–9.
148. Shimamoto T, Ohyashiki JH, Ohyashiki K. Methylation of p15(INK4b) and E-cadherin genes is independently correlated with poor prognosis in acute myeloid leukemia. *Leuk Res* 2005;29(6):653–9.
149. Tessema M, Langer F, Dingemann J, Ganser A, Kreipe H, Lehmann U. Aberrant methylation and impaired expression of the p15(INK4b) cell cycle regulatory gene in chronic myelomonocytic leukemia (CMML). *Leukemia* 2003;17(5):910–8.
150. Child FJ, Scarisbrick JJ, Calonje E, Orchard G, Russell-Jones R, Whittaker SJ. Inactivation of tumor suppressor genes p15(INK4b) and p16(INK4a) in primary cutaneous B cell lymphoma. *J Invest Dermatol* 2002;118(6):941–8.
151. Herman JG, Civin CI, Issa JP, Collector MI, Sharkis SJ, Baylin SB. Distinct patterns of inactivation of p15INK4B and p16INK4A characterize the major types of hematological malignancies. *Cancer Res* 1997;57(5):837–41.
152. Drexler HG. Review of alterations of the cyclin-dependent kinase inhibitor INK4 family genes p15, p16, p18 and p19 in human leukemia-lymphoma cells. *Leukemia* 1998;12(6):845–59.
153. Fujita M, Enomoto T, Haba T, et al. Alteration of p16 and p15 genes in common epithelial ovarian tumors. *Int J Cancer* 1997;74(2):148–55.
154. Liu Z, Wang LE, Wang L, et al. Methylation and messenger RNA expression of p15INK4b but not p16INK4a are independent risk factors for ovarian cancer. *Clin Cancer Res* 2005;11(13):4968–76.
155. Furonaka O, Takeshima Y, Awaya H, Ishida H, Kohno N, Inai K. Aberrant methylation of p14(ARF), p15(INK4b) and p16(INK4a) genes and location of the primary site in pulmonary squamous cell carcinoma. *Pathol Int* 2004;54(8):549–55.
156. Ogino A, Yoshino A, Katayama Y, et al. The p15(INK4b)/p16(INK4a)/RB1 pathway is frequently deregulated in human pituitary adenomas. *J Neuropathol Exp Neurol* 2005;64(5):398–403.
157. Perrone F, Tabano S, Colombo F, et al. p15INK4b, p14ARF, and p16INK4a inactivation in sporadic and neurofibromatosis type 1-related malignant peripheral nerve sheath tumors. *Clin Cancer Res* 2003;9(11):4132–8.
158. Le Frere-Belda MA, Cappellen D, Daher A, et al. p15(INK4b) in bladder carcinomas: decreased expression in superficial tumours. *Br J Cancer* 2001;85(10):1515–21.
159. Nishimura N, Furukawa Y, Sutheesophon K, et al. Suppression of ARG kinase activity by STI571 induces cell cycle arrest through up-regulation of CDK inhibitor p18/INK4c. *Oncogene* 2003;22(26):4074–82.
160. Yokota T, Matsuzaki Y, Sakai T. Trichostatin A activates p18INK4c gene: differential activation and cooperation with p19INK4d gene. *FEBS Lett* 2004;574(1–3):171–5.
161. Uziel T, Zindy F, Xie S, et al. The tumor suppressors Ink4c and p53 collaborate independently with patched to suppress medulloblastoma formation. *Genes Dev* 2005;19(22):2656–67.
162. Tschan MP, Peters UR, Cajot JF, Betticher DC, Fey MF, Tobler A. The cyclin-dependent kinase inhibitors p18INK4c and p19INK4d are highly expressed in CD34+ progenitor and acute myeloid leukaemic cells but not in normal differentiated myeloid cells. *Br J Haematol* 1999;106(3):644–51.
163. Ceruti JM, Scassa ME, Flo JM, Varone CL, Canepa ET. Induction of p19INK4d in response to ultraviolet light improves DNA repair and confers resistance to apoptosis in neuroblastoma cells. *Oncogene* 2005;24(25):4065–80.
164. Weiss RH. p21Waf1/Cip1 as a therapeutic target in breast and other cancers. *Cancer Cell* 2003;4(6):425–9.

165. Zhang W, Kornblau SM, Kobayashi T, Gambel A, Claxton D, Deisseroth AB. High levels of constitutive WAF1/Cip1 protein are associated with chemoresistance in acute myelogenous leukemia. *Clin Cancer Res* 1995;1(9):1051–7.
166. Steinman RA, Johnson DE. p21WAF1 prevents down-modulation of the apoptotic inhibitor protein c-IAP1 and inhibits leukemic apoptosis. *Mol Med* 2000;6(9):736–49.
167. Roman-Gomez J, Castillejo JA, Jimenez A, et al. 5' CpG island hypermethylation is associated with transcriptional silencing of the p21(CIP1/WAF1/SDI1) gene and confers poor prognosis in acute lymphoblastic leukemia. *Blood* 2002;99(7):2291–6.
168. Powell BL, van Staveren IL, Roosken P, Grieu F, Berns EM, Iacopetta B. Associations between common polymorphisms in TP53 and p21WAF1/Cip1 and phenotypic features of breast cancer. *Carcinogenesis* 2002;23(2):311–5.
169. Keshava C, Frye BL, Wolff MS, McCanlies EC, Weston A. Waf-1 (p21) and p53 polymorphisms in breast cancer. *Cancer Epidemiol Biomarkers Prev* 2002;11(1):127–30.
170. Ralhan R, Agarwal S, Mathur M, Wasylyk B, Srivastava A. Association between polymorphism in p21(Waf1/Cip1) cyclin-dependent kinase inhibitor gene and human oral cancer. *Clin Cancer Res* 2000;6(6):2440–7.
171. Bahl R, Arora S, Nath N, Mathur M, Shukla NK, Ralhan R. Novel polymorphism in p21(waf1/cip1) cyclin dependent kinase inhibitor gene: association with human esophageal cancer. *Oncogene* 2000;19(3):323–8.
172. Winters ZE, Leek RD, Bradburn MJ, Norbury CJ, Harris AL. Cytoplasmic p21WAF1/CIP1 expression is correlated with HER-2/neu in breast cancer and is an independent predictor of prognosis. *Breast Cancer Res* 2003;5(6):R242–9.
173. Martin-Caballero J, Flores JM, Garcia-Palencia P, Serrano M. Tumor susceptibility of p21(Waf1/Cip1)-deficient mice. *Cancer Res* 2001;61(16):6234–8.
174. Topley GI, Okuyama R, Gonzales JG, Conti C, Dotto GP. p21(WAF1 /Cip1) functions as a suppressor of malignant skin tumor formation and a determinant of keratinocyte stem-cell potential. *Proc Natl Acad Sci USA* 1999;96(16):9089–94.
175. Jackson RJ, Adnane J, Coppola D, Cantor A, Sebt SM, Pledger WJ. Loss of the cell cycle inhibitors p21(Cip1) and p27(Kip1) enhances tumorigenesis in knockout mouse models. *Oncogene* 2002;21(55):8486–97.
176. Blain SW, Scher HI, Cordon-Cardo C, Koff A. p27 as a target for cancer therapeutics. *Cancer Cell* 2003;3(2):111–5.
177. Slingerland J, Pagano M. Regulation of the cdk inhibitor p27 and its deregulation in cancer. *J Cell Physiol* 2000;183(1):10–7.
178. Cordon-Cardo C, Koff A, Drobnjak M, et al. Distinct altered patterns of p27KIP1 gene expression in benign prostatic hyperplasia and prostatic carcinoma. *J Natl Cancer Inst* 1998;90(17):1284–91.
179. Park MS, Rosai J, Nguyen HT, Capodiec P, Cordon-Cardo C, Koff A. p27 and Rb are on overlapping pathways suppressing tumorigenesis in mice. *Proc Natl Acad Sci USA* 1999;96(11):6382–7.
180. Di Cristofano A, De Acetis M, Koff A, Cordon-Cardo C, Pandolfi PP. Pten and p27KIP1 cooperate in prostate cancer tumor suppression in the mouse. *Nat Genet* 2001;27(2):222–4.
181. Martins CP, Berns A. Loss of p27(Kip1) but not p21(Cip1) decreases survival and synergizes with MYC in murine lymphomagenesis. *EMBO J* 2002;21(14):3739–48.
182. Sotillo R, Renner O, Dubus P, et al. Cooperation between Cdk4 and p27kip1 in tumor development: a preclinical model to evaluate cell cycle inhibitors with therapeutic activity. *Cancer Res* 2005;65(9):3846–52.
183. Kondo M, Suzuki H, Ueda R, Takagi K, Takahashi T, Takahashi T. Parental origin of 11p15 deletions in human lung cancer. *Oncogene* 1994;9(10):3063–5.
184. Hatada I, Mukai T. Genomic imprinting of p57KIP2, a cyclin-dependent kinase inhibitor, in mouse. *Nat Genet* 1995;11(2):204–6.
185. Hatada I, Ohashi H, Fukushima Y, et al. An imprinted gene p57KIP2 is mutated in Beckwith-Wiedemann syndrome. *Nat Genet* 1996;14(2):171–3.
186. Hatada I, Inazawa J, Abe T, et al. Genomic imprinting of human p57KIP2 and its reduced expression in Wilms' tumors. *Hum Mol Genet* 1996;5(6):783–8.
187. Kondo M, Matsuoka S, Uchida K, et al. Selective maternal-allele loss in human lung cancers of the maternally expressed p57KIP2 gene at 11p15.5. *Oncogene* 1996;12(6):1365–8.

188. Bourcigaux N, Gaston V, Logie A, Bertagna X, Le Bouc Y, Gicquel C. High expression of cyclin E and G1 CDK and loss of function of p57KIP2 are involved in proliferation of malignant sporadic adrenocortical tumors. *J Clin Endocrinol Metab* 2000;85(1):322–30.
189. Oya M, Schulz WA. Decreased expression of p57(KIP2)mRNA in human bladder cancer. *Br J Cancer* 2000;83(5):626–31.
190. Sato N, Matsubayashi H, Abe T, Fukushima N, Goggins M. Epigenetic down-regulation of CDKN1C/p57KIP2 in pancreatic ductal neoplasms identified by gene expression profiling. *Clin Cancer Res* 2005;11(13):4681–8.
191. Hannon GJ, Beach D. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* 1994;371(6494):257–61.
192. Fernandez-Pol JA, Talkad VD, Klos DJ, Hamilton PD. Suppression of the EGF-dependent induction of c-myc proto-oncogene expression by transforming growth factor beta in a human breast carcinoma cell line. *Biochem Biophys Res Commun* 1987;144(3):1197–205.
193. Warner BJ, Blain SW, Seoane J, Massague J. Myc downregulation by transforming growth factor beta required for activation of the p15(Ink4b) G(1) arrest pathway. *Mol Cell Biol* 1999;19(9):5913–22.
194. Peukert K, Staller P, Schneider A, Carmichael G, Hanel F, Eilers M. An alternative pathway for gene regulation by Myc. *EMBO J* 1997;16(18):5672–86.
195. Salghetti SE, Kim SY, Tansey WP. Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc. *EMBO J* 1999;18(3):717–26.
196. Dimberg A, Oberg F. Retinoic acid-induced cell cycle arrest of human myeloid cell lines. *Leuk Lymphoma* 2003;44(10):1641–50.
197. Gartel AL, Radhakrishnan SK. Lost in transcription: p21 repression, mechanisms, and consequences. *Cancer Res* 2005;65(10):3980–5.
198. Wu S, Cetinkaya C, Munoz-Alonso MJ, et al. Myc represses differentiation-induced p21CIP1 expression via Miz-1-dependent interaction with the p21 core promoter. *Oncogene* 2003;22(3): 351–60.
199. Vaque JP, Navascues J, Shiio Y, et al. Myc antagonizes Ras-mediated growth arrest in leukemia cells through the inhibition of the Ras-ERK-p21Cip1 pathway. *J Biol Chem* 2005;280(2):1112–22.
200. O'Hagan RC, Ohh M, David G, et al. Myc-enhanced expression of Cull1 promotes ubiquitin-dependent proteolysis and cell cycle progression. *Genes Dev* 2000;14(17):2185–91.
201. Minella AC, Clurman BE. Mechanisms of tumor suppression by the SCF(Fbw7). *Cell Cycle* 2005;4(10):1356–9.
202. Wang W, Ungermannova D, Chen L, Liu X. A negatively charged amino acid in Skp2 is required for Skp2-Cks1 interaction and ubiquitination of p27Kip1. *J Biol Chem* 2003;278(34):32390–6.
203. Shapira M, Ben-Izhak O, Bishara B, et al. Alterations in the expression of the cell cycle regulatory protein cyclin kinase subunit 1 in colorectal carcinoma. *Cancer* 2004;100(8):1615–21.
204. Masuda TA, Inoue H, Nishida K, et al. Cyclin-dependent kinase 1 gene expression is associated with poor prognosis in gastric carcinoma. *Clin Cancer Res* 2003;9(15):5693–8.
205. Kitajima S, Kudo Y, Ogawa I, et al. Role of Cks1 overexpression in oral squamous cell carcinomas: cooperation with Skp2 in promoting p27 degradation. *Am J Pathol* 2004;165(6):2147–55.
206. Inui N, Kitagawa K, Miwa S, et al. High expression of Cks1 in human non-small cell lung carcinomas. *Biochem Biophys Res Commun* 2003;303(3):978–84.
207. Hattori T, Kitagawa K, Uchida C, Oda T, Kitagawa M. Cks1 is degraded via the ubiquitin-proteasome pathway in a cell cycle-dependent manner. *Genes Cells* 2003;8(11):889–96.
208. Ganoth D, Bornstein G, Ko TK, et al. The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27. *Nat Cell Biol* 2001;3(3):321–4.
209. Spruck C, Strohmaier H, Watson M, et al. A CDK-independent function of mammalian Cks1: targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. *Mol Cell* 2001;7(3):639–50.
210. Hao B, Zheng N, Schulman BA, et al. Structural basis of the Cks1-dependent recognition of p27(Kip1) by the SCF(Skp2) ubiquitin ligase. *Mol Cell* 2005;20(1):9–19.

13

Interplay Between γ H2AX and 53BP1 Pathways in DNA Double-Strand Break Repair Response

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SUMMARY

One of the most fascinating themes in the biology of double-strand breaks (DSBs) is that chromatin is emerging as a multifunctional player in the DSB damage response. The phosphorylation of H2AX on Ser 139, named γ H2AX, is an early response to the generation of DNA DSBs and extends along megabase-long domains, both sites of the lesion, supporting amplification of signal transduction pathways. In parallel, 53BP1 accumulates on damaged chromatin to interface between methylated histone residues and proteins that belong to the signal-transduction pathways, mediating cell-cycle arrest or apoptosis. Interestingly, the two pathways crosstalk at the chromatin level.

Key Words: H2AX; 53BP1; double-strand breaks; DNA repair; chromatin.

1. GENERAL CHARACTERISTICS OF DOUBLE-STRAND BREAK DAMAGE RESPONSE NETWORKS

Within the living cells, genetic information encoded in DNA sequences is constantly under threat by various genotoxic stresses. Of the various types of DNA lesions that arise, double-strand breaks (DSBs) are the most damaging. In mammalian cells, DSB can result from exogenous agents, such as background radiation and environmental mutagens, or arise from endogenous sources as metabolically produced free radicals. In addition, DSB may emanate as DNA intermediates, normal or aberrant, in several specialized cellular functions, such as DNA replication, V(D)J recombination, meiotic recombination, class switching, and apoptosis.

The consequences of DSBs are of major cost to the cells. If left unrepaired, even one DSB can be lethal. Loss of continuity between the DNA fragment and the centromere

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Table 1
Classification of DNA repair factors according their role in DSB cellular response.

<i>Factor</i>	<i>Participation in multiprotein complex</i>	<i>Function</i>	<i>Role in DSB response</i>
γ H2AX	Nucleosome	Chromatin structure	Chromatin mark, transducer
Methyl-H3 Lys 79 and relevant residues	Nucleosome	Chromatin structure	Chromatin mark
53BP1	–	Activator, adaptor–mediator	Sensor, transducer
MDC1	–	Activator, adaptor–mediator	Transducer
Mre11	MRN	Nuclease	Sensor, effector
Rad50	MRN	DNA structural protein	Effector
NBS1	MRN	Activator, adaptor–mediator	Transducer
ATM	Homodimer	Kinase	Sensor (?), proximal kinase
RAD17	Several complexes	Chromatin-binding protein	Sensor
ATRIP	ATR–ATRIP complex	Adaptor–mediator	Transducer
ATR	ATR–ATRIP complex	Kinase	Proximal kinase
DNA-PKcs	DNA-PK	Kinase	Proximal kinase
Ku 80/70	DNA-PK	Sensor	Sensor, transducer
Chk1	–	Kinase	Effector kinase
Chk2	–	Kinase	Effector kinase
BRCA1	BRCA1–BARD1 and several other complexes	E3 ubiquitin ligase, scaffold protein, etc.	Transducer, effector
BRCA2	BASK and several other complexes	Scaffold protein, etc.	Transducer
P53	–	Transcription factor	Effector
Cdc25A	–	Phosphatase (?)	Effector
Cdc25C	–	Phosphatase	Effector
RAD17	Several complexes	Chromatin binding	Sensor
ARTEMIS	–	Nuclease	Effector
XRCC4	XRCC4–LIG4 complex	Adaptor (?)	Adaptor (?)
LIG4	XRCC4–LIG4 complex	Ligase	Effector
DSS1	19S proteasome	Catalytic subunit	Effector
SMC1	Cohesin complex	Structural protein	Effector

during mitosis can result in deletions of a large proportion of the genetic information passed to the progeny cells. If not properly repaired, DSBs can cause alterations in the DNA sequence, chromosomal translocations, genomic instability, and eventually neoplastic transformation. Thus, it is not surprising that the DSB damage response is a highly sophisticated set of reactions that channels cellular activities toward three directions: DNA repair, cell-cycle arrest, and apoptosis.

Two major pathways orchestrate the DSB repair in mammalian cells, one based on homologous recombination (HR), and the other on non-homologous end joining (NHEJ). HR promotes accurate repair by copying information from an intact homologous DNA template, preferentially the sister chromatid when available, and predominates in S/G2 phases (1). NHEJ is independent of homology, or utilizes microhomology to join broken ends, is error-prone, and predominates in G1 (2–4). To provide damaged cells sufficient time to repair, DNA DSB repair systems initiate signal-transduction pathways to activate G1/S, intra-S, and G2/M cell-cycle checkpoints. In the presence of irreparable or excessive damage, checkpoint signaling can also induce apoptotic cell death pathways (5).

To accomplish its multifaceted role, DSB damage response is organized in networks that set in motion cellular subroutines. These networks operate via protein-protein interactions that are communicated by post-translational modifications, mainly phosphorylation and dephosphorylation (6).

Conceptually, the DSB damage response networks need to serve three major necessities: the recognition of the damaged site, the efficient amplification of the damage signal and its transmission to other cell compartments, and the adequate commencing of cellular subroutines that are going to carry out the end results. Reflecting these necessities, the proteins that have evolved to participate in these networks fall into three general classes: sensors, transducers, and effectors. These days, ongoing research reveals that DSB damage response proteins may participate in more than one class, demonstrating that the DSB damage response networks are characterized by elevated sophistication (Table 1).

2. CHROMATIN-BASED EVENTS IN RESPONSE TO DSBs

The lodging of genetic material in the nucleus of eukaryotic cells requires an extreme compaction of DNA that is achieved at different levels of chromatin folding. At the nucleosome level, 147 base pairs of DNA are wrapped 1.7 times around the histone octamer. Further compaction is achieved by the linker histone H1. Nucleosome arrays are further folded into progressively higher-order structures, with the support of non-histone structural proteins. Although the structure of the nucleosome is well characterized, less is known about the molecular nature of more highly folded structures. In this chapter, we focus on two chromatin-based events, γ H2AX and 53BP1 focus formation, and their role in the biology of DSBs.

After irradiation, DSB lesions can be visualized as distinct formations called foci, when immunocytochemistry methods are combined with epifluorescent microscopy (7). Currently it is becoming clear that DSB-dependent foci are dynamic chromatin structures juxtaposed to the lesion, where repair, signal transduction, chromatin, and structural proteins are bound onto DNA. Many repair and signaling factors are known to translocate to DSB-dependent foci in a time-dependent manner. These factors are

afterward released from the focus to perform functions in the nucleoplasm or cytoplasm. Experimental evidence have substantiated the classification of these factors to the three categories mentioned above, sensors, transducers, and effectors.

Foci facilitate repair and amplification of the checkpoint signal by orchestrating ordered recruitment, assembly, and activation of further repair and signaling proteins; focus constitution varies considerably. At a given time, different factors can be detected in a focus population within the same cell, as they accommodate different repair complexes depending on the repair system involved, the cell-cycle phase, or the timing of the recruitment and release process. Although the damage response events that occur in a cell are not restricted to the sites of lesion, chromatin-based foci events appear to be crucial in the organization and monitoring of the repair process that takes place at the damaged sites.

2.1. The Biology of γ H2AX Foci

2.1.1. γ H2AX Foci

The “higher-order chromatin domains model” of γ -phosphorylated chromatin

The first indication that higher-order chromatin structures are involved in the biology of DSBs was in the case of the phosphorylation of the histone variant H2AX (8). Upon the introduction of DSBs into genomic DNA, a characteristic SQ motif that is accommodated in the C-terminus of the histone H2AX becomes phosphorylated in nucleosome arrays that span a distance of up to megabase-long domains (7).

When immunocytochemistry methods are applied to irradiated cells, and cell specimens are observed under the fluorescent microscope, γ H2AX forms large, bright, and discrete foci at a random distribution throughout the nucleus but not within the nucleoli area. Focus formation pattern follows fast kinetics; γ H2AX foci appear as small and numerous within 1–3 min, become fewer in number but larger and better detected at 15 min, stay steady in size and number between 15 and 60 min, decrease in number at 180 min, and eventually almost disappear at 24–48 h. Various normal and cancer cell lines as well as living organisms respond by the formation of γ H2AX to lethal and non-lethal amounts of ionizing radiation (IR) (7).

The demonstration of precise γ H2AX localization to the sites of DNA DSBs was achieved by the means of a laser scissors experiment, where DSBs were introduced through a pulsed laser microbeam driven along a predetermined course. Subsequent immunocytochemistry showed that γ H2AX forms precisely along this track of DSBs (7).

Remarkably, the phosphorylation of this characteristic SQ motif is conserved over evolution and is part of the DSB damage response. The histone SQ motif is present in different species but resides on different histone variants, all members of the H2A family. In mammals, *Xenopus laevis*, and *Tetrahymena thermophila*, the SQ motif resides on H2AX, in *Drosophila melanogaster* on H2AvD, and in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* on H2A1 and H2A2 (9). In this chapter, for simplicity and clarity purposes, we are going to use indistinguishably the H2AX designation, when we refer to general, conserved features of this cellular response, except where otherwise indicated.

In mammals, the percentage of H2AX with respect to total H2A differs between cell lines, spanning from 2.5 to 30%, whereas in yeast, the ortholog H2A comprises 95%

of the whole complement. Exploiting this divergence, the Bonner laboratory noted a very interesting correlation: although a different amount of H2AX is phosphorylated among different cell lines, the percentage of γ H2AX versus H2A per DSB is constant. On the basis of this correlation, the Bonner laboratory proposed a model that predicted that megabase-long domains in chromatin become γ -phosphorylated per DSB (8) (Fig. 1A and B).

The expansion of γ -phosphorylated chromatin along megabase-long domains model is based on average values. It predicts a distribution of γ -phosphorylated nucleosomes along an average domain size rather than a “fixed” length on chromatin. In mitotic *Muntiacus muntjak* cells from cultures that were previously exposed to sublethal amounts of IR, γ H2AX foci form band-like structures on chromosome arms (7). From a different end, immunoprecipitation experiments in yeast with a γ H2AX antibody revealed that γ -modified chromatin extends in the range of several hundred kilobases (10). In budding yeast, it has been showed that a single DSB induces the γ -formation of an approximately 100-kb domain around the lesion (11). It is very likely that these domains reveal higher-order chromatin structures that may be characteristic of the particular species. Yet, little is known about the organization of these chromatin domains.

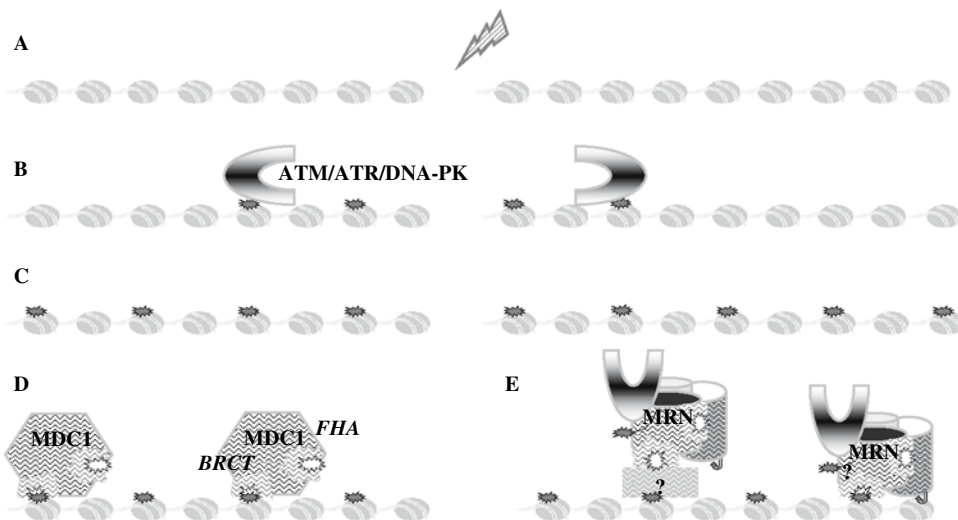


Fig. 1. γ -Phosphorylation alters the affinity properties of megabase-long chromatin domains. When double-stranded DNA damage occurs by irradiation (A) or other factors, multiple members of the phosphatidylinositol-3 (PI3) kinases family, namely ataxia telangiectasia mutated (ATM), ATM and Rad3 related (ATR), and DNA-dependent protein kinase (DNA-PK), redundantly phosphorylate Ser 139 residue of the histone H2AX (γ H2AX) (B). γ -phosphorylation expands along megabase-long chromatin domains, reveals the existence of higher-order chromatin structures that are involved in the biology of double-strand break (DSB) repair, alters the affinity properties of the affected chromatin, and depicts a biological amplification mechanism as DSB sites are surrounded by thousands of γ -modified nucleosomes (C). MDC1 translocates to the lesions sites and binds to γ H2AX through its BRCA1 carboxy-terminal (BRCT) domains (D). The MRN complex (Mre11, Rad50, and NBS1) translocates to the lesions sites and binds to damaged DNA through its Mre11 subunit. Forkhead-associated (FHA) domains of NBS1 may facilitate a direct or indirect association of MRN with γ H2AX. Subsequently, NBS1 recruits and activates ATM at the damage site (E).

The expansion of γ -phosphorylated chromatin along megabase-long domains depicts a biological amplification mechanism where the DSB site is surrounded by thousands of γ -modified nucleosomes (Fig. 1). A practical implication of this amplification is the notion that even one DSB can be visualized by immunocytochemistry methods. Indeed, during V(D)J recombination, recombination-activating gene (RAG)-mediated cleavage generates DBSs between immunoglobulins and T-cell receptor loci (12,13,14). In developing thymocytes, γ H2AX forms nuclear foci that colocalize with the T-cell receptor locus, as determined by immunofluorescence in situ hybridization (14).

This result also demonstrates that immunocytochemistry with γ H2AX antibodies is a powerful tool as it can detect the presence of only one DSB per nucleus.

2.1.2. KINASES RESPONSIBLE FOR γ -PHOSPHORYLATION OF H2AX

Phosphorylation of H2AX at Ser 139 is governed by multiple kinases that are members of the phosphatidylinositol-3 (PI3) family, namely ataxia telangiectasia mutated (ATM, ATM and Rad3 related (ATR), and DNA-dependent protein kinase (DNA-PK). In *S. cerevisiae*, Tel1 and Mec1, the yeast homologs to ATM and ATR, respectively, are also involved in γ -phosphorylation of H2A, which is the yeast homolog to H2AX (9,15). When yeast strains that bear deletions in *tell1* or/and *mec1* genes are subjected to methyl methane-sulfonate (MMS) treatment that involves generation of putative DSBs, γ -phosphorylation of H2A is impaired (16). A low-level signal is present in the *mec1* or *tell1* null single mutants, but no signal is detectable in *tell1/mec1* double null (16). The above results indicate that both kinases are involved in γ H2A phosphorylation and have overlapping roles. In addition, immunoprecipitated Mec1 from yeast cell extracts can phosphorylate a C-terminal yeast H2A peptide *in vitro*, indicating that Ser 129 of the SQ motif of the yeast H2A is a direct target for Mec1 (10).

In human cells, ATM seems to be the major kinase that controls γ -phosphorylation (17–19). ATM knockout cells exhibit impaired γ -H2AX focus formation that can be further eliminated by low-concentration treatment of wortmannin, indicating a redundant role of DNA-PK and/or ATR (20). Low doses of IR activate ATM to γ -phosphorylate H2AX, whereas at higher doses, other kinases contribute or substitute. Fluorescent microscopy of ATM colocalization at the sites of DSBs has been problematic because of the abundance of the former molecules throughout the nucleus. Retention of the ATM molecules at DSBs and colocalization with γ H2AX are shown only after in situ extraction of the unbound ATM molecules before immunocytochemistry (21). In accordance with the *in vivo* experiments, immunoprecipitated ATM phosphorylates the SQ motif of H2AX on Ser 139 *in vitro* (17).

Under hypoxic conditions, H2AX is γ -phosphorylated in an ATR-dependent manner (22). In S phase of the cell cycle, ATR is the kinase to take over γ -phosphorylation, in response to replication arrest and the consequent generation of DSBs, or upon formation of topoisomerase I cleavage complexes after collision of DNA replication forks (23,24). Nevertheless, ATM and ATR exhibit extended redundancy as these kinases are known to have several targets in common, including H2AX (25).

The role of DNA-PK in γ -phosphorylation of H2AX is not yet well understood. Although crude extracts of DNA-PK phosphorylate the SQ motif of H2AX on Ser 139 *in vitro*, several rodent cell lines that are deficient either in the DNA-PK catalytic subunit or in the Ku antigen exhibit no detectable defects in γ -phosphorylation of

H2AX (8,26). However, it has been reported that DNA-PK plays a redundant role in several cases as shown in astrocytoma M059J cell line (7,27) and upon formation of topoisomerase I cleavage complexes at replication forks in S phase (24).

By fluorescence microscopy, DNA-PK immunocytochemistry reveals a diffuse pattern throughout the nucleus in both non-irradiated and irradiated cells. However, upon ionizing irradiation, the DNA-PK catalytic subunit becomes autophosphorylated on threonine 2609 and colocalizes with γ H2AX in distinct foci (28). Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA DSBs, but the involvement of γ H2AX in this mechanism is not clear yet.

H2AX was found to be essentially immobile in chromatin (29). N-terminal fusion constructs of H2AX with green fluorescent protein (GFP) were used to study their diffusional mobility in transient and stable cell transfections. In the absence or presence of DSBs, only a small fraction of GFP-H2AX was redistributed after photo-bleaching. This fact suggests that a phosphorylation–dephosphorylation cycle takes place during the induction and disappearance of foci rather than that a diffusion-exchange mechanism (29).

2.1.3. THE γ H2AX DSB-REPAIR PATHWAY

Colocalization and interactions of repair and signaling factors with γ H2AX foci

Many components of the DNA damage response, including ATM, 53BP1, MDC1, MRN complex, BRCA1, and SMC1, form IR-induced foci (IRIF) that colocalize with γ H2AX foci (9,30–32). These factors participate in HR and NHEJ, indicating that γ H2AX plays a role in both repair systems in mammals. It has been demonstrated that there is a time-dependent sequential assembly of repair factors and signal mediators on γ H2AX foci (27). When cells are treated with wortmannin, a known inhibitor of the PI3 kinase family, γ H2AX focus formation is abolished and 53BP1, MNR, and BRCA1 foci are severely impaired. Along the same line, in H2AX^{-/-} cells, initial migration of 53BP1 and MNR to IRIF is not totally abrogated, but further accumulation is diminished (33). This phenotype rises the question as to whether H2AX is a crucial component of double-strand repair and what role it plays in this response.

The Nussenzweig laboratory has proposed the “two-stage recruitment model,” according to which γ H2AX does not constitute the primary signal that is required for the redistribution of repair complexes to damaged chromatin, but functions as a platform to concentrate repair factors to the vicinity of DNA lesions and to promote interactions between multicomponent complexes (33). The accumulation of repair and signaling factors in proximity to a DSB would facilitate an amplification step of signal transduction and checkpoint pathways, particularly in the case where low numbers of foci per nucleus are present in cells. γ H2AX also modulates the accumulation of repair/signaling proteins in chromatin regions distal to a DSB, following their initial, H2AX-independent migration to DSBs. The retention and subsequent increase in the local concentration of factors may be mediated through weak interactions between the SQ motif in the H2AX tail, thousands of which are modified by phosphorylation, and specific domains of repair/signaling proteins.

This model is also consistent with the finding that H2AX^{-/-} cells exhibit reduced ability to arrest the cell cycle at low doses of IR (33). In the case when only a few DSBs are generated in the nucleus, the DNA repair factors that are modified to transduce the signal are limited, and signal amplification at IRIF becomes essential. On the

contrary, the haploinsufficiency of H2AX^{+/-} mice (13,34), which is based on dosage dependence of the *H2AX* gene, could be explained with the hypothesis that chromatin of H2AX^{+/-} cells, which comprises sparsely γ H2AX-containing nucleosomes, would not mediate efficient concentration of soluble DNA repair factors on IRIF and further amplification of the signal. Notably, in experimental models where Ser 136 and Ser 139 were substituted with glutamic acid, IRIF fail to form (34). Moreover, these cells exhibit enhanced sensitivity to IR comparable with the wild type, indicating that the actual phosphoserine 139 is essential for biological activity and cannot be substituted.

2.1.3.1. γ H2AX Interactions with MDC1. The mediator of DNA damage checkpoint protein 1 (MDC1) is a novel nuclear protein that contains a forkhead-associated (FHA) domain and two BRCA1 carboxy-terminal (BRCT) domains and possesses *in vitro* DNA binding activity (35). MDC1 also presents 20 potential ATM/ATR consensus target phosphorylation motifs (Ser/Thr-Gln), located throughout its N-terminal half, and another 19 consecutive imperfect repeats in its central region (36,37).

In irradiated cultured human cells, MDC1 translocates to the lesion sites rapidly to form foci. MDC1 foci colocalize extensively with γ H2AX foci and exhibit similar kinetics (37–39). It has been shown that MDC1 localization to the DSB sites is abolished in H2AX^{-/-} mouse embryonic fibroblasts (36). In support of this observation, MDC1 foci are also abolished in human cells where H2AX expression had been down-regulated by small interfering RNA (siRNA). These experimental results demonstrate that MDC1 focus formation is strictly dependent on γ H2AX and place these factors in the same pathway (40,41).

BRCT domains are considered to account for the focus formation of MDC1, as in experiments where these domains were deleted, MDC1 foci were compromised (42). As it has been shown that BRCT domains recognize phosphopeptides (43), it is reasonable to speculate that these domains recognize the γ H2AX tail and drive toward the MDC1 focus formation (Fig. 1). In support to this speculation, *in vitro* experiments have shown that a H2AX C-terminus peptide phosphorylated on Ser 139 interacts with MDC1 in human cell extracts, whereas the equivalent non-phosphorylated peptide does not (44).

Down-regulation of MDC1 expression levels by siRNA renders cells hypersensitive to IR (36). At the cellular level, MDC1 down-regulated cells exhibit defects in IR-induced G2/M and intra-S phase checkpoints and show reduced levels of IR-induced apoptosis (45). At the molecular level, the MRN complex and BRCA1 fail to efficiently accumulate in IRIF (40,46). 53BP1 accumulation at sites of DNA damage was found to be MDC1 independent by three different research groups (36,38,47) and partially dependent by another (39).

2.1.3.2. γ H2AX Interactions with MRN Complex. Colocalization of γ H2AX foci with the three-protein MRN complex is evident in IRIF linking H2AX with both DNA-repair and checkpoint cell-cycle responses (24,27,48). Mre11, Rad50, and NBS1 play a crucial role in the biology of DSB repair and operate together in a form of a stable complex (49).

Mre11 has a C-terminal DNA-binding domain and possesses a 3'–5' exonuclease activity, a single-strand endonuclease activity, and a limited DNA unwinding activity (50). Mre11 is characterized by high affinity for aberrant DNA structures.

Rad50 is an ATPase with a characterized structural role; Rad50 binds DNA through a globular domain, whereas the coiled-coil regions of Rad50 form an extended intramolecular flexible arm. It has been shown by force scanning microscopy that Mre11 and Rad50 heterodimers seriate along broken DNA fragments (51). The intramolecular flexible arm of Rad50 on one DNA fragment “weaves” with the reciprocal Rad50 arm of a distant DNA fragment to hold the two molecules together. It is postulated that Rad50 gives the MRN complex the ability to tether sister chromatids together *in vivo* (52).

The NBS1 role as a mediator in the DSB damage response is well established (53). NBS1 protein has an N-terminal FHA domain and a BRCT domain (54). In H2AX knockout cells, NBS1 foci (and presumably MRN complex foci) are impaired but not abolished, indicating that γ H2AX does not comprise the primary signal for the recruitment of MRN complex at the lesion sites (33). These results indicate that the MRN complex has properties of a sensor protein through the DNA-binding domain of Mre11 (49).

γ H2AX-modified chromatin plays a crucial role in the accumulation of NBS1 at the lesion sites (40). *In vivo* disruption of FHA domains abrogates its interaction with γ -modified chromatin, indicating a direct or indirect association of NBS1 with γ H2AX (54) (Fig. 1). Moreover, NBS1 interaction with γ H2AX is independent of hMre11 or BRCA1 (55). Interestingly, MDC1-depleted cells fail to accumulate the MRN complex in the vicinity of the DSB damaged sites, indicating that this interaction is mediated by MDC1 (see Section 2.1.3.3.).

Several consensus ATM phosphorylation SQ motifs are located within the central region of NBS1 (56). In particular, Ser 278, Ser 343, and Ser 397 are phosphorylated by ATM in response to irradiation. In cells that express mutations at these sites to prevent phosphorylation, no difference was observed in the ability of NBS1 to form foci. Although the role of these phosphorylation events is not clear yet, it has been postulated that they function downstream to mediate the signal to other ATM substrates (55,57–59).

It has been demonstrated that migration of NBS1 molecules occurs independently of their phosphorylation status. A strong indication that NBS1 phosphorylation takes place at the foci, where ATM also migrates in response to DSB, is derived from studies in cells where NBS1 was fused to histone H2B. It was shown that NBS1-H2B molecules were phosphorylated after irradiation only at the lesion sites and not in the residual, undamaged chromatin (40).

NBS1 has an additional function, as activator of ATM. ATM autophosphorylation in response to IR is profoundly impaired in human Mre11ATLD1-expressing cells that contain a C-terminally truncated Mre11 domain (58,60,61).

2.1.3.3. Tripartite Interactions Between γ H2AX, MDC1, and MRN Complex.

At present, the interaction between MDC1 and MRN complex is being actively investigated. Down-regulation of MDC1 by siRNA reveals a similar phenotype as in the H2AX knockout and knockdown cells, characterized by impaired, but not abolished NBS1 focus formation (36,38,62). Taking into account that MDC1 focus formation is strictly dependent on γ H2AX (see Section 2.1.3.3.), it is reasonable to speculate that the contribution of γ H2AX to the accumulation of MRN complex at the lesion sites is mediated by MDC1 (Fig. 2). Consistent with its role as a mediator, FHA domains of

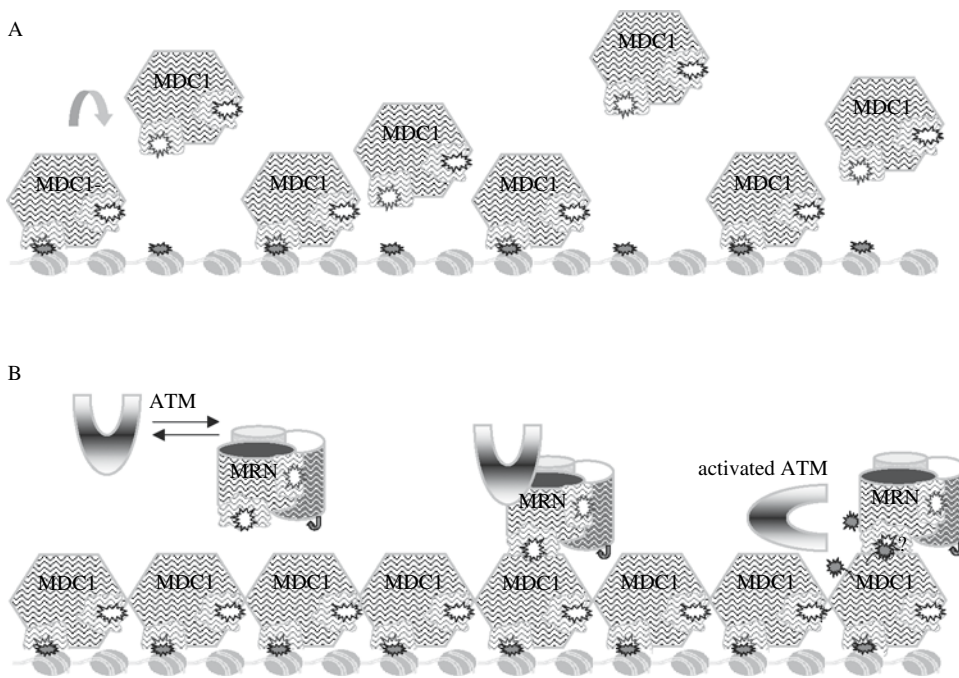


Fig. 2. MDC1 and MRN create a reinforcement loop to enhance ATM activity on γ -modified chromatin. MDC1 relocates to γ H2AX foci and binds to phosphorylated Ser 139 through its BRCA1 carboxy-terminal (BRCT) domains (A), where it creates a scaffold to facilitate anchorage of MRN complex (B). In turn, MRN complex enhances ATM activity to facilitate phosphorylation of repair factors, including MRN and MDC1. Multiple phosphorylation of MDC1 may promote multimerization of this protein through its forkhead-associated (FHA) domains and facilitates anchorage of other repair factors that are subsequently recruited to the lesion sites.

MDC1 may be involved in the multimerization of this protein on the DSB-modified chromatin. It is possible that MDC1 multimerization could function to create a scaffold on γ -modified chromatin, where the MRN complex and other checkpoint and DNA repair proteins could anchor and interact with other factors (37,38,40) (Fig. 2).

In cells expressing FHA-disrupted NBS1, NBS1 accumulation was abrogated, but microfocus-like structures were formed along the DSB path that was generated by microlaser (40,47,54). These structures are MDC1 independent, demonstrating that there is a small region at the lesion sites where NBS1 associates in a FHA-independent way. Whether these results indicate that NBS1 binds directly to naked DNA or interacts with γ -modified chromatin through alternative means remains to be experimentally approached (Fig. 1).

MDC1 becomes phosphorylated in response to IR in an ATM-dependent manner. This phosphorylation is partially affected in H2AX^{-/-} MEFs, and NBS cells, suggesting that both NBS1 and γ H2AX participate in a feedback loop reaction involving ATM activation (41,55). These results are consistent with a model according to which MDC1 relocates to γ H2AX foci, where it attracts the MRN complex. In turn, NBS1 activates ATM that is also relocated there, which subsequently phosphorylates MDC1 (Fig. 2). In support to this model, γ -phosphorylation is also affected in MDC1-depleted cells (35). However, the possibility that MDC1 phosphorylation could

occur in parallel at the nucleoplasm by other mechanisms cannot be excluded from these experiments.

In addition to its role as mediator, MDC1 has a function as an activator to ATM as well (see Section 3).

2.2. *The Biology of 53BP1 Foci*

2.2.1. 53BP1 Foci

53BP1 was originally identified during a yeast two-hybrid screen for p53-interacting proteins (63). The sequence similarity of 53BP1 with the yeast DNA damage checkpoint proteins Rad9 and Crb2/Rhp9 and the localization of 53BP1 to sites of IRIF suggested that 53BP1 would function in the DNA DSB checkpoint pathway. The first experimental evidence showing that 53BP1 is involved in the cellular response to DNA damage was its localization to sites of DSBs in cells exposed to IR (64). Upon DNA damage, 53BP1 relocates to discrete nuclear foci that represent sites of DNA lesions and become hyperphosphorylated (65,66). The notion that 53BP1 foci mark sites of DNA DSBs has been documented by colocalization experiments of 53BP1 with γ H2AX and other repair factors known to form foci (62,64–68).

In G1, 53BP1 exists in a diffuse nuclear pattern as well as in large nuclear “dots.” In S phase, 53BP1 can be found in a discrete, punctuate pattern. The nuclear distribution pattern of 53BP1 in G2 cells appeared in two forms, one similar to S phase but with fewer foci and one that exhibited few, if any, large dots. 53BP1 foci form within minutes of irradiation, and at doses of IR as low as 0.5 Gy. 53BP1 foci colocalize with γ H2AX and exhibit kinetics parallel to γ H2AX focus formation (64,69). The number of 53BP1 foci increases linearly over time, reaching a maximum at about 15–30 min, and then steadily decreases to baseline levels within the next 16 h. The number of 53BP1 foci, about 20 per cell per Gy of IR, closely parallels the number of DSBs. In addition, the kinetics of resolution of 53BP1 foci is very similar to the kinetics of DNA DSB repair following IR, indicating that there is a relationship between completion of repair and disappearance of 53BP1 foci (64,70).

Several domains have been characterized in 53BP1: a 53BP1 focus formation region (FFR), two tandem tudor folds, two tandem BRCT domains, and an 8-kDa light chain (LC8)-binding domain (71–76). A nuclear localization signal is also present. A 53BP1 FFR has been mapped at residues 1052–1639 (69,77). It was previously shown that γ H2AX coimmunoprecipitates with 53BP1 from irradiated cells (69). Interestingly, in a pull-down assay, where six different 53BP1 GST fragments spanning the entire 53BP1 protein were incubated with immobilized C-terminus H2AX peptides, only the fragments 956–1354 showed strong interaction with the γ -phosphorylated one (72,78). In contrast, no binding was detected to the non-phosphorylated peptide bearing identical sequence.

At the C-terminus of the FFR, there are two tandem tudor folds that consist of a 50 amino acid long stretch (72,76). Although not well established, Tudor domains seem to play a role in protein–protein interactions through methylated residues. In support to this notion, tudor folds have structural similarities to chromo domains, that are known to bind to histone tails that contain methylated lysines (see Section 2.2.2.).

The FFR also includes a region required for 53BP1 kinetochore localization in mitotic cells (amino acids 1220–1601) (76). In a different set of experiments

where various GFP-tagged 53BP1 truncations were expressed, the kinetochore-binding domain has been mapped to a 380 residue portion of the protein that excludes the nuclear localization signal and the BRCT motifs (79). It was shown that this region is responsible for loading 53BP1 to kinetochores in prophase and release by mid-anaphase (79). Recently, it was shown that the kinetochore localization region is also essential for 53BP1 focus formation in response to DNA damage, suggesting that both events might be regulated in a similar fashion.

An LC8-binding domain of 53BP1 has been mapped to a short peptide segment immediately next to N-terminal to the kinetochore localization region (75). Unlike other known LC8-binding proteins, 53BP1 contains two distinct LC8-binding motifs that are arranged in tandem. The LC8-binding domain is completely separated from the p53-binding domain in 53BP1. As the LC8 of dynein binds to 53BP1, it has been proposed that 53BP1 can potentially act as an adaptor to assemble p53 to the dynein complex (75).

BRCT domains are a common protein–protein interaction motif that are present in proteins involved in the DNA damage response. 53BP1 has two tandem BRCT domains (amino acids 1714–1850 and 1865–1972, respectively) (64,66,73). A role for 53BP1 in a DNA damage response pathway was first proposed based on the similarity of its BRCT domains to the BRCT domains present in the *S. cerevisiae* Rad9 and *S. pombe* Crb2/Rhp9 proteins (64,71,72). Rad9 in *S. cerevisiae* is required for cell-cycle arrest in response to DNA damage and becomes phosphorylated by Mec1. After phosphorylation, Rad9 interacts with Rad53, which is the homolog of the Chk2 kinase in humans, and this interaction is required for the activation of Rad53.

A comparison of the structure of the BRCT region of 53BP1 with the BRCT tandem repeats reveals that the interdomain interface and linker regions are remarkably well conserved. The crystal structure of the 53BP1 BRCT tandem repeats in complex with the DNA-binding domain of p53 shows that the two tandem BRCT repeats pack extensively through an interface that also involves the inter-repeat linker (72,74). The first BRCT repeat and the linkers together bind p53 on the region that overlaps with the DNA-binding surface of p53 and involves p53 residues that are mutated in cancer and are important for DNA binding.

It has been suggested that the interaction of p53 with 53BP1 reflects the putative adaptor function of 53BP1; 53BP1 may recruit p53 to sites of DSBs, thereby facilitating its ATM-dependent phosphorylation (72). However, this is not the only possible scenario. Recent experimental results suggest that BRCT domains may have phosphopeptide-binding activity, depending on the structure in the vicinity (43). Given the absence of p53 in yeast and the evolutionary conservation of the BRCT domains of 53BP1 in all eukaryotes, the core function of the 53BP1 BRCT domains could relate to some function of 53BP1 that are independent of p53 (72,78,80,81). Because the BRCT domains are not required for localization of 53BP1 to sites of DNA DSBs, it is likely that they play a role in ATM activation, directly or indirectly (41,71,82). The identification of a physiological relevant phospholigand of the 53BP1 BRCT domains will shed light to this question.

Fifteen potential phosphorylation sites (15AQ) have been mapped on 53BP1 N-terminal region, some of which are known to be targeted during the DNA damage response (66). Indeed, 53BP1 becomes hyperphosphorylated on its N-terminus in

an ATM-depended manner in response to IR and mediates DNA damage-signaling pathways in mammalian cells (41,66,78,81,82).

2.2.2. RECRUITMENT OF 53BP1 TO SITES OF DNA DSBs

As mentioned in Sections 2.1.3. and 2.1.3.1., in cells that lack histone H2AX, or where the phosphorylation of H2AX is abrogated, 53BP1 is initially recruited to the damaged chromatin, but cannot be retained (33). A number of laboratories have addressed the question regarding the nature of the factor that recruits 53BP1 to DSBs sites (47,72). The initial recruitment of 53BP1 to the site of a DSB does not seem to require ATM, NBS1, or DNA-PK, since in cells deficient in these proteins, 53BP1 localizes to sites of DSBs with normal kinetics (33). Recently, a role of histone methylation in the DSB damage response has been discovered. The Halazonetis laboratory showed that in human cells, 53BP1 binds to methylated H3 (72). *In vitro* experiments using residues that form the walls of the pocket between the tudor folds showed that the 53BP1 tudor domain binds histone H3 methylated on Lys 79 (72). By deletion analysis, these residues were also shown to be required for recruitment of 53BP1 to DSBs *in vivo*. Competition experiments with H3 peptides showed that the H3 peptide with dimethylated Lys 79 competed with the native histone H3 (72). The corresponding non-methylated peptide did not compete, whereas the peptides with mono-methylated and dimethylated Lys 27, or Arg 26 competed at lower efficiency (72). The enzyme that methylates Lys 79 in human cells is DOT1, an evolutionary conserved methyltransferase. Suppression of DOT1 by siRNA resulted in suppression of H3 methylation on Lys 79 and minimized the 53BP1 recruitment to the lesion sites.

One possible explanation for the reduced affinity of 53BP1 to the damaged chromatin when abolished either the relevant methylated sites or the γ -phosphorylation could be that the tudor domain and the phosphopeptide-binding region act in a cooperative way. As mentioned earlier in Section 2.2.1., the tudor domain and the phosphopeptide-binding region reside in proximity within the FFR of 53BP1, supporting further this hypothesis (Fig. 3).

Surprisingly, the levels of H3 Lys 79 methylation were unaltered upon DSB damage (72). To resolve this discrepancy, the following model was proposed by the Halazonetis laboratory. The introduction of DSBs into chromatin results in disruption of nucleosome stacking, which leads to revelation of H3 Lys 79 and possibly other methylated residues of the histones in the core particle, resulting in exposure of binding sites for 53BP1 and its recruitment to the DSB sites. According to this proposed mechanism, 53BP1 can “sense” DSB lesions through changes in higher-order chromatin structure and participates as a sensor in the DSB damage response (72).

This model, based on the results from human cells, has been supported by parallel work in *S. cerevisiae* and *S. pombe*. In *S. cerevisiae*, Rad9, a DNA damage checkpoint protein that has a sequence similarity to human 53BP1, interacted with native H3 (83). Deletion of DOT1 in yeast resulted in radiation sensitivity and a DSB checkpoint defect. In the fission yeast *S. pombe*, it was demonstrated that Set9, a previously uncharacterized SET domain protein, is responsible for H4 Lys 20 methylation (84). Interestingly, this methylation does not have any apparent role in the regulation of gene expression or heterochromatin function. However, loss of Set9 activity or mutation of H4 Lys 20 markedly impairs cell survival after genotoxic challenge and compromises the ability of cells to maintain checkpoint-mediated cell-cycle arrest. Furthermore,

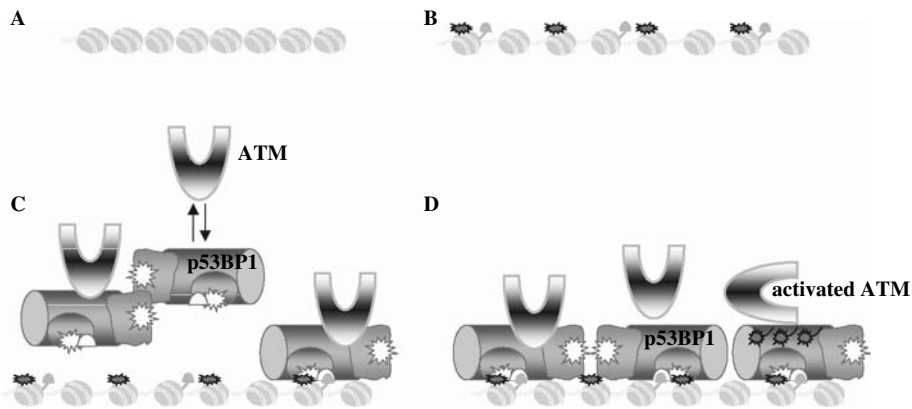


Fig. 3. Recruitment of 53BP1 to damaged sites by chromatin γ -phosphorylation and methylation. Collectively with γ -phosphorylation, the introduction of double-strand breaks (DSBs) into chromatin results in disruption of nucleosome stacking (**A and B**), which leads to revelation of methylated H3 lysine 79 and possibly other methylated residues of the histones in the core particle (**B**). 53BP1 relocates to the damaged chromatin where it binds through its focus formation region (FFR). FFR includes a phosphopeptide-binding site and a tudor domain. In the absence of γ H2AX or the relevant methylated residues, 53BP1 is recruited to the DSBs sites but not retained, indicating that FFR facilitates cooperative binding (**C**). 53BP1 creates a scaffold on damaged chromatin to recruit and activate ATM. Activated ATM phosphorylates repair factors including p53BP1 (**D**).

genetic experiments have demonstrated that Set9 is required for Crb2 localization to sites of DNA damage (84). Although H4 Lys 20 is a different methylation site from H3 Lys 79, it seems that these sites play the same role with respect to the 53BP1/Crb2 recruitment mechanism. This could be explained if the affinity of these factors is not restricted to one methylated site, or multiple histone “marks” may contribute to 53BP1/Crb2 recruitment.

It needs to be mentioned also that BLM helicase has been reported to recruit 53BP1 to DSB lesions independently of its helicase activity (67). Interestingly, a deficiency of the BLM helicase has been reported that markedly increases cancer incidence in humans (85).

3. INTERPLAY BETWEEN γ H2AX AND 53BP1

To recapitulate, the introduction of DSBs into DNA results in long stretches of chromatin that are marked by two histone modifications: histone methylation (mainly the H3 Lys 79 in mammals and H3 Lys 20 in *S. pombe*) and the γ -phosphorylation of H2AX. In turn, this DSB-modified chromatin initiates two branches of the DSB damage response: one by the 53BP1 recruitment by methylated H3 Lys 79 (or relevant residues) and the other by the recruitment of MDC1/MRN complex by γ H2AX (31,33).

The γ H2AX and the 53BP1 branches do, however, clearly interact with each other. The lack of retention of 53BP1 in H2AX^{-/-} cells may indicate a direct mechanism where the FFR of 53BP1 facilitates cooperative binding of Ser 139 phospho groups and H3 Lys 79 methyl groups. Nevertheless, γ H2AX seems to play a role in accumulation of 53BP1 by indirect mechanisms as well.

Notably, phosphorylation of 53BP1 is reduced by 40% in H2AX^{-/-} cells, 1 h after low-dose irradiation (86). Although it is not clear whether 53BP1 is phosphorylated only when bound to damaged DNA, these results suggest that γ H2AX creates a reinforcement loop to 53BP1 phosphorylation. Notably, in AT cells, 53BP1 relocalizes at DSBs, indicating that ATM-dependent phosphorylation of 53BP1 is not required for 53BP1 focus formation (69). What role this phosphorylation plays is however not clear yet.

Several lines of evidence have shown that 53BP1 is required for a subset of ATM-dependent phosphorylation events (71). An antibody that specifically recognizes the phospho-Ser/Thr-Gln epitope generated by ATM/ATR phosphorylation showed reduced immunofluorescence reactivity in cells treated with 53BP1 siRNA (41,82). However, the magnitude of the defects observed in this system was smaller than that observed in AT cells, indicating the existence of additional 53BP1 network branches that lead to ATM-dependent phosphorylation. In addition, specific antibodies against SMC1 phospho-Ser 966 and phospho-Ser 957 and Chk2 phospho-Ser 33 and phospho-Ser 35 showed suppressed phosphorylation in 53BP1 knockdown cells (82). The above mentioned phosphorylation events have been taken as an indication that 53BP1 functions downstream of ATM activation, as a mediator to several ATM substrates. In support of this conclusion, it has been shown that 53BP1 is required for efficient accumulation of p53 to DSB sites and subsequent p53 activation (81).

According to this line of evidence, 53BP1 functions by creating a scaffold on the revealed methylated H3 Lys 79 chromatin that is further stabilized by interactions with γ H2AX to recruit ATM substrates. Recruitment of ATM to the lesion sites would facilitate the phosphorylation of these substrates. This model is consistent with our current understanding of Rad9 function in budding yeast; Rad9 is phosphorylated by Mec1 and binds to Rad53 to recruit it to the lesion sites (72).

The same experimental results could, however, be interpreted in a reverse way. 53BP1 may function upstream of ATM to activate it, in response to DSBs. In fact, a factor that recruits and possibly activates ATM to the DSBs could be 53BP1, as 53BP1 physically interacts with ATM in irradiated, but not in non-irradiated cells, as shown by coprecipitation experiments (71). Consistent with this hypothesis is the observation that 53BP1 forms foci at ATM^{-/-}, indicating that it functions upstream of ATM activation (71). According to this model, 53BP1 accumulation to the lesion sites would facilitate the recruitment of ATM, where the ATM substrates would be recruited by γ H2AX.

The Halazonetis laboratory has proposed a solution that combines features of two previous opposing models. According to this group, 53BP1 acts both as an activator of ATM and as an adaptor and/or a mediator. 53BP1 functions creating a feedback loop upstream of ATM by activating this kinase, and also downstream of ATM by facilitating the ability of ATM to phosphorylate its substrates, such as Chk1 and Chk2 (72). A dual function of 53BP1 as an activator and adaptor is certainly possible, given that ATM phosphorylates 53BP1 in response to DNA damage at Ser 25 and most likely at other sites as well. Whether these phosphorylation events are critical for phosphorylation of ATM substrates still remains to be demonstrated (87).

To develop experimental support for the “activator” function of 53BP1, the Halazonetis laboratory addressed the question whether 53BP1 contributes to the activation of ATM. It has been demonstrated by the Kastan laboratory that ATM can be activated at a distance from chromatin, in response to the DSB (88). This research

group has shown that ATM is located at the nucleoplasm and exists in a dimer/oligomer form in an inactive form. Upon chromatin aberrations, ATM responds by autophosphorylation on Ser 1981. This phosphorylation disrupts the oligomer form and renders ATM active by exposing its catalytic site (88). Remarkably, ATM is very sensitive in “sensing” chromatin irregularities, even when they are not derived from DSBs, indicating a wide-ranging role of this kinase in response to chromatin aberrations. Whether ATM directly senses these DSB-induced chromatin alterations, or requires DNA “sensors” to transmit the damage signal is not clear.

To test the hypothesis that 53BP1 plays a role in ATM activation, the Halazonetis group addressed the question whether 53BP1 contributes to the phosphorylation of ATM at Ser 1981, by suppressing 53BP1 (72). In contrast to what they were expected, their first observation indicated that there is no effect on ATM Ser 1981 phosphorylation in 53BP1 knockdown cells.

Analyzing further into their experimental results, they took into consideration that ATM is activated also by NBS1, as was shown by different research groups (41,56,57,59). The MCD1/MRN complex is recruited to the lesion sites, and this response is mediated by γ H2AX, independently to 53BP1 (59). This could provide an explanation to the discrepancy between their working hypothesis and their experimental results, as the MCD1/MRN contribution could overpower the effect on ATM Ser 1981 phosphorylation.

Exploring the possibility that there are conditions where the two pathways do not overlap, the recruitment of both factors to IRIF was monitored at high and low doses of irradiation (41). The results show that exposure of cells to low dose of IR exhibited recruitment of 53BP1 but not that of MCD1/MRN complex. This piece of evidence emphasizes the notion that 53BP1 plays an essential role at low doses of irradiation (41).

Further pursuing the same question, these investigators investigated the possibility that there is crosstalk between the 53BP1 and the MDC1/MRN pathway. At low-dose irradiation, suppression of MDC1 had no effect on 53BP1 recruitment, but surprisingly, suppression of 53BP1 compels recruitment of MCD1/MRN complex to IRIF (41). Similar results were observed with fibroblasts derived from an individual with XPC (xeroderma pigmentosum group C), in which suppression of 53BP1 resulted in increased localization of MDC1 and NBS1 (41). The increased localization of NBS1 was accompanied by increased phosphorylation of Ser 343, suggesting that in the absence of 53BP1, a greater pool of NBS1 molecules is recruited (41).

Finally, and in accordance with their previous experiments, the Halazonetis group showed that suppression of 53BP1 in NBS1-deficient cells revealed a defect in ATM phosphorylation at low doses of irradiation (41). In addition, downstream targets of ATM, specifically Chk2 Thr 68 and SMC Ser 957, were also found to exhibit impaired phosphorylation. This experimental evidence supports a model according to which 53BP1 has a dual role as an activator and as an adaptor and/or a mediator in the DSB damage response (Fig. 3).

A different line of experiments from the Lukas laboratory indicates another relationship between 53BP1 and MDC1 at later time points post-irradiation. When the departure of 53BP1 from γ H2AX foci was monitored by real-time microscopy, it was evident that this departure is accelerated in MDC1-depleted cells (47). In these experiments, GFP-tagged 53BP1 was observed to leave the γ H2AX foci after 7–12 h post-irradiation. Surprisingly, in MDC1 knockdown cells, the departure time was reduced to less than 6 h post-irradiation while γ H2AX was still present at the sites

of the lesion (47). It has been suggested by these investigators that phosphorylation of MDC1 at foci renders it able to interact directly with 53BP1, or alternatively, with remodeling factors that stabilize chromatin conformation changes that facilitate DSB repair (47).

4. OPEN QUESTIONS AND PERSPECTIVES

DSB-dependent foci are dynamic chromatin structures that facilitate repair and amplification of checkpoint signals by orchestrating a time-dependent ordered recruitment, assembly, and activation of sensors, transducers, and effectors during the DSB damage response. Focus constitution varies considerably at a given time, as different factors can be detected on a focus population within a particular cell, as they accommodate different repair complexes depending on the repair system involved, the cell-cycle phase, or the severity of the damage. Although the damage response events that occur in a cell are not restricted to the sites of lesion, but take place at the nucleoplasm and cytoplasm as well, focus events appear to be crucial in monitoring the repair process that take place at the damaged sites.

During this dynamic series of events, a very reliable point of reference that marks the existence of DSBs from the very early events until the restoration of the damage is γ H2AX. It is widely accepted that γ H2AX plays a central role in the repair of DSB. Three main pieces of evidence substantiate this conclusion: (i) the early appearance of γ H2AX foci after induction of DSB, prior to all other known proteins that form foci; (ii) the delayed disappearance of γ H2AX foci that is concomitant with restoration of the lesion (iii) the abrogation of accumulation of several proteins at the lesion sites in H2AX knockout and knockdown systems, whereas ablation of other proteins that form foci does not have a similar impact. As mentioned in Section 2.1.3, 53BP1 and MRN form minute foci in H2AX knockout cells, where further accumulation of these molecules is abrogated. This evidence poses a theoretical question: how sensors such as 53BP1 and MRN are unable to recognize their primary signal in chromatin structures all along the damaged chromatin domains in H2AX knockout cells?

It is reasonable to speculate that γ H2AX may recruit other factors that facilitate chromatin changes along the broken fiber that in turn would expose methylated sites for 53BP1 to accumulate or perhaps other structures relevant to MRN recognition. Candidates for this role have recently emerged. Chromatin-remodeling subunits, known to be otherwise involved in transcription, have been shown to be recruited by the γ -phosphorylated chromatin during the DSB repair process. It remains to be experimentally tested whether they indeed play a role in this context or not.

The overwhelming evidence for the critical function of γ H2AX in the DSB response, do not necessarily require acceptance of a “hierarchical” model that operates in signal transduction pathways that are initiated by chromatin lesions. The fact that 53BP1 recognizes a different type of histone modification, that of methylated H3 Lys 79, and initiates a different branch in the DSB damage response, strongly suggests that the chromatin-initiated network of interactions is “relational” rather than “hierarchical”. The existence of an interplay between methylation and 53BP1 from one end and γ H2AX and MDC1/MRN from the other supports this notion. It is expected that in the near future, additional factors and interactions will be identified, providing an improved understanding of the role that γ H2AX, 53BP1, MDC1 and MRN may play in this highly sophisticated network of DSB damage response.

REFERENCES

1. van den Bosch M, Lohman PH, Pastink A. DNA double-strand break repair by homologous recombination. *Biol Chem* 2002;383(6):873–92.
2. Rothkamm K, Kruger I, Thompson LH, Lobrich M. Pathways of DNA double-strand break repair during the mammalian cell cycle. *Mol Cell Biol* 2003;23(16):5706–15.
3. Weterings E, van Gent DC. The mechanism of non-homologous end-joining: a synopsis of synapsis. *DNA Repair (Amst)* 2004;3(11):1425–35.
4. Lees-Miller SP, Meek K. Repair of DNA double strand breaks by non-homologous end joining. *Biochimie* 2003;85(11):1161–73.
5. Kaina B. DNA damage-triggered apoptosis: critical role of DNA repair, double-strand breaks, cell proliferation and signaling. *Biochem Pharmacol* 2003;66(8):1547–54.
6. Jackson SP. Sensing and repairing DNA double-strand breaks. *Carcinogenesis* 2002;23(5):687–96.
7. Rogakou EP, Boon C, Redon C, Bonner WM. Megabase chromatin domains involved in DNA double-strand breaks *in vivo*. *J Cell Biol* 1999;146(5):905–16.
8. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* 1998;273(10):5858–68.
9. Redon C, Pilch D, Rogakou E, Sedelnikova O, Newrock K, Bonner W. Histone H2A variants H2AX and H2AZ. *Curr Opin Genet Dev* 2002;12(2):162–9.
10. Shroff R, Arbel-Eden A, Pilch D, Ira G, Bonner WM, Petrini JH, Haber JE, Lichten M. Distribution and dynamics of chromatin modification induced by a defined DNA double-strand break. *Curr Biol* 2004;14(19):1703–11.
11. Unal E, Arbel-Eden A, Sattler U, et al. DNA damage response pathway uses histone modification to assemble a double-strand break-specific cohesin domain. *Mol Cell* 2004;16(6):991–1002.
12. Bassing CH, Alt FW. The cellular response to general and programmed DNA double strand breaks. *DNA Repair (Amst)* 2004;3(8–9):781–96.
13. Bassing CH, Suh H, Ferguson DO, et al. Histone H2AX: a dosage-dependent suppressor of oncogenic translocations and tumors. *Cell* 2003;114(3):359–70.
14. Chen HT, Bhandoola A, Difilippantonio MJ, et al. Response to RAG-mediated VDJ cleavage by NBS1 and gamma-H2AX. *Science* 2000;290(5498):1962–5.
15. Nakamura TM, Du LL, Redon C, Russell P. Histone H2A phosphorylation controls Crb2 recruitment at DNA breaks, maintains checkpoint arrest, and influences DNA repair in fission yeast. *Mol Cell Biol* 2004;24(14):6215–30.
16. Downs JA, Lowndes NF, Jackson SP. A role for *Saccharomyces cerevisiae* histone H2A in DNA repair. *Nature* 2000;408(6815):1001–4.
17. Burma S, Chen BP, Murphy M, Kurimasa A, Chen DJ. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J Biol Chem* 2001;276(45):42462–7.
18. Shiloh Y. ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer* 2003;3(3):155–68.
19. Riballo E, Kuhne M, Rief N, et al. A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci. *Mol Cell* 2004;16(5):715–24.
20. Stiff T, O’Driscoll M, Rief N, Iwabuchi K, Lobrich M, Jeggo PA. ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. *Cancer Res* 2004;64(7):2390–6.
21. Andegeko Y, Moyal L, Mittelman L, Tsarfaty I, Shiloh Y, Rotman G. Nuclear retention of ATM at sites of DNA double strand breaks. *J Biol Chem* 2001;276(41):38224–30.
22. Hammond EM, Dorie MJ, Giaccia AJ. ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. *J Biol Chem* 2003;278(14):12207–13.
23. Ward IM, Chen J. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *J Biol Chem* 2001;276(51):47759–62.
24. Furuta T, Takemura H, Liao ZY, et al. Phosphorylation of histone H2AX and activation of Mre11, Rad50, and Nbs1 in response to replication-dependent DNA double-strand breaks induced by mammalian DNA topoisomerase I cleavage complexes. *J Biol Chem* 2003;278(22):20303–12.
25. Koundrioukoff S, Polo S, Almouzni G. Interplay between chromatin and cell cycle checkpoints in the context of ATR/ATM-dependent checkpoints. *DNA Repair (Amst)* 2004;3(8–9):969–78.
26. Fernandez-Capetillo O, Lee A, Nussenzweig M, Nussenzweig A. H2AX: the histone guardian of the genome. *DNA Repair (Amst)* 2004;3(8–9):959–67.

27. Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol* 2000;10(15):886–95.
28. Chan DW, Chen BP, Prithivirajasingh S, et al. Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA double-strand breaks. *Genes Dev* 2002;16(18):2333–8.
29. Siino JS, Nazarov IB, Svetlova MP, et al. Photobleaching of GFP-labeled H2AX in chromatin: H2AX has low diffusional mobility in the nucleus. *Biochem Biophys Res Commun* 2002;297(5):1318–23.
30. Sedelnikova OA, Pilch DR, Redon C, Bonner WM. Histone H2AX in DNA damage and repair. *Cancer Biol Ther* 2003;2(3):233–5.
31. Pilch DR, Sedelnikova OA, Redon C, Celeste A, Nussenzweig A, Bonner WM. Characteristics of gamma-H2AX foci at DNA double-strand breaks sites. *Biochem Cell Biol* 2003;81(3):123–9.
32. Fernandez-Capetillo O, Celeste A, Nussenzweig A. Focusing on foci: H2AX and the recruitment of DNA-damage response factors. *Cell Cycle* 2003;2(5):426–7.
33. Celeste A, Fernandez-Capetillo O, Kruhlak MJ, et al. Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. *Nat Cell Biol* 2003;5(7):675–9.
34. Celeste A, Difilippantonio S, Difilippantonio MJ, et al. H2AX haploinsufficiency modifies genomic stability and tumor susceptibility. *Cell* 2003;114(3):371–83.
35. Goldberg M, Stucki M, Falck J, et al. MDC1 is required for the intra-S-phase DNA damage checkpoint. *Nature* 2003;421(6926):952–6.
36. Stewart GS, Wang B, Bignell CR, Taylor AM, Elledge SJ. MDC1 is a mediator of the mammalian DNA damage checkpoint. *Nature* 2003;421(6926):961–6.
37. Stucki M, Jackson SP. MDC1/NFBD1: a key regulator of the DNA damage response in higher eukaryotes. *DNA Repair (Amst)* 2004;3(8–9):953–7.
38. Xu X, Stern DF. NFBD1/MDC1 regulates ionizing radiation-induced focus formation by DNA checkpoint signaling and repair factors. *FASEB J* 2003;17(13):1842–8.
39. Peng A, Chen PL. NFBD1, like 53BP1, is an early and redundant transducer mediating Chk2 phosphorylation in response to DNA damage. *J Biol Chem* 2003;278(11):8873–6.
40. Lukas C, Melander F, Stucki M, et al. Mdc1 couples DNA double-strand break recognition by Nbs1 with its H2AX-dependent chromatin retention. *EMBO J* 2004;23(13):2674–83.
41. Mochan TA, Venere M, DiTullio RA Jr, Halazonetis TD. 53BP1 and NFBD1/MDC1-Nbs1 function in parallel interacting pathways activating ataxia-telangiectasia mutated (ATM) in response to DNA damage. *Cancer Res* 2003;63(24):8586–91.
42. Shang YL, Boderer AJ, Chen PL. NFBD1, a novel nuclear protein with signature motifs of FHA and BRCT, and an internal 41-amino acid repeat sequence, is an early participant in DNA damage response. *J Biol Chem* 2003;278(8):6323–9.
43. Manke IA, Lowery DM, Nguyen A, Yaffe MB. BRCT repeats as phosphopeptide-binding modules involved in protein targeting. *Science* 2003;302(5645):636–9.
44. Lee MS, Edwards RA, Thede GL, Glover JN. Structure of the BRCT repeat domain of MDC1 and its specificity for the free COOH-terminal end of the {gamma}-H2AX histone tail. *J Biol Chem* 2005;280(37):32053–6.
45. Peng A, Chen PL. NFBD1/Mdc1 mediates ATR-dependent DNA damage response. *Cancer Res* 2005;65(4):1158–63.
46. Lee AC, Fernandez-Capetillo O, Pisupati V, Jackson SP, Nussenzweig A. Specific association of mouse MDC1/NFBD1 with NBS1 at sites of DNA-damage. *Cell Cycle* 2005;4(1):177–82.
47. Bekker-Jensen S, Lukas C, Melander F, Bartek J, Lukas J. Dynamic assembly and sustained retention of 53BP1 at the sites of DNA damage are controlled by Mdc1/NFBD1. *J Cell Biol* 2005;170(2):201–11.
48. D'Amours D, Jackson SP. The Mre11 complex: at the crossroads of DNA repair and checkpoint signalling. *Nat Rev Mol Cell Biol* 2002;3(5):317–27.
49. Petrini JH, Stracker TH. The cellular response to DNA double-strand breaks: defining the sensors and mediators. *Trends Cell Biol* 2003;13(9):458–62.
50. Costanzo V, Robertson K, Bibikova M, et al. Mre11 protein complex prevents double-strand break accumulation during chromosomal DNA replication. *Mol Cell* 2001;8(1):137–47.
51. Moreno-Herrero F, de Jager M, Dekker NH, Kanaar R, Wyman C, Dekker C. Mesoscale conformational changes in the DNA-repair complex Rad50/Mre11/Nbs1 upon binding DNA. *Nature* 2005;437(7057):440–3.

52. Williams RS, Tainer JA. A nanomachine for making ends meet: MRN is a flexing scaffold for the repair of DNA double-strand breaks. *Mol Cell* 2005;19(6):724–6.
53. Kobayashi J, Antocchia A, Tauchi H, Matsuura S, Komatsu K. NBS1 and its functional role in the DNA damage response. *DNA Repair (Amst)* 2004;3(8–9):855–61.
54. Kobayashi J, Tauchi H, Sakamoto S, et al. NBS1 localizes to gamma-H2AX foci through interaction with the FHA/BRCT domain. *Curr Biol* 2002;12(21):1846–51.
55. Kang J, Ferguson D, Song H, et al. Functional Interaction of H2AX, NBS1, and p53 in ATM-dependent DNA damage responses and tumor suppression. *Mol Cell Biol* 2005;25(2):661–70.
56. Horejsi Z, Falck J, Bakkenist CJ, Kastan MB, Lukas J, Bartek J. Distinct functional domains of Nbs1 modulate the timing and magnitude of ATM activation after low doses of ionizing radiation. *Oncogene* 2004;23(17):3122–7.
57. Difilippantonio S, Celeste A, Fernandez-Capetillo O, et al. Role of Nbs1 in the activation of the Atm kinase revealed in humanized mouse models. *Nat Cell Biol* 2005;7(7):675–85.
58. Lee JH, Paull TT. Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex. *Science* 2004;304(5667):93–6.
59. Uziel T, Lerenthal Y, Moyal L, Andegeko Y, Mittelman L, Shiloh Y. Requirement of the MRN complex for ATM activation by DNA damage. *EMBO J* 2003;22(20):5612–21.
60. Girard PM, Riballo E, Begg AC, Waugh A, Jeggo PA. Nbs1 promotes ATM dependent phosphorylation events including those required for G1/S arrest. *Oncogene* 2002;21(27):4191–9.
61. You Z, Chahwan C, Bailis J, Hunter T, Russell P. ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1. *Mol Cell Biol* 2005;25(13):5363–79.
62. Lee AC, Fernandez-Capetillo O, Pisupati V, Jackson SP, Nussenzweig A. Specific association of mouse MDC1/NFBD1 with NBS1 at sites of DNA-damage. *Cell Cycle* 2005;4(1):177–82.
63. Iwabuchi K, Bartel PL, Li B, Marraccino R, Fields S. Two cellular proteins that bind to wild-type but not mutant p53. *Proc Natl Acad Sci USA* 1994;91(13):6098–102.
64. Schultz LB, Chehab NH, Malikzay A, Halazonetis TD. p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. *J Cell Biol* 2000;151(7):1381–90.
65. Anderson L, Henderson C, Adachi Y. Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. *Mol Cell Biol* 2001;21(5):1719–29.
66. Rappold I, Iwabuchi K, Date T, Chen J. Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. *J Cell Biol* 2001;153(3):613–20.
67. Sengupta S, Robles AI, Linke SP, et al. Functional interaction between BLM helicase and 53BP1 in a Chk1-mediated pathway during S-phase arrest. *J Cell Biol* 2004;166(6):801–13.
68. Riehle CT, Peterson C, Lu T, Hittelman WN, Carpenter PB, Legerski RJ. hSnm1 colocalizes and physically associates with 53BP1 before and after DNA damage. *Mol Cell Biol* 2002;22(24):8635–47.
69. Ward IM, Minn K, Jorda KG, Chen J. Accumulation of checkpoint protein 53BP1 at DNA breaks involves its binding to phosphorylated histone H2AX. *J Biol Chem* 2003;278(22):19579–82.
70. Kao GD, McKenna WG, Guenther MG, Muschel RJ, Lazar MA, Yen TJ. Histone deacetylase 4 interacts with 53BP1 to mediate the DNA damage response. *J Cell Biol* 2003;160(7):1017–27.
71. Mochan TA, Venere M, DiTullio RA Jr, Halazonetis TD. 53BP1, an activator of ATM in response to DNA damage. *DNA Repair (Amst)* 2004;3(8–9):945–52.
72. Huyen Y, Zgheib O, Ditullio RA Jr, et al. Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. *Nature* 2004;432(7015):406–11.
73. Joo WS, Jeffrey PD, Cantor SB, Finnin MS, Livingston DM, Pavletich NP. Structure of the 53BP1 BRCT region bound to p53 and its comparison to the Brcal BRCT structure. *Genes Dev* 2002;16(5):583–93.
74. Derbyshire DJ, Basu BP, Serpell LC, et al. Crystal structure of human 53BP1 BRCT domains bound to p53 tumour suppressor. *EMBO J* 2002;21(14):3863–72.
75. Lo KW, Kan HM, Chan LN, et al. The 8 kDa dynein light chain binds to 53BP1 and mediates DNA damage-induced p53 nuclear accumulation. *J Biol Chem* 2005;280(9):8172–9.
76. Charier G, Couprie J, Alpha-Bazin B, et al. The Tudor tandem of 53BP1: a new structural motif involved in DNA and RG-rich peptide binding. *Structure (Camb)* 2004;12(9):1551–62.
77. Iwabuchi K, Basu BP, Kysela B, et al. Potential role for 53BP1 in DNA end-joining repair through direct interaction with DNA. *J Biol Chem* 2003;278(38):36487–95.
78. Fernandez-Capetillo O, Chen HT, Celeste A, et al. DNA damage-induced G2-M checkpoint activation by histone H2AX and 53BP1. *Nat Cell Biol* 2002;4(12):993–7.

79. Jullien D, Vagnarelli P, Earnshaw WC, Adachi Y. Kinetochores localise the DNA damage response component 53BP1 during mitosis. *J Cell Sci* 2002;115(Pt 1):71–9.
80. Abraham RT. Checkpoint signalling: focusing on 53BP1. *Nat Cell Biol* 2002;4(12):E277–9.
81. Wang B, Matsuoka S, Carpenter PB, Elledge SJ. 53BP1, a mediator of the DNA damage checkpoint. *Science* 2002;298(5597):1435–8.
82. DiTullio RA Jr, Mochan TA, Venere M, et al. 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. *Nat Cell Biol* 2002;4(12):998–1002.
83. Wysocki R, Javaheri A, Allard S, Sha F, Cote J, Kron SJ. Role of dot1-dependent histone H3 methylation in G1 and S phase DNA damage checkpoint functions of Rad9. *Mol Cell Biol* 2005;25(19):8430–43.
84. Sanders SL, Portoso M, Mata J, Bahler J, Allshire RC, Kouzarides T. Methylation of histone H4 lysine 20 controls recruitment of Crb2 to sites of DNA damage. *Cell* 2004;119(5):603–14.
85. Onclercq-Delic R, Calsou P, Delteil C, Salles B, Papadopoulou D, Amor-Gueret M. Possible anti-recombinogenic role of Bloom's syndrome helicase in double-strand break processing. *Nucleic Acids Res* 2003;31(21):6272–82.
86. Celeste A, Petersen S, Romanienko PJ, et al. Genomic instability in mice lacking histone H2AX. *Science* 2002;296(5569):922–7.
87. Zgheib O, Huyen Y, DiTullio RA Jr, et al. ATM signaling and 53BP1. *Radiother Oncol* 2005;76(2):119–22.
88. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 2003;421(6922):499–506n.

14

DNA-Dependent Protein Kinase in Repair, Apoptosis, Telomere Maintenance, and Chemotherapy

Lawrence F. Povirk, PhD

SUMMARY

DNA-dependent protein kinase (DNA-PK) is a high-molecular-weight kinase that binds to and is activated by free double-stranded DNA ends. The primary function of DNA-PK is to promote DNA end synapsis and regulate DNA end processing in the nonhomologous end-joining pathway of double-strand break repair. This regulation is mediated in part by DNA-PK-catalyzed phosphorylation of both itself and the Artemis nuclease, which trims heavily damaged ends that cannot otherwise be joined. In mice, DNA-PK is also required for radiation-stimulated apoptosis, mediated by direct phosphorylation of p53. At telomeres, DNA-PK cooperates with the telomere structural protein TRF2 in a capping function that is essential for preventing chromosome end fusions. The molecular phosphorylation targets for this and other DNA-PK-mediated signaling pathways remain to be identified. In principle, DNA-PK inhibitors should enhance radiotherapy and some forms of cancer chemotherapy, but such inhibitors may have significant toxicities due to the telomeric and other functions of DNA-PK.

Key Words: End joining; radiation; double-strand breaks; DNA repair; Ku; Artemis; repair foci.

1. BIOCHEMICAL PROPERTIES OF DNA-DEPENDENT PROTEIN KINASE

DNA-dependent protein kinase (DNA-PK) was discovered 20 years ago by Anderson and colleagues as a high-molecular-weight serine/threonine kinase activated by DNA ends (1). The DNA end-binding heterodimer Ku, consisting of 70- and 86-kDa subunits, was discovered a few years earlier as an autoantigen in scleroderma polymyositis overlap syndrome (2). Although the 465-kDa polypeptide containing the DNA-PK active site, designated DNA-PKcs, binds to and is activated by DNA ends, its affinity for DNA is low, and under physiological conditions, DNA-PK activity is largely

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dependent on recruitment of DNA-PKcs to DNA ends by Ku (3–5). X-ray crystallography indicates that, when Ku binds to DNA, the DNA helix passes through an orifice in the Ku heterodimer for which the fit is extremely tight, much like a nut on a bolt (6). Ku prefers to bind to the extreme end of DNA, but DNA-PKcs, upon recruitment by Ku, replaces Ku at the tip of the DNA end, forcing Ku to translate inwardly along the DNA by approximately 10 bp (7). The complete DNA-PK holoenzyme sequesters about 28 bp at a DNA end, completely protecting it from DNase digestion (7,8). Although DNA-PKcs also has a central orifice large enough to accommodate double-helical DNA (9), a recent structural study by electron microscopy suggested that, in the ternary DNA/Ku/DNA-PKcs complex, DNA is not inserted into this orifice (10).

DNA-PK is a member of a family of high-molecular-weight protein kinases that are involved in the earliest stages of DNA damage recognition and are more closely homologous to phosphatidylinositol 3-kinase (PIK) than to other protein kinases (11). Other members include the ataxia-telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) kinases as well as their yeast equivalents Tel1 and Mec1, respectively.

The mechanism by which double-stranded DNA ends activate DNA-PK kinase activity is not entirely understood; however, kinetic data suggest that synapsis of two DNA ends is required for activation (12). Based on studies with substrates bearing partially noncomplementary terminal nucleotides, it has been proposed that DNA-PK may denature several base pairs at a DNA end and that the threading of these single-strand termini into cavities in the DNA-PKcs protein may trigger kinase activation (13,14). However, this mechanism cannot be the only means of DNA-PK activation, as under appropriate conditions, DNA-PK can also be activated by double-strand DNA terminated with hairpin loops as well as by single-strand DNA with extensive secondary structure (e.g., single-strand circles from M13 virions, which contain multiple hairpin loops) (15–17). Protein phosphorylation is favored when DNA-PK and the target protein are able to bind to the same DNA molecule, and thus proteins that bind single-strand DNA are favored targets when DNA-PK is activated by M13 DNA (16). Phosphorylation of some target proteins *in vitro* can only be detected under conditions that minimize DNA-PKcs autophosphorylation and inactivation, e.g., very low ATP concentrations.

In vitro, a broad range of DNA-binding proteins are phosphorylated by DNA-PK, preferentially at SQ and TQ dipeptides (18–20). However, only for a very few proteins has DNA-PK-mediated phosphorylation been demonstrated to be functionally relevant. There is substantial substrate overlap between DNA-PK and the related ATM kinase, and this overlap has complicated efforts to define the specific role of each (19,20). Although DNA-PK is directly activated by DNA ends and is much more abundant than ATM kinase (21), ATM kinase plays a much more prominent role in triggering the overall cellular response to double-strand breaks (DSBs) than does DNA-PK (22,23). In particular, cell-cycle arrest appears to be completely dependent on ATM kinase, with little or no involvement of DNA-PK (24–28). Nevertheless, at least in some cells, DNA-PK plays an important role in DSB-induced apoptosis. In addition, DNA-PK plays a critical, though as yet not clearly defined role in telomere maintenance, distinct from its role in repair.

2. DIRECT ROLE IN DNA DSB REPAIR

DNA-PK is essential for efficient DSB repair by nonhomologous DNA end joining (NHEJ). DNA-PK-deficient mouse and human cells fail to repair a significant fraction of DSBs, even after several days (29–31), and such cells are twofold to fourfold more sensitive to ionizing radiation than normal cells (31,32), with even greater hypersensitivity for cells synchronized in G₀/G₁ phase (33). There appear to be at least five roles for DNA-PK in this DSB repair pathway (Fig. 1). First, DNA-PK binding initially

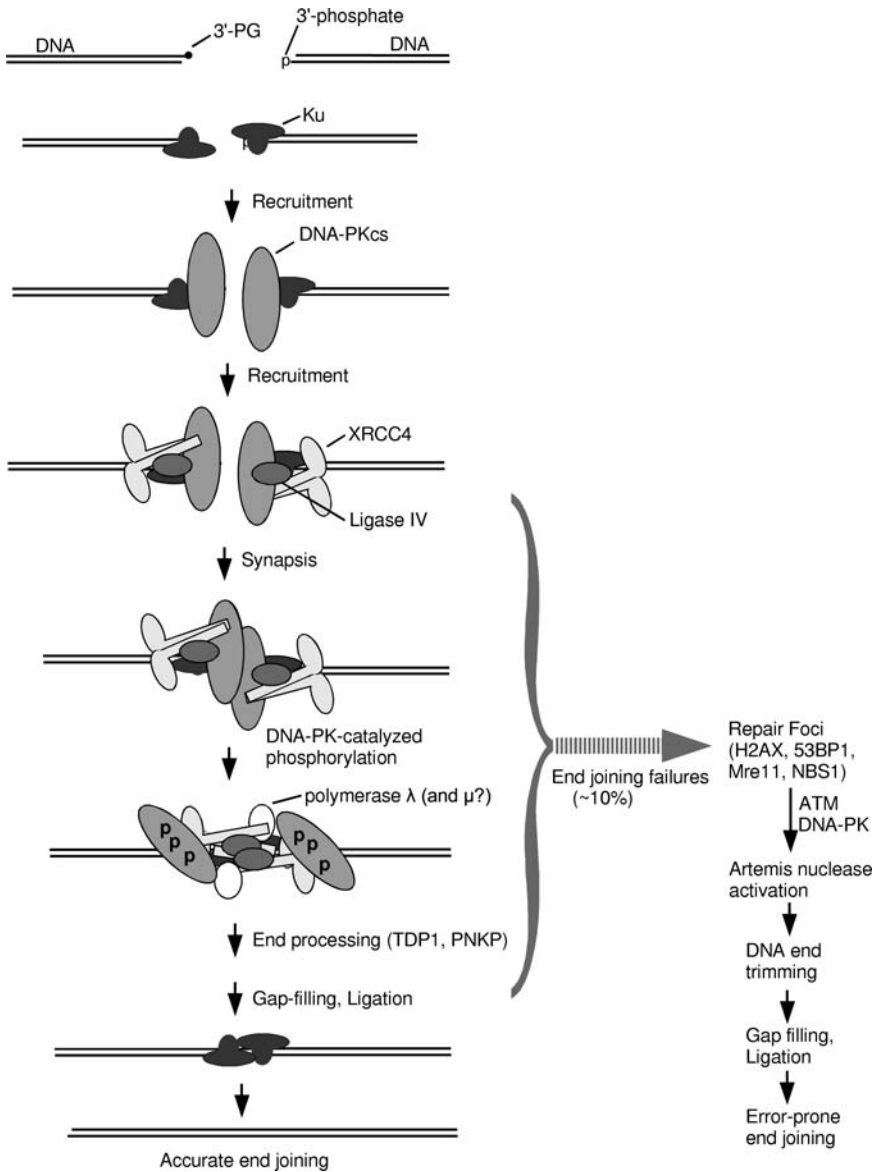


Fig. 1. The nonhomologous end-joining pathway of DNA double-strand break repair. DNA-dependent protein kinase (DNA-PK) protects the DNA ends, recruits XRCC4/DNA ligase IV complex, promotes synapsis, and phosphorylates both itself and the Artemis nuclease.

protects free DNA ends from exonucleolytic degradation (8,34). Second, it promotes synapsis of two DSB ends (12). Third, it aids in recruiting the XRCC4/DNA ligase IV complex (35,36), which is required for fill-in of missing bases in the aligned ends by DNA polymerases λ and μ (37–39) as well as for the final ligation. Fourth, it activates the Artemis nuclease by phosphorylating it at multiple sites in the C-terminal domain (40). Activated Artemis trims long 3' and 5' overhangs, and this trimming is apparently essential for repair of a subset of DSBs ($\sim 10\%$ in the case of ionizing radiation) that are rejoined relatively slowly (38,41,42). Fifth, once two DNA ends are aligned, DNA-PKcs phosphorylates itself (probably in *trans*) at a cluster of SQ/TQ sites (T2609, S2612, T2620, S2624, T2638, T2647, and S2056) near the middle of the polypeptide (43–45). This autophosphorylation promotes a rearrangement that makes DNA ends more accessible, allowing DNA end processing, gap-filling, and ligation to proceed (8,38,46–48). Thus, DNA-PK may serve to delay end processing until two broken DNA ends have been optimally aligned, thus reducing the possibility of deletions resulting from nucleolytic degradation (47). In addition, DNA-PK and ATM kinase appear to be redundantly capable of phosphorylating histone H2AX in chromatin in the vicinity of each DSB, an event that occurs within minutes of irradiation (49–51). Finally, *in vitro* studies suggest that DNA-PK-mediated phosphorylation of histone H1 may be required for rejoining of DSBs in chromatin (52).

Chinese hamster ovary cells harboring DNA-PKcs alleles with alanine substitutions at the T2609, T2638, or S2056 position are slightly radiosensitive, but a mutant with alanine substitutions at multiple sites in the central SQ/TQ cluster is more radiosensitive than any of the single-site mutants, suggesting that autophosphorylation of multiple sites is important for repair (43,44). A mutant with six of these sites mutated is more radiosensitive than the parent cell line that lacks DNA-PK entirely (44), suggesting that nonphosphorylatable DNA-PKcs interferes with residual repair that would otherwise occur even in its absence. Foci of T2609-phosphorylated and S2056-phosphorylated DNA-PK can be detected following exposure to ionizing radiation (43,45), suggesting that although mechanistically a single DNA-PK complex per end would appear to be sufficient for end joining, aggregates of multiple DNA-PK complexes accumulate and become phosphorylated in the vicinity of at least some DSBs. S2056-phosphorylated DNA-PKcs can be detected in extracts containing a mixture of kinase-dead and S2056A-mutated alleles, confirming that DNA-PK, like ATM kinase (53), is autophosphorylated in *trans*, at least *in vitro*. Protein phosphatase 5 interacts with and dephosphorylates DNA-PKcs. Either elimination or overexpression of protein phosphatase 5 results in radiosensitivity, suggesting that optimal repair requires a balance between phosphorylation and dephosphorylation of DNA-PKcs (54).

The same basic end-joining pathway involving DNA-PK, Artemis, XRCC4, and DNA ligase IV is utilized in V(D)J recombination, wherein antibody and T-cell receptor genes are created by cleavage and joining of individual V, D, and J segments (55,56). In this process, Artemis nuclease is absolutely required for opening the hairpin loops formed during initial cleavage by RAG1/RAG2 (41). It is presumably because of this requirement that a genetic defect in DNA-PK confers murine severe combined immune deficiency (SCID) (57), whereas lack of Artemis results in human SCID (58).

Diverse cytological and biochemical data suggest that whenever a DSB is formed, there is an initial window of opportunity (~ 1 h) during which the break can be repaired by simple end joining involving Ku, DNA-PK, XRCC4, and DNA ligase IV (30,59).

This rapid component of end joining probably incorporates conservative end processing by TDP1 and PNKP as well as gap-filling by DNA polymerases λ and μ , although this has yet to be directly demonstrated. Any break that cannot be rejoined by this mechanism, either because of a refractory end structure or because it lacks an end-joining partner, invokes a slower and more complex process characterized by the accumulation of multiple additional factors in “repair foci” (59–62). In G1/G0 cells, these persistent breaks are repaired almost exclusively by an ATM kinase-dependent NHEJ subpathway, incorporating nonconservative end processing by Artemis but still requiring the core NHEJ components as well as many proteins typically found in repair foci, i.e., 53BP1, Mre11, NBS1, and H2AX (42). In late S and G2, many if not most breaks in replicated DNA that escape the rapid phase of NHEJ may instead be channeled into repair by homologous recombination with the sister chromatid (63), but the detailed mechanisms by which these unrepaired DSBs are assigned to slow NHEJ or to homologous recombination remain to be elucidated.

3. DNA-PK AND APOPTOSIS

SCID mice, which harbor a C-terminal truncation of DNA-PKcs, have very low levels of DNA-PKcs protein and at least 100-fold lower levels of DNA-PK activity than normal mice (57,64,65). Attempts to assess the effect of this DNA-PK deficiency on apoptosis yielded diverse results, with reports of a slight reduction in radiation-induced apoptosis in mouse spleen (66), enhanced spontaneous apoptosis and normal radiation-induced apoptosis in intestinal crypt cells (67), and enhanced radiation-induced apoptosis in thymocytes (68) and cultured fibroblasts (69). Moreover, concomitant elimination of p53 appeared to enhance the tumorigenic effect of the SCID mutation, and this effect was attributed to abrogation of apoptosis in p53^{-/-} but not in p53^{+/+} SCID cells (70). Thus, these results suggest that there is little, if any, impairment of the apoptotic response as a result of the SCID-associated DNA-PKcs mutation; however, because of the residual truncated DNA-PKcs protein in SCID cells, the results do not rigorously exclude a role for DNA-PK in apoptosis.

In irradiated mice with a complete homozygous knockout of DNA-PKcs, apoptosis in thymocytes was reduced approximately fivefold compared with that in normal mice (28), and in embryonic fibroblasts from the same knockout mice, radiation-induced apoptosis was completely abolished (71). This loss of apoptosis was accompanied by a marked decrease in induction of both p53 and the proapoptotic effector Bax. There appeared to be a distinct bifurcation of p53 function, wherein Bax induction and apoptosis were dependent on DNA-PK but not ATM kinase, whereas p21^{waf1} induction and cell-cycle arrest were dependent on ATM kinase but not DNA-PK, while all these effects were dependent on p53 (28). Studies in murine cell extracts suggest that this DNA-PK-dependent apoptosis reflects ATM kinase-independent phosphorylation of p53, first by DNA-PK at serine 18 (murine S18, equivalent to S15 in human p53), and then by Chk2 at additional, as yet undefined sites (72). Upon phosphorylation, p53 acquires sequence-specific DNA-binding capacity, but it was not determined whether this phosphorylation is sufficient for transcriptional activation. Nevertheless, the hypothesis that DNA-PK and Chk2 cooperate to produce a proapoptotic, phosphorylated form of p53 is also supported by the finding that apoptosis is similarly attenuated in thymocytes and embryonic fibroblasts derived from Chk2 knockout mice (72,73).

There is conflicting evidence regarding whether DNA-PK plays a similar role in apoptosis in human cells. No instances of human SCID associated with DNA-PK mutations have been reported, suggesting that inactivating mutations of DNA-PK are likely embryonic lethal in humans. Thus, studies of human DNA-PK function have centered on comparisons between the human glioma cell lines M059J and M059K. Although these cells were derived from the same tumor, M059K cells have normal DNA-PK function, whereas M059J cells harbor a frameshift mutation in DNA-PKcs and contain no detectable DNA-PK activity or DNA-PKcs protein (74). However, interpretation of data obtained from these cells is complicated by the fact that M059J cells also show abnormally low levels of ATM kinase expression (75).

In extracts of M059J and M059K cells, activation of constitutively expressed p53 for sequence-specific DNA binding shows dependence on Chk2 and DNA-PK, similar to that seen in mouse cells (72). However, despite lack of DNA-PK, M059J cells showed greater apoptosis following ionizing radiation than did M059K cells (76). Similarly, microinjection of an antibody that binds DNA-PK and blocks DNA-PK-mediated DNA end joining increased radiation-induced cell killing and apoptosis (77). Moreover, in HCT116 colon carcinoma cells, homozygous disruption of Chk2 by homologous recombination did not block either fluorouracil-induced p53-dependent apoptosis or activation of p53 for DNA binding. Although this result could be specific for this particular cell type and/or damaging agent, the result nevertheless calls into question whether the proapoptotic activation of p53 by a combination of DNA-PK and Chk2, as described in mouse cells, is important for DNA damage-induced apoptosis in human cells. On the contrary, apoptosis induced by the protein kinase C inhibitor staurosporine was greater in M059K than in M059J cells (78), suggesting that it was at least partially DNA-PK-dependent. In human myeloid leukemia cells treated with the nucleoside analogue gemcitabine, which causes interruption of DNA chain elongation, Ku, DNA-PKcs, and p53 were found in a putative sensor complex bound to gemcitabine-containing DNA, and formation of this complex was accompanied by p53 phosphorylation on S15 (79). These results provide circumstantial evidence of a role for DNA-PK in gemcitabine-induced apoptosis, although a requirement for DNA-PK was not explicitly demonstrated. In human macrophages, DNA-PK is required for activation of Akt (by phosphorylation on T308 and S473) in response to bacterial DNA, suggesting that in some instances DNA-PK can mediate antiapoptotic pro-survival signaling (80,81). DNA-PK-mediated pro-survival signaling through NF- κ B in response to DNA damage has also been reported (82). Overall, the evidence suggests that although DNA-PK is involved in proapoptotic signaling in some situations in human cells, its role in apoptosis may be less critical than in mouse cells. It appears that the proapoptotic effect of persistent DSBs (due to lack of repair) in DNA-PK-deficient human cells usually outweighs any deficiency in DNA-PK-mediated apoptotic signaling, at least for agents that directly induce DSBs.

It has been noted that both DNA-PKcs and Ku are substrates for caspase 3 and are efficiently cleaved at an early stage of apoptosis (83,84). Although the functional significance of this phenomenon remains to be demonstrated, such cleavage could in principle contribute to cell disintegration by preventing the rejoining of the DSBs induced by apoptotic endonucleases.

4. DNA-PK, TELOMERES, AND SENESCENCE

At each tip of each eukaryotic chromosome, telomeric DNA is incorporated into a looped structure that prevents DSB repair mechanisms from joining chromosomes end-to-end (85,86). In principle, this structure should preclude binding by Ku in its canonical binding mode, i.e., threading onto a double-stranded DNA end. Nevertheless, both Ku and DNA-PKcs are bound at telomeres (although not necessarily in association with each other) (87–89), and both are required for proper telomere function (90–92). Telomeric Ku is not bound directly to telomeric DNA but rather to TRF1, a primary structural component of the telomere (92). DNA-PKcs may be bound to the telomere through KIP, which was originally identified as a DNA-PKcs-binding protein and subsequently shown to bind to the reverse transcriptase subunit of telomerase (93).

Although $Terc^{-/-}$ knockout mice, which lack the catalytic subunit of telomerase, can survive for several generations, telomeres in stem cells of these mice become progressively shorter with each generation and the mice begin to develop senescence-like phenotypes (94,95), as do $Terc^{-/-}$ embryonic stem cells after long-term culture (96). In double-knockout $Terc^{-/-}$ DNA-PKcs $^{-/-}$ mice, telomere loss and onset of age-associated pathology (such as atrophy in a variety of tissues) are accelerated, and the mice are completely infertile by the second generation (97,98). These results suggest that DNA-PKcs contributes to maintenance of stable telomere length, although it has not yet been determined whether DNA-PKcs kinase activity is required for this function. It was proposed that in $Terc^{+/+}$ cells, the accelerated telomere loss associated with DNA-PKcs deficiency was reversed by telomerase. In human HeLa cells, RNAi-mediated knockdown of Ku86 (~50%) also resulted in telomere loss, as well as telomere fusions and an increase in spontaneous apoptosis (99).

Murine embryonic fibroblasts and other cells derived from SCID mice as well as from mice with knockouts in Ku80, Ku70, or DNA-PKcs all show elevated levels of telomere fusions (detected cytogenetically), even when they have wild-type telomerase and normal telomere length (90,91,100). Thus, the end-protection function of the telomere requires both Ku and DNA-PKcs, and the requirement for DNA-PKcs is not solely due to its role in maintaining telomere length. High levels of telomere fusions are also induced by the DNA-PK inhibitor IC86621, suggesting that the kinase activity of DNA-PK is required for normal telomere function (101). Intriguingly, the levels of telomere fusions induced by IC86621 were much higher than levels in cells that lack DNA-PK entirely. IC86621-induced fusions, like those seen in DNA-PKcs- or TRF2-deficient cells (102), involved only telomeres with ends replicated by leading-strand synthesis and were exclusively of the chromatid type. These results suggest that DNA-PKcs activity is required for TRF2-mediated telomere capping following replication. Thus, even though the normal looped telomere structure is not expected to activate DNA-PKcs, the double-stranded end transiently exposed at the telomere by replication could account for the DNA-PK activation that is apparently required for this capping function. Paradoxically, whereas lack of DNA-PK promotes fusions in cells with telomeres of normal length, DNA-PK is required for the telomere fusions and subsequent apoptosis induced by critically short telomeres (97). Thus, the telomere fusions in DNA-PKcs-deficient cells must be formed by some aberrant end-joining pathway that does not require DNA-PKcs.

5. DNA-PK AND CHEMOTHERAPY/RADIOTHERAPY

DNA-PK-deficient cells, including cells containing catalytically inactive DNA-PK (103), are radiosensitive; therefore, specific inhibitors of DNA-PK are expected to confer radiosensitivity. The most frequently used DNA-PK inhibitor is wortmannin, a fungal toxin that attaches covalently at the ATP-binding site of PIK and all PIK-related protein kinases, irreversibly inactivating kinase activity. Pretreatment with wortmannin inhibits DSB repair and renders cells about threefold more sensitive to ionizing radiation (104–106). Although PIK is about 100 times more sensitive to wortmannin than are DNA-PK and ATM kinase, the fact that DNA-PK-deficient cells are not radiosensitized argues that DNA-PK is the target for radiosensitization. Like genetic NHEJ deficiency, wortmannin treatment eliminates split-dose recovery (104,105), that is, the wortmannin-treated cells sustain the same degree of cell killing when a given dose of radiation is divided into two fractions separated by several hours as when the same amount of radiation is administered in a single dose. Sensitization to radiation and DSB-inducing chemotherapeutic agents (e.g., bleomycin and the topoisomerase II inhibitor etoposide) has also been achieved by siRNA-mediated silencing of DNA-PKcs (107), expression of a dominant-negative Ku fragment (108), and suppression of Ku expression with antisense oligonucleotides (109,110). In cases where cross-sensitivity to other agents has been tested, there appears to be little or no sensitization to cross-linking agents such as cisplatin and mitomycin C (109), consistent with a specific effect on DSB repair.

Unexpectedly, mouse and hamster cells deficient in Ku or DNA-PK were found to be more resistant than normal cells to cisplatin. This difference was only seen when the cells were treated at high density and was eliminated by chemical or genetic disruption of gap junctions (111). Thus, these results suggest a cell-interdependent mode of killing involving a death signal that requires DNA-PK kinase activity and intercellular communication through gap junctions. Although this phenomenon has not been demonstrated in human cells, it could in principle explain some clinical studies in which higher DNA-PK levels were correlated with better response to cytotoxic cancer therapy. For example, in one study, abundance of DNA-PK in individual esophageal tumors was associated with improved response to combined chemotherapy/radiotherapy (107). Similarly, in leukemic cells derived from patients with chronic lymphocytic leukemia, sensitivity to doxorubicin and etoposide was correlated with high DNA-PKcs expression (112).

In view of its multiple roles in DSB repair, telomere maintenance, and apoptosis (Fig. 2), DNA-PK could be a reasonable candidate for pharmacological or genetic manipulation in cancer radiotherapy and chemotherapy. However, attempts at pharmacologically maximizing DNA-PK-mediated apoptosis will, at a minimum, require a much more detailed understanding of the specific apoptotic pathways involved as well as some means of constitutively activating DNA-PK or increasing its expression. Moreover, much of the evidence for the proapoptotic functions of DNA-PK (including gap junction-mediated cytotoxicity) has come from rodent cells, and most of these findings have yet to be confirmed in human cells.

A more promising strategy is the development of new small-molecule DNA-PK inhibitors that are much more specific than wortmannin and have little effect on related kinases such as ATM or PI3K. In principle, these agents can be used to block DSB repair in tumors and thus potentiate cell killing by ionizing radiation and DSB-inducing

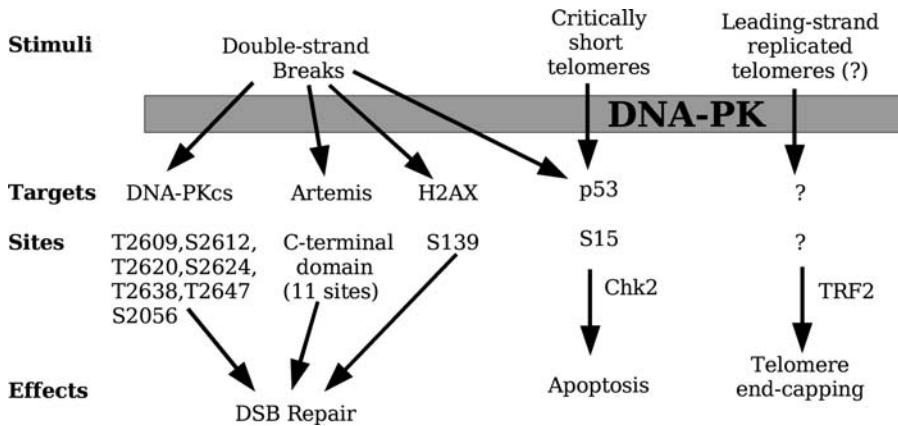


Fig. 2. Signaling pathways mediated by DNA-dependent protein kinase (DNA-PK).

chemotherapeutic agents. Preclinical radiosensitization/chemosensitization studies with two such inhibitors, IC87361 and NU7441, have yielded quite encouraging initial results. IC87361 markedly increased the radiosensitivity of human melanoma and lung cancer cells in culture and increased radiation-induced growth delay in xenografts in mice (113). NU7441 similarly increased sensitivity of human colon cancer cells to both radiation and the topoisomerase II inhibitor etoposide and enhanced etoposide-induced growth delay in xenografts (114). As predicted, sensitization by NU7441 was accompanied by a marked increase in the persistence of radiation- and etoposide-induced DSBs, as judged by γ H2AX foci. Neither of these DNA-PK inhibitors produced any obvious acute toxicity, suggesting that the potentially toxic effects of DNA-PK inhibition may be tolerable for the short periods of exposure during chemotherapy/radiotherapy, at least in mice. However, the significant apparent differences between rodent and human cells in Ku and DNA-PK function (8,18,48,115) raise the possibility that some human toxicities might not be manifest in animal models. DNA-PK inhibitors would presumably block repair in normal cells as well, so it is likely that there will be at least some increase in toxicity of radiotherapy and chemotherapy toward normal tissue as well, and indeed the combination of NU7441 and etoposide resulted in some loss of body weight (114). Moreover, if, as expected, these inhibitors promote telomere dysfunction, the possibility of carcinogenesis as a long-term side effect must be considered. Nevertheless, results obtained thus far suggest that these agents warrant further development and testing.

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REFERENCES

1. Walker AI, Hunt T, Jackson RJ, Anderson CW: Double-stranded DNA induces the phosphorylation of several proteins including the 90 000 mol. wt. heat-shock protein in animal cell extracts. *EMBO J* 1985; 4:139-145.

2. Mimori T, Akizuki M, Yamagata H, Inada S, Yoshida S, Homma M: Characterization of a high molecular weight acidic nuclear protein recognized by autoantibodies in sera from patients with polymyositis-scleroderma overlap. *J Clin Invest* 1981; 68:611–620.
3. Yaneva M, Kowalewski T, Lieber MR: Interaction of DNA-dependent protein kinase with DNA and with Ku: biochemical and atomic-force microscopy studies. *EMBO J* 1997; 16:5098–5112.
4. West RB, Yaneva M, Lieber MR: Productive and nonproductive complexes of Ku and DNA-dependent protein kinase at DNA termini. *Mol Cell Biol* 1998; 18:5908–5920.
5. Hammarsten O, Chu G: DNA-dependent protein kinase: DNA binding and activation in the absence of Ku. *Proc Natl Acad Sci USA* 1998; 95:525–530.
6. Walker JR, Corpina RA, Goldberg J: Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature* 2001; 412:607–614.
7. Yoo S, Dynan WS: Geometry of a complex formed by double strand break repair proteins at a single DNA end: recruitment of DNA-PKcs induces inward translocation of Ku protein. *Nucleic Acids Res* 1999; 27:4679–4686.
8. Lee JW, Inamdar KV, Hannah MF, Lees-Miller SP, Povirk LF: DNA end sequestration by DNA-dependent protein kinase and joining of sterically constrained substrates in whole-cell extracts. *Environ Mol Mutagen* 2003; 42:279–287.
9. Leuther KK, Hammarsten O, Kornberg RD, Chu G: Structure of DNA-dependent protein kinase: implications for its regulation by DNA. *EMBO J* 1999; 18:1114–1123.
10. Spagnolo L, Rivera-Calzada A, Pearl LH, Llorca O: Three-dimensional structure of the human DNA-PKcs/Ku70/Ku80 complex assembled on DNA and its implications for DNA DSB repair. *Mol Cell* 2006; 22:511–519.
11. Keith CT, Schreiber SL: PIK-related kinases: DNA repair, recombination, and cell cycle checkpoints. *Science* 1995; 270:50–51.
12. DeFazio LG, Stansel RM, Griffith JD, Chu G: Synapsis of DNA ends by DNA-dependent protein kinase. *EMBO J* 2002; 21:3192–3200.
13. Hammarsten O, DeFazio LG, Chu G: Activation of DNA-dependent protein kinase by single-stranded DNA ends. *J Biol Chem* 2000; 275:1541–1550.
14. Martensson S, Hammarsten O: DNA-dependent protein kinase catalytic subunit. Structural requirements for kinase activation by DNA ends. *J Biol Chem* 2002; 277:3020–3029.
15. Soubeyrand S, Schild-Poulter C, Hache RJ: Structured DNA promotes phosphorylation of p53 by DNA-dependent protein kinase at serine 9 and threonine 18. *Eur J Biochem* 2004; 271:3776–3784.
16. Soubeyrand S, Torrance H, Giffin W, Gong W, Schild-Poulter C, Hache RJ: Activation and autoregulation of DNA-PK from structured single-stranded DNA and coding end hairpins. *Proc Natl Acad Sci USA* 2005; 98: 9605–9610.
17. Morozov VE, Falzon M, Anderson CW, Kuff EL: DNA-dependent protein kinase is activated by nicks and larger single-stranded gaps. *J Biol Chem* 1994; 269:16684–16688.
18. Anderson CW, Lees-Miller SP: The nuclear serine/threonine protein kinase DNA-PK. *Crit Rev Eukaryot Gene Expr* 1992; 2:283–314.
19. Kim ST, Lim DS, Canman CE, Kastan MB: Substrate specificities and identification of putative substrates of ATM kinase family members. *J Biol Chem* 1999; 274:37538–37543.
20. O'Neill T, Dwyer AJ, Ziv Y, et al.: Utilization of oriented peptide libraries to identify substrate motifs selected by ATM. *J Biol Chem* 2000; 275:22719–22727.
21. Goodarzi AA, Lees-Miller SP: Biochemical characterization of the ataxia-telangiectasia mutated (ATM) protein from human cells. *DNA Repair* 2004; 3:753–767.
22. Goodarzi AA, Block WD, Lees-Miller SP: The role of ATM and ATR in DNA damage-induced cell cycle control. *Prog Cell Cycle Res* 2003; 5:393–411.
23. Shiloh Y: ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer* 2003; 3:155–168.
24. Jimenez GS, Bryntesson F, Torres-Arzayus MI, et al.: DNA-dependent protein kinase is not required for the p53-dependent response to DNA damage. *Nature* 1999; 400:81–83.
25. Burma S, Kurimasa A, Xie G, et al.: DNA-dependent protein kinase-independent activation of p53 in response to DNA damage. *J Biol Chem* 1999; 274:17139–17143.
26. Rathmell WK, Kaufmann WK, Hurt JC, Byrd LL, Chu G: DNA-dependent protein kinase is not required for accumulation of p53 or cell cycle arrest after DNA damage. *Cancer Res* 1997; 57:68–74.

27. Jhappan C, Yusufzai TM, Anderson S, Anver MR, Merlino G: The p53 response to DNA damage *in vivo* is independent of DNA-dependent protein kinase. *Mol Cell Biol* 2000; 20:4075–4083.
28. Wang S, Guo MF, Ouyang HF, et al.: The catalytic subunit of DNA-dependent protein kinase selectively regulates p53-dependent apoptosis but not cell-cycle arrest. *Proc Natl Acad Sci USA* 2002; 97:1584–1588.
29. Virsik-Kopp P, Rave-Frank M, Hofman-Huther H, Schmidberger H: Role of DNA-dependent protein kinase in the process of radiation-induced aberration formation. *Int J Radiat Biol* 2004; 80:125–133.
30. DiBiase SJ, Zeng ZC, Chen R, Hyslop T, Curran WJ Jr, Iliakis G: DNA-dependent protein kinase stimulates an independently active, nonhomologous, end-joining apparatus. *Cancer Res* 2000; 60:1245–1253.
31. Errami A, He DM, Friedl AA, et al.: XR-C1, a new CHO cell mutant which is defective in DNA-PKcs, is impaired in both V(D)J coding and signal joint formation. *Nucleic Acids Res* 1998; 26:3146–3153.
32. Lees-Miller SP, Godbout R, Chan DW, et al.: Absence of p350 subunit of DNA-activated protein kinase from a radiosensitive human cell line. *Science* 1995; 267:1183–1185.
33. Whitmore GF, Varghese AJ, Gulyas S: Cell cycle responses of two X-ray sensitive mutants defective in DNA repair. *Int J Radiat Biol* 1989; 56:657–665.
34. Calsou P, Frit P, Humbert O, Muller C, Chen DJ, Salles B: The DNA-dependent protein kinase catalytic activity regulates DNA end processing by means of Ku entry into DNA. *J Biol Chem* 1999; 274:7848–7856.
35. Calsou P, Delteil C, Frit P, Drouet J, Salles B: Coordinated assembly of Ku and p460 subunits of the DNA-dependent protein kinase on DNA ends is necessary for XRCC4-ligase IV recruitment. *J Mol Biol* 2003; 326:93–103.
36. Hsu H-L, Yannone SM, Chen DJ: Defining the interactions between DNA-PK and ligase IV/XRCC4. *DNA Repair* 2001; 1:225–235.
37. Lee JW, Blanco L, Zhou T, et al.: Implication of DNA polymerase lambda in alignment-based gap filling for nonhomologous DNA end joining in human nuclear extracts. *J Biol Chem* 2004; 279:805–811.
38. Ma Y, Lu H, Tippin B, et al.: A biochemically defined system for mammalian nonhomologous DNA end joining. *Mol Cell* 2004; 16:701–713.
39. Mahajan KN, Nick McElhinny SA, Mitchell BS, Ramsden DA: Association of DNA polymerase mu (pol μ) with Ku and ligase IV: role for pol μ in end-joining double-strand break repair. *Mol Cell Biol* 2002; 22:5194–5202.
40. Ma Y, Pannicke U, Lu H, Niewolik D, Schwarz K, Lieber MR: The DNA-PKcs phosphorylation sites in human Artemis. *J Biol Chem* 2005; 280:33839–33846.
41. Ma Y, Pannicke U, Schwarz K, Lieber MR: Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. *Cell* 2002; 108:781–794.
42. Riballo E, Kuhne M, Rief N, et al.: A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci. *Mol Cell* 2004; 16:715–724.
43. Chan DW, Chen BP, Prithivirajasingh S, et al.: Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA double-strand breaks. *Genes Dev* 2002; 16:2333–2338.
44. Ding Q, Reddy YV, Wang W, et al.: Autophosphorylation of the catalytic subunit of the DNA-dependent protein kinase is required for efficient end processing during DNA double-strand break repair. *Mol Cell Biol* 2003; 23:5836–5848.
45. Chen BP, Chan DW, Kobayashi J, et al.: Cell cycle dependence of DNA-PK phosphorylation in response to DNA double strand breaks. *J Biol Chem* 2005; 280:14709–14715.
46. Reddy YV, Ding Q, Lees-Miller SP, Meek K, Ramsden DA: Non-homologous end joining requires that the DNA-PK complex undergo an autophosphorylation-dependent rearrangement at DNA ends. *J Biol Chem* 2004; 279:39408–39413.
47. Gu XY, Weinfeld M, Povirk LF: Implication of DNA-dependent protein kinase in an early, essential, local phosphorylation event during end-joining of DNA double-strand breaks *in vitro*. *Biochemistry* 1998; 37:9827–9835.
48. Chen S, Inamdar KV, Pfeiffer P, et al.: Accurate *in vitro* end-joining of a DNA double-strand break with partially cohesive 3'-overhangs and 3'-phosphoglycolate termini: Effect of Ku on repair fidelity. *J Biol Chem* 2001; 276:24323–24330.

49. Wang H, Wang M, Bocker W, Iliakis G: Complex H2AX phosphorylation patterns by multiple kinases including ATM and DNA-PK in human cells exposed to ionizing radiation and treated with kinase inhibitors. *J Cell Physiol* 2005; 202:492–502.
50. Park EJ, Chan DW, Park JH, Oettinger MA, Kwon J: DNA-PK is activated by nucleosomes and phosphorylates H2AX within the nucleosomes in an acetylation-dependent manner. *Nucleic Acids Res* 2003; 31:6819–6827.
51. Stiff T, O'Driscoll M, Rief N, Iwabuchi K, Lobrich M, Jeggo PA: ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. *Cancer Res* 2004; 64:2390–2396.
52. Kysela B, Chovanec M, Jeggo PA: Phosphorylation of linker histones by DNA-dependent protein kinase is required for DNA ligase IV-dependent ligation in the presence of histone H1. *Proc Natl Acad Sci USA* 2005; 102:1877–1882.
53. Bakkenist CJ, Kastan MB: DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 2003; 421:499–506.
54. Wechsler T, Chen BP, Harper R, et al.: DNA-PKcs function regulated specifically by protein phosphatase 5. *Proc Natl Acad Sci USA* 2004; 101:1247–1252.
55. Jackson SP, Jeggo PA: DNA double-strand break repair and V(D)J recombination: involvement of DNA-PK. *Trends Biochem Sci* 1995; 20:412–415.
56. Smider V, Chu G: The end-joining reaction in V(D)J recombination. *Semin Immunol* 1997; 9:189–197.
57. Vlahos CJ, Matter WF, Hui KY, Brown RF: A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* 1994; 269:5241–5248.
58. Moshous D, Callebaut I, de Chasseval R, et al.: Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency. *Cell* 2001; 105:177–186.
59. Lisby M, Rothstein R: DNA damage checkpoint and repair centers. *Curr Opin Cell Biol* 2004; 16:328–334.
60. Maser RS, Monsen KJ, Nelms BE, Petrini JH: hMre11 and hRad50 nuclear foci are induced during the normal cellular response to DNA double-strand breaks. *Mol Cell Biol* 1997; 17:6087–6096.
61. Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM: A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol* 2000; 10:886–895.
62. Bradbury JM, Jackson SP, Fernandez-Capetillo O, Celeste A, Nussenzweig A: The complex matter of DNA double-strand break detection Focusing on foci: H2AX and the recruitment of DNA-damage response factors. *Cell Cycle* 2003; 2:426–427.
63. Thompson LH, Schild D: Homologous recombinational repair of DNA ensures mammalian chromosome stability. *Mutat Res* 2001; 477:131–153.
64. Kirchgessner CU, Patil CK, Evans JW, et al.: DNA-dependent kinase (p350) as a candidate gene for the murine SCID defect. *Science* 1995; 267:1178–1183.
65. Blunt T, Gell D, Fox M, et al.: Identification of a nonsense mutation in the carboxyl-terminal region of DNA-dependent protein kinase catalytic subunit in the scid mouse. *Proc Natl Acad Sci USA* 1996; 93:10285–10290.
66. Takahashi A, Asakawa I, Yuki K, et al.: Radiation-induced apoptosis in the scid mouse spleen after low dose-rate irradiation. *Int J Radiat Biol* 2002; 78:689–693.
67. Gurley KE, Vo K, Kemp CJ: DNA double-strand breaks, p53, and apoptosis during lymphomagenesis in scid/scid mice. *Cancer Res* 1998; 58:3111–3115.
68. Candeias SM, Durum SK, Muegge K: p53-dependent apoptosis and transcription of p21waf/cip1/sdi1 in SCID mice following gamma-irradiation. *Biochimie* 1997; 79:607–612.
69. Meng H, Terado T, Kimura H: Apoptosis induced by X-rays and chemical agents in murine fibroblastic cell lines with a defect in repair of DNA double-strand breaks. *Int J Radiat Biol* 1998; 73:503–510.
70. Nacht M, Strasser A, Chan YR, et al.: Mutations in the p53 and SCID genes cooperate in tumorigenesis. *Genes Dev* 1996; 10:2055–2066.
71. Woo RA, Jack MT, Xu Y, Burma S, Chen DJ, Lee PW: DNA damage-induced apoptosis requires the DNA-dependent protein kinase, and is mediated by the latent population of p53. *EMBO J* 2002; 21:3000–3008.
72. Jack MT, Woo RA, Motoyama N, Takai H, Lee PW: DNA-dependent protein kinase and checkpoint kinase 2 synergistically activate a latent population of p53 upon DNA damage. *J Biol Chem* 2004; 279:15269–15273.

73. Hirao A, Kong YY, Matsuoka S, et al.: DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science* 2000; 287:1824–1827.
74. Anderson CW, Dunn JJ, Freimuth PI, Galloway AM, Allalunis-Turner MJ: Frameshift mutation in PRKDC, the gene for DNA-PKcs, in the DNA repair-defective, human, glioma-derived cell line M059J. *Radiat Res* 2001; 156:2–9.
75. Chan DW, Gately DP, Urban S, et al.: Lack of correlation between ATM protein expression and tumour cell radiosensitivity. *Int J Radiat Biol* 1998; 74:217–224.
76. Holgersson A, Jernberg AR, Persson LM, et al.: Low and high LET radiation-induced apoptosis in M059J and M059K cells. *Int J Radiat Biol* 2003; 79:611–621.
77. Li S, Takeda Y, Wragg S, Barrett J, Phillips A, Dynan WS: Modification of the ionizing radiation response in living cells by an scFv against the DNA-dependent protein kinase. *Nucleic Acids Res* 2003; 31:5848–5857.
78. Chen GG, Sin FL, Leung BC, Ng HK, Poon WS: Glioblastoma cells deficient in DNA-dependent protein kinase are resistant to cell death. *J Cell Physiol* 2005; 203:127–132.
79. Achanta G, Pelicano H, Feng L, Plunkett W, Huang P: Interaction of p53 and DNA-PK in response to nucleoside analogues: potential role as a sensor complex for DNA damage. *Cancer Res* 2001; 61:8723–8729.
80. Dragoi AM, Fu X, Ivanov S, et al.: DNA-PKcs, but not TLR9, is required for activation of Akt by CpG-DNA. *EMBO J* 2005; 24:779–789.
81. Chu W, Gong X, Li Z, et al.: DNA-PKcs is required for activation of innate immunity by immunostimulatory DNA. *Cell* 2000; 103:909–918.
82. Panta GR, Kaur S, Cavin LG, et al.: ATM and the catalytic subunit of DNA-dependent protein kinase activate NF-kappaB through a common MEK/extracellular signal-regulated kinase/p90(rsk) signaling pathway in response to distinct forms of DNA damage. *Mol Cell Biol* 2004; 24:1823–1835.
83. Le Romancer M, Cosulich SC, Jackson SP, Clarke PR: Cleavage and inactivation of DNA-dependent protein kinase catalytic subunit during apoptosis in *Xenopus* egg extracts. *J Cell Sci* 1996; 109:3121–3127.
84. Casciola-Rosen L, Nicholson DW, Chong T, et al.: Apopain/ CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. *J Exp Med* 1996; 183:1957–1964.
85. Greider CW: Telomere length regulation. *Annu Rev Biochem* 1996; 65:337–365.
86. Griffith JD, Comeau L, Rosenfield S, et al.: Mammalian telomeres end in a large duplex loop. *Cell* 1999; 97:503–514.
87. Hsu HL, Gilley D, Blackburn EH, Chen DJ: Ku is associated with the telomere in mammals. *Proc Natl Acad Sci USA* 1999; 96:12454–12458.
88. Hande MP: DNA repair factors and telomere-chromosome integrity in mammalian cells. *Cytogenet Genome Res* 2004; 104:116–122.
89. Burma S, Chen DJ: Role of DNA-PK in the cellular response to DNA double-strand breaks. *DNA Repair* 2004; 3:909–918.
90. Goytisolo FA, Samper E, Edmonson S, Taccioli GE, Blasco MA: The absence of the DNA-dependent protein kinase catalytic subunit in mice results in anaphase bridges and in increased telomeric fusions with normal telomere length and G-strand overhang. *Mol Cell Biol* 2001; 21:3642–3651.
91. Gilley D, Tanaka H, Hande MP, et al.: DNA-PKcs is critical for telomere capping. *Proc Natl Acad Sci USA* 2001; 98:15084–15088.
92. Hsu HL, Gilley D, Galande SA, et al.: Ku acts in a unique way at the mammalian telomere to prevent end joining. *Genes Dev* 2000; 14:2807–2812.
93. Lee GE, Yu EY, Cho CH, Lee J, Muller MT, Chung IK: DNA-protein kinase catalytic subunit-interacting protein KIP binds telomerase by interacting with human telomerase reverse transcriptase. *J Biol Chem* 2004; 279:34750–34755.
94. Blasco MA, Lee HW, Hande MP, et al.: Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* 1997; 91:25–34.
95. Rudolph KL, Chang S, Lee HW, et al.: Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell* 1999; 96:701–712.
96. Niida H, Matsumoto T, Satoh H, et al.: Severe growth defect in mouse cells lacking the telomerase RNA component. *Nat Genet* 1998; 19:203–206.

97. Espejel S, Franco S, Sgura A, et al.: Functional interaction between DNA-PKcs and telomerase in telomere length maintenance. *EMBO J* 2002; 21:6275–6287.
98. Espejel S, Klatt P, Menissier-de Murcia J, et al.: Impact of telomerase ablation on organismal viability, aging, and tumorigenesis in mice lacking the DNA repair proteins PARP-1, Ku86, or DNA-PKcs. *J Cell Biol* 2004; 167:627–638.
99. Jaco I, Munoz P, Blasco MA: Role of human Ku86 in telomere length maintenance and telomere capping. *Cancer Res* 2004; 64:7271–7278.
100. Bailey SM, Meyne J, Chen DJ, et al.: DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes. *Proc Natl Acad Sci USA* 1999; 96:14899–14904.
101. Bailey SM, Brenneman MA, Halbrook J, Nickoloff JA, Ullrich RL, Goodwin EH: The kinase activity of DNA-PK is required to protect mammalian telomeres. *DNA Repair* 2005; 3:225–233.
102. Bailey SM, Cornforth MN, Kurimasa A, Chen DJ, Goodwin EH: Strand-specific postreplicative processing of mammalian telomeres. *Science* 2001; 293:2462–2465.
103. Peterson SR, Stackhouse M, Waltman MJ, Chen F, Sato K, Chen DJ: Characterization of two DNA double-stranded break repair-deficient cell lines that express inactive DNA-dependent protein kinase catalytic subunits. *J Biol Chem* 1997; 272:10227–10231.
104. Chernikova SB, Wells RL, Elkind MM: Wortmannin sensitizes mammalian cells to radiation by inhibiting the DNA-dependent protein kinase-mediated rejoining of double-strand breaks. *Radiat Res* 1999; 151:159–166.
105. Hosoi Y, Miyachi H, Matsumoto Y, et al.: A phosphatidylinositol 3-kinase inhibitor wortmannin induces radioresistant DNA synthesis and sensitizes cells to bleomycin and ionizing radiation. *Int J Cancer* 1998; 78:642–647.
106. Okayasu R, Suetomi K, Ullrich RL: Wortmannin inhibits repair of DNA double-strand breaks in irradiated normal human cells. *Radiat Res* 1998; 149:440–445.
107. Collis SJ, Swartz MJ, Nelson WG, Dewese TL: Enhanced radiation and chemotherapy-mediated cell killing of human cancer cells by small inhibitory RNA silencing of DNA repair factors. *Cancer Res* 2003; 63:1550–1554.
108. Kim CH, Park SJ, Lee SH: A targeted inhibition of DNA-dependent protein kinase sensitizes breast cancer cells following ionizing radiation. *J Pharmacol Exp Ther* 2002; 303:753–759.
109. Omori S, Takiguchi Y, Suda A, et al.: Suppression of a DNA double-strand break repair gene, Ku70, increases radio- and chemosensitivity in a human lung carcinoma cell line. *DNA Repair* 2002; 1:299–310.
110. Belenkov AI, Paiement JP, Panasci LC, Monia BP, Chow TY: An antisense oligonucleotide targeted to human Ku86 messenger RNA sensitizes M059K malignant glioma cells to ionizing radiation, bleomycin, and etoposide but not DNA cross-linking agents. *Cancer Res* 2002; 62:5888–5896.
111. Jensen R, Glazer PM: Cell-interdependent cisplatin killing by Ku/DNA-dependent protein kinase signaling transduced through gap junctions. *Proc Natl Acad Sci USA* 2004; 101:6134–6139.
112. Eriksson A, Lewensoh R, Larsson R, Nilsson A: DNA-dependent protein kinase in leukaemia cells and correlation with drug sensitivity. *Anticancer Res* 2002; 22:1787–1793.
113. Shinohara ET, Geng L, Tan J, et al.: DNA-dependent protein kinase is a molecular target for the development of noncytotoxic radiation-sensitizing drugs. *Cancer Res* 2005; 65:4987–4992.
114. Zhao Y, Thomas HD, Batey MA, et al.: Preclinical evaluation of a potent novel DNA-dependent protein kinase inhibitor NU7441. *Cancer Res* 2006; 66:5354–5362.
115. Li G, Nelsen C, Hendrickson EA: Ku86 is essential in human somatic cells. *Proc Natl Acad Sci USA* 2002; 99:832–837.

IV

RESISTANCE AND SENSITIZATION

15

Resistance/Signaling Pathways

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SUMMARY

Prior to the early 1990s, the mechanisms by which growth factors and cytokines modulate cell behavior were largely unknown. In the mid-1980s, with the discovery of the first mitogen-activated protein kinase (MAPK) pathway, and with subsequent discoveries of other MAPK family pathways in the early 1990s, our understanding of the hormonal control of cell biology was provided with a greater degree of molecular underpinning. In light of these findings, the ability of cytotoxic drugs and ionizing radiation to control the activity of MAPK family (and other) signaling pathways was first investigated in the mid-1990s. It was discovered that radiation and many noxious drugs, in a cell-type-dependent manner, can activate multiple intracellular signal transduction pathways: the activation of some pathways has been reported to be DNA-damage-dependent, that of others by generation of lipids such as ceramide, whereas others have been noted to be dependent on mitochondria-derived reactive oxygen/nitrogen species and the activation of growth factor receptor tyrosine kinases. The precise roles of growth factor receptors and signal transduction pathways in cellular responses following exposure to noxious stresses are presently under intense investigation. Generally, in a cell-type and dose-dependent manner, inhibition of the extracellular-regulated kinase 1/2 (ERK1/2), and to a greater extent phosphatidylinositol 3 kinase (PI3K)/AKT, pathways can enhance cell killing. The modulation of cell survival by the ERK1/2 and AKT pathways has been correlated to the expression of mutant active RAS isoforms and to growth factor receptors of the ERBB and insulin/insulin-like growth factor (IGF) families. The activation of the c-Jun NH₂-terminal kinase 1/2 (JNK1/2), ERK1/2, and PI3K/AKT pathways in tumor cells has also been linked to the expression of paracrine ligands—ligands that can promote cell growth and survival after exposure to a noxious stress and ligands that are generally only expressed at high levels in transformed cells. This chapter will discuss the signaling pathways activated by cytotoxic drugs and ionizing radiation and the roles each pathway can play in cellular responses of tumor cells.

Key Words: Radiation; signaling; kinase; phosphatase; reactive oxygen/nitrogen species; receptor.

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1. SELF-LIMITING EFFECTS OF CANCER THERAPIES: AGENTS THAT CAUSE INCOMPLETE KILLING AND THE COMPENSATORY ACTIVATION OF GROWTH FACTOR RECEPTORS AND SURVIVAL SIGNALING PATHWAYS

As judged by the findings of many groups using a wide variety of noxious xenobiotic agents, cellular stresses cause pleiotropic activation of multiple receptor proteins in the plasma membranes of cells. The activation of death receptors by xenobiotic agents, such as the FAS and tumor necrosis factor α (TNF α) receptors, has been linked to the generation of ceramide, resulting in the clustering of activated death receptors in the plasma membrane, and the activation of apoptotic pathways. However, lethal cellular stresses also promote activation of growth factor receptors that when activated by their natural ligands are normally linked to cell growth and survival. Hence, conflicting signals are generated.

For example, xenobiotic agents can activate ERBB family receptors, and signaling by ERBB family of receptors is, in general, believed to be pro-proliferative and cytoprotective (1,2). Because both ERBB receptor expression and autocrine growth factor levels are often increased in carcinoma cells compared with normal tissue, many laboratories have studied signaling by the ERBB family in tumor cell growth and survival control after exposure to therapeutic agents. Thus, when signaling from ERBB family receptors is blocked, by use of inhibitory antibodies (e.g., C225, 4D5 Herceptin, and monoclonal antibody 806), small molecular weight inhibitors of receptor tyrosine kinases [e.g., PD183805 (also called CI1033), PKI166, AG1478, PD153035, ZD1839, PD169414, OSI774, AG825, and AG879], dominant-negative truncated receptors (e.g., dominant-negative EGFR-CD533), or antisense approaches (antisense EGFR), that tumor cell growth can be reduced and the sensitivity of these cells to being killed by noxious stresses increased (3–21).

In part, the above findings can be explained by the fact that growth factor receptors, including ERBB1, ERBB2, ERBB3, VEGF-R (Flt-1), and the insulin-like growth factor-1 (IGF-1), are all known to be activated, in a cell-type-dependent manner, after radiation/drug exposure (22–24). Recent publications have tended to argue that reactive oxygen and nitrogen species (ROS/RNS) play an important role in the activation of ERBB family receptors and the signaling by protective downstream pathways following exposure to ionizing radiation (25,26). In the case of receptor tyrosine kinases, the primary target of ROS/RNS is most likely to be protein tyrosine phosphatase (PTPase) enzymes that contain an ROS/RNS-sensitive cysteine residue within their active site, which is a residue that is essential for phosphatase activity (27). As PTPases are approximately 100-fold more active as enzymes than protein kinases, a small reduction in cellular PTPase activity will result in an increase in the steady-state tyrosine phosphorylation of the protein kinases, for example, receptor tyrosine kinases, whose phosphorylation the PTPases act to suppress. Many cytotoxic drugs also have been shown to directly or indirectly generate ROS/RNS as part of the mechanism by which they kill both non-transformed and tumor cells, for example, cisplatin, histone deacetylase inhibitors, rituximab, doxorubicin, and NSAIDs (28–32). Hence, both cytotoxic drugs and radiation have the potential to both kill tumor cells and simultaneously activate protective pathways, for example, ERBB receptor signaling into downstream survival signal transduction pathways, by which their toxicity is blunted.

In addition to a direct effect on the receptor tyrosine kinases themselves, the actions of ERBB receptor autocrine ligands have also been shown to play important roles in the activation of the downstream protective pathways following exposure to noxious stresses. For example, transforming growth factor alpha (TGF α) has been shown to mediate secondary activation of ERBB1 and the downstream extracellular-regulated kinase 1/2 (ERK1/2), AKT, and c-Jun NH₂-terminal kinase 1/2 (JNK1/2) pathways after irradiation of several carcinoma cell lines (33,34). Radiation caused an ERK1/2-dependent cleavage of pro-TGF α in the plasma membrane within 2 h that led to its release into the growth media (35). Similar findings have been noted by other groups (36,37). In addition, signaling by RAS proteins, ERK1/2, and p53 can, over many hours/days in various cell systems, act to increase the expression of autocrine factors such as HB-EGF and epiregulin (38).

2. A PARADIGM FOR DOWNSTREAM PROTECTIVE PATHWAYS: THE “CLASSICAL” MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY, ERK1/2

“MAP-2 kinase” was first reported by the laboratory of Dr Thomas Sturgill in 1986 (39). This protein kinase was originally described as a 42-kDa insulin-stimulated protein kinase activity whose tyrosine phosphorylation increased after insulin exposure and which phosphorylated the cytoskeletal protein MAP-2. Contemporaneous studies from the laboratory of Dr Melanie Cobb identified an additional 44-kDa isoform of this enzyme, termed ERK1 (40). As many growth factors and mitogens could activate these enzymes, the acronym for this enzyme was subsequently changed to denote mitogen-activated protein kinase (MAPK). Additional studies demonstrated that the p42 (ERK2) and p44 (ERK1) MAPKs regulated another protein kinase activity (p90^{sk}) and that they were themselves regulated by protein kinase activities designated MKK1/2 [MAPK kinase (MAP2K)] also termed MEK1/2. MKK1 and MKK2 were also regulated by reversible phosphorylation. The protein kinase responsible for catalyzing MKK1/2 activation was initially described as the proto-oncogene RAF-1 (41). This was soon followed by another MEK1/2 activating kinase, termed MEKK1, which was a mammalian homolog with similarity to the yeast *Ste 11* and *Byr 2* genes (42). However, further studies have shown that the primary function of MEKK1 is to regulate the JNK1/2, rather than the ERK1/2, pathway (43).

Plasma membrane receptors transduce signals through the membrane to its inner leaflet, leading to the recruitment and activation of guanine nucleotide exchange factors, which increases the amount of GTP bound to membrane-associated GTP-binding proteins, in particular RAS family proteins (44–46) (Fig. 1). There are four widely recognized isoforms of RAS: Harvey (H), Kirsten (K4A and K4B), and Neuroblastoma (N) (47). Receptor-stimulated guanine nucleotide exchange of “RAS” to the GTP-bound form permits RAF proteins and P110 phosphatidylinositol 3 kinase (PI3K) to associate with “RAS”, resulting in kinase translocation to the plasma membrane environment where activation of these kinases takes place. RAS contains a GTPase activity that converts bound GTP to GDP resulting in inactivation of the RAS molecule. Transforming/oncogenic mutation of RAS results in a loss of the protein’s inherent GTPase activity, generating a constitutively active RAS molecule that can lead to elevated activity within downstream signaling modules, notably the ERK1/2 and PI3K pathways.

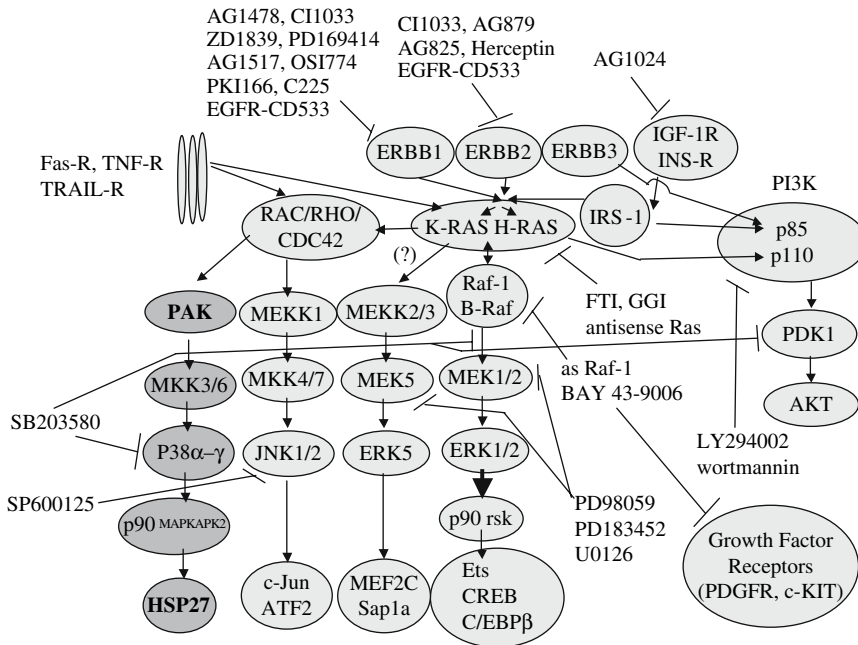


Fig. 1. Some of the characterized signal transduction pathways in mammalian cells. Growth factor receptors, for example, the ERBB family, and insulin receptor can transmit radiation-induced signals down through GTP-binding proteins into multiple intracellular signal transduction pathways. Predominant among these pathways are the mitogen-activated protein kinase (MAPK) superfamily of cascades (ERK1/2, ERK5, JNK, and p38) and the phosphatidylinositol 3 kinase (PI3K) pathway. Growth factor receptors, RAS proteins, and downstream pathways are often partially or constitutively activated in tumor cells, and inhibitors have been developed to block the function of these molecules thereby slowing cell growth and promoting cell death responses. Multiple inhibitors for the ERBB family receptors have been developed. Inhibitors of RAS farnesylation (and geranylgeranylation) are in clinical trials as are inhibitors of the ERK1/2/5 pathway. It should be noted that MEK1/2 inhibitors also are capable of inhibiting the “Big” MAPK pathway through blocking activation of MEK5.

Mutated active H-RAS was one of the first oncogenes discovered, in bladder cancer cells (48), and approximately one-third of human cancers have RAS mutations, primarily the K-RAS isoforms. Early studies examining RAS transformation noted that RAS proteins were prenylated and proteolytically processed causing their translocation into the plasma membranes of cells (49). RAS proteins that could not be prenylated were not located in membranes and could not cause transformation. Subsequently, pharmaceutical companies developed inhibitors of RAS prenylation [farnesyltransferase inhibitor (FTI) and geranylgeranyltransferase inhibitor (GGTI)] that block the prenylation of RAS proteins as well as other small GTP-binding proteins including RAC/RHO/CDC42 (50). Antisense techniques have also been used to manipulate RAS expression (51). Inhibition of small GTP-binding protein prenylation has been shown to both growth arrest and radiosensitize tumor cells in a mutated active RAS-independent fashion, arguing that RAS may not be the primary target of this therapeutic approach (52). In the clinic, as single agents, FTIs have not proven highly successful, and the long-term future of such agents remains unclear. It is possible that FTIs in

combination with cytotoxic agents, whose activity they may magnify, may represent the likeliest avenue for the use of FTIs in the clinic.

RAF-1 is a member of a family of serine–threonine protein kinases comprising also B-RAF and A-RAF. All “RAF” family members can phosphorylate MKK1/2 and activate the ERK1/2 pathway (53). Thus, the “RAF” kinases act at the level of an MAPK kinase kinase (MAP3K). The NH₂ domain of RAF-1 can reversibly interact with Ras family members in the plasma membrane, and the ability of RAF-1 to associate with RAS is dependent on the RAS molecule being in the GTP-bound state (54). Additional protein serine/threonine and tyrosine phosphorylations are also known to play a role increasing RAF-1 activity when in the plasma membrane environment (55,56). The initial biochemical analyses of purified RAF-1 demonstrated constitutive Y340–Y341 phosphorylation when the protein was co-expressed with SRC. PI3K-dependent phosphorylation of S338 may facilitate RAF-1 tyrosine phosphorylation by *src* family members, leading to full activation of RAF-1 (57,58). Of note, activating B-RAF mutations have more recently been shown to play an important role in tumorigenesis in melanoma, thyroid, colorectal, and cholangio-carcinoma, and B-RAF is now considered to be a genuine proto-oncogene, with transforming potential after its mutation (59–62).

Phosphorylation of RAF-1 at S259 by either the AKT or the cAMP protein kinase [protein kinase A (PKA)] can inhibit RAF-1 activity and its activation by upstream stimuli (63–66). Phosphorylation of RAF-1 at S43 by cAMP-dependent protein kinase (PKA) inhibits the interaction of RAF-1 with RAS molecules, thereby blocking RAF-1 translocation to the plasma membrane and its RAS-dependent activation (67). In contrast to RAF-1, the B-RAF isoform does not contain an equivalent to S43 but contains multiple sites of AKT (and potentially PKA)-mediated phosphorylation in addition to the B-RAF equivalent of S259 (68). B-RAF can be activated by both RAS and cAMP through the RAP1 GTPase (69,70). Thus, the regulation of the MEK1/2–ERK1/2 pathway is very complex, and in some cell types, may be both inhibited by cAMP/PKA, through RAF-1, and being stimulated by cAMP/RAP1, through B-RAF (71).

As there are several “RAF” isoforms, it is likely that inhibition of one “RAF” family member, for example, by a specific antisense approach, will not have a profound prolonged growth inhibitory or drug/radiation sensitizing effect on tumor cells because of a compensatory utilization of another “RAF” family member. In light of this, although antisense oligonucleotides to RAF-1 have been shown *in vitro* and *in vivo* to enhance the radiosensitivity of tumors cells (72), such studies have not been highly successful in the clinic because of poor response rates. The development of a relatively non-specific small molecule Raf-1/B-Raf inhibitor is thus more likely to have therapeutic usefulness (73,74).

3. DOWNSTREAM PROTECTIVE PATHWAYS: PI3K/AKT SIGNALING AND THE INTERACTIONS OF PI3K/AKT SIGNALING WITH RAF/ERK1/2 PATHWAY SIGNALING

PI3K enzymes consist of two subunits, a catalytic p110 subunit and a regulatory p85 subunit, and several different classes of PI3K enzymes exist (75,76). The p85 subunit of PI3K enzymes contains a phospho-tyrosine (SH2) binding domain (77). The major

catalytic function of the PI3K enzymes is in the p110 subunit that acts to phosphorylate inositol phospholipids [phosphatidyl inositol 4,5 *bis*-phosphate (PIP₂)] at the third position within the inositol sugar ring. Mitogens such as TGF α and heregulin stimulate tyrosine phosphorylation of ERBB family receptors, providing acceptor sites for the SH2 domain of p85 (78,79). Binding of p85 to active ERBB receptors (predominantly ERBB3) results in p110 PI3K activation. Other studies have suggested that mutant oncogenic H-RAS, or serpentine receptors that are stimulated by mitogens, that the p110 subunit of PI3K can directly bind to RAS-GTP, leading to catalytic activation of the kinase (80–82) (Fig. 1).

The molecule inositol 3,4,5 trisphosphate is an acceptor site in the plasma membrane for molecules that contain a pleckstrin-binding domain (PH domain), in particular, the protein kinases PDK1 and AKT (also called PKB) (83). PDK1 is proposed to phosphorylate and activate AKT. Signaling by PDK1 to AKT and by PDK1 and AKT downstream to other protein kinases such as PKC isoforms, GSK3, mTOR, p70^{S6K}, and p90^{rsk}, has been shown to play a key role in mitogenic and metabolic responses of cells as well as protection of cells from noxious stresses (84–88).

The anti-apoptotic role of the PI3K/AKT pathway has been well documented by many investigators in response to numerous noxious stimuli, and in some cell types, the anti-apoptotic effects of ERBB signaling have been attributed to activation of the PI3K/AKT pathway (89,90). ERBB signaling to PI3K/AKT has been proposed to enhance the expression of the mitochondrial anti-apoptosis proteins BCL-X_L and MCL-1 and caspase inhibitor proteins such as c-FLIP isoforms (91–93). Enhanced expression of BCL-X_L and MCL-1 will protect cells from apoptosis through the intrinsic/mitochondrial pathway, whereas expression of c-FLIP isoforms will block killing from the extrinsic pathway through death receptors (94). In addition, AKT has been shown to phosphorylate BAD and human pro-caspase 9, thereby rendering these proteins inactive in apoptotic processes (95,96). Inhibitors of ERBB signaling have been shown to decrease the activity of the PI3K/AKT pathway in various cell types and to increase the sensitivity of cells to a wide range of toxic stresses including cytotoxic drugs and radiation (97). Activation of AKT was shown to protect cells from death in the presence of ERBB receptor inhibition (98). These findings strongly argue that PI3K/AKT signaling is a key cyto-protective response in many cell types downstream of ERBB family receptors.

Inhibition of PI3K signaling in cells has been achieved by using several drugs, notably wortmannin and LY294002. Wortmannin inhibits PI3K isoforms with IC₅₀ values in the low nanometer range and LY294002 with IC₅₀ values in the low micrometer range (99). More recently, inhibitors of AKT function (through both the PH domain of AKT and the ATP binding site) as well as inhibitors of PDK-1 (also, primarily through the PH domain of PDK-1) have been developed (100–103). The therapeutic usefulness of PI3K inhibitors, and potentially those of AKT and PDK-1, may be limited because of systemic toxicities and effects on cellular glucose metabolism. For example, wortmannin was noted to cause hemorrhages, and the development of effective PI3K inhibitors without dose-limiting side effects is being undertaken by many pharmaceutical companies.

4. DOWNSTREAM CYTOTOXIC PATHWAYS: THE C-JUN KINASE AND STRESS-ACTIVATED PATHWAY, JNK1/2/3

JNK1 and JNK2 were initially described biochemically to be stress-induced protein kinase activities that phosphorylated the NH₂ terminus of the transcription factor c-JUN; hence, the pathway is often called the stress-activated protein kinase (SAPK) pathway (104–106). Multiple stresses increase JNK1/2 (and the subsequently discovered JNK3) activity including UV and γ irradiation, cytotoxic drugs, bile acids, and compounds that primarily generate ROS (e.g., H₂O₂). Phosphorylation of the NH₂-terminal sites Ser63 and Ser73 in c-JUN increases its ability to transactivate AP-1 enhancer elements in the promoters of many genes (107,108). It has been suggested that JNK1/2 can phosphorylate the NH₂ terminus of c-MYC, potentially playing a role in both proliferative and apoptotic signaling (109). In a similar manner to the previously described ERK1/2 MAPK pathway, JNK1/2 activities were regulated by dual threonine and tyrosine phosphorylation that were found to be catalyzed by a protein kinase analogous to MKK1/2, termed stress-activated extracellular-regulated kinase 1 (SEK1), also called MKK4 (110,111). An additional isoform of MKK4, termed MKK7, was subsequently discovered. As in the case of MKK1 and MKK2, MKK4 and MKK7 were regulated by dual serine phosphorylation. Recent studies have also indicated that AKT can phosphorylate and inhibit the activity of MKK4, demonstrating cross-talk between the PI3K and JNK pathways (112).

In contrast to the ERK1/2 pathway, however, which appears to primarily utilize the three protein kinases of the “RAF” family to activate MKK1/2, at least 10 protein kinases are known to phosphorylate and activate MKK4/7, including the Ste 11/Byr2 homologs MKKK1–4, as well as proteins such as TAK-1 and TPL-2 (113–115). Cleavage of MEKK1 by caspase molecules into a constitutively active molecule may play an amplifying role in the execution of apoptotic processes (116).

Upstream of the MAP3K enzymes are another layer of JNK1/2 pathway protein kinases, for example, Ste20 homologs and low molecular weight GTP-binding proteins of the RHO family, in particular CDC42 and RAC1 (117–119) (Fig. 1). It is not clear how growth factor receptors, for example, ERBB1, activate the RHO family low molecular weight GTP-binding proteins; one mechanism may be through the RAS proto-oncogene, whereas others have suggested through PI3K and/or PKC isoforms (120). In addition, others have shown that agonists acting through the TNF α and FAS receptors, through sphingomyelinase enzymes generating the messenger ceramide, can activate the JNK1/2 pathway by mechanisms that may act through RHO family GTPases (121).

Thus, there appear to be multiple distinct mechanisms by which toxic drugs and radiation could activate the JNK1/2 pathway. Initial reports demonstrated that ceramide generation and that the clustering of death receptors on the plasma membrane of cells played an important role in JNK1/2 activation (122–125). This was linked to a pro-apoptotic role for JNK1/2 signaling. Other studies have argued that JNK1/2 activation can be dependent on DNA damage and activation of the ataxia telangiectasia mutated (ATM) and c-ABL proteins (126–128). Studies by our group of laboratories have shown that low-dose radiation activates JNK1/2 in two waves in carcinoma cells. The first wave of JNK1/2 activation was dependent on activation of the TNF α receptor, whereas the second wave of JNK1/2 activity was dependent on ERBB1 and autocrine TGF α . It is

also possible that JNK1/2 activation could be a secondary event to the loss of protective pathway activities, and this could occur through two mechanisms: (i) drug/radiation causes a loss of protective pathway activity resulting in the loss of a negative regulatory signal, which suppresses pro-apoptotic JNK1/2 signaling, for example, loss of JNK phosphatase expression and loss of MKK4 inhibitory phosphorylation; and (ii) JNK1/2 activation is dependent on the subsequent activation of effector pro-caspases: cleavage of the upstream activator MEKK1 can lead to constitutive activation of this enzyme and the downstream JNK1/2 pathway, which in some cell types plays a key role in full commitment to apoptotic cell death (129–131).

5. A SIMPLISTIC SCENARIO FOR CELL SURVIVAL SIGNALING AFTER EXPOSURE TO A NOXIOUS STRESS: INHIBITION OF RAF/MEK/ERK1/2 FUNCTION AND/OR PI3K/AKT FUNCTION TO CIRCUMVENT PROTECTIVE SIGNALING PROCESSES INDUCED BY CYTOTOXIC INSULTS THUS PROMOTES DRUG/RADIATION LETHALITY

Data from several groups have argued that a key protective pathway, responsible for blunting the toxicity of therapeutic drugs, downstream of plasma membrane receptors is the PI3K pathway. Inhibition of p110 PI3K function by using the inhibitors LY294002 and wortmannin radio- and chemo-sensitizes tumor cells of multiple diverse expressing mutant RAS molecules or wild-type RAS molecules that are constitutively active (132–135). It is possible that these inhibitors may also exert a portion of their sensitizing properties by inhibiting proteins with PI3K-like kinase domains such as ATM, ATR, and DNA-PK proteins whose function is to modulate cell survival after DNA damage (136–138). Dominant-negative AKT and small molecule inhibitors of AKT have also been shown to sensitize tumor cells exposed to various noxious agents, suggesting that AKT is a key downstream target for cytoprotective PI3K signaling (100–103).

In many cell types, ERK1/2 signaling does not appear to play a role in controlling cell sensitivity to cytotoxic drugs or ionizing radiation, and in some cells, elevated ERK1/2 signaling has been linked to the promotion of cell killing. However, others have argued that the duration of suppression of ERK1/2 activity may play an important role in whether signaling by this pathway has protective or toxic effects (139). The dual positive and negative nature of ERK1/2 signaling in the control of cell survival has also been observed for other DNA-damaging agents such as adriamycin and UV radiation (140–142).

The mechanisms by which PI3K, AKT, and ERK1/2 signaling protect cells from toxic stresses are multi-layered. The activity and activation of apoptotic cysteine proteases has been shown by many groups to be modulated directly and indirectly by PI3K and AKT signaling (143). For example, activation of PI3K/AKT can promote phosphorylation and inactivation of caspase 9 and BAD, increase expression of inhibitor of apoptosis proteins (IAPs: XIAP), and enhance expression of the caspase 8 inhibitors c-FLIP_{L/S} (144–147). Similarly, ERK1/2 signaling has been shown to promote phosphorylation and inactivation of caspase 9, BAD, BIM, and the FAS receptor, increase expression of Bcl_{XL}, and elevate expression of the caspase 8 inhibitors

c-FLIP_{L/S} (148–153). AKT and ERK1/2 have also been shown in a cell-type-dependent fashion to suppress the generation of ROS and the toxic lipid ceramide (154,155).

Hence, as toxic (therapeutic) stresses increase activation of pro-apoptotic pathways, they simultaneously activate parallel survival pathways such as AKT and ERK1/2 that act to blunt apoptotic pathway function. This has been exploited in the laboratory with excellent success in many tumor cell types and in the clinic with more modest success, for example, the combination of ionizing radiation with PI3K or MEK1/2 inhibitors or cytotoxic drugs with PI3K or MEK1/2 inhibitors. A portion of the reason why inhibiting one survival pathway or one survival growth factor receptor has not proven to be “the magic bullet” in the clinic is because tumor cells are metastable with respect to their survival signaling. For example, inhibition of the estrogen receptor in established breast cancer cells *in vitro* can generate a surviving line which is dependent on ERK1/2 signaling, rather than estrogen receptor signaling, for cell growth and survival (156,157). Inhibition of ERK1/2 signaling in turn promotes outgrowth of tumor cells that are dependent on PI3K signaling for growth and survival and not the estrogen receptor or ERK1/2 pathway. Thus, inhibition of two (or more) parallel survival pathways may be required to promote prolonged tumor cell killing *in vivo*.

An example of how simultaneous inhibition of several survival pathways kills tumor cells can be found in the combination of inhibitors of the ERK1/2 pathway with the Chk1/PKC/PDK-1 inhibitor UCN-01 (158–160). Treatment of tumor cells with UCN-01 causes activation of the ERK1/2 pathway. Inhibition of the induced ERK1/2 activity by using various MEK1/2 inhibitors results in a rapid apoptotic response, dependent on mitochondrial dysfunction and the release of cytochrome c into the cytosol. Other groups have combined tyrosine kinase inhibitors with inhibitors of the protective mTOR pathway to achieve similar results (161). An additional example of this concept is the combination of the cyclin-dependent kinase inhibitor flavopiridol with the PI3K inhibitor LY294002. Treatment of leukemia cells with flavopiridol causes compensatory activation of the PI3K survival pathway: simultaneous inhibition of kinases using flavopiridol and LY294002 results in a synergistic increase in cell killing (162).

Activation of the JNK1/2 pathway has been in general linked by many groups to cell-killing processes; however, it is of note that activation of the JNK1/2 pathway *per se* does not induce cell death, for example, by growth factors. Intense activation of JNK1/2 for a short period of time (~ 1 h) or prolonged low levels of JNK1/2 activity can cause proliferation and/or differentiation through modulation of transcription factors, for example, AP-1 complex, whereas prolonged intense activation of the JNK1/2 pathway has been linked to cell killing (122–131,163). Activation of JNK1/2 can promote mitochondrial injury, that is, BAX association with mitochondria, as well as facilitate death receptor signaling by multiple mechanisms and has been strongly linked to the toxicity of ionizing radiation (164,165). The toxicity of JNK1/2 pathway signaling is also regulated by the relative activities of protective signaling pathways such as PI3K-AKT and ERK1/2 (166, reviewed in ref. 1). Thus, activation of JNK1/2 may not cause cell death when AKT and ERK1/2 remain active: conversely, activation of JNK1/2 in the presence of inhibited or inactivated ERK1/2 and AKT promotes cell killing.

Collectively, these findings argue that the future of signaling modulators in cancer therapy may be linked to a careful appreciation of how compensatory signaling pathways

interact, so that multiple inhibitors can be combined in a relatively precise manner to achieve a maximum amount of tumor cell killing while preventing the activation of survival pathways that will lead to loss of tumor control and therapeutic failure.

6. CONCLUSIONS

The manipulation of signal transduction pathways to reduce cell survival after exposure of cells to noxious insults has been extensively explored over the last 10 years. Some of these concepts are slowly being translated into the clinic, for example, combining ERBB1 or ERBB2 inhibitors with radiotherapy or drugs such as cisplatin or paclitaxel. Many of the initial studies have proven less successful in patients than was expected based on *in vitro* and orthotopic animal tumor model systems, in part because tumor cell signaling is metastable, and inhibition of one signaling survival pathway can lead to the activation of a compensatory survival signaling module. The development of therapies that combine kinase inhibitors in a specific manner to cause cell death, combined with traditional modalities such as radiotherapy, may represent the future of therapeutic intervention based on the inhibition of multiple survival signaling pathways.

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REFERENCES

1. Grant S, Qiao L, Dent P. Roles of ERBB family receptor tyrosine kinases, and downstream signaling pathways, in the control of cell growth and survival. *Front. Biosci.* 2002; 7: d376–d389.
2. Schmidt-Ullrich RK, Dent P, Grant S, Mikkelsen RB, Valerie K. Signal transduction and cellular radiation responses. *Radiat. Res.* 2000; 153: 245–257.
3. Mendelsohn J. The epidermal growth factor receptor as a target for cancer therapy. *Endocr. Relat. Cancer* 2001; 8: 3–9.
4. Ross JS, Fletcher JA. The HER-2/neu oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy. *Stem Cells* 1998; 16: 413–428.
5. Mishima K, Johns TG, Luwor RB, Scott AM, Stockert E, Jungbluth AA, Ji XD, Suvarna P, Volland JR, Old LJ, Huang HJ, Cavenee WK. Growth suppression of intracranial xenografted glioblastomas overexpressing mutant epidermal growth factor receptors by systemic administration of monoclonal antibody (mAb) 806, a novel monoclonal antibody directed to the receptor. *Cancer Res.* 2001; 61: 5349–5354.
6. Erlichman C, Boerner SA, Hallgren CG, Spieker R, Wang XY, James CD, Scheffer GL, Maliepaard M, Ross DD, Bible KC, Kaufmann SH. The HER tyrosine kinase inhibitor CI1033 enhances cytotoxicity of 7-ethyl-10-hydroxycamptothecin and topotecan by inhibiting breast cancer resistance protein-mediated drug efflux. *Cancer Res.* 2001; 61: 739–748.
7. Bruns CJ, Solorzano CC, Harbison MT, Ozawa S, Tsan R, Fan D, Abbruzzese J, Traxler P, Buchdunger E, Radinsky R, Fidler IJ. Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res.* 2000; 60: 2926–2935.
8. Suzuki K, Kodama S, Watanabe M. Extremely low-dose ionizing radiation causes activation of mitogen-activated protein kinase pathway and enhances proliferation of normal human diploid cells. *Cancer Res.* 2001; 61: 5396–5401.

9. Wakita H, Takigawa M. Activation of epidermal growth factor receptor promotes late terminal differentiation of cell-matrix interaction-disrupted keratinocytes. *J. Biol. Chem.* 1999; 274: 37285–37291.
10. Barker AJ, Gibson KH, Grundy W, Godfrey AA, Barlow JJ, Healy MP, Woodburn JR, Ashton SE, Curry BJ, Scarlett L, Henthorn L, Richards L. Studies leading to the identification of ZD1839 (IRESSA): an orally active, selective epidermal growth factor receptor tyrosine kinase inhibitor targeted to the treatment of cancer. *Bioorg. Med. Chem. Lett.* 2001; 11: 1911–1914.
11. Vincent PW, Bridges AJ, Dykes DJ, Fry DW, Leopold WR, Patmore SJ, Roberts BJ, Rose S, Sherwood V, Zhou H, Elliott WL. Anticancer efficacy of the irreversible EGFR tyrosine kinase inhibitor PD 0169414 against human tumor xenografts. *Cancer Chemother. Pharmacol.* 2000; 45: 231–238.
12. Hidalgo M, Siu LL, Nemunaitis J, Rizzo J, Hammond LA, Takimoto C, Eckhardt SG, Tolcher A, Britten CD, Denis L, Ferrante K, Von Hoff DD, Silberman S, Rowinsky EK. Phase I and pharmacologic study of OSI-774, an epidermal growth factor receptor tyrosine kinase inhibitor, in patients with advanced solid malignancies. *J. Clin. Oncol.* 2001; 19: 3267–3279.
13. Fernandes A, Hamburger AW, Gerwin BI. ErbB-2 kinase is required for constitutive stat 3 activation in malignant human lung epithelial cells. *Int. J. Cancer* 1999; 83: 564–570.
14. Yeh S, Lin HK, Kang HY, Thin TH, Lin MF, Chang C. From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. *Proc. Natl. Acad. Sci. U. S. A.* 1999; 96: 5458–5463.
15. Reardon DB, Contessa JN, Mikkelsen RB, Valerie K, Amir C. Dominant negative EGFR-CD533 and inhibition of MAPK modify JNK1 activation and enhance radiation toxicity of human mammary carcinoma cells. *Oncogene* 1999; 18: 4756–4766.
16. Jones FE, Stern DF. Expression of dominant-negative ErbB2 in the mammary gland of transgenic mice reveals a role in lobuloalveolar development and lactation. *Oncogene* 1999; 18: 3481–3490.
17. Ram TG, Schelling ME, Hosick HL. Blocking HER-2/HER-3 function with a dominant negative form of HER-3 in cells stimulated by heregulin and in breast cancer cells with HER-2 gene amplification. *Cell Growth Differ.* 2000; 11: 173–183.
18. Park JS, Carter S, Reardon DB, Schmidt-Ullrich RK, Dent P, Fisher PB. Roles for basal and stimulated p21(Cip-1/WAF1/MDA6) expression and mitogen-activated protein kinase signaling in radiation-induced cell cycle checkpoint control in carcinoma cells. *Mol. Biol. Cell* 1999; 10: 4231–4246.
19. Schattenberg JM, Wang Y, Rigoli RM, Koop DR, Czaja MJ. CYP2E1 overexpression alters hepatocyte death from menadione and fatty acids by activation of ERK1/2 signaling. *Hepatology* 2004; 39: 444–455.
20. Wagner M, Cao T, Lopez ME, Hope C, van Nostrand K, Kobrin MS, Fan HU, Buchler MW, Korc M. Expression of a truncated EGF receptor is associated with inhibition of pancreatic cancer cell growth and enhanced sensitivity to cisplatin. *Int. J. Cancer.* 1996; 68: 782–787.
21. Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, Schiff R. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. *J. Natl. Cancer Inst.* 2004; 96: 926–935.
22. Contessa JN, Hampton J, Lammering G, Mikkelsen RB, Dent P, Valerie K, Schmidt-Ullrich RK. Ionizing radiation activates Erb-B receptor dependent Akt and p70 S6 kinase signaling in carcinoma cells. *Oncogene* 2002; 21: 4032–4041.
23. Geng L, Donnelly E, McMahon G, Lin PC, Sierra-Rivera E, Oshinka H, Hallahan DE. Inhibition of vascular endothelial growth factor receptor signaling leads to reversal of tumor resistance to radiotherapy. *Cancer Res.* 2001; 61: 2413–2419.
24. Wen B, Deutsch E, Marangoni E, Frasca V, Maggiora L, Abdulkarim B, Chavaudra N, Bourhis J. Tyrphostin AG 1024 modulates radiosensitivity in human breast cancer cells. *Br. J. Cancer* 2001; 85: 2017–2021.
25. Leach JK, Van Tuyle G, Lin PS, Schmidt-Ullrich R, Mikkelsen RB. Ionizing radiation-induced, mitochondria-dependent generation of reactive oxygen/nitrogen. *Cancer Res.* 2001; 61: 3894–3901.
26. Mikkelsen RB, Wardman P. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. *Oncogene* 2003; 22: 5734–5754.
27. Huang HL, Fang LW, Lu SP, Chou CK, Luh TY, Lai MZ. DNA-damaging reagents induce apoptosis through reactive oxygen species-dependent Fas aggregation. *Oncogene* 2003; 22: 8168–8177.

28. Ravid A, Rocker D, Machlenkin A, Rotem C, Hochman A, Kessler-Icekson G, Liberman UA, Koren R. 1, 25-Dihydroxyvitamin D₃ enhances the susceptibility of breast cancer cells to doxorubicin-induced oxidative damage. *Cancer Res.* 1999; 59: 862–867.
29. Rosato RR, Grant S. Histone deacetylase inhibitors in cancer therapy. *Cancer Biol. Ther.* 2003; 2: 30–37.
30. Yang B, Keshelava N, Anderson CP, Reynolds CP. Antagonism of buthionine sulfoximine cytotoxicity for human neuroblastoma cell lines by hypoxia is reversed by the bioreductive agent tirapazamine. *Cancer Res.* 2003; 63: 1520–1526.
31. Bellosillo B, Villamor N, Lopez-Guillermo A, Marce S, Esteve J, Campo E, Colomer D, Montserrat E. Complement-mediated cell death induced by rituximab in B-cell lymphoproliferative disorders is mediated *in vitro* by a caspase-independent mechanism involving the generation of reactive oxygen species. *Blood* 2003; 98: 2771–2777.
32. Gomez-Lechon MJ, Ponsoda X, O'Connor E, Donato T, Castell JV, Jover R. Diclofenac induces apoptosis in hepatocytes by alteration of mitochondrial function and generation of ROS. *Biochem. Pharmacol.* 2003; 66: 2155–2167.
33. Dent P, Reardon DB, Park JS, Bowers G, Logsdon C, Valerie K, Schmidt-Ullrich R. Radiation-induced release of transforming growth factor alpha activates the epidermal growth factor receptor and mitogen-activated protein kinase pathway in carcinoma cells, leading to increased proliferation and protection from radiation-induced cell death. *Mol. Biol. Cell* 1999; 10: 2493–2506.
34. Hagan M, Wang L, Hanley JR, Park JS, Dent P. Ionizing radiation-induced mitogen-activated protein (MAP) kinase activation in DU145 prostate carcinoma cells: MAP kinase inhibition enhances radiation-induced cell killing and G₂/M-phase arrest. *Radiat. Res.* 2000; 153: 371–383.
35. Yacoub A, Park JS, Qiao L, Dent P, Hagan MP. MAPK dependence of DNA damage repair: ionizing radiation and the induction of expression of the DNA repair genes XRCC1 and ERCC1 in DU145 human prostate carcinoma cells in a MEK1/2 dependent fashion. *Int. J. Radiat. Biol.* 2001; 77: 1067–1078.
36. Grana TM, Sartor CI, Cox AD. Epidermal growth factor receptor autocrine signaling in RIE-1 cells transformed by the Ras oncogene enhances radiation resistance. *Cancer Res.* 2003; 63: 7807–7814.
37. Gangarosa LM, Sizemore N, Graves-Deal R, Oldham SM, Der CJ, Coffey RJ. A raf-independent epidermal growth factor receptor autocrine loop is necessary for Ras transformation of rat intestinal epithelial cells. *J. Biol. Chem.* 1997; 272: 18926–18931.
38. Baba I, Shirasawa S, Iwamoto R, Okumura K, Tsunoda T, Nishioka M, Fukuyama K, Yamamoto K, Mekada E, Sasazuki T. Involvement of deregulated epiregulin expression in tumorigenesis *in vivo* through activated Ki-Ras signaling pathway in human colon cancer cells. *Cancer Res.* 2000; 60: 6886–6889.
39. Sturgill TW, Ray LB. Muscle proteins related to microtubule associated protein-2 are substrates for an insulin-stimulatable kinase. *Biochem. Biophys. Res. Commun.* 1986; 134: 565–571.
40. Boulton TG, Cobb MH. Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies. *Cell Regul.* 1991; 2: 357–371.
41. Dent P, Haser W, Haystead TA, Vincent LA, Roberts TM, Sturgill TW. Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and *in vitro*. *Science* 1992; 257: 1404–1407.
42. Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ, Johnson GL. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science* 1993; 260: 315–319.
43. Yan M, Dai T, Deak JC, Kyriakis JM, Zon LI, Woodgett JR, Templeton DJ. Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature* 1994; 37: 2798–2800.
44. Li W, Liang X, Kellendonk C, Poli V, Taub R. STAT3 contributes to the mitogenic response of hepatocytes during liver regeneration. *J. Biol. Chem.* 2002; 277: 28411–28417.
45. Okumura K, Shirasawa S, Nishioka M, Sasazuki T. Activated Ki-RAS suppresses 12-O-tetradecanoylphorbol-13-acetate-induced activation of the c-Jun NH₂-terminal kinase pathway in human colon cancer cells. *Cancer Res.* 1999; 59: 2445–2450.
46. Olivier JP, Raabe T, Henkemeyer M, Dickson B, Mbamalu G, Margolis B, Schlessinger J, Hafen E, Pawson T. A Drosophila SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. *Cell* 1993; 73: 179–191.
47. Reuther GW, Der CJ. The RAS branch of small GTPases: RAS family members don't fall far from the tree. *Curr. Opin. Cell Biol.* 2000; 12: 157–165.

48. Feramisco JR, Clark R, Wong G, Arnheim N, Milley R, McCormick F. Transient reversion of ras oncogene-induced cell transformation by antibodies specific for amino acid 12 of ras protein. *Nature* 1985; 314: 639–642.
49. Willumsen BM, Papageorge AG, Hubbert N, Bekesi E, Kung HF, Lowy DR. Transforming p21 ras protein: flexibility in the major variable region linking the catalytic and membrane-anchoring domains. *EMBO J.* 1985; 4: 2893–2896.
50. Zhu K, Hamilton AD, Sebt SM. Farnesyltransferase inhibitors as anticancer agents: current status. *Curr. Opin. Investig. Drugs* 2003; 4: 1428–1435.
51. Stahel RA, Zangemeister-Wittke U. Antisense oligonucleotides for cancer therapy-an overview. *Lung Cancer* 2003; 41 Suppl 1: S81–S8.
52. Lebowitz PF, Prendergast GC. Non-Ras targets of farnesyltransferase inhibitors: focus on Rho. *Oncogene* 1998; 17: 1439–1445.
53. Tombes RM, Auer KL, Mikkelsen R, Valerie K, Wymann MP, Marshall CJ, McMahon M, Dent P. The mitogen-activated protein (MAP) kinase cascade can either stimulate or inhibit DNA synthesis in primary cultures of rat hepatocytes depending upon whether its activation is acute/phasic or chronic. *Biochem. J.* 1998; 330: 1451–1460.
54. Moodie SA, Willumsen BM, Weber MJ, Wolfman A. Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* 1993; 260: 1658–1661.
55. Dent P, Reardon DB, Morrison DK, Sturgill TW. Regulation of Raf-1 and Raf-1 mutants by Ras-dependent and Ras-independent mechanisms *in vitro*. *Mol. Cell Biol.* 1995; 15: 4125–4135.
56. Fabian JR, Daar IO, Morrison DK. Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. *Mol. Cell Biol.* 1993; 13: 7170–7179.
57. King AJ, Wireman RS, Hamilton M, Marshall MS. Phosphorylation site specificity of the Pak-mediated regulation of Raf-1 and cooperativity with Src. *FEBS Lett.* 2001; 497: 6–14.
58. Marais R, Light Y, Paterson HF, Mason CS, Marshall CJ. Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. *J. Biol. Chem.* 1997; 272: 4378–4383.
59. Hubbard SR. Oncogenic mutations in B-Raf: some losses yield gains. *Cell* 2004; 116: 764–766.
60. O'Neill E, Kolch W. Conferring specificity on the ubiquitous Raf/MEK signalling pathway. *Br. J. Cancer* 2004; 90: 283–288.
61. Mercer KE, Pritchard CA. Raf proteins and cancer: B-Raf is identified as a mutational target. *Biochim. Biophys. Acta* 2003; 1653: 25–40.
62. Karasarides M, Chiloeches A, Hayward R, Niculescu-Duvaz D, Scanlon I, Friedlos F, Ogilvie L, Hedley D, Martin J, Marshall CJ, Springer CJ, Marais R. B-RAF is a therapeutic target in melanoma. *Oncogene* 2004; 23: 6292–6298.
63. Dhillon AS, Pollock C, Steen H, Shaw PE, Mischak H, Kolch W. Cyclic AMP-dependent kinase regulates Raf-1 kinase mainly by phosphorylation of serine 259. *Mol. Cell Biol.* 2002; 22: 3237–3246.
64. Reusch HP, Zimmermann S, Schaefer M, Paul M, Moelling K. Regulation of Raf by Akt controls growth and differentiation in vascular smooth muscle cells. *J. Biol. Chem.* 2001; 276: 33630–33637.
65. Yip-Schneider MT, Miao W, Lin A, Barnard DS, Tzivion G, Marshall MS. Regulation of the Raf-1 kinase domain by phosphorylation and 14-3-3 association. *Biochem. J.* 2000; 351: 151–159.
66. Zimmermann S, Moelling K. Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science* 1999; 286: 1741–1744.
67. Wu J, Dent P, Jelinek T, Wolfman A, Weber MJ, Sturgill TW. Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3', 5'-monophosphate. *Science* 1993; 262: 1065–1069.
68. Guan KL, Figueroa C, Brtva TR, Zhu T, Taylor J, Barber TD, Vojtek AB. Negative regulation of the serine/threonine kinase B-Raf by Akt. *J. Biol. Chem.* 2000; 275: 27354–27359.
69. Klinger M, Kudlacek O, Seidel MG, Freissmuth M, Sexl V. MAP kinase stimulation by cAMP does not require RAP1 but SRC family kinases. *J. Biol. Chem.* 2002; 277: 32490–32497.
70. York RD, Molliver DC, Grewal SS, Stenberg PE, McCleskey EW, Stork PJ. Role of phosphoinositide 3-kinase and endocytosis in nerve growth factor-induced extracellular signal-regulated kinase activation via Ras and Rap1. *Mol. Cell Biol.* 2000; 20: 8069–8083.
71. Brummer T, Shaw PE, Reth M, Misawa Y. Inducible gene deletion reveals different roles for B-Raf and Raf-1 in B-cell antigen receptor signalling. *EMBO J.* 2002; 21: 5611–5622.
72. Gokhale PC, Zhang C, Newsome JT, Pei J, Ahmad I, Rahman A, Dritschilo A, Kasid UN. Pharmacokinetics, toxicity, and efficacy of ends-modified raf antisense oligodeoxyribonucleotide encapsulated in a novel cationic liposome. *Clin. Cancer Res.* 2002; 8: 3611–3621.

73. Hilger RA, Kredke S, Hedley D, Moeller JG, Bauer RJ, Stellberg W, Seeber S, Scheulen ME, Strumberg D. ERK1/2 phosphorylation: a biomarker analysis within a phase I study with the new Raf kinase inhibitor BAY43-9006. *Int. J. Clin. Pharmacol. Ther.* 2002; 40: 567–568.
74. Mross K, Steinbild S, Baas F, Reil M, Buss P, Mersmann S, Voliotis D, Schwartz B, Brendel E. Drug-drug interaction pharmacokinetic study with the Raf kinase inhibitor (RKI) BAY 43-9006 administered in combination with irinotecan (CPT-11) in patients with solid tumors. *Int. J. Clin. Pharmacol. Ther.* 2001; 41: 618–619.
75. Vanhaesebroeck B, Alessi DR. The PI3K-PDK1 connection: more than just a road to PKB. *Biochem. J.* 2000; 346: 561–576.
76. Wymann MP, Pirola L. Structure and function of phosphoinositide 3-kinases. *Biochim. Biophys. Acta* 1998; 1436: 127–150.
77. Ching TT, Lin HP, Yang CC, Oliveira M, Lu PJ, Chen CS. Specific binding of the C-terminal Src homology 2 domain of the p85alpha subunit of phosphoinositide 3-kinase to phosphatidylinositol 3,4,5-trisphosphate. Localization and engineering of the phosphoinositide-binding motif. *J. Biol. Chem.* 2001; 276: 43932–43938.
78. Lee H, Akita RW, Sliwkowski MX, Maihle NJ. A naturally occurring secreted human ErbB3 receptor isoform inhibits heregulin-stimulated activation of ErbB2, ErbB3, and ErbB4. *Cancer Res.* 2001; 61: 4467–4473.
79. Yu CF, Roshan B, Liu ZX, Cantley LG. ERK regulates the hepatocyte growth factor-mediated interaction of Gab1 and the phosphatidylinositol 3-kinase. *J. Biol. Chem.* 2001; 276: 32552–32558.
80. Van-Weering DH, de Rooij J, Marte B, Downward J, Bos JL, Burgering BM. Protein kinase B activation and lamellipodium formation are independent phosphoinositide 3-kinase-mediated events differentially regulated by endogenous Ras. *Mol. Cell Biol.* 1998; 18: 1802–1811.
81. Gu H, Maeda H, Moon JJ, Lord JD, Yoakim M, Nelson BH, Neel BG. New role for Shc in activation of the phosphatidylinositol 3-kinase/Akt pathway. *Mol. Cell Biol.* 2000; 20: 7109–7120.
82. Rubio I, Rodriguez-Viciano P, Downward J, Wetzker R. Interaction of Ras with phosphoinositide 3-kinase gamma. *Biochem. J.* 1997; 326: 891–895.
83. Filippa N, Sable CL, Hemmings BA, Van Obberghen E. Effect of phosphoinositide-dependent kinase 1 on protein kinase B translocation and its subsequent activation. *Cell Biol.* 2000; 20: 5712–5721.
84. Balendran A, Hare GR, Kieloch A, Williams MR, Alessi DR. Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C (PKC) isoforms. *FEBS Lett.* 2000; 484: 217–223.
85. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 1995; 378: 785–789.
86. Alessi DR, Caudwell FB, Andjelkovic M, Hemmings BA, Cohen P. Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Lett.* 1996; 399: 333–338.
87. Podsypanina K, Lee RT, Politis C, Hennessy I, Crane A, Puc J, Neshat M, Wang H, Yang L, Gibbons J, Frost P, Dreisbach V, Blenis J, Gaciong Z, Fisher P, Sawyers C, Hedrick-Ellenson L, Parsons R. An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten^{+/-} mice. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 2001; 98: 10320–10325.
88. Dickson LM, Lingohr MK, McCuaig J, Hugl SR, Snow L, Kahn BB, Myers MG, Rhodes CJ. Differential activation of protein kinase B and p70(S6)K by glucose and insulin-like growth factor 1 in pancreatic beta-cells (INS-1). *J. Biol. Chem.* 2001; 276: 21110–21120.
89. Kainulainen V, Sundvall M, Maatta JA, Santiestevan E, Klagsbrun M, Elenius K. A natural ErbB4 isoform that does not activate phosphoinositide 3-kinase mediates proliferation but not survival or chemotaxis. *J. Biol. Chem.* 2000; 27: 8641–8649.
90. Daly JM, Olayioye MA, Wong AM, Neve R, Lane HA, Maurer FG, Hynes NE. NDF/heregulin-induced cell cycle changes and apoptosis in breast tumour cells: role of PI3 kinase and p38 MAP kinase pathways. *Oncogene* 1999; 18: 3440–3451.
91. Leverrier Y, Thomas J, Mathieu AL, Low W, Blanquier B, Marvel J. Role of PI3-kinase in Bcl-X induction and apoptosis inhibition mediated by IL-3 or IGF-1 in Baf-3 cells. *Cell Death Differ.* 1999; 6: 290–296.
92. Kuo ML, Chuang SE, Lin MT, Yang SY. The involvement of PI 3-K/Akt-dependent up-regulation of Mcl-1 in the prevention of apoptosis of Hep3B cells by interleukin-6. *Oncogene* 2001; 20: 677–685.

93. Panka DJ, Mano T, Suhara T, Walsh K, Mier JW. Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. *J. Biol. Chem.* 2001; 276: 6893–6896.
94. Suhara T, Mano T, Oliveira BE, Walsh K. Phosphatidylinositol 3-kinase/Akt signaling controls endothelial cell sensitivity to Fas-mediated apoptosis via regulation of FLICE-inhibitory protein (FLIP). *Circ. Res.* 2001; 89: 13–19.
95. Li Y, Tennekoon GI, Birnbaum M, Marchionni MA, Rutkowski JL. Neuregulin signaling through a PI3K/Akt/Bad pathway in Schwann cell survival. *Mol. Cell Neurosci.* 2001; 17: 761–767.
96. Fujita E, Jinbo A, Matuzaki H, Konishi H, Kikkawa U, Momoi T. Akt phosphorylation site found in human caspase-9 is absent in mouse caspase-9. *Biochem. Biophys. Res. Commun.* 1999; 264: 550–555.
97. Pianetti S, Arsura M, Romieu-Mourez R, Coffey RJ, Sonenshein GE. Her-2/neu overexpression induces NF-kappaB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of I kappa B-alpha that can be inhibited by the tumor suppressor PTEN. *Oncogene* 2001; 20: 1287–1299.
98. Cuello M, Ettenberg SA, Clark AS, Keane MM, Posner RH, Nau MM, Dennis PA, Lipkowitz S. Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2. *Cancer Res.* 2001; 61: 4892–4900.
99. Cuenda A, Alessi DR. Use of kinase inhibitors to dissect signaling pathways. *Methods Mol. Biol.* 2000; 99: 161–175.
100. Castillo SS, Brognard J, Petukhov PA, Zhang C, Tsurutani J, Granville CA, Li M, Jung M, West KA, Gills JG, Kozikowski AP, Dennis PA. Preferential inhibition of Akt and killing of Akt-dependent cancer cells by rationally designed phosphatidylinositol ether lipid analogues. *Cancer Res.* 2004; 64: 2782–2792.
101. Kondapaka SB, Singh SS, Dasmahapatra GP, Sausville EA, Roy KK. Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation. *Mol. Cancer Ther.* 2003; 2: 1093–1103.
102. Yang L, Dan HC, Sun M, Liu Q, Sun XM, Feldman RI, Hamilton AD, Polokoff M, Nicosia SV, Herlyn M, Sebti SM, Cheng JQ. Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of akt signaling with antitumor activity in cancer cells overexpressing akt. *Cancer Res.* 2004; 64: 4394–4399.
103. Zhu J, Huang JW, Tseng PH, Yang YT, Fowble J, Shiau CW, Shaw YJ, Kulp SK, Chen CS. From the cyclooxygenase-2 inhibitor celecoxib to a novel class of 3-phosphoinositide-dependent protein kinase-1 inhibitors. *Cancer Res.* 2004; 64: 4309–4318.
104. Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, Davis RJ. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* 1994; 76: 1025–1037.
105. Derijard B, Raingeaud J, Barrett T, Wu IH, Han J, Ulevitch RJ, Davis RJ. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science* 1995; 267: 682–685.
106. Hibi M, Lin A, Smeal T, Minden A, Karin M. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.* 1993; 7: 2135–2148.
107. Davis RJ. Signal transduction by the c-Jun N-terminal kinase. *Biochem. Soc. Symp.* 1999; 64: 1–12.
108. Eferl R, Sibilina M, Hilberg F, Fuchsbichler A, Kufferath I, Guertl B, Zenz R, Wagner EF, Zatloukal K. Functions of c-Jun in liver and heart development. *J. Cell Biol.* 1999; 145: 1049–1061.
109. Noguchi K, Kitanaka C, Yamana H, Kokubu A, Mochizuki T, Kuchino Y. Regulation of c-Myc through phosphorylation at Ser-62 and Ser-71 by c-Jun N-terminal kinase. *J. Biol. Chem.* 1999; 274: 32580–32587.
110. Lin A, Minden A, Martinetto H, Claret FX, Lange-Carter C, Mercurio F, Johnson GL, Karin M. Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. *Science* 1995; 268: 286–290.
111. Tournier C, Whitmarsh AJ, Cavanagh J, Barrett T, Davis RJ. The MKK7 gene encodes a group of c-Jun NH2-terminal kinase kinases. *Mol. Cell Biol.* 1999; 19: 1569–1581.
112. Park HS, Kim MS, Huh SH, Park J, Chung J, Kang SS, Choi EJ. Akt (protein kinase B) negatively regulates SEK1 by means of protein phosphorylation. *J. Biol. Chem.* 2002; 277: 2573–2578.
113. Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ, Johnson GL. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science* 1993; 260: 315–319.

114. Schlesinger TK, Bonvin C, Jarpe MB, Fanger GR, Cardinaux JR, Johnson GL, Widmann C. Apoptosis stimulated by the 91-kDa caspase cleavage MEKK1 fragment requires translocation to soluble cellular compartments. *J. Biol. Chem.* 2002; 277: 10283–10291.
115. Yan M, Dai T, Deak JC, Kyriakis JM, Zon LI, Woodgett JR, Templeton DJ. Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature* 1994; 37: 2798–2800.
116. Widmann C, Gerwins P, Johnson NL, Jarpe MB, Johnson GL. MEK kinase 1, a substrate for DEVD-directed caspases, is involved in genotoxin-induced apoptosis. *Mol. Cell Biol.* 1998; 18: 2416–2429.
117. Frost JA, Xu S, Hutchison MR, Marcus S, Cobb MH. Actions of Rho family small G proteins and p21-activated protein kinases on mitogen-activated protein kinase family members. *Mol. Cell Biol.* 1996; 16: 3707–3713.
118. Graves JD, Draves KE, Gotoh Y, Krebs EG, Clark EA. Both phosphorylation and caspase-mediated cleavage contribute to regulation of the Ste20-like protein kinase Mst1 during CD95/Fas-induced apoptosis. *J. Biol. Chem.* 2001; 276: 14909–14915.
119. Yustein JT, Li D, Robinson D, Kung HJ. KFC, a Ste20-like kinase with mitogenic potential and capability to activate the SAPK/JNK pathway. *Oncogene* 2000; 19: 710–718.
120. Timokhina I, Kissel H, Stella G, Besmer P. Kit signaling through PI 3-kinase and Src kinase pathways: an essential role for Rac1 and JNK activation in mast cell proliferation. *EMBO J.* 1998; 17: 6250–6262.
121. Lu Y, Settleman J. The role of rho family GTPases in development: lessons from *Drosophila melanogaster*. *Mol. Cell Biol. Res. Commun.* 1999; 1: 87–94.
122. Rosette C, Karin M. Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. *Science* 1996; 274: 1194–1197.
123. Cremesti A, Paris F, Grassme H, Holler N, Tschopp J, Fuks Z, Gulbins E, Kolesnick R. Ceramide enables fas to cap and kill. *J. Biol. Chem.* 2001; 276: 23954–23961.
124. Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z, Kolesnick RN. Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature* 1996; 380: 75–79.
125. Herr I, Wilhelm D, Bohler T, Angel P, Debatin KM. Activation of CD95 (APO-1/Fas) signaling by ceramide mediates cancer therapy-induced apoptosis. *EMBO J.* 1997; 16: 6200–6208.
126. Kharbanda S, Yuan ZM, Weichselbaum R, Kufe D. Activation of CD95 (APO-1/Fas) signaling by ceramide mediates cancer therapy-induced apoptosis. *Oncogene* 1998; 17: 3309–3318.
127. Bar-Shira A, Rashi-Elkeles S, Zlochover L, Moyal L, Smorodinsky NI, Seger R, Shiloh Y. ATM-dependent activation of the gene encoding MAP kinase phosphatase 5 by radiomimetic DNA damage. *Oncogene* 2002; 21: 849–855.
128. Zhang Y, Ma WY, Kaji A, Bode AM, Dong Z. Requirement of ATM in UVA-induced signaling and apoptosis. *J. Biol. Chem.* 2002; 277: 3124–3131.
129. Widmann C, Johnson NL, Gardner AM, Smith RJ, Johnson GL. Potentiation of apoptosis by low dose stress stimuli in cells expressing activated MEK kinase 1. *Oncogene* 1997; 15: 2439–2447.
130. Widmann C, Gerwins P, Johnson NL, Jarpe MB, Johnson GL. MEK kinase 1, a substrate for DEVD-directed caspases, is involved in genotoxin-induced apoptosis. *Mol. Cell Biol.* 1998; 18: 2416–2429.
131. Schlesinger TK, Bonvin C, Jarpe MB, Fanger GR, Cardinaux JR, Johnson GL, Widmann C. Apoptosis stimulated by the 91-kDa caspase cleavage MEKK1 fragment requires translocation to soluble cellular compartments. *J. Biol. Chem.* 2002; 277: 10283–10291.
132. Gupta AK, Bakanauskas VJ, Cerniglia GJ, Cheng Y, Bernhard EJ, Muschel RJ, McKenna WG. The Ras radiation resistance pathway. *Cancer Res.* 2001; 61: 4278–4282.
133. Gupta AK, McKenna WG, Weber CN, Feldman MD, Goldsmith JD, Mick R, Machtay M, Rosenthal DI, Bakanauskas VJ, Cerniglia GJ, Bernhard EJ, Weber RS, Muschel RJ. Local recurrence in head and neck cancer: relationship to radiation resistance and signal transduction. *Clin. Cancer Res.* 2002; 8: 885–892.
134. Gupta AK, Bernhard EJ, Bakanauskas VJ, Wu J, Muschel RJ, McKenna WG. RAS-Mediated radiation resistance is not linked to MAP kinase activation in two bladder carcinoma cell lines. *Radiat. Res.* 2000; 154: 64–72.
135. Grana TM, Rusyn EV, Zhou H, Sartor CI, Cox AD. Ras mediates radioresistance through both phosphatidylinositol 3-kinase-dependent and Raf-dependent but mitogen-activated protein

- kinase/extracellular signal-regulated kinase kinase-independent signaling pathways. *Cancer Res.* 2002; 62: 4142–4150.
136. Lali FV, Hunt AE, Turner SJ, Foxwell BM. The pyridinyl imidazole inhibitor SB203580 blocks phosphoinositide-dependent protein kinase activity, protein kinase B phosphorylation, and retinoblastoma hyperphosphorylation in interleukin-2-stimulated T cells independently of p38 mitogen-activated protein kinase. *J. Biol. Chem.* 2000; 275: 7395–7402.
 137. Rane MJ, Coxon PY, Powell DW, Webster R, Klein JB, Pierce W, Ping P, McLeish KR. p38 Kinase-dependent MAPKAPK-2 activation functions as 3-phosphoinositide-dependent kinase-2 for Akt in human neutrophils. *J. Biol. Chem.* 2001; 276: 3517–3523.
 138. Zhang Y, Dong Z, Nomura M, Zhong S, Chen N, Bode AM, Dong Z. Signal transduction pathways involved in phosphorylation and activation of p70S6K following exposure to UVA irradiation. *J. Biol. Chem.* 2001; 276: 20913–20923.
 139. Qiao L, Yacoub A, McKinstry R, Park JS, Caron R, Fisher PB, Hagan MP, Grant S, Dent P. Pharmacologic inhibitors of the mitogen activated protein kinase cascade have the potential to interact with ionizing radiation exposure to induce cell death in carcinoma cells by multiple mechanisms. *Cancer Biol. Ther.* 2002; 1: 168–176.
 140. Pardo OE, Arcaro A, Salerno G, Raguz S, Downward J, Seckl MJ. Fibroblast growth factor-2 induces translational regulation of Bcl-XL and Bcl-2 via a MEK-dependent pathway: correlation with resistance to etoposide-induced apoptosis. *J. Biol. Chem.* 2002; 277: 12040–12046.
 141. Tang D, Wu D, Hirao A, Lahti JM, Liu L, Mazza B, Kidd VJ, Mak TW, Ingram AJ. ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. *J. Biol. Chem.* 2002; 277: 12710–12717.
 142. Kitagawa D, Tanemura S, Ohata S, Shimizu N, Seo J, Nishitai G, Watanabe T, Nakagawa K, Kishimoto H, Wada T, Tezuka T, Yamamoto T, Nishina H, Katada T. Activation of extracellular signal-regulated kinase by ultraviolet is mediated through Src-dependent epidermal growth factor receptor phosphorylation. Its implication in an anti-apoptotic function. *J. Biol. Chem.* 2002; 277: 366–371.
 143. Wetzker R, Rommel C. Phosphoinositide 3-kinases as targets for therapeutic intervention. *Curr. Pharm. Des.* 2004; 10: 1915–1922.
 144. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. Regulation of cell death protease caspase-9 by phosphorylation. *Science* 1998; 282: 1318–1321.
 145. del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 1997; 278: 687–689.
 146. Tran J, Rak J, Sheehan C, Saibil SD, LaCasse E, Korneluk RG, Kerbel RS. Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 1999; 264: 781–788.
 147. Panka DJ, Mano T, Suhara T, Walsh K, Mier JW. Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. *J. Biol. Chem.* 2001; 276: 6893–6896.
 148. Allan LA, Morrice N, Brady S, Magee G, Pathak S, Clarke PR. Inhibition of caspase-9 through phosphorylation at Thr 125 by ERK MAPK. *Nat. Cell Biol.* 2003; 5: 647–654.
 149. Scheid MP, Duronio V. Dissociation of cytokine-induced phosphorylation of Bad and activation of PKB/akt: involvement of MEK upstream of Bad phosphorylation. *Proc. Natl. Acad. Sci. U. S. A.* 1998; 95: 7439–7444.
 150. Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ. Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. *J. Biol. Chem.* 2003; 278: 18811–18816.
 151. Meng XW, Chandra J, Loegering D, Van Becelaere K, Kottke TJ, Gore SD, Karp JE, Sebolt-Leopold J, Kaufmann SH. Central role of Fas-associated death domain protein in apoptosis induction by the mitogen-activated protein kinase kinase inhibitor CI-1040 (PD184352) in acute lymphocytic leukemia cells *in vitro*. *J. Biol. Chem.* 2003; 278: 47326–47339.
 152. Jost M, Huggett TM, Kari C, Rodeck U. Matrix-independent survival of human keratinocytes through an EGF receptor/MAPK-kinase-dependent pathway. *Mol. Biol. Cell* 2001; 12: 1519–1527.
 153. Qiao L, Studer E, Leach K, McKinstry R, Gupta S, Decker R, Kukreja R, Valerie K, Nagarkatti P, El Deiry W, Molkenin J, Schmidt-Ullrich R, Fisher PB, Grant S, Hylemon PB, Dent P. Deoxycholic acid (DCA) causes ligand-independent activation of epidermal growth factor receptor (EGFR) and FAS receptor in primary hepatocytes: inhibition of EGFR/mitogen-activated protein kinase-signaling module enhances DCA-induced apoptosis. *Mol. Biol. Cell* 2001; 12: 2629–2645.

154. Lee SE, Chung WJ, Kwak HB, Chung CH, Kwack KB, Lee ZH, Kim HH. Tumor necrosis factor- α supports the survival of osteoclasts through the activation of Akt and ERK. *J. Biol. Chem.* 2001; 276: 49343–49349.
155. Yoon SO, Yun CH, Chung AS. Dose effect of oxidative stress on signal transduction in aging. *Mech. Ageing Dev.* 2002; 123: 1597–1604.
156. Gee JM, Harper ME, Hutcheson IR, Madden TA, Barrow D, Knowlden JM, McClelland RA, Jordan N, Wakeling AE, Nicholson RI. The anti-epidermal growth factor receptor agent gefitinib (ZD1839/Iressa) improves antihormone response and prevents development of resistance in breast cancer *in vitro*. *Endocrinology* 2003; 144: 5105–5117.
157. Hutcheson IR, Knowlden JM, Madden TA, Barrow D, Gee JM, Wakeling AE, Nicholson RI. Oestrogen receptor-mediated modulation of the EGFR/MAPK pathway in tamoxifen-resistant MCF-7 cells. *Breast Cancer Res. Treat.* 2003; 81: 81–93.
158. McKinstry R, Qiao L, Yacoub A, Dai Y, Decker R, Holt S, Hagan MP, Grant S, Dent P. Inhibitors of MEK1/2 interact with UCN-01 to induce apoptosis and reduce colony formation in mammary and prostate carcinoma cells. *Cancer Biol. Ther.* 2002; 1: 243–253.
159. Dai Y, Yu C, Singh V, Tang L, Wang Z, McKinstry R, Dent P, Grant S. Pharmacological inhibitors of the mitogen-activated protein kinase (MAPK) kinase/MAPK cascade interact synergistically with UCN-01 to induce mitochondrial dysfunction and apoptosis in human leukemia cells. *Cancer Res.* 2001; 61: 5106–5115.
160. Dai Y, Landowski TH, Rosen ST, Dent P, Grant S. Combined treatment with the checkpoint abrogator UCN-01 and MEK1/2 inhibitors potently induces apoptosis in drug-sensitive and -resistant myeloma cells through an IL-6-independent mechanism. *Blood* 2002; 100: 3333–3343.
161. Mohi MG, Boulton C, Gu TL, Sternberg DW, Neuberger D, Griffin JD, Gilliland DG, Neel BG. Combination of rapamycin and protein tyrosine kinase (PTK) inhibitors for the treatment of leukemias caused by oncogenic PTKs. *Proc. Natl. Acad. Sci. U. S. A.* 2004; 101: 3130–3135.
162. Yu C, Rahmani M, Dai Y, Conrad D, Krystal G, Dent P, Grant S. The lethal effects of pharmacological cyclin-dependent kinase inhibitors in human leukemia cells proceed through a phosphatidylinositol 3-kinase/Akt-dependent process. *Cancer Res.* 2003; 63: 1822–1833.
163. Kayahara M, Wang X, Tournier C. Selective regulation of c-jun gene expression by mitogen-activated protein kinases via the 12-*o*-tetradecanoylphorbol-13-acetate- responsive element and myocyte enhancer factor 2 binding sites. *Mol. Cell Biol.* 2005; 25: 3784–3792.
164. Papadakis ES, Finegan KG, Wang X, Robinson AC, Guo C, Kayahara M, Tournier C. The regulation of Bax by c-Jun N-terminal protein kinase (JNK) is a prerequisite to the mitochondrial-induced apoptotic pathway. *FEBS Lett.* 2006; 580: 1320–1326.
165. Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA, Davis RJ. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science* 2000; 288: 870–874.
166. Molton SA, Todd DE, Cook SJ. Selective activation of the c-Jun N-terminal kinase (JNK) pathway fails to elicit Bax activation or apoptosis unless the phosphoinositide 3'-kinase (PI3K) pathway is inhibited. *Oncogene* 2003; 22: 4690–4701.

16

Ceramide and Multidrug Resistance

Myles C. Cabot, PhD

SUMMARY

Chemotherapy resistance represents a formidable barrier to the successful treatment of advancing cancer, and patients and oncologists would welcome its possible correction. Unfortunately, many frontline chemotherapeutic agents drive biochemical events that promote tumor cell resistance. Averting these events is key to halting and perhaps reversing this process. Only recently have we begun to appreciate the role of lipids in both chemotherapy response mechanisms and resistance process. Because much emphasis is being placed on “targeted therapeutics,” we believe that targeting the enzymes of lipid metabolism represents a promising direction in the treatment of cancer. This chapter explains how the harnessing of ceramide metabolism could be key in tempering multidrug resistance.

Key Words: Ceramide; chemotherapy; multidrug resistance; glucosylceramide synthase.

1. INTRODUCTION

Drug and biocide resistance arising in biological systems is widespread in nature. Organisms develop defense mechanisms against deleterious effects of myriad toxins. Posing a serious threat to human health and the environment are drug-resistant bacteria and viruses, pesticide-resistant insects, and herbicide-resistant plants. Many human cancers are also drug resistant; although some such as melanoma can be intrinsically resistant (1), cancers more often acquire resistance through selection pressure in the face of adversity, for example, chemotherapy.

Poor response to chemotherapy is a major clinical problem, and drug resistance is usually the cause (2,3). Approximately 40% of cancer patients with resectable disease and 80% of cancer patients with unresectable disease have a poor response to chemotherapy and radiotherapy. In breast cancer alone, nearly 50% of patients demonstrate primary and/or secondary resistance to doxorubicin (4). In prostate and ovarian malignancies, the prevalence of drug resistance prevents significant cure with current chemotherapeutic regimens; in melanoma, which is characterized by high risk for early metastasis, the commonly employed frontline anticancer drugs do not improve prognosis (5). In small cell lung cancer, acquired resistance to multiple drugs has

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reduced the cure rate of chemotherapy to less than 10% (6). A clinically effective means of tempering or reversing resistance would significantly improve response to chemotherapy and thereby decrease the mortality of human cancer. This chapter will explore how interfering with ceramide metabolism might modulate tumor cell resistance to chemotherapy and thereby serve as the basis for clinically effective approaches to drug-resistant cancer.

2. CERAMIDE IN GROWTH ARREST AND CELLULAR SENESENCE

To the small fraternity of lipid biochemists worldwide, news that ceramide was involved in the killing action of chemotherapy drugs brought a resurgence in cancer studies. In the not-too-distant past, life was composed of proteins, lipids, carbohydrates, and nucleic acids; today, these elements are being redefined by knockouts, clones, and chain reactions, leaving little room for mere fat. Lipids, like Mahler, can be difficult to appreciate—unless they prove effective in the treatment of cancer.

Ceramides or “*N*-acylsphingosines” are neutral lipids that can be either generated by a *de novo* pathway through condensation of serine and palmitoyl CoA and a series of enzymatic steps or formed by degradation of sphingomyelin through the sphingomyelinase pathway (Fig. 1). The amide-linked acyl moieties of ceramide can consist of various chain lengths of typically 16–26 carbons. Ceramides influence numerous cellular

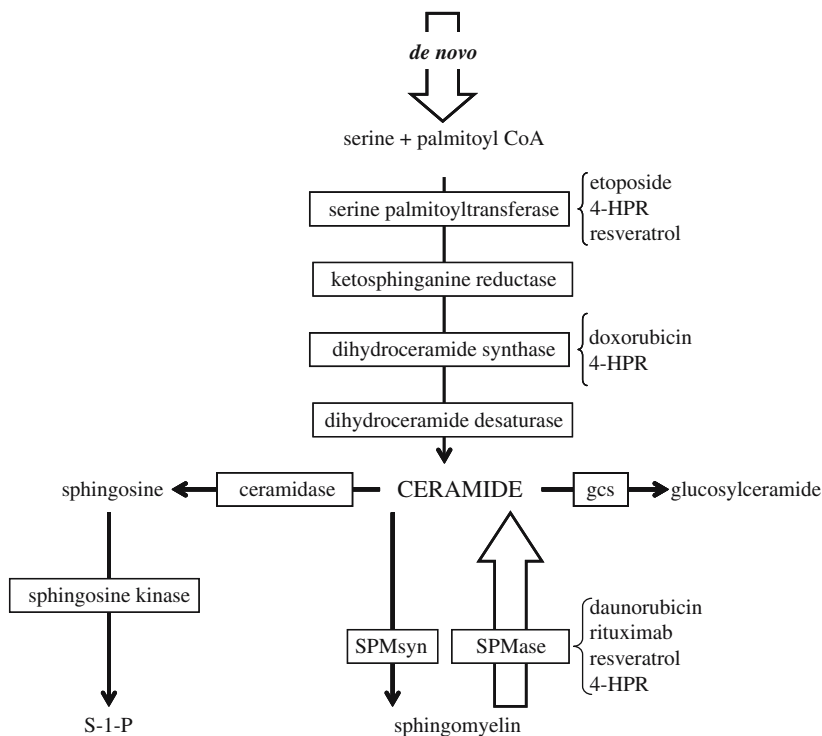


Fig. 1. Ceramide metabolism. Shown at top and bottom (bold arrows) are *de novo* and sphingomyelinase (SPMase) pathways of ceramide formation. The points of action of a few selected anticancer agents are shown in braces. All enzymes are shown in boxes. S-1-P, sphingosine-1-phosphate; GCS, glucosylceramide synthase.

responses, among them growth arrest, cell death pathways, chemotherapy responses, and cellular senescence. Cellular senescence, which is characterized by an irreversible growth phase, can be prompted by ceramide. Mouton and Venable (7) demonstrated, using human diploid fibroblasts, that ceramide induces biochemical changes in character with senescence when administered to low-passage cells. In particular, they showed that ceramide (short-chain C₆ analog) exposure induced expression of beta-galactosidase, a biochemical marker of senescence. Similarly, in young proliferating fibroblasts, beta-galactosidase can be induced by exposure to ceramide, tumor necrosis factor alpha (TNF α), or okadaic acid (8). Administration of exogenous ceramide to fibroblasts and endothelial cells provided data to suggest that differences in the regulation of apoptosis are associated with senescence in different histotypes (9). In a study by Obeid and colleagues (10), C₆-ceramide-induced G0/G1 arrest of the cell cycle in Wi38 human diploid fibroblasts was accompanied by inhibition of cyclin-dependent kinase 2 by way of activation of p21, a class of cyclin-dependent kinase inhibitory proteins. Using breast cancer as a model, Gewirtz and colleagues (11) studied the role of ceramide in blocking escape from radiation-induced cell death. MCF-7 cells exposed to fractionated radiation undergo a senescence-like growth arrest but then recover proliferative capacity. This recovery, it was shown, could be blocked, and cell killing by radiation could be potentiated by the addition of ceramide-generating vitamin D₃ analogs (11). In a recent study by Venable et al. (12), the authors showed that increases in senescence-associated ceramide appear to be associated with the action of neutral sphingomyelinase. Finally, in a very interesting study of age-related acceleration of apoptosis, Perez et al. (13) showed in a mouse model that elevated oocyte death of aged females is coincident with enhanced ceramide sensitivity.

3. CERAMIDE SIGNALING PATHWAYS

Excellent reviews are available on second-messenger signaling events associated with ceramide's action (14,15); therefore, we will briefly caption some of the paramount ceramide targets. Protein phosphatases 1 and 2A are activated by ceramide (16); these phosphatases regulate both growth and apoptosis. Downstream targets include Bad (17), Bax (18), Akt (19), and Bcl2 (20). Kinase suppressor of ras (KSR) represents another target of ceramide through the intermediary action of protein phosphatase 2A (21,22). Protein kinase C (PKC) translocation is also mediated by ceramide, and several members of the PKC family are affected, including PKC α , δ , ϵ , ξ , and β 2 (23–28).

4. CERAMIDE GENERATION BY CHEMOTHERAPEUTIC AGENTS

Many anticancer chemotherapeutics activate enzymes that catalyze ceramide production (reviewed in ref. 29). Ceramide can be produced *de novo* by steps that begin with the enzyme serine palmitoyltransferase and end with the enzyme dihydroceramide desaturase; alternatively, ceramide can be produced by hydrolysis of sphingomyelin (30–33), which cleaves the phosphocholine group from the ceramide backbone. As shown in Fig. 1, some drugs may have more than one effect on ceramide metabolism: 4-HPR (fenretinide) activates serine palmitoyltransferase, dihydroceramide synthase, and sphingomyelinase (34,35). Anthracyclines such as doxorubicin and daunorubicin can also stimulate both *de novo* ceramide production (36,37) and sphingomyelin hydrolysis (38), although these pathways may be cell-type specific. Resveratrol, a

hydroxylated stilbene found in skins of red grapes, stimulates serine palmitoyltransferase and neutral sphingomyelinase, promoting ceramide production and apoptosis in metastatic breast cancer cells (39), whereas etoposide, an epipodophyllotoxin derived from the May apple and used in the treatment of lymphoma, leukemia, and small cell lung carcinoma, promotes ceramide formation de novo during apoptosis (40). Laurent and colleagues (41) have linked the antiproliferative activity of rituximab, a chimeric human immunoglobulin G1 anti-CD20 monoclonal antibody used to treat B-cell lymphoma, to activation of acid sphingomyelinase. Findings that anticancer agents promoted formation of ceramide, a proapoptotic messenger (42–44), heralded a wave of enthusiasm and in-depth studies on the role of ceramide and glycolipid metabolism in cancer treatment (29,45–48).

5. DRUG RESISTANCE IN CANCER

Cancer cells develop multiple mechanisms to evade drug toxicity (2,49,50). The biology underlying drug resistance in cancer cells encompasses multiple factors including overexpression of P-glycoprotein (P-gp) and multidrug resistance-associated protein (2,51), changes in topoisomerase II (52), and modifications in glutathione *S*-transferase activity (53). Chemoresistance also may be related to the expression of important apoptosis-associated proteins such as the bcl-2 family of proteins (54) and the tumor suppressor protein p53 (55), synthesis of vaults (56), and overexpression of caveolae (57). Many recent studies (58–62) suggest that the dysfunctional metabolism of ceramide contributes to multidrug resistance. For example, the glycosylated form of ceramide (glucosylceramide) is elevated in multidrug-resistant cancer cells (58,59,63,64); this might be expected to dull ceramide's cytotoxic impact by sequestration of the apoptosis inducer (ceramide) as a glycolipid. In other work, Kok and colleagues (65) demonstrated altered glycolipid metabolism in multidrug-resistant ovarian carcinoma; Sonnino et al. (66) reported similar findings in 4-HPR-resistant ovarian cancer cells. The work of Okazaki et al. (67) revealed a strong connection between low ceramide levels and elevated levels of glucosylceramide synthase (GCS) and sphingomyelin synthase in doxorubicin-resistant leukemia cells. These reports of changes in the cerebroside metabolism of multidrug-resistant cells were followed by studies showing that GCS influences cellular responses to chemotherapy. Thus, GCS is a likely target for manipulating chemoresponse.

The remaining sections of this chapter focus on ceramide metabolism through GCS and its relationship to chemotherapy resistance, with particular emphasis on a new concept: the connection between natural product chemotherapy, ceramide metabolism, and expression of P-gp, product of the *MDR1* gene. One of the most consistent alterations in drug resistance is overexpression of P-gp (2). This membrane-resident protein, which has a mass of 170 kDa, functions as an energy-dependent pump that reduces the intracellular concentration of anticancer drugs, chiefly natural product agents including anthracyclines, *Vinca* alkaloids, and paclitaxel.

6. GCS AND CELLULAR RESPONSE TO CHEMOTHERAPY

Resistance of cancer cells to chemotherapy is associated not only with elevated levels of cerebroside and ganglioside (58,59,63–66) but also with enhanced expression of GCS (68,69). Interestingly, overexpression of GCS and *MDR1* coincides in multidrug-resistant breast cancer, leukemia, melanoma, colon cancer, and epidermoid carcinoma

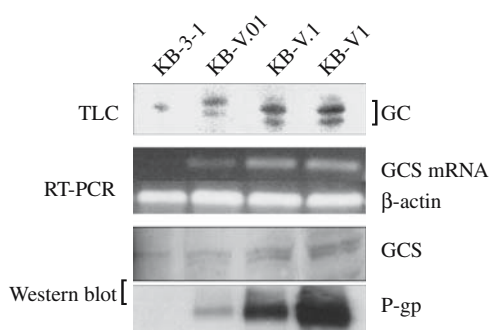


Fig. 2. Ceramide glycosylation and MDR1 expression increase in parallel under chemotherapy selection pressure. KB-3-1 and vinblastine-resistant sublines were analyzed for glucosylceramide (GC) content by thin-layer chromatography (TLC) and autoradiography, and GC synthase (GCS) and MDR1 expression by reverse transcriptase–polymerase chain reaction (RT–PCR) and Western blot. GC migrates as a doublet because of heterogeneity in chain length of the ceramide moiety. Reproduced with permission (69).

(head/neck) cells (69). This relationship was shown quite elegantly in the head/neck carcinoma cell line KB-3-1 and in vinblastine-resistant sublines KB-V.01, KB-V.1, and KB-V1, listed in order of increasing multidrug resistance (Fig. 2). As illustrated, glucosylceramide, GCS mRNA, GCS protein, and P-gp increased with increases in vinblastine selection pressure. This clearly shows that selection pressure for natural product drug resistance not only activates the *MDR1* gene (2,70,71) but also enhances ceramide metabolism through GCS. Kok et al. (72) have reported similar findings in HT29 colon cancer cells selected for colchicine resistance. In their study, increases in glucosylceramide coincided with upregulation of MRP1; however, GCS expression and activity were not upregulated in the colchicine-resistant model.

Altering GCS activity, either chemically or genetically, has been helpful in illustrating the impact of ceramide metabolism on cellular response to anticancer drugs. In breast cancer cells, for example, sensitivity to anthracyclines, *Vinca* alkaloids, and taxanes can be decreased or increased by manipulating GCS expression or activity (61,68,73). Transfection of drug-sensitive MCF-7 breast cancer cells with GCS cDNA using a Tet-on expression system (74) conferred resistance to doxorubicin (61). Resistance closely paralleled the level of expression of GCS, which could be manipulated by doxycycline addition (61). Enforced overexpression of GCS also potentiates resistance to TNF α (62), a tumor-killing cytokine that regulates apoptosis through ceramide generation from sphingomyelin (75). Conversely, GCS antisense transfection of multidrug-resistant MCF-7-AdrR breast cancer cells knocks down high GCS levels and greatly increases cell sensitivity to doxorubicin, vinblastine, and paclitaxel (68). Antisense GCS (asGCS) transfection also can decrease the growth rate of neuroepithelioma cells and reduce the tumorigenicity of melanoma cells (76,77); recently antisense oligodeoxyribonucleotides to GCS have been used to enhance doxorubicin sensitivity in multidrug-resistant breast and ovarian cancer cells (78).

These studies on the genetic manipulation of ceramide metabolism highlight the importance of GCS in regulation of cellular response to chemotherapy. This relationship has been reinforced by investigations that used chemical inhibitors of GCS, such as 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), which is a structural analog of the natural GCS substrate (79). PPMP and related agents enhance sensitivity

to ceramide-generating anticancer agents (doxorubicin, paclitaxel, and vincristine) in multidrug-resistant breast cancer, neuroblastoma, and leukemia cells (80–82). In neuroblastoma cells, *D,L-threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) as a single agent caused only a transient elevation in ceramide; however, when PDMP was combined with paclitaxel, ceramide levels remained high (81). In vincristine-resistant leukemia cells, the combination of *D,L-threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (PPPP) and vincristine raised ceramide levels above those observed with vincristine alone and enhanced apoptosis (82). Nicholson et al. (83) have demonstrated that PPMP and PPPP preferentially kill drug-resistant variants of KB-3-1 cells; this suggests that manipulation of glycolipid levels could be key in targeting drug resistance.

We have shown that the cytotoxicity of 4-HPR, a potent ceramide generator (34,35), can be enhanced in 10 different tumor cell lines by the addition of PPMP and other modulators of ceramide metabolism (84). Interestingly, a combination of three inhibitors of sphingolipid metabolism elevates ceramide levels and reverses resistance to radiation in squamous cell carcinoma (85), suggesting that the manipulation of ceramide metabolism may offer new opportunities for overcoming radioresistant cancer. Kolesnick and Fuks (86) review radiation- and ceramide-induced cell death.

Results with a few GCS inhibitors such as the imido sugar derivative *N*-nonyl-deoxygalactonojirimycin, used at millimolar concentrations (87), and a few cancer cell types such as GM95 mouse melanoma (88) do not support the targeting of ceramide metabolism to enhance radiotherapy and chemotherapy. However, most reports demonstrate that the sequestering of ceramide by GCS leads to chemotherapy resistance; conversely, inhibiting glycosylation of ceramide potentiates downstream cell death programs. The following section examines mechanisms by which ceramide might promote drug resistance.

7. INFLUENCE OF CERAMIDE ON MULTIDRUG RESISTANCE

The ability of chemotherapeutic agents to activate ceramide formation through *de novo* and sphingomyelinase pathways has been well documented, as has the ability of ceramide to promote apoptosis. However, the influence of ceramide on multidrug resistance is far from understood. Recent reports show that ceramide influences gene expression. For example, ceramide upregulates sphingomyelinase (89), p21 (90), and cyclooxygenase-2 expression (91) and downregulates glutathione *S*-transferase expression (92). Ceramide also enhances GCS expression in cultured cells (93,94), prompting Shayman and colleagues (93) to speculate that exogenous sphingomyelinase and other agents that produce large increases in ceramide also induce elevations in GCS, because of increased availability of the enzyme's lipoidal substrate. Because some anticancer agents are potent ceramide generators, we examined whether these drugs affected GCS expression.

Doxorubicin activates ceramide production in a number of systems (29). We have recently shown that treatment of breast cancer cells with either C_6 -ceramide or doxorubicin increases GCS expression (Liu et al., manuscript submitted for publication). This indicates that levels of GCS message can be enhanced by ceramide, the GCS substrate, and by chemotherapeutic agents that generate ceramide. We also evaluated breast cancer cells transiently transfected with GCS promoter pGCS-Luc1 (95). GCS

promoter activity increased approximately fourfold in cells exposed to C₆-ceramide and twofold in cells exposed to doxorubicin, but it remained unchanged in cells exposed to C₆-dihydroceramide, the saturated precursor of ceramide (96). These data suggest that ceramide's influence on the expression of GCS might drive cellular resistance to ceramide-generating anticancer agents.

8. GCS AND THE *MDR1* PHENOTYPE

Studies using asGCS and other GCS blockers indicate that ceramide glycosylation influences chemosensitivity by affecting both ceramide residence time and P-gp function (68,73,76,77,97,98). We reported that the use of asGCS to stably transfect multidrug-resistant MCF-7-AdrR cells, which are rich in GCS and P-gp, decreased GCS expression, enhanced chemotherapy uptake, and greatly diminished the expression of *MDR1*, based on mRNA and P-gp protein (98). These data suggest an association between GCS and glycolipids and *MDR1* expression. Ladisch et al. (77) found that asGCS transfection of melanoma cells reduced cellular glycolipids levels, GM3 ganglioside in particular, and inhibited melanoma formation in mice. In our model, asGCS transfection caused a fourfold reduction in ganglioside levels of MCF-7-AdrR cells (98). To determine whether the loss of *MDR1* was a clonal artifact, we treated MCF-7-AdrR cells for prolonged periods with either PPMP or tamoxifen, specific and nonspecific inhibitors of GCS, respectively (79,80,99): both agents produced striking decreases in *MDR1* expression (Fig. 3). With tamoxifen, the reduction was time-dependent; by 72 h, mRNA levels were minimal. The high levels of P-gp in MCF-7-AdrR cells are much greater than those that are clinically relevant (100). For this reason perhaps, and because of slow protein turnover, treatment of MCF-7-AdrR cells with PPMP did not significantly reduce P-gp levels. However, we showed a 50% reduction in P-gp levels of multidrug-resistant KB-V0.01 cells treated for 7 days with PPMP (unpublished data, Cabot laboratory).

Use of the PDMP to reduce glucosylceramide levels in human leukemia cells resulted in elevated ceramide levels and in increased rhodamine-123 uptake (97). Similarly, in doxorubicin-resistant ovarian cancer cells, PPMP markedly enhanced rhodamine-123 uptake and also inhibited *MDR1* expression at the mRNA level (101). Verapamil, an agent long known to enhance cell sensitivity to chemotherapy (102), decreased *MDR1* gene transcription in multidrug-resistant leukemia cells (103). We have reported that several of the classical P-gp blocking agents as well block glycolipid metabolism (80,99). In a study of vincristine-resistant epidermoid carcinoma cells, Zhang and

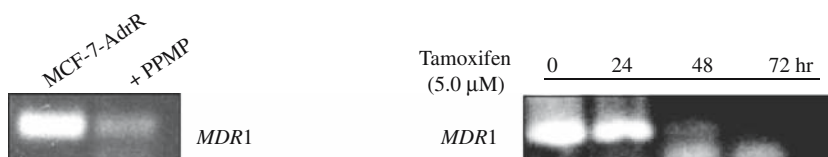


Fig. 3. Influence of glucosylceramide synthase (GCS) blocking agents, 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) and tamoxifen, on *MDR1* expression in multidrug-resistant human breast cancer cells. Cultured MCF-7-AdrR cells were exposed to either *D-threo* PPMP (5.0 μM) for 48 h or tamoxifen (5.0 μM) for times shown. *MDR1* mRNA was evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR).

coworkers (104) found that expression of GCS was associated with multidrug resistance and that inhibition of GCS with PPMP and verapamil reduced *MDR1* gene expression. Therefore, whether by antisense downregulation or chemical inhibition, these works show that targeting GCS influences *MDR1* expression and draw a curious parallel between drug-resistance modifiers, glycosphingolipids, and the multidrug-resistant phenotype.

9. CONCLUSIONS

Although numerous studies have demonstrated that GCS influences cellular response to anticancer agents, the mechanisms remain uncertain. Most studies on ceramide, chemotherapy, and drug resistance emphasize circumvention of apoptosis by GCS-mediated sequestering of ceramide (Fig. 4, top). Chemotherapy enhances ceramide formation; increased levels of ceramide lead to apoptosis and/or enhanced expression of GCS. GCS is a scavenger of ceramide; high levels of GCS therefore can counteract apoptosis by sequestration of ceramide in the form of glucosylceramide. Chemoresistance can be circumvented in some systems by inclusion of GCS blocking agents.

GCS appears to have dual roles in multidrug resistance. It dulls the cytotoxic response to ceramide-generating chemotherapeutics, and it may also enhance expression of the *MDR1* phenotype. As shown in Fig. 4, we propose that GCS upregulates *MDR1* through either cerebroside-driven (glucosylceramide) or ganglioside-driven pathways; upregulation of *MDR1* then leads to increased production of P-gp. Overall, the data suggest that the acquisition of multidrug resistance is associated, in part, with changes in cellular glycolipids. A functional role for gangliosides in multidrug resistance has recently been noted (97), and other works have proposed that drug sensitivity and metastatic potential are linked to ganglioside metabolism (83–109). We already know that chemotherapeutic agents can directly activate the *MDR1* gene (70,71); Fig. 4 introduces another pathway that emphasizes GCS as a potential upstream target for downstream drug resistance.

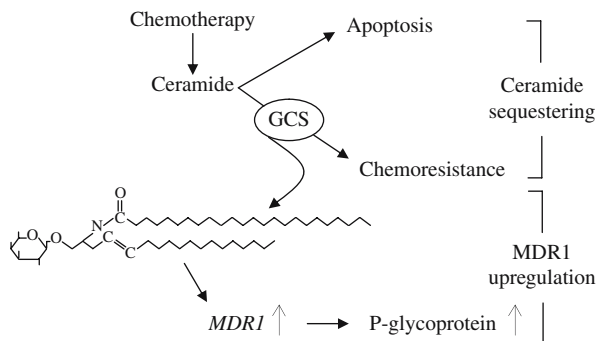


Fig. 4. Suggested mechanisms for the impact of glucosylceramide synthase (GCS) on cell response to chemotherapy. Chemotherapy enhances formation of ceramide (Fig. 1), which signals events leading to apoptosis and/or upregulates expression of “its enzyme,” GCS. The latter step can produce chemoresistance through ceramide sequestering in the form of glucosylceramide (molecular structure shown). Glucosylceramide and/or other cerebroside and gangliosides upregulate *MDR1* expression leading to production of P-glycoprotein.

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REFERENCES

1. Morton DL, Essner R, Kirkwood JM, Wollman RC. Malignant melanoma. In: Kufe DW, Pollock RE, Weichselbaum RR, Bast RC, Gansler TS, Holland JF, Frei E, Eds. *Cancer Medicine*. Sixth edition. BC Decker, Ontario, Canada, 2003:1973–1995.
2. Gottesman MM. Mechanisms of cancer drug resistance. *Annu Rev Med* 2002;53:615–627.
3. Sikic BI. Modulation of multidrug resistance: a paradigm for translational clinical research. *Oncology (Williston Park)* 1999;13:183–187.
4. Kourousis C, Kakolyris S, Androulakis N, Heras P, Vlachonicolis J, Vamvakas L, Vlata M, Hatzidaki D, Samonis G, Georgoulis V. Salvage chemotherapy with paclitaxel, vinorelbine, and cisplatin (PVC) in anthracycline-resistant advanced breast cancer. *Am J Clin Oncol* 1998;21:226–232.
5. Serrone L, Hersey P. The chemoresistance of human malignant melanoma: an update. *Melanoma Res* 1999;9:51–58.
6. Mirski SE, Cole SP. Multidrug resistance in small cell lung cancer. In: Bernal SD, Ed. *Drug Resistance in Oncology*. Marcel Dekker, New York, 1997:27–77.
7. Mouton RE, Venable ME. Ceramide induces expression of the senescence histochemical marker, beta-galactosidase, in human fibroblasts. *Mech Ageing Dev* 2000;113:169–181.
8. DeJesus V, Rios I, Davis C, Chen Y, Calhoun D, Zakeri Z, Hubbard K. Induction of apoptosis in human replicative senescent fibroblasts. *Exp Cell Res* 2002;274:92–99.
9. Hampel B, Malisan F, Niederegger H, Testi R, Jansen-Durr P. Differential regulation of apoptotic cell death in senescent human cells. *Exp Gerontol* 2004;39:1713–1721.
10. Lee JY, Bielawska AE, Obeid LM. Regulation of cyclin-dependent kinase 2 activity by ceramide. *Exp Cell Res* 2000;261:303–311.
11. DeMasters GA, Gupta MS, Jones KR, Cabot M, Wang H, Gennings C, Park M, Bratland A, Ree AH, Gewirtz DA. Potentiation of cell killing by fractionated radiation and suppression of proliferative recovery in MCF-7 breast tumor cells by the Vitamin D3 analog EB 1089. *J Steroid Biochem Mol Biol* 2004;92:365–374.
12. Venable ME, Webb-Froehlich LM, Sloan EF, Thomley JE. Shift in sphingolipid metabolism leads to an accumulation of ceramide in senescence. *Mech Ageing Dev* 2006;127:473–480.
13. Perez GI, Jurisicova A, Matikainen T, Moriyama T, Kim MR, Takai Y, Pru JK, Kolesnick RN, Tilly JL. A central role for ceramide in the age-related acceleration of apoptosis in the female germline. *FASEB J* 2005;19:860–862.
14. Ruvolo PP. Ceramide regulates cellular homeostasis via diverse stress signaling pathways. *Leukemia* 2001;15:1153–1160.
15. Ruolo PP. Intracellular signal transduction pathways activated by ceramide and its metabolites. *Pharmacol Res* 2003;47:383–392.
16. Chalfant CE, Kishikawa K, Mumby MC, Kamibayashi C, Bielawska A, Hannun YA. Long chain ceramides activate protein phosphatase-1 and protein phosphatase-2A. Activation is stereospecific and regulated by phosphatidic acid. *J Biol Chem* 1999;274:20313–20317.
17. Chiang CS, Kanies C, Kim KW, Fang WB, Parkhurst C, Xie M, Henry T, Yang E. Protein phosphatase 2A dephosphorylation of phosphoserine 112 plays the gatekeeper role for BAD-mediated apoptosis. *Mol Cell Biol* 2003;23:6350–6362.

18. Xin M, Deng X. Protein phosphatase 2A enhances Bax's proapoptotic function through dephosphorylation. *J Biol Chem* 2006;281:18859–18867.
19. Yellaturu CR, Bhanoori M, Neeli I, Rao GN. N-Ethylmaleimide inhibits platelet-derived growth factor BB-stimulated Akt phosphorylation via activation of protein phosphatase 2A. *J Biol Chem* 2002;277:40148–40155.
20. Ruvolo PP, Deng X, Ito T, Carr BK, May WS. Ceramide induces Bcl2 dephosphorylation via a mechanism involving mitochondrial PP2A. *J Biol Chem* 1999;274:20296–20300.
21. Zhang Y, Yao B, Delikat S, Bayoumy S, Lin XH, Basu S, McGinley M, Chan-Hui PY, Lichenstein H, Kolesnick R. Kinase suppressor of Ras is ceramide-activated protein kinase. *Cell* 1997;89:63–72.
22. Yao B, Zhang Y, Delikat S, Mathias S, Basu S, Kolesnick R. Phosphorylation of Raf by ceramide-activated protein kinase. *Nature* 1995;378:307–310.
23. Kajimoto T, Shirai Y, Sakai N, Yamamoto T, Matsuzaki H, Kikkawa U, Saito N. Ceramide-induced apoptosis by translocation, phosphorylation, and activation of protein kinase Cdelta in the Golgi complex. *J Biol Chem* 2004;279:12668–12676.
24. Aschrafi A, Franzen R, Shabahang S, Fabbro D, Pfeilschifter J, Huwiler A. Ceramide induces translocation of protein kinase C-alpha to the Golgi compartment of human embryonic kidney cells by interacting with the C2 domain. *Biochim Biophys Acta* 2003;1634:30–39.
25. Kashiwagi K, Shirai Y, Kuriyama M, Sakai N, Saito N. Importance of C1B domain for lipid messenger-induced targeting of protein kinase C. *J Biol Chem* 2002;277:18037–18045.
26. Becker KP, Kitatani K, Idkowiak-Baldys J, Bielawski J, Hannun YA. Selective inhibition of juxtacellular translocation of protein kinase C beta11 by a negative feedback mechanism involving ceramide formed from the salvage pathway. *J Biol Chem* 2005;280:2606–2612.
27. Becker KP, Hannun YA. cPKC-dependent sequestration of membrane-recycling components in a subset of recycling endosomes. *J Biol Chem* 2003;278:52747–52754.
28. Wang G, Silva J, Krishnamurthy K, Tran E, Condie BG, Bieberich E. Direct binding to ceramide activates protein kinase Czeta before the formation of a pro-apoptotic complex with PAR-4 in differentiating stem cells. *J Biol Chem* 2005;280:26415–26424.
29. Senchenkov A, Litvak DA, Cabot MC. Targeting ceramide metabolism—a strategy for overcoming drug resistance. *J Natl Cancer Inst* 2001;93:347–357.
30. Spiegel S, Foster D, Kolesnick R. Signal transduction through lipid second messengers. *Curr Opin Cell Biol* 1996;8:159–167.
31. Merrill AH Jr, Schmelz EM, Dillehay DL, Spiegel S, Shayman JA, Schroeder JJ, Riley RT, Voss KA, Wang E. Sphingolipids—the enigmatic lipid class: biochemistry, physiology, and pathophysiology. *Toxicol Appl Pharmacol* 1997;142:208–225.
32. Mathias S, Pena LA, Kolesnick RN. Signal transduction of stress via ceramide. *Biochem J* 1998;335:465–480.
33. Hannun YA, Luberto C, Argraves KM. Enzymes of sphingolipid metabolism: from modular to integrative signaling. *Biochemistry* 2001;40:4893–4903.
34. Wang H, Maurer BJ, Reynolds CP, Cabot MC. N-(4-hydroxyphenyl)retinamide elevates ceramide in neuroblastoma cell lines by coordinate activation of serine palmitoyltransferase and ceramide synthase. *Cancer Res* 2001;61:5102–5105.
35. Lovat PE, Di Sano F, Corazzari M, Fazi B, Donnorso RP, Pearson AD, Hall AG, Redfern CP, Piacentini M. Gangliosides link the acidic sphingomyelinase-mediated induction of ceramide to 12-lipoxygenase-dependent apoptosis of neuroblastoma in response to fenretinide. *J Natl Cancer Inst* 2004;96:1288–1299.
36. Bose R, Verheij M, Haimovitz-Friedman A, Scotto K, Fuks Z, Kolesnick R. Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals. *Cell* 1995;82:405–414.
37. Lucci A, Han TY, Liu YY, Giuliano AE, Cabot MC. Modification of ceramide metabolism increases cancer cell sensitivity to cytotoxics. *Int J Oncol* 1999;15:541–546.
38. Jaffrezou JP, Levade T, Bettaieb A, Andrieu N, Bezombes C, Maestre N, Vermeersch S, Rousse A, Laurent G. Daunorubicin-induced apoptosis: triggering of ceramide generation through sphingomyelin hydrolysis. *EMBO J* 1996;15:2417–2424.
39. Scarlatti F, Sala G, Somenzi G, Signorelli P, Sacchi N, Ghidoni R. Resveratrol induces growth inhibition and apoptosis in metastatic breast cancer cells via de novo ceramide signaling. *FASEB J* 2003;17:2339–2341.

40. Perry DK, Carton J, Shah AK, Meredith F, Uhlinger DJ, Hannun YA. Serine palmitoyltransferase regulates de novo ceramide generation during etoposide-induced apoptosis. *J Biol Chem* 2000;275:9078–9084.
41. Bezombes C, Grazide S, Garret C, Fabre C, Quillet-Mary A, Muller S, Jaffrezou JP, Laurent G. Rituximab antiproliferative effect in B-lymphoma cells is associated with acid-sphingomyelinase activation in raft microdomains. *Blood* 2004;104:1166–1173.
42. Hannun YA, Obeid LM. Mechanisms of ceramide-mediated apoptosis. *Adv Exp Med Biol* 1997;407:145–149.
43. Jaffrezou JP, Laurent G, Levade T. Ceramide in regulation of apoptosis. Implication in multitoxicant resistance. *Subcell Biochem* 2002;36:269–284.
44. Ruvolo PP. Intracellular signal transduction pathways activated by ceramide and its metabolites. *Pharmacol Res* 2003;47:383–392.
45. Reynolds CP, Maurer BJ, Kolesnick RN. Ceramide synthesis and metabolism as a target for cancer therapy. *Cancer Lett* 2004;206:169–180.
46. Radin NS. Killing tumours by ceramide-induced apoptosis: a critique of available drugs. *Biochem J* 2003;371:243–256.
47. Radin NS. Poly-drug cancer therapy based on ceramide. *Eksp Onkol* 2004;26:3–10.
48. Kok JW, Sietsma H. Sphingolipid metabolism enzymes as targets for anticancer therapy. *Curr Drug Targets* 2004;5:375–382.
49. Bernal SD, Ed. *Drug Resistance in Oncology*. Marcel Dekker, New York, 1997.
50. Kellen JA, Ed. *Alternative mechanisms of multidrug resistance in cancer*. Birkhäuser, Boston, MA, 1995.
51. Krishnamachary N, Center MS. The MRP gene associated with a non-P-glycoprotein multidrug resistance encodes a 190-kDa membrane bound glycoprotein. *Cancer Res* 1993;53:3658–3661.
52. Deffie AM, Batra JK, Goldenberg GJ. Direct correlation between DNA topoisomerase II activity and cytotoxicity in adriamycin-sensitive and -resistant P388 leukemia cell lines. *Cancer Res* 1989;49:58–62.
53. Morrow CS, Cowan KH. Glutathione S-transferases and drug resistance. *Cancer Cells* 1990;2:15–22.
54. Reed JC. Regulation of apoptosis by bcl-2 family proteins and its role in cancer and chemoresistance. *Curr Opin Oncol* 1995;7:541–546.
55. Mueller H, Eppenberger U. The dual role of mutant p53 protein in chemosensitivity of human cancers. *Anticancer Res* 1996;16:3845–3848.
56. Kickhoefer VA, Rajavel KS, Scheffer GL, Dalton WS, Scheper RJ, Rome LH. Vaults are up-regulated in multidrug-resistant cancer cell lines. *J Biol Chem* 1998;273:8971–8974.
57. Lavie Y, Fiucci G, Liscovitch M. Up-regulation of caveolae and caveolar constituents in multidrug-resistant cancer cells. *J Biol Chem* 1998;273:32380–32383.
58. Lavie Y, Cao H, Bursten SL, Giuliano AE, Cabot MC. Accumulation of glucosylceramides in multidrug-resistant cancer cells. *J Biol Chem* 1996;271:19530–19536.
59. Lucci A, Cho WI, Han TY, Giuliano AE, Morton DL, Cabot MC. Glucosylceramide: a marker for multiple-drug resistant cancers. *Anticancer Res* 1998;18:475–480.
60. Cai Z, Bettaieb A, Mahdani NE, Legres LG, Stancou R, Masliah J, Chouaib S. Alteration of the sphingomyelin/ceramide pathway is associated with resistance of human breast carcinoma MCF7 cells to tumor necrosis factor-alpha-mediated cytotoxicity. *J Biol Chem* 1997;272:6918–6926.
61. Liu YY, Han TY, Giuliano AE, Cabot MC. Expression of glucosylceramide synthase, converting ceramide to glucosylceramide, confers adriamycin resistance in human breast cancer cells. *J Biol Chem* 1999;274:1140–1146.
62. Liu YY, Han TY, Giuliano AE, Ichikawa S, Hirabayashi Y, Cabot MC. Glycosylation of ceramide potentiates cellular resistance to tumor necrosis factor-alpha-induced apoptosis. *Exp Cell Res* 1999;252:464–470.
63. Sietsma H, Veldman RJ, Kok JW. The involvement of sphingolipids in multidrug resistance. *J Membr Biol* 2001;181:153–162.
64. Morjani H, Aouali N, Belhoussine R, Veldman RJ, Levade T, Manfait M. Elevation of glucosylceramide in multidrug-resistant cancer cells and accumulation in cytoplasmic droplets. *Int J Cancer* 2001;94:157–165.
65. Veldman RJ, Klappe K, Hinrichs J, Hummel I, van der Schaaf G, Sietsma H, Kok JW. Altered sphingolipid metabolism in multidrug-resistant ovarian cancer cells is due to uncoupling of glycolipid biosynthesis in the Golgi apparatus. *FASEB J* 2002;16:1111–1113.

66. Prinetti A, Basso L, Appierto V, Villani MG, Valsecchi M, Loberto N, Prioni S, Chigorno V, Cavadini E, Formelli F, Sonnino S. Altered sphingolipid metabolism in N-(4-hydroxyphenyl)-retinamide-resistant A2780 human ovarian carcinoma cells. *J Biol Chem* 2003;278:5574–5583.
67. Itoh M, Kitano T, Watanabe M, Kondo T, Yabu T, Taguchi Y, Iwai K, Tashima M, Uchiyama T, Okazaki T. Possible role of ceramide as an indicator of chemoresistance: decrease of the ceramide content via activation of glucosylceramide synthase and sphingomyelin synthase in chemoresistant leukemia. *Clin Cancer Res* 2003;9:415–423.
68. Liu YY, Han TY, Giuliano AE, Cabot MC. Ceramide glycosylation potentiates cellular multidrug resistance. *FASEB J* 2001;15:719–730.
69. Gouaze V, Yu JY, Bleicher RJ, Han TY, Liu YY, Wang H, Gottesman MM, Bitterman A, Giuliano AE, Cabot MC. Overexpression of glucosylceramide synthase and P-glycoprotein in cancer cells selected for resistance to natural product chemotherapy. *Mol Cancer Ther* 2004;3:633–639.
70. Kohno K, Sato S, Takano H, Matsuo K, Kuwano M. The direct activation of human multidrug resistance gene (MDR1) by anticancer agents. *Biochem Biophys Res Commun* 1989;165:1415–1421.
71. Chaudhary PM, Roninson IB. Induction of multidrug resistance in human cells by transient exposure to different chemotherapeutic drugs. *J Natl Cancer Inst* 1993;85:632–639.
72. Klappe K, Hinrichs JW, Kroesen BJ, Sietsma H, Kok JW. MRP1 and glucosylceramide are coordinately over expressed and enriched in rafts during multidrug resistance acquisition in colon cancer cells. *Int J Cancer* 2004;110:511–522.
73. Liu YY, Han TY, Giuliano AE, Hansen N, Cabot MC. Uncoupling ceramide glycosylation by transfection of glucosylceramide synthase antisense reverses adriamycin resistance. *J Biol Chem* 2000;275:7138–7143.
74. Liu YY, Cabot MC. Development of a mammalian Tet-on expression cell line: glucosylceramide synthase regulates TNF-alpha-induced apoptosis. *Methods Mol Biol* 2004;249:177–192.
75. Pena LA, Fuks Z, Kolesnick R. Stress-induced apoptosis and the sphingomyelin pathway. *Biochem Pharmacol* 1997;53:615–621.
76. Di Sano F, Di Bartolomeo S, Fazi B, Fiorentini C, Matarrese P, Spinedi A, Piacentini M. Antisense to glucosylceramide synthase in human neuroepithelioma affects cell growth but not apoptosis. *Cell Death Differ* 2002;9:693–695.
77. Deng W, Li R, Guerrero M, Liu Y, Ladisch S. Transfection of glucosylceramide synthase antisense inhibits mouse melanoma formation. *Glycobiology* 2002;12:145–152.
78. Liu YY, Han TY, Yu JY, Bitterman A, Le A, Giuliano AE, Cabot MC. Oligonucleotides blocking glucosylceramide synthase expression selectively reverse drug resistance in cancer cells. *J Lipid Res* 2004;45:933–940.
79. Abe A, Inokuchi J, Jimbo M, Shimeno H, Nagamatsu A, Shayman JA, Shukla GS, Radin NS. Improved inhibitors of glucosylceramide synthase. *J Biochem (Tokyo)* 1992;111:191–196.
80. Lavie Y, Cao H, Volner A, Lucci A, Han TY, Geffen V, Giuliano AE, Cabot MC. Agents that reverse multidrug resistance, tamoxifen, verapamil, and cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells. *J Biol Chem* 1997;272:1682–1687.
81. Sietsma H, Veldman RJ, Kolk D, Ausema B, Nijhof W, Kamps W, Vellenga E, Kok JW. 1-phenyl-2-decanoylamino-3-morpholino-1-propanol chemosensitizes neuroblastoma cells for taxol and vincristine. *Clin Cancer Res* 2000;6:942–948.
82. Olshefski RS, Ladisch S. Glucosylceramide synthase inhibition enhances vincristine-induced cytotoxicity. *Int J Cancer* 2001;93:131–138.
83. Nicholson KM, Quinn DM, Kellett GL, Warr JR. Preferential killing of multidrug-resistant KB cells by inhibitors of glucosylceramide synthase. *Br J Cancer* 1999;81:423–430.
84. Maurer BJ, Melton L, Billups C, Cabot MC, Reynolds CP. Synergistic cytotoxicity in solid tumor cell lines between N-(4-hydroxyphenyl)retinamide and modulators of ceramide metabolism. *J Natl Cancer Inst* 2000;92:1897–1909.
85. Alphonse G, Bionda C, Aloy MT, Ardail D, Rousson R, Rodriguez-Lafrasse C. Overcoming resistance to gamma-rays in squamous carcinoma cells by poly-drug elevation of ceramide levels. *Oncogene* 2004;23:2703–2715.
86. Kolesnick R, Fuks Z. Radiation and ceramide-induced apoptosis. *Oncogene* 2003;22:5897–5906.
87. Norris-Cervetto E, Callaghan R, Platt FM, Dwek RA, Butters TD. Inhibition of glucosylceramide synthase does not reverse drug resistance in cancer cells. *J Biol Chem* 2004;279:40412–40418.

88. Veldman RJ, Mita A, Cuvillier O, Garcia V, Klappe K, Medin JA, Campbell JD, Carpentier S, Kok JW, Levade T. The absence of functional glucosylceramide synthase does not sensitize melanoma cells for anticancer drugs. *FASEB J* 2003;17:1144–1146.
89. Murate T, Suzuki M, Hattori M, Takagi A, Kojima T, Tanizawa T, Asano H, Hotta T, Saito H, Yoshida S, Tamiya-Koizumi K. Up-regulation of acid sphingomyelinase during retinoic acid-induced myeloid differentiation of NB4, a human acute promyelocytic leukemia cell line. *J Biol Chem* 2002;277:9936–9943.
90. Kim WH, Kang KH, Kim MY, Choi KH. Induction of p53-independent p21 during ceramide-induced G1 arrest in human hepatocarcinoma cells. *Biochem Cell Biol* 2000;78(2):127–135.
91. Chen CC, Sun YT, Chen JJ, Chang YJ. Tumor necrosis factor-alpha-induced cyclooxygenase-2 expression via sequential activation of ceramide-dependent mitogen-activated protein kinases, and IkkappaB kinase 1/2 in human alveolar epithelial cells. *Mol Pharmacol* 2001;59:493–500.
92. Park IN, Cho IJ, Kim SG. Ceramide negatively regulates glutathione S-transferase gene transactivation via repression of hepatic nuclear factor-1 that is degraded by the ubiquitin proteasome system. *Mol Pharmacol* 2004;65:1475–1484.
93. Abe A, Radin NS, Shayman JA. Induction of glucosylceramide synthase by synthase inhibitors and ceramide. *Biochim Biophys Acta* 1996;1299:333–341.
94. Komori H, Ichikawa S, Hirabayashi Y, Ito M. Regulation of UDP-glucose:ceramide glucosyltransferase-1 by ceramide. *FEBS Lett* 2000;475:247–250.
95. Ichikawa S, Ozawa K, Hirabayashi Y. Molecular cloning and characterization of the mouse ceramide glucosyltransferase gene. *Biochem Biophys Res Commun* 1998;253:707–711.
96. Liu YY, Yu JY, Gouaze V, Hansen N, Giuliano AE, Hirabayashi Y, Cabot MC. Doxorubicin drives drug resistance in breast cancer cells through ceramide-upregulated gene expression. *Proc Am Assoc Cancer Res* 2003;44:847 [Abst #3703].
97. Plo I, Lehne G, Beckstrom KJ, Maestre N, Bettaieb A, Laurent G, Lautier D. Influence of ceramide metabolism on P-glycoprotein function in immature acute myeloid leukemia KG1a cells. *Mol Pharmacol* 2002;62:304–312.
98. Gouaze V, Liu Y-Y, Yu JY, Prickett CS, Giuliano AE, Cabot MC. Blockers of glycolipid metabolism diminish expression of the multidrug resistance gene (MDR1) and enhance chemotherapy sensitivity (abstract). *FASEB J* 2004;18:C51.
99. Cabot MC, Giuliano AE, Volner A, Han TY. Tamoxifen retards glycosphingolipid metabolism in human cancer cells. *FEBS Lett* 1996;394:129–131.
100. Mechetner E, Kyshtoobayeva A, Zonis S, Kim H, Stroup R, Garcia R, Parker RJ, Fruehauf JP. Levels of multidrug resistance (MDR1) P-glycoprotein expression by human breast cancer correlate with *in vitro* resistance to taxol and doxorubicin. *Clin Cancer Res* 1998;4:389–398.
101. Yuan Y, Wang L, Zhang J. [Modulation of multidrug resistance by 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol in SKOV3-adriamycin-resistant cell line]. *Zhonghua Fu Chan Ke Za Zhi* 2001;36:493–495 [Chinese].
102. Cornwell MM, Pastan I, Gottesman MM. Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. *J Biol Chem* 1987;262:2166–2170.
103. Muller C, Goubin F, Ferrandis E, Cornil-Scharwitz I, Bailly JD, Bordier C, Benard J, Sikic BI, Laurent G. Evidence for transcriptional control of human *mdr1* gene expression by verapamil in multidrug-resistant leukemic cells. *Mol Pharmacol* 1995;47:51–56.
104. Yang Q, Zhang J, Wang SM, Zhang JR. [Expression of glucosylceramide synthase mRNA in vincristine-resistant KBV200 cell line in association with multidrug resistance]. *Di Yi Jun Yi Da Xue Xue Bao* 2004;24:779–781 [Chinese].
105. Nohara K, Wang F, Spiegel S. Glycosphingolipid composition of MDA-MB-231 and MCF-7 human breast cancer cell lines. *Breast Cancer Res Treat* 1998;48:149–157.
106. Kato T, Wang Y, Yamaguchi K, Milner CM, Shineha R, Satomi S, Miyagi T. Overexpression of lysosomal-type sialidase leads to suppression of metastasis associated with reversion of malignant phenotype in murine B16 melanoma cells. *Int J Cancer* 2001;92:797–804.
107. Fukumoto H, Nishio K, Ohta S, Hanai N, Fukuoka K, Ohe Y, Sugihara K, Kodama T, Saijo N. Effect of a chimeric anti-ganglioside GM2 antibody on ganglioside GM2-expressing human solid tumors *in vivo*. *Int J Cancer* 1999;82:759–764.

108. Mazzoni A, Trave F, Fabbri M, Leto G, Ghidoni R. Membrane gangliosides and immuno-mediated cytolysis in drug sensitive and treatment-induced multidrug resistant human ovarian cancer cells. *Anticancer Res* 1991;11:2181–2185.
109. Fukumoto H, Nishio K, Ohta S, Hanai N, Saijo N. Reversal of adriamycin resistance with chimeric anti-ganglioside GM2 antibody. *Int J Cancer* 1996;67:676–680.

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Chemo- and Radiosensitization Through Inhibition of PI3K/Akt Signaling

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SUMMARY

Tumorigenesis and tumor progression are the consequences of disturbed balance between cell proliferation, differentiation, and programmed cell death. Additional processes including loss of cellular polarity, increased motility, and invasiveness lead to metastases resulting in spread of the tumor beyond the original site and account for the majority of morbidity and mortality from cancer. The phosphatidylinositol 3-kinase (PI3K) pathway, composed of multiple intracellular signaling proteins, controls various pivotal cellular functions related to cancer biology including cell proliferation, cell survival, migration, and vascularization. Major components of this pathway, including PI3K isoforms, phosphatase and *tensin* homolog (PTEN), phosphoinositide-dependent kinase 1 (PDK1), Akt, and mTOR, are frequently dysregulated by mutation, amplification, or rearrangement in multiple cancer lineages. These genetic aberrations render cancer cells sensitive to the inhibition of PI3K pathway at various levels. Targeting the PI3K/Akt pathway demonstrates marked efficacy in preclinical models. The PI3K signaling pathway has emerged as a high-quality therapeutic target in cancer treatment.

Key Words: Phosphatidylinositol 3-kinase; Akt; signal transduction; molecular-targeted therapy; chemosensitization; radiosensitization.

1. INTRODUCTION

Phosphatidylinositol 3-kinases (PI3Ks) are a group of lipid and protein kinases, catalyzing phosphorylation of membrane phosphatidylinositols (PtdIns) at the D3 position of the inositol ring. The resulting products of PI3K, D3-phosphoinositols

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PtdIns(3,4)P₂, PtdIns(3,5)P₂, and PtdIns(3,4,5)P₃ play a critical role in the regulation of cellular signaling networks (1). There are multiple PI3K isoforms present in the mammalian genome. On the basis of their *in vitro* substrate selectivity, these isoforms can be divided into four classes (2,3). Class I PI3Ks efficiently phosphorylate PtdIns(4)P and PtdIns(4,5)P₂ *in vitro*; however, the latter may present as a preferable substrate *in vivo*. Class I PI3Ks are heterodimers composed of a p110 kDa catalytic subunit and an adaptor/regulatory subunit. The Class I PI3Ks are further categorized into two subclasses, classes IA and IB, based on structural and functional differences. Class IA consists of three p110 catalytic isoforms, p110 α , β , and δ , constitutively associated with a regulatory subunit, which can be p85 α , β , p55 α , p50 α , or p55 γ . The regulatory subunits of Class IA contain two Src-homology 2 (SH2) domains and one SH3 domain. The SH2 domains bind to specific phosphorylated tyrosine residues in receptor proteins and in other signaling molecules. SH3 domains mediate protein–protein interaction through proline-rich sequences. Class IA PI3K isoforms transduce signals from tyrosine kinase-coupled receptors, including those with intrinsic tyrosine kinase activities. Class IB PI3K, of which the sole member is p110 γ , associates with a p101 regulatory subunit. p110 γ can be activated *in vitro* by the $\beta\gamma$ subunits of heterotrimeric G proteins. Therefore, Class IB PI3K primarily transduces signals from G-protein-coupled receptors, although these receptors can also activate Class IA PI3K through recruitment and activation of intracellular tyrosine kinases, particularly Src. Class IB PI3K also transduces signals of Rac, Rho, and cdc42 small GTPases.

Class II PI3Ks exclusively phosphorylate PtdIns and PtdIns(4)P *in vitro*. Class II isoforms, PI3K-C2 α , β , and γ , are larger molecules (~200 kDa) whose defining feature consists of a C-terminus C2 domain with no adaptor/regulatory subunits. The C2 domain binds to phospholipids *in vitro* in a Ca²⁺-independent manner. Class II PI3Ks are predominantly associated with the cellular membrane fraction and can be activated by tyrosine kinase-coupled receptors, such as insulin receptor (INR), epithelial growth factor receptor (EGFR), or platelet-derived growth factor receptor (PDGFR). However, the activation mechanisms, the *in vivo* substrates, and the cellular functions of Class II PI3Ks remain to be fully elucidated.

Class III PI3K, a constitutively active enzyme that phosphorylates only PtdIns as a substrate *in vitro*, is likely responsible for the generation of the majority of PtdIns(3)P in resting cells. A single Class III PI3K catalytic subunit has been identified in all eukaryotic species, which is a homolog of Vps34P in yeast and of Age 1 in *Caenorhabditis elegans*. Class III PI3K regulates the trafficking of proteins through lysosomes or vacuoles. Class III PI3K has recently been implicated in the regulation of autophagy, an evolutionarily conserved process whereby cells utilize intracellular energy sources to survive during environmental stress. Prolonged autophagy, however, results in programmed cell death. In contrast to apoptosis where a role in chemotherapy and radiation therapy-induced cell death is well documented, the role of autophagy in therapy-induced death is under investigation. Our recent data indicate that both entry into and survival during autophagy are highly regulated processes with multiple mediators in addition to cellular responses to stress.

Class IV PI3Ks are composed of lipid and protein kinases whose catalytic domains closely resemble those of other PI3Ks. Among these enzymes are PI4K, phosphoinositide phosphate (PIP) kinase, DNA-dependent protein kinase (DNA-PK), ataxia teleangiectasia mutated (ATM), ataxia teleangiectasia related (ATR), and mTOR. Class

IV PI3Ks play an important role in the regulation of the phosphoinositide cycle, DNA repair, and protein synthesis. Furthermore, in contrast to Classes I–III PI3Ks, which are primarily lipid kinases that can phosphorylate proteins in close physical complexes, several members of the Class IV PI3Ks such as DNA-PK, ATM, ATR, and mTOR are exclusively protein kinases.

2. PI3K/AKT SIGNALING IN CELL BIOLOGY

PI3K signaling mechanisms and their functional processes have been most extensively studied for Class IA PI3Ks. Under resting conditions, p85 regulatory subunits bind and inhibit p110 catalytic activity while stabilizing p110. This creates a pool of p110 poised to be activated by removal of the inhibitory constraints of p85. When a GFR is triggered by its ligand, such as insulin, EGF, or PDGF, the receptor kinase is activated, causing a rapid increase in tyrosine phosphorylation on the receptor or other intracellular proteins. PI3Ks are recruited to the activation site through an interaction of the SH2 domain in PI3K regulatory subunits with tyrosine-phosphorylated residues. This interaction relieves the inhibitory effect of the p85 subunit on p110 catalytic activity resulting in the accumulation of PI3K enzymatic products PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ at the cellular membrane (4,5). The action of PI3K is directly opposed by the tumor suppressor phosphatase and *tensin* homolog (PTEN, also known as MMAC1 and TEP). PTEN encodes a dual specificity phosphatase capable of dephosphorylating PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ at the D3 position. Therefore, PTEN functions as the key negative regulator of PI3K signaling (6–10). Two other phosphatases, SHIP1 and SHIP2, dephosphorylate the 5' site of the inositol ring generating PtdIns(3,4)P₂, which regulates a separate subset of downstream signaling molecules.

Accumulated D3 phosphatidylinositols at the cellular plasma membrane bind to a subset of pleckstrin homology (PH) domains and recruit PH domain-containing signaling molecules to the activation nidus. A smaller subset of FYVE, Phox (PX), C1, C2, and other lipid-binding domains also bind D3 phosphatidylinositols. Among PH domain-containing proteins, the serine/threonine kinases Akt (also called protein kinase B, PKB) and phosphoinositide-dependent kinase 1 (PDK1) are of particular interest in transducing PI3K signaling as they have been demonstrated to be the primary downstream mediators of D3 phosphatidylinositols by genetic screens in the model organisms *C. elegans* and *Drosophila melanogaster*. PDK1 possesses an N-terminal catalytic domain and a C-terminal PH domain. Binding to D3 phosphoinositides enables PDK1 to colocalize with its cellular substrates at the membrane, such as Akt, and other AGC family protein kinases including PKA, p70S6K, p90RSK, and PKC. In this regard, PDK1 is an important mediator of PI3K signaling (2,11).

Akt isoforms (Akt1, Akt2, and Akt3 in the human proteome) are composed of an N-terminal PH domain, a central kinase catalytic domain, and a C-terminal hydrophobic regulatory domain. Binding of the PH domain to membrane phosphoinositides not only brings Akt into the proximity of PDK1 but also triggers a conformational change in Akt, allowing PDK1 access to the catalytic loop of Akt. PDK1 phosphorylates Akt on its Thr308 activation site, and further phosphorylation on Ser473, in the C-terminal regulatory domain, is required for full activation of Akt (2,12,13). The identity of the kinase (PDK-2) that phosphorylates Ser473 has been elusive, although integrin-linked

kinase (ILK), Akt itself, PKC β II, and PKC α have been proposed as possible kinases. Recently, the mTOR–riCTOR complex, but not the mTOR–raptor complex, has been demonstrated as a kinase phosphorylating Akt at Ser473 (14).

The biological consequences of Akt activation in cancer are significant and contribute directly to the survival, proliferation (an increase in cell number), and growth (an increase in size) of these cells. In fact, a role for Akt has been well established in nearly all the collective changes that occur genetically/epigenetically in cells during tumorigenesis, called the six “Hallmarks of Cancer” outlined by Hanahan and Weinberg (15,16). The gain of unlimited replicative ability of tumor cells has been suggested to occur through enhancement of telomerase activity and putative phosphorylation of hTERT by Akt (17). PI3K and Akt also regulate small G proteins contributing to changes in cellular polarity, motility, and invasiveness. Akt has been implicated in binding to intracellular SMADs relieving the growth inhibitory effects of TGF β .

Akt delivers a broad survival signal through phosphorylation of multiple downstream targets including transcription factors and signaling proteins (Fig. 1). Phosphorylation of Forkhead transcription factors (AFX, FKHR, and FKHL1) by Akt results in their cytoplasmic retention through interaction with 14-3-3 family proteins, which blocks the transcription of proapoptotic genes including *FasL* and *Bim* (18,19). Additionally, Akt phosphorylates cyclic AMP-response element-binding protein (CREB), which increases

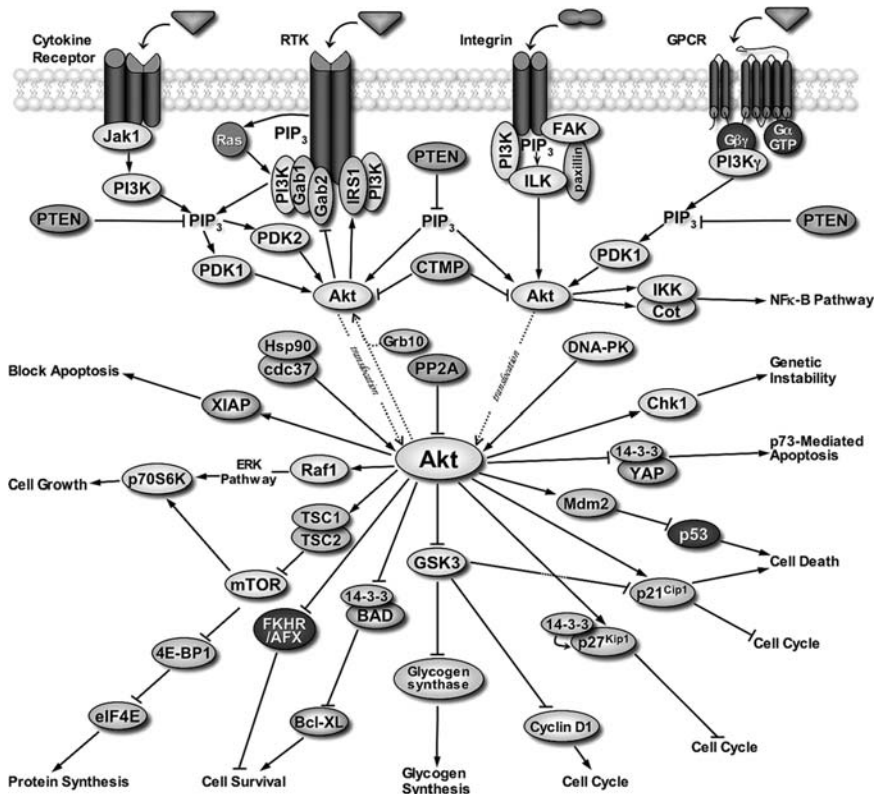


Fig. 1. The PI3K/Akt signaling pathway. Adapted by L. Nolden from <http://www.cellsignal.com> Product Pathways.

the transcription of antiapoptotic genes such as *Bcl-2* and *Akt* itself (20–22). Phosphorylation of I- κ B kinase by Akt causes degradation of I- κ B, promotes nuclear translocation and activation of NF- κ B, and induces transcription of prosurvival genes including *Bcl-XL* (23–25), caspase inhibitors, and c-Myb (26). Akt phosphorylates the ubiquitin ligase MDM2 and promotes its nuclear translocation thereby destabilizing p53. The loss of p53 alters DNA damage and stress-induced cell death allowing replication and survival of cells with DNA aberrations, thus potentially contributing to tumorigenesis (27,28). Akt directly phosphorylates the BCL2 family member, pro-apoptotic protein BAD (at Ser136), and promotes its association with 14-3-3 proteins, releasing BAD from BCL2 or BCL-XL complexes thus increasing cell survival (29). Similarly, Akt phosphorylates apoptosis signal-regulating kinase-1 (ASK-1) and reduces its ability to regulate stress- and cytokine-induced cell death (30,31). Taken together, the activation of Akt downregulates the expression or activity of multiple pro-apoptotic proteins, whereas it upregulates those of antiapoptotic proteins.

Akt promotes cell-cycle progression through its deregulation of cell-cycle checkpoint controls, particularly at the G1/S transition. Akt phosphorylates cyclin-dependent kinase inhibitors p21^{Cip1/WAF1} (on Thr45) and p27^{Kip1} (on Thr157) inducing the cytoplasmic retention of these molecules and preventing their interaction with nuclear effectors, conferring a proliferative advantage (32). In addition, Akt phosphorylates GSK3- α (on Ser21) and GSK3- β (on Ser9), thereby decreasing their respective activities. Inactivation of GSK3 by Akt enhances glycogen synthesis, a key step in tumor development. Inhibition of GSK3 also stabilizes cyclin D1 and Myc, two proteins that drive S-phase entry. Furthermore, phosphorylation and cytoplasmic retention of Forkhead receptor transcription factor releases the repression of cyclin D1 expression, contributing to the overall upregulation of cyclin D1. Tumor cell growth (size), which is required for cellular division, occurs through mTOR leading to increased protein synthesis. Recent studies have revealed that Akt activity also regulates cell-cycle transition through G2/M. Increased Akt activity is present during the G2/M phase in epithelial cells. Akt promotes G2/M cell-cycle progression by inducing phosphorylation-dependent 14-3-3 θ binding and cytoplasmic localization of WEE1Hu, thus preventing inhibitory phosphorylation of cdc2 (33). In addition, phosphorylation of Chk1 peptide by Akt indicates the existence of crosstalk between the PI3K/Akt pathway and key regulators of the DNA damage checkpoint machinery (34).

mTOR (also known as FRAP1 or RAFT1), a central regulator of cell growth, belongs to Class IV of the PI3K family and also function as a major downstream effector of Akt (35). Its Ser2448 residue can be directly phosphorylated and activated by Akt (36–38). Recent studies reveal that the tuberous sclerosis complex (TSC)-1/2, PTEN, and LKB1 tumor suppressor proteins also modulate mTOR activity (39,40). Intriguingly, mutational inactivation of TSC, PTEN, and LKB1 results in a series of hamartoma syndromes, tuberous sclerosis, Cowden's syndrome, and Peutz-Jaegers' syndrome, characterized by an increased propensity to develop tumors in particular cell lineages. These experiments of nature provide genetic evidence that these proteins regulate a similar set of biochemical and functional processes. TSC2 contains multiple potential Akt phosphorylation sites. Akt phosphorylates and inhibits TSC, which subsequently derepresses the small GTP-binding protein Rheb leading to the activation of mTOR. In the contrasting situation of energy depletion, changes in the intracellular ATP/AMP ratio induce the activation of AMP-activated protein kinase (AMPK),

a process largely dependent on the activity of the upstream kinase LKB1. AMPK phosphorylation activates TSC1/2, thereby decreasing mTOR function downstream. mTOR regulates protein synthesis by activating the p70 ribosomal S6 kinase (p70S6K) and inhibiting the elongation-initiation factor 4E binding protein-1 (4E-BP1). Activated p70S6K phosphorylates S6 and enhances translation initiation of mRNA that has 5'-terminal oligopyrimidine tracts. 4E-BP1 acts as a translational repressor of mRNA that bears a 5' CAP structure and inhibits, through direct binding, the function of elongation factor eIF4E. Phosphorylation of 4E-BP1 by mTOR decreases the association of 4E-BP1 with eIF4E, thus initiating mRNA translation. Protein synthesis is a process, in large part, controlling cell growth and aberrations in protein synthesis contribute to the high growth rate of cancer cells (41,42).

3. DYSREGULATION OF PI3K/AKT SIGNALING IN CANCER

Deregulation of the PI3K/Akt pathway can confer a number of the functional capabilities required for cells to undergo tumorigenesis. As discussed in Section 2, activation of Akt promotes cellular survival, stimulates nutrient uptake and glycolysis, and leads to substantive growth and proliferation of the cell. Its phosphorylation of downstream effectors reduces transcription of proapoptotic genes while promoting the transcription of prosurvival factors, rendering the cell insensitive to normal apoptotic and senescent program. Furthermore, the PI3K/Akt pathway regulates cellular motility, production of proteases, as well as proangiogenic factors leading to the development of tumor vasculature.

The importance of Akt as a major hub and interchange between numerous signaling cascades positions it and its component pathway members as a prime group of molecular targets in cancers where this pathway is overactive. Interestingly, Akt itself is amplified in only a small group of cancers, and only rare mutations in the *Akt* gene have been reported to date. Hyperactivity through this network appears to occur, rather, through sustained activation of growth factor RTKs, mutational activation of upstream regulators such as Ras, loss of the primary negative regulator PTEN, or through mutation, overexpression, rearrangement, and subsequent constitutive activation of both the catalytic and the regulatory subunits of PI3K.

3.1. Alterations Upstream of PI3K/Akt in Cancer

Overexpression and constitutive upregulation of cell surface receptors in various cancers leads to ligand-dependent stimulation of receptor tyrosine kinases, G-protein-coupled receptors, and cytokine and integrin receptors, subsequently increasing downstream signaling through PI3K/Akt (12,43). This sustained activation through upstream effectors results in hyperactivated signaling through PI3K and has been shown to contribute to the pathophysiology of a number of malignancies.

A well-examined case in point is that of the erbB2 (*Her2/neu*) tyrosine kinase receptor (44–48). ErbB2 homodimerizes and heterodimerizes with other members of the human EGFR (HER/EGFR) family to elicit a broad spectrum of downstream signaling effects dictating cell growth, antiapoptosis, and cell invasion (44). ErbB2 is overexpressed as a result of gene amplification in various cancers, including breast (45). Overexpressed erbB2 has been shown to be constitutively associated with erbB3 in primary breast tumors, breast cancer cell lines, and transgenic mouse models, and

these heterodimers strongly activate the PI3K/Akt pathway (46,47). This powerful stimulation occurs through the ability of erbB3, through seven tyrosine residues, to bind the SH2 domains of the PI3K regulatory subunit p85 thus enabling the survival and proliferation of erbB2-overexpressing tumor cells (48).

Activating mutations in Ras, an upstream regulator of PI3K, occur in nearly a quarter of all human malignancies and lead to receptor-independent, constitutive upregulation of the PI3K/Akt pathway (15,49,50). Ras directly binds and activates the catalytic subunit of PI3K, p110, and although Ras signaling through other cascades, such as mitogen-activated protein kinase (MAPK), has been implicated in tumor cell resistance to radiotherapy and chemotherapy, PI3K has been identified as the key downstream mediator of Ras-induced resistance (51).

3.2. Alterations of PI3K, Akt, and PTEN in Cancer

Evidence of the significant contribution of aberrant PI3K/Akt signaling to tumorigenesis can be inferred from the sheer number of diverse malignancies with mutations, amplifications, deletions, and/or loss of signaling of PI3K pathway components (reviewed in refs 1 and 52). The PI3K/Akt signaling cascade has been implicated in as many as 30% of all human malignancies (53–57). Activation of PI3K/Akt in ovarian cancer, for example, has been shown to be as high as 60–70% in samples directly isolated from patients (58).

3.2.1. PTEN

Loss of the PTEN tumor suppressor can occur through mutation (59,60), deletion (61), epigenetic silencing (promoter methylation) (62), transcriptional silencing (63), or protein instability (62). Mutation of PTEN is one of the most prevalent events in most human cancers, rivaling the mutational frequency of p53 or Rb (64). Loss of PTEN leads to hyperactivation of the PI3K/Akt pathway, drives inappropriate proliferation, and confers resistance to apoptosis in tumor cells (65,66). Germline mutations in PTEN give rise to autosomal dominant hamartomatous tumor syndromes such as Cowden's disease and Bannayan–Riley–Ruvalcaba syndrome (67,68). Constitutive activation of Akt resulting from loss-of-function mutations in PTEN is common in sporadic glioblastoma (69,70), prostate cancer (71–73), melanoma (74), and endometrial carcinoma (75). Additionally, PTEN mutations contribute to a small portion of lung cancer (76), breast cancer (9,10,61), and lymphomas (77).

3.2.2. PI3K SUBUNITS

Overactivation of PI3K in human cancer has been shown to occur through mutations in the p110 and p85 subunits, rearrangement of p85, as well as overexpression through genetic amplification (56,78–80). Somatic mutations in *PIK3CA*, the gene encoding the p110 α catalytic subunit of PI3K, are prevalent in a high percentage of tumors, such as breast (40%) (54,55,81), colorectal cancers (CRCs) (32%) (56), glioblastoma (27%) (70), and others (53,82,83), and likely contribute directly to tumor progression (55). Study in colorectal tumors showed that mutations of *PIK3CA* were primarily found in high-grade, advanced tumors, indicating that these mutations may be important in progression to aggressive and/or invasive

phenotypes. When three of the most frequent mutations were individually introduced in chicken embryo fibroblasts, the resulting mutant p110 α , but not wild-type p110 α , was sufficient to cause oncogenic transformation, constitutively activate Akt and downstream pathway signaling, and increase phosphorylation of p70S6K and 4E-BP1 (84). Additionally, activating mutations in *PIK3R1*, encoding the p85 subunit, have been observed in ovarian and colon carcinomas, and rearrangements have been identified in lymphomas (78).

The *PIK3CA* gene is located on chromosome 3q26, a region that is frequently amplified in human tumors including breast (27%) (85), ovarian (40%) (86), and cervical (50%) (87) carcinomas. Amplification of this region occurs frequently in low-grade and low-stage ovarian tumors and is likely indicative of key early events in the development of this disease (79,80). In lung cancer, specific study of the *PIK3CA* locus during progression revealed its amplification in high-grade, but not low-grade, preinvasive lesions (88). In both cases, gain of PI3K function through amplification of this region is thought to imply a commitment to tumor progression.

3.2.3. AKT

Rare mutations in the *Akt* gene have only recently been described, but amplification of Akt in human malignancies is a known event leading to hyperactivation of downstream signaling. The family is composed of three isoforms: Akt1/PKB α , Akt2/PKB β , and Akt3/PKB γ (66); each Akt is derived from distinct genes and amplified in various tumors, with each isoform appearing to have selective effects in different types of cancer. Akt1 is rarely amplified, reported only in glioblastoma and gastric carcinomas (70,89), but overactivation of Akt1 has been observed in a high percentage of prostate, breast, and ovarian carcinomas, and this increase in Akt1 kinase activity is associated with poor prognosis (90). Amplification of the 19q13 chromosomal region, which includes Akt2, occurs in approximately 25% of ovarian cancers (58,91–93), particularly high-grade aggressive tumors (94), and is also amplified in breast and pancreatic tumors (10%) (91,94,95). Somatic mutation of Akt2 has been reported in breast and pancreatic carcinomas (85). Interestingly, the Akt2 kinase is activated in nearly 40% of ovarian tumors (58). Overexpression of Akt2 in NIH 3T3 cells is oncogenic (96), and breast cancer cells expressing Akt2 exhibit increased metastatic potential *in vivo* (97). Likewise, Akt3 is suggested to contribute to the aggressive clinical phenotype of estrogen-negative breast and androgen-insensitive prostate cancers as both Akt3 mRNA levels are elevated and enzymatic capacity is increased in these cell types (98). Genetic amplification of *Akt3* has not been reported. The three Akt isoforms are amplified with comparatively less frequency than other pathway components, and constitutive upstream signaling is more frequently causative of the hyperactive state of Akt observed in malignancies.

3.3. Downstream Alterations

Alterations of downstream targets of Akt in cancer are less thoroughly described to date. Loss of heterozygosity (LOH) and subsequent mutation of the tumor suppressor *TSC1* contributes to sporadic tumor formation in the bladder (99). Amplification of *RPS6KB1* (*p70S6K*) has been reported in breast cancer (~30%), but it is unclear whether these increases occur independently or as a consequence of the frequent

17q22-24 chromosomal amplification (particularly in BRCA1/2-associated tumors), where the neighboring *ERBB2* could be the driver of the amplicon (100). Whatever the mechanism, amplification is associated with overexpression of p70S6K in these tumors. As patient tumors are better characterized with increasing resolution at the DNA, RNA, and protein level, our understanding of the number of genetic aberrations and the complexity of changes that can lead to dysregulation of this pathway in various cancers will expand. Additionally, aberrations in unidentified components of the pathway and upstream/downstream pathway crosstalk have yet to be elucidated.

3.4. Activation of PI3K/Akt Pathway and Correlation to Therapy Resistance

Refractoriness to anticancer therapy is often due to failure of the apoptotic program, gain of proliferative capacity, growth and decreased sensitivity to antigrowth signals in tumor cells, all events that can be mediated through the activation of Akt and its downstream substrates. Akt activity, as reflected by phosphorylation, is linked to poor disease prognosis in many cancer lineages (101–105), and several studies have examined the contribution of the PI3K/Akt pathway toward targeted/radio/chemotherapeutic tumor resistance (106–108).

In breast cancer, loss of PTEN in patients with erbB2-expressing tumors predicts resistance to trastuzumab (Herceptin), an erbB2-targeting antibody (109). Combinatorial therapeutics such as trastuzumab plus paclitaxel overcome this resistance through a more effective inhibition of Akt phosphorylation and pathway activation (110). In one-third of patients with estrogen receptor α (ER α)-positive tumors, de novo or acquired resistance develops and resistance to the standard adjuvant therapy tamoxifen is predicted by the activation of Akt (111). This has led to an ongoing phase III trial of inhibition of mTOR (the only target with validated drugs currently available) and inhibition of estrogen signaling. Models of ER α -positive tumors overexpressing erbB2 *in vivo* revealed that tamoxifen treatment led to growth stimulation and de novo resistance, in part, through activation of the Akt pathway. In MCF7 breast adenocarcinoma cell lines, overexpression of erbB2 in the presence of erbB3 led to a PI3K-dependent increase in Akt phosphorylation and conferred increased chemotherapeutic resistance against multiple agents including doxorubicin, paclitaxel, 5-FU, etoposide, and camptothecin (112). Studies of radioresistance in breast cancer cells have also uncovered a causal role for PI3K/Akt, and combination with a PI3K inhibitor, *in vitro*, led to sensitization of cells to radiation treatment (113).

Non-small cell lung cancer (NSCLC) cell lines with high levels of PI3K/Akt activity have shown increased cellular resistance to cisplatin, paclitaxel, VP16, and ionizing radiation (106). Combination of a specific PI3K inhibitor potentiates both chemo- and radiotherapy in resistant NSCLC (106). mTOR, an effector downstream of Akt (Fig. 1), is targeted by rapamycin (Sirolimus) and its derivatives, which are currently in clinical trials. Indeed, these are the only selective PI3K pathway inhibitors in current clinical trials. Resistance to rapamycin was studied *in vitro* in NSCLC and attributed to PI3K/Akt stimulation. Despite inhibition of mTOR signaling by rapamycin, a negative-feedback mechanism leads to the increase in phosphorylation of both Akt and eIF4E, and this resistance is attenuated through combination with a PI3K inhibitor (114). Resistance to gefitinib (Iressa) in advanced NSCLC and imatinib mesylate (Gleevec) in SCLC have also been attributed to activation of Akt or loss of PTEN expression (in the case of gefitinib) (115,116). Inhibition of the PI3K/Akt pathway through the

use of inhibitors sensitizes both NSCLC and SCLC to traditional chemotherapeutic agents (106,117).

Increased signaling and activation of Akt, either before or after treatment, may indicate resistance to therapy. Paradoxically, however, high Akt activity is associated with increased patient responses to gefitinib in lung cancer likely as a surrogate for the presence of activating mutations in the EGFR targeted by gefitinib. Broadly combining PI3K/Akt inhibitors with standard therapeutics to synergize treatment effects and/or overcome issues of treatment resistance has also been suggested in ovarian (118,119), prostate (120,121), bladder (49,122), colon carcinomas (121), as well as glioma (123), among others (124). Possible mechanisms for synergistic effects include enhancement of apoptosis and attenuation of PI3K-like kinases DNA-PK, ATM, and ATR (activated by DNA damage/radiation) by agents that target the PI3K kinase family. However, it is important to note that many of these studies are based on the actions of LY294002 and wortmannin, which inhibit multiple members of the PI3K kinase family with high efficiency. Inhibition at the level of Akt compared with downstream inhibition (e.g., mTOR) confers a theoretical advantage because of the bulk of the downstream network branches that would be targeted (Fig. 1); thus, the PI3K/Akt signaling network may be more extensively and effectively inhibited when the pathway is targeted at the level of PI3K or Akt.

4. CANCER THERAPEUTICS TARGETING PI3K/AKT SIGNALING

Overactivation of the PI3K/Akt pathway is frequently a major driver of tumor progression. In addition, the PI3K/Akt pathway contributes to the development of resistance to chemotherapy and radiation treatment (121,125,126). Activation of the PI3K/Akt pathway in neoplastic tissues, resulting from a genetic aberration of the cancer cells or resulting from DNA damage or other mechanisms of activation induced by radiation and/or chemotherapy treatments, mediates survival and results in resistance to radiation and chemotherapy treatments. As tumors with overactive PI3K/Akt signaling are often rendered resistant to apoptosis, inhibition of this pathway may be necessary to induce tumor regression in response to chemotherapy and/or radiotherapy.

Small-molecule drugs have been developed and are in development to target the PI3K/Akt pathway at every level, including upstream of PI3K at the level of GFRs and downstream at the level of Akt targets.

4.1. Inhibition of PI3K

Several cancer models have indicated that inhibition of PI3K increases response to chemotherapy and/or radiation.

Wortmannin, a small-molecule PI3K inhibitor not used clinically because of its insolubility in aqueous conditions and low stability, has demonstrated efficacy in sensitizing multiple tumor cell lines to both ionizing radiation and bleomycin treatment (127). Inhibition of PI3K with LY294002, another PI3K inhibitor not used clinically because of short half-life, enhances paclitaxel-induced apoptosis in ovarian cancer models (119).

Cancer cells of the pancreas often express constitutively active Ki-Ras and overexpress multiple receptor tyrosine kinases, two genetic aberrations that may upregulate activity of the PI3K/Akt pathway (128). Two pancreatic adenocarcinoma cell lines shown to be resistant to treatment with the chemotherapeutic gemcitabine (a DNA

chain terminator) showed substantial enhancement of apoptosis when treated with PI3K inhibitors wortmannin and LY294002 (128).

Recently, isoform-specific PI3K inhibitors have been identified (129). The development of isoform-specific PI3K inhibitors will facilitate analysis of distinct isoform-specific cell-signaling pathways in tumorigenesis. Furthermore, the discovery and characterization of novel PI3K inhibitors may provide compounds suitable for clinical use. These isoform-specific inhibitors offer the opportunity to compare the efficacy and toxicity of inhibition of specific PI3K isoforms to that of pan-PI3K inhibitors. As clinical utility reflects the balance between efficacy and toxicity, there may be advantages to isoform-specific inhibitors. Indeed, as indicated in Section 3.2, *PIK3CA*, which produces p110 α , is selectively targeted in cancer, suggesting it is an optimal therapeutic target. Furthermore, insulin signal appears to process primarily through p110 β , suggesting that a selective p110 α inhibitor may have decreased toxicity.

4.2. Inhibition of Akt

Activated PI3K facilitates subsequent activation of Akt, which in turn phosphorylates many substrates to effect cell proliferation, cell-cycle progression, and cell survival.

Currently there are four Akt inhibitors in clinical trials. The first of these is perifosine, which like other members of the alkylphospholipid (APL) class of compounds, cause dose-dependent inhibition of Akt phosphorylation, although the exact mechanism of action is not known. APLs have, however, been demonstrated to bind to PH domains and to selectively interfere with the PH domain of Akt potentially providing selectivity. Miltefosine, another APL, is used topically to treat cutaneous breast cancer metastases (130). This efficacy may be due to the high frequency of *PIK3CA* mutations in breast cancer. European clinical studies evaluating miltefosine as an oral anticancer agent were terminated at phase II because of cumulative gastrointestinal side effects (130). Perifosine was better tolerated than miltefosine in preclinical studies and is currently in phase I/II trials (130).

A number of pan-Akt inhibitors have been developed. These demonstrate a high degree of efficacy *in vitro* inducing apoptosis in multiple cell lineages, particularly those with abnormalities in the PI3K pathway. Compound XL418 and compound GSK690693 have recently entered Phase I studies. Additionally, a series of potential isoform-specific inhibitors has recently been identified.

Intriguingly, a number of drugs that are in use in other diseases or that have been assessed because of potential other mechanisms have been demonstrated to inhibit Akt signaling. These include triciribine (131) and more recently amiloride (132). Whether these molecules inhibit Akt in patients and are useful probes of the function of the PI3K pathway will require further investigation. A Phase I trial is in progress to evaluate triciribine in patients with metastatic cancer whose tumors are p-Akt positive.

4.3. Inhibition of Hsp90

Hsp90 ensures proper folding and stability of many proteins including HER2, Erk, Akt, mutant B-Raf, and mutant p53. Radicol and geldamycins, such as 17-allylamino 17-demethoxygeldanamycin (17AAG), inhibit Hsp90 by binding to the ATPase domain of Hsp90 (133), thus destabilizing proteins that depend on Hsp90 for proper form and function (130). These proteins are then depleted through the ubiquitin-proteasome

pathway. In this manner, 17AAG modulates the PI3K/Akt pathway (130). Phases I and II studies of 17AAG are in progress. In these studies, 17AAG induces loss of both total Akt and phospho-Akt from tumors, suggesting that targeting the PI3K pathway may contribute to their function.

4.4. Inhibition of PDK1 Activation of Akt

PDK1 phosphorylation of Akt on its Threonine 308 residue is required for Akt activation. Inhibition of PDK1 is thus an attractive strategy for inhibiting the survival pathways mediated through Akt. Studies have shown that inhibition of PDK1 with UCN-01 results in inhibition of Akt and cell growth (130). UCN-01 also enhances the antitumor effects of several chemotherapeutic cancer agents including antimetabolites, camptothecins, and cisplatin (130). Phase I and Phase II clinical trials with UCN-01 have been carried out in patients with refractory solid tumors (134), clinical chronic leukemia (135), and chronic lymphoma and Phase II clinical. Whether the effects of UCN-01 in patients are a consequence of inhibition of PDK1 and Akt is unknown, however, because of the pleiomorphic action of UCN-01.

4.5. Targeting Ras, Rac, and Rho

4.5.1. INHIBITION OF PI3K/AKT WITH PRENYLATION INHIBITORS

G proteins, such as Ras, Rac, and Rho, are important mediators of signal transduction at the cell membrane and are anchored to the membrane by a post-translationally added prenyl group. Ras, a G-protein activated in many cancers, for example, is generally prenylated with a farnesyl group, and other important G proteins such as Rac and Rho are prenylated with a geranylgeranyl group (136). As prenylation of these important signaling molecules is essential to their function, inhibitors of the farnesyltransferase and geranylgeranyltransferase I enzymes that mediate prenylation have been developed as anticancer agents (137).

Inhibition of PI3K/Akt signaling has been demonstrated as a critical target for farnesyltransferase inhibitor-induced apoptosis (138) and geranylgeranyltransferase I inhibitor (GGTI)-induced apoptosis (139). FTI-277, a farnesyltransferase inhibitor, has been shown to inhibit growth-factor-induced PI3K/Akt2 activation *in vitro* and *in vivo* (138). Furthermore, ectopic expression of wild-type Akt2 sensitizes cells to FTI-277 (138). Two GGITs, GGTI-298 and GGTI-2166, have been shown to induce apoptosis in both cisplatin-sensitive and cisplatin-resistant human ovarian epithelial cancer cells by inhibition of the PI3K/Akt pathway as well as the survivin pathway (139), which is essential for proper cell division and also inhibits apoptosis (140). As upregulation of Akt and survivin are frequently associated with chemoresistance, FTIs and GGITs could prove to be valuable agents to overcome resistance to chemotherapy. Several prenylation inhibitors are at varying stages of clinical development (141–143).

4.5.2. ADDITIONAL RAS INHIBITORS

Short antisense synthetic oligonucleotides are another treatment in development for targeting activated Ras (143). ISIS 2503, an antisense oligonucleotide targeting H-Ras, is in phase II clinical trials (143,144). Whether this will inhibit downstream activation of PI3K and Akt needs to be assessed in patient samples.

4.6. Inhibition of mTOR Activity

Akt activation results in subsequent activation of mTOR, a key mediator of protein translation, cell size, and cell-cycle progression. Phosphorylation of Akt, mTOR, and 4E-BP1 increases progressively from normal breast epithelium to hyperplasia and from abnormal hyperplasia to tumor invasion. Akt-induced drug resistance can be mediated by mTOR, indicated by reversal of tumor chemoresistance upon treatment with mTOR inhibitors (130).

The current mTOR inhibitors consist of rapamycin and related analogs. These proteins interfere with the activity of the mTOR–raptor complex but not the mTOR–riCTOR complex blocking-specific functions of mTOR. Indeed, rapalogs block mTOR-mediated p70S6K phosphorylation without interfering with phosphorylation of the PDK2 site of Akt or of the effects of mTOR on 4E-BP1 and eIF4E (14). This may contribute to the relative non-toxicity of rapamycin and its analogs. However, it may also limit the efficacy of rapalogs in the treatment of cancer patients.

Treatment of cancer cells (*in vitro*) with an inhibitor of mTOR, rapamycin, yields a higher rate of cell death when used in conjunction with traditional cytotoxic agents, such as cisplatin, camptothecin, 5-fluorouracil, and cyclophosphamide (145). Rapamycin analogs with better pharmacological properties such as CCI779 and RAD001 have recently been developed. Currently, CCI779 and RAD001 are in phase II and Phase III clinical trials.

Feedback downregulation of Akt activity through mTOR/p70S6K-dependent loss of IRS-1 expression is well-established (146) and thus, potential upregulation of Akt signaling with mTOR inhibitors must be carefully evaluated. A treatment regimen combining an mTOR inhibitor with an inhibitor of Akt may provide for greater therapeutic efficacy.

4.7. Targeting EGFR Family Receptor-Mediated PI3K/Akt and MAPK Signaling

Much of cell signaling in epithelial cells is initiated through activation of epidermal growth factor receptors. The EGFR family includes EGFR (HER1/erbB1), HER2/neu (erbB2), HER3/erbB3, and HER4/erbB4. Binding of a ligand to the receptor facilitates its homo- and heterodimerization with other EGFR family members and subsequent activation of the receptor's tyrosine kinase located on the cytosolic side of the cell membrane. HER3 lacks intrinsic tyrosine kinase activity resulting in its functioning primarily as a linker molecule. Intriguingly, HER2, which lacks a functional ligand binding site, is constitutively in the active conformation resulting in its being the preferred binding partner for other members of the EGFR family. The tyrosine kinase activity results in autophosphorylation and subsequent activation of downstream signaling pathways that regulate cell proliferation and survival, such as the PI3K/Akt pathway and the Ras/Raf/MAPK pathway, which shows significant crosstalk with the PI3K/Akt pathway.

The EGFR pathway has been shown to contribute to radiation and chemotherapeutic resistance in many tumor types (147–150). DNA damage can activate EGFR and downstream pathways (151), and it has been observed that both radiation and chemotherapy can activate EGFR signaling, likely the reason for increased cross-resistance between sequential administration of radiation and chemotherapy observed

in glioblastoma cells (152). It appears that Ras-mediated activation of PI3K/Akt and MAPK (p44/p42), through EGFR, may play a critical role in mediating this effect (152).

Antitumor drugs targeting the EGFR family of tyrosine kinase receptors have been developed, and more are in development. These agents include monoclonal antibodies that antagonize the receptors as well as other chemical inhibitors of the tyrosine kinase receptors.

Cetuximab, an anti-EGFR monoclonal antibody in clinical use, is approved for treatment of CRC and NSCLC and is in clinical trials for treatment of pancreatic, head and neck, breast, renal, and prostate cancers (153).

ZD1839 (also known as gefitinib and Iressa), a reversible EGFR inhibitor in clinical use, is approved for treatment of NSCLC and is in clinical development for use in head and neck, CRC, breast, gastrointestinal, prostate, and esophageal cancers and has shown clinical activity in NSCLC and prostate cancer (153). Its failure to increase patient survival in clinical trials, however, has resulted in most patients being transferred to a related inhibitor, erlotinib, which has demonstrated activity. Subset analysis indicates importantly that gefitinib does have high activity in patients with activating mutations in the EGFR, amplification of the EGFR, or phosphorylation of Akt (123,154). These may represent a surrogate for constitutive activation of the EGFR and “addiction” of tumor cells to signaling through the EGFR pathway.

Unlike the other EGFR family members, which have a series of ligands that trigger homo- and heterodimer formation upon binding, the HER2 receptor has no ligands. Its activation occurs through heterodimer formation with other EGFR family members or through spontaneous homodimer formation under conditions of marked overexpression (155). High levels of HER2 result in the progression of breast cancer tumorigenesis and metastasis and may also protect cancer cells from traditional cytotoxic therapies (125). Trastuzumab (anti-HER2 monoclonal antibody), a drug in clinical use that inhibits breast cancer cell proliferation (in tumors overexpressing HER2) but does not induce apoptosis alone, was shown to enhance radiation-induced apoptosis of human breast cancer cells (*in vitro*) in a HER2-level-dependent manner (125).

Phosphorylation of at least two important HER2 downstream molecules, Akt and MAPK, is increased in breast cancer cells with high levels of HER2, and cells are resistant to radiotherapy as shown by reduced induction of apoptosis (125). *In vitro* exposure of these cells to trastuzumab downregulated the levels of HER2, reduced phosphorylation levels of Akt and MAPK, and sensitized these cells to radiotherapy (125). On the basis of the results of chemical inhibition of each of the PI3K and MAPK pathways, the radiosensitization induced by PI3K inhibition was much stronger relative to MAPK inhibition, suggesting that the PI3K pathway may be the major pathway for trastuzumab-mediated radiosensitization of breast cancer cells (125). Recent studies suggest that patients with decreased PTEN levels are less responsive to trastuzumab (110). *In vitro* studies suggest that trastuzumab results in inactivation of HER2 and dissociation of Src from the HER2 activation complex (110). Src-dependent phosphorylation sites on PTEN (156) are dephosphorylated, increasing PTEN activity. In patients with low PTEN levels, it is hypothesized that insufficient PTEN is present to mediate the effects of trastuzumab and that high phospho-Akt levels persist, leading to drug resistance (110).

As described previously in this subsection, gefitinib is selective for EGFR; however, as the members of the EGFR family can form heterodimers, drugs targeting one

receptor may be effective in cells overexpressing other members of the EGFR family of receptors (157). In a panel of human breast cancer and other epithelial tumor cell lines, HER2-overexpressing tumors were particularly sensitive to gefitinib. Growth inhibition of these tumor cell lines was associated with the dephosphorylation of EGFR, HER2, and HER3, accompanied by loss of association of HER3 with PI3K, and downregulation of Akt activity (157). Erlotinib (Tarceva), another EGFR tyrosine kinase inhibitor in clinical use, has been shown to enhance radiosensitivity (158). A series of reversible and non-reversible pan-EGFR inhibitors are currently under development and evaluation.

4.8. Patient Selection

The PI3K/Akt pathway is targeted genomically in multiple tumor lineages. Indeed, as described in Section 3.2.1, the PI3K pathway is mutationally activated in tumors with a greater frequency than any other pathway. Studies of trastuzumab in breast cancer, erlotinib and gefitinib in lung cancer, retinoic acid in PMML, imatinib mesylate in CML, gastrointestinal stromal tumors, dermatofibrosarcoma protuberans, and hyper-eosinophil syndrome have demonstrated that mutational activation, genomic amplification, or genomic rearrangement identifies a population of patients most likely to respond to therapeutic manipulation of the target. Indeed, modeling of the registration trial for trastuzumab suggests that the efficacy of trastuzumab would not have been detected had all breast cancer patients rather than just those with overexpression of HER2 been treated. This suggests that targeting therapy against the PI3K/Akt pathway in patients with genetic abnormalities in the pathway will result in greater efficacy, a greater likelihood of demonstrating activity, a decreased number of patients and decreased time of enrollment in trials, and thus decreased trial costs and more rapid implementation of effective drugs. Furthermore, in terms of the interests of pharmaceutical companies, this will result in protection of patent life. Thus, selection of patients with mutations in *PIK3CA*, *PTEN*, or other pathway components is likely to result in greater efficacy. At a minimum, patient samples should be collected before and after treatment to allow identification of patients likely to respond in subsequent clinical studies.

5. CONCLUSION

The emerging data strongly support a central role for aberrant PI3K/Akt pathway activation in many human cancers. The extended reach of this network, through its evergrowing list of substrates together with its direct effects toward cell survival, growth, motility, invasion, neovascularization, and proliferation, makes the PI3K/Akt pathway an attractive target for cancer treatment. Robust PI3K/Akt signaling in various tumor lineages has been linked to chemo- and radiotherapeutic resistance. PI3K/Akt inhibitors used in parallel to standard therapy have proven synergistic and effective in counteracting resistance in the preclinical setting. These demonstrate the greatest activity in models with genetic aberrations in the PI3K pathway, suggesting that selection of patients based on these aberrations is likely to identify those most likely to benefit from therapy. Targeting this central player in pathogenesis is a desirable approach that has become well accepted as a cancer therapeutic strategy. As such, multiple compounds targeting different components of the PI3K/Akt pathway have

entered or will begin clinical trials, either as single agents or in combination regimen with conventional cytotoxic agents. However, the utility of a drug is based on its therapeutic index, the ratio between efficacy and toxicity. As the PI3K/Akt pathway plays a critical role in normal cellular physiology, some components of the PI3K pathway may prove to be non-druggable. Thus, it will be important to develop inhibitors against multiple components of the pathway to identify targets with an adequate therapeutic index. These questions will only be answered with the development of pharmacologically relevant, highly selective drugs targeting components of the PI3K pathway.

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REFERENCES

1. Cantley LC. The phosphoinositide 3-kinase pathway. *Science* 2002;296(5573):1655–7.
2. Lu Y, Wang H, Mills GB. Targeting PI3K-AKT pathway for cancer therapy. *Rev Clin Exp Hematol* 2003;7(2):205–28.
3. Vanhaesebroeck B, Waterfield MD. Signaling by distinct classes of phosphoinositide 3-kinases. *Exp Cell Res* 1999;253(1):239–54.
4. Cuevas BD, Lu Y, Mao M, et al. Tyrosine phosphorylation of p85 relieves its inhibitory activity on phosphatidylinositol 3-kinase. *J Biol Chem* 2001;276(29):27455–61.
5. Chan TO, Rodeck U, Chan AM, et al. Small GTPases and tyrosine kinases coregulate a molecular switch in the phosphoinositide 3-kinase regulatory subunit. *Cancer Cell* 2002;1(2):181–91.
6. Li DM, Sun H. TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Res* 1997;57(11):2124–9.
7. Li J, Yen C, Liaw D, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 1997;275(5308):1943–7.
8. Steck PA, Pershouse MA, Jasser SA, et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* 1997;15(4):356–62.
9. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 1998;273(22):13375–8.
10. Lu Y, Lin YZ, LaPushin R, et al. The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells. *Oncogene* 1999;18(50):7034–45.
11. Alessi DR. Discovery of PDK1, one of the missing links in insulin signal transduction. Colworth Medal Lecture. *Biochem Soc Trans* 2001;29(Pt 2):1–14.
12. Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002;2(7):489–501.
13. Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal* 2002;14(5):381–95.
14. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 2005;307(5712):1098–101.
15. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100(1):57–70.
16. Testa JR, Bellacosa A. AKT plays a central role in tumorigenesis. *Proc Natl Acad Sci USA* 2001;98(20):10983–5.
17. Kang SS, Kwon T, Kwon DY, Do SI. Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J Biol Chem* 1999;274(19):13085–90.

18. Brunet A, Bonni A, Zigmond MJ, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 1999;96(6):857–68.
19. Dijkers PF, Medema RH, Lammers JW, Koenderman L, Coffey PJ. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol* 2000;10(19):1201–4.
20. Maira SM, Galetic I, Brazil DP, et al. Carboxyl-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane. *Science* 2001;294(5541):374–80.
21. Pugazhenth S, Nesterova A, Sable C, et al. Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. *J Biol Chem* 2000;275(15):10761–6.
22. Du K, Montminy M. CREB is a regulatory target for the protein kinase Akt/PKB. *J Biol Chem* 1998;273(49):32377–9.
23. Romashkova JA, Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* 1999;401(6748):86–90.
24. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 1999;401(6748):82–5.
25. Barkett M, Gilmore TD. Control of apoptosis by Rel/NF-kappaB transcription factors. *Oncogene* 1999;18(49):6910–24.
26. Lauder A, Castellanos A, Weston K. c-Myb transcription is activated by protein kinase B (PKB) following interleukin 2 stimulation of T cells and is required for PKB-mediated protection from apoptosis. *Mol Cell Biol* 2001;21(17):5797–805.
27. Mayo LD, Donner DB. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc Natl Acad Sci USA* 2001;98(20):11598–603.
28. Zhou BP, Liao Y, Xia W, Zou Y, Spohn B, Hung MC. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. *Nat Cell Biol* 2001;3(11):973–82.
29. Datta SR, Dudek H, Tao X, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 1997;91(2):231–41.
30. Kim AH, Khursigara G, Sun X, Franke TF, Chao MV. Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. *Mol Cell Biol* 2001;21(3):893–901.
31. Ichijo H, Nishida E, Irie K, et al. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* 1997;275(5296):90–4.
32. Zhou BP, Liao Y, Xia W, Spohn B, Lee MH, Hung MC. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat Cell Biol* 2001;3(3):245–52.
33. Katayama K, Fujita N, Tsuruo T. Akt/protein kinase B-dependent phosphorylation and inactivation of WEE1Hu promote cell cycle progression at G2/M transition. *Mol Cell Biol* 2005;25(13):5725–37.
34. Shtivelman E, Sussman J, Stokoe D. A role for PI 3-kinase and PKB activity in the G2/M phase of the cell cycle. *Curr Biol* 2002;12(11):919–24.
35. Schmelzle T, Hall MN. TOR, a central controller of cell growth. *Cell* 2000;103(2):253–62.
36. Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J* 1999;344(Pt 2):427–31.
37. Sekulic A, Hudson CC, Homme JL, et al. A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. *Cancer Res* 2000;60(13):3504–13.
38. Brunn GJ, Williams J, Sabers C, Wiederrecht G, Lawrence JC Jr, Abraham RT. Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. *EMBO J* 1996;15(19):5256–67.
39. Shaw RJ, Bardeesy N, Manning BD, et al. The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell* 2004;6(1):91–9.
40. Corradetti MN, Inoki K, Bardeesy N, DePinho RA, Guan KL. Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. *Genes Dev* 2004;18(13):1533–8.
41. Li Y, Corradetti MN, Inoki K, Guan KL. TSC2: filling the GAP in the mTOR signaling pathway. *Trends Biochem Sci* 2004;29(1):32–8.
42. Inoki K, Corradetti MN, Guan KL. Dysregulation of the TSC-mTOR pathway in human disease. *Nat Genet* 2005;37(1):19–24.
43. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. *Nature* 2001;411:355–65.

44. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: Receptor heterodimerization in development and cancer. *EMBO J* 2000;19:3159–67.
45. Harari D, Yarden Y. Molecular mechanisms underlying ErbB2/HER2 action in breast cancer. *Oncogene* 2000;19(53):6102–14.
46. Siegel PM, Ryan ED, Cardiff RD, Muller WJ. Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: Implications for human breast cancer. *EMBO J* 1999;18:2149–64.
47. Zhou BP, Hu MC, Miller SA, et al. HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-kappaB pathway. *J Biol Chem* 2000;275(11):8027–31.
48. Prigent SA, Gullick WJ. Identification of c-erbB-3 binding sites for phosphatidylinositol 3-kinase and SHC using an EGF receptor/c-erbB-3 chimera. *EMBO J* 1994;13:2831–41.
49. Rodriguez-Viciana P, Warne PH, Khwaja A, et al. Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* 1997;89:457–67.
50. Kauffmann-Zeh A, Rodriguez-Viciana P, Ulrich E, et al. Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature* 1997;385(6616):544–8.
51. Gupta AK, Bakanauskas VJ, Cerniglia GJ, et al. The Ras radiation resistance pathway. *Cancer Res* 2001;61(10):4278–82.
52. Mitsiades CS, Mitsiades N, Koutsilieris M. The Akt pathway: molecular targets for anti-cancer drug development. *Curr Cancer Drug Targets* 2004;4(3):235–56.
53. Broderick DK, Di C, Parrett TJ, et al. Mutations of PIK3CA in anaplastic oligodendrogliomas, highgrade astrocytomas, and medulloblastomas. *Cancer Res* 2004;64:5048–50.
54. Campbell IG, Russell SE, Choong DY, et al. Mutation of the PIK3CA gene in ovarian and breast cancer. *Cancer Res* 2004;64:7678–81.
55. Bachman KE, Argani P, Samuels Y, et al. The PIK3CA gene is mutated with high frequency in human breast cancers. *Cancer Biol Ther* 2004;3(8):772–5.
56. Samuels Y, Wang Z, Bardelli A, et al. High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004;304(5670):554.
57. Samuels Y, Velculescu VE. Oncogenic mutations of PIK3CA in human cancers. *Cell Cycle* 2004;3(10):1221–4.
58. Yuan ZQ, Sun M, Feldman RI, et al. Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. *Oncogene* 2000;19(19):2324–30.
59. Ali IU, Schriml LM, Dean M. Mutational spectra of PTEN/MMAC1 gene: a tumor suppressor with lipid phosphatase activity. *J Natl Cancer Inst* 1999;91(22):1922–32.
60. Nassif NT, Lobo GP, Wu X, et al. PTEN mutations are common in sporadic microsatellite stable colorectal cancer. *Oncogene* 2004;23(2):617–28.
61. Garcia JM, Silva JM, Dominguez G, et al. Allelic loss of the PTEN region (10q23) in breast carcinomas of poor pathophenotype. *Breast Cancer Res Treat* 1999;57(3):237–43.
62. Goel A, Arnold CN, Niedzwiecki D, et al. Frequent inactivation of PTEN by promoter hypermethylation in microsatellite instability-high sporadic colorectal cancers. *Cancer Res* 2004;64(9):3014–21.
63. Whang YE, Wu X, Suzuki H, et al. Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. *Proc Natl Acad Sci USA* 1998;95:5246–50.
64. Cantley LC, Neel BG. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci USA* 1999;96:4240–5.
65. Stambolic V, Suzuki A, de la Pompa JL, et al. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 1998;95(1):29–39.
66. Datta SR, Brunet A, Greenberg ME. Cellular survival: A play in three Akts. *Genes Dev* 1999;13:2905–27.
67. Marsh DJ, Dahia PL, Coulon V, et al. Allelic imbalance, including deletion of PTEN/MMAC1, at the Cowden disease locus on 10q22-23, in hamartomas from patients with Cowden syndrome and germline PTEN mutation. *Genes Chromosomes Cancer* 1998;21(1):61–9.
68. Marsh DJ, Coulon V, Lunetta KL, et al. Mutation spectrum and genotype-phenotype analyses in Cowden disease and Bannayan-Zonana syndrome, two hamartoma syndromes with germline PTEN mutation. *Hum Mol Genet* 1998;7(3):507–15.

69. Rasheed BK, Wiltshire RN, Bigner SH, Bigner DD. Molecular pathogenesis of malignant gliomas. *Curr Opin Oncol* 1999;11:162–7.
70. Knobbe CB, Reifenberger G. Genetic alterations and aberrant expression of genes related to the phosphatidylinositol-3 – kinase/protein kinase B (Akt) signal transduction pathway in glioblastomas. *Brain Pathol* 2003;13:507–18.
71. Ittmann MM. Chromosome 10 alterations in prostate adenocarcinoma. *Oncol Rep* 1998;5:1329–35.
72. Suzuki H, Freije D, Nusskern DR, et al. Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. *Cancer Res* 1998;58(2):204–9.
73. Cairns P, Okami K, Halachmi S, et al. Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. *Cancer Res* 1997;57(22):4997–5000.
74. Guldberg P, Thor Straten P, Birck A, Ahrenkiel V, Kirkin AF, Zeuthen J. Disruption of the MMAC1/PTEN gene by deletion or mutation is a frequent event in malignant melanoma. *Cancer Res* 1997;57(17):3660–3.
75. Risinger JI, Hayes AK, Berchuck A, Barrett JC. PTEN/MMAC1 mutations in endometrial cancers. *Cancer Res* 1997;57(21):4736–8.
76. Marsit CJ, Zheng S, Aldape K, et al. PTEN expression in non-small-cell lung cancer: evaluating its relation to tumor characteristics, allelic loss, and epigenetic alteration. *Hum Pathol* 2005;36(7):768–76.
77. Sakai A, Thieblemont C, Wellmann A, Jaffe ES, Raffeld M. PTEN gene alterations in lymphoid neoplasms. *Blood* 1998;92(9):3410–5.
78. Philp AJ, Campbell IG, Leet C, et al. The phosphatidylinositol 3'-kinase p85alpha gene is an oncogene in human ovarian and colon tumors. *Cancer Res* 2001;61(20):7426–9.
79. Gray JW, Suzuki S, Kuo WL, et al. Specific keynote: genome copy number abnormalities in ovarian cancer. *Gynecol Oncol* 2003;88(1 Pt 2):S16–21.
80. Mills GB, Lu Y, Fang X, et al. The role of genetic abnormalities of PTEN and the phosphatidylinositol 3-kinase pathway in breast and ovarian tumorigenesis, prognosis, and therapy. *Semin Oncol* 2001;28(5 Suppl 16):125–41.
81. Lee JW, Soung YH, Kim SY, et al. PIK3CA gene is frequently mutated in breast carcinomas and hepatocellular carcinomas. *Oncogene* 2005;24:1477–80.
82. Woenckhaus J, Steger K, Werner E, et al. Genomic gain of PIK3CA and increased expression of p110alpha are associated with progression of dysplasia into invasive squamous cell carcinoma. *J Pathol* 2002;198(3):335–42.
83. Byun DS, Cho K, Ryu BK, et al. Frequent monoallelic deletion of PTEN and its reciprocal association with PIK3CA amplification in gastric carcinoma. *Int J Cancer* 2003;104(3):318–27.
84. Kang S, Bader AG, Vogt PK. Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. *Proc Natl Acad Sci USA* 2005;102:802–7.
85. The Wellcome Trust Sanger Institute. *Cancer Genome Project: COSMIC Database* [3rd Oct 2005]: <http://www.sanger.ac.uk/genetics/CGP/cosmic/>.
86. Shayesteh L, Lu Y, Kuo WL, et al. PIK3CA is implicated as an oncogene in ovarian cancer. *Nat Genet* 1999;21(1):99–102.
87. Ma YY, Wei SJ, Lin YC, et al. PIK3CA as an oncogene in cervical cancer. *Oncogene* 2000;19(23):2739–44.
88. Massion PP, Taflan PM, Shyr Y, et al. Early involvement of the phosphatidylinositol 3-kinase/Akt pathway in lung cancer progression. *Am J Respir Crit Care Med* 2004;170:1088–94.
89. Staal SP. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: Amplification of AKT1 in a primary human gastric adenocarcinoma. *Proc Natl Acad Sci USA* 1987;84:5034–7.
90. Sun M, Wang G, Paciga JE, et al. AKT1/PKBalpha kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. *Am J Pathol* 2001;159(2):431–7.
91. Ruggeri BA, Huang L, Wood M, Cheng JQ, Testa JR. Amplification and overexpression of the AKT2 oncogene in a subset of human pancreatic ductal adenocarcinomas. *Mol Carcinog* 1998;21(2):81–6.
92. Liu AX, Testa JR, Hamilton TC, Jove R, Nicosia SV, Cheng JQ. AKT2, a member of the protein kinase B family, is activated by growth factors, v-Ha-ras, and v-src through phosphatidylinositol 3-kinase in human ovarian epithelial cancer cells. *Cancer Res* 1998;58(14):2973–7.
93. Cheng JQ, Godwin AK, Bellacosa A, et al. AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. *Proc Natl Acad Sci USA* 1992;89(19):9267–71.

94. Bellacosa A, de Feo D, Godwin AK, Bell DW, Cheng JQ, Altomare DA, Wan M, Dubeau L, Scambia G, Masciullo V, et al. Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *Int J Cancer* 1995;64(4):280–5.
95. Cheng JQ, Ruggeri B, Klein WM, et al. Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. *Proc Natl Acad Sci USA* 1996;93(8):3636–41.
96. Cheng JQ, Altomare DA, Klein MA, et al. Transforming activity and mitosis-related expression of the AKT2 oncogene: evidence suggesting a link between cell cycle regulation and oncogenesis. *Oncogene* 1997;14(23):2793–801.
97. Arboleda MJ, Lyons JF, Kabbinar FF, et al. Overexpression of AKT2/protein kinase Bbeta leads to up-regulation of beta1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells. *Cancer Res* 2003;63(1):196–206.
98. Nakatani K, Thompson DA, Barthel A, et al. Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. *J Biol Chem* 1999;274(31):21528–32.
99. Knowles MA, Hornigold N, Pitt E. Tuberous sclerosis complex (TSC) gene involvement in sporadic tumours. *Biochem Soc Trans* 2003;31(Pt 3):597–602.
100. Couch FJ, Wang XY, Wu GJ, Qian J, Jenkins RB, James CD. Localization of PS6K to chromosomal region 17q23 and determination of its amplification in breast cancer. *Cancer Res* 1999;59(7):1408–11.
101. Ermoian RP, Furniss CS, Lamborn KR, et al. Dysregulation of PTEN and protein kinase B is associated with glioma histology and patient survival. *Clin Cancer Res* 2002;8(5):1100–6.
102. Lee JI, Soria JC, Hassan KA, et al. Loss of PTEN expression as a prognostic marker for tongue cancer. *Arch Otolaryngol Head Neck Surg* 2001;127(12):1441–5.
103. Nam SY, Lee HS, Jung GA, et al. Akt/PKB activation in gastric carcinomas correlates with clinicopathologic variables and prognosis. *APMIS* 2003;111(12):1105–13.
104. Perez-Tenorio G, Stal O. Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. *Br J Cancer* 2002;86:540–5.
105. Yamamoto S, Tomita Y, Hoshida Y, et al. Prognostic significance of activated Akt expression in pancreatic ductal adenocarcinoma. *Clin Cancer Res* 2004;10(8):2846–50.
106. Brognard J, Clark AS, Ni Y, Dennis PA. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. *Cancer Res* 2001;61(10):3986–97.
107. Clark AS, West K, Streicher S, Dennis PA. Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. *Mol Cancer Ther* 2002;1:707–17.
108. Tanno S, Yanagawa N, Habiro A, et al. Serine/threonine kinase AKT is frequently activated in human bile duct cancer and is associated with increased radioresistance. *Cancer Res* 2004;64(10):3486–90.
109. Nagata Y, Lan KH, Zhou X, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell* 2004;6(2):117–27.
110. Klos KS, Zhou X, Lee S, et al. Combined trastuzumab and paclitaxel treatment better inhibits ErbB-2-mediated angiogenesis in breast carcinoma through a more effective inhibition of Akt than either treatment alone. *Cancer* 2003;98(7):1377–85.
111. Kirkegaard T, Witton CJ, McGlynn LM, et al. AKT activation predicts outcome in breast cancer patients treated with tamoxifen. *J Pathol* 2005;207(2):139–46.
112. Knuefermann C, Lu Y, Liu B, et al. HER2/PI-3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells. *Oncogene* 2003;22(21):3205–12.
113. Liang K, Jin W, Knuefermann C, et al. Targeting the phosphatidylinositol 3-kinase/Akt pathway for enhancing breast cancer cells to radiotherapy. *Mol Cancer Ther* 2003;2(4):353–60.
114. Sun SY, Rosenberg LM, Wang X, et al. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res* 2005;65(16):7052–8.
115. Kokubo Y, Gemma A, Noro R, et al. Reduction of PTEN protein and loss of epidermal growth factor receptor gene mutation in lung cancer with natural resistance to gefitinib (IRESSA). *Br J Cancer* 2005;92(9):1711–9.
116. Tsurutani J, West KA, Sayyah J, Gills JJ, Dennis PA. Inhibition of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway but not the MEK/ERK pathway attenuates laminin-mediated small cell lung cancer cellular survival and resistance to imatinib mesylate or chemotherapy. *Cancer Res* 2005;65(18):8423–32.

117. Krystal GW, Sulanke G, Litz J. Inhibition of phosphatidylinositol 3-kinase-Akt signaling blocks growth, promotes apoptosis, and enhances sensitivity of small cell lung cancer cells to chemotherapy. *Mol Cancer Ther* 2002;1(11):913–22.
118. Lee S, Choi EJ, Jin C, Kim DH. Activation of PI3K/Akt pathway by PTEN reduction and PIK3CA mRNA amplification contributes to cisplatin resistance in an ovarian cancer cell line. *Gynecol Oncol* 2005;97(1):26–34.
119. Hu L, Hofmann J, Lu Y, Mills GB, Jaffe RB. Inhibition of phosphatidylinositol 3'-kinase increases efficacy of paclitaxel in *in vitro* and *in vivo* ovarian cancer models. *Cancer Res* 2002;62(4):1087–92.
120. Lee JT Jr, Steelman LS, McCubrey JA. Phosphatidylinositol 3'-kinase activation leads to multidrug resistance protein-1 expression and subsequent chemoresistance in advanced prostate cancer cells. *Cancer Res* 2004;64(22):8397–404.
121. Coffey JC, Wang JH, Smith MJ, et al. Phosphoinositide 3-kinase accelerates postoperative tumor growth by inhibiting apoptosis and enhancing resistance to chemotherapy-induced apoptosis. Novel role for an old enemy. *J Biol Chem* 2005;280(22):20968–77.
122. Gupta AK, Cerniglia GJ, Mick R, et al. Radiation sensitization of human cancer cells *in vivo* by inhibiting the activity of PI3K using LY294002. *Int J Radiat Oncol Biol Phys* 2003;56(3):846–53.
123. Haas-Kogan DA, Prados MD, Tihan T, et al. Epidermal growth factor receptor, protein kinase B/Akt, and glioma response to erlotinib. *J Natl Cancer Inst* 2005;97(12):880–7.
124. Burchert A, Wang Y, Cai D, et al. Compensatory PI3-kinase/Akt/mTor activation regulates imatinib resistance development. *Leukemia* 2005;19(10):1774–82.
125. Liang K, Lu Y, Jin W, Ang KK, Milas L, Fan Z. Sensitization of breast cancer cells to radiation by trastuzumab. *Mol Cancer Ther* 2003;2:1113–20.
126. Grandage VL, Gale RE, Linch DC, Khwaja A. PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-kappaB, Map kinase and p53 pathways. *Leukemia* 2005;19(4):586–94.
127. Hosoi Y, Miyachi H, Matsumoto Y, et al. A phosphatidylinositol 3-kinase inhibitor wortmannin induces radioresistant DNA synthesis and sensitizes cells to bleomycin and ionizing radiation. *Int J Cancer* 1998;78(5):642–7.
128. Ng SS, Tsao M, Chow S, Hedley DW. Inhibition of phosphatidylinositol 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells. *Cancer Res* 2000;60:5451–5.
129. Knight ZA, Chiang GG, Alaimo PJ, Kenski DM, Ho CB, Coan K, Abraham RT, Shokat KM: Isoform-specific phosphoinositide 3-kinase inhibitors from an arylmorpholine scaffold. *Bioorg Med Chem* 2004;12:4749–59.
130. Wendel HG, De Stanchina E, Fridman JS, et al. Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. *Nature* 2004;428(6980):332–7.
131. Yang L, Dan HC, Sun M, et al. Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt. *Cancer Res* 2004;64(13):4394–9.
132. Kim KM, Lee YJ. Amiloride augments TRAIL-induced apoptotic death by inhibiting phosphorylation of kinases and phosphatases associated with the PI3K-Akt pathway. *Oncogene* 2005;24(3):355–66.
133. Tago K, Tsukahara F, Naruse M, Yoshioka T, Takano K. Hsp90 inhibitors attenuate effect of dexamethasone on activated NF-kB and AP-1. *Life Sci* 2004;74:1981–92.
134. Dees EC, Baker SD, O'Reilly S, et al. A phase I and pharmacokinetic study of short infusions of UCN-01 in patients with refractory solid tumors. *Clin Cancer Res* 2005;11(2 Pt 1):664–71.
135. Wendtner CM, Eichhorst BF, Hallek MJ. Advances in chemotherapy for chronic lymphocytic leukemia. *Semin Hematol* 2004;41:224–33.
136. Cohen LH, Pieterman E, van Leeuwen REW, et al. Inhibitors of prenylation of Ras and other G-proteins and their application as therapeutics. *Biochem Pharmacol* 2000;60:1061–8.
137. Sebt SM, Hamilton AD. Farnesyltransferase and geranylgeranyltransferase I inhibitors and cancer therapy: Lessons from mechanism and bench-to bedside translational studies. *Oncogene* 2000;19:6584–93.
138. Jiang K, Coppola D, Crespo NC, et al. The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. *Mol Cell Biol* 2000;20(1):139–48.
139. Dan HC, Kun J, Coppola D, et al. Phosphatidylinositol-3-OH kinase/AKT and survivin pathways as critical targets for geranylgeranyltransferase I inhibitor-induced apoptosis. *Oncogene* 2004;23:706–15.

140. Johnson ME, Howerth EW. Survivin: a bifunctional inhibitor of apoptosis protein. *Vet Pathol* 2004;41:599–607.
141. Lobell RB, Omer CA, Abrams MT, et al. Evaluation of farnesyl:protein transferase inhibitor combinations in preclinical models. *Cancer Res* 2001;61:8758–68.
142. Morgan MA, Wegner J, Aydilek E, Ganser A, Reuter CW. Synergistic cytotoxic effects in myeloid leukemia cells upon treatment with farnesyltransferase and geranylgeranyl transferase-I inhibitors. *Leukemia* 2003;17:1508–20.
143. Vara JAF, Casado E, Castro J, Cejas P, Belda-Iniesta C, Gonzalez-Baron M. PI3K/Akt signaling pathway and cancer. *Cancer Treat Rev* 2004;30:193–204.
144. Alberts SR, Schroeder M, Erlichman C, et al. Gemcitabine and ISIS-2503 for patients with locally advanced or metastatic pancreatic adenocarcinoma: a North Central Cancer Treatment Group phase II trial. *J Clin Oncol* 2004;22:4944–50.
145. Newton HB. Molecular neuro-oncology and development of targeted therapeutic strategies for brain tumors. *Expert Rev Anticancer Ther* 2004;4:105–28.
146. O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, Lane H, Hofmann F, Hicklin DJ, Ludwig DL, Baselga J, Rosen N. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res* 2006;66(3):1500–8.
147. Baselga J, Pfister D, Cooper MR, et al. Phase I studies of anti-epidermal growth factor receptor chimeric antibody C225 alone and in combination with cisplatin. *J Clin Oncol* 2000;18(4):904–14.
148. Hidalgo M, Siu LL, Nemunaitis J, et al. Phase I and pharmacologic study of OSI-774, an epidermal growth factor receptor tyrosine kinase inhibitor, in patients with advanced solid malignancies. *J Clin Oncol* 2001;19(13):3267–79.
149. Robert F, Ezekiel MP, Spencer SA, et al. Phase I study of anti-epidermal growth factor receptor antibody cetuximab in combination with radiation therapy in patients with advanced head and neck cancer. *J Clin Oncol* 2001;19(13):3234–43.
150. Huang SM, Li J, Armstrong EA, Harari PM. Modulation of radiation response and tumor-induced angiogenesis after epidermal growth factor receptor inhibition by ZD1839 (Iressa). *Cancer Res* 2002;62(15):4300–6.
151. Benhar M, Engelberg D, Levitzki A. Cisplatin-induced activation of the EGF receptor. *Oncogene* 2002;21(57):8723–31.
152. Chakravarti A, Chakladar A, Delaney M, Latham D, Loeffler J. The epidermal growth factor receptor pathway mediates resistance to sequential administration of radiation and chemotherapy in primary human glioblastoma cells in a RAS-dependent manner. *Cancer Res* 2002;62:4307–15.
153. Herbst RS, Fukuoka M, Baselga J. Gefitinib—a novel targeted approach to treating cancer. *Nat Rev Cancer* 2004;4(12):956–65.
154. Haas-Kogan DA, Prados MD, Lamborn KR, Tihan T, Berger MS, Stokoe D. Biomarkers to predict response to epidermal growth factor receptor inhibitors. *Cell Cycle* 2005;4(10):1369–72.
155. Baselga J. A new anti-ErbB2 strategy in the treatment of cancer: prevention of ligand-dependent ErbB2 receptor heterodimerization. *Cancer Cell* 2002;2:93–5.
156. Lu Y, Yu Q, Liu JH, et al. Src family protein-tyrosine kinases alter the function of PTEN to regulate phosphatidylinositol 3-kinase/AKT cascades. *J Biol Chem* 2003;278(41):40057–66.
157. Moasser MM, Basso A, Averbuch SD, Rosen N. The tyrosine kinase inhibitor ZD1839 (“Iressa”) inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. *Cancer Res* 2001;61:7184–8.
158. Chinnaiyan P, Huang S, Vallabhaneni G, et al. Mechanisms of enhanced radiation response following epidermal growth factor receptor signaling inhibition by erlotinib (Tarceva). *Cancer Res* 2005;65(8):3328–35.

18

The Advancement of Epidermal Growth Factor Receptor Inhibitors in Cancer Therapy

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SUMMARY

Epidermal growth factor receptor (EGFR) signaling is involved in various cellular processes ranging from normal growth and differentiation to oncogenesis. Targeted disruption of this pathway affords therapeutic potential in oncology, particularly in combination with conventional anticancer treatment modalities. Preclinical data across a spectrum of tumor model systems show promise for this approach, and a broad series of clinical trials that examine EGFR inhibition are currently underway or recently complete. Insights gained from early preclinical and clinical experience regarding mechanisms of EGFR action and response to inhibitors are helping to shape the rational incorporation of these new agents into modern cancer therapy.

Key Words: Epidermal growth factor receptor; radiation; apoptosis; small molecule inhibitors; monoclonal antibodies.

1. INTRODUCTION

Conventional cancer treatment involves the judicious application of surgery, radiation, and chemotherapy. Although incremental advances continue to emerge using these classic treatment modalities, increased knowledge regarding specific cellular, molecular, and genetic mechanisms of tumor initiation, growth, and progression has stimulated opportunities for new cancer treatment approaches. Improved understanding of molecular pathways involved in tumor cell growth has yielded a class of cancer therapeutic agents capable of more specific disruption of critical cellular proteins. This approach is commonly referred to as molecularly targeted biologic therapy, because unique biologic features of the underlying malignancy are exploited for therapeutic effect. As validation of this strategy, several molecularly targeted anticancer therapies have recently completed clinical trial testing and are currently entering clinical practice.

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This chapter will focus on a particularly promising molecular targeted therapy—epidermal growth factor receptor (EGFR)-signaling inhibition. The EGFR was identified as a potential anticancer target more than two decades ago following recognition that EGFR signaling is an important regulator of tumor cell growth (1). Several strategies to inhibit EGFR signaling have been subsequently tested in preclinical systems, including anti-EGFR monoclonal antibodies (mAbs) to the extracellular receptor domain, small molecule tyrosine kinase inhibitors (TKIs) targeting the intracellular kinase domain, mRNA disruption, and gene targeting (2). Advances in high throughput pharmacologic screening of mAb production have facilitated the development of an array of molecules with therapeutic potential. Two broad classes of agents that modulate EGFR signaling have emerged in clinical oncology practice: mAbs and TKIs.

To date, clinical testing of anti-EGFR agents has resulted in the approval of one mAb, cetuximab (Erbix, ImClone Systems, New York, New York), and two TKIs, gefitinib (Iressa, Astra Zeneca, London, England) and erlotinib (Tarceva, OSI/Genentech, South San Francisco, California). Although not all clinical trials have shown positive results to date, EGFR-signaling inhibition as a therapeutic approach has been validated for several clinical applications including chemotherapy-refractory colon cancer, lung cancer, and advanced head and neck cancer (3–5). Efforts to study the efficacy of these agents across a variety of other cancer disease sites are ongoing.

This chapter will focus on the preclinical development of EGFR-signaling inhibitors and examine current clinical indications for their use. Current hypotheses regarding the anticancer activity of EGFR-signaling inhibition will be presented, with particular focus on the interaction between EGFR signaling and the balance between cell survival and cell death.

2. EGFR STRUCTURE AND FUNCTION

2.1. Structure

Growth factors are soluble effector molecules of cellular proliferation, differentiation, adhesion, survival, and migration. Growth factor receptors therefore play a key role in transmitting signals from the extracellular compartment to the intracellular compartment. The EGFR was one of the earliest growth factor receptors to be characterized and sequenced (6,7). This 170-kDa glycoprotein belongs to a family of four closely related transmembrane receptors named the ErbB or HER family. Receptor structure across the family is conserved, with a characteristic cysteine-rich extracellular ligand-binding domain, a hydrophobic transmembrane domain, and a cytoplasmic tyrosine kinase domain (8,9) (Fig. 1). Family members share considerable homology in the kinase domains but are divergent in the extracellular and C-terminal domains. As the prototypical ErbB family member, the EGFR is also known as ErbB1 or HER1. The remaining family members include ErbB2 (HER2/neu), Erb3 (HER3), and ErbB4 (HER4) (10,11). ErbB family members are expressed across a wide array of tissues in a temporally and spatially distinct manner. More than 10 ligands are known to bind to the EGFR, including epidermal growth factor (EGF), transforming growth factor- α (TGF- α), heparin-binding EGF, amphiregulin, betacellulin, epiregulin, and neuregulin (9,12,13).

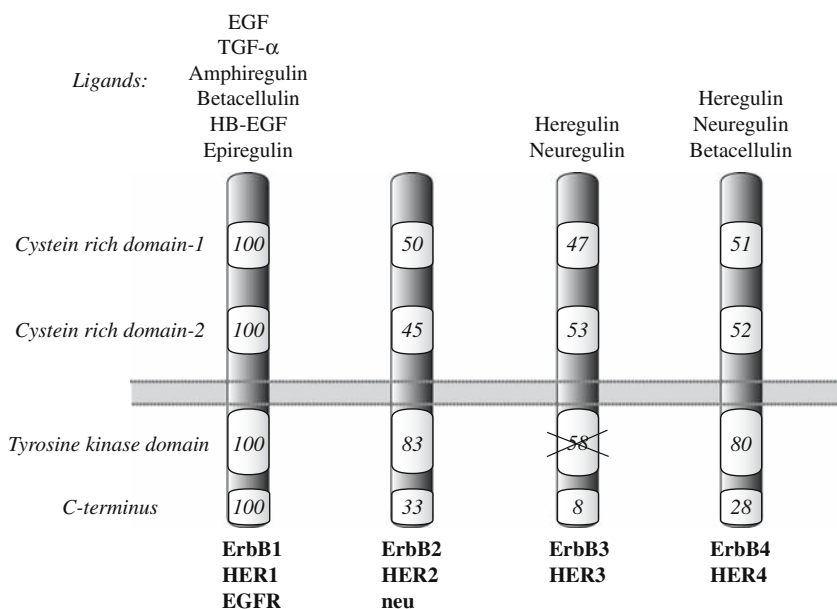


Fig. 1. Diagram demonstrating the ligands, domain structure, degree of homology, and naming convention of ErbB family members. Numbers denote the degree of homology relative to ErbB1/EGFR, expressed as a percentage. The “X” over the tyrosine kinase domain of ErbB3 indicates the lack of tyrosine kinase activity for this monomer. Adapted with permission (170).

2.2. Signaling

Signaling through the EGFR begins when a ligand binds to an ErbB monomer, triggering receptor homodimerization or heterodimerization and subsequent autophosphorylation within the cytoplasmic domain (8). Phosphorylation occurs on specific tyrosine residues and creates binding sites for Src-homology 2 and phosphotyrosine-binding domain-containing proteins, which serve as adaptors of downstream proteins involved in signal transduction. This horizontally and vertically organized network amplifies and integrates the extracellular ligand-binding events, conveying these signals to the nucleus, where genes involved in cellular proliferation and differentiation are regulated (11,14–16). Termination of the ligand-binding-initiated signal can be accomplished by endocytosis of the phosphorylated receptor–ligand complex (11).

The ErbB-signaling network has been conceptualized as a multilayer network consisting of an input layer, a signal-processing layer, and an output layer (11,16) (Fig. 2). Ligand–receptor binding is contained within the input layer. Signaling diversity within this layer arises from the different combinations of ligand–receptor interaction as well as the dimerization status (homodimer or heterodimer) of the receptor (10). Heterodimerization leads to stronger mitogenic cues, thought to result from additional phosphotyrosine residues available for receptor stabilization (17). Attenuation of signaling and altered lysosomal degradation also contribute to heterodimer signal diversity (18,19). The formation of homodimers or heterodimers is influenced by the bivalency of ErbB ligands, the differential binding affinities of these ligands, and the pH stability of the ligand–receptor complex (20–24). The signal-processing layer comprises the effector molecules downstream of the receptor. Several important

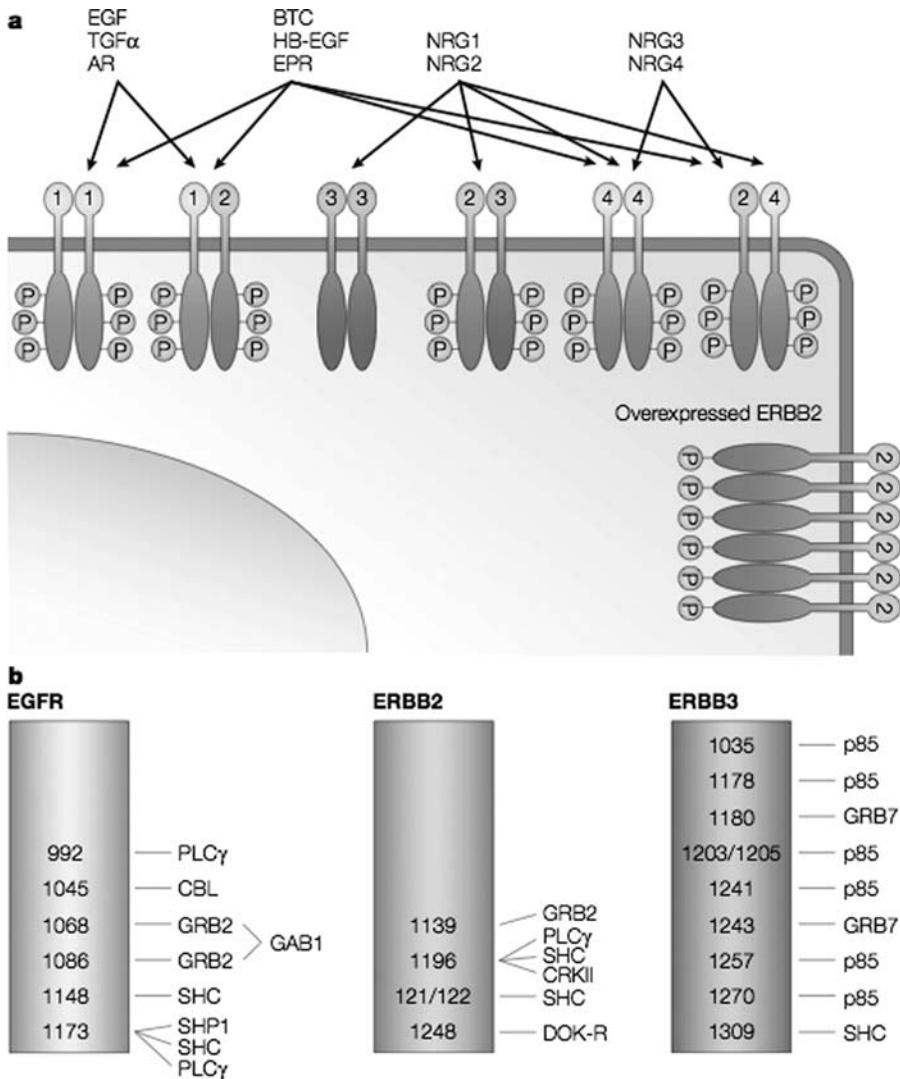


Fig. 2. (A) Epidermal growth factor family members are ligands for ErbB receptors and interact in multiple distinct homodimer and heterodimer configurations. (B) Receptor–ligand interaction induces autophosphorylation at a variety of sites within the intracellular receptor domain, leading to activation of multiple downstream signaling pathways. Used with permission (16).

signal amplification pathways are utilized, including the Ras/mitogen-activated protein kinase (MAPK), phospholipase C (PLC) γ /protein kinase C (PKC), phosphatidylinositol 3'-kinase (PI3K)/Akt, and the Jak–Stat pathways (9,25,26). Vertical transmission of signals to the nucleus as well as horizontal crosstalk between pathways amplifies and contextualizes the signal, ultimately leading to activation of nuclear transcription factors. Gene transcription in turn drives the production of proteins that mediate cellular responses including proliferation, migration, adhesion, differentiation, and apoptosis. Collectively, these diverse downstream processes comprise the network's output layer (11,16). An appreciation for the complexity of the EGFR-signaling network in the larger context of growth factor signaling is central to our understanding of organ

(and organism) development. Insights into the function of these networks will continue to yield critical information regarding the molecular pathogenesis of cancer.

3. EGFR—SIGNIFICANCE IN ONCOLOGY

3.1. Pathological Basis of EGFR Signaling During Carcinogenesis

As signaling through the EGFR is required for normal cellular proliferation, survival, adhesion, migration, and differentiation, dysregulation of this pathway can lead to oncogenesis (9,27,28). This may occur by several mechanisms that ultimately lead to hyperactivation of the EGFR and its downstream effectors. Hyperactivation of the EGFR can result from either autocrine or paracrine secretory loops, or through constitutive activation of the receptor regardless of ligand interaction. Secretory loops may become established when the EGFR receptor is overexpressed and ligands are overproduced by either the tumor or the supporting stroma (27). Receptor activation may also result from a mutated receptor, as evidenced by the extracellular-domain truncated receptor EGFRvIII, which displays constitutive tyrosine kinase activity in the absence of ligand binding (29).

Supporting the link between EGFR hyperactivation and cancer, EGFR overexpression, gene amplification, mutation, and rearrangement have been demonstrated in malignant gliomas and several other human malignancies including head and neck, lung, and breast cancers (30). Although the EGFR is normally expressed by an array of cell types, there is variability in the reported incidence of receptor overexpression or dysregulation in human malignancies (31,32) (Table 1). In clinical series of epithelial malignancies, the number of tumors with dysregulated EGFR expression ranges from 30 to 50%, variability that may well reflect a lack of standardization in measurement

Table 1
Epidermal Growth Factor Receptor (EGFR) Expression and Potential Prognostic Significance in Human Cancers, Adapted with Permission (169)

<i>Tumor type</i>	<i>Expression (%)</i>	<i>Prognostic significance</i>
NSCLC	40–80	Shorter overall survival and higher metastases
Head and neck	80–100	Shorter disease-free and overall survival
Renal cell	50–90	Poor prognosis, shorter survival in positive tumors
Bladder	31–48	More prevalent in recurrent invasive tumors
Gastric	40	Shorter survival in tumors positive for TGF- α and EGFR
Pancreatic	30–50	Coexpression with ligands associated with reduced survival
Colon	25–77	Higher proportion of tumor cells expressing EGFR associated with poor patient outcome
Breast	14–91	Associated with worse overall survival and steroid receptor-negative tumors
Ovary	35–70	Associated with reduced disease-free survival, overall survival, and drug resistance
Glioma	40–50	Most common tumor exhibiting EGFRvIII mutation

NSCLC, non-small-cell lung cancer; TGF, transforming growth factor.

techniques (33–35). A review of over 20,000 patients across 200 studies for whom EGFR expression data were available determined the prognostic significance of EGFR overexpression was highest in head and neck, ovarian, cervical, bladder and esophageal cancers. More moderate prognostic links were found for gastric, breast, endometrial, and colorectal cancers, whereas non-small-cell lung cancer (NSCLC) prognosis was only weakly associated with EGFR expression levels (32). Cellular response to EGFR inhibitors is also heterogeneous. Preclinical studies with 60 cell lines found that a high level of EGFR expression level alone did not predict well for cellular sensitivity to an array of EGFR inhibitors (36).

The EGFR promotes the establishment and maintenance of the transformed phenotype through several downstream effector pathways (Fig. 3). Activation of the Ras–MAPK pathway ultimately leads to phosphorylation of Erk1/2 and the ribosomal p70-S6 kinase. These in turn activate a variety of transcription factors known to play a role in cellular transformation, including AP1, E2F, and Sp1. The PI3K/Akt pathway activates an array of proteins, primarily through the action of Akt. The proapoptotic protein Bad is a downstream target of the Akt kinase. When Bad is phosphorylated, interaction with Bcl-2 and Bcl-X is impaired, thereby promoting cell survival. Akt furthermore promotes cell cycle progression by downregulating the cyclin-dependent kinase inhibitor p27^{KIP1} and initiates protein translation by activating mammalian target of rapamycin (mTOR) and the initiation factor 4E (17). The PLC/PKC pathway also promotes cellular growth by mediating calcium release from the endoplasmic reticulum, which in turn activates calcium-dependent protein kinases and phosphatases (17).

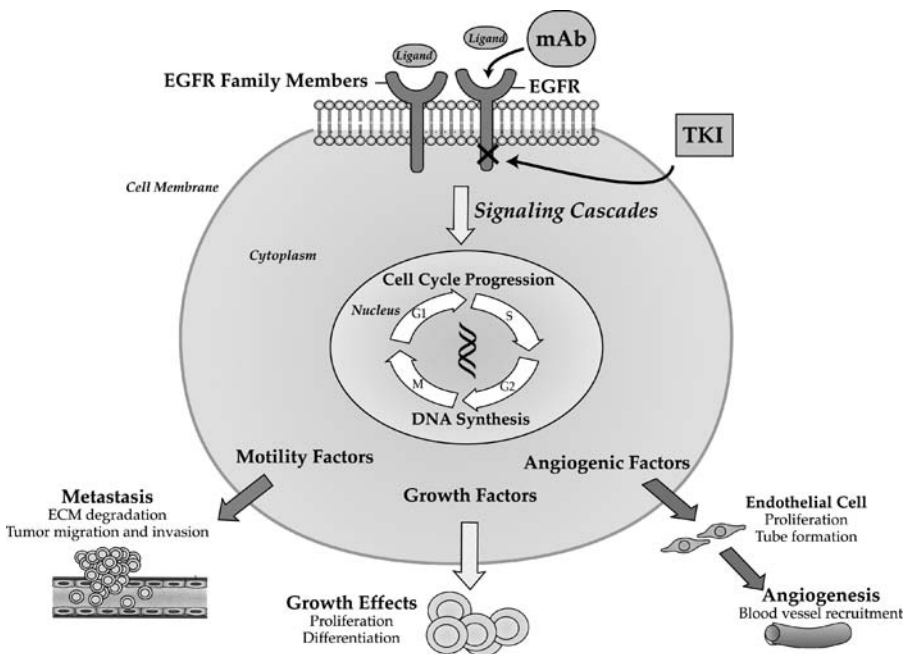


Fig. 3. Schematic of the epidermal growth factor receptor (EGFR) family and downstream pathways. Therapeutic targets for anti-EGFR mAbs and tyrosine kinase inhibitors (TKIs) include motility, growth and angiogenic factors. Adapted with permission (171).

4. PRECLINICAL STUDIES

4.1. Proliferation/Cell Cycle Control

The cellular machinery involved in cell cycle progression offers an attractive therapeutic target for cancer therapy (37). EGF is known to induce cyclin D1 expression, a protein that is required for progression from the G₁ to S phase. Studies of EGFR-signaling inhibition with cetuximab or gefitinib have demonstrated proliferation inhibition in cultured epithelial tumor cells. A primary mechanism of growth arrest is blockade of cell cycle progression at G₁ (38–45). Other mechanisms include accumulation of hypophosphorylated retinoblastoma (Rb) protein and the cyclin-dependent kinase (CDK2) inhibitor p27^{KIP1}. Accumulation of these two proteins leads to a reduction in CDK2 activity, which in turn inhibits cell cycle transition from the G₁ to S phase. Further growth arrest occurs by inhibiting CDK2 phosphorylation of Rb, which decreases E2F-mediated transcription (45–47). These results have been corroborated *in vivo* with xenograft studies in which EGFR inhibitor treatment resulted in increased expression of p27^{KIP1} (44,48). Additionally, *in vitro* experiments have demonstrated multiple cyclin-dependent kinases that are inhibited by disruption of EGFR signaling. These include CDK4, CDK6, and cyclin-D3 and lend support to a more widespread involvement of EGF signaling in cell cycle progression (49).

4.2. Apoptosis

Harnessing intrinsic cell death programs for therapeutic advantage represents another promising anticancer strategy. The EGFR-signaling axis is an important target in this regard, as EGF acts as a survival factor by inhibiting apoptosis and promoting tumor growth (50–52). The downstream effectors PI3K/Akt and MEK/Erk appear to be important mediators of EGF-induced cell survival (53). Preclinical studies of targeted EGFR-signaling inhibition demonstrate that this approach can induce or promote apoptosis in human tumor cells. As an example of this effect, treatment of human NSCLC or DiFi (colon cancer) cells in culture induces apoptosis (46,54,55). This observation is not limited to anti-EGFR antibodies, as EGFR TKIs are also effective inducers of apoptosis (56–58). Interestingly, EGFR co-targeting with a combined mAb and TKI enhanced apoptotic cell death over either strategy alone (59,60). Taken together, these findings suggest that in some cancers, EGFR inhibition leads to promotion of apoptosis resulting from activation of proapoptotic pathways, inhibition of antiapoptotic pathways, or a combination of both mechanisms.

In head and neck cancer cell lines, cetuximab triggers a rise in Bax and decrease in *bcl-2* expression (46). In normal keratinocytes, EGFR blockade led to increased expression of the *bcl-2* homolog *Bcl-x(L)* (61). Subcellular localization of Bax is also impacted by EGFR blockade, with relocation of Bax from the cytosol to the nucleus (62). Co-targeting two proapoptotic molecules, *bcl-2* and protein kinase A, in concert with EGFR-signaling inhibition led to increased apoptosis compared with each individual inhibitor, demonstrating that EGFR-signaling inhibition may be augmented by inhibition of other targets (63). Induction of apoptosis in cancer cell lines by EGFR inhibition strategies is not universal, however, as the prostate cancer cell line DU-145 does not appear to undergo apoptosis after exposure to cetuximab (43). Therefore, although not universally observed, EGFR-signaling inhibition appears to induce or promote apoptosis in a substantial proportion of human tumor cell lines.

4.3. Angiogenesis

The concept that new vessel formation (angiogenesis) is necessary for tumor growth was first proposed in 1971 (64). Angiogenesis has subsequently evolved into an important target for anticancer therapy. The formation of new blood vessels is orchestrated by several angiogenic factors, including basic fibroblast growth factor (bFGF), cytokines such as interleukin-8 (IL-8), and vascular endothelial growth factor (VEGF) (65–69). These factors are produced either by tumor cells themselves or by stromal cells including endothelial cells, fibroblasts, smooth muscle cells, or pericytes. Evidence suggests that, in addition to the aforementioned factors, signaling through the EGFR axis plays a role in the regulation of angiogenesis (48,70–73). EGF and TGF- α are known to stimulate angiogenesis, and EGFR is present in vascular endothelial cells (67,74,75). At the cellular level, activation of EGFR by EGF and TGF- α results in increased VEGF expression (76–79). Transcription of VEGF has been linked to TGF- α and is mediated by AP1/AP2 and early growth response gene 1 (EGR-1) (80). Furthermore, inhibitors of EGFR signaling result in the downregulation of VEGF, IL-8, and bFGF in a variety of cancer cell lines (48,81–83). EGFR-blocking strategies, therefore, in addition to their direct antiproliferative and proapoptotic effects, may also inhibit the growth of tumors by limiting tumor vessel formation.

4.4. Metastasis

EGFR expression has been correlated with cellular invasion or metastasis in a variety of model systems. In NSCLC lines, overexpression of EGFR and/or its ligands, as well as several metalloproteinases (MMPs), is associated with an invasive phenotype (84–88). An increase in motility and invasive potential has been demonstrated for EGF, independent of its mitogenic effects (77,87,89). In an *in vitro* model of breast cancer cell motility, macrophages were found to stimulate invasion by paracrine release of EGF (90). Autocrine effects have also been observed for a prostate cancer cell line, with an increase in invasive potential mediated by EGFR-induced urokinase-type plasminogen activator (91). Additionally, EGFR levels have been directly correlated with invasive potential in NSCLC lines (87). These effects are likely mediated through MMPs, as EGFR levels correlate directly with MMP-9 expression and vary inversely with tissue inhibitor of MMP (87). EGFR inhibition strategies diminish tumor cell invasion and downregulate MMP-9, likely by decreasing activation of MAPK and Akt (72,87,92,93). Similar findings have emerged from xenograft models. A model of human transitional cell carcinoma of the bladder treated with cetuximab led to downregulation of MMP-9 mRNA in a dose-dependent manner with complete abrogation of lymph node metastases (48). A combined antimetastasis and antiangiogenic effect was observed in a xenograft model of pancreatic cancer treated with cetuximab alone or in combination with gemcitabine (94).

4.5. Radiation Interactions

A promising role for EGFR-signaling inhibition combined with radiation therapy has emerged over the last several years. Radiation exposure induces cell death by physical means, resulting primarily from an accumulation of lethal DNA-damaging events. Strategies directed at the inhibition of other cellular targets may provide additive or synergistic therapeutic value in combination with radiation. The EGFR pathway

in particular may serve as an important mediator of radiation resistance, as several studies have demonstrated a link between EGFR expression level and cellular resistance to radiation (95–97). Supporting this hypothesis, cancer cell lines transfected with EGFR expression vectors are more radioresistant than their parental lines (98–100). Furthermore, the degree of radioresistance correlates positively with the magnitude of EGFR overexpression (101). EGFR levels and activation status may affect tumor cell survival during radiation, as EGFR and TGF- α are induced following radiation exposure (102,103). Additionally, overexpression of the constitutively active EGFRvIII also leads to enhanced radioresistance (104). Taken together, these preclinical results provide a rationale for EGFR-signaling inhibition during radiotherapy (83).

Several *in vitro* studies of EGFR blockade have demonstrated radiosensitization using a variety of experimental approaches. A broad spectrum of human cancer cells demonstrates radiosensitization with either anti-EGFR mAbs or TKIs (40,46,82,105–108). Co-targeting EGFR signaling and the downstream effectors MEK and PI3K results in synergistic radiosensitization (109). Enhanced radiation-induced apoptosis with EGFR blockade has been observed, suggesting a mechanism of EGFR-mediated radiosensitization (46,110). Data from *in vivo* xenograft models demonstrate a similar positive interaction between EGFR-signaling inhibition and irradiation (83,111–114). Well-established squamous cell carcinoma (SCC) xenografts treated with cetuximab and radiation results in potent regression of tumors in athymic mice over a 100-day follow-up period (83). A threefold enhancement in A431 tumor regression with cetuximab following single-fraction radiation exposure has been demonstrated (111). Similar results have been obtained for both gefitinib and erlotinib. Treatment of head and neck cancer xenografts with gefitinib or erlotinib plus radiation was significantly more effective than either modality alone, potentially reflecting impaired DNA repair capacity in tumors exposed to EGFR inhibitors (40,115). These data lend strong evidence that in selected preclinical tumor models, EGFR-signaling inhibition interacts positively, and perhaps synergistically, with ionizing radiation.

4.6. Chemotherapy Interactions

Considerable evidence exists regarding the capacity for EGFR-signaling inhibition to enhance cytotoxic chemotherapy in both *in vitro* and *in vivo* model systems. Favorable interactions exist for cetuximab and a variety of chemotherapeutic agents, including cisplatin, doxorubicin, paclitaxel, gemcitabine, irinotecan, and topotecan (94,116–121). Complementary data exist for the TKI gefitinib, in which favorable interactions with the aforementioned compounds exist, in addition to carboplatin, oxaliplatin, docetaxel, and etoposide (81,122,123). These favorable effects appear to exist over a broad range of cell and tumor types, including those derived from colon, pancreas, prostate, bladder, lung, head and neck, breast, and vulva (124–126). Erlotinib combined with chemotherapy has been studied in preclinical models of lung, pancreas, and head and neck cancers. When administered in combination with cisplatin and gemcitabine, erlotinib more effectively induced xenograft growth arrest than when either chemotherapy or erlotinib was administered alone (127–129). These promising preclinical data confirm that EGFR-signaling inhibition strategies may serve as clinically useful adjuncts to chemotherapy.

4.7. Biologic Therapy Interactions

Preclinical and clinical models of EGFR-signaling inhibition combined with radiation and chemotherapy have shown considerable promise; however, many patients in clinical trials still do not demonstrate a favorable response. One hypothesis for this observation is that tumors may rely on several aberrant signaling pathways for their growth advantage. This has prompted investigators to combine EGFR-signaling inhibition and other biologically targeted therapies in an effort to increase antitumor efficacy. Targeting both EGFR and HER2 pathways represents one such approach, as both ErbB family members share common downstream signaling mechanisms. Breast cancer cells expressing the estrogen receptor demonstrated growth inhibition with a combined EGFR/HER2 inhibition approach, greater than with either inhibitor alone (130). Similar antiproliferative effects were observed for this combination in hormone-refractory prostate cancer cells treated with radiation (131). Dual EGFR/HER2 targeting has also been validated in colon cancer cell lines, but only lines exhibiting autocrine EGFR release were found to be sensitive to HER2-signaling blockade (132). A dual EGFR/HER2 TKI, lapatinib (GW572016), has demonstrated cytostatic and proapoptotic effects in breast and head and neck cancer cell lines as well as radiosensitizing effects in breast cancer lines (108,133). Dual-agent EGFR inhibition combining an anti-EGFR mAb and TKI has undergone preclinical testing, with enhanced antiproliferative, proapoptotic, and radiosensitizing effects observed for this combination over either agent alone (59,60) (Fig. 4). These findings support the hypothesis that single-agent EGFR inhibitor strategies may not be sufficient to control the growth of many complex tumors, providing further rationale for combined molecular inhibitor approaches.

Targeting the EGFR- and additional growth factor-signaling pathways represents an attractive therapeutic strategy, as signaling through parallel pathways may confer resistance to EGFR inhibitor therapy and the disruption of multiple pathways may yield improved antitumor efficacy. One of the most promising dual-pathway approaches combines targeted disruption of EGFR signaling and angiogenesis. As an example, the small molecule AE788 (Novartis, Basel, Switzerland), which inhibits both EGFR and the VEGF receptor (VEGFR), has been shown effective in blocking EGF- and VEGF-mediated proliferation in a variety of cell lines and exhibits antitumor and antiangiogenic effects in xenograft tumor models (134,135). Similar results have been observed with the dual EGFR/VEGFR inhibitor ZD6474 (Astra Zeneca) (136,137). Targeting the platelet-derived growth factor receptor, an important therapy for chronic myelogenous leukemia, has also been combined with EGFR-signaling inhibition in at least one preclinical study. In a model of prostate cancer metastasis, co-targeting both receptors was found to inhibit metastatic bone involvement (138). Dual targeting of the insulin-like growth factor-1 receptor (IGF-1R) and EGFR signaling has also shown promise in a variety of preclinical tumor models (139,140). In conclusion, inhibition of EGFR signaling combined with other targeted therapies is yielding positive preclinical results with promise for eventual clinical application.

In head and neck cancer, co-targeting EGFR signaling and signal transducers and activators of transcription (STAT) is under current investigation. STAT3 has become a particular focus of attention as a direct mediator of EGFR-induced cell cycle progression and apoptosis inhibitor (141). The intracellular domain of EGFR physically interacts with STAT3 in the nucleus, leading to transcriptional activation of inducible

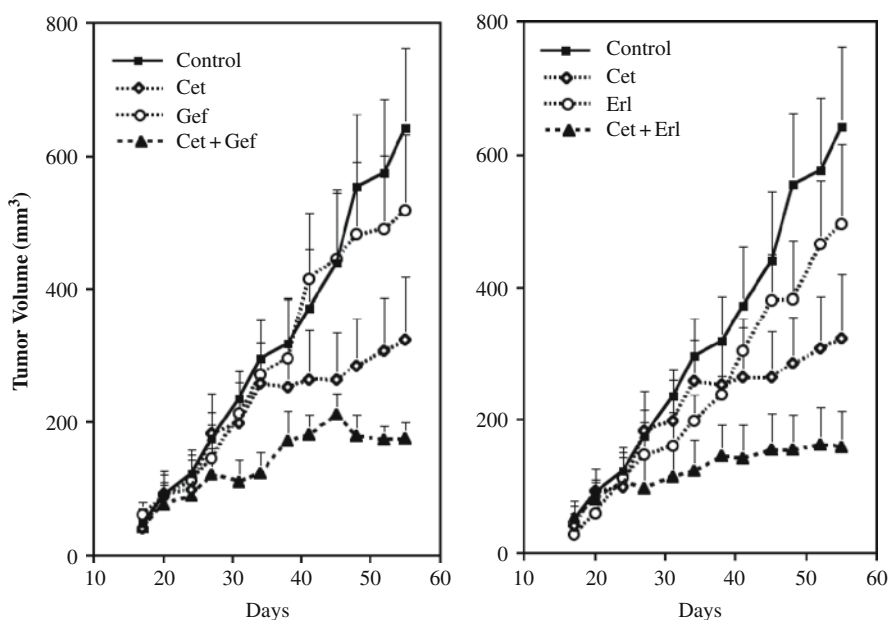


Fig. 4. Antitumor activity of cetuximab in combination with gefitinib or erlotinib in H226 tumor xenografts. After establishment of grafts, animals received either control or experimental treatments twice weekly for a total of eight treatments. Values indicate mean tumor size ($n = 8$ per group). Adapted with permission (59).

nitric oxide synthase (iNOS) (142). Other reports suggest that inhibitors of c-Met and ErbB3 may interact favorably with EGFR inhibition strategies, as these molecules are overexpressed in cell lines resistant to EGFR-signaling inhibition (143,144).

5. CLINICAL STUDIES

The two dominant EGFR inhibition strategies under clinical investigation include the anti-EGFR mAbs and TKIs. Although EGFR-signaling inhibition is successfully accomplished with either class of EGFR inhibitor, several important distinctions between them exist. The pharmacokinetic profiles of mAbs and TKIs vary widely. As they are large macromolecular proteins, mAbs are susceptible to degradation in the gastrointestinal tract, and therefore, oral administration is not effective. mAbs have relatively long half-lives and thus weekly intravenous infusion is the preferred method of clinical administration. The TKIs, by contrast, are small molecules that are effectively absorbed across the gastrointestinal tract; thus oral administration is routine and the short half-life requires daily administration. Specific mechanisms of action for each anti-EGFR class also vary in several subtle but important ways. The mAbs target the extracellular ligand-binding domain of EGFR, whereas the TKIs interact with the catalytic domain in the intracellular compartment. Antibodies binding to the extracellular domain prompt receptor internalization and may stimulate host immune responses to contribute to the therapeutic outcome. TKIs show some degree of cross-reactivity to a variety of tyrosine kinases, which may contribute to their ultimate clinical impact.

Anti-EGFR mAbs and TKIs have been evaluated in clinical trials, both as single agents and in combination with conventional radiation or chemotherapy. Combination strategies are a logical choice due to the non-overlapping action mechanisms and toxicity profiles of targeted EGFR-signaling inhibition and conventional radiation or chemotherapy. The preclinical data summarized above provide strong support for clinical trials examining combination therapies. Selected clinical trials of EGFR inhibitors are briefly reviewed.

5.1. mAb EGFR Inhibitors

5.1.1. CETUXIMAB

Cetuximab, formerly known as IMC-225 or C225, is a chimeric mouse anti-EGFR mAb that received US Food and Drug Administration (FDA) approval in 2004 for the treatment of irinotecan-refractory colorectal cancer. Cetuximab is the most widely studied anti-EGFR mAb and is the first agent in this class approved for clinical use. Early phase I and I/II trials demonstrated the safety of cetuximab alone or in combination with cytotoxic chemotherapy for patients with metastatic squamous cell carcinoma of the head and neck, colorectal cancer, and NSCLC (145–148).

Efficacy for concomitant cetuximab and chemotherapy was established in a phase II trial in colorectal cancer patients. Patients with metastatic colon cancer refractory to irinotecan ($n = 121$) received concurrent cetuximab and irinotecan, with partial responses observed in 23% and stable disease in 31% (4). A larger European phase II study (the Bowel Oncology with Cetuximab Antibody (BOND) trial) examined the combination of cetuximab and irinotecan ($n = 218$) versus cetuximab alone ($n = 111$) for patients with EGFR-positive irinotecan-refractory metastatic colon cancer (149). Response rates were higher for the combined therapy group (22.9%) versus the cetuximab monotherapy group (10.8%). Median survival was also higher in the combined group (8.6 months versus 6.9 months), although this did not reach statistical significance. Two phase III studies have been initiated in the USA. The Erbitux Plus Irinotecan in Colorectal Cancer (EPIC) trial will examine the combination of cetuximab and irinotecan versus irinotecan monotherapy as second-line treatment for patients with metastatic EGFR-positive colorectal cancer. The Cetuximab plus FOLFOX for Colorectal Cancer (EXPLORE) trial will determine whether patients with EGFR-positive metastatic colorectal cancer will benefit from concurrent cetuximab and 5-fluorouracil/leucovorin/oxaliplatin (FOLFOX) compared with the standard FOLFOX regimen.

Results of an international phase III randomized trial of cetuximab in advanced head and neck cancer have been reported (3). In this study, 424 patients received either definitive radiation therapy alone or the same treatment with concurrent cetuximab. At a median follow-up of 54 months, near doubling of the median survival was observed in patients receiving cetuximab versus patients receiving radiation therapy alone (49 versus 29 months, $p = 0.03$). Statistically significant increases in locoregional control and progression-free survival were also reported for the group receiving cetuximab. This landmark trial provided the first demonstration of clinical efficacy for combined EGFR-signaling inhibition and radiation therapy, providing rationale for further clinical investigation with a variety of anti-EGFR compounds. Furthermore, this is the first study to demonstrate a statistically significant survival benefit for an

anti-EGFR antibody. As a result, in March 2006, the US FDA granted full regulatory approval for the use of cetuximab combined with radiation in the treatment of locoregionally advanced head and neck cancer.

5.1.2. PANITUMUMAB

Panitumumab (rHuMAB-EGFr, Amgen, Thousand Oaks, California), formerly known as ABX-EGF, is a fully human anti-EGFR mAb that has undergone early clinical trials testing in prostate cancer (150). Interestingly, panitumumab appeared to inhibit angiogenesis in this study, validating preclinical models linking EGFR inhibition and angiogenesis. A phase II trial of panitumumab monotherapy in patients with renal cell carcinoma refractory to IL-2/interferon- α demonstrated two objective responses out of 58 patients, with 58% of patients experiencing stable disease. A multicenter phase II trial of panitumumab monotherapy was conducted in 44 patients with metastatic colorectal cancer (151). Stable disease was observed in 52%, whereas 9% had a partial response and 39% experienced disease progression. Phase II trials of panitumumab monotherapy are ongoing in hormone-resistant prostate cancer, colorectal cancer (first line), and NSCLC. A phase III trial of third-line panitumumab in chemotherapy-refractory metastatic colorectal cancer was opened in 2004.

5.1.3. OTHER MABS

Several additional anti-EGFR mAbs are currently under development. The majority of these mAbs are fully human, as opposed to the first generation chimeric antibody cetuximab. Advances in antibody-screening technology have made the selection of high-affinity fully human antibodies more feasible, further lessening the potential for a host immune response directed against the mAb. Several bispecific antibodies, co-targeting EGFR and other growth factor receptors, are also in development (Table 2).

5.2. TKI EGFR Inhibitors

5.2.1. GEFITINIB

Originally known as ZD1839, gefitinib is approved in the USA for single-agent use in the treatment of chemotherapy-refractory NSCLC. Gefitinib was the first anti-EGFR TKI approved by the FDA for use in cancer therapy. Gefitinib is an effective inhibitor of EGFR kinase activity, with an IC_{50} in the nanomolar range *in vitro*; however,

Table 2
Epidermal Growth Factor Receptor (EGFR) Monoclonal Antibodies in Clinical Trials

<i>Agent</i>	<i>Type</i>	<i>Generic/trade name</i>	<i>Institution</i>
IMC-C225	Chimeric IgG1	cetuximab/Erbitux	ImClone/BMS/Merck
ABX-EGF	Fully human IgG2	panitumumab	Abgenix/Amgen
EMD 72000	Humanized IgG1		EMD Pharms/Merck
MDX-447	Bispecific EGFR/FcRy1	HuMab-Mouse	Medarex/Merck
h-R3	Humanized	TheraCIM	YM Biosciences/CIM
Mab 806	Anti-EGFR VIII		Ludwig Institute

higher *in vivo* concentrations may be required because of competition with intracellular ATP (152). Gefitinib is also known to inhibit the HER2 kinase, albeit at levels 100-fold greater than those required for EGFR-signaling inhibition (126,152,153).

A number of single-agent phase II clinical trials have been carried out using gefitinib in a range of solid tumor malignancies including head and neck cancer, renal cell carcinoma, and advanced breast cancer (154–159). Most notably, the Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL-1) and IDEAL-2 trials in NSCLC validated the single-agent efficacy of gefitinib in patients with refractory NSCLC, resulting in FDA approval. Two subsequent phase III trials, Iressa NSCLC Trial Assessing Combination Treatment (INTACT-1) ($n = 1093$) and INTACT-2 ($n = 1037$), failed to demonstrate a significant benefit for the concurrent addition of gefitinib to standard chemotherapy in advanced lung cancer (160,161). The lack of efficacy in these large phase III trials was disappointing in light of the positive results seen in the phase II trials. Some have argued that by not requiring verification of EGFR positivity for entrance into the trial, potential drug effects may have been diluted, even though quantitative EGFR status has not yet been linked to EGFR inhibitor response (157,162).

With the knowledge that a subset of patients with lung cancer exhibited dramatic responses to gefitinib, the existence of EGFR mutations was hypothesized. Two independent groups subsequently demonstrated the presence of multiple in-frame deletions or amino acid substitutions in the EGFR tyrosine kinase domain that strongly predict for positive response to gefitinib (163,164). Interestingly, these mutations were found to be most prevalent in patients who were Japanese, female, and non-smokers and whose tumor histologies were bronchoalveolar or adenocarcinomas with bronchoalveolar features. Efforts are underway to identify additional EGFR mutations and to determine whether EGFR catalytic domain mutations may ultimately serve as a clinical tool in the selection of patients for EGFR TKI therapy.

5.2.2. ERLOTINIB

Erlotinib, also known as OSI-774, was the second EGFR TKI approved by the FDA for clinical application. Like gefitinib, erlotinib is a potent inhibitor of EGFR autophosphorylation, with an IC_{50} in the nanomolar range *in vitro*. Similar to gefitinib, erlotinib demonstrates activity against HER2 and has been found to be particularly effective at inhibiting signaling through the mutant EGFRvIII receptor (165,166). Two phase III trials in NSCLC were conducted with a design similar to that of the INTACT-1 and INTACT-2 trials for gefitinib. The Tarceva Lung Cancer Investigation (TALENT) and Tarceva Responses in Conjunction with Paclitaxel and Carboplatin (TRIBUTE) trials examined the efficacy of adding concurrent erlotinib to standard chemotherapy in advanced lung cancer (167,168). As with the INTACT trials, primary survival endpoints were not significantly improved with the addition of erlotinib, although in the TRIBUTE study, the secondary endpoint of time to symptomatic progression was improved. The failure of these trials to demonstrate a benefit for erlotinib in NSCLC parallels findings from the INTACT trials, with similar concerns regarding the difficulty in achieving statistical significance for primary clinical endpoints in the absence of more stringent patient selection.

The National Cancer Institute of Canada Clinical Trials Group recently concluded a 700-patient phase III trial of erlotinib monotherapy versus placebo in second- or third-line metastatic NSCLC. Whereas the INTACT and TALENT/TRIBUTE trials

Table 3
Epidermal Growth Factor Receptor (EGFR) Tyrosine Kinase Inhibitors (TKIs)
in Clinical Trials

<i>Agent</i>	<i>Type</i>	<i>Generic/trade name</i>	<i>Institution</i>
ZD1839	erbB1	Gefitinib/Iressa	AstraZeneca
OSI-774	erbB1	Erlotinib/Tarceva	OSI/Genentech/Roche
CI-1033	pan erbB	Canertinib	Pfizer
EKB-569	erbB1/2		Wyeth Ayerst
GW2016	erbB1/2	Lapatinib	GlaxoSmithKline
PKI-166	erbB1/2		Novartis

studied whether adding concurrent gefitinib or erlotinib to chemotherapy was effective in advanced (non-metastatic) NSCLC, the Canadian trial examined erlotinib as monotherapy in the setting of chemotherapy-refractory metastatic disease. Unlike the previous trials, the primary endpoint of overall survival was significantly improved for patients receiving erlotinib compared with those receiving placebo (6.7 versus 4.7 months). Additionally, the secondary endpoints of time to symptomatic progression, progression-free survival and response rate were all statistically significant (5).

5.2.3. OTHER TKIs

In addition to gefitinib and erlotinib, several additional TKIs are under preclinical and clinical development (Table 3). Generally, these inhibitors have been developed to target the EGFR as well as other ErbB family members, most notably ErbB2. Canertinib (CI-1033, Pfizer Pharmaceuticals, New York, New York) is unique in this regard as it is a pan-ErbB family inhibitor capable of influencing all four ErbB family receptors.

6. CONCLUSION

Molecularly targeted biologic therapy offers the promise of new treatment tools that may be used alone or in combination with conventional cancer therapy modalities. EGFR-signaling inhibition represents one of the emerging strategies within this new cancer treatment paradigm. Our improved understanding of normal and dysregulated EGFR signaling provides sound rationale for targeted disruption of this growth receptor. Dysregulated EGFR signaling impacts a variety of oncogenic processes including proliferation, differentiation, migration, metastasis, apoptosis, and angiogenesis. Disruption of EGFR signaling has gained strong validation in preclinical tumor models and is undergoing extensive clinical testing. The results to date suggest that targeted EGFR inhibition will offer a valuable new therapeutic approach for selected patients with head and neck cancer, colorectal cancer, and NSCLC. The recent identification of EGFR mutations conferring sensitivity to TKIs has set the foundation for continued investigation of more specific molecular predictors of disease response. Ultimately, approaches such as DNA microarray and proteomic analyses may enable tumors to be better characterized at the molecular level, uncovering a “molecular phenotype” of EGFR inhibitor response that may be used to more rationally select patients for anti-EGFR therapies.

REFERENCES

1. Gill GN, Kawamoto T, Cochet C, et al. Monoclonal anti-epidermal growth factor receptor antibodies which are inhibitors of epidermal growth factor binding and antagonists of epidermal growth factor binding and antagonists of epidermal growth factor-stimulated tyrosine protein kinase activity. *J Biol Chem* 1984; 259, 7755–7760.
2. Kim ES, Khuri FR, Herbst RS. Epidermal growth factor receptor biology (IMC-C225). *Curr Opin Oncol* 2001; 13, 506–513.
3. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med* 2006; 354, 567–578.
4. Saltz L, Rubin M, Hochster H, et al. Cetuximab (IMC-C225) plus irinotecan (CPT-11) is active in CPT-11-refractory colorectal cancer (CRC) that expresses epidermal growth factor receptor (EGFR). *Proc Am Soc Clin Oncol* 2001; 20, Abstract 7.
5. Shepherd F, Pereira J, Ciuleanu E, et al. A randomized placebo-controlled trial of erlotinib in patients with advanced non-small cell lung cancer (NSCLC) following failure of 1st line or 2nd line chemotherapy. A National Cancer Institute of Canada Clinical Trials Group (NCIC CTG) trial. *Proc Am Soc Clin Oncol* 2004; 22, 7022.
6. Cohen S, Carpenter G. Human epidermal growth factor: isolation and chemical and biological properties. *Proc Natl Acad Sci USA* 1975; 72, 1317–1321.
7. Ullrich A, Coussens L, Hayflick JS, et al. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 1984; 309, 418–425.
8. Carpenter G, Cohen S. Epidermal growth factor. *J Biol Chem* 1990; 265, 7709–7712.
9. Wells A. EGF receptor. *Int J Biochem Cell Biol* 1999; 31, 637–643.
10. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J* 2000; 19, 3159–3167.
11. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001; 2, 127–137.
12. Toyoda H, Komurasaki T, Uchida D, Morimoto S. Distribution of mRNA for human epiregulin, a differentially expressed member of the epidermal growth factor family. *Biochem J* 1997; 326, 69–75.
13. Watanabe T, Shintani A, Nakata M, et al. Recombinant human betacellulin. Molecular structure, biological activities, and receptor interaction. *J Biol Chem* 1994; 269, 9966–9973.
14. Bogdan S, Klambt C. Epidermal growth factor receptor signaling. *Curr Biol* 2001; 11, R292–R295.
15. Moghal N, Sternberg PW. Multiple positive and negative regulators of signaling by the EGF-receptor. *Curr Opin Cell Biol* 1999; 11, 190–196.
16. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 2005; 5, 341–354.
17. Marmor MD, Skaria KB, Yarden Y. Signal transduction and oncogenesis by ErbB/HER receptors. *Int J Radiat Oncol Biol Phys* 2004; 58, 903–913.
18. Lenferink AE, Pinkas-Kramarski R, van de Poll ML, et al. Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. *EMBO J* 1998; 17, 3385–3397.
19. Worthyly R, Opreko LK, Wiley HS. ErbB-2 amplification inhibits down-regulation and induces constitutive activation of both ErbB-2 and epidermal growth factor receptors. *J Biol Chem* 1999; 274, 8865–8874.
20. Beerli RR, Hynes NE. Epidermal growth factor-related peptides activate distinct subsets of ErbB receptors and differ in their biological activities. *J Biol Chem* 1996; 271, 6071–6076.
21. French AR, Tadaki DK, Niyogi SK, Lauffenburger DA. Intracellular trafficking of epidermal growth factor family ligands is directly influenced by the pH sensitivity of the receptor/ligand interaction. *J Biol Chem* 1995; 270, 4334–4340.
22. Riese DJ 2nd, Bermingham Y, van Raaij TM, Buckley S, Plowman GD, Stern DF. Betacellulin activates the epidermal growth factor receptor and erbB-4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-beta. *Oncogene* 1996; 12, 345–353.
23. Tzahar E, Moyer JD, Waterman H, et al. Pathogenic poxviruses reveal viral strategies to exploit the ErbB signaling network. *EMBO J* 1998; 17, 5948–5963.
24. Waterman H, Sabanai I, Geiger B, Yarden Y. Alternative intracellular routing of ErbB receptors may determine signaling potency. *J Biol Chem* 1998; 273, 13819–13827.

25. Carpenter G. The EGF receptor: a nexus for trafficking and signaling. *Bioessays* 2000; 22, 697–707.
26. Grant S, Qiao L, Dent P. Roles of ERBB family receptor tyrosine kinases, and downstream signaling pathways, in the control of cell growth and survival. *Front Biosci* 2002; 7, d376–d389.
27. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 1995; 19, 183–232.
28. Woodburn JR. The epidermal growth factor receptor and its inhibition in cancer therapy. *Pharmacol Ther* 1999; 82, 241–250.
29. Moscatello DK, Montgomery RB, Sundareshan P, McDanel H, Wong MY, Wong AJ. Transformatonal and altered signal transduction by a naturally occurring mutant EGF receptor. *Oncogene* 1996; 13, 85–96.
30. Rasheed BK, Wiltshire RN, Bigner SH, Bigner DD. Molecular pathogenesis of malignant gliomas. *Curr Opin Oncol* 1999; 11, 162–167.
31. Arteaga CL. Epidermal growth factor receptor dependence in human tumors: more than just expression? *Oncologist* 2002; 7 Suppl 4, 31–39.
32. Nicholson RI, Gee JM, Harper ME. EGFR and cancer prognosis. *Eur J Cancer* 2001; 37 Suppl 4, S9–S15.
33. Aaronson SA. Growth factors and cancer. *Science* 1991; 254, 1146–1153.
34. Earp HS 3rd, Calvo BF, Sartor CI. The EGF receptor family – multiple roles in proliferation, differentiation, and neoplasia with an emphasis on HER4. *Trans Am Clin Climatol Assoc* 2003; 114, 315–333; discussion 333–334.
35. Grunwald V, Hidalgo M. Developing inhibitors of the epidermal growth factor receptor for cancer treatment. *J Natl Cancer Inst* 2003; 95, 851–867.
36. Bishop PC, Myers T, Robey R, et al. Differential sensitivity of cancer cells to inhibitors of the epidermal growth factor receptor family. *Oncogene* 2002; 21, 119–127.
37. Giordano A, Rustum YM, Wenner CE. Cell cycle: molecular targets for diagnosis and therapy: tumor suppressor genes and cell cycle progression in cancer. *J Cell Biochem* 1998; 70, 1–7.
38. Anderson NG, Ahmad T, Chan K, Dobson R, Bundred NJ. ZD1839 (Iressa), a novel epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, potently inhibits the growth of EGFR-positive cancer cell lines with or without erbB2 overexpression. *Int J Cancer* 2001; 94, 774–782.
39. Chan KC, Knox WF, Gandhi A, Slamon DJ, Potten CS, Bundred NJ. Blockade of growth factor receptors in ductal carcinoma in situ inhibits epithelial proliferation. *Br J Surg* 2001; 88, 412–418.
40. Huang SM, Li J, Armstrong EA, Harari PM. Modulation of radiation response and tumor-induced angiogenesis after epidermal growth factor receptor inhibition by ZD1839 (Iressa). *Cancer Res* 2002; 62, 4300–4306.
41. Kiyota A, Shintani S, Mihara M, et al. Anti-epidermal growth factor receptor monoclonal antibody 225 upregulates p27(KIP1) and p15(INK4B) and induces G1 arrest in oral squamous carcinoma cell lines. *Oncology* 2002; 63, 92–98.
42. Lichtner RB, Menrad A, Sommer A, Klar U, Schneider MR. Signaling-inactive epidermal growth factor receptor/ligand complexes in intact carcinoma cells by quinazoline tyrosine kinase inhibitors. *Cancer Res* 2001; 61, 5790–5795.
43. Peng D, Fan Z, Lu Y, DeBlasio T, Scher H, Mendelsohn J. Anti-epidermal growth factor receptor monoclonal antibody 225 up-regulates p27KIP1 and induces G1 arrest in prostatic cancer cell line DU145. *Cancer Res* 1996; 56, 3666–3669.
44. Shintani S, Li C, Mihara M, et al. Gefitinib ('Iressa', ZD1839), an epidermal growth factor receptor tyrosine kinase inhibitor, up-regulates p27KIP1 and induces G1 arrest in oral squamous cell carcinoma cell lines. *Oral Oncol* 2004; 40, 43–51.
45. Wu X, Rubin M, Fan Z, et al. Involvement of p27KIP1 in G1 arrest mediated by an anti-epidermal growth factor receptor monoclonal antibody. *Oncogene* 1996; 12, 1397–1403.
46. Huang SM, Bock JM, Harari PM. Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radiosensitivity in squamous cell carcinomas of the head and neck. *Cancer Res* 1999; 59, 1935–1940.
47. Raben D, Helfrich BA, Chan D, Johnson G, Bunn PAJ. ZD1839, a selective epidermal growth factor receptor tyrosine kinase inhibitor, alone and in combination with radiation and chemotherapy as a new therapeutic strategy in non-small cell lung cancer. *Semin Oncol* 2002; 29, 37–46.
48. Perrotte P, Matsumoto T, Inoue K, et al. Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res* 1999; 5, 257–265.

49. Chang GC, Hsu SL, Tsai JR, et al. Molecular mechanisms of ZD1839-induced G1-cell cycle arrest and apoptosis in human lung adenocarcinoma A549 cells. *Biochem Pharmacol* 2004; 68, 1453–1464.
50. Gibson EM, Henson ES, Haney N, Villanueva J, Gibson SB. Epidermal growth factor protects epithelial-derived cells from tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by inhibiting cytochrome c release. *Cancer Res* 2002; 62, 488–496.
51. Gibson S, Tu S, Oyer R, Anderson SM, Johnson GL. Epidermal growth factor protects epithelial cells against Fas-induced apoptosis. Requirement for Akt activation. *J Biol Chem* 1999; 274, 17612–17618.
52. Takeuchi K, Ito F. Suppression of adriamycin-induced apoptosis by sustained activation of the phosphatidylinositol-3'-OH kinase-Akt pathway. *J Biol Chem* 2004; 279, 892–900.
53. Ramljak D, Coticchia CM, Nishanian TG, et al. Epidermal growth factor inhibition of c-Myc-mediated apoptosis through Akt and Erk involves Bcl-xL upregulation in mammary epithelial cells. *Exp Cell Res* 2003; 287, 397–410.
54. Modjtahedi H, Affleck K, Stubberfield C, Dean C. EGFR blockade by tyrosine kinase inhibitor or monoclonal antibody inhibits growth, directs terminal differentiation and induces apoptosis in the human squamous cell carcinoma HN5. *Int J Oncol* 1998; 13, 335–342.
55. Wu X, Fan Z, Masui H, Rosen N, Mendelsohn J. Apoptosis induced by an anti-epidermal growth factor receptor monoclonal antibody in a human colorectal carcinoma cell line and its delay by insulin. *J Clin Invest* 1995; 95, 1897–1905.
56. Gilmore AP, Valentijn AJ, Wang P, et al. Activation of BAD by therapeutic inhibition of epidermal growth factor receptor and transactivation by insulin-like growth factor receptor. *J Biol Chem* 2002; 277, 27643–27650.
57. Karnes WEJ, Weller SG, Adjei PN, et al. Inhibition of epidermal growth factor receptor kinase induces protease-dependent apoptosis in human colon cancer cells. *Gastroenterology* 1998; 114, 930–939.
58. Moyer JD, Barbacci EG, Iwata KK, et al. Induction of apoptosis and cell cycle arrest by CP-358,774, an inhibitor of epidermal growth factor receptor tyrosine kinase. *Cancer Res* 1997; 57, 4838–4848.
59. Huang S, Armstrong EA, Benavente S, Chinnaiyan P, Harari PM. Dual-agent molecular targeting of the epidermal growth factor receptor (EGFR): combining anti-EGFR antibody with tyrosine kinase inhibitor. *Cancer Res* 2004; 64, 5355–5362.
60. Matar P, Rojo F, Cassia R, et al. Combined epidermal growth factor receptor targeting with the tyrosine kinase inhibitor gefitinib (ZD1839) and the monoclonal antibody cetuximab (IMC-C225): superiority over single-agent receptor targeting. *Clin Cancer Res* 2004; 10, 6487–6501.
61. Jost M, Class R, Kari C, Jensen PJ, Rodeck U. A central role of Bcl-X(L) in the regulation of keratinocyte survival by autocrine EGFR ligands. *J Invest Dermatol* 1999; 112, 443–449.
62. Mandal M, Adam L, Mendelsohn J, Kumar R. Nuclear targeting of Bax during apoptosis in human colorectal cancer cells. *Oncogene* 1998; 17, 999–1007.
63. Tortora G, Caputo R, Damiano V, et al. Combined targeted inhibition of bcl-2, bcl-XL, epidermal growth factor receptor, and protein kinase A type I causes potent antitumor, apoptotic, and antiangiogenic activity. *Clin Cancer Res* 2003; 9, 866–871.
64. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971; 285, 1182–1186.
65. Bouma-ter Steege JC, Mayo KH, Griffioen AW. Angiostatic proteins and peptides. *Crit Rev Eukaryot Gene Expr* 2001; 11, 319–334.
66. Eisma RJ, Spiro JD, Kreutzer DL. Role of angiogenic factors: coexpression of interleukin-8 and vascular endothelial growth factor in patients with head and neck squamous carcinoma. *Laryngoscope* 1999; 109, 687–693.
67. Fox SB, Gatter KC, Harris AL. Tumour angiogenesis. *J Pathol* 1996; 179, 232–237.
68. Shemirani B, Crowe DL. Head and neck squamous cell carcinoma lines produce biologically active angiogenic factors. *Oral Oncol* 2000; 36, 61–66.
69. Smith BD, Haffty BG, Sasaki CT. Molecular markers in head and neck squamous cell carcinoma: their biological function and prognostic significance. *Ann Otol Rhinol Laryngol* 2001; 110, 221–228.
70. Ellis LM. Epidermal growth factor receptor in tumor angiogenesis. *Hematol Oncol Clin North Am* 2004; 18, 1007–21, viii.
71. Harris VK, Coticchia CM, Kagan BL, Ahmad S, Wellstein A, Riegel AT. Induction of the angiogenic modulator fibroblast growth factor-binding protein by epidermal growth factor is mediated through both MEK/ERK and p38 signal transduction pathways. *J Biol Chem* 2000; 275, 10802–10811.

72. Huang SM, Li J, Harari PM. Molecular inhibition of angiogenesis and metastatic potential in human squamous cell carcinomas after epidermal growth factor receptor blockade. *Mol Cancer Ther* 2002; 1, 507–514.
73. Petit AM, Rak J, Hung MC, et al. Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells *in vitro* and *in vivo*: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* 1997; 151, 1523–1530.
74. Kim SJ, Uehara H, Karashima T, Shepherd DL, Killion JJ, Fidler IJ. Blockade of epidermal growth factor receptor signaling in tumor cells and tumor-associated endothelial cells for therapy of androgen-independent human prostate cancer growing in the bone of nude mice. *Clin Cancer Res* 2003; 9, 1200–1210.
75. Schreiber AB, Winkler ME, Derynck R. Transforming growth factor-alpha: a more potent angiogenic mediator than epidermal growth factor. *Science* 1986; 232, 1250–1253.
76. Bancroft CC, Chen Z, Yeh J, et al. Effects of pharmacologic antagonists of epidermal growth factor receptor, PI3K and MEK signal kinases on NF-kappaB and AP-1 activation and IL-8 and VEGF expression in human head and neck squamous cell carcinoma lines. *Int J Cancer* 2002; 99, 538–548.
77. O-charoenrat P, Rhys-Evans P, Modjtahedi H, Eccles SA. Vascular endothelial growth factor family members are differentially regulated by c-erbB signaling in head and neck squamous carcinoma cells. *Clin Exp Metastasis* 2000; 18, 155–161.
78. O-charoenrat P, Rhys-Evans PH, Archer DJ, Eccles SA. C-erbB receptors in squamous cell carcinomas of the head and neck: clinical significance and correlation with matrix metalloproteinases and vascular endothelial growth factors. *Oral Oncol* 2002; 38, 73–80.
79. Ravindranath N, Wion D, Brachet P, Djakiew D. Epidermal growth factor modulates the expression of vascular endothelial growth factor in the human prostate. *J Androl* 2001; 22, 432–443.
80. Gille J, Swerlick RA, Caughman SW. Transforming growth factor-alpha-induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP-2-dependent DNA binding and transactivation. *EMBO J* 1997; 16, 750–759.
81. Ciardiello F, Caputo R, Bianco R, et al. Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin Cancer Res* 2001; 7, 1459–1465.
82. Hirata A, Ogawa S, Kometani T, et al. ZD1839 (Iressa) induces antiangiogenic effects through inhibition of epidermal growth factor receptor tyrosine kinase. *Cancer Res* 2002; 62, 2554–2560.
83. Huang SM, Harari PM. Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. *Clin Cancer Res* 2000; 6, 2166–2174.
84. Helliwell TR. Molecular markers of metastasis in squamous carcinomas. *J Pathol* 2001; 194, 289–293.
85. Juarez J, Clayman G, Nakajima M, et al. Role and regulation of expression of 92-kDa type-IV collagenase (MMP-9) in 2 invasive squamous-cell-carcinoma cell lines of the oral cavity. *Int J Cancer* 1993; 55, 10–18.
86. Kusakawa J, Harada H, Shima I, Sasaguri Y, Kameyama T, Morimatsu M. The significance of epidermal growth factor receptor and matrix metalloproteinase-3 in squamous cell carcinoma of the oral cavity. *Eur J Cancer B Oral Oncol* 1996; 32B, 217–221.
87. O-charoenrat P, Modjtahedi H, Rhys-Evans P, Court WJ, Box GM, Eccles SA. Epidermal growth factor-like ligands differentially up-regulate matrix metalloproteinase 9 in head and neck squamous carcinoma cells. *Cancer Res* 2000; 60, 1121–1128.
88. Quon H, Liu FF, Cummings BJ. Potential molecular prognostic markers in head and neck squamous cell carcinomas. *Head Neck* 2001; 23, 147–159.
89. Price JT, Wilson HM, Haites NE. Epidermal growth factor (EGF) increases the *in vitro* invasion, motility and adhesion interactions of the primary renal carcinoma cell line, A704. *Eur J Cancer* 1996; 32A, 1977–1982.
90. Wyckoff J, Wang W, Lin EY, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res* 2004; 64, 7022–7029.
91. Mamoune A, Kassis J, Kharait S, et al. DU145 human prostate carcinoma invasiveness is modulated by urokinase receptor (uPAR) downstream of epidermal growth factor receptor (EGFR) signaling. *Exp Cell Res* 2004; 299, 91–100.

92. Kruger JS, Reddy KB. Distinct mechanisms mediate the initial and sustained phases of cell migration in epidermal growth factor receptor-overexpressing cells. *Mol Cancer Res* 2003; 1, 801–809.
93. O-charoenrat P, Wongkajornsilp A, Rhys-Evans PH, Eccles SA. Signaling pathways required for matrix metalloproteinase-9 induction by betacellulin in head-and-neck squamous carcinoma cells. *Int J Cancer* 2004; 111, 174–183.
94. Bruns CJ, Harbison MT, Davis DW, et al. Epidermal growth factor receptor blockade with C225 plus gemcitabine results in regression of human pancreatic carcinoma growing orthotopically in nude mice by antiangiogenic mechanisms. *Clin Cancer Res* 2000; 6, 1936–1948.
95. Contessa JN, Reardon DB, Todd D, et al. The inducible expression of dominant-negative epidermal growth factor receptor-CD533 results in radiosensitization of human mammary carcinoma cells. *Clin Cancer Res* 1999; 5, 405–411.
96. Sheridan MT, O'Dwyer T, Seymour CB, Mothersill CE. Potential indicators of radiosensitivity in squamous cell carcinoma of the head and neck. *Radiat Oncol Investig* 1997; 5, 180–186.
97. Zhu A, Shaeffer J, Leslie S, Kolm P, El-Mahdi AM. Epidermal growth factor receptor: an independent predictor of survival in astrocytic tumors given definitive irradiation. *Int J Radiat Oncol Biol Phys* 1996; 34, 809–815.
98. Bonner JA, Buchsbaum DJ, Rogers BE, et al. Adenoviral vector-mediated augmentation of epidermal growth factor receptor (EGFr) enhances the radiosensitization properties of anti-EGFr treatment in prostate cancer cells. *Int J Radiat Oncol Biol Phys* 2004; 58, 950–958.
99. Bonner JA, Buchsbaum DJ, Russo SM, et al. Anti-EGFR-mediated radiosensitization as a result of augmented EGFR expression. *Int J Radiat Oncol Biol Phys* 2004; 59, 2–10.
100. Liang K, Ang KK, Milas L, Hunter N, Fan Z. The epidermal growth factor receptor mediates radioresistance. *Int J Radiat Oncol Biol Phys* 2003; 57, 246–254.
101. Akimoto T, Hunter NR, Buchmiller L, Mason K, Ang KK, Milas L. Inverse relationship between epidermal growth factor receptor expression and radiocurability of murine carcinomas. *Clin Cancer Res* 1999; 5, 2884–2890.
102. Kavanagh BD, Lin PS, Chen P, Schmidt-Ullrich RK. Radiation-induced enhanced proliferation of human squamous cancer cells *in vitro*: a release from inhibition by epidermal growth factor. *Clin Cancer Res* 1995; 1, 1557–1562.
103. Schmidt-Ullrich RK, Mikkelsen RB, Dent P, et al. Radiation-induced proliferation of the human A431 squamous carcinoma cells is dependent on EGFR tyrosine phosphorylation. *Oncogene* 1997; 15, 1191–1197.
104. Lammering G, Hewit TH, Valerie K, et al. EGFRvIII-mediated radioresistance through a strong cytoprotective response. *Oncogene* 2003; 22, 5545–5553.
105. Balaban N, Moni J, Shannon M, Dang L, Murphy E, Goldkorn T. The effect of ionizing radiation on signal transduction: antibodies to EGF receptor sensitize A431 cells to radiation. *Biochim Biophys Acta* 1996; 1314, 147–156.
106. Nasu S, Ang KK, Fan Z, Milas L. C225 antiepidermal growth factor receptor antibody enhances tumor radiocurability. *Int J Radiat Oncol Biol Phys* 2001; 51, 474–477.
107. Solomon B, Hagekyriakou J, Trivett MK, Stacker SA, McArthur GA, Cullinane C. EGFR blockade with ZD1839 (“Iressa”) potentiates the antitumor effects of single and multiple fractions of ionizing radiation in human A431 squamous cell carcinoma. Epidermal growth factor receptor. *Int J Radiat Oncol Biol Phys* 2003; 55, 713–723.
108. Zhou H, Kim YS, Peletier A, McCall W, Earp HS, Sartor CI. Effects of the EGFR/HER2 kinase inhibitor GW572016 on EGFR- and HER2-overexpressing breast cancer cell line proliferation, radiosensitization, and resistance. *Int J Radiat Oncol Biol Phys* 2004; 58, 344–352.
109. Akimoto T, Nonaka T, Harashima K, Ishikawa H, Sakurai H, Mitsuhashi N. Selective inhibition of survival signal transduction pathways enhanced radiosensitivity in human esophageal cancer cell lines *in vitro*. *Anticancer Res* 2004; 24, 811–819.
110. Bonner JA, Raisch KP, Trummell HQ, et al. Enhanced apoptosis with combination C225/radiation treatment serves as the impetus for clinical investigation in head and neck cancers. *J Clin Oncol* 2000; 18, 47S–53S.
111. Milas L, Mason K, Hunter N, et al. *In vivo* enhancement of tumor radioresponse by C225 antiepidermal growth factor receptor antibody. *Clin Cancer Res* 2000; 6, 701–708.
112. Nakata E, Hunter N, Mason K, Fan Z, Ang KK, Milas L. C225 antiepidermal growth factor receptor antibody enhances the efficacy of docetaxel chemoradiotherapy. *Int J Radiat Oncol Biol Phys* 2004; 59, 1163–1173.

113. She Y, Lee F, Chen J, et al. The epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 selectively potentiates radiation response of human tumors in nude mice, with a marked improvement in therapeutic index. *Clin Cancer Res* 2003; 9, 3773–3778.
114. Williams KJ, Telfer BA, Stratford IJ, Wedge SR. ZD1839 ('Iressa'), a specific oral epidermal growth factor receptor-tyrosine kinase inhibitor, potentiates radiotherapy in a human colorectal cancer xenograft model. *Br J Cancer* 2002; 86, 1157–1161.
115. Chinnaiyan P, Huang S, Vallabhaneni G, et al. Mechanisms of enhanced radiation response following epidermal growth factor receptor signaling inhibition by erlotinib (Tarceva). *Cancer Res* 2005; 65, 3328–3335.
116. Ciardiello F, Bianco R, Damiano V, et al. Antitumor activity of sequential treatment with topotecan and anti-epidermal growth factor receptor monoclonal antibody C225. *Clin Cancer Res* 1999; 5, 909–916.
117. Fan Z, Baselga J, Masui H, Mendelsohn J. Antitumor effect of anti-epidermal growth factor receptor monoclonal antibodies plus cis-diamminedichloroplatinum on well established A431 cell xenografts. *Cancer Res* 1993; 53, 4637–4642.
118. Inoue K, Slaton JW, Perrotte P, et al. Paclitaxel enhances the effects of the anti-epidermal growth factor receptor monoclonal antibody ImClone C225 in mice with metastatic human bladder transitional cell carcinoma. *Clin Cancer Res* 2000; 6, 4874–4884.
119. Mendelsohn J, Fan Z. Epidermal growth factor receptor family and chemosensitization. *J Natl Cancer Inst* 1997; 89, 341–343.
120. Prewett MC, Hooper AT, Bassi R, Ellis LM, Waksal HW, Hicklin DJ. Enhanced antitumor activity of anti-epidermal growth factor receptor monoclonal antibody IMC-C225 in combination with irinotecan (CPT-11) against human colorectal tumor xenografts. *Clin Cancer Res* 2002; 8, 994–1003.
121. Shin DM, Donato NJ, Perez-Soler R, et al. Epidermal growth factor receptor-targeted therapy with C225 and cisplatin in patients with head and neck cancer. *Clin Cancer Res* 2001; 7, 1204–1213.
122. Ciardiello F, Caputo R, Bianco R, et al. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* 2000; 6, 2053–2063.
123. Sirotnak FM, Zakowski MF, Miller VA, Scher HI, Kris MG. Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin Cancer Res* 2000; 6, 4885–4892.
124. Bundred NJ, Chan K, Anderson NG. Studies of epidermal growth factor receptor inhibition in breast cancer. *Endocr Relat Cancer* 2001; 8, 183–189.
125. Ling YH, Donato NJ, Perez-Soler R. Sensitivity to topoisomerase I inhibitors and cisplatin is associated with epidermal growth factor receptor expression in human cervical squamous carcinoma ME180 sublines. *Cancer Chemother Pharmacol* 2001; 47, 473–480.
126. Moulder SL, Yakes FM, Muthuswamy SK, Bianco R, Simpson JF, Arteaga CL. Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells *in vitro* and *in vivo*. *Cancer Res* 2001; 61, 8887–8895.
127. Higgins B, Kolinsky K, Smith M, et al. Antitumor activity of erlotinib (OSI-774, Tarceva) alone or in combination in human non-small cell lung cancer tumor xenograft models. *Anticancer Drugs* 2004; 15, 503–512.
128. Ng SS, Tsao MS, Nicklee T, Hedley DW. Effects of the epidermal growth factor receptor inhibitor OSI-774, Tarceva, on downstream signaling pathways and apoptosis in human pancreatic adenocarcinoma. *Mol Cancer Ther* 2002; 1, 777–783.
129. Pollack VA, Savage DM, Baker DA, et al. Inhibition of epidermal growth factor receptor-associated tyrosine phosphorylation in human carcinomas with CP-358,774: dynamics of receptor inhibition *in situ* and antitumor effects in athymic mice. *J Pharmacol Exp Ther* 1999; 291, 739–748.
130. Warburton C, Dragowska WH, Gelmon K, et al. Treatment of HER-2/neu overexpressing breast cancer xenograft models with trastuzumab (Herceptin) and gefitinib (ZD1839): drug combination effects on tumor growth, HER-2/neu and epidermal growth factor receptor expression, and viable hypoxic cell fraction. *Clin Cancer Res* 2004; 10, 2512–2524.
131. Formento P, Hannoun-Levi JM, Fischel JL, Magne N, Etienne-Grimaldi MC, Milano G. Dual HER 1–2 targeting of hormone-refractory prostate cancer by ZD1839 and trastuzumab. *Eur J Cancer* 2004; 40, 2837–2844.

132. Kuwada SK, Scaife CL, Kuang J, et al. Effects of trastuzumab on epidermal growth factor receptor-dependent and -independent human colon cancer cells. *Int J Cancer* 2004; 109, 291–301.
133. Xia W, Mullin RJ, Keith BR, et al. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* 2002; 21, 6255–6263.
134. Traxler P, Allegrini PR, Brandt R, et al. AEE788: a dual family epidermal growth factor receptor/Erbb2 and vascular endothelial growth factor receptor tyrosine kinase inhibitor with antitumor and antiangiogenic activity. *Cancer Res* 2004; 64, 4931–4941.
135. Yigitbasi OG, Younes MN, Doan D, et al. Tumor cell and endothelial cell therapy of oral cancer by dual tyrosine kinase receptor blockade. *Cancer Res* 2004; 64, 7977–7984.
136. Ciardiello F, Caputo R, Damiano V, et al. Antitumor effects of ZD6474, a small molecule vascular endothelial growth factor receptor tyrosine kinase inhibitor, with additional activity against epidermal growth factor receptor tyrosine kinase. *Clin Cancer Res* 2003; 9, 1546–1556.
137. Wedge SR, Ogilvie DJ, Dukes M, et al. ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. *Cancer Res* 2002; 62, 4645–4655.
138. Kim SJ, Uehara H, Yazici S, et al. Simultaneous blockade of platelet-derived growth factor-receptor and epidermal growth factor-receptor signaling and systemic administration of paclitaxel as therapy for human prostate cancer metastasis in bone of nude mice. *Cancer Res* 2004; 64, 4201–4208.
139. Burtrum D, Zhu Z, Lu D, et al. A fully human monoclonal antibody to the insulin-like growth factor I receptor blocks ligand-dependent signaling and inhibits human tumor growth in vivo. *Cancer Res* 2003; 63, 8912–8921.
140. Goetsch L, Gonzalez A, Leger O, et al. A recombinant humanized anti-insulin-like growth factor receptor type I antibody (h7C10) enhances the antitumor activity of vinorelbine and anti-epidermal growth factor receptor therapy against human cancer xenografts. *Int J Cancer* 2005; 113, 316–328.
141. Grandis JR, Drenning SD, Chakraborty A, et al. Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor-mediated cell growth *in vitro*. *J Clin Invest* 1998; 102, 1385–1392.
142. Lo HW, Hsu SC, Ali-Seyed M, et al. Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. *Cancer Cell* 2005; 7, 575–589.
143. Benavente S, Armstrong E, Hsu KT, Huang S, Harari PM. Array-based identification of genes and proteins associated with resistance to EGFR inhibitors. *AACR* 2006; 1, Abstract 1246.
144. Gondi V, Huang S, Benavente S, Armstrong E, Harari PM. Potential mechanisms of acquired resistance to EGFR inhibitors. *AACR* 2006; 1, Abstract 1244.
145. Kelly K, Hanna N, Rosenberg A, Bunn PAJ. A multicenter phase I/II study of cetuximab in combination with paclitaxel and carboplatin in untreated patients with stage IV non-small cell lung cancer. *Proc Am Soc Clin Oncol* 2003; 22, 2592.
146. Robert F, Blumenschein G, Dicke K, Tseng J, Saleh MN. Phase IB/IIA study of anti-epidermal growth factor receptor antibody, cetuximab, in combination with gemcitabine/carboplatin in patients with advanced stage IV non-small cell lung cancer. *Proc Am Soc Clin Oncol* 2003; 22, 2587.
147. Saltz LB, Meropol NJ, Loehrer PJS, Needle MN, Kopit J, Mayer RJ. Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. *J Clin Oncol* 2004; 22, 1201–1208.
148. Vega-Villegas E, Awada R, Mesia L, Geoffrois L, Borel C. A phase I study of cetuximab in combination with cisplatin or carboplatin and 5-FU in patients with recurrent or metastatic squamous cell carcinoma of the head and neck. *Proc Am Assoc Cancer Res* 2003; 22, 2020.
149. Cunningham D, Humblet Y, Siena D, et al. Cetuximab (C225) alone or in combination with irinotecan (CPT-11) in patients with epidermal growth factor receptor (EGFR)-positive, irinotecan-refractory metastatic colorectal cancer (MCRC). *Proc Am Soc Clin Oncol* 2003; 22, Abstract 1012.
150. Wang P, Fredlin P, Davis CG, Yang X-D. Human anti-EGF receptor monoclonal antibody ABX-EGF: a potential monotherapy for the treatment of prostate cancer. *Proc Am Soc Clin Oncol* 2003; 22, Abstract 4525.
151. Meropol NJ, Berlin J, Hecht JR, et al. Multicenter study of ABX-EGF monotherapy in patients with metastatic colorectal cancer. *Proc Am Soc Clin Oncol* 2003; 22, Abstract 1026.
152. Woodburn JR, Kendrew J, Fennell M, Wakeling AE. ZD1839 ('IRESSA'), a selective epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI): inhibition of c-fos mRNA, an inter-

- mediate marker of EGFR activation, correlates with tumor growth inhibition. *Proc Am Assoc Cancer Res* 2000; 41, Abstract 2552.
153. Arteaga CL, Johnson DH. Tyrosine kinase inhibitors-ZD1839 (Iressa). *Curr Opin Oncol* 2001; 13, 491–498.
 154. Baselga J, Albanell J, Ruiz A, et al. Phase II and tumor pharmacodynamic study of gefitinib (ZD1839) in patients with advanced breast cancer. *Proc Am Soc Clin Oncol* 2003; 22, Abstract 24.
 155. Cohen EE, Stenson K, Gustin DM, et al. A phase II study of 250-mg gefitinib (ZD1839) monotherapy in recurrent or metastatic squamous cell carcinoma of the head and neck (SCCHN). *Proc Am Soc Clin Oncol* 2003; 22, Abstract 2021.
 156. Cohen EE, Rosen F, Dekker A, et al. Phase II study of ZD1839 (Iressa) in recurrent or metastatic squamous cell carcinoma of the head and neck (SCCHN). *Proc Am Soc Clin Oncol* 2002; 21, Abstract 899.
 157. Dawson NA, Guo C, Zak R, et al. A phase II trial of ZD1839 in stage IV and recurrent renal cell carcinoma. *Proc Am Soc Clin Oncol* 2003; 22, Abstract 1623.
 158. Fountzilias G, Pectasides D, Skarlos DV, et al. Paclitaxel, carboplatin and gefitinib (Iressa, ZD1839) as first-line chemotherapy in patients with advanced breast cancer: a phase II study. *26th San Antonio Breast Cancer Symposium* 2003; Abstract 357.
 159. Robertson JFR, Gutteridge E, Cheung KL, et al. Gefitinib (ZD1839) is active in acquired tamoxifen (TAM)-resistant oestrogen receptor (ER)-positive and ER-negative breast cancer: results from a phase II study. *Proc Am Soc Clin Oncol* 2003; 22, Abstract 23.
 160. Giaccone G, Johnson DH, Manegold C. A phase III clinical trial of ZD1839 (“Iressa”) in combination with gemcitabine and cisplatin in chemotherapy-naïve patients with advanced non-small cell lung cancer (INTACT 1). *Ann Oncol* 2002; 13, Abstract 40.
 161. Johnson DH, Herbst RS, Giaccone G. ZD1839 (“Iressa”) in combination with paclitaxel and carboplatin in chemotherapy-naïve patients with advanced non-small cell lung cancer (NSCLC): results from a phase III clinical trial (INTACT 2). *Ann Oncol* 2002; 13, Abstract 4680.
 162. Dancey JE, Freidlin B. Targeting epidermal growth factor receptor—are we missing the mark? *Lancet* 2003; 362, 62–64.
 163. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004; 350, 2129–2139.
 164. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; 304, 1497–1500.
 165. Akita RW, Sliwkowski MX. Preclinical studies with Erlotinib (Tarceva). *Semin Oncol* 2003; 30, 15–24.
 166. Iwata KK, Provoncha K, Gibson N. Inhibition of mutant EGFRvIII transformed cells by tyrosine kinase inhibitor OSI-774 (Tarceva). *Proc Am Soc Clin Oncol* 2002; 21, Abstract 79.
 167. Gatzemeier U, Pluzanska A, Szczesna A. Results of a phase III trial of erlotinib (OSI-774) combined with cisplatin and gemcitabine (GC) chemotherapy in advanced non-small cell lung cancer (NSCLC). *Proc Am Soc Clin Oncol* 2004; 23, Abstract 7010.
 168. Herbst RS, Prager D, Hermann R. TRIBUTE – a phase III trial of erlotinib HCl (OSI-774) combined with carboplatin and paclitaxel (CP) chemotherapy in advanced non-small cell lung cancer (NSCLC). *Proc Am Soc Clin Oncol* 2004; 23, Abstract 7011.
 169. Ritter CA, Arteaga CL. The epidermal growth factor receptor-tyrosine kinase: a promising therapeutic target in solid tumors. *Semin Oncol* 2003; 30, 3–11.
 170. Harari PM, Huang S, Herbst RS, Quon H. *Molecular Targeting of the Epidermal Growth Factor Receptor in Head and Neck Cancer*. Lippincott, Williams and Wilkins, 2003.
 171. Harari PM, Huang SM. Radiation response modification following molecular inhibition of epidermal growth factor receptor signaling. *Semin Radiat Oncol* 2001; 11, 281–289.

V

ESTABLISHED CANCER THERAPIES

19

Antimetabolites

Janet A. Houghton, PhD

SUMMARY

Cell death has been divided into two main types: programmed cell death (PCD), in which the cell plays an active role, and passive (necrotic) cell death. PCD has been categorized into different subtypes: apoptosis, anoikis, mitotic catastrophe, autophagy, paraptosis, and cytoplasmic (for which the mechanism of molecular activation remains unknown) (1). Senescence arrest, accelerated senescence, and differentiation are also responses that can be induced in response to DNA-damaging agents. Considerable attention has been given to the study of apoptotic responses following DNA damage induced in cancer cells by chemotherapeutic agents. Apoptosis may occur as a primary event following chemotherapy in which genes that regulate apoptosis will influence the outcome of therapy or alternatively as an event secondary to the induction of lethal damage that involves the subsequent processing of cellular damage (2,3). In the former case, apoptosis may occur early within the first few hours of treatment as observed in cells of hematopoietic origin, whereas for solid tumors, the outcome is less clear (4). The particular type of response induced is highly dependent on the agent and dose employed, the type of DNA damage induced, as well as the genetic and cellular phenotypes. It has been proposed that apoptosis may play a lesser role in tumor response to radiation in comparison with the induction of cell death through mitotic catastrophe or a senescence-like irreversible growth arrest (5). However, in comparison with the induction of apoptosis, there is a lack of as much definitive information on other cell death processes that occur in cancer cells in response to chemotherapeutic agents, including antimetabolites. This chapter reviews what is known about these processes at the present time in response to experimental or clinically used agents that are analogs of 5-fluorouracil (FUra), cytidine (Cyd) or purines, hydroxyurea, or that belong to the family of folate antagonists.

Key Words: Antimetabolites; cell death; apoptosis; mitotic catastrophe; necrosis; senescence.

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1. PROGRAMMED CELL DEATH

1.1. Apoptosis (Type I)

It has become widely accepted that DNA-damaging agents, including antimetabolites, induce cell death in neoplastic cells generally by triggering apoptosis. The susceptibility of cells to apoptosis depends upon the status of genes in regulating critical components of the cell death machinery (6), which in turn is influenced by signaling events that result in a complex system of interacting signals that determine the ultimate outcome of the treatment response (7).

1.1.1. FURA, ANALOGS, AND FOLATE-BASED INHIBITORS OF THYMIDYLATE SYNTHASE

The vast majority of studies with antimetabolites of this class has been conducted with FUra itself, 5-fluorodeoxyuridine (FdUrd, floxuridine), capecitabine (xeloda, a pro-drug for FUra), raltitrexed [tomudex, ZD1694, a folate-based inhibitor of thymidylate synthase (TS)], or ZD9331 (BGC9331). FUra and FdUrd have been widely used in the treatment of colorectal cancer (8–10) and are extensively metabolized inside the cell. The metabolite 5-fluorodeoxyuridylylate inhibits TS in the presence of 5,10-methylene tetrahydrofolate ($\text{CH}_2\text{-H}_4\text{-PteGlu}$), resulting in depletion of thymidine-5'-triphosphate (dTTP) as well as elevation in 2'-deoxyadenosine-5'-triphosphate (dATP) (11), induction of DNA damage (11), and S-phase arrest (12,13). DNA-directed effects may also include misincorporation of 2'-deoxyuridine 5'-triphosphate (dUTP) or 5-fluoro dUTP (FdUTP) into DNA (14,15). Alternatively, incorporation of 5-fluorouridine triphosphate (FUTP) into RNA interferes with RNA processing and is the primary mechanism by which toxicity of FUra is induced in normal Gastrointestinal (GI) tissues (16,17), in contrast to DNA-directed cytotoxicity that is important in chemotherapeutic response (15,18,19). Apoptosis has been studied extensively in response to TS inhibitors and can be influenced by the status of the p53 tumor-suppressor gene. S-phase arrest occurs rapidly in response to TS inhibition (13,20). In HCT116 cells that express wt p53, cells progress to the G2-phase of the cell cycle, and apoptosis occurs rapidly. In contrast to the presence of mp53, S-phase arrest is maintained during drug exposure, after which time delayed apoptosis is evident (20). Adenoviral transduction of p53 sensitizes cells to FUra-induced apoptosis (13,21). P53 appears to influence the kinetics of apoptosis induction but not overall response as determined by clonogenic survival (2,13). FUra-resistant cell lines have demonstrated reduced apoptosis (22).

The cyclin-dependent kinase inhibitor p21^{Waf1} has demonstrated a significant role in influencing sensitivity to TS inhibitors, influencing both apoptosis (20,23) and clonogenic survival (20), in a p53-independent manner. Using isogenic HCT116 cell lines, parental cells were highly sensitive to ZD9331, in contrast to p21^{Waf1} $-/-$ cells that were completely resistant (20). Both cell lines accumulated in S-phase by 24 h after ZD9331 exposure; however, wt cells progressed to G2 and underwent apoptosis prior to mitosis in contrast to p21^{Waf1} $-/-$ cells that re-entered a normal cell cycle. Furthermore, selective induction of p21^{Waf1} in RKO cells was sufficient to induce apoptosis (20). S-phase arrest appears to occur through Chk1-mediated proteolysis of Cdc25A (24,25), leading to inhibition of Cdk2.

Chemotherapeutic agents have generally been considered to activate the intrinsic apoptotic pathway. It has been reported that caspase-8 (26–28) and/or caspase-3 (27,28) have been directly activated by FUra, and inhibitors of caspase activation can influence the extent of apoptosis induced (13,29,30). However, after co-incubation with the pancaspase inhibitor Z-VAD-fmk, only partial but not complete protection from thymineless stress-induced apoptosis (31) or loss in clonogenic survival (31,32) was demonstrated in colon carcinoma cells. Caspase inhibitors can block the appearance of the apoptotic phenotype but may delay rather than prevent cell death (33,34). Whether the ensuing death is a form of apoptosis or occurs through an alternative mechanism still remains to be clarified.

Several studies have now demonstrated the involvement of the extrinsic pathway of apoptosis in response to TS inhibitors. The Fas death receptor, belonging to the tumor necrosis factor (TNF) receptor superfamily, is critical in the induction of thymineless stress-induced cell death in TS⁻ cells (35) (Fig. 1), as a component in the mechanism of FUra-induced cell death both *in vitro* (36–38) and *in vivo* (39) or in capecitabine-induced apoptosis (40). Figure 1 demonstrates that in the presence of the NOK-1 antibody that blocks the interaction between Fas and FasL, protection is afforded from the induction of cell death in TS⁻ cells following thymidine (dThd) deprivation, in contrast to co-incubation with an IgG1 isotype-matched control antibody or control cells. Fas is downregulated in expression in approximately 50% of colon carcinomas (41,42); however, the cytokine interferon- γ upregulates the expression of Fas at the cell surface and sensitizes cells to both Fas-mediated and FUra-induced cell death (36). Agents that target Fas, including the cytolytic anti-Fas antibody CH-11, induce synergistic cytotoxicity (apoptosis and clonogenic survival) when combined with FUra (36,38,43). In the presence of wtp53, FUra induces the expression of Fas (36,38). The cytotoxic ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also belonging to the TNF family of death receptors and their ligands, induces a synergistic interaction in combination with FUra both *in vitro* (44,45) and *in vivo* (46), which

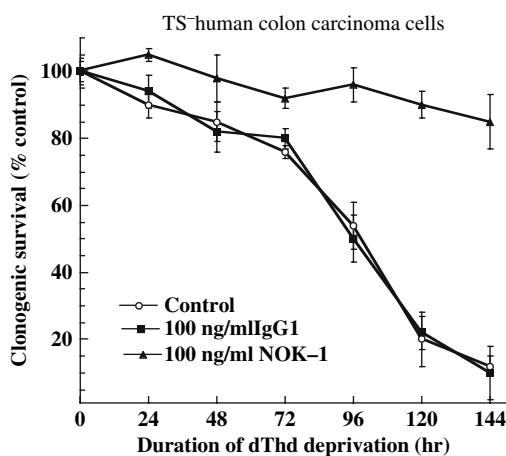


Fig. 1. The extrinsic pathway of apoptosis through the Fas death receptor is involved in the induction of thymineless death in thymidylate synthase (TS)⁻ cells, determined by clonogenic survival assay (35).

may involve upregulated expression of TRAIL receptors (47), enhanced formation of a death-inducing signaling complex (DISC) (44,45), or downregulated expression of cellular FLICE inhibitory protein (c-FLIP) (43,44). Elimination of the function of DR5 in HCT116 cells conferred resistance to FUra *in vivo* (48). Genes that are known to directly regulate apoptosis at other sites can influence sensitivity to this class of agent. Drug resistance has been determined in several studies to be influenced by the constitutive expression of nuclear factor NF- κ B (49–51). Downregulation of NF- κ B (50–52) or Bcl-2 (53) or overexpression of Bax (54,55) sensitized cells to FUra-induced or raltitrexed-induced apoptosis. Furthermore, Bcl-w was found to be an important determinant of FUra sensitivity in intestinal epithelia (56). In human head and neck squamous cell carcinoma xenografts, overexpression of Bax induced apoptosis in response to raltitrexed as well as enhancing tumor regression *in vivo* (54) (Fig. 2). Current data suggest that at least under certain conditions, apoptosis is an important determinant of the cytotoxic activity of TS inhibitors both *in vitro* and *in vivo*, in experimental models.

A third general pathway for the induction of apoptosis originates from the endoplasmic reticulum, resulting in the activation of caspase-12 and caspase-9 (57–59); however, a potential role in antimetabolite-induced apoptosis has not yet been established.

Expression of other genes that are involved in sensitization to TS inhibitor-induced apoptosis includes overexpression of the p16^{INK4A} tumor-suppressor gene (60), oncogenes (HPV16 E6/E7) (61) or E2F-1 (62), c-myb (where cells from c-myb^{-/-} mice were hypersensitive to FUra) (63), or treatment with exogenous sphingomyelin (64).

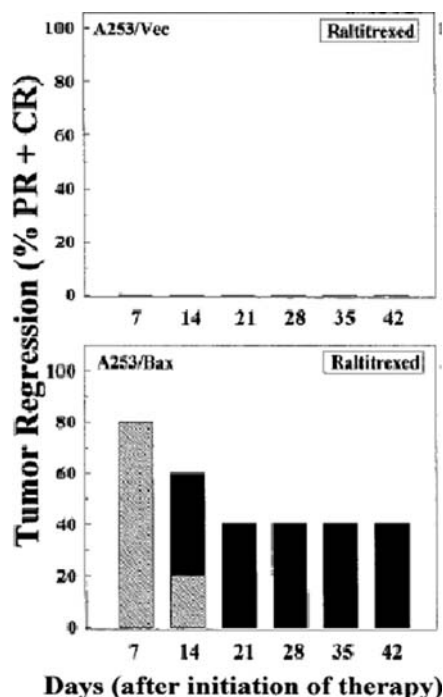


Fig. 2. Tumor responses (partial response+complete response) (PR+CR) in nude mice bearing A253/Vec and A253/Bax xenografts induced by raltitrexed at the maximum tolerated dose (MTD). Each group represents 10–20 animals from two to four independent experiments. CR rates ($p < 0.001$) (54).

In contrast, HaRas overexpression conferred resistance to FUra-induced apoptosis (65), likely mediated through the PI-3K signaling pathway (66), and Cox-2 overexpression has attenuated FUra-induced apoptosis (67,68). DNA mismatch repair (MMR) can detect and initiate responses to many lesions in DNA, including those induced by FUra. MMR-deficient HCT116 cells were significantly more resistant to fluoropyrimidines than MMR-proficient cells, as determined by clonogenic survival, reflecting tolerance to DNA damage (69,70). Although DNA fragmentation was detected and effects were dThd reversible, cytotoxicity did not appear to be reflected in the extent of apoptosis induced in this system.

Clinically, the role of apoptosis regulatory proteins in overall response and survival following treatment with fluoropyrimidines is being evaluated, and this chapter will focus on their expression in colorectal cancer. Few studies have evaluated the correlation between Bcl-2 expression and response of colorectal cancers to chemotherapy. Schneider et al. (71) did not find a correlation between Bcl-2 expression and either response to FUra-based therapy or overall survival in advanced colorectal carcinoma. However, Bcl-2-negative lesions were more frequent among patients who reached an objective clinical response when treated with FUra-containing regimens (72). Bax mutations and hence reduced Bax expression occur in colon carcinomas (73–75). In stage III primary colorectal carcinomas, low Bax expression was a negative prognostic factor in these tumors (76), and in patients undergoing surgery for advanced colorectal cancer, patients with Bax⁺ primary tumors had significantly better prognosis (5-year survival) than patients with Bax⁻ tumors (77). Low Bax expression was an independent prognostic marker in patients with metastatic disease of the liver (78). For the IAP family member survivin, expression occurs in 53–61% of adenocarcinomas (79–81) and has demonstrated significant correlations with survival rates in colorectal cancers (79,81). High survivin expression has been associated with reduced apoptosis in rectal cancer and may predict for reduced disease-free survival after preoperative radiochemotherapy and surgical resection (80). Elevated survivin/Fas ratios have predicted for recurrent disease in pediatric patients with renal cell tumors (82) and for a more aggressive behavioral pattern in neuroblastomas as well as for worse outcome (83). Downregulation of survivin has sensitized tumor cells to chemotherapy (84), although little is currently known concerning survivin as a prognostic factor in response to fluoropyrimidine therapy. Thus, Bax and survivin are examples of genes that are critical in the regulation of apoptosis and clinically may influence treatment response and outcome.

1.1.2. CYD ANALOGS

Agents of this class include gemcitabine (2',2'-difluorodeoxycytidine, dFdC, Gemzar), Ara-C (cytosine arabinoside, cytarabine), 2-chlorodeoxyadenosine (2-CdA), and 5-aza-2'-deoxycytidine (5-dAzaC, decitabine, DAC). Ara-C is widely used in the treatment of leukemias, the predominant mechanism for cytotoxicity involving incorporation of the drug into DNA during replication following metabolism by dCyd kinase (85). Gemcitabine, used widely in the treatment of lung cancers, is also metabolized intracellularly by dCyd kinase. Decreased dCyd kinase expression is associated with resistance to both agents (86–88), whereas other genes including the M2 subunit of ribonucleotide reductase (RRM2) (89), or the human equilibrative nucleoside transporter 1 (87), are also associated with gemcitabine resistance. Sensitivity to agents of this class is enhanced in the presence of wtp53 (88,90,91).

Cyd analogs appear to utilize predominantly the intrinsic apoptotic pathway requiring mitochondrial involvement in the induction of apoptosis, caspase activation, which is inhibited in the presence of high levels of Bcl-2 or Bcl-xL (92–95) or potentiated when these proteins are downregulated (96–98) and appears independent of Mcl-1 (99). The ratio of Bax/Bcl-2 has been predictive for the sensitivity of pancreatic carcinoma cell lines to gemcitabine (100). As with TS inhibitors, mitochondrial damage and apoptosis appear to be delayed, but not eliminated, by overexpression of Bcl-2 or Bcl-xL (94). This is consistent with studies that have demonstrated that Bcl-2 can protect cells from apoptosis but not overall loss in clonogenic survival, likely because of the occurrence of dual modes of cell death (101). Z-VAD-fmk has exerted similar effects in drug-induced cytotoxic responses (102).

The intrinsic pathway of apoptosis induced by Cyd analogs is also influenced by secondary signaling pathways that regulate the overall cytotoxic response. Thus, PI-3K (103–105), MAP kinase (MAPK) (106–108), or protein kinase C (PKC) (109) influence sensitivity to Cyd analogs. Inhibition of Src tyrosine kinase sensitizes pancreatic carcinoma cells to gemcitabine (110,111). Constitutive activation of NF- κ B is linked to drug resistance (112–114), and proteasomal synthesis inhibitors that prevent the function of NF- κ B (115–117) sensitize cells to these agents. Regarding the role of death receptors in apoptosis induced by Cyd analogs, there is no evidence that these proteins are directly involved (118,119), although TRAIL can cooperate with gemcitabine to amplify apoptotic signaling and apoptosis through the mitochondria (120).

There is little information available from clinical studies on the prognostic value of expression of apoptosis regulatory proteins in response to gemcitabine or Ara-C administered alone. However, in preclinical studies, Colo357-bax tumors overexpressing Bax in SCID mice demonstrated 12-fold greater volume regression than Colo357-wt tumors (92). Gemcitabine combined with the Src tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*] pyrimidine (PP2) significantly reduced tumor masses and prevented the onset of metastatic disease in an orthotopic model (110). Furthermore, gemcitabine in combination with the proteasomal synthesis inhibitor Velcade inhibited the growth of non-small cell lung cancer (NSCLC) xenografts (115) or in combination with bortezomib inhibited the growth of bladder carcinoma xenografts (117). These studies suggest that *in vitro* approaches that target pathways of apoptosis in response to Cyd analogs have relevance to the overall cytotoxic response *in vivo*.

An alternate strategy for Cyd analogs is the use of 5-azacytidine or 5-dAzaC as demethylating agent to reverse the documented methylation of critical regions of genes in human cancers, thereby causing the re-expression of these genes after several days (121). To this end, 5-dAzaC appears to be more effective. Clinically, these agents have been used in the treatment of acute myeloid leukemia (AML) and acute myelodysplastic syndromes (122,123). 5-dAzaC has enhanced cellular sensitivity to DNA-damaging agents, including cisplatin, doxorubicin, VP-16, and temozolomide (124–126). Considerable focus has been placed on the ability of 5-dAzaC to induce the re-expression of critical caspases involved in the induction of apoptosis. Cell lines or tumors derived from aggressive neuroblastomas with amplification of *n*-Myc demonstrate deletion or silencing of the caspase-8 gene primarily through methylation of the promoter region, independent of caspase-9 or Apaf-1.

Treatment with 5-dAzaC or re-introduction of caspase-8 to these cells conferred sensitivity to Fas-induced apoptosis, to TRAIL-induced apoptosis, and to chemotherapeutic agents (127–130). Caspase-8 expression is also lost in primitive neuroectodermal tumors (131). 5-dAzaC has also been used to upregulate the expression of caspase-9 in human lung carcinoma cells to enhance p53-induced apoptosis (132) and to upregulate caspase-1 expression in renal cancer cells both *in vitro* and *in vivo*, which slows tumor growth indicating that caspase-1 may be involved in oncogenesis (133).

1.1.3. PURINE ANALOGS, HYDROXYUREA, AND METHOTREXATE

Three analogs of adenosine including 2'-deoxycoformycin (dCF), 2-CdA, and fludarabine (9- β -D-arabinosyl-2-fluoroadenine) are in current clinical use for the treatment of lymphoproliferative diseases. dCF inhibits the enzyme adenosine deaminase [reviewed in (134)], resulting in the cellular accumulation of dAdo and dATP, which induces cytotoxicity. In contrast, 2-CdA and fludarabine are metabolized by dCyd kinase to nucleoside triphosphates, incorporated into DNA, induce S-phase arrest, and are cytotoxic to dividing cells [reviewed in (135,136)], influenced by the ratio of dCyd kinase/5'-nucleotidase (137). Both agents also induce cytotoxicity in non-dividing cells, which may involve reduction in RNA synthesis, and depletion of cellular nicotinamide adenine dinucleotide (NAD) and ATP [reviewed in (138)]. The induction of apoptosis has been established for both agents in leukemic cells (139–142). Although these agents activate caspases (143), following co-incubation with purine analogs and Z-VAD-fmk, secondary necrosis was detected, and the overall induction of cell death was not inhibited (144) (Fig. 3). In cells from patients with B-cell chronic lymphocytic leukemia (B-CLL), 2-CdA induced translocation of apoptosis inducing factor (AIF) from the mitochondria to the nucleus. In the presence of caspase inhibitors, development of the classic apoptotic phenotype was prevented; however, loss in mitochondrial membrane potential, AIF apoptosis inducing factor translocation, and cell death still proceeded (145).

The S-phase-specific cytotoxicity in dividing ML-1 cells involves activation of Jun N-terminal kinase1 (JNK-1) (146). Cells expressing wtp53 are more sensitive to purine analogs (147), and apoptosis induced by these agents is also influenced by NF-KB (148), Bcl-xL (149), and the ratio of Bax/Bcl-2 (150). Of interest, the *ex vivo* response to fludarabine of blasts derived from CLL patients correlated with downregulated expression of the survival factor BFL-1 (151) but did not correlate with Bax expression levels (152). Furthermore, failure of CLL patients to achieve complete remission correlated with high levels of Mcl-1 expression (153).

The thiopurine antimetabolites, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG), are analogs of the purine nucleosides hypoxanthine and guanine, respectively, and are important agents in the treatment of acute lymphoblastic leukemia (ALL) [reviewed in (154,155)]. Anabolism to ribonucleotides involves hypoxanthine–guanine phosphoribosyltransferase (HGPRT), or alternatively, 6-MP and 6-TG are S-methylated by thiopurine S-methyltransferase to yield nucleobases that are inactive (156). The cytotoxicity of 6-MP and 6-TG is considered to occur through incorporation into DNA resulting in interference with the function of DNA polymerases, ligases, and endonucleases and may involve DNA MMR (157). Thiopurines inhibit *de novo* purine synthesis (158), and decreased HGPRT determines acquired resistance (159). 6-MP induces apoptosis in activated B lymphocytes (160) and in hamster fibrosarcoma cells (161). Apoptosis

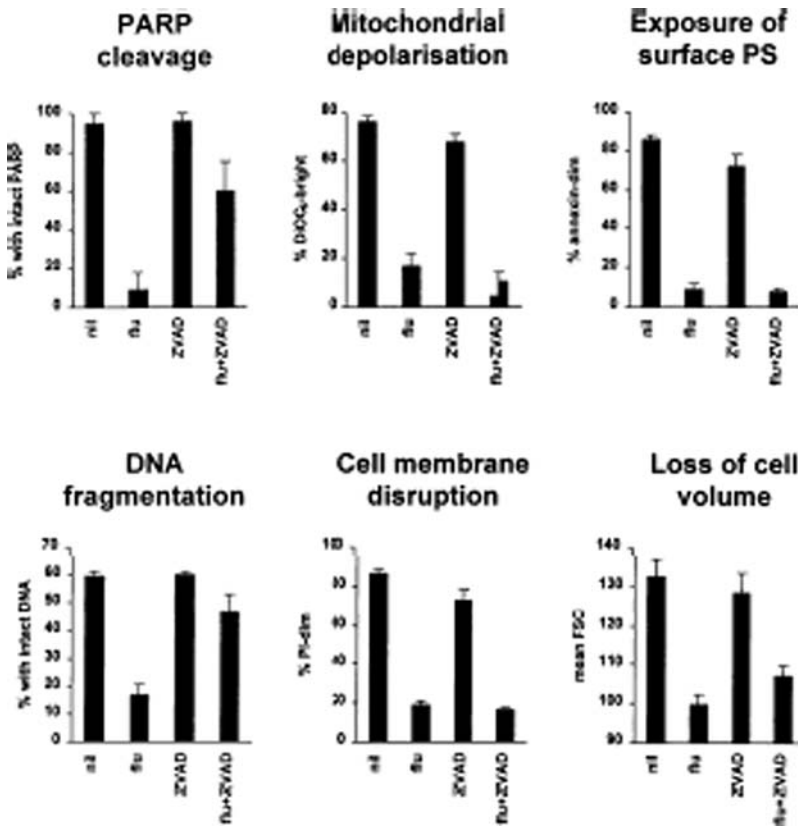


Fig. 3. Effect of Z-VAD-fmk on fludarabine cytotoxicity in lymphocytic leukemia (CLL) cells. Fludarabine induced extensive killing as measured by all six criteria. Poly (ADP-ribose) polymerase (PARP) cleavage and DNA fragmentation indicate that cell death occurred by apoptosis, whereas cell membrane disruption indicates that the majority of apoptotic cells had undergone secondary necrosis. Z-VAD-fmk produced selective inhibition of PARP cleavage and DNA fragmentation (144).

was induced rapidly in activated B cells (within 6 h) and was associated with decreased activity of nitric oxide synthase and decreased Bcl-2/Bax that would otherwise promote cell survival in mature B lymphocytes (162).

Hydroxyurea (HU) is commonly used for the treatment of chronic myeloid leukemia (CML), polycythemia vera, and thrombocytopenia (163). HU depletes dNTP pools by inhibiting RR, thereby inhibiting the conversion of ribonucleotides to deoxyribonucleotides, inducing DNA damage, and preventing DNA replication (164). HU has also been shown to induce apoptosis (165).

Methotrexate (MTX) and its polyglutamates are inhibitors of dihydrofolate reductase (DHFR) and other folate metabolizing enzymes, thereby causing the depletion of reduced folates, dTTP, purines, inhibition of DNA synthesis, and cell death. Intrinsic and acquired resistance mechanisms both *in vitro* and *in vivo* include defective transport, amplification, or altered MTX affinity for DHFR, decreased MTX polyglutamates, or elevated MRP that enhances drug efflux [reviewed in (166)]. MTX has been shown to induce cell death through apoptosis in fibrosarcoma cells (161), CHO cells (167), HL60 and Jurkat leukemia cells (168,169), breast cancer cells (170), and intestinal epithelial cells characterized by the sequential activation of caspase-9,

caspase-2, and caspase-3 prior to DNA fragmentation (171). In breast cancer cells, this process may be dependent on caspase-3 but independent of p53 (170). In other cell lines, however, MTX did not appear to induce apoptosis (169,172). Studies suggest that in contrast to FUra and other agents, MTX may not induce a synergistic interaction when combined with TRAIL (173) or anti-Fas Abs (174). MTX is used extensively in combination with other agents in the treatment of ALL and in solid tumors.

Although metabolic processes that influence cytotoxicity and drug resistance have been extensively studied for these agents, the induction of apoptosis and the regulatory mechanisms involved in apoptosis are not well documented, although apoptosis has been conclusively demonstrated in certain systems. Furthermore, combination chemotherapy approaches used in clinical therapy make cell death mechanisms in response to individual agents of these groups difficult to determine.

1.2. Anoikis

Anoikis constitutes apoptosis induced in response to lack of adhesion or inappropriate adhesion in cell–matrix interactions and is characterized by all of the features that govern apoptosis (175). It occurs mainly in epithelial cells to prevent cellular shedding and to assure proper developmental positioning of the cells in specialized structures. Anoikis utilizes predominantly the Fas pathway in inducing caspase-dependent apoptosis, FAS-associated death domain (FADD), and also integrin receptors (176). However, the involvement of anoikis in antimetabolite-specific treatment responses has not been well investigated.

1.2.1. MITOTIC CATASTROPHE

An emerging literature suggests that alternative mechanisms of programmed cell death (PCD), independent of caspase activation, can be invoked by chemotherapeutic agents and include mitotic catastrophe, autophagy, or paraptosis. In addition to the mitochondria, lysosomes and the endoplasmic reticulum can play a key role in these processes, resulting in the release of proteases [reviewed in (177)].

Mitotic cell death or mitotic catastrophe leads to the formation of interphase cells with multiple micronuclei. This may also occur following aberrant reentry of tumor cells into cycle after prolonged growth arrest. Mitotic catastrophe can occur in apoptosis-competent cells prior to the induction of apoptosis, although apoptosis is not required to elicit cell death. Checkpoint deficiencies in tumor cells promote mitotic catastrophe [reviewed in (178)]. In the case of antimetabolites that predominantly inhibit DNA synthesis, this form of cell death could conceivably result from the premature induction of mitosis before the appropriate completion of S-phase or G2-phase as observed for other forms of cellular damage following premature chromatin condensation (179,180). The G2 checkpoint appears critical in preventing mitotic cell death in cells treated with DNA-damaging agents. The vast majority of studies that have determined the induction of mitotic catastrophe in neoplastic cells have utilized the DNA-damaging agents doxorubicin (181), bleomycin (182), or etoposide (101) or the microtubule poison taxol (183). Cells that die through mitotic catastrophe do in general not demonstrate DNA ladder formation or DNA strand breaks detectable by TUNEL staining (178) and die in the presence of overexpressed Bcl-2 that inhibits apoptosis but not loss in clonogenic survival following drug treatment (101). Very few studies have addressed this form of cell death following treatment with

Table 1
Effects of Different Agents on HT1080 3'SS6 Cells

<i>Agent</i>	<i>ID₈₅</i>	<i>% SA-β-gal+</i>	<i>% micronucleated</i>	<i>% sub-G₁</i>
None		1	1.5	0.4
Doxorubicin	30 nM	79	45	10
Aphidicolin	200 ng/ml	64	45	10
Cisplatin	2.2 μM	55	47	9.3
γ-irradiation	1300 rad	36	63	3.2
Cytarabine	1.5 μM	23	64	7.5
Etoposide	900 nM	15	55	7.5
Taxol	5 ng/ml	9.1	66	7.1
Vincristine	1.5 nM	3.1	62	13

Reproduced from (181).

antimetabolites. In one study, cytarabine (1.5 μM) induced micronucleation in 64% of HT1080 cells in the absence of apoptosis, indicative of mitotic death equivalent to that observed following treatment with taxol or γ-irradiation (181) (Table 1). However, following Fura treatment of HeLa or H1299 human lung cancer cells, downregulation of Chk1 expression by Chk1siRNA abrogated Fura-induced S-phase arrest and potentiated Fura-induced cell death following premature chromatin condensation, M-phase progression, and the induction of apoptosis in the presence of a 2N DNA content. This is in contrast to abrogation of the S-phase checkpoint during treatment with CPT-11 that resulted in mitotic catastrophe and cell death in the presence of a 4N DNA content (25). HCT116 cells treated with the TS inhibitor ZD9331 accumulated in S-phase and subsequently progressed to G2 where they underwent apoptosis prior to M, in contrast to HCT116 p21^{-/-} cells that demonstrated more prolonged accumulation in S-phase followed by cell-cycle re-entry in the absence of apoptosis (20). Although overexpression of Bcl-2 or treatment with Z-VAD-fmk, that inhibit apoptosis, can have less effect on the overall response to TS inhibitors as determined by clonogenic survival assay (51), the contribution of mitotic cell death to this class of agent and other antimetabolites remains to be unequivocally determined.

1.3. Autophagy (Type II) and Paraptosis

Autophagy is characterized by sequestration of bulk cytoplasm and organelles into autophagic vesicles with subsequent degradation by the cellular lysosomal system (177). It can be downregulated by mutation in phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and activation of Akt signaling (184), induced in the presence of DN-Akt and regulated by target of rapamycin (TOR) (185). Autophagy is often observed when apoptosis is suppressed (186). Suppression of autophagy may be involved in the process of tumorigenesis (187). Drugs including vinblastine, rapamycin, and the estrogen antagonist tamoxifen can induce autophagy (185); however, the contribution of this mode of cell death to overall response has yet to be determined, and no studies linking this form of cell death to the cytotoxic response of antimetabolites have yet been reported.

Paraptosis is characterized by progressive swelling of the mitochondria and the endoplasmic reticulum, followed by cytoplasmic vacuolation (177). This form of cell

death is caspase-independent and can be triggered by the TNF receptor family member TAJ/TROY (188) and the IGFIR (189); however, its role in cell death induced by DNA-damaging agents including antimetabolites has not yet been established.

2. PASSIVE (NECROSIS) CELL DEATH

Necrosis is characterized by swelling of the cytoplasm and mitochondria, rupture of the plasma membrane, loss of chromatin (190,191), and DNA fragmentation (191,192), and *in vivo* induces an inflammatory response (176). Peters et al. (30) demonstrated morphologic evidence of necrosis in the absence of apoptosis in WiDr colon carcinoma cells following treatment with FUra or ZD1694. An additional study employing electron microscopy of MKN45 gastric cancer cells demonstrated mixed patterns of cell death in the population following FUra treatment, including two distinct patterns of necrosis (191). As mentioned earlier, necrosis has been detected as a secondary form of cell death in response to purine analogs (144) (Fig. 3). This mechanism of cell death induction has not been well documented in response to antimetabolites, however, when present may contribute to the overall survival of cell populations.

3. SENESENCE ARREST AND DIFFERENTIATION

Replicative senescence limits the proliferative life span of normal cells (193), whereas accelerated senescence, which is more rapid, can be induced by treatment with DNA-damaging agents (194) or introduction of Ras or Raf oncogenic mutants into normal cells (195,196). The phenotype is characterized by enlargement and flattening of cell shape, increased granularity, and expression of senescence-associated β -galactosidase (SA- β -gal). Terminal arrest requires the function of p53, p21^{Waf1}, p16^{INK4A}, and the shortening of telomeres [reviewed in (197,198)].

Earlier studies of differentiation induction by antimetabolites have most frequently involved leukemic cells following treatment with Cyd analogs and are most likely linked to the induction of cellular senescence. Thus, Ara-C induced differentiation of U937 leukemic cells involving p21^{Cip1} (199), and a Cyd deaminase-resistant analog of Ara-C induced differentiation in human myeloid leukemic cell lines *in vitro* and was active in Ara-C-resistant solid tumors *in vivo* (200). In HT1080 cells, senescence induction was weaker and also independent from mitotic cell death following treatment with Ara-C in the absence of apoptosis (193). In the human B-cell lymphoma cell line BL36, treatment with 5-AzaC decreased cell growth following cell-cycle arrest at the G0/G1-phase, and cells demonstrated phenotypic changes consistent with a differentiated phenotype (201). 5-dAzaC induced re-expression of p16^{INK4A} and the expression of SA- β -gal in hepatocellular carcinoma cells (202). Furthermore, MLL cells were effectively induced to differentiate by all-trans retinoic acid or dihydroxyvitamin D3 in the presence of 5-dAzaC, which effectively induced the expression of p16^{INK4A} (203).

Cellular senescence has also been induced by BrdUrd (204,205) or HU (206) in several cell lines and by MTX in MCF-7 cells dependent on p53 and caspase function (170). Inhibition of caspase function has switched doxorubicin-induced apoptosis to senescence, although this has not yet been demonstrated for antimetabolites (207). In one study utilizing tumor samples from patients receiving neoadjuvant therapy for breast cancer that involved treatment with cyclophosphamide, doxorubicin, and FUra, SA- β -gal staining was observed in 41% of the specimens and was associated

with low p53 staining (wtp53) and high expression of p16^{INK4A} (208). Hence, it is evident that cell death processes in addition to apoptosis can be induced in the same cell population by antimetabolites and that cellular senescence may play a role in overall response to agents in preclinical model systems and also clinically.

4. CONCLUSIONS

Although the mechanisms that induce cell death following exposure of cancer cells to DNA-damaging agents both *in vitro* and *in vivo* are still being determined, it is evident that the mechanism most studied in response to antimetabolites is the induction of apoptosis. It is clear that apoptosis can be responsible for cell death in cultured cell models and in preclinical models, and clinically, the expression of certain genes that regulates apoptotic responses has yielded prognostic significance in certain studies. However, data generated in the presence of overexpression of Bcl-2 or Bcl-xL, or during co-exposure to inhibitors of caspase activation, indicate that in other systems, the kinetics of induction of cell death may be delayed but not prevented, suggesting that apoptosis may not contribute significantly to the overall survival of a population following drug treatment. Other mechanisms of induction of cell death have been identified in response to antimetabolites, including mitotic catastrophe, necrosis, and senescence, and mixed forms of cell death have also been identified within the same cell population. This field of research is an emerging area in our understanding of the mechanisms by which specific forms of DNA damage kill cells, and a considerable amount is still to be learned about cellular responses of cancer cells induced following DNA damage by the various classes of antimetabolites.

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REFERENCES

1. Castro-Obregon S, Rao RV, del Rio G, Chen SF, Poksay KS, Rabizadeh S, Vesce S, Zhang XK, Swanson RA, Bredesen DE. Alternative, nonapoptotic programmed cell death. *J Biol Chem* 2004; 279:17543–17553.
2. Brown JM, Wouters BG. Apoptosis, p53, and tumor cell sensitivity to anticancer agents. *Cancer Res* 1999; 59:1391–1399.
3. Tannock IF, Lee C. Evidence against apoptosis as a major mechanism for reproductive cell death following treatment of cell lines with anti-cancer drugs. *Br J Cancer* 2001; 84:100–105.
4. Brown M, Wilson G. Apoptosis genes and resistance to cancer therapy. *Cancer Biol Ther* 2003; 2:477–490.
5. Gudkov A, Komarova EA. The role of p53 in determining sensitivity to radiotherapy. *Nat Rev* 2003; 3:117–129.
6. Green DR, McGahon A, Martin SJ. Regulation of apoptosis by oncogenes. *J Cell Biochem* 1996; 60:33–38.
7. Gajewski TF, Thompson CB. Apoptosis meets signal transduction: elimination of a BAD influence. *Cell* 1996; 87:589–592.
8. Sobrero AF, Aschele C, Bertino JR. Fluorouracil in colorectal cancer – a tale of two drugs: implications for biochemical modulation. *J Clin Oncol* 1997; 15:368–381.

9. van Laar JA, Rustum YM, Ackland SP, van Groeningen CJ, Peters GJ. Comparison of 5-fluoro-2'-deoxyuridine with 5-fluorouracil and their role in the treatment of colorectal cancer. *Eur J Cancer* 1998; 34:296–306.
10. Schwartzberg L, Petak I, Stewart C, Turner PK, Ashley J, Tillman DM, Douglas L, Mihalik R, Weir A, Tauer K, Shope S, Houghton JA. Modulation of the Fas signaling pathway by interferon- γ in therapy of colon cancer: phase I trial and correlative studies of interferon- γ , 5-fluorouracil and leucovorin. *Clin Cancer Res* 2002; 8:2488–2498.
11. Houghton JA, Tillman DM, Harwood FG. The ratio of dATP/dTTP influences the commitment of human colon carcinoma cells to thymineless death. *Clin Cancer Res* 1995; 1:723–730.
12. Maybaum J, Ullman B, Mandel HG, Day JL, Sadee W. Regulation of RNA- and DNA-directed actions of 5-fluoropyrimidines in mouse T-lymphoma (S-49) cells. *Cancer Res* 1980; 40:4209–4215.
13. Petak I, Tillman DM, Houghton JA. P53-dependence of Fas induction and acute apoptosis in response to 5-fluorouracil-leucovorin in human colon carcinoma cell lines. *Clin Cancer Res* 2000; 6:4432–4441.
14. Parsels LA, Parsels JD, Wagner LM, Loney TL, Radany EH, Maybaum J. Mechanism and pharmacological specificity of dUTPase-mediated protection from DNA damage and cytotoxicity in human tumor cells. *Cancer Chemother Pharmacol* 1998; 42:357–362.
15. Grem JL. 5-Fluorouracil: forty plus and still ticking. A review of its preclinical and clinical development. *Invest New Drugs* 2000; 18:299–313.
16. Houghton JA, Houghton PJ, Wooten RS. Mechanism of induction of gastrointestinal toxicity in the mouse by 5-fluorouracil, 5-fluorouridine and 5-fluoro-2'-deoxyuridine. *Cancer Res* 1979; 39:2406–2413.
17. Pritchard DM, Watson AJM, Potten CS, Jackman AL, Hickman JA. Inhibition by uridine but not thymidine of p53-dependent intestinal apoptosis initiated by 5-fluorouracil: evidence for the involvement of RNA perturbation. *Proc Natl Acad Sci USA* 1997; 94:1795–1799.
18. Rustum YM, Cao S. New drugs in colorectal cancer: preclinical studies. *Semin Oncol* 1999; 26:612–620.
19. Sobrero A, Guglielmi A, Grossi F, Puglisi F, Aschele C. Mechanism of action of fluoropyrimidines: relevance to the new developments in colorectal cancer chemotherapy. *Semin Oncol* 2000; 27 (5 Suppl 10):72–77.
20. Geller JI, Szekely-Szucs K, Petak I, Doyle B, Houghton JA. P21^{Cip1} is a critical mediator of the cytotoxic action of thymidylate synthase (TS) inhibitors in colorectal carcinoma cells. *Cancer Res* 2004; 64:6296–6303.
21. Eisold S, Linnebacher M, Ryschich E, Antolovic D, Hinz U, Klar E, Schmidt J. The effect of adenovirus expressing wild-type p53 on 5-fluorouracil chemosensitivity is related to p53 status in pancreatic cancer cell lines. *World J Gastroenterol* 2004; 10:3583–3589.
22. de Angelis PM, Fjell B, Kravik KL, Haug T, Tunheim SH, Reichelt W, Beigi M, Clausen OP, Galteland E, Stokke T. Molecular characterizations of derivatives of HCT116 colorectal cancer cells that are resistant to the chemotherapeutic agent 5-fluorouracil. *Int J Oncol* 2004; 24:1279–1288.
23. Li WW, Fan J, Hochhauser D, Bertino JR. Overexpression of p21waf1 leads to increased inhibition of E2F-1 phosphorylation and sensitivity to anticancer drugs in retinoblastoma-negative human sarcoma cells. *Cancer Res* 1997; 57:2193–2199.
24. Parsels LA, Parsels JD, Tai DC-H, Coughlin DJ, Maybaum J. 5-Fluoro-2'-deoxyuridine-induced cdc25A accumulation correlates with premature mitotic entry and clonogenic death in human colon cancer cells. *Cancer Res* 2004; 64:6588–6594.
25. Xiao Z, Xue J, Sowin TJ, Rosenberg SH, Zhang H. A novel mechanism of checkpoint abrogation conferred by chk1 downregulation. *Oncogene* 2005; 24:1403–1411.
26. Engels IH, Szczepynska A, Stroh C, Lauber K, Berg C, Schwenzer R, Wajant H, Janicke RU, Porter AG, Belka C, Gregor M, Schulze-Osthoff K, Wesselborg S. Caspase-8/FLICE functions as an executioner caspase in anticancer drug-induced apoptosis. *Oncogene* 2000; 19:4563–4573.
27. Adachi Y, Taketani S, Oyaizu H, Ikebukuro K, Tokunaga R, Ikehara S. Apoptosis of colorectal adenocarcinoma induced by 5-FU and/or IFN- γ through caspase 3 and caspase 8. *Int J Oncol* 1999; 15:1191–1196.
28. Wu XX, Kakehi Y, Mizutani Y, Lu J, Terachi T, Ogawa O. Activation of caspase-3 in renal cell carcinoma cells by anthracyclines or 5-fluorouracil. *Int J Oncol* 2001; 19:19–24.
29. Petak I, Tillman DM, Harwood FG, Houghton JA. Fas-dependent and -independent mechanisms of cell death following DNA damage in human colon carcinoma cells. *Cancer Res* 2000; 60:2643–2650.

30. Peters GJ, van Triest B, Backus HHJ, Kuiper CM, van der Wilt CL, Pinedo HM. Molecular downstream events and induction of thymidylate synthase in mutant and wild-type p53 colon cancer cell lines after treatment with 5-fluorouracil and the thymidylate synthase inhibitor raltitrexed. *Eur J Cancer* 2000; 36:916–924.
31. Geller J, Petak I, Szekely Szucs K, Nagy K, Tillman DM, Houghton JA. Interferon- γ -induced sensitization of colon carcinomas to ZD9331 targets caspases, downstream of Fas, independent of mitochondrial signaling and the IAP survivin. *Clin Cancer Res* 2003; 9:6504–6515.
32. Petak I, Tillman DM, Harwood FG, Houghton JA. Fas-dependent and -independent mechanisms of cell death following DNA damage in human colon carcinoma cells. *Cancer Res* 2000; 60:2643–2650.
33. Green DR, Beere HM. Killers or clean-up crew: how central are the central mechanisms of apoptosis. In: *Apoptosis and Cancer Therapy*, Hickman JA and Dive C (eds.), Totowa, NJ: Humana Press Inc., 1999; 157–174.
34. McCarthy NJ, Whyte MKB, Gilbert CS, Evan GI. Inhibition of ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak. *J Cell Biol* 1997; 136:215–227.
35. Houghton JA, Harwood FG, Tillman DM. Thymineless death in colon carcinoma cells is mediated via Fas signaling. *Proc Natl Acad Sci USA* 1997; 94:8144–8149.
36. Tillman DM, Petak I, Houghton JA. A Fas-dependent component in 5-fluorouracil/leucovorin-induced cytotoxicity in colon carcinoma cells. *Clin Cancer Res* 1999; 5: 425–430.
37. Eichhorst ST, Muller M, Li-Weber M, Schulze-Bergkamen H, Angel P, Krammer PH. A novel AP-1 element in the CD95 ligand promoter is required for induction of apoptosis in hepatocellular carcinoma cells upon treatment with anticancer drugs. *Mol Cell Biol* 2000; 20:7826–7837.
38. Longley DB, Allen WL, McDermott U, Wilson TR, LAtif T, Boyer J, Lynch M, Johnston PG. The roles of thymidylate synthase and p53 in regulating Fas-mediated apoptosis in response to antimetabolites. *Clin Cancer Res* 2004; 10:3562–3571.
39. Eichhorst ST, Muerkoster S, Weigand MA, Krammer PH. The chemotherapeutic drug 5-fluorouracil induces apoptosis in mouse thymocytes *in vivo* via activation of the CD95(APO-1/Fas) system. *Cancer Res* 2001; 61:243–248.
40. Cicolini J, Fina F, Bezulier K, Giacometti S, Roussel M, Evrard A, Cuq P, Romain S, Martin P-M, Aubert C. Transmission of apoptosis in human colorectal tumor cells exposed to capecitabine, xeloda, is mediated via Fas. *Mol Cancer Ther* 2002; 1:923–927.
41. Moller P, Koretz K, Leithauser F, Bruderlein S, Henne C, Quentmeier A, Krammer PH. Expression of APO-1 (CD95), a member of the NGF/TNF receptor superfamily, in normal and neoplastic colon epithelium. *Int J Cancer* 1994; 57: 371–377.
42. Leithauser F, Dhein J, Mechtersheimer G, Koretz K, Bruderlein S, Henne C, Schmidt A, Debatin KM, Krammer PH, Moller P. Constitutive and induced expression of APO-1, a new member of the nerve growth factor/tumor necrosis factor receptor superfamily, in normal and neoplastic cells. *Lab Invest* 1993; 69:415–429.
43. Iwase M, Watanabe H, kondo G, Ohashi M, Nagumo M. Enhanced susceptibility of oral squamous cell carcinoma cell lines to FAS-mediated apoptosis by cisplatin and 5-fluorouracil. *Int J Cancer* 2003; 106:619–625.
44. Ganten TM, Haas TL, Sykora J, Stahl H, Sprick MR, Fas SC, Krueger A, Weigand MA, Grosse-Wilde A, Stremmel W, Krammer PH, Walczak H. Enhanced caspase-8 recruitment to and activation at the DISC is critical for sensitization of human hepatocellular carcinoma cells to TRAIL-induced apoptosis by chemotherapeutic drugs. *Cell Death Diff* 2004; 11:S86–S96.
45. Lacour S, Micheau O, Hammann A, Drouineaud V, Tschopp J, Solary E, Dimanche-Boitrel M-T. Chemotherapy enhances TNF-related apoptosis-inducing ligand DISC assembly in HT29 human colon cancer cells. *Oncogene* 2003; 22:1807–1816.
46. Naka T, Sugamura K, Hylander BL, Widmer MB, Rustum YM, Repasky EA. Effects of tumor necrosis factor-related apoptosis-inducing ligand alone and in combination with chemotherapeutic agents on patients' colon tumors grown in SCID mice. *Cancer Res* 2002; 62:5800–5806.
47. Yamamoto T, Nagano H, Sakon M, Wada H, Eguchi H, Kondo M, Damdinsuren B, Ota H, Nakamura M, Wada H, Marubashi S, Miyamoto A, Dono K, Umeshita K, Nakamori S, Yagita H, Monden M. Partial contribution of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor pathway to antitumor effects of interferon-alpha/5-fluorouracil against hepatocellular carcinoma. *Clin Cancer Res* 2004; 10:7884–7895.

48. Wang S, El-Deiry WS. Inducible silencing of KILLER/DR5 *in vivo* promotes bioluminescent colon tumor xenograft growth and confers resistance to chemotherapeutic agent 5-fluorouracil. *Cancer Res* 2004; 64:6666–6672.
49. Wang W, Cassidy J. Constitutive nuclear factor-kappa B mRNA, protein overexpression and enhanced DNA-binding activity in thymidylate synthase inhibitor-resistant tumour cells. *Br J Cancer* 2003; 88:624–629.
50. Wang W, McLeod HL, Cassidy J. Disulfiram-mediated inhibition of NF- κ B activity enhances cytotoxicity of 5-fluorouracil in human colorectal cancer cell lines. *Int J Cancer* 2003; 104:504–511.
51. Voboril R, Hochwald SN, Li J, Brank A, Weberova J, Wessels F, Moldawer LL, Camp ER, MacKay SL. Inhibition of NF-kappa B augments sensitivity to 5-fluorouracil/folinic acid in colon cancer. *J Surg Res* 2004; 120:178–188.
52. Uetsuka H, Haisa M, Kimura M, Gunduz M, Kaneda Y, Ohkawa T, Takaoka M, Murata T, Nobuhisa T, Yamatsuji T, Matsuoka J, Tanaka N, Naomoto Y. Inhibition of inducible NF-kappaB activity reduces chemoresistance to 5-fluorouracil in human stomach cancer cell line. *Exp Cell Res* 2003; 10:27–35.
53. Yang JH, Fenf F, Qian H, Cheng H. Chemosensitization of breast carcinoma cells with the use of bcl-2 antisense oligonucleotide. *Breast* 2004; 13:227–231.
54. Guo B, Cao S, Toth K, Azrak RG, Rustum YM. Overexpression of Bax enhances antitumor activity of chemotherapeutic agents in human head and neck squamous cell carcinoma. *Clin Cancer Res* 2000; 6:718–724.
55. Xu ZW, Freiss H, Buchler MW, Solioz M. Overexpression of Bax sensitizes human pancreatic cancer cells to apoptosis induced by chemotherapeutic agents. *Cancer Chemother Pharmacol* 2002; 49:504–510.
56. Pritchard DM, Print C, O'Reilly L, Adams JM, Potten CS, Hickman JA. Bcl-w is an important determinant of damage-induced apoptosis in epithelia of small and large intestine. *Oncogene* 2000; 19:3955–3959.
57. Yuan J, Yanker BA. Caspase activity sows the seeds of neuronal death. *Nat Cell Biol* 1999; 1: E44–E45.
58. Rao RV, Castro-Obregon S, Frankowski H, Schuler M, Stoka V, Del Rio G, Bredesen DE, Ellerby HM. Coupling endoplasmic reticulum stress to the cell death program. An Apaf-1-independent intrinsic pathway. *J Biol Chem* 2002; 277:21836–21842.
59. Morishima N, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y. An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12. *J Biol Chem* 2002; 277:34287–34294.
60. Halloran CM, Ghaneh P, Shore S, Greenhalf W, Zumstein L, Wilson D, Neoptolemos JP, Costello E. 5-Fluorouracil or gemcitabine combined with adenoviral-mediated reintroduction of p16INK4A greatly enhanced cytotoxicity in Panc-1 pancreatic adenocarcinoma cells. *J Gene Med* 2004; 6: 514–525.
61. Hsu LC, Lee RM, White RL. The HPV16 E6/E7 oncogene sensitizes human ovarian surface epithelial cells to low-dose but not high-dose 5-FU and 5-FUdR. *Biochem Biophys Res Commun* 2004; 320:249–255.
62. Elliott MJ, Farmer MR, Atienza C, Stilwell A, Dong YB, Yang HL, Wong SL, McMasters KM. E2F-1 gene therapy induces apoptosis and increases chemosensitivity in human pancreatic carcinoma cells. *Tumour Biol* 2002; 23:76–86.
63. Ramsay RG, Micallef S, Lightowler S, Mucenski ML, Mantamadiotis T, Bertoncello I. C-myc heterozygous mice are hypersensitive to 5-fluorouracil and ionizing radiation. *Mol Cancer Res* 2004; 2:354–361.
64. Modrak DE, Rodriguez MD, Goldenberg DM, Lew W, Blumenthal RD. Sphingomyelin enhances chemotherapy efficacy and increases apoptosis in human colonic tumor xenografts. *Int J Oncol* 2002; 20:379–384.
65. Tseng YS, Tzeng CC, Chiu AW, Lin CH, Won SJ, Wu IC, Liu HS. Ha-ras overexpression mediated cell apoptosis in the presence of 5-fluorouracil. *Exp Cell Res* 2003; 288:403–414.
66. Jin W, Wu L, Liang K, Liu B, Lu Y, Fan Z. Roles of the PI-3K and MEK pathways in Ras-mediated chemoresistance in breast cancer cells. *Br J Cancer* 2003; 89:185–191.
67. Sun Y, Tang XM, Half E, Kuo MT, Sinicrope FA. Cyclooxygenase-2 overexpression reduces apoptotic susceptibility by inhibiting the cytochrome c-dependent apoptotic pathway in human colon cancer cells. *Cancer Res* 2002; 62:6323–6328.

68. Chen WS, Liu JH, Liu JM, Lin JK. Sequence-dependent effect of a cyclooxygenase-2 inhibitor on topoisomerase I inhibitor and 5-fluorouracil-induced cytotoxicity of colon cancer cells. *Anticancer Drugs* 2004; 15:287–294.
69. Meyers M, Wagner MW, Hwang H-S, Kinsella TJ, Boothman DA. Role of the hMLH1 DNA mismatch repair protein in fluoropyrimidine-mediated cell death and cell cycle responses. *Cancer Res* 2001; 61:5193–5201.
70. Meyers M, Hwang A, Wagner MW, Boothman DA. Special section: impact of the environment on colon cancer (II): roles of DNA mismatch repair in apoptotic responses to therapeutic agents. *Environ Mol Mutagen* 2004; 44:249–264.
71. Schneider HJ, Sampson SA, Cunningham D, Norman AR, Andreyev HJ, Tilsed JV, Clarke PA. Bcl-2 expression and response to chemotherapy in colorectal adenocarcinomas. *Br J Cancer* 1997; 75:427–431.
72. Paradiso A, Simone G, Lena MD, Leone B, Vallejo C, Lacava J, Dellapasqua S, Daidone MG, Costa A. Expression of apoptosis-related markers and clinical outcome in patients with advanced colorectal cancer. *Br J Cancer* 2001; 84:651–658.
73. Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC, Perucho M. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* 1997; 275:967–969.
74. Ouyang H, Furukawa T, Abe T, Kato Y, Horii A. The BAX gene, the promoter of apoptosis, is mutated in genetically unstable cancers of the colorectum, stomach, and endometrium. *Clin Cancer Res* 1998; 4:1071–1074.
75. Ionov Y, Yamamoto H, Krajewski S, Reed JC, Perucho M. Mutational inactivation of the proapoptotic gene BAX confers selective advantage during tumor clonal evolution. *Proc Natl Acad Sci USA* 2000; 97:10872–10877.
76. Schelwies K, Sturm I, Grabowski P, Scherubl H, Schindler I, Hermann S, Stein H, Huhr H-J, Riecken EO, Zeitz M, Dorken B, Daniel PT. Analysis of P53/BAX in primary colorectal carcinoma: low BAX protein expression is a negative prognostic factor in UICC stage III tumors. *Int J Cancer* 2002; 99: 589–596.
77. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 1995; 80:293–299.
78. Sturm I, Kohne C-H, Wolff G, Petrowsky H, Hillebrand T, Hauptmann S, Lorenz M, Dorken B, Daniel PT. Analysis of the p53/BAX pathway in colorectal cancer: low BAX is a negative prognostic factor in patients with resected liver metastases. *J Clin Oncol* 1999; 17:1364–1374.
79. Sarela A, Scott N, Ramsdale J, Markham AF, Guilou PJ. Immunohistochemical detection of the anti-apoptosis protein, survivin, predicts survival after curative resection of stage II colorectal carcinomas. *Ann Surg Oncol* 2001; 8:305–310.
80. Rodel F, Hoffmann J, Grabenbauer GG, Papadopoulos T, Weiss C, Gunther K, Schick C, Sauer R, Rodel C. High survivin expression is associated with reduced apoptosis in rectal cancer and may predict disease-free survival after preoperative radiochemotherapy and surgical resection. *Strahlenther Onkol* 2002; 178:426–435.
81. Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N. Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. *Cancer Res* 1998; 58:5071–5074.
82. Takamizawa S, Scott D, Wen J, Grundy P, Bishop W, Kimura K, Sandler A. The survivin:fas ratio in pediatric renal tumors. *J Pediatr Surg* 2001; 36:37–42.
83. Sandler A, Scott D, Azuhata T, Takamizawa S, O'Dorisio S. The survivin:Fas ratio is predictive of recurrent disease in neuroblastoma. *J Pediatr Surg* 2002; 37:507–511.
84. Olie RA, Simoes-Wust AP, Baumann B, Leech SH, Fabbro D, Stahel RA, Zangemeister-Wittke U. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cells to chemotherapy. *Cancer Res* 2000; 60:2805–2809.
85. Kufe D, Spriggs D, Egan EM, Munroe D. Relationships among Ara-CTP pools, formation of (Ara-C) DNA, and cytotoxicity of human leukemic cells. *Blood* 1984; 64: 54–58.
86. Mansson E, Paul A, Lofgren C, Ullberg K, Paul C, Eriksson S, Albertioni F. Cross-resistance to cytosine arabinoside in a multidrug-resistant human promyelocytic cell line selected for resistance to doxorubicin: implications for combination chemotherapy. *Br J Haematol* 2001; 114:557–565.
87. Aciwa H, Oguri T, Sato S, Maeda H, Niimi T, Ueda R. Determinants of sensitivity and resistance to gemcitabine: the roles of human equilibrative nucleoside transporter 1 and deoxycytidine kinase in non-small cell lung cancer. *Cancer Sci* 2004; 95:753–757.

88. Galmarini CM, Clarke ML, Jordheim L, Santos CL, Cros E, Mackey JR, Dumontet C. Resistance to gemcitabine in a human follicular lymphoma cell line is due to partial deletion of the deoxycytidine kinase gene. *BMC Pharmacol* 2004; 4:8.
89. Duxbury MS, Hirouchi I, Zinner MJ, Ashley SW, Whang EE. RNA interference targeting the M2 subunit of ribonucleotide reductase enhances pancreatic adenocarcinoma chemosensitivity to gemcitabine. *Oncogene* 2004; 23:1539–1548.
90. Kanno S, Higurashi A, Watanabe Y, Shouji A, Asou K, Ishikawa M. Susceptibility to cytosine arabinoside (Ara-C)-induced cytotoxicity in human leukemia cell lines. *Toxicol Lett* 2004; 152: 149–158.
91. Chen M, Hough AM, Lawrence TS. The role of p53 in gemcitabine-mediated cytotoxicity and radio sensitization. *Cancer Chemother Pharmacol* 2000; 45:369–374.
92. Schniewind B, Christgen M, Kurdow R, Haye S, Kremer B, Kalthoff H. Resistance of pancreatic cancer to gemcitabine treatment is dependent on mitochondria-mediated apoptosis. *Int J Cancer* 2004; 109:182–188.
93. Wang Z, Wang S, Dai Y, Grant S. Brostatin 1 increases 1-β-D-arabinofuranosylcytosine-induced cytochrome c release and apoptosis in human leukemia cells ectopically expressing Bcl-xL. *J Pharmacol Exp Ther* 2002; 301:568–577.
94. Tang L, Boise LH, Dent P, Grant S. Potentiation of 1-β-D-arabinofuranosylcytosine-mediated mitochondrial damage and apoptosis in human leukemia cells (U937) overexpressing Bcl-2 by the kinase inhibitor 7-hydroxystaurosporine (UCN-01). *Biochem Pharmacol* 2000; 60:1445–1456.
95. Kim CN, Wang X, Huang Y, Ibrado AM, Liu L, Fang G, Bhalla K. Overexpression of Bcl-x(L) inhibits Ara-C-induced mitochondrial loss of cytochrome c and other perturbations that activate the molecular cascade of apoptosis. *Cancer Res* 1997; 57:3115–3120.
96. Chang G-C, Hsu S-L, Tsai J-R, Wu W-J, Chen C-Y, Sheu G-T. Extracellular signal-regulated kinase activation and Bcl-2 downregulation mediate apoptosis after gemcitabine treatment partly via a p53-independent pathway. *Eur J Pharmacol* 2004; 502:169–183.
97. Xu Z, Freiss H, Solioz M, Aebi S, Korc M, Kleef J, Buchler MW. Bcl-x(L) antisense oligonucleotides induce apoptosis and increase sensitivity of pancreatic cancer cells to gemcitabine. *Int J Cancer* 2001; 94:268–274.
98. Konopleva M, Tari AM, Estrov Z, Harris D, Xie Z, Zhao S, Lopez-Berestein G, Andreeff M. Liposomal Bcl-2 antisense oligonucleotides enhance proliferation, sensitize acute myeloid leukemia to cytosine-arabioside, and induce apoptosis independent of other antiapoptotic proteins. *Blood* 2001; 95:3929–3938.
99. Vrana JA, Bieszczyk CK, Cleveland ES, Ma Y, Park JP, Mohandas TK, Craig RW. An MCL-1-overexpressing Burkitt lymphoma subline exhibits enhanced survival on nexpopure to serum deprivation, topoisomerase inhibitors, or staurosporine but remains sensitive to 1-beta-D-arabinofuranosylcytosine. *Cancer Res* 2002; 62:892–900.
100. Shi X, Liu S, Kleeff J, Freiss H, Buchler MW. Acquired resistance of pancreatic cancer cells towards 5-fluorouracil and gemcitabine is associated with altered expression of apoptosis-regulating genes. *Oncology* 2002; 62:354–362.
101. Lock RB and Stribinskene L. Dual modes of cell death induced by etoposide in human epithelial tumor cells allow Bcl-2 to inhibit apoptosis without affecting clonogenic survival. *Cancer Res* 1996; 56:4006–4012.
102. Amarante-Mendes GP, Finucane DM, Martin SJ, Cotter TG, Salvesen GS, Green DR. Anti-apoptotic oncogenes prevent caspase-dependent and independent commitment for cell death. *Cell Death Diff* 1998; 4:298–306.
103. Ng SSW, Tsao M-S, Chow S, Hadley DW. Inhibition of phosphatidylinositol 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells. *Cancer Res* 2000; 60:5451–5455.
104. Ng SS, Tsao MS, Nicklee T, Hedley DW. Wortmannin inhibits pkb/akt phosphorylation and promotes gemcitabine antitumor activity in orthotopic human pancreatic cancer xenografts in immunodeficient mice. *Clin Cancer Res* 2001; 7:3269–3275.
105. Yokoi K, Fidler IJ. Hypoxia increases resistance of human pancreatic cancer cells to apoptosis induced by gemcitabine. *Clin Cancer Res* 2004; 10:2299–2306.
106. Yu C, Wang Z, Dent P, Grant S. MEK1/2 inhibitors promote Ara-C-induced apoptosis but not loss in ΔΨ_m in HL-60 cells. *Biochem Biophys Res Commun* 2001; 286:1011–1018.
107. Milella M, Kornblau SM, Estrov Z, Carter BZ, Lapillonne H, Harris D, Konopleva M, Zhao S, Estey E, Andreeff M. Therapeutic targeting of the MEKK/MAPK signal transduction module in acute myeloid leukemia. *J Clin Invest* 2001; 108:851–859.

108. Habiro A, Tanno S, Koizumi K, Izawa T, Nakano Y, Osanai M, Mizukami Y, Okumura T, Kohgo Y. Involvement of p38 mitogen-activated protein kinase in gemcitabine-induced apoptosis in human pancreatic cancer cells. *Biochem Biophys Res Commun* 2004; 316:71–77.
109. Jarvis WD, Fornari FA, Tombes RM, Erukulla RK, Bittman R, Schwartz GK, Dent P, Grant S. Evidence of involvement of mitogen-activated protein kinase, rather than stress-activated protein kinase, in potentiation of 1- β -D-arabinofuranosylcytosine-induced apoptosis by interruption of protein kinase C signaling. *Mol Pharmacol* 1998; 54:844–856.
110. Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. Inhibition of Src tyrosine kinase impairs inherent and acquired gemcitabine resistance in human pancreatic adenocarcinoma cells. *Clin Cancer Res* 2004; 10:2307–2318.
111. Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. SiRNA directed against s-Src enhances pancreatic adenocarcinoma cell gemcitabine chemosensitivity. *J Am Coll Surg* 2004; 198:953–959.
112. Arlt A, Gehrz A, Muerkoster S, Vorndamm J, Kruse ML, Folsch UR, Schafer H. Role of NF-kappaB and Akt/PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death. *Oncogene* 2003; 22:3243–3251.
113. Jones DR, Broad RM, Comeau LD, Parsons SJ, Mayo MW. Inhibition of nuclear factor kappaB chemosensitizes non-small cell lung cancer through cytochrome c release and caspase activation. *J Thorac Cardiovasc Surg* 2002; 123:310–317.
114. Romano MF, Lamberti A, Bisogni R, Tassone P, Pagnini D, Storti G, Del Vecchio L, Turco MC, Venuta S. Enhancement of cytosine arabinoside-induced apoptosis in human myeloblastic leukemia cells by NF-kappa B-Rel-specific decoy oligodeoxynucleotides. *Gene Ther* 2000; 7:1234–1237.
115. Denlinger CE, Rundall BK, Keller MD, Jones DR. Proteasome inhibition sensitizes non-small-cell lung cancer to gemcitabine-induced apoptosis. *Ann Thorac Surg* 2004; 78:1207–1214.
116. Zheng B, Georgakis GV, Li Y, Bharti A, McConkey D, Aggarwal BB, Younes A. Induction of cell cycle arrest and apoptosis by the proteasome inhibitor PS-341 in Hodgkin disease cell lines is independent of inhibitor of nuclear factor- κ B mutations or activation of the CD30, CD40, and RANK receptors. *Clin Cancer Res* 2004; 10:3207–3215.
117. Kamat AM, Karashima T, Davis DW, Lashinger L, Bar-Eli M, Millikan R, Shen Y, Dinney CPN, McConkey DJ. The proteasome inhibitor bortezomib synergizes with gemcitabine to block the growth of human 253JB-V bladder tumors *in vivo*. *Mol Cancer Ther* 2004; 3:279–290.
118. Meli M, Tolomeo M, D'Alessandro N, Grimaudo S, Notarbartolo M, Papoff G, Ruberti G, Rausa L, Dusonchet L. Resistance to gemcitabine in a lymphoma cell line resistant to Fas-mediated apoptosis. *Anticancer Res* 2004; 24:851–857.
119. Ferreira CG, Tolis C, Span SW, Peters GJ, van Lopik T, Kummer AJ, Pinedo HM, Giaccone G. Drug-induced apoptosis in lung cancer cells is not mediated by the Fas/FasL (CD95/APO1) signaling pathway. *Clin Cancer Res* 2000; 6:203–212.
120. Liu W, Bodle E, Chen JY, Gao M, Rosen GD, Broaddus VC. Tumor necrosis factor-related apoptosis-inducing ligand and chemotherapy cooperate to induce apoptosis in mesothelioma cell lines. *Am J Respir Cell Mol Biol* 2001; 25:111–118.
121. Fojo T, Bates S. Strategies for reversing drug resistance. *Oncogene* 2003; 22:7512–7523.
122. Glover AB, Leyland-Jones BR, Chun HG, Davies B, Hoth DF. Acacitidine: 10 years later. *Cancer Treat Rep* 1987; 71:737–746.
123. Momparler RL, Cote S, Eliopoulos N. Pharmacological approach for optimization of the dose schedule of 5-aza-2'-deoxycytidine (decitabine) for the therapy of leukemia. *Leukemia* 1997; 11:175–180.
124. Durant ST, Morris MM, Illand M, McKay HJ, McCormick C, Hirst GL, Borts RH, Brown R. Dependence on RAD52 and RAD1 for anticancer drug resistance mediated by inactivation of mismatch repair genes. *Curr Biol* 1999; 9:51–54.
125. Plumb JA, Strathdee G, Sludden J, Kaye SB, Brown R. Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. *Cancer Res* 2000; 60:6039–6044.
126. Strathdee G, MacKean MJ, Illand M, Brown R. A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer. *Oncogene* 1999; 18:2335–2341.
127. Teitz T, Lahti JM, Kidd VJ. Aggressive childhood neuroblastomas do not express caspase-8: an important component of programmed cell death. *J Mol Med* 2001; 79:428–436.
128. Eggert A, Grotzer MA, Zuzak TJ, Wiewrodt BR, Ho R, Ikegaki N, Brodeur GM. Resistance to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression. *Cancer Res* 2001; 61:1314–1319.

129. Hopkins-Donaldson S, Bodmer J-L, Bourland KB, Brognara CB, Tschopp J, Gross N. Loss of caspase-8 expression in neuroblastoma is related to malignancy and resistance to TRAIL-induced apoptosis. *Med Pediatr Oncol* 2000; 35:608–611.
130. Fulda S, Kufer MU, Meyer E, van Halen F, Dockhorn-Dworniczak B, Debatin K-M. Sensitization for death receptor- or drug-induced apoptosis by re-expression of caspase-8 through demethylation or gene transfer. *Oncogene* 2001; 20:5865–5877.
131. Grotzer MA, Eggert A, Zuzak TJ, Janss Aj, Marwaha S, Wiewrodt BR, Ikegaki N, Brodeur GM, Phillips PC. Resistance to TRAIL-induced apoptosis in primitive neuroectodermal brain tumor cells correlates with a loss of caspase-8 expression. *Oncogene* 2000; 19:4604–4610.
132. Gomyo Y, Sasaki J, Branch C, Roth JA, Mukhopadhyay T. 5-Aza-2'-deoxycytidine upregulates caspase-9 expression cooperating with p53-induced apoptosis in human lung cancer cells. *Oncogene* 2004; 23:6779–6787.
133. Ueki T, Takeuchi T, Nishimatsu H, Kajiwara T, Moriyama N, Narita Y, Kawabe K, Ueki K, Kitamura T. Silencing of the caspase-1 gene occurs in murine and human renal cancer cells and causes solid tumor growth *in vivo*. *Int J Cancer* 2001; 91:673–679.
134. Cheson BD, Grever MR. Clinical development of 2' deoxycoformycin. In: *Nucleoside Analogs in Cancer Therapy*, Cheson BD, Keating MJ, Plunket W (eds), New York: Marcel Dekker, 1997; 95–112.
135. Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues: mechanisms of drug resistance and reversal strategies. *Leukemia* 2001; 15:875–890.
136. Pettitt A. mechanism of action of purine analogues in chronic lymphocytic leukaemia. *Br J Hematol* 2003; 121:692–702.
137. Kawasaki H, CARRERA CJ, Piro LD, Saven A, Kipps TJ, Carson DA. Relationship of deoxycytidine kinase and cytoplasmic 5'-nucleotidase to the chemotherapeutic efficacy of 20 chlorodeoxyadenosine. *Blood* 1993; 81:597–601.
138. Seto S, CARRERA CJ, Kubota M, Wasson DB, Carson DA. Mechanism of deoxyadenosine and 2-chlorodeoxyadenosine toxicity to nondividing human lymphocytes. *J Clin Invest* 1985; 75: 377–383.
139. Robertson LE, Chubb S, Meyn RE, Story M, Ford R, Hittelman WN, Plunkett W. Induction of apoptotic cell death in chronic lymphocytic leukemia by 2-chloro-2'-deoxyadenosine and 9-beta-D-arabinosyl-2-fluoroadenine. *Blood* 1993; 81:143–150.
140. Huang P, Plunkett W. Fludarabine- and gemcitabine-induced apoptosis: incorporation of analogs into DNA as a critical event. *Cancer Chemther Pharmacol* 1995; 36:181–188.
141. Huang P, Robertson LE, Wright LS, Plunkett W. High molecular weight DNA fragmentation: a critical event in nucleoside analogue-induced apoptosis in leukemia cells. *Clin Cancer Res* 1995; 1:1005–1013.
142. Huang P, Ballal K, Plunkett W. Biochemical characterization of the protein activity responsible for high molecular weight DNA fragmentation during drug-induced apoptosis. *Cancer Res* 1997; 57:3407–3414.
143. Belosillo B, Dalmau M, Colomer D, Gil J. Involvement of CED-3/ICE proteases in the apoptosis of B-chronic lymphocytic leukemia cells. *Blood* 1997; 89:3378–3384.
144. Pettitt AR, Cawley JC. Caspases influence the mode but not the extent of cell death induced by purine analogues in chronic lymphocytic leukaemia. *Br J Haematol* 2000; 109:800–804.
145. Perez-Galan P, Marzo I, Giraldo P, Rubio-Felix D, Lasiera P, Larrad L, Anel A, Naval J. Role of caspases and apoptosis-inducing factor (AIF) in cladribine-induced apoptosis of B cell chronic lymphocytic leukemia. *Leukemia* 2002; 16:2106–114.
146. Sampath D, Plunkett W. The role of c-Jun kinase in the apoptotic response to nucleoside analogue-induced DNA damage. *Cancer Res* 2000; 60:6408–6415.
147. Achanta G, Pelicano H, Feng L, Plunkett W, Huang P. Interaction of p53 and DNA-PK in response to nucleoside analogues: potential role as a sensor complex for DNA damage. *Cancer Res* 2001; 61:8723–8729.
148. Romano MF, Lamberti A, Turco MC, Venuta S. CD40 and B chronic lymphocytic leukemia cell response to fludarabine: the influence of NF-kappaB/Rel transcription factors on chemotherapy-induced apoptosis. *Leuk Lymph* 2000; 36:255–262.
149. de la Fuente MT, Casanove B, Moyano JV, Garcia-Gila M, Sanz L, Garcia-Marco J, Silva A, Garcia-Pardo A. Engagement of alpha4beta1 integrin by fibronectin induces *in vitro* resistance of B chronic lymphocytic leukemia cells to fludarabine. *J Leukoc Biol* 2002; 71:495–502.

150. Pepper C, Thomas A, Hidalgo de Quintana J, Davies S, Hoy T, Bentley P. Pleiotropic drug resistance in B-cell chronic lymphocytic leukaemia: the role of Bcl-2 family dysregulation. *Leuk Res* 1999; 23:1007–1014.
151. Morales AA, Olsson A, Celsing F, Osterborg A, Jondal M, Osorio LM. High expression of bfl-1 contributes to the apoptosis resistant phenotype in B-cell chronic lymphocytic leukemia. *Int J Cancer* 2005; 113:730–737.
152. Bosanquet AG, Sturm I, Wieder T, Essmann F, Bosanquet MI, Head DJ, Dorken B, Daniel PT. Bax expression correlates with cellular drug sensitivity to doxorubicin, cyclophosphamide and chlorambucil but not fludarabine, cladribine or corticosteroids in B cell chronic lymphocytic leukemia. *Leukemia* 2002; 16:1035–1044.
153. Kitada S, Andersen J, Akar S, Zapata JM, Takayama S, Krajewski S, Wang HG, Zhang X, Bullrich F, Croce CM, Rai K, Hines J, Reed JC. Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with *in vitro* and *in vivo* chemoresponses. *Blood* 1998; 91:3379–3389.
154. Estlin EJ. Optimizing antimetabolite-based chemotherapy for the treatment of childhood acute lymphoblastic leukaemia. *Br J Haematol* 2000; 110:29–40.
155. Estlin EJ. Continuing therapy for childhood acute lymphoblastic leukaemia: clinical and cellular pharmacology of methotrexate, 6-mercaptopurine and 6-thioguanine. *Cancer Treat Rev* 2001; 27: 351–363.
156. Krynetski EY, Krynetskaia NF, Yanishevski Y, Evans WE. Methylation of mercaptopurine, thioguanine, and their nucleotide metabolites by heterologously expressed human thiopurine S-methyltransferase. *Mol Pharmacol* 1995; 47:1141–1147.
157. Fink D, Abei S, Howell SB. The role of DNA mismatch repair in drug resistance. *Clin Cancer Res* 1998; 4:1–6.
158. Hande KR, Garrow JC. Purine antimetabolites. In: *Cancer Chemotherapy and Biotherapy, Principle and Practice*, 2nd ed, Chabner BA, Longo DL (eds), Philadelphia, PA: Lippincott JB & Company, 1996; 235–252.
159. Zimm S, Johnson GE, Chabner BA, Poplack DG. Cellular pharmacokinetics of mercaptopurine in human neoplastic cells and cell lines. *Cancer Res* 1985; 45:4156–4161.
160. Hortelano S, Dallaporta B, Zamzami N, Hirsch T, Susin SA, Marzo I, Bosca L, Kroemer G. Nitric oxide induces apoptosis via triggering mitochondrial permeability transition. *FEBS Lett* 1997; 410:373–377.
161. el Alaoui S, Lawry J, Griffin M. The cell cycle and induction of apoptosis in a hamster fibrosarcoma cell line treated with anti-cancer drugs: its importance to solid tumour chemotherapy. *J Neurooncol* 1997; 31:195–207.
162. Hortelano S, Bosca L. 6-Mercaptopurine decreases the Bcl-2/Bax ratio and induces apoptosis in activated splenic B lymphocytes. *Mol Pharmacol* 1997; 51:414–421.
163. Young HS, Khan AS, Kendra JR, Coulson IH. The cutaneous side-effects of hydroxyurea. *Clin Lab Haematol* 2000; 22:229–232.
164. Severin I, Padieu M, Lhuguenot J-C, Chagnon M-C. Toxic interaction between hydroxyurea and 1-β-D-arabino-furanosylcytosine on the DNA of a human hepatoma cell line (HEPG2). *Toxicol Lett* 2003; 145:303–311.
165. Huyghe P, Dassonneville L, Fenaux P, Bailly C. Hydroxyurea-induced apoptosis in an EBV-immortalized lymphoblastoid cell line. *Oncol Res* 2004; 14:235–245.
166. Longo-Sorbello GSA, Bertino JR. Current understanding of methotrexate pharmacology and efficacy in acute leukemias. Use of newer antifolates in clinical trials. *Hematologica* 2001; 86:121–127.
167. Barry MA, Behnke CA, Eastman A. Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. *Biochem Pharmacol* 1990; 40:2353–2362.
168. Kaufmann S. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin and other cytotoxic anticancer drugs: a cautionary note. *Cancer Res* 1989; 49:5870–5878.
169. de Silva CP, de Oliveira CR, de Conceicao PL. Apoptosis as a mechanism of cell death induced by different chemotherapeutic drugs in human leukemic T-lymphocytes. *Biochem Pharmacol* 1996; 51:1331–1340.
170. Hattangadi DK, DeMasters GA, Walker TD, Jones KR, Di X, Newsham IF, Gewirtz DA. Influence of p53 and caspase 3 activity on cell death and senescence in response to methotrexate in the breast tumor cell. *Biochem Pharmacol* 2004; 68:1699–1708.

171. Papaconstantinou HT, Xie C, Zhang W, Ansari NH, Hellmich MR, Townsend CM, Ko TC. The role of caspases in methotrexate-induced gastrointestinal toxicity. *Surgery* 2001; 859–865.
172. Caltayud S, Warner TD, Mitchell JA. Modulation of colony stimulating factor release and apoptosis in human colon cancer cells by anticancer drugs. *Br J Cancer* 2002; 86:1316–1321.
173. Evdokiou A, Bouralexix S, Atkins GJ, Chai F, Hay S, Clayer M, Findlay DM. Chemotherapeutic agents sensitize osteogenic sarcoma cells, but not normal human bone cells, to Apo2L/TRAIL-induced apoptosis. *Int J Cancer* 2002; 99:491–504.
174. Mizutani Y, Yoshida O. Effect of anticancer agents on Fas-mediated cytotoxicity against bladder cancer cells. *J Infect Chemother* 1999; 5:139–143.
175. Valentijn AJ, Zouq N, Gilmore AP, Anoikis. *Biochem Soc Trans* 2004; 32:421–425.
176. Sreedhar AS, Csermely P. Heat shock proteins in the regulation of apoptosis: new strategies in tumor therapy. A comprehensive review. *Pharmacol Ther* 2004; 101:227–257.
177. Broker LE, Kruyt FAE, Giaccone G. Cell death independent of caspases: a review. *Clin Cancer Res* 2005; 11:3155–3162.
178. Roninson IB, Broude EV, Chang B-D. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist Updates* 2001; 4:303–313.
179. Ianzini F, Mackey MA. Spontaneous premature chromatin condensation and mitotic catastrophe following irradiation of HeLa S3 cells. *Int J Radiat Biol* 1997; 72:409–421.
180. Mackey MA, Zhang XF, Hunt CR, Sullivan SJ, Blum J, Laszlo A. Uncoupling of M-phase kinase activation from the completion of S-phase by heat shock. *Cancer Res* 1996; 56:1770–1774.
181. Chang B-D, Broude EV, Dokmanovic M, Zhu H, Ruth A, Xuan Y, Kandel ES, Lausch E, Christov K, Roninson IB. A senescence-like phenotype distinguishes tumor cells that undergo terminal proliferation arrest after exposure to anticancer agents. *Cancer Res* 1999; 59:3761–3767.
182. Touneki O, Pron G, Belehradek J, Mir LM. Bleomycin, an apoptosis-mimetic drug that induces two types of cell death depending on the number of molecules internalized. *Cancer Res* 1993; 53: 5462–5469.
183. Torres K, Horwitz SB. Mechanisms of taxol-induced cell death are concentration-dependent. *Cancer Res* 1998; 58:3620–3626.
184. Arico S, Petiot A, Bauvy C, Dubbelhuis PF, Meijer AJ, Codogno P, Ogier-Denis E. The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *J Biol Chem* 2001; 276:35243–35246.
185. Shintani T, Klionsky DJ. Autophagy in health and disease: a double-edged sword. *Science* 2004; 306:990–995.
186. Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science* 2004; 304:1500–1502.
187. Nelson DA, White E. Exploiting different ways to die. *Genes Dev* 2004; 18:1223–1226.
188. Wang Y, Li X, Wang L, Ding P, Zhang Y, Han W, Ma D. An alternative form of paraptosis-like cell death, triggered by TAJ/TROY and enhanced by PDCD5 overexpression. *J Cell Sci* 2004; 117:1525–1532.
189. Sperandio S, Poksay K, de Belle I, Lafuente MJ, Liu B, Nasir J, Bredesen DE. Paraptosis: mediation by MAP kinases and inhibition by AIP-1/Alix. *Cell Death Diff* 2004; 11:1066–1075.
190. Majno G, Joris I. Apoptosis, oncosis, and necrosis: an overview of cell death. *Am J Pathol* 1995; 146:3–15.
191. Matsuo A, Watanabe A, Takahashi T, Futamura M, Mori S, Sugiyama Y, Takahashi Y, Saji S. A simple method for classification of cell death by use of thin layer collagen gel for the detection of apoptosis and/or necrosis after cancer chemotherapy. *Jpn J Cancer Res* 2001; 92:813–820.
192. Columbano A. Cell death: current difficulties in discriminating apoptosis from necrosis in the context of pathological processes *in vivo*. *J Cell Biochem* 1995; 58:181–190.
193. Chang B-D, Xuan Y, Broude EV, Zhu H, Schott B, Fang J, Roninson IB. Role of p53 and p21^{waf1/cip1} in senescence-like terminal proliferation arrest induced in human tumor cells by chemotherapeutic drugs. *Oncogene* 1999; 18:4808–4818.
194. Robles SJ, Adams GR. Agents that cause DNA double strand breaks lead to p16INK4a enrichment and the premature senescence of normal fibroblasts. *Oncogene* 1998; 16:1113–1123.
195. Zhu J, Woods D, McMahon M, Bishop JM. Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev* 1998; 12:2997–3007.
196. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 1997; 88:593–602.

197. Blagosklonny MV. Prospective strategies to enforce selectively cell death in cancer cells. *Oncogene* 2004; 23:2967–2975.
198. Roninson IB. Tumor cell senescence in cancer treatment. *Cancer Res* 2003; 63:2705–2715.
199. Wang Z, Wang S, Fisher PB, Dent P, Grant S. Evidence of a functional role for the cyclin-dependent kinase inhibitor p21CIP1 in leukemic cell (U937) differentiation induced by low concentrations of 1-beta-D-arabinofuranosylcytosine. *Differentiation* 2000; 66:1–13.
200. Niitsu N, Ishii Y, Matsuda A, Honma Y. Induction of differentiation of acute promyelocytic leukemia cells by a cytidine deaminase-resistant analogue of 1-beta-D-arabinofuranosylcytosine, 1-(2-deoxy-2-methylene-beta-D-erythro-pentofuranosyl)cytidine. *Cancer Res* 2001; 61:178–185.
201. Poirier F, Bourin P, Bladier D, Joubert-Caron R, Caron M. Effect of 5-azacytidine and galectin-1 on growth and differentiation of the human b lymphoma cell line bl36. *Cancer Cell Int* 2001; 1:2.
202. Suh SI, Pyun HY, Cho JW, Baek WK, Park JB, Kwon T, Park JW, Suh MH, Carson DA. 5-Aza-2'-deoxycytidine leads to down-regulation of aberrant p16INK4A RNA transcripts and restores the functional retinoblastoma protein pathway in hepatocellular carcinoma cell lines. *Cancer Lett* 2000; 160:81–88.
203. Niitsu N, Hayashi Y, Sugita K, Honma Y. Sensitization by 5-aza-2'-deoxycytidine of leukaemia cells with MLL abnormalities to induction of differentiation by all-trans retinoic acid and 1alpha, 25-dihydroxyvitamin D3. *Br J Haematol* 2001; 112:315–326.
204. Michishita E, Nakabayashi K, Suzuki T, Kaul SC, Ogino H, Fujii M, Mitsui Y, Ayusawa D. 5-Bromodeoxyuridine induces senescence-like phenomena in mammalian cells regardless of cell type or species. *J Biochem (Tokyo)* 1999; 126:1052–1059.
205. Suzuki T, Minagawa S, Michishita E, Ogino H, Fujii M, Mitsui Y, Ayusawa D. Induction of senescence-associated genes by 5-bromodeoxyuridine in HeLa cells. *Exp Gerontol* 2001; 36: 465–474.
206. Kwak IH, Kim HS, Choi OR, Ryu MS, Lim IK. Nuclear accumulation of globular actin as a cellular senescence marker. *Cancer Res* 2004; 64:572–580.
207. Rebbaa A, Zheng X, Chou PM, Mirkin BL. Caspase inhibition switches doxorubicin-induced apoptosis to senescence. *Oncogene* 2003; 22:2805–2811.
208. te Poele RH, Okorokov AL, Jardine L, Cummings J, Joel SP. DNA damage is able to induce senescence in tumor cells *in vitro* and *in vivo*. *Cancer Res* 2002; 62:1876–1883.

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Topoisomerase I Poisons and Apoptotic Topoisomerase I-DNA Complexes

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SUMMARY

Topoisomerase I (Top1), an abundant nuclear enzyme expressed throughout the cell cycle, relaxes DNA supercoiling by forming transient covalent DNA cleavage complexes. We will review three situations leading to enhanced cellular Top1 cleavage complexes. First, Top1 cleavage complexes can be stabilized by camptothecins, which are referred to as Top1 poisons and among the most efficient inducers of apoptosis. The second mechanism is related to exogenous and endogenous DNA lesions that enhance Top1 cleavage complexes. Lastly, Top1 cleavage complexes form during programmed cell death and are then referred to as “apoptotic Top1 cleavage complexes.”

Key Words: DNA topoisomerase I; Top1 poisons; Top1 cleavage complexes; camptothecins; apoptosis; mitochondria; arsenic trioxide; staurosporine.

1. INTRODUCTION

DNA topoisomerase I (Top1) is an ubiquitous and essential enzyme as it relaxes DNA supercoiling ahead of replication and transcription complexes (1–3). DNA relaxation proceeds through induction of transient single-strand breaks, thereby allowing rotation of the DNA double helix around the intact phosphodiester bonds opposite to the enzyme-mediated DNA cleavages. Once the DNA is relaxed, Top1 readily religates the break and restores intact duplex DNA. Under normal conditions, the covalent Top1-cleaved DNA intermediates, referred to as “cleavage complexes,” are transient. At a given time point, their number remains very low as the DNA religation (“closing”) step proceeds much faster than the DNA cleavage (“nicking”) step. There are three situations leading to enhanced cellular Top1 cleavage complexes (Fig. 1). First, Top1 cleavage complexes can be stabilized by “Top1 poisons,” including camptothecin and

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Apoptosis, Senescence, and Cancer

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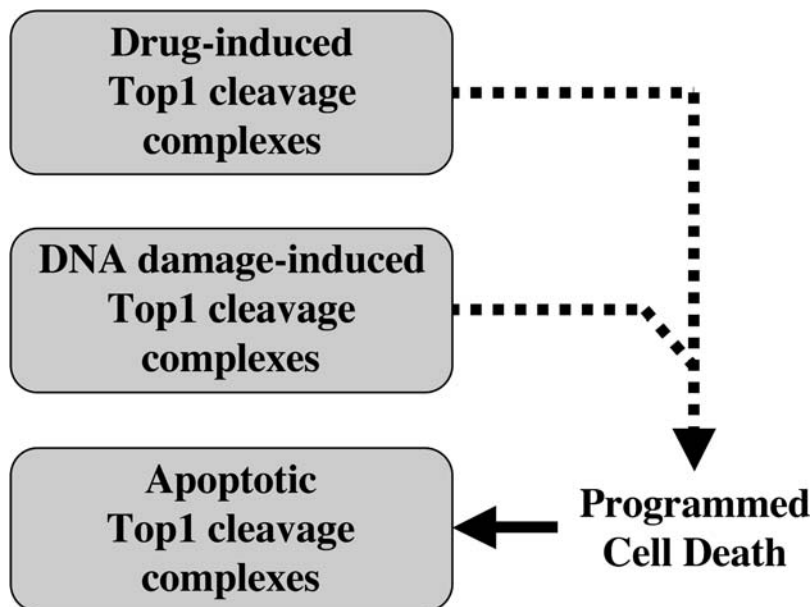


Fig. 1. Induction of cellular Topoisomerase I (Top1) cleavage complexes. Top1 cleavage complexes can be stabilized/induced (i) by cytotoxic drugs, referred to as “Top1 poisons”; (ii) by endogenous and exogenous DNA damage; and (iii) during cell death by apoptosis (apoptotic Top1 cleavage complexes). Drug-induced and DNA damage-induced Top1 cleavage complexes can trigger apoptosis and generate apoptotic Top1 cleavage complexes.

its chemotherapeutic derivatives that bind specifically at the Top1–DNA interface and trap the cleavage complexes by preventing the DNA religation step (for review, see ref. 4). Secondly, endogenous and exogenous DNA damage, including oxidized bases, abasic sites, mismatches, strand breaks and carcinogenic adducts (5–7), can interfere with Top1’s DNA nicking-closing activities (for review, see ref. 8). Lastly, Top1 cleavage complexes form during apoptotic cell death that occurs in response to a wide range of stimuli. These complexes, which are likely a general response of cells undergoing apoptosis, are referred to as “apoptotic Top1 cleavage complexes” (for review, see ref. 9). Connections exist between these three situations as drug-induced and DNA damage-induced Top1 cleavage complexes can trigger apoptosis, thus generating apoptotic Top1 cleavage complexes (Fig. 1). Moreover, apoptotic Top1 cleavage complexes are probably due, at least in part, to oxidative DNA damage.

This chapter focuses first on camptothecins and non-camptothecins Top1 poisons. We will present a common molecular mechanism for the stabilization of Top1–DNA complexes by Top1 poisons, which is referred to as the “5′-end misalignment model.” We will describe the apoptotic pathways activated by Top1 poisons. Emphasis will be given to the transmission of the apoptotic signal from the nucleus (DNA lesions) to the cytoplasm (mitochondrial permeabilization). Lastly, we will review the current knowledge on the formation and functional relevance of apoptotic Top1 cleavage complexes. The biochemistry and cellular biology of Top1, the DNA damaging lesions

leading to Top1 cleavage complexes and the DNA repair pathways elicited by Top1 cleavage complexes have been recently reviewed elsewhere and will be only mentioned here (1–3).

2. NOVEL TOPOISOMERASE I POISONS

Camptothecin is a natural compound isolated from *Camptotheca acuminata* (Fig. 2). The sodium salt camptothecin was found to be clinically active but its use was discontinued in the 1970s because of severe side effects (10). In 1985, camptothecin was shown to specifically poison DNA Top1 (11), which generated great interest and provided new opportunities for finding more efficient and less toxic analogs of camptothecin.

2.1. Camptothecin Derivatives

Two water-soluble camptothecin derivatives are currently used for the treatment of human cancers: Irinotecan (CPT-11, Camptosar®) for advanced colorectal carcinomas and Topotecan (Hycamtin®) for ovarian cancers (12). A number of other camptothecin derivatives are in clinical trials: 9-nitrocamptothecin (SuperGen) (13), exatecan mesylate (DX-8951f) (14), Afeletecan® (Bayer AG), CKD-602 (Chong Kun Dang Pharmaceutical Corp.), DRF-1042 (Dr. Reddys Research Foundation), PEG-camptothecin (Prothecan®, Enzon Inc.), MAG-camptothecin (PNU-166148, Pharmacia), ST1481 (Sigma-Tau Healthsci SpA), Homa-copolymer-camptothecin (University of London) and Karenitecin® (12).

Homocamptothecins retain potent Top1 inhibition (15–18) and differ from camptothecin by the presence of an additional methylene group in the E-ring, which limits the conversion to the inactive carboxylate. Conversely, the inactive carboxylate of homocamptothecins cannot be converted to the lactone once the E-ring is open (16). The binding of homocamptothecins in the Top1–DNA complex is likely to be similar to the binding of camptothecins as Top1 mutations that confer resistance to camptothecins confer cross-resistance to homocamptothecins (15). However, because of their greater potency, homocamptothecins remain more active in camptothecin-resistant cells (15). The difluorohomocamptothecin derivative BN-80915 (diflomotecan), currently tested in clinical trials, is more potent than SN-38 (active metabolite of CPT-11) and produces more stable cellular cleavage complexes (19).

The camptothecin derivatives presently in the clinic have two major limitations: (i) at physiological pH, the labile alpha hydroxylactone, which is essential for camptothecin activity (20), is in equilibrium with its inactive (carboxylate) form, which binds to

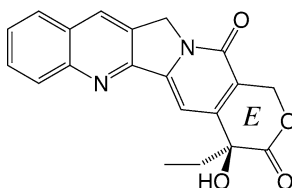


Fig. 2. Structure of camptothecin

serum albumin (21); (ii) the camptothecin-trapped cleavage complexes reverse within minutes after drug removal, which imposes long and/or repeated infusions for cancer treatment.

2.2. Non-Camptothecin Topoisomerase I Poisons: Polyheterocyclic Aromatic Inhibitors

The indolocarbazoles represent the most advanced class of non-camptothecin derivatives in terms of chemotype, clinical development and structure activity (22–24). Among the Top1 inhibitor, indolocarbazole derivatives, NB-506 and J-107088, have recently been selected for clinical trials. Like camptothecins, indolocarbazoles prevent the religation of a subset of Top1 cleavage complexes. By contrast to camptothecin, indolocarbazole generally binds to DNA by intercalation, and the DNA sequence selectivity of these cleavages differ from the pattern of cleavage sites induced by camptothecins (25–28).

A second class of non-camptothecins polyheterocyclic aromatic inhibitors is the indenoisoquinolines. The synthesis of the indenoisoquinoline NSC-314622 was first reported in 1978 (29). Consecutively, a series of indenoisoquinolines were synthesized and found to possess significant anticancer activity (30,31). However, little was known about their anticancer mechanism until recently when a COMPARE analysis of cytotoxicity in the National Cancer Institute (NCI) *in vitro* Anticancer Drug Discovery Screen of 60 cell lines revealed that NSC-314622 was a Top1 inhibitor (32). The patterns of DNA breaks produced by Top1 in the presence of indenoisoquinolines differ from camptothecins. Because of their novel structure, a large number of indenoisoquinolines have been synthesized and tested for Top1 inhibition and antiproliferative activity in the NCI cell screen over the past 3 years (33–35). Generally, the indenoisoquinolines that inhibit Top1 are cytotoxic in the NCI cell lines (33–35). Antitumor activity is also observed for some of these compounds in animal models (35). Indenoisoquinolines are in preclinical development, and current efforts are focusing on testing the antitumor activity of selected indenoisoquinolines (e.g., compound MJ-III-65) in animal models. A co-crystal structure of indenoisoquinolines in the Top1-DNA complex has recently been obtained (36).

The pyrroloquinazolinoquinoline alkaloid luotonin A and its derivatives were reported recently to poison also Top1 (37). Luotonins and camptothecins are structurally related and share the same sequence selectivity of DNA cleavage by Top1. However, luotonins are less efficient than camptothecins at trapping of Top1 cleavage complex and show only weak antiproliferative activity.

Other polyheterocyclic Top1 poisons include nitidine, coralyne, berberine and benzo[a]acridine derivatives. These compounds share a common heterocyclic ring system and generally bind to DNA by intercalation. Although some of them exhibit antiproliferative activity, to the best of our knowledge, they are not in clinical development (for further details, see ref. 22).

2.3. Non-Camptothecin Topoisomerase I Poisons: Benzimidazoles and Minor Groove Ligands

The bis-benzimidazole dyes, Hoechst 33342, and its parent compound Hoechst 33258 (NSC-32291, pibenzimol) represent a structurally unique class of Top1

poisons. Ho-33342 is commonly used for histochemical staining and flow cytometry analysis of DNA content. Hoechst 33342 and 33258 reversibly trap Top1 cleavage complexes with different sequence selectivity than camptothecins (38). They both bind to Adenine, Thymine (AT)-rich sequences, causing widening of the DNA minor groove (39). However, minor groove binding is not sufficient for Top1 trapping as distamycin, berenil and netropsin do not poison Top1 (40). Hoechst 33342 also disrupts TATA box-binding protein/TATA box element binding (40), suggesting other targets besides Top1.

In recent years, a series of benzimidazoles (41), bibenzimidazoles (42) and terbenzimidazole derivatives (43,44) have been studied with modifications of the 5-position or 2''-position of terbenzimidazoles. A number of 5-substituted terbenzimidazoles can poison Top1 in biochemical assays. 5-phenyl-terbenzimidazole (5PTB) is the most effective in cell culture assays (43,44). Studies with poly(dA).poly(dT) duplex DNA suggest that 5PTB intercalates and binds in the DNA minor groove (44).

Ecteinascidin-743 (Et-743, NSC-648766) is a potent antitumor agent from the Caribbean tunicate *Ecteinascidia turbinata*. Et-743 is in phase II and III clinical trials, with remarkable activity in soft tissue sarcomas and solid tumors including ovarian carcinoma (45,46). Et-743 traps Top1 cleavage complexes *in vitro* and in cancer cells (47,48). The distribution of the drug-induced Top1 sites is different for Et-743 and camptothecin (48). A derivative of Et-743, phthalascidin (Pt-650), was also found to poison Top1 cleavage complexes *in vitro* and in cells (47). However, Top1 is probably not the primary cellular target of Et-743 as the drug remains cytotoxic in yeast with a deletion in the *Top1* gene (49) and in mammalian cells deficient in Top1 (50). Also, Top1 inhibition is only detectable at micromolar concentrations exceeding pharmacologically active concentrations (51,52). Recent studies revealed that Et-743 acts by a novel mechanism of action: poisoning of transcription-coupled nucleotide excision repair (53).

2.4. Top1 Cleavage Complexes Induced by DNA-Damaging Agents

Chemotherapeutic agents that damage DNA can also trap Top1 (for details, see ref. 8). Incorporation of the nucleoside analogs cytosine arabinoside, gemcitabine (2'-difluorocytosine) and 5-fluorouracil (54) immediately downstream from a Top1 cleavage complex prevent the Top1-mediated DNA religation (55,56). Alkylating agents, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, also trap Top1 (57). The resistance of Top1-deficient cells to these drugs (56,57) suggests the contribution of Top1 poisoning to their antiproliferative activity. Besides chemotherapeutic agents, Top1 can be trapped by naturally occurring endogenous and carcinogenic DNA lesions, ranging from UV dimers (58), oxidative base lesions, base mismatches and abasic sites (59), DNA strand breaks (60), the carcinogenic adducts, N6-ethenoadenine (61), benzo[*a*]pyrene diol epoxide and formaldehyde adducts (6,7) (for review, see ref. 8). It is not known how frequently such Top1 cleavage complexes form. However, the ubiquitous expression of Tdp1 (tyrosyl-DNA phosphodiesterase), an enzyme specifically involved in the repair of Top1-DNA covalent complexes (for review, see ref. 3), suggests selective pressure for removing Top1 cleavage complexes, and therefore their natural occurrence.

3. MOLECULAR MODEL FOR TOPOISOMERASE I INHIBITION: MISALIGNMENT OF THE 5'-HYDROXYL END OF THE CLEAVED DNA AND INTERFACIAL INHIBITION

3.1. Binding of Camptothecins and Polycyclic/Heterocyclic Poisons to the Top1–DNA Complex

Camptothecins are non-competitive inhibitors of Top1. They inhibit Top1 by binding both to Top1 and the cleaved DNA (20,62). Consequently, they uncouple the Top1's nicking-closing reaction by preventing the DNA religation ("closing") step. This unique mode of action defines the concept, referred to as "interfacial inhibition" (63,64), that it is possible to interfere with two macromolecules (i.e., Top1 and DNA) to stabilize their interaction. This concept is a paradigm for drug discovery as one of the present objectives in drug development is to interfere with macromolecule interactions. According to this concept, it is conceivable to look for drugs that prevent the dissociation of two macromolecules rather than inhibiting their binding.

Top1 cleavage complexes are not equally trapped by camptothecins. Trapping is most effective at DNA sequences with a T at the 3'-end of the scissile DNA strand, which corresponds to the DNA end covalently linked to Top1 [position -1 (Fig. 3A)],

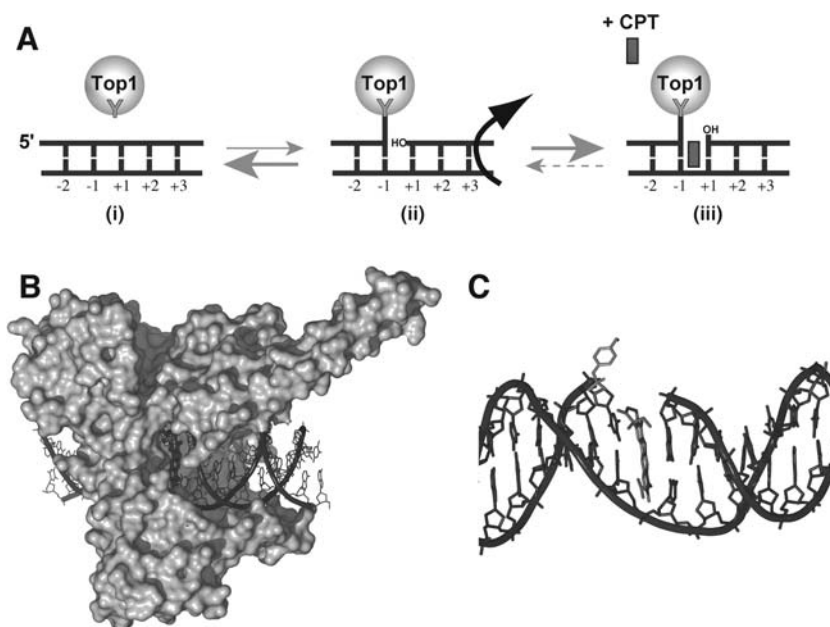


Fig. 3. Interfacial inhibition of Topoisomerase I (Top1) cleavage complex by camptothecins. (A) Schematic representation of the Top1 catalytic cycle with the cleavage complex (panel ii) allowing DNA relaxation by rotation of the cleaved DNA strand around the intact strand at the break site. Camptothecin (CPT) and its clinical derivatives (topotecan) trap the cleavage complex and shift the normal DNA nicking-closing equilibrium. Y = tyrosine. (B, C) Crystal structure of topotecan (or CPT) bound to the Top1 cleavage complex. Top1 is shown surrounding the DNA and topotecan (at the center) is shown at the interface between Top1 and the DNA. In (C), only the tyrosine of Top1 that binds to the broken DNA is shown. Topotecan is shown intercalated between the base pairs flanking the Top1 cleavage site.

and a G at the 5'-end of the broken DNA [position +1 (Fig. 3A)]. It is this DNA sequence dependence that led to the hypothesis that camptothecin formed a ternary complex with Top1 and the DNA by binding at the Top1–DNA interface (65). This hypothesis was further strengthened by the finding that a derivative of camptothecin with an alkylating group at position 7 can form an adduct with the +1 guanine (at the N3 position) in the presence of active Top1 (66).

It has now been shown that camptothecins stabilize Top1 cleavage complexes by forming a ternary complex, Top1–DNA–drug. In the topotecan co-crystal with an irreversible Top1 cleavage complex (67) (Fig. 3B and C) and in the proposed models, the camptothecin polycyclic rings intercalate at the Top1–DNA interface between the bases that flank the DNA cleavage site generated by Top1 (20,65,66,68–70). Camptothecin prevents therefore the DNA religation by keeping the 5'-end of the broken DNA out of alignment with the Top1–DNA phosphotyrosyl bond that needs to be attacked by the 5'-hydroxyl of the broken DNA during religation (Fig. 3).

Recently, experiments with intercalating ligands demonstrated position-specific trapping of Top1 cleavage complexes by polycyclic hydrocarbons (benzo[*a*]pyrene diol epoxide adducts) intercalated between the bases that flank the Top1 cleavage site or that are immediately downstream from the cleavage site (5,71). A unifying model is that the polycyclic aromatics (camptothecins, indolocarbazoles, indenoisoquinolines, luotonins, coralyne, berberine and nitidine derivatives) bind to a common site in the Top1–DNA complex by stacking (intercalating) either on the 5'-side or the 3'-side of the base pair immediately downstream [position +1 (Fig. 3A)] from the Top1 cleavage site (70). The differences in DNA cleavage patterns (i.e., differential intensity of cleavage at any given site) between compounds might be due to specific interactions between particular drugs and the bases flanking the Top1 cleavage site (70).

A potential exception to this model has been proposed for nogalamycin (72), which traps Top1 cleavage complexes by intercalating away from the Top1 cleavage site and by inducing a local bent downstream from the Top1 cleavage, which interferes with DNA religation. Thus, nogalamycin bound to a Top1–DNA complex may act similarly to minor groove ligands.

3.2. General Model for Top1 Poisoning: “5'-Terminus Misalignment”

Minor groove binding drugs (such as benzimidazoles and Et-743) poison Top1 by binding immediately downstream (3') from the cleaved DNA strand. This would alter the structure of the DNA downstream from the cleavage site, preventing DNA religation by Top1. Trapping of Top1 by base modifications at position +1 (for review, see ref. 8) results probably also in structural modifications of the broken end downstream from the Top1 cleavage site. Together, the molecular observations presented above lead to a relatively simple and general mechanism for the trapping of Top1 cleavage complexes: presence of a ligand that either intercalates or binds to the minor groove, or presence of DNA modifications that result in a misalignment of the 5'-hydroxyl DNA terminus, and interfere with the religation of Top1 cleavage complexes. As indicated at the beginning of this section, the inhibitors act in a non-competitive manner by preventing the dissociation of Top1–DNA complex.

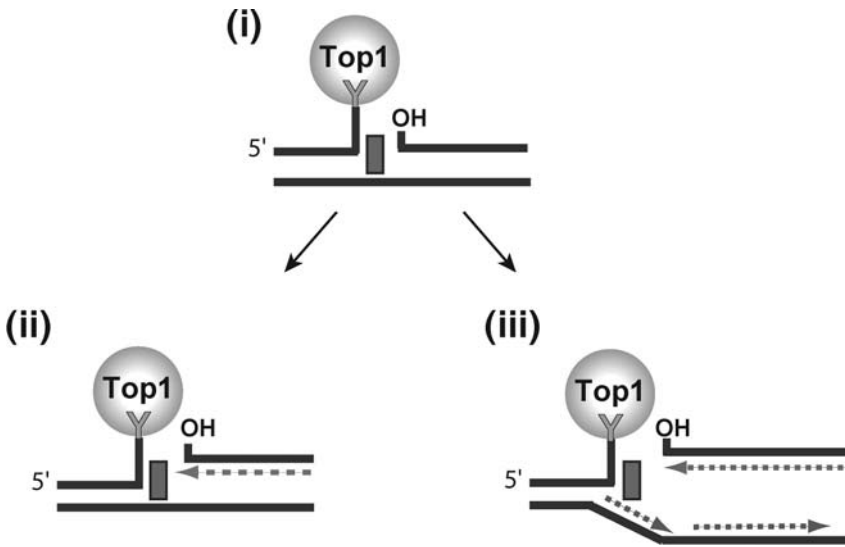


Fig. 4. Conversion of Topoisomerase I (Top1) cleavage complexes into DNA damage during replication and transcription. (i) Schematic representation of a Top1 cleavage complex trapped by camptothecin (rectangle). Top1 is covalently bound to the 3'-end of the broken DNA. The other end is a 5'-hydroxyl (OH). (ii) Conversion of the cleavage complex into an irreversible covalent Top1-DNA complex by a colliding transcription complex (left dashed arrow represents RNA). (iii) Conversion of the cleavage complex on the leading strand into an irreversible covalent Top1-DNA complex associated with a replication DNA double-strand break by a colliding replication fork (left dashed arrow represents leading replication; right dashed arrows represent lagging replication).

4. CELLULAR LESIONS INDUCED BY TOPOISOMERASE I POISONS

Top1 cleavage complexes are readily reversible after camptothecin removal, and short exposures to camptothecin (for less than 1 h) are relatively non-cytotoxic. Persistent drug exposure is required to induce apoptosis, as Top1 cleavage complexes are converted into irreversible DNA lesions by cellular metabolism (Fig. 4). DNA double-strand breaks (DSBs) are generated during replication (73). During transcription, collision of the elongating RNA polymerase II with a Top1 cleavage complexes on the leading strand can induce a duplex DNA-RNA and generates in all cases an irreversible DNA lesions (74). Transcription-dependent apoptosis appears mainly mediated by p53 (for review, see ref. 75).

Although in most cancer cells, the cytotoxicity of camptothecin appears primarily related to replication-mediated DSBs (11,76), the protection of cells by the DNA polymerase inhibitor aphidicolin is generally limited. A replication-independent cytotoxicity can be observed in non-replicating cells such as neurons (77) and lymphocytes (Sordet and Pommier, unpublished).

5. SENSING DNA DAMAGE INDUCED BY TOPOISOMERASE I POISONS: ATM, ATR AND DNA-PK

Proteins and protein complexes, called “DNA sensors,” bind to (or at the proximity of) DNA lesions and activate checkpoint and repair proteins. ATR, ataxia-telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK) are serine/threonine kinases known to be activated by Top1 poisons-mediated DNA damage.

ATM responds preferentially to DSBs (78). However, the activation of ATM does not strictly require its physical interaction with DNA breaks. Alteration of chromatin structures induced by interruptions of DNA integrity might generate the initial signal for ATM activation (79). Such changes in chromatin topology induce trans-phosphorylation (on S1981), which dissociates ATM dimers into active ATM monomers (79). Recent reports suggest that the protein phosphatase-5 and phosphatase-2A might be involved in ATM activation (80,81). Activated monomeric ATM phosphorylates nucleoplasmic substrates such as p53 (79,82). Nbs1, Brca1 and other BRCT proteins are believed to recruit ATM substrates, including Chk2. ATM can also phosphorylate the BRCT proteins (83) such as Nbs1, Brca1, Smc1, 53BP1 and Mdc1.

ATR (ataxia-telangiectasia and RAD3 related) is activated by replication-associated DNA damage (84). According to the current model, the binding of ATR to stressed replication forks is mediated by replication protein A (RPA). The persistent and longer stretches of single-stranded DNA associated with stalled replication forks are coated by RPA polymers that serve as a template for the recruitment of the ATR-interacting protein-ATR complex (85). The concomitant (but ATR-independent) loading of RAD17, the 9-1-1 complex (Hus1, Rad1 and Rad9) and claspin cooperates for full activation of ATR.

DNA-PK consists of the Ku heterodimer (Ku70/80) and the catalytic serine/threonine kinase subunit (DNA-PKcs). Whether DNA-PKcs requires the binding of Ku proteins to the DNA ends for activation is still a matter of debate. Autophosphorylation of DNA-PKcs also seems to be required for efficient DNA repair, in spite of a loss of kinase activity (86). In addition, Ku70 has been recently involved in apoptosis (see Section 7.2.).

The above-mentioned DNA sensors activate cellular effectors leading to cell cycle arrest, DNA repair and/or apoptosis. The following sections focus on the apoptotic pathways elicited by Top1 poisons-mediated DNA damage. The DNA repair and checkpoint pathways have been described elsewhere (3).

6. MITOCHONDRIAL AND PLASMA MEMBRANE RECEPTOR PATHWAYS ARE ACTIVATED BY TOPOISOMERASE I POISONS

Camptothecins are among the most efficient inducers of apoptosis. They activate the mitochondrial “intrinsic” pathway and, in some cell types, the transmembrane “extrinsic” pathway. Both pathways lead to full caspase activation. Caspases belong to two groups: initiator and effector caspases. Initiator caspases are activated as they bind to adapter molecules, after which, they activate effector caspases. The initiator caspase for the mitochondrial pathway is caspase-9 (and possibly caspase-2), whereas the initiator caspases for the transmembrane pathways are caspase-8 and caspase-10. Both pathways share the effector caspases (caspase-3, caspase-6 and caspase-7). These latter enzymes cleave intracellular targets, leading to biochemical and morphological changes that characterize apoptosis.

6.1. The Mitochondrial “Intrinsic” Pathway

Most chemotherapeutic drugs, including Top1 poisons, increase the permeability of the outer mitochondrial membrane as they induce apoptosis (87). This permeabilization causes bioenergetic failure and permits the release of soluble molecules from the intermembrane space of the mitochondria to the cytosol. These molecules

include apoptosis-inducing factor (AIF) and endonuclease G that migrate to the nucleus to condense chromatin and cleave DNA, respectively. Other mitochondrial proteins cooperate in the cytosol to activate a cascade of caspases (cytochrome c, Smac/DIABLO and HtrA2/Omi) (87). Cytochrome c induces oligomerization of apoptosis protease activating factor-1 (Apaf-1) in the presence of ATP (89). Apaf-1 oligomers recruit procaspase-9 in a complex called the “apoptosome” where juxtaposition of procaspase-9 molecules results in autoactivation (90,91). Mature caspase-9 activates additional caspase-9 molecules as well as caspase-3 and caspase-7. In turn, caspase-3 activates downstream caspases in a proteolytic cascade (92). Smac/DIABLO and HtrA2/Omi, released simultaneously with cytochrome c, neutralize proteins of the inhibitor of apoptosis protein (IAP) family. Some of these IAPs associate to procaspase-9, caspase-3 and caspase-7 to inhibit their proteolytic activity, possibly by triggering their proteosomal degradation after ubiquitinylation (93).

The Bcl-2 family of proteins controls, at least in part, the release of the above-mentioned mitochondrial proteins (94,95). This family includes more than 30 anti-apoptotic and pro-apoptotic molecules characterized by the presence of 1–4 conserved Bcl-2 homology (BH) domains (BH1–BH4). Schematically, the anti-apoptotic proteins of the family such as Bcl-2 itself and Bcl-xL inhibit mitochondrial permeabilization, whereas the pro-apoptotic “multidomains,” mainly Bax and Bak, exert the opposite effect. The BH3-only proteins (including Bid, Bad, Bim, Noxa, Puma and many others) act as sensors of cellular damage. Each of them is activated by a specific cell damage and translocates to mitochondria to promote their permeabilization by activating Bax and Bak proteins and/or by inhibiting Bcl-2 and Bcl-xL (96).

6.2. The Transmembrane “Extrinsic” Pathway

In some cell types, apoptotic response to Top1 poisons implicates the plasma membrane receptor Fas (APO-1/CD95). Crosslinking of Fas by its natural ligand (FasL) induces clustering of Fas, which in turn recruits the adapter Fas-associated death domain (FADD) (97) and procaspase-8 (98) to form the death-inducing signaling complex (DISC). When oligomerized in the DISC, procaspase-8 auto-activates and releases mature caspase-8 (99), which induces apoptosis by two different pathways depending on the cell type (100). In type I cells, large amounts of active caspase-8 formed at the DISC induce direct cleavage/activation of procaspase-3 and the downstream caspase cascade independently of mitochondria. In type II cells, small amounts of DISC and active caspase-8 are insufficient to directly activate procaspase-3 but sufficient to cleave the “BH3-only protein” Bid, generating an active fragment (tBid) that migrates to the mitochondria and activates the mitochondrial death pathway (101,102).

Top1 poisons are among the various anticancer drugs that activate the death receptor pathway by enhancing the expression of Fas and FasL (103–105). At least in some cell types, this up-regulation is transcription dependent and implicates p53 (106). Interaction of FasL with Fas at the cell surface defines an autocrine/paracrine pathway similar to that observed in activation-induced cell death in T lymphocytes. However, the role of FasL in drug-induced apoptosis is probably not essential because antagonist antibodies or molecules that prevent FasL interaction with Fas (103,107) do not suppress apoptosis. Anticancer drugs can induce Fas clustering at the cell surface of tumor cells in the absence of FasL (108). This clustering could take place in lipid rafts at the plasma membrane (109). However, apoptosis induced by chemotherapeutic

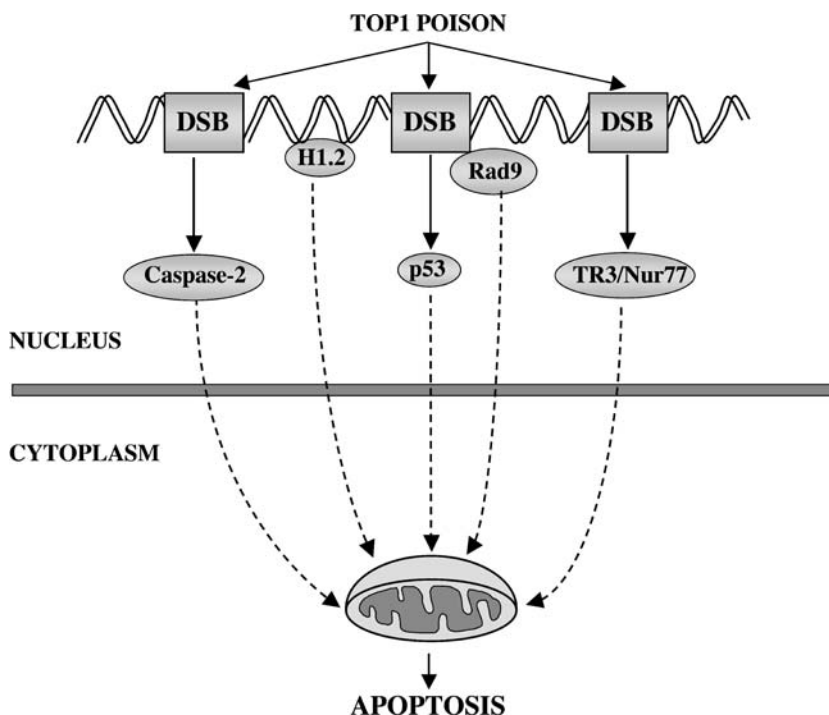


Fig. 5. Overview of the transduction of Topoisomerase I (Top1)-mediated DNA damage signals from the nucleus to mitochondria. Top1-mediated DNA damage induces the nucleo-cytoplasmic redistribution of caspase-2, histone H1.2, p53, Rad9 and TR3/Nur77, which promote mitochondrial permeability and apoptosis. See text for further details. Dashed lines represent nucleo-cytoplasmic redistribution.

drugs is not altered in embryonic fibroblasts from FADD (*110*) and caspase-8 (*111*) knockout mice indicating only a partial role for the death receptor pathway in response to chemotherapeutic agents.

In summary, Top1 poisons generally induce apoptosis by the mitochondrial pathway. This pathway can be amplified, at least in some cell types, by the transmembrane pathway. To the best of our knowledge, Top1 poisons and other genotoxic agents (e.g., ionizing radiation) activate the same transductional pathways to permeabilize mitochondria during apoptosis and have been therefore extensively reviewed (*112,113*). The following section will provide examples of molecules that transmit apoptotic signals from the nucleus (DSBs) to the cytoplasm (mitochondria) (Fig. 5).

7. FROM DNA DAMAGE IN THE NUCLEUS TO APOPTOSIS IN THE CYTOPLASM

As we discussed in Section 5., ATM (and also ATR and DNA-PK) responds preferentially to DSBs. Top1 poisons generate DSBs during replication. Four known substrates of ATM are implicated in apoptosis: p53, Chk2, E2F1 and c-Abl (<http://discover.nci.nih.gov/mim/>) (see also ref. 87). ATM phosphorylates/activates directly p53 and E2F1 thereby enhancing their stability and transcriptional activity.

ATM can also activate p53 and E2F1 indirectly by phosphorylating/activating the checkpoint kinase Chk2. The third Chk2 substrate implicated in apoptosis is promyelocytic leukemia (PML). ATM also phosphorylates/activates c-Abl. PML-mediated, c-Abl-mediated and E2F1-mediated apoptosis can occur through both p53-dependent and p53-independent pathways. Thus, apoptosis induced by DNA damage can schematically be divided into a p53-dependent manner and/or p53-independent manner.

7.1. p53-Dependent Apoptosis Induced by DNA Damage

p53 is a transcription factor (for review, see ref. 114) for a set of pro-apoptotic proteins from the Bcl-2 family (Bax, Bid, Noxa and Puma) that promote mitochondrial permeabilization, and thereby the release of cytochrome c. p53 also induces ASC (apoptosis-associated speck-like protein) that relocalizes Bax to mitochondria (115). Several other mitochondria-targeting proteins are induced by p53. Some of these, such as ferrodoxin reductase, are involved in the production of reactive oxygen species (ROS), while others permeabilize mitochondria either directly or indirectly by less understood mechanisms. These proteins include p53AIP (p53-regulated apoptosis-inducing protein-1), mitochondrial chloride intracellular channel-4 (116), PIDD (p53-induced protein with a death domain; implicated in caspase-2 activation; see below), and histone H1.2 (see below). p53 also transactivates components of the transmembrane “extrinsic” pathway (e.g., Fas/CD95, DR5 and PERP), proteins located to the endoplasmic reticulum (see ref. 117), caspase-6 and the procaspase-9 adapter Apaf-1. p53 also represses the transcription of the anti-apoptotic proteins Bcl-2 and survivin (an IAP that prevents caspase activity and regulates cell cycle).

p53 also induces apoptosis in a transcription-independent manner by directly targeting mitochondria (Fig. 5). After DNA damage, a fraction of p53 is exported from the nucleus and binds to the outer mitochondrial membrane (118). Mitochondrial p53 binds to Bcl-2 and Bcl-xL and neutralizes their inhibitory effect on Bak (and Bax), resulting in Bak oligomerization and subsequent mitochondrial permeabilization (118). Bcl-2 and Bcl-xL interact with the DNA-binding region of p53, the same region that harbors the vast majority of “hot spots” mutations in human cancer cells. Thus, some p53 mutations that interfere with DNA binding also interfere with p53 binding to Bcl-2 and Bcl-xL.

It has been proposed that p53 triggers a rapid first wave (within minutes) of transcription-independent apoptosis that precedes a second slower/delayed (within hours) transcription-dependent wave. The importance of p53 mitochondrial translocation in the response to DNA damage is controversial. The direct mitochondrial effect of p53 is cell type dependent, as wild-type p53 in primary fibroblasts does not translocate to mitochondria whereas wild-type p53 in thymocytes does. The molecular basis of this difference is not known. In addition, p53 itself can bind to DNA strand breaks (119) and might therefore be involved also in DNA damage detection/repair.

Histone H1.2 also relocalizes to mitochondria following DSBs (Fig. 5). This process requires p53 but is independent of p53 transcriptional activity (120,121). H1 is the histone subunit that binds the chromatin linker region between the nucleosomes. Humans and mice have eight histone *H1* genes. Among them, all of the somatic H1s (H1.1–H1.5) are ubiquitously expressed in all tissues throughout development. After DSBs, all nuclear histone H1 variants relocate partially to the cytoplasm. However, only H1.2 is able to trigger cytochrome c release from mitochondria. The mechanism for

the nucleo-cytoplasmic redistribution of H1.2 is unclear as H1.2 does not undergo any obvious post-translational modification, neither change in expression. DSBs themselves or the subsequent p53-dependent repair process may cause H1.2 release from chromatin to the nucleoplasm and subsequently in the cytoplasm. At the mitochondrial level, H1.2 induces conformational activation and oligomerization of Bak. In mitochondria isolated from Bak-deficient cells, H1.2 fails to induce cytochrome c release. The mechanism for H1.2 to cause the conformational change of Bak and the subsequent cytochrome c release remains to be determined. H1.2 appears to respond specifically to DSB-induced apoptosis as down-regulation of H1.2 by antisense RNA or small interfering RNA (siRNA) reduces apoptosis induced by X-rays or the Top2 inhibitor etoposide, but not by tumor necrosis factor- α or UV radiations. Also, thymocytes and cells in the small intestine from H1.2-deficient mice show strong resistance to X-ray-induced apoptosis.

Procaspase-2 might also link DNA damage and mitochondria (122) (Fig. 5). The "PIDDosome," a molecular complex containing PIDD, whose expression is induced by p53, and RAIDD/CRAIDD, an adaptor protein with a death domain, activate procaspase-2 in the nucleus (123). Increased PIDD expression results in spontaneous activation of procaspase-2 and sensitization to apoptosis by genotoxic stimuli (123). In the nucleus, juxtaposition of procaspase-2 molecules (dimerization) results in auto-cleavage/activation (124), a similar mechanism of activation to the initiator procaspase-8 and procaspase-9 (see Section 6.). Release of mature caspase-2 can stimulate directly mitochondrial release of cytochrome c. This process requires the processing of procaspase-2 but not its enzymatic activity and is also independent of Bax, Bak and Bcl-2 (125).

p53 can activate multiple pathways besides apoptosis, including cell cycle arrest/checkpoint, DNA repair, senescence and angiogenesis. Both cell cycle arrest and apoptosis prevent replication of damaged DNA and represent a coherent response for p53 as "the guardian of the genome." However, the p53-mediated cell cycle arrest response might in some cases antagonize p53-mediated apoptotic response. For instance, activation of *p21^{CIP1}*, which was among the first isolated p53-dependent genes, induces both cell cycle arrest in response to low doses of camptothecin and blocks DNA damage-induced apoptosis (126,127). p53 may selectively induce apoptosis in cells with elevated E2F1 activity, such as pRb-deficient cells. p53 binds to the cyclin A box of E2F1, and this complex induces apoptosis when cyclin A is low (128). E2F1, like p53, is negatively regulated by Mdm2, and both E2F1 and p53 are up-regulated in response to DNA damage (129). p53 may also specifically promote apoptosis when it is transcribed as a N-terminal truncated variant, designated p53/47. In contrast to p53, p53/47 lacks the Mdm2-binding domain. Thus Mdm2 expression increases the ratio p53/47 to p53. p53/47 has a different gene expression profile: up-regulation of Bax and down-regulation of p21 (130). It is therefore possible that specific modifications of p53 might selectively activate apoptotic or cell cycle arrest genes. Depending on the cellular context, p53 might selectively activate one set of genes or the other. The pleiotropic regulation of p53 (for details, see ref. 131) could allow fine tuned adjustments of p53 levels and p53 phosphorylation (depending on the intensity of DNA damage), which could account for the selectivity of p53 for transactivating cell cycle arrest and/or apoptotic genes.

7.2. p53-Independent Apoptosis Induced by DNA Damage

In spite of the apparent pivotal role of p53 in apoptosis (132,133), p53-null cells such as HL60 and U937 human leukemic cells undergo apoptosis readily in response to DNA-damaging agents (134–136). In these cells, the p53-related protein p73 does not compensate the lack of p53 as apoptosis is also transcription independent. Moreover, more than 50% of human tumors contain mutated and defective p53. Although such tumors might be defective in their apoptotic response *in vivo*, experiments performed in cell cultures demonstrate that these tumors can undergo apoptosis in response to Top1 poisons (137). p53-independent apoptosis involves the receptor TR3 and the checkpoint kinase Rad9 that target directly mitochondria in response to DNA damage and also the DNA repair protein Ku70. Other p53-independent pathways include Chk2, E2F1, PML and c-Abl (for review, see ref. 87).

The orphan receptor TR3 (also called Nur77 or nerve growth factor-induced protein B) is a transcription factor of the steroid/thyroid receptor superfamily. TR3 is involved in promoting cell proliferation (138). It is also a critical inducer of apoptosis. TR3 gene is rapidly induced by different apoptosis-inducing agents, and overexpression of a dominant-negative TR3 protein (139) or inhibition of TR3 expression by antisense TR3 mRNA (140) inhibits apoptosis. By contrast, constitutive expression of TR3 induces apoptosis (141). Although the mitogenic effect of TR3 occurs in the nucleus through target gene regulation, the pro-apoptotic effect of TR3 occurs in the cytoplasm independently of its transactivating activity. In response to apoptotic stimuli (including DSBs), TR3 translocates from the nucleus to mitochondria where it induces the mitochondrial release of cytochrome c (142) (Fig. 5). Despite lacking classical mitochondria-targeting sequences, TR3 relocates to mitochondria by binding to the Bcl-2 N-terminal loop region. The TR3-Bcl-2 interaction induces a Bcl-2 conformational change that exposes its BH3 domain (143). TR3 could therefore act on mitochondria by converting Bcl-2 from an anti-apoptotic to a pro-apoptotic member of the Bcl-2 family.

Another molecule that directly targets mitochondria is the cell cycle checkpoint Rad9 (Fig. 5). Rad9 is loaded as a complex with Hus1 and Rad1 (known as 9-1-1 complex) onto damaged chromatin by a clamp loader consisting of Rad17 and replication factor C (144). The 9-1-1 complex promotes the phosphorylation/activation of Chk1 by ATR, which in turn induces cell cycle arrest. Following DNA damage, Rad9 can also migrate from the nucleus to mitochondria. The BH3-like domain of Rad9 interacts with Bcl-2 and Bcl-xL, which induces apoptosis (145). Dual phosphorylation of Rad9 by c-Abl (146) (which is itself activated by ATM) and PKC δ (147) may be required for Rad9 to bind Bcl-2 and Bcl-xL. In summary, Rad9 seems to have two opposite functions: (i) cell survival by activating Chk1 and cell cycle arrest and (ii) cell death by activating the pro-apoptotic Bcl-2 proteins and mitochondrial pathway. The predominant role of Rad9 is likely to promote cell survival after Top1 poison-mediated DNA damage as *Rad9*^{-/-} ES cells are hypersensitive to camptothecin (148).

Ku70 is another molecule involved in both DNA repair and apoptosis. In addition to its nuclear localization, Ku70 is present in the cytoplasm where it binds to Bax, preventing Bax mitochondrial localization and pro-apoptotic activity (149,150). Bax-mediated apoptosis is suppressed by overexpression of Ku70. By contrast, Bax-mediated apoptosis is enhanced by down-regulation of Ku70. Upon apoptotic stimuli, Bax is released from Ku70 (150). Acetylation of Ku70 by p300/CBP and PCAF/GCN5 may induce this dissociation (151). As a result, the N-terminus region of Bax is

exposed, allowing Bax to form a large complex, called the “baxosome,” containing Bax itself, BH3-only proteins, cardiolipin, and probably other unidentified proteins. In the baxosome, Bax undergoes conformational changes, allowing its insertion into the outer membrane mitochondrial, where it promotes the release of cytochrome c and other pro-apoptotic molecules. Because Ku70 accumulates following DSBs, it is possible that Ku70 would prevent Bax from inducing a premature apoptosis (if the cell can still repair). However, beyond a certain threshold of DNA damage, Ku70 would release Bax to induce apoptosis.

The relative importance of these pathways in p53-deficient cells is not known. It will be interesting to use genetically altered cells and/or selective pharmacological inhibitors to determine the relative contribution of each of these pathways.

8. FORMATION OF TOP1 CLEAVAGE COMPLEXES DURING APOPTOSIS

Besides their role in the initiation of apoptosis (see Section 7.), stabilization of Top1 cleavage complexes occurs in cells undergoing apoptosis (152). “Apoptotic Top1 cleavage complexes” have been detected in different human cell lines exposed to a wide range of stimuli, which by themselves do not act directly as Top1 inhibitors. Such stimuli include arsenic trioxide (As_2O_3) (153), staurosporine (154), etoposide, Fas-L (CD95-L, Apo-1L) or TRAIL (Apo-2L) (Sordet and Pommier, unpublished), indicating that formation of Top1 cleavage complexes is likely a general response of cells undergoing programmed cell death.

The formation of apoptotic Top1 cleavage complexes is, at least in part, related to DNA alterations that occur during apoptosis and interfere with Top1’s nicking-closing activities (see Section 2.4.). Indeed, As_2O_3 and staurosporine induce oxidative DNA lesions as a result of ROS production, and modulation of ROS levels was directly correlated with the formation of Top1 cleavage complexes (153,154). Accordingly, cellular exposure to H_2O_2 also induces Top1 cleavage complexes (155). Thus, As_2O_3 and staurosporine (as well as a wide range of apoptotic stimuli) induce the generation of ROS that damage DNA (oxidized bases and abasic sites), thereby generating Top1 cleavage complexes in apoptotic cells (Fig. 6). It is also possible that some of the DNA breaks produced by apoptotic nucleases such as DFF40/CAD (156), endonuclease G (157), or Ape1 (158) contribute to the trapping of Top1 cleavage complexes (60).

Mitochondria are likely to participate in the production of the ROS and the Top1 cleavage complexes during apoptosis as Bcl-2 overexpression prevents their formation (153). Bcl-2 prevents the permeabilization of the outer mitochondrial membrane, and therefore, the release of cytochrome c and the downstream activation of caspases (see Section 6.1.). Activated caspase-3 feeds back on permeabilized mitochondria, which further dissipates the mitochondrial transmembrane potential ($\Delta\psi_m$) and induces the further accumulation of intracellular ROS (159,160). Caspase activation is involved in the generation of the apoptotic cleavage complexes as the general caspase inhibitor z-VAD-fmk prevents As_2O_3^- and staurosporine-induced ROS and Top1 cleavage complexes (153,154). Activation of caspases could therefore generate the ROS that lead to Top1 cleavage complexes during apoptosis (Fig. 6).

A critical question arising from these studies centers on defining the role of apoptotic Top1 cleavage complexes. Top1 down-regulation by siRNA in human cells

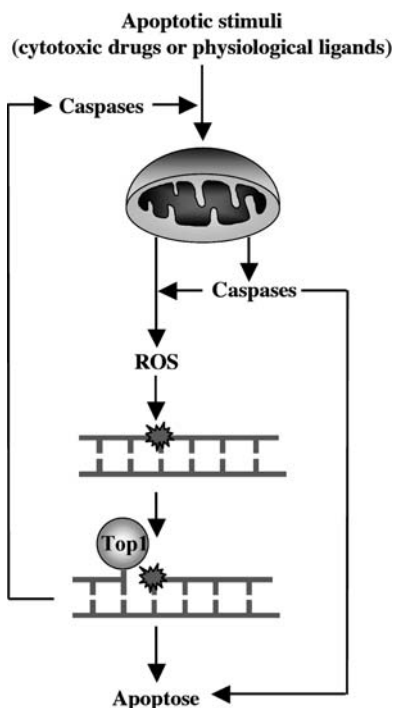


Fig. 6. Formation of apoptotic Topoisomerase I (Top1) cleavage complexes. Apoptotic stimuli (cytotoxic drugs or physiological ligands) induce the generation of reactive oxygen species (ROS) from mitochondria. ROS produce oxidative DNA lesions, which in turn generate Top1 cleavage complexes. These cleavage complexes might participate in apoptosis by generating DNA strand breaks and degrading chromatin. The Top1 cleavage complexes could also engage the apoptotic machinery in trans by activating caspases.

reduces DNA fragmentation induced by As_2O_3 and staurosporine (153,154). Also, mouse P388/CPT45 cells lacking Top1 show a reduction in apoptotic DNA fragmentation when exposed to As_2O_3 or staurosporine as compared to P388 cells expressing Top1 (153,154). Lastly, ectopic expression of Top1 in *Saccharomyces cerevisiae* confers sensitivity to H_2O_2 (155). Thus, apoptotic Top1 cleavage complexes are likely to contribute to a programmed nuclear dismantling.

These findings raise the possibility that Top1, which is abundant and essential in mammalian cells, could also participate in apoptosis by engaging the apoptotic machinery in trans, as trapping of Top1 (for instance, by camptothecins) is among the most efficient inducers of apoptosis (87) (<http://discover.nci.nih.gov/pommier/pommier.htm>). Thus, apoptotic Top1 cleavage complexes could serve to amplify the apoptotic process initiated by chemotherapeutic drugs and possibly by physiological ligands such as Fas-L and TRAIL.

9. CONCLUSION

Cellular responses to Top1 poisons include DNA repair, cell cycle arrest and/or apoptosis and thus determine cell survival or cell death. It is becoming increasingly clear that cell cycle checkpoint and DNA repair pathways such as the ATM/Chk2,

Rad9 (9.1.1) and Ku70 pathways are also connected to the apoptotic pathways. A more complete molecular interaction network connecting these pathways will emerge in the near future. One of the challenge is to understand how these pathways are integrated and how in the presence of extensive DNA damage, the same DNA damage sensors and checkpoints that stop cell cycle progression and promote DNA repair can activate apoptosis. Thus, a promising new area of research is the elucidation of the relationships between specific DNA lesions, sensor proteins, checkpoints, DNA repair and apoptosis. Integration of these pathways in comprehensive molecular interaction maps (87,131, 161–168) (see also <http://discover.nci.nih.gov/mim>) should reveal the interplay between the cellular determinants of cellular response to topoisomerase inhibitors and other types of DNA damages. They should also provide opportunities to develop novel therapeutic strategies and markers to better predict and follow tumor responses to therapeutic agents.

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REFERENCES

1. Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol* 2002; 3:430–440.
2. Champoux JJ. DNA TOPOISOMERASES: structure, function, and mechanism. *Annu Rev Biochem* 2001; 70:369–413.
3. Pommier Y, Redon C, Rao VA, et al. Repair of and checkpoint response to topoisomerase I-mediated DNA damage. *Mutat Res* 2003; 532:173–203.
4. Pommier Y. Camptothecins and topoisomerase I: a foot in the door. Targeting the genome beyond topoisomerase I with camptothecins and novel anticancer drugs: importance of DNA replication, repair and cell cycle checkpoints. *Curr Med Chem Anticancer Agents* 2004; 4:429–434.
5. Pommier Y, Kohlhagen G, Laco GS, Sayer JM, Kroth H, Jerina DM. Position-specific trapping of topoisomerase I-DNA cleavage complexes by the intercalated benzo[a]pyrene diol epoxide adducts at the 6-amino group of adenine. *Proc Natl Acad Sci USA* 2000; 97:10739–10744.
6. Pommier Y, Kohlhagen G, Pourquier P, Sayer JM, Kroth H, Jerina DM. Benzo[a]pyrene epoxide adducts in DNA are potent inhibitors of a normal topoisomerase I cleavage site and powerful inducers of other topoisomerase I cleavages. *Proc Natl Acad Sci USA* 2000; 97:2040–2045.
7. Antony S, Theruvathu JA, Brooks PJ, Leshner DT, Redinbo M, Pommier Y. Enhancement of camptothecin-induced topoisomerase I cleavage complexes by the acetaldehyde adduct N2-ethyl-2'-deoxyguanosine. *Nucleic Acids Res* 2004; 32:5685–5692.
8. Pourquier P, Pommier Y. Topoisomerase I-mediated DNA damage. *Adv Cancer Res* 2001; 80:189–216.
9. Sordet O, Khan QA, Pommier Y. Apoptotic topoisomerase I-DNA complexes induced by oxygen radicals and mitochondrial dysfunction. *Cell Cycle* 2004; 3:1095–1097.
10. Wall ME, Wani MC. Camptothecin and taxol: discovery to clinic – thirteenth Bruce F. Cain Memorial Award lecture. *Cancer Res* 1995; 55:753–760.
11. Hsiang YH, Hertzberg R, Hecht S, Liu LF. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J Biol Chem* 1985; 260:14873–14878.
12. Garcia-Carbonero R, Supko JG. Current perspectives on the clinical experience, pharmacology, and continued development of the camptothecins. *Clin Cancer Res* 2002; 8:641–661.
13. Konstadoulakis MM, Antonakis PT, Tsiabloulis BG, et al. A phase II study of 9-nitrocamptothecin in patients with advanced pancreatic adenocarcinoma. *Cancer Chemother Pharmacol* 2001; 48:417–420.
14. Verschraegen CF, Levenback C, Vincent M, et al. Phase II study of intravenous DX-8951f in patients with advanced ovarian, tubal, or peritoneal cancer refractory to platinum, taxane, and topotecan. *Ann N Y Acad Sci* 2000; 922:349–351.

15. Urasaki Y, Takebayashi Y, Pommier Y. Activity of a novel camptothecin analogue, homocamptothecin, in camptothecin-resistant cell lines with topoisomerase I alterations. *Cancer Res* 2000; 60:6577–6580.
16. Lesueur-Ginot L, Demarquay D, Kiss R, et al. Homocamptothecin, an E-ring modified camptothecin with enhanced lactone stability, retains topoisomerase I-targeted activity and antitumor properties. *Cancer Res* 1999; 59:2939–2943.
17. Lavergne O, Lesueur-Ginot L, Pla Rodas F, et al. Homocamptothecins: synthesis and antitumor activity of novel E-ring- modified camptothecin analogues. *J Med Chem* 1998; 41:5410–5419.
18. Philippart P, Harper L, Chaboteaux C, et al. Homocamptothecin, an E-ring-modified camptothecin, exerts more potent antiproliferative activity than other topoisomerase I inhibitors in human colon cancers obtained from surgery and maintained *in vitro* under histotypical culture conditions. *Clin Cancer Res* 2000; 6:1557–1562.
19. Larsen AK, Gilbert C, Chyzak G, et al. Unusual Potency of BN 80915, a novel fluorinated E-ring modified camptothecin, toward human colon carcinoma cells. *Cancer Res* 2001; 61:2961–2967.
20. Jaxel C, Kohn KW, Wani MC, Wall ME, Pommier Y. Structure-activity study of the actions of camptothecin derivatives on mammalian topoisomerase I: evidence for a specific receptor site and a relation to antitumor activity. *Cancer Res* 1989; 49:1465–1469.
21. Burke TG, Mi Z, M. The structural basis of camptothecin interactions with human serum albumin: impact on drug stability. *J Med Chem* 1994; 37:40–46.
22. Meng L-H, Liao Z-H, Pommier Y. Non-camptothecin DNA topoisomerase I inhibitors in cancer chemotherapy. *Curr Top Med Chem* 2003; 3:305–320.
23. Long BH, Balasubramanian BN. Non-camptothecin topoisomerase I active compounds as potential anticancer agents. *Exp Opin Ther Patents* 2000; 10:655–686.
24. Prudhomme M. Recent developments of rebeccamycin analogues as topoisomerase I inhibitors and antitumor agents. *Curr Med Chem* 2000; 7:1189–1212.
25. Ren J, Bailly C, Chaires JB. NB-506, an indolocarbazole topoisomerase I inhibitor, binds preferentially to triplex DNA. *FEBS Lett* 2000; 470:355–359.
26. Urasaki Y, Laco G, Takebayashi Y, Bailly C, Kohlhagen G, Pommier Y. Use of camptothecin-resistant mammalian cell lines to evaluate the role of topoisomerase I in the antiproliferative activity of the indolocarbazole, NB-506, and its topoisomerase I binding site. *Cancer Res* 2001; 61:504–508.
27. Bailly C, Riou J-F, Colson P, Houssier C, Rodriguez-Pereira E, Prudhomme M. DNA cleavage by topoisomerase I in the presence of indolocarbazole derivatives of rebeccamycin. *Biochemistry* 1997; 36:3917–3929.
28. Fukasawa K, Komatani H, Hara Y, et al. Sequence-selective DNA cleavage by a topoisomerase I poison, NB-506. *Int J Cancer* 1998; 75:145–150.
29. Cushman M, Cheng L. Total synthesis of nitidine chloride. *J Org Chem* 1978; 43:286–288.
30. Cushman M, Mohan P, Smith EC. Synthesis and biological activity of structural analogues of the anticancer benzophenanthridine alkaloid nitidine chloride. *J Med Chem* 1984; 27:544–547.
31. Cushman M, Mohan P. Synthesis and antitumor activity of structural analogues of the anticancer benzophenanthridine alkaloid fagaronine chloride. *J Med Chem* 1985; 28:1031–1036.
32. Kohlhagen G, Paull K, Cushman M, Nagafuji P, Pommier Y. Protein-linked DNA strand breaks induced by NSC 314622, a non-camptothecin topoisomerase I poison. *Mol Pharmacol* 1998; 54: 50–58.
33. Cushman M, Jayaraman M, Vroman JA, et al. Synthesis of new Indeno[1,2-c]isoquinolines: cytotoxic non-camptothecin topoisomerase I inhibitors. *J Med Chem* 2000; 43:3688–3698.
34. Strumberg D, Pommier Y, Paull K, Jayaraman M, Nagafuji P, Cushman M. Synthesis of cytotoxic indenoisoquinoline topoisomerase I poisons. *J Med Chem* 1999; 42:446–457.
35. Jayaraman M, Fox BM, Hollingshead M, Kohlhagen G, Pommier Y, Cushman M. Synthesis of new dihydroindeno[1,2-c]isoquinoline and indenoisoquinolinium chloride topoisomerase I inhibitors having high *in vivo* anticancer activity in the hollow fiber animal model. *J Med Chem* 2002; 45:242–249.
36. Staker BL, Feese MD, Cushman M, et al. Structures of three classes of anticancer agents bound to the human topoisomerase I-DNA covalent complex. *J Med Chem* 2005; 48:2336–2345.
37. Cagir A, Jones SH, Gao R, Eisenhauer BM, Hecht SM, Luotonen A. A naturally occurring human DNA topoisomerase I poison. *J Am Chem Soc* 2003; 125:13628–13629.

38. Chen AY, Yu C, Bodley A, Peng LF, Liu LF. A new mammalian DNA topoisomerase I poison Hoechst 33342: cytotoxicity and drug resistance in human cell cultures. *Cancer Res* 1993; 53: 1332–1337.
39. Beerman TA, McHugh MM, Sigmund R, Lown JW, Rao KE, Bathini Y. Effects of analogs of the DNA minor groove binder Hoechst 33258 on topoisomerase II and I mediated activities. *Biochim Biophys Acta* 1992; 1131:53–61.
40. Zhang X, Kiechle F. Hoechst 33342-induced apoptosis is associated with decreased immunoreactive topoisomerase I and topoisomerase I-DNA complex formation. *Ann Clin Lab Sci* 2001; 31:187–198.
41. Kim JS, Sun Q, Gatto B, et al. Structure-activity relationships of benzimidazoles and related heterocycles as topoisomerase I poisons. *Bioorg Med Chem* 1996; 4:621–630.
42. Jin S, Kim JS, Sim SP, et al. Heterocyclic bibenzimidazole derivatives as topoisomerase I inhibitors. *Bioorg Med Chem Lett* 2000; 10:719–723.
43. Rangarajan M, Kim JS, Jin S, et al. 2''-Substituted 5-phenylterbenzimidazoles as topoisomerase I poisons. *Bioorg Med Chem* 2000; 8:1371–1382.
44. Pilch DS, Xu Z, Sun Q, LaVoie EJ, Liu LF, Breslauer KJ. A terbenzimidazole that preferentially binds and conformationally alters structurally distinct DNA duplex domains: a potential mechanism for topoisomerase I poisoning. *Proc Natl Acad Sci USA* 1997; 94:13565–13570.
45. Verschraegen CF, Glover K. ET-743 (PharmaMar/NCI/Ortho Biotech). *Curr Opin Investig Drugs* 2001; 2:1631–1638.
46. Aune GJ, Furuta T, Pommier Y. Ecteinascidin 743: a novel anticancer drug with a unique mechanism of action. *Anticancer Drugs* 2002; 13:545–555.
47. Martinez EJ, Owa T, Schreiber SL, Corey EJ. Phthalascidin, a synthetic antitumor agent with potency and mode of action comparable to ecteinascidin 743. *Proc Natl Acad Sci USA* 1999; 96:3496–3501.
48. Takebayashi Y, Pourquier P, Yoshida A, Kohlhagen G, Pommier Y. Poisoning of human DNA topoisomerase I by ecteinascidin 743, an anticancer drug that selectively alkylates DNA in the minor groove. *Proc Natl Acad Sci USA* 1999; 96:7196–7201.
49. Damia G, Silvestri S, Carrassa L, et al. Unique pattern of ET-743 activity in different cellular systems with defined deficiencies in DNA-repair pathways. *Int J Cancer* 2001; 92:583–588.
50. Takebayashi Y, Goldwasser F, Urasaki Y, Kohlhagen G, Pommier Y. Ecteinascidin 743 induces protein-linked DNA breaks in human colon carcinoma HCT116 cells and is cytotoxic independently of topoisomerase I expression. *Clin Cancer Res* 2001; 7:185–191.
51. Ryan DP, Supko JG, Eder PP, et al. Phase I and pharmacokinetic study of ecteinascidin 743 administered as a 72-hour continuous intravenous infusion in patients with solid malignancies. *Clin Cancer Res* 2001; 7:231–242.
52. Taamma A, Misset JL, Riofrio M, et al. Phase I and pharmacokinetic study of ecteinascidin-743, a new marine compound, administered as a 24-hour continuous infusion in patients with solid tumors. *J Clin Oncol* 2001; 19:1256–1265.
53. Takebayashi Y, Pourquier P, Zimonjic DB, et al. Antiproliferative activity of ecteinascidin 743 is dependent upon transcription-coupled nucleotide-excision repair. *Nat Med* 2001; 7:961–966.
54. Liao ZY, Sordet O, Zhang HL, Kohlhagen G, Antony S, Gmeiner WH, Pommier Y. A novel polypyrimidine antitumor agent FdUMP[10] induces thymineless death with topoisomerase I-DNA complexes. *Cancer Res* 2005; 65:4844–4851.
55. Pourquier P, Gioffre C, Kohlhagen G, et al. Gemcitabine (2', 2'-difluoro-2'-deoxycytidine), an antimetabolite that poisons topoisomerase I. *Clin Cancer Res* 2002; 8:2499–2504.
56. Pourquier P, Takebayashi Y, Urasaki Y, Gioffre C, Kohlhagen G, Pommier Y. Induction of topoisomerase I cleavage complexes by 1-β-D-arabinofuranosylcytosine (Ara-C) *in vitro* and in ara-C-treated cells. *Proc Natl Acad Sci USA* 2000; 97:1885–1890.
57. Pourquier P, Waltman JL, Urasaki Y, et al. Topoisomerase I-mediated cytotoxicity of N-methyl-N'-nitro-N-nitrosoguanidine: trapping of topoisomerase I by the O6-methylguanine. *Cancer Res* 2001; 61:53–58.
58. Lanza A, Tornatelli S, Rodolfo C, Scanavini MC, Pedrini AM. Human DNA topoisomerase I-mediated cleavages stimulated by ultraviolet light-induced DNA damage. *J Biol Chem* 1996; 271:6978–6986.
59. Pourquier P, Ueng L-M, Fertala J, et al. Induction of reversible complexes between eukaryotic DNA topoisomerase I and DNA-containing oxidative base damages. *J Biol Chem* 1999; 274:8516–8523.

60. Pourquier P, Pilon AA, Kohlhagen G, Mazumder A, Sharma A, Pommier Y. Trapping of mammalian topoisomerase I and recombinations induced by damaged DNA containing nicks or gaps. Importance of DNA end phosphorylation and camptothecin effects. *J Biol Chem* 1997; 272: 26441–26447.
61. Pourquier P, Bjornsti M-A, Pommier Y. Induction of topoisomerase I cleavage complexes by the vinyl chloride adduct, 1,N⁶-ethenoadenine. *J Biol Chem* 1998; 273:27245–27249.
62. Hertzberg RP, Caranfa MJ, Hecht SM. On the mechanism of topoisomerase I inhibition by camptothecin: Evidence for binding to an enzyme-DNA complex. *Biochemistry* 1989; 28:4629–4638.
63. Pommier Y, Marchand C. Interfacial inhibitors of protein-DNA interactions. *Curr Med Chem Anticancer Agents* 2005; 5:421–429.
64. Pommier Y, Cherfils J. Interfacial protein inhibition: a nature's paradigm for drug discovery. *Trends Pharmacol Sci* 2005; 26:138–145.
65. Jaxel C, Capranico G, Kerrigan D, Kohn KW, Pommier Y. Effect of local DNA sequence on topoisomerase I cleavage in the presence or absence of camptothecin. *J Biol Chem* 1991; 266: 20418–20423.
66. Pommier Y, Kohlhagen G, Kohn F, Leteurtre F, Wani MC, Wall ME. Interaction of an alkylating camptothecin derivative with a DNA base at topoisomerase I-DNA cleavage sites. *Proc Natl Acad Sci USA* 1995; 92:8861–8865.
67. Staker BL, Hjerrild K, Feese MD, Behnke CA, Burgin AB, Jr., Stewart L. The mechanism of topoisomerase I poisoning by a camptothecin analog. *Proc Natl Acad Sci USA* 2002; 99: 15387–15392.
68. Redinbo MR, Stewart L, Kuhn P, Champoux JJ, Hol WGJ. Crystal structure of human topoisomerase I in covalent and noncovalent complexes with DNA. *Science* 1998; 279:1504–1513.
69. Kerrigan JE, Pilch DS. A structural model for the ternary cleavable complex formed between human topoisomerase I, DNA, and camptothecin. *Biochemistry* 2001; 40:9792–9798.
70. Laco G, Collins JR, Luke BT, et al. Human topoisomerase I inhibition: docking camptothecin and derivatives into a structure-based active site model. *Biochemistry* 2002; 41:1428–1435.
71. Pommier Y, Kohlhagen G, Laco GS, Kroth H, Sayer JM, Jerina DM. Different effects on human topoisomerase I by minor groove and intercalated deoxyguanosine adducts derived from two polycyclic aromatic hydrocarbon diol epoxides at or near a normal cleavage site. *J Biol Chem* 2002; 277:13666–13672.
72. Sim SP, Pilch DS, Liu LF. Site-specific topoisomerase I-mediated DNA cleavage induced by nogalamycin: a potential role of ligand-induced DNA bending at a distal site. *Biochemistry* 2000; 39:9928–9934.
73. Strumberg D, Pilon AA, Smith M, Hickey R, Malkas L, Pommier Y. Conversion of topoisomerase I cleavage complexes on the leading strand of ribosomal DNA into 5'-phosphorylated DNA double-strand breaks by replication runoff. *Mol Cell Biol* 2000; 20:3977–3987.
74. Wu J, Liu LF. Processing of topoisomerase I cleavable complexes into DNA damage by transcription. *Nucleic Acids Res* 1997; 25:4181–4186.
75. Ljungman M, Lane DP. Transcription - guarding the genome by sensing DNA damage. *Nat Rev Cancer* 2004; 4:727–737.
76. Goldwasser F, Shimizu T, Jackman J, et al. Correlations between S- and G2-phase arrest and cytotoxicity of camptothecin in human colon carcinoma cells. *Cancer Res* 1996; 56:4430–4437.
77. Stefanis L, Park DS, Friedman WJ, Greene LA. Caspase-dependent and -independent death of camptothecin-treated embryonic cortical neurons. *J Neurosci* 1999; 19:6235–6247.
78. Kurz EU, Lees-Miller SP. DNA damage-induced activation of ATM and ATM-dependent signaling pathways. *DNA Repair (Amst)* 2004; 3:889–900.
79. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 2003; 421:499–506.
80. Ali A, Zhang J, Bao S, et al. Requirement of protein phosphatase 5 in DNA-damage-induced ATM activation. *Genes Dev* 2004; 18:249–254.
81. Goodarzi AA, Jonnalagadda JC, Douglas P, et al. Autophosphorylation of ataxia-telangiectasia mutated is regulated by protein phosphatase 2A. *EMBO J* 2004; 23:4451–4461.
82. Banin S, Moyal L, Shieh S, et al. Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* 1998; 281:1674–1677.
83. Kitagawa R, Bakkenist CJ, McKinnon PJ, Kastan MB. Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-BRCA1 pathway. *Genes Dev* 2004; 18:1423–1438.

84. Lupardus PJ, Byun T, Yee MC, Hekmat-Nejad M, Cimprich KA. A requirement for replication in activation of the ATR-dependent DNA damage checkpoint. *Genes Dev* 2002; 16:2327–2332.
85. Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* 2003; 300:1542–1548.
86. Burma S, Chen DJ. Role of DNA-PK in the cellular response to DNA double-strand breaks. *DNA Repair (Amst)* 2004; 3:909–918.
87. Sordet O, Khan QA, Kohn KW, Pommier Y. Apoptosis induced by topoisomerase inhibitors. *Curr Med Chem Anticancer Agents* 2003; 3:271–290.
88. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science* 2004; 305:626–629.
89. Li P, Nijhawan D, Budihardjo I, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; 91:479–489.
90. Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES. Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol Cell* 1998; 1:949–957.
91. Saleh A, Srinivasula SM, Acharya S, Fishel R, Alnemri ES. Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. *J Biol Chem* 1999; 274:17941–17945.
92. Slee EA, Harte MT, Kluck RM, et al. Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol* 1999; 144:281–292.
93. Vaux DL, Silke J. Mammalian mitochondrial IAP binding proteins. *Biochem Biophys Res Commun* 2003; 304:499–504.
94. Sharpe JC, Arnould D, Youle RJ. Control of mitochondrial permeability by Bcl-2 family members. *Biochim Biophys Acta* 2004; 1644:107–113.
95. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2002; 2:647–656.
96. Bouillet P, Strasser A. BH3-only proteins – evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. *J Cell Sci* 2002; 115:1567–1574.
97. Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 1995; 81:505–512.
98. Medema JP, Scaffidi C, Kischkel FC, et al. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J* 1997; 16:2794–2804.
99. Salvesen GS, Dixit VM. Caspase activation: the induced-proximity model. *Proc Natl Acad Sci USA* 1999; 96:10964–10967.
100. Scaffidi C, Fulda S, Srinivasan A, et al. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 1998; 17:1675–1687.
101. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998; 94:491–501.
102. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 1998; 94:481–490.
103. Shao RG, Cao CX, Nieves-Neira W, Dimanche-Boitrel MT, Solary E, Pommier Y. Activation of the Fas pathway independently of Fas ligand during apoptosis induced by camptothecin in p53 mutant human colon carcinoma cells. *Oncogene* 2001; 20:1852–1859.
104. Chatterjee D, Schmitz I, Krueger A, et al. Induction of apoptosis in 9-nitrocamptothecin-treated DU145 human prostate carcinoma cells correlates with de novo synthesis of CD95 and CD95 ligand and down-regulation of c-FLIP(short). *Cancer Res* 2001; 61:7148–7154.
105. Ciusani E, Perego P, Carenini N, et al. Fas/CD95-mediated apoptosis in human glioblastoma cells: a target for sensitisation to topoisomerase I inhibitors. *Biochem Pharmacol* 2002; 63:881–887.
106. Muller M, Wilder S, Bannasch D, et al. p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. *J Exp Med* 1998; 188:2033–2045.
107. Micheau O, Solary E, Hammann A, Martin F, Dimanche-Boitrel MT. Sensitization of cancer cells treated with cytotoxic drugs to fas-mediated cytotoxicity. *J Natl Cancer Inst* 1997; 89:783–789.
108. Micheau O, Solary E, Hammann A, Dimanche-Boitrel MT. Fas ligand-independent, FADD-mediated activation of the Fas death pathway by anticancer drugs. *J Biol Chem* 1999; 274:7987–7992.
109. Lacour S, Hammann A, Grazide S, et al. Cisplatin-induced CD95 redistribution into membrane lipid rafts of HT29 human colon cancer cells. *Cancer Res* 2004; 64:3593–3598.

110. Yeh WC, Pompa JL, McCurrach ME, et al. FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. *Science* 1998; 279:1954–1958.
111. Varfolomeev EE, Schuchmann M, Luria V, et al. Targeted disruption of the mouse caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity* 1998; 9:267–276.
112. Zhivotovsky B, Kroemer G. Apoptosis and genomic instability. *Nat Rev Mol Cell Biol* 2004; 5:752–762.
113. Norbury CJ, Zhivotovsky B. DNA damage-induced apoptosis. *Oncogene* 2004; 23:2797–2808.
114. Fridman JS, Lowe SW. Control of apoptosis by p53. *Oncogene* 2003; 22:9030–9040.
115. Ohtsuka T, Ryu H, Minamishima YA, et al. ASC is a Bax adaptor and regulates the p53-Bax mitochondrial apoptosis pathway. *Nat Cell Biol* 2004; 6:121–128.
116. Fernandez-Salas E, Suh KS, Speransky VV, et al. mtCLIC/CLIC4, an organellar chloride channel protein, is increased by DNA damage and participates in the apoptotic response to p53. *Mol Cell Biol* 2002; 22:3610–3620.
117. Bourdon JC, Renzing J, Robertson PL, Fernandes KN, Lane DP. Scotin, a novel p53-inducible proapoptotic protein located in the ER and the nuclear membrane. *J Cell Biol* 2002; 158:235–246.
118. Mihara M, Erster S, Zaika A, et al. p53 has a direct apoptogenic role at the mitochondria. *Mol Cell* 2003; 11:577–590.
119. Bakalkin G, Yakovleva T, Selivanova G, et al. p53 binds single-stranded DNA ends and catalyzes DNA renaturation and strand transfer. *Proc Natl Acad Sci USA* 1994; 91:413–417.
120. Konishi A, Shimizu S, Hirota J, et al. Involvement of histone H1.2 in apoptosis induced by DNA double-strand breaks. *Cell* 2003; 114:673–688.
121. Yan N, Shi Y. Histone H1.2 as a trigger for apoptosis. *Nat Struct Biol* 2003; 10:983–985.
122. Lassus P, Opitz-Araya X, Lazebnik Y. Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* 2002; 297:1352–1354.
123. Tinel A, Tschopp J. The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. *Science* 2004; 304:843–846.
124. Baliga BC, Colussi PA, Read SH, Dias MM, Jans DA, Kumar S. Role of prodomain in importin-mediated nuclear localization and activation of caspase-2. *J Biol Chem* 2003; 278:4899–4905.
125. Robertson JD, Gogvadze V, Kropotov A, Vakifahmetoglu H, Zhivotovsky B, Orrenius S. Processed caspase-2 can induce mitochondria-mediated apoptosis independently of its enzymatic activity. *EMBO Rep* 2004; 5:643–648.
126. Han Z, Wei W, Dunaway S, et al. Role of p21 in apoptosis and senescence of human colon cancer cells treated with camptothecin. *J Biol Chem* 2002; 277:17154–17160.
127. Gupta M, Fan S, Zhan Q, Kohn KW, O'Connor PM, Pommier Y. Inactivation of p53 increases the cytotoxicity of camptothecin in human colon HCT116 and breast MCF-7 cancer cells. *Clin Cancer Res* 1997; 3:1653–1660.
128. Hsieh JK, Yap D, O'Connor DJ, et al. Novel function of the cyclin A binding site of E2F in regulating p53-induced apoptosis in response to DNA damage. *Mol Cell Biol* 2002; 22:78–93.
129. Blattner C, Sparks A, Lane D. Transcription factor E2F-1 is upregulated in response to DNA damage in a manner analogous to that of p53. *Mol Cell Biol* 1999; 19:3704–3713.
130. Yin Y, Stephen CW, Luciani MG, Fahraeus R. p53 stability and activity is regulated by Mdm2-mediated induction of alternative p53 translation products. *Nat Cell Biol* 2002; 4:462–467.
131. Kohn KW, Pommier Y. Molecular interaction map of the p53 and Mdm2 logic elements, which control the off-on switch of p53 in response to DNA damage. *Biochem Biophys Res Commun* 2005; 331:816–827.
132. Hickman ES, Moroni MC, Helin K. The role of p53 and pRB in apoptosis and cancer. *Curr Opin Genet Dev* 2002; 12:60–66.
133. Gottlieb TM, Oren M. p53 and apoptosis. *Semin Cancer Biol* 1998; 8:359–368.
134. Sordet O, Bettaieb A, Bruey JM, et al. Selective inhibition of apoptosis by TPA-induced differentiation of U937 leukemic cells. *Cell Death Differ* 1999; 6:351–361.
135. Sordet O, Rebe C, Leroy I, et al. Mitochondria-targeting drugs arsenic trioxide and lonidamine bypass the resistance of TPA-differentiated leukemic cells to apoptosis. *Blood* 2001; 97:3931–3940.
136. Shimizu T, Pommier Y. Camptothecin-induced apoptosis in p53-null human leukemia HL60 cells and their isolated nuclei: effects of the protease inhibitors Z-VAD-fmk and dichloroisocoumarin suggest an involvement of both caspases and serine proteases. *Leukemia* 1997; 11:1238–1244.

137. Nieves-Neira W, Pommier Y. Apoptotic response to camptothecin and 7-hydroxystaurosporine (UCN-01) in the 8 human breast cancer cell lines of the NCI Anticancer Drug Screen: multifactorial relationships with topoisomerase I, protein kinase C, Bcl-2, p53, MDM-2 and caspase pathways. *Int J Cancer* 1999; 82:396–404.
138. Kolluri SK, Bruey-Sedano N, Cao X, et al. Mitogenic effect of orphan receptor TR3 and its regulation by MEKK1 in lung cancer cells. *Mol Cell Biol* 2003; 23:8651–8667.
139. Woronicz JD, Calnan B, Ngo V, Winoto A. Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas. *Nature* 1994; 367:277–281.
140. Liu ZG, Smith SW, McLaughlin KA, Schwartz LM, Osborne BA. Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77. *Nature* 1994; 367:281–284.
141. Xue Y, Chomez P, Castanos-Velez E, Biberfeld P, Perlmann T, Jondal M. Positive and negative thymic selection in T cell receptor-transgenic mice correlate with Nur77 mRNA expression. *Eur J Immunol* 1997; 27:2048–2056.
142. Li H, Kolluri SK, Gu J, et al. Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3. *Science* 2000; 289:1159–1164.
143. Lin B, Kolluri SK, Lin F, et al. Conversion of Bcl-2 from protector to killer by interaction with nuclear orphan receptor Nur77/TR3. *Cell* 2004; 116:527–540.
144. Roos-Mattjus P, Vroman BT, Burtelow MA, Rauen M, Eapen AK, Karnitz LM. Genotoxin-induced Rad9-Hus1-Rad1 (9-1-1) chromatin association is an early checkpoint signaling event. *J Biol Chem* 2002; 277:43809–43812.
145. Komatsu K, Miyashita T, Hang H, et al. Human homologue of *S. pombe* Rad9 interacts with BCL-2/BCL-xL and promotes apoptosis. *Nat Cell Biol* 2000; 2:1–6.
146. Yoshida K, Komatsu K, Wang HG, Kufe D. c-Abl tyrosine kinase regulates the human Rad9 checkpoint protein in response to DNA damage. *Mol Cell Biol* 2002; 22:3292–3300.
147. Yoshida K, Wang HG, Miki Y, Kufe D. Protein kinase Cdelta is responsible for constitutive and DNA damage-induced phosphorylation of Rad9. *EMBO J* 2003; 22:1431–1441.
148. Loegering D, Arlander SJ, Hackbarth J, et al. Rad9 protects cells from topoisomerase poison-induced cell death. *J Biol Chem* 2004; 279:18641–18647.
149. Nothwehr SF, Martinou JC. A retention factor keeps death at bay. *Nat Cell Biol* 2003; 5:281–283.
150. Sawada M, Sun W, Hayes P, Leskov K, Boothman DA, Matsuyama S. Ku70 suppresses the apoptotic translocation of Bax to mitochondria. *Nat Cell Biol* 2003; 5:320–329.
151. Cohen HY, Lavu S, Bitterman KJ, et al. Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. *Mol Cell* 2004; 13:627–638.
152. Sordet O, Khan QA, Pommier Y. Apoptotic topoisomerase I-DNA complexes induced by oxygen radicals and mitochondrial dysfunction. *Cell Cycle* 2004; 3.
153. Sordet O, Liao Z, Liu H, et al. Topoisomerase I-DNA complexes contribute to arsenic trioxide-induced apoptosis. *J Biol Chem* 2004; 279:33968–33975.
154. Sordet O, Khan QA, Plo I, et al. Apoptotic topoisomerase I-DNA complexes induced by staurosporine-mediated oxygen radicals. *J Biol Chem* 2004; 279:50499–50504.
155. Daroui P, Desai SD, Li TK, Liu AA, Liu LF. Hydrogen peroxide induces topoisomerase I-mediated DNA damage and cell death. *J Biol Chem* 2004; 279:14587–14594.
156. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 1998; 391:43–50.
157. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* 2001; 412:95–99.
158. Yoshida A, Urasaki Y, Waltham M, et al. Human apurinic/aprimidinic endonuclease (Ape1) and its N-terminal truncated form (AN34) are involved in DNA fragmentation during apoptosis. *J Biol Chem* 2003; 278:37768–37776.
159. Ricci JE, Gottlieb RA, Green DR. Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. *J Cell Biol* 2003; 160:65–75.
160. Ricci JE, Munoz-Pinedo C, Fitzgerald P, et al. Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain. *Cell* 2004; 117:773–786.
161. Aladjem MI, Pasa S, Parodi S, Weinstein JN, Pommier Y, Kohn KW. Molecular interaction maps—a diagrammatic graphical language for bioregulatory networks. *Sci STKE* 2004; 2004:pe8.

162. Kohn KW. Functional capabilities of molecular network components controlling the mammalian G1/S cell cycle phase transition. *Oncogene* 1998; 16:1065–1075.
163. Kohn KW. Molecular interaction map of the mammalian cell cycle control and DNA repair systems. *Mol Biol Cell* 1999; 10:2703–2734.
164. Kohn KW, Aladjem MI, Pasa S, Parodi S, Pommier Y. Cell cycle control: molecular interaction map. *Nature Encyclopedia of the Human Genome* 2004; 1:457–474.
165. Kohn KW, Riss J, Aprelikova O, Weinstein JN, Pommier Y, Barrett JC. Properties of switch-like bioregulatory networks studied by simulation of the hypoxia response control system. *Mol Biol Cell* 2004; 15:3042–3052.
166. Pommier Y, Kohn KW. Cell cycle and checkpoints in oncology: new therapeutic targets. *Med Sci (Paris)* 2003; 19:173–186.
167. Pommier Y, Yu Q, Kohn KW. Novel targets in the cell cycle and cell cycle checkpoints. In: Kerr DJ, ed. *Anticancer Drug Development*. San Diego: Academic Press, 2002:13–30.
168. Reinhold WC, Kouros-Mehr H, Kohn KW, et al. Apoptotic susceptibility of cancer cells selected for camptothecin resistance: gene expression profiling, functional analysis, and molecular interaction mapping. *Cancer Res* 2003; 63:1000–1011.

21

Perturbations of Cellular Functions by Topoisomerase II Inhibitors

All Roads Lead to Cell Death?

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SUMMARY

DNA topoisomerase II inhibitors such as doxorubicin, daunorubicin, and etoposide are among the most widely used anticancer agents for treatment of both solid tumors and hematological malignancies. Clinically active topoisomerase II inhibitors are able to induce different types of cell death/growth arrest including apoptosis, mitotic catastrophe, and necrosis as well as drug-induced pseudo-senescence (DIPS), autophagy, and differentiation. Topoisomerase II may itself modulate the cell death processes induced by other classes of anticancer agents. Topoisomerase II is involved in the formation of high-molecular-weight apoptotic DNA fragments both directly and indirectly through its interaction with the caspase-activated DNase (CAD) nuclease. Topoisomerase II can also regulate the occurrence of mitotic catastrophe through its capacity to modulate mitotic entry and cell cycle progression in early mitosis.

Key Words: Topoisomerase II; anticancer agents; doxorubicin; etoposide; cell death; growth arrest; apoptosis; necrosis; mitotic catastrophe; drug-induced pseudo-senescence; autophagy.

1. DRUG-INDUCED CELL DEATH

Our understanding of the different types of cell death/proliferation arrest induced by anticancer agents and other cellular stress has substantially improved during recent years. However, the experimental evidence has also contributed to an increasing realization of the complexity of these cellular processes. Before reviewing our current knowledge about the mechanisms of cell death/proliferation arrest induced by topoisomerase II inhibitors, we like to discuss a number of factors that play an important role in the cellular response to anticancer agents. This includes tissue and cell type,

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the identity of the cytotoxic agent, and pharmacological factors such as dose and exposure time.

Cells can die or become growth-arrested by a number of different mechanisms including apoptosis, necrosis, mitotic catastrophe, drug-induced pseudo-senescence (DIPS), autophagy, and differentiation. However, all these processes are not necessarily operational in all cells and tissue types. Even a given cell death process induced by the same agent may not occur the same way in different tissues. For example, early work on apoptosis focused on thymocytes or cell lines like the widely used HL-60 human leukemia cell model, in which extensive endonucleosomal degradation could be observed within a few hours after drug exposure. By contrast, for solid tumor cell lines, even massive apoptosis is usually a much slower process appearing 12–24 h after drug exposure and which may not necessarily be accompanied by endonucleosomal DNA fragmentation.

Although most of the current literature classifies alkylating agents, antimetabolites, topoisomerase inhibitors, and even irradiation under the global term “DNA-damaging agents,” there are substantial differences between the different classes of agents as well as between agents of the same class. As an example, the topoisomerase II inhibitor doxorubicin is able to induce several types of cell death as well as DIPS and differentiation. By contrast, amsacrine, another topoisomerase II inhibitor, is principally a strong inducer of apoptotic cell death. In this respect, it is interesting that doxorubicin shows major activity toward different types of solid tumors including breast cancer whereas the clinical activity of amsacrine is basically limited to leukemias.

An often neglected parameter in the literature is the importance of pharmacological factors such as dose and exposure time. It is well established that a short-term exposure to a high concentration of an anticancer agent may not necessarily result in the same biological effects as a sustained exposure to lower doses of the same drug, as observed clinically for the antimetabolite 5-fluorouracil. Furthermore, at comparable exposure times, a given drug may induce different types of cell death dependent on the drug concentration. For example, in HL-60 myeloid leukemia cells, daunorubicin induced rapid apoptosis at 0.5–1 μM but mitotic catastrophe and delayed apoptotic cell death at 0.1 μM . These concentrations are all within the clinical dose range (1). This concept is further illustrated in Fig. 1.

At low drug concentrations, exposure to topoisomerase II inhibitors is accompanied by reversible cell cycle arrest, by DNA repair, or, if the DNA lesions are incompletely repaired, by mutagenesis. At higher drug concentrations, we may observe delayed

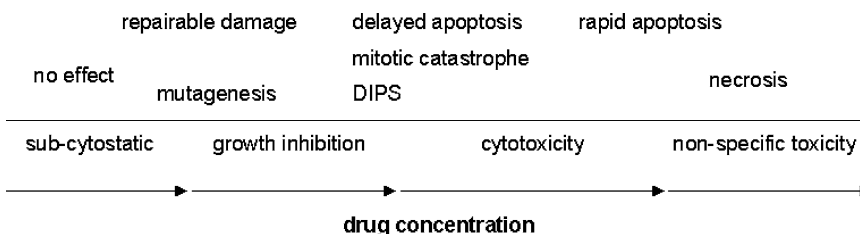


Fig. 1. Different cellular effects induced by topoisomerase II inhibitors as a function of drug concentration.

apoptosis, mitotic catastrophe, and DIPS. Finally, high drug concentrations usually lead to rapid apoptosis or necrosis. Taken together, we can conclude that the cellular outcome of exposure to genotoxic anticancer agents such as topoisomerase II inhibitors depends on both the genetic and epigenetic background (cell or tissue type, oncogenic alterations) and on pharmacological parameters such as the nature of the drug, dose, and exposure time.

2. TOPOISOMERASE II AND ITS INHIBITORS

The nuclear enzyme DNA topoisomerase II regulates DNA topology and chromatin organization in living cells (2). In higher eukaryotes, there are two isoforms of topoisomerase II, alpha and beta, that are coded by different genes and have partially different cellular functions. However, both isoforms of the enzyme are able to relieve torsional stress in DNA by controlled breakage-reunion of both DNA strands and a passage of a DNA segment through the strand break. Topoisomerase II is absolutely essential for chromatin condensation and separation of sister chromatids during mitosis (for recent reviews, see 3 and 4).

2.1. Inhibition of Topoisomerase II Leads to DNA Damage

All topoisomerase II-directed agents interfere with at least one step of the catalytic cycle and are thus classified as topoisomerase II inhibitors. DNA topoisomerase II inhibitors are among the most widely used anticancer agents and show clinical activity toward both solid tumors and hematological malignancies. Clinically used topoisomerase II inhibitors principally act by stabilizing a covalent DNA–topoisomerase II complex (the so-called cleavable complex) with nuclear DNA. These compounds are often classified as topoisomerase II poisons. Although the cleavable complexes themselves are reversible and disappear when the drug is removed, collision between the complexes and ongoing macromolecular synthesis can give rise to secondary DNA damage like single- and double-strand breaks that trigger diverse cellular responses such as cell cycle arrest, DNA repair, cell death pathways, and altered transcription (reviewed in 5). It is currently believed that the cytotoxic and antitumor effects of topoisomerase II-directed agents are principally mediated by the induction of cleavable complexes and are further influenced by three factors: where in the genome the complexes are formed, how many complexes are present, and how long they last. It has long been debated whether topoisomerase II inhibitors preferentially induce cleavable complexes and/or secondary DNA damage in specific regions of the genome, such as Matrix Associated Region (MAR) sequences (6). Quantitative and qualitative differences in DNA damage may also be a result of the capacity of the drug to inhibit either one or both isoforms of DNA topoisomerase II (7). Specific cleavage at defined sites of the DNA could explain why different topoisomerase II inhibitors results in differential effects on the DNA transcription patterns.

A second group of topoisomerase II inhibitors is the so-called catalytic inhibitors. This group is a heterogeneous group of compounds that are able to inhibit the catalytic activity of topoisomerase II by a variety of mechanisms other than cleavable complex formation. This class of compounds has been extensively reviewed recently (8). The most widely studied catalytic inhibitor is ICRF-187 (dexrazoxane) and structurally

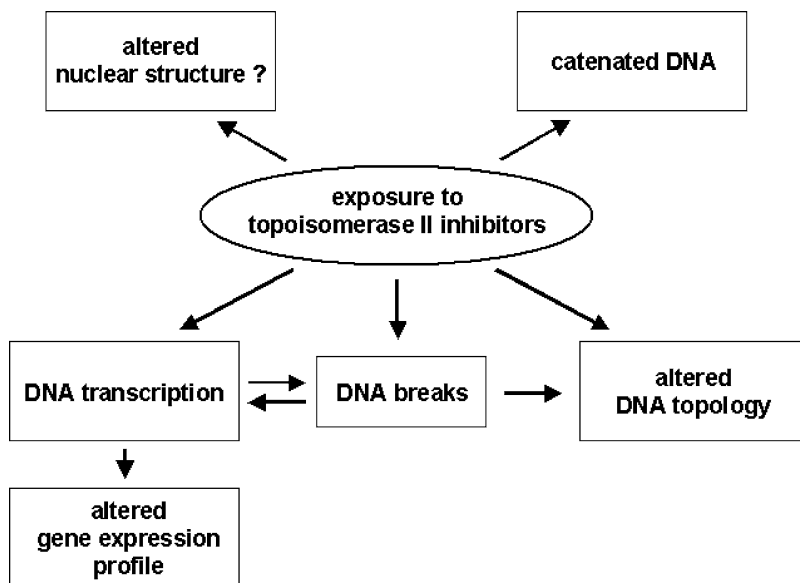


Fig. 2. Changes in DNA structure and function in cells exposed to topoisomerase II inhibitors.

related bisdioxopiperazine derivatives that are able to stabilize non-covalent topoisomerase II complexes on DNA (9). Although less genotoxic than the topoisomerase II poisons, the ICRF compounds are also able to induce DNA damage and chromosomal aberrations either as a result of secondary damage following collision with macromolecular synthesis machineries or as a result of aberrant mitosis (10).

2.2. Interference with Biological Functions of Topoisomerase II

In addition to inducing DNA damage, topoisomerase II inhibitors are likely to interfere with the normal biological functions of topoisomerase II as outlined in Fig. 2. These principally include its role as a transcriptional regulator, its contribution to the DNA damage response, and its mitotic activities.

2.2.1. TRANSCRIPTIONAL MODULATION

When immobilized on the DNA, the drug-associated topoisomerase II is no longer able to relax or decatenate DNA, which results in alterations of local topology and nuclear architecture. The role of DNA topology on transcription is incompletely understood in higher eukaryotes but has been extensively characterized in yeast and bacteria, in which it plays a major role in the expression of certain genes (11). Importantly, this group includes genes coding for proteins involved in the stress response to external factors such as changes in pH, oxygen, and osmolarity, as well as gene products required for invasion. Exposure of mammalian cells to the topoisomerase II inhibitor etoposide stimulates the expression of interferon regulatory factor-7 (IRF-7), which plays an important role in the development and maturation of the immune system (12), and of molecules involved in cell death and survival signaling. For example, although etoposide treatment of human HL-60 leukemia cells resulted in practically unchanged expression of cell death receptor-3 (DR-3), DR-4,

and FAS, the expression of silencers of death domain (SODD) and FADD-like-il converting enzyme (caspase-8) (FLICE) proteins, which serve as inhibitors of the cell death receptor signaling pathways, decreased substantially (13). This was accompanied by aggregation of DR3 and DR4, but not FAS, into high-molecular-weight death complexes at the cell surface (13). In glioma cells, etoposide was reported to upregulate the expression of DR-5 and downregulate the caspase-8 inhibitors Cellular caspase-8 (FLICE)-like inhibitory protein (c-FLIPs) (14).

2.2.2. INDUCTION OF THE DNA DAMAGE RESPONSE

Topoisomerase II is able to form molecular complexes with a number of proteins involved in cell cycle and survival signaling. These include the p53 oncogene suppressor, the BRCA1 C-terminus (BRCT) containing protein TopBP1, 14-3-3 ϵ , the mitotic protein kinase CDK1 (also called Cdc2 kinase), and the survival protein kinase CK2 (reviewed in 3). Topoisomerase II is involved in chromatin condensation during apoptotic execution which, in part, is mediated by its interaction with the caspase-activated DNase (CAD) (15). Topoisomerase II alpha serves as a nuclear scaffold for CAD during apoptosis, thereby concentrating CAD at specific regions of the chromatin, most likely at the base of the chromatin loops. At the same time, the DNA cleavage activities of topoisomerase II are activated by the physical interaction with CAD, and simultaneous inhibition of topoisomerase II alpha and caspases was reported to completely abolish apoptotic chromatin condensation in isolated HeLa cell nuclei (15).

2.2.3. MITOTIC FUNCTIONS OF TOPOISOMERASE II

Topoisomerase II is known to play several essential roles during late G2 and early mitosis. First, topoisomerase II is required for premitotic chromosome individualization, the process by which different chromosomes become untangled and converted into individual units (16). The protein also plays a direct role in early chromosome condensation by a so far poorly understood mechanism that, at least in part, seems to rely on protein–protein and DNA–protein interactions (17).

In addition, topoisomerase II alpha is needed for recruitment of CDK1 to the chromatin of mitotic chromosomes (18) and thus for mitotic phosphorylation of other chromatin-associated proteins. Finally, topoisomerase II is required for the separation of intertwined sister chromatids in prometaphase (19,20). Recently, different genetic models have shown that the alpha isoform of topoisomerase II directly influences the cell cycle progression in G2 and during early mitosis, because downregulation of topoisomerase II alpha, but not topoisomerase II beta, protein levels results in delayed G2 transit and prolonged early mitosis (21,22). These studies strongly suggest that topoisomerase II might, at least in part, control the onset of mitotic catastrophe by regulating the fraction of cells entering mitosis.

3. CELL DEATH AND CELL GROWTH ARREST

3.1. Morphology of Tumor Cells Treated with Topoisomerase II Inhibitors

The morphology associated with different types of cell death and growth arrest in tumor cells following exposure to topoisomerase II inhibitors is illustrated in Fig. 3. After treatment with topoisomerase II inhibitors, cells (Fig. 3A) may die by

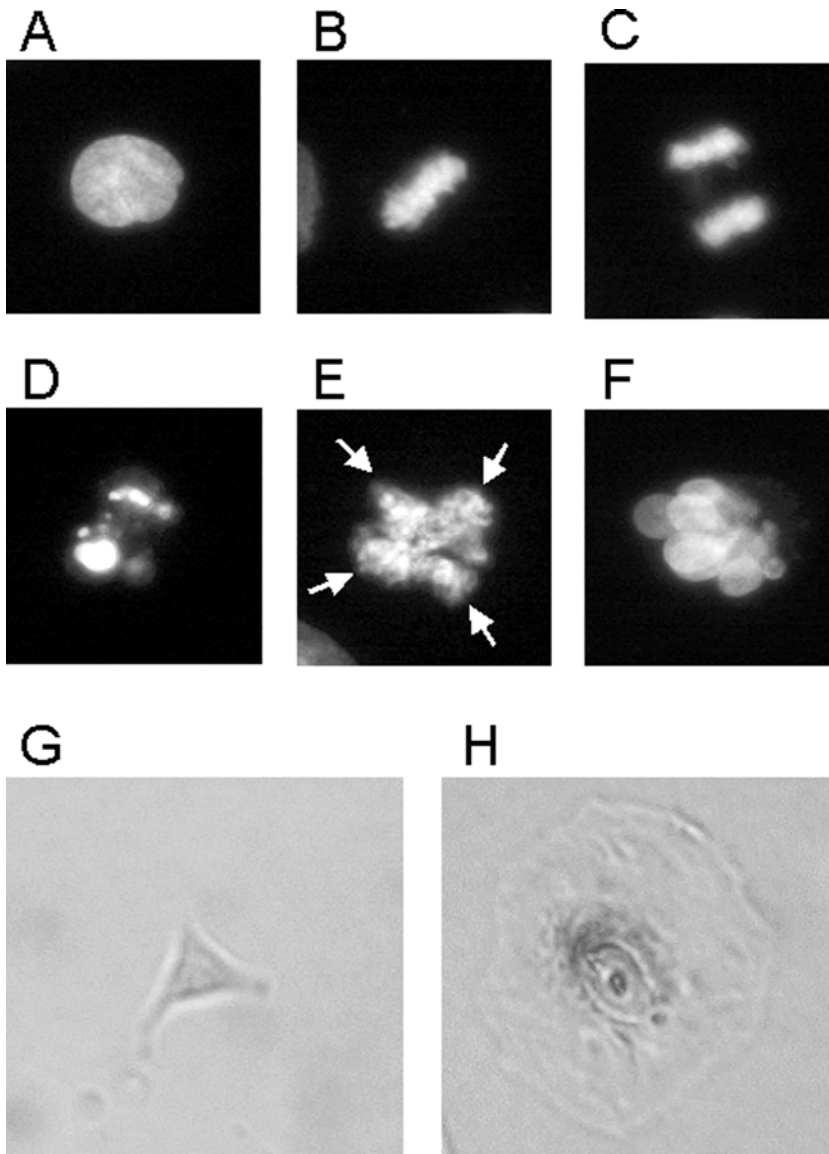


Fig. 3. Nuclear morphology of untreated A549 carcinoma cells (panels A–C). A549 cells treated with the topoisomerase II inhibitor triazoloacridone c-1305 (panels D–F). After prolonged drug exposure, cells (G) become enlarged and flat and express senescence-associated-B-galactosidase (H). Original magnifications $\times 200$ (A–F) and $\times 50$ (G and H).

apoptosis, characterized by fragmentation of the nucleus into discrete fragments of highly condensed chromatin (Fig. 3D). Damaged cells may also progress into mitosis (for comparison, normal metaphase and anaphase are shown in Fig. 3B and C, respectively) and undergo mitotic catastrophe that is characterized by the presence of highly entangled and non-segregated chromosomes (Fig. 3E), frequently with attempted segregation into multiple poles (arrows) and appearance of cells with segmented nuclei (Fig. 3F). After prolonged drug exposure, some cells (Fig. 3G) become very big and

flat with an extended lysosomal compartment and biochemical markers of cellular senescence such as senescence-associated- β -galactosidase (SA- β -Gal) with optimum activity at pH 6 (Fig. 3H). This phenotype corresponds to DIPS cells. Original magnifications are $\times 200$ (pictures Fig. 3A–F) and $\times 50$ (Fig. 3G and H).

3.2. Death by Apoptosis and Necrosis

As discussed above (See Section 1), treatment of tumor cells with topoisomerase II inhibitors may result in different cellular effects depending on the dose that is applied (Fig. 1). At sufficiently high drug concentrations, cells mostly die by activation of an endogenous cell death program, characterized by the presence of distinct morphological and biochemical alterations that collectively are called apoptosis (23–25). Apoptosis can be a rapid process occurring a few hours after drug treatment or a delayed process happening after one or more cell divisions. Biochemical changes that are associated with apoptosis include activation of specific proteases (caspases), DNA fragmentation, and cleavage of a subset of proteins with important cellular functions, such as DNA repair [e.g., poly(ADP-ribose)polymerase (PARP) and DNA-dependent protein kinase (DNA-PK)], or structural proteins (e.g., lamins, topoisomerase II alpha or actin). These biochemical changes are accompanied by specific morphological features such as chromatin condensation, nuclear fragmentation, cellular shrinking, and formation of cell fragments called apoptotic bodies that *in vivo* are engulfed by neighboring cells or macrophages to prevent local inflammation. Apoptosis is a highly controlled process, and it is increasingly evident that the final outcome of drug treatment depends on a delicate balance between pro- and anti-apoptotic signaling (26). Therefore, the susceptibility of tumor cells to either undergo apoptosis or escape apoptotic death pathways is an important determinant in the response to antitumor agents.

It should be stressed that although induction of apoptosis by topoisomerase II inhibitors has been particularly well documented in the literature, other cell death pathways are also activated including mitotic catastrophe, autophagy (discussed in sections 3.3 and 3.5), and necrosis. To add to the complexity, some of the biochemical and morphological features of apoptotic cells have also been described for other types of cell death. For example, non-random DNA fragmentation and caspase activation have been observed in cells undergoing mitotic catastrophe (27,28). Likewise, rapid and massive apoptosis may appear indistinguishable from necrosis because of the lack of healthy neighboring cells and local macrophages that can engulf the apoptotic bodies of the dying cells.

Interestingly, the choice between apoptotic and necrotic cell death is partly determined by the intracellular levels of available ATP. It has been reported that etoposide treatment was accompanied by necrosis under ATP-depleting conditions whereas apoptosis was induced at higher ATP levels (29). Interestingly, modulation of cellular ATP levels by 2-deoxy-D-glucose, an inhibitor of the glycolytic ATP production, in etoposide-treated human glioma and squamous carcinoma cells was accompanied by enhanced cytotoxicity suggesting that necrosis may, at least in some situations, be an efficient mediator of etoposide toxicity (30). Classically, necrosis is considered as a non-specific death mechanism, in contrast to programmed apoptotic cell death. However, increasing evidence suggest that necrosis may also be governed by an intrinsic cell death program (31,32).

PARP appears as a crucial player in the choice between the apoptotic and the necrotic cell death program. Topoisomerase-mediated DNA strand breaks could lead to activation of PARP and subsequent depletion of intracellular ATP due to production of energy-consuming ADP-ribose oligomers at the expense of the PARP substrate, NAD⁺ (33 and references therein). In support of this hypothesis, it has been reported that cells with low PARP activity are resistant to etoposide (34,35). Interestingly, the influence of PARP is closely monitored in apoptotic cells where PARP is inactivated by caspase-specific cleavage, thus maintaining cellular ATP levels sufficiently high to carry out the energy-dependent apoptotic process.

3.3. Mitotic Catastrophe

3.3.1. DEFINITIONS AND MECHANISMS

Several different definitions of mitotic catastrophe are used in the literature. Usually, it refers to a type of cell death that occurs during mitosis, most frequently during the metaphase to anaphase transition (36), and this is the definition that we use here. However, some define mitotic catastrophe as a case of aborted mitosis resulting in aneuploidy (28). Mitotic catastrophe may also be defined as aberrant chromosome segregation and/or cytokinesis resulting in the formation of G1 cells with a tetraploid DNA content (37).

Mitotic cell death is due to activation of the apoptotic program by either caspase-independent mechanisms (38) or by a classical apoptotic pathway associated with caspase-2/3 activation and/or mitochondrial membrane permeabilization (39). In the presence of strong survival signaling, cells will not die immediately but may stay viable for prolonged periods of time in a multinucleated, aneuploid or polyploid state. It is unclear what eventually happens with cells that survive abnormal chromosome segregation, but the presence of polyploid cells is often followed by the appearance of long-term survivors and is thus associated with drug resistance (1,40). Interestingly, at least in some situations, the polyploid cells are able to reconstitute mitosis and return to the normal diploid state by the process of de-polyploidization (41) or by an unusual process of cell division called neosis, which is characterized by karyokinesis through nuclear budding followed by asymmetric, intracellular cytokinesis (42).

Classical topoisomerase II inhibitors (etoposide, daunorubicin, and doxorubicin) have been shown to induce mitotic catastrophe in human tumor cells, in which apoptosis was inhibited by overexpression of Bcl-2 (43), in cells overexpressing P-glycoprotein (1) or when the functioning of the DNA damage checkpoint has been perturbed by checkpoint abrogators such as caffeine (44) or by genetic modifications, such as inactivation of 14-3-3 σ (45). Recent data have shown that the imidazoacridone C-1311, a topoisomerase II inhibitor associated with the induction of very low levels of cleavable complexes, produced mitotic cell death in human colon carcinoma HT-29 cells (46). Other studies have shown that inhibition of topoisomerase II in Ptk1 cells by the catalytic inhibitor ICRF-187 had dramatic effect on chromosome condensation and chromosome distribution during mitosis (47). Massive chromosome bridges were formed in cells treated with ICRF-187, and the chromatin mass was unable to segregate during metaphase/anaphase transition resulting in unequal distribution of DNA between daughter cells. Interestingly, in many cases, all DNA was transferred to one nucleus, which caused production of polyploid cells with a 4N DNA content. It was concluded

that accumulation of cells with a 4N DNA content following treatment with ICRF compounds may reflect either a G2 arrest or incomplete mitotic chromosome segregation. It should be stressed that mitotic catastrophe is not restricted to topoisomerase II inhibitors but is also induced by other DNA-damaging agents such as irradiation and cisplatin (48–50) and by microtubule inhibitors (51,52).

3.3.2. ROLES OF CHECKPOINT AND SURVIVAL SIGNALING

It is evident that a critical aspect of mitotic catastrophe is checkpoint failure in G2 or during early mitosis, which permits cells with damaged DNA to reach metaphase. In agreement, exposure of etoposide-treated cells with G2 abrogators such as caffeine led to mitotic death and increased cytotoxicity (44). However, once damaged cells reach metaphase, the remaining mitotic checkpoints most likely enforce the mitotic catastrophe. Strong metaphase checkpoints including the classical spindle assembly checkpoint and the mitotic DNA damage checkpoint (53) result in prolonged mitotic arrest of DNA-damaged cells that subsequently die directly out of metaphase. In agreement, abrogation of the spindle checkpoint of DNA damaged cells was accompanied by escape from mitotic cell death and abnormal mitosis (54). Recent results suggest the presence of an additional checkpoint controlling mitotic exit by preventing cytokinesis. This checkpoint would favor mitotic cell death by preventing mitotic exit (55).

Besides checkpoint function, survival signaling seems to be important for cells to survive abnormal chromosome segregation. This explains why overexpression of Bcl2 or survivin may rescue cells from mitotic cell death following treatment with topoisomerase II inhibitors despite abnormal chromosome segregation (56).

The role of oncogene suppressors such as p53 in mitotic catastrophe is much less clear although it is often reported that mitotic catastrophe preferentially occurs in cells with non-functional p53 (36,57). However, a recently identified suppressor of mitotic catastrophe is the *Xenopus* ortholog of 53BP1, a BRCT protein previously identified in humans through its ability to bind the p53 (58). Furthermore, mitotic catastrophe due to Chk1 deficiency seems to be mediated by a p53-dependent pathway that involves both the ataxia-telangiectasia-mutated (ATM) and the ATM- and Rad3-related (ATR) checkpoint kinases (59). Whether the same mechanisms are operative following DNA damage induced by topoisomerase II inhibitors remains to be established.

In conclusion, the survival of cells with damaged DNA during mitosis depends on many factors including (i) the different checkpoints and adaptation processes controlling the entry, transit, and exit from mitosis (generally, early mitotic checkpoints will tend to prevent mitotic catastrophe whereas late mitotic checkpoints will facilitate the mitotic catastrophe), (ii) proteins such as topoisomerase II that are needed for normal mitotic functions, and (iii) the expression of survival factors and survival signaling that can inhibit the induction and execution of apoptosis during mitosis.

3.4. Pseudo-Senescence (DIPS) Induced by Topoisomerase II Inhibitors

3.4.1. DEFINITIONS AND MARKERS

Multicellular organisms have evolved different mechanisms to prevent the development of cancer originating in cells with deleterious DNA mutations. There are basically two ways of eliminating such cells, of which the best understood is the

induction of cell death. The second pathway is the induction of cellular senescence (irreversible growth arrest) after a well-defined number of cell population doublings before cells actually become cancerous.

The terms “cellular senescence” and “replicative senescence” refer to the state where normal somatic cells stop proliferating due to critical telomere shortening. Importantly, DNA damage induced by oxidative stress, UV irradiation, DNA-alkylating agents, histone deacetylase inhibitors, or DNA topoisomerase II inhibitors may also lead to irreversible growth arrest with a phenotype resembling cellular senescence (60). In this review, we refer to this process as DIPS. DIPS principally occurs in tumor cells, usually happens after only one or two cell divisions, and, in striking contrast to replicative senescence, is independent of telomere length. It is currently not known whether DIPS is controlled by pathways that overlap with those of replicative senescence or, alternatively, is mechanistically different from replicative senescence despite the morphological and biochemical similarities.

Many anticancer agents including the topoisomerase II inhibitors doxorubicin, daunorubicin, and ICRF-193 are able to induce DIPS at relatively low doses that do not lead to apoptosis (36,61). This is observed not only for tumor cells *in vitro* but also in animal models (36) and in cancer patients (62). Importantly, studies with the alkylating agent cyclophosphamide have shown animals with tumors able to undergo drug-induced senescence have a much better prognosis following chemotherapy than those harboring tumors with senescence defects (63). These findings suggest that DIPS may be an important factor in the tumor response to chemotherapy. However, this has been difficult to verify in the clinic, because the most frequently used biochemical assay, SA- β -Gal staining (with optimum pH of 6) is not always reliable, especially in tissue samples (62).

DIPS induced by topoisomerase II inhibitors, such as doxorubicin, is observed both in cells with functional and non-functional p53 (50), although the senescence phenotype is attenuated in p53 and p21 null cells (36). This suggests that DIPS is favored by functional p53 and/or p21 although the two gene products are not absolutely required. DIPS may also be favored by the presence of functional P16, because SA- β -Gal-positive breast cancers cells treated with doxorubicin *in vitro* or *in vivo* were characterized by high levels of p16 (62). However, many cell lines that are used for studies on DIPS *in vitro* and *in vivo* have inactivated P16, usually by hypermethylation of the promoter region. Thus, it seems that p16 is not required for induction of DIPS by antitumor agents although it might still be necessary for the maintenance of DIPS-associated growth arrest. Together, these findings suggest that even modest changes in the expression of genes such as p53, p21, and p16 may determine whether the drug-treated cells are directed toward apoptosis, mitotic catastrophe, or DIPS.

The relationship between cell death and DIPS is unclear. Most likely, DIPS is able to suppress the apoptotic program. Recent studies have shown that caspase inhibitors may switch drug-induced apoptosis to an alternative type of “default death” which resembles DIPS. Treatment of human neuroblastoma cells with combinations of doxorubicin and caspase-3 inhibitors led to increased p21/WAF1 expression and enhanced SA- β -Gal (64). Moreover, usually prolonged growth arrest following treatment with DNA damaging agents is accompanied by cell death. Because cells undergoing DIPS can stay viable for extended time periods, there must be a mechanism which blocks pro-apoptotic signaling or provides survival signals to these cells.

Another important question is whether antitumor agents are able to induce DIPS in both normal and tumor cells. Although DIPS can be observed in both normal and transformed cells *in vitro*, analysis of tissue samples from cancer patients treated with the CAF regimen (cyclophosphamide, doxorubicin, 5-fluorouracil) showed exclusively tumor-specific SA- β -Gal staining with no detectable staining of neighboring normal cells (62). This suggests that *in vivo*, DIPS preferentially occurs in tumor cells.

3.4.2. DIPS-ASSOCIATED PATHWAYS

Although DIPS typically is induced by DNA-damaging agents, it is not clear which type of DNA damage (single-stranded or double-stranded DNA breaks, chromosome breaks) triggers DIPS. It has been reported that permanent growth arrest of human fibroblasts is limited to agents creating double-strand DNA breaks (65). This is in line with data obtained for senescing human cells that accumulated unrepaired DNA double-strand breaks (66). Interestingly, DNA damage foci in senescent cells co-localize with telomeric DNA (67). Thus it is possible that although cells undergoing DIPS show unchanged telomere length, DNA damage induced by antitumor drugs (such as topoisomerase II inhibitors) may lead to inactivation (uncapping) of telomeres. This could in turn activate a permanent DNA damage response state leading to growth arrest with markers of senescent cells.

Another fascinating possibility is that DIPS might be associated with changes in adenine thymidine (AT)-rich nuclear scaffold sequences that are preferential binding sites for topoisomerase II. It has been reported that 5-bromodeoxyuridine induces a senescence-like phenotype in HeLa cells, which is strongly potentiated by AT-binding ligands such as Hoechst 33258 (68). Concomitant gene expression analysis by DNA microarray technology showed that the genes most affected by AT-binding ligands were located on or near Giemsa-dark bands (i.e., heterochromatin) of human chromosomes. Interestingly, it has been postulated that packaging of proliferation-promoting genes into repressive heterochromatin is a key step in cellular senescence (69). Topoisomerase II may thus be directly involved in turning off proliferation-promoting genes and/or switching on senescence-associated genes by mediating the chromatin condensation and matrix association.

Finally, it should be recalled that at least some topoisomerase II inhibitors, including doxorubicin, produce oxidative stress in tumor cells that could lead to indirect DNA damage (70). It is well established that oxidative stress can lead to stress-induced premature senescence (SIPS) in normal cells (71). Although the relation between DIPS and SIPS is not known, it is likely that doxorubicin-induced DIPS is, at least in part, mediated by the induction of oxidative stress. Furthermore, hydrogen peroxide, which is produced during oxidative stress, has been shown to be potent inhibitor of topoisomerase II (72). Although speculative, we suggest that the potency of doxorubicin as a DIPS-inducing agent may be linked to its ability to induce formation of covalent DNA-topoisomerase II complexes both directly and indirectly through formation of hydrogen peroxide.

3.4.3. REVERSIBILITY OF DIPS

One of the current dogmas is that once cells become senescent, they never return to the proliferative state or, in other words, that cellular senescence is irreversible. Although this appears to be true for replicative senescence, this issue has only

recently been addressed for DIPS following exposure to two different topoisomerase II inhibitors, ICRF-193 (73) and doxorubicin (74). Studies with human embryonic fibroblast cells showed that ICRF-193-treated cells resumed proliferation after an initial growth arrest (73). More recent data revealed that treatment of MCF-7 human breast carcinoma cells with a clinically relevant dose of doxorubicin was accompanied by the appearance of senescence-resistant cell clones, which express high levels of CDK1 (74). This study as well as others (75) suggest a new mechanism of tumor cell resistance to topoisomerase II inhibitors and probably also to other DNA-damaging agents, by avoiding drug-induced senescence associated with the maintenance of high CDK1 levels/activity. These results show that at least in some cases, drug-induced senescence may be followed by resumption of cellular proliferation. This issue is particularly important because induction of DIPS has been proposed as an alternative approach to treat human cancers.

3.5. Induction of Autophagy by Topoisomerase II Inhibitors

Autophagy is a process during which a cell degrades its cytoplasmic material, including organelles, in their lysosomes (for recent review, see 76). This unusual pathway is activated during stress conditions such as nutrient- or amino acid-deprivation and likely serves to provide substrates for energy metabolism and protein synthesis by degradation of the cytoplasmic components. In this way, autophagy provides a short-term survival mechanisms under unfavorable growth conditions. Recent results suggested a dual role for autophagy including both protection from apoptotic cell death and execution of alternative ways of cell death (77). Autophagic cell death was observed after treatment of cells with inactivated Bax and Bak genes with etoposide (78). Interestingly, drug-induced autophagy in these cells was not simply a consequence of the lack of apoptosis as no autophagy was detected in cells deficient in two other pro-apoptotic factors, Apaf-1 and caspase 9, nor in cells treated with etoposide and the pan-caspase inhibitor zVAD (78). Cell death with signs of autophagy was also observed in epirubicin-treated breast cancer cells and was further enhanced by addition of medroxyprogesterone acetate (79). These results show that at least in some situations, autophagy may substitute for apoptosis in drug-treated tumor cells. Much work is still needed to clarify the role and regulation of autophagy during drug-induced cell death, and in particular the relationship between autophagy and apoptotic pathways. It can be proposed that autophagy initiates the death pathway but requires the apoptotic machinery to finalize the cell kill. In this respect, it is interesting that some of the proteins that regulate autophagy have also been shown to interact with pro-apoptotic factors including FADD and Atg5 (80) as well as Bcl-2/Bcl-X_L and Beclin-1 (81). Autophagy could benefit cellular survival by removal of damaged cell components following DNA damage (82), and from this perspective, manipulation of autophagy has the potential to improve anticancer therapeutics.

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REFERENCES

1. Come MG, Skladanowski A, Larsen AK, Laurent G. Dual mechanism of daunorubicin-induced cell death in both sensitive and MDR-resistant HL-60 cells. *Br J Cancer* 1999;79:1090–1097.
2. Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol* 2002;3:430–440.
3. Larsen AK, Escargueil AE, Skladanowski A. From DNA damage to G2 arrest: the many roles of topoisomerase II. *Prog Cell Cycle Res* 2003;5:295–300.
4. Swedlow JR and Hirano T. The making of the mitotic chromosome: modern insights into classical questions. *Mol Cell* 2003;11:557–569.
5. Larsen AK, Skladanowski A. Cellular resistance to topoisomerase-targeted drugs: from drug uptake to cell death. *Biochim Biophys Acta* 1998;1400:257–274.
6. Miassod R, Razin SV, Hancock R. Distribution of topoisomerase II-mediated cleavage sites and relation to structural and functional landmarks in 830 kb of *Drosophila* DNA. *Nucleic Acids Res* 1997;25:2041–2046.
7. Binaschi M, Farinosi R, Borgnetto ME, Capranico G. *In vivo* site specificity and human isoenzyme selectivity of two topoisomerase II-poisoning anthracyclines. *Cancer Res* 2000;60:3770–3776.
8. Larsen AK, Escargueil AE, Skladanowski A. Catalytic topoisomerase II inhibitors in cancer therapy. *Pharmacol Ther* 2003;99:167–181.
9. Roca J, Ishida R, Berger JM, Andoh T, Wang JC. Antitumor bisdioxopiperazines inhibit yeast DNA topoisomerase II by trapping the enzyme in the form of a closed protein clamp. *Proc Natl Acad Sci USA* 1994;91:1781–1785.
10. Wang L, Eastmond D, Catalytic inhibitors of topoisomerase II are DNA-damaging agents: induction of chromosomal damage by merbarone and ICRF-187. *Environ Mol Mutagen* 2002;39:348–356.
11. Larsen AK, Lemke K, Oestergaard V, Andersen AH, Vekris A, Haaz MC, Robert J, Escargueil AE and Skladanowski A. Topoisomerases and transcriptional regulation. *Eur J Cancer* 2002;38(Suppl):S71.
12. Lu R, Moore PA, Pitha PM. Stimulation of IRF-7 gene expression by tumor necrosis factor alpha: requirement for NFkappa B transcription factor and gene accessibility. *J Biol Chem* 2002;277:16592–16598.
13. Bergeron S, Beauchemin M, Bertrand R. Camptothecin- and etoposide-induced apoptosis in human leukemia cells is independent of cell death receptor-3 and -4 aggregation but accelerates tumor necrosis factor-related apoptosis-inducing ligand-mediated cell death. *Mol Cancer Ther* 2004;3:1659–1669.
14. Song JH, Song DK, Pyrzynska B, Petruk KC, Van Meir EG, Hao C. TRAIL triggers apoptosis in human malignant glioma cells through extrinsic and intrinsic pathways. *Brain Pathol* 2003;3:539–553.
15. Durrieu F, Samejima K, Fortune JM, Kandels-Lewis S, Osheroff N, Earnshaw WC. DNA topoisomerase II alpha interacts with CAD nuclease and is involved in chromatin condensation during apoptotic execution. *Curr Biol* 2000;10:923–926.
16. Gimenez-Abian JF, Clarke DJ, Devlin J, Gimenez-Abian MI, De la Torre C, Johnson RT, Mullinger AM, Downes CS. Premitotic chromosome individualization in mammalian cells depends on topoisomerase II activity. *Chromosoma* 2000;109:235–244.
17. Adachi Y, Luke M, Laemmli UK. Chromosome assembly *in vitro*: topoisomerase II is required for condensation. *Cell* 1991;64:137–148.
18. Escargueil AE, Plisov SY, Skladanowski A, Borgne A, Meijer L, Gorbisky GJ, Larsen AK. Recruitment of cdc2 kinase by DNA topoisomerase II is coupled to chromatin remodeling. *FASEB J* 2001;15:2288–2290.
19. Gimenez-Abian JF, Clarke DJ, Mullinger AM, Downes CS, Johnson RT. A postprophase topoisomerase II-dependent chromatid core separation step in the formation of metaphase chromosomes. *J Cell Biol* 1995;131:7–17.
20. Losada A, Hirano M, Hirano T. Cohesin release is required for sister chromatid resolution, but not for condensin-mediated compaction, at the onset of mitosis. *Genes Dev* 2002;16:3004–3016.
21. Sakaguchi A, Kikuchi A. Functional compatibility between isoform alpha and beta of type II DNA topoisomerase. *J Cell Sci* 2004;117(Pt 7):1047–1054.
22. Skladanowski A, Come MG, Sabisz M, Escargueil AE, Larsen AK. Specific downregulation of the mitotic regulator DNA topoisomerase II alpha in vincristine-resistant cells leads to prolonged G2 transit and increased survival to microtubule-interacting agents. *Mol Pharmacol* 2005;68:625–634.
23. Skladanowski A, Konopa J. Adriamycin and daunomycin induce programmed cell death (apoptosis) in tumour cells. *Biochem Pharmacol* 1993;46: 375–382.

24. Morris SM, Domon OE, McGarrity LJ, Chen JJ, Casciano DA. Programmed cell death and mutation induction in AHH-1 human lymphoblastoid cells exposed to m-amsa. *Mutat Res* 1995;329:79–96.
25. Negri C, Bernardi R, Donzelli M, Scovassi AI. Induction of apoptotic cell death by DNA topoisomerase II inhibitors. *Biochimie* 1995;77:893–899.
26. Norbury CJ, Zhivotovsky B. DNA damage-induced apoptosis. *Oncogene* 2004;12;23:2797–2808.
27. Demarcq C, Bunch RT, Creswell D, Eastman A. The role of cell cycle progression in cisplatin-induced apoptosis in Chinese hamster ovary cells. *Cell Growth Differ* 1994;5:983–993.
28. Castedo M, Perfettini JL, Roumier T, Valent A, Raslova H, Yakushijin K, Horne D, Feunteun J, Lenoir G, Medema R, Vainchenker W, Kroemer G. Mitotic catastrophe constitutes a special case of apoptosis whose suppression entails aneuploidy. *Oncogene* 2004;23:4362–4370.
29. Eguchi Y, Shimizu S, Tsujimoto Y. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. *Cancer Res* 1997;57:1835–1840.
30. Dwarakanath BS, Khaitan D, Ravindranath T. 2-deoxy-d-glucose enhances the cytotoxicity of topoisomerase inhibitors in human tumor cell lines. *Cancer Biol Ther* 2004;3:864–870.
31. Nicotera P, Melino G. Regulation of the apoptosis-necrosis switch. *Oncogene* 2004;23:2757–2765.
32. Etinger AL, Thompson CB. Death by design: apoptosis, necrosis and autophagy. *Curr Opin Cell Biol* 2004;16:663–669.
33. Ha HC, Snyder SH. Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. *Proc Natl Acad Sci USA* 1999;96:13978–13982.
34. Whitacre CM, Hashimoto H, Tsai ML, Chatterjee S, Berger SJ, Berger NA. Involvement of NAD-poly(ADP-ribose) metabolism in p53 regulation and its consequences. *Cancer Res* 1995;55:3697–3701.
35. Bowman KJ, Newell DR, Calvert AH, Curtin NJ. Differential effects of the poly (ADP-ribose) polymerase (PARP) inhibitor NU1025 on topoisomerase I and II inhibitor cytotoxicity in L1210 cells *in vitro*. *Br J Cancer* 2001;84:106–112.
36. Roninson IB, Broude EV, Chang B-D. If not apoptosis, than what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist Updat* 2002;4:303–313.
37. Andreassen PR, Lacroix FB, Lohez OD, Margolis RL. Neither p21WAF1 nor 14-3-3sigma prevents G2 progression to mitotic catastrophe in human colon carcinoma cells after DNA damage, but p21WAF1 induces stable G1 arrest in resulting tetraploid cells. *Cancer Res* 2001;61:7660–7668.
38. Mansilla S, Priebe W, Portugal J. Mitotic catastrophe results in cell death by caspase-dependent and caspase-independent mechanisms. *Cell Cycle* 2006;5:53–60.
39. Castedo M, Perfettini JL, Roumier T, Yakushijin K, Horne D, Medema R, Kroemer G. The cell cycle checkpoint kinase Chk2 is a negative regulator of mitotic catastrophe. *Oncogene* 2004;23:4353–4361.
40. Roberts JR, Allison DC, Donehower RC, Rowinsky EK. Development of polyploidization in taxol-resistant human leukemia cells *in vitro*. *Cancer Res* 1990;50:710–716.
41. Erenpreisa JA, Cragg MS, Fringes B, Sharakhov I, Illidge TM. Release of mitotic descendants by giant cells from irradiated Burkitt's lymphoma cell line. *Cell Biol Int* 2000;24:635–648.
42. Sundaram M, Guernsey DL, Rajaraman MM, Rajaraman R. Neosis: a novel type of cell division in cancer. *Cancer Biol Ther* 2004;3:207–218.
43. Lock RB, Stribinskiene L. Dual modes of death induced by etoposide in human epithelial tumor cells allow Bcl-2 to inhibit apoptosis without affecting clonogenic survival. *Cancer Res* 1996;56:4006–4012.
44. Lock RB, Galperina OV, Feldhoff RC, Rhodes LJ. Concentration-dependent differences in the mechanism by which caffeine potentiates etoposide cytotoxicity in HeLa cells. *Cancer Res* 1994;54:4933–4939.
45. Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B. 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* 1999;401:616–620.
46. Hyzy M, Bozko P, Konopa J, Skladanowski A. Antitumour imidazoacridone C-1311 induces cell death by mitotic catastrophe in human colon carcinoma cells. *Biochem Pharmacol* 2005;69:801–809.
47. Gorbisky GJ. Cell cycle progression and chromosome segregation in mammalian cells cultured in the presence of the topoisomerase II inhibitors ICRF-187 [(+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane; ADR-529] and ICRF-159 (Razoxane). *Cancer Res* 1994;54:1042–1048.
48. Bernhard EJ, Muschel RJ, Bakanauskas VJ, McKenna WG. Reducing the radiation-induced G2 delay causes HeLa cells to undergo apoptosis instead of mitotic death. *Int J Radiat Biol* 1996;69:575–584.
49. Ianzini F, Mackey MA. Delayed DNA damage associated with mitotic catastrophe following X-irradiation of HeLa S3 cells. *Mutagenesis* 1998;13:337–344.

50. Chang BD, Broude EV, Dokmanovic M, Zhu H, Ruth A, Xuan Y, Kandel ES, Lausch E, Christov K, Roninson IB. A senescence-like phenotype distinguishes tumor cells that undergo terminal proliferation arrest after exposure to anticancer agents. *Cancer Res* 1999;59:3761–3767.
51. Chen JG, Horwitz SB. Differential mitotic responses to microtubule-stabilizing and -destabilizing drugs. *Cancer Res* 2002;62:1935–1938.
52. Burns TF, Fei P, Scata KA, Dicker DT, El-Deiry WS. Silencing of the novel p53 target gene Snk/Plk2 leads to mitotic catastrophe in paclitaxel (taxol)-exposed cells. *Mol Cell Biol* 2003;23:5556–5571.
53. Bayart E, Grigorieva O, Leibovitch S, Onclercq-Delic R, Amor-Gueret M. A major role for mitotic CDC2 kinase inactivation in the establishment of the mitotic DNA damage checkpoint. *Cancer Res* 2004;64:8954–8959.
54. Nitta M, Kobayashi O, Honda S, Hirota T, Kuninaka S, Marumoto T, Ushio Y, Saya H. Spindle checkpoint function is required for mitotic catastrophe induced by DNA-damaging agents. *Oncogene* 2004;23:6548–6558.
55. Huang X, Tran T, Zhang L, Hatcher R, Zhang P. DNA damage-induced mitotic catastrophe is mediated by the Chk1-dependent mitotic exit DNA damage checkpoint. *Proc Natl Acad Sci USA* 2005;102:1065–1070.
56. Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: a molecular definition. *Oncogene* 2004;23:2825–2837.
57. Erenpreisa J, Cragg MS. Mitotic death: a mechanism of survival? A review. *Cancer Cell Int* 2001;1:1.
58. Xia Z, Morales JC, Dunphy WG, Carpenter PB. Negative cell cycle regulation and DNA damage-inducible phosphorylation of the BRCT protein 53BP1. *J Biol Chem* 2001;276:2708–2718.
59. Niida H, Tsuge S, Katsuno Y, Konishi A, Takeda N, Nakanishi M. Depletion of Chk1 leads to premature activation of Cdc2-cyclin B and mitotic catastrophe. *J Biol Chem* 2005;280:39246–39252.
60. Shay JW, Roninson IB. Hallmarks of senescence in carcinogenesis and cancer therapy. *Oncogene* 2004;23:2919–2933.
61. Mansilla S, Pina B, Portugal J. Daunorubicin-induced variations in gene transcription: commitment to proliferation arrest, senescence and apoptosis. *Biochem J* 2003;372:703–711.
62. te Poele RH, Okorokov AL, Jardine L, Cummings J, Joel SP. DNA damage is able to induce senescence in tumor cells *in vitro* and *in vivo*. *Cancer Res* 2002;62:1876–1883.
63. Schmitt CA, Fridman JS, Yang M, Lee S, Baranov E, Hoffman RM, Lowe SW. A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. *Cell* 2002;109:335–346.
64. Rebbaa A, Zheng X, Chou PM, Mirkin BL. Caspase inhibition switches doxorubicin-induced apoptosis to senescence. *Oncogene* 2003;22:2805–2811.
65. Robles SJ, Buehler PW, Negrusz A, Adami GR. Permanent cell cycle arrest in asynchronously proliferating normal human fibroblasts treated with doxorubicin or etoposide but not camptothecin. *Biochem Pharmacol* 1999;58:675–685.
66. Sedelnikova OA, Horikawa I, Zimonjic DB, Popescu NC, Bonner WM, Barrett JC. Senescing human cells and ageing mice accumulate DNA lesions with unreparable double-strand breaks. *Nat Cell Biol* 2004;6:168–170.
67. Zglinicki T, Saretzki G, Ladhoff J, Fagagna F, Jackson SP. Human cell senescence as a DNA damage response. *Mech Ageing Dev* 2005;126:111–117.
68. Suzuki T, Michishita E, Ogino H, Fujii M, Ayusawa D. Synergistic induction of the senescence-associated genes by 5-bromodeoxyuridine and AT-binding ligands in HeLa cells. *Exp Cell Res* 2002;276:174–184.
69. Schulz L, Tyler J. Heterochromatin focuses on senescence. *Mol Cell* 2005;17:168–170.
70. Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol* 1999;57:727–741.
71. Toussaint O, Royer V, Salmon M, Remacle J. Stress-induced premature senescence and tissue ageing. *Biochem Pharmacol* 2002;64:1007–1009.
72. Li TK, Chen AY, Yu C, Mao Y, Wang H, Liu LF. Activation of topoisomerase II-mediated excision of chromosomal DNA loops during oxidative stress. *Genes Dev* 1999;13:1553–1560.
73. Michishita E, Nakabayashi K, Ogino H, Suzuki T, Fuji M, Ayusawa D. DNA topoisomerase inhibitors induce reversible senescence in normal human fibroblasts. *Biochem Biophys Res Commun* 1998;253:667–671.

74. Elmore LW, Di X, Dumur C, Holt SE, Gewirtz DA. Evasion of a single-step, chemotherapy-induced senescence in breast cancer cells: implications for treatment response. *Clin Cancer Res* 2005;11:2637–2643.
75. Roberson RS, Kussick SJ, Vallieres E, Chen SY, Wu DY. Escape from therapy-induced accelerated cellular senescence in p53-null lung cancer cells and in human lung cancers. *Cancer Res* 2005;65:2795–803.
76. Baehrecke EH. Autophagy: dual roles in life and death? *Nat Rev Mol Cell Biol* 2005; 6:505–510.
77. Eskelinen EL. Doctor Jekyll and Mister Hyde: autophagy can promote both cell survival and cell death. *Cell Death Differ* 2005;12(Suppl 2):1468–1472.
78. Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB, Tsujimoto Y. Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. *Nat Cell Biol* 2004;6:1221–1228.
79. Bilir A, Altinoz MA, Erkan M, Ozmen V, Aydinler A. Autophagy and nuclear changes in FM3A breast tumor cells after epirubicin, medroxyprogesterone and tamoxifen treatment *in vitro*. *Pathobiology* 2001;69:120–126.
80. Pyo JO, Jang MH, Kwon YK, Lee HJ, Jun JI, Woo HN, Cho DH, Choi B, Lee H, Kim JH, Mizushima N, Oshumi Y, Jung YK. Essential roles of Atg5 and FADD in autophagic cell death: dissection of autophagic cell death into vacuole formation and cell death. *J Biol Chem* 2005;280:20722–20729.
81. Liang XH, Kleeman LK, Jiang HH, Gordon G, Goldman JE, Berry G, Herman B, Levine B. Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. *J Virol* 1998;72:8586–8596.
82. Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D, Yahalom J. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* 2001;61:439–444.

22

The Significance of Poly-Targeting in Apoptosis Induction by Alkylating Agents and Platinum Drugs

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SUMMARY

Apoptosis is believed to be an important aspect of the anticancer potency of alkylating agents (AAs) and platinum (Pt) complexes. Despite the high clinical utility of these classes of drugs, the nature and determinants of the apoptotic sensitivity/resistance of cancer cells to these agents are not completely understood. One underappreciated aspect is the wide and variable spectrum of cellular targets of AAs and Pt drugs and the complexity of the responses to poly-targeted insults. This chapter discusses the heterogeneity of targeting profiles for diverse drug types and the interdependence of apoptotic routes elicited by the damage to various cellular targets. Although many of these agents target DNA, DNA damage is not the only cause of their apoptotic effects. Drugs that alkylate proteins are strongly apoptotic, even if they do not react with DNA. The ability of alkylating and Pt drugs to damage and inactivate specific proteins and to globally distort the state of the proteome needs to be considered as a self-standing apoptotic stimulus and a factor that enhances lethal responses to DNA damage. Particular emphasis is placed on the significance of drug effects on redox-regulating proteins of the thioredoxin family. Disruption of protein redox homeostasis is likely to be critical for death/survival in response to poly-targeted alkylating and Pt drugs. Differential distortion of redox regulation is suggested as a molecular basis underlying the demonstrated potential of specific drugs such as irifolven and oxaliplatin to promote apoptosis in cancer cells while sparing normal cells.

Key Words: Alkylating drugs; platinum drugs; DNA adducts; protein alkylation; protein targeting; apoptosis; redox regulation.

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1. INTRODUCTION

Alkylating agents (AAs) and platinum compounds (Pt drugs) are among the most useful clinical anticancer drugs (Fig. 1, Table 1). These categories include such widely used drugs as nitrogen mustards, nitrosoureas, mitomycins, cisplatin and the newer Pt analogs. All the main clinical AA/Pt drugs share the ability to damage cellular DNA. DNA lesions are widely accepted as the critical factor in the ability of AA/Pt drugs to kill cancer cells, including specifically the proapoptotic potential (1,2).

Apoptosis is thought to represent a pharmacologically relevant outcome for a majority, if not all, of the anticancer AA/Pt drugs (3–6). Yet, even a cursory review of the literature reveals a multitude of disparate, sometimes seemingly incongruent observations on apoptotic potency of these drugs in cancer cells. The magnitude of apoptotic responses to AA/Pt agents and their timing strongly depend on a specific drug, cell type, culture conditions and cell position in the cell cycle (7). Although a

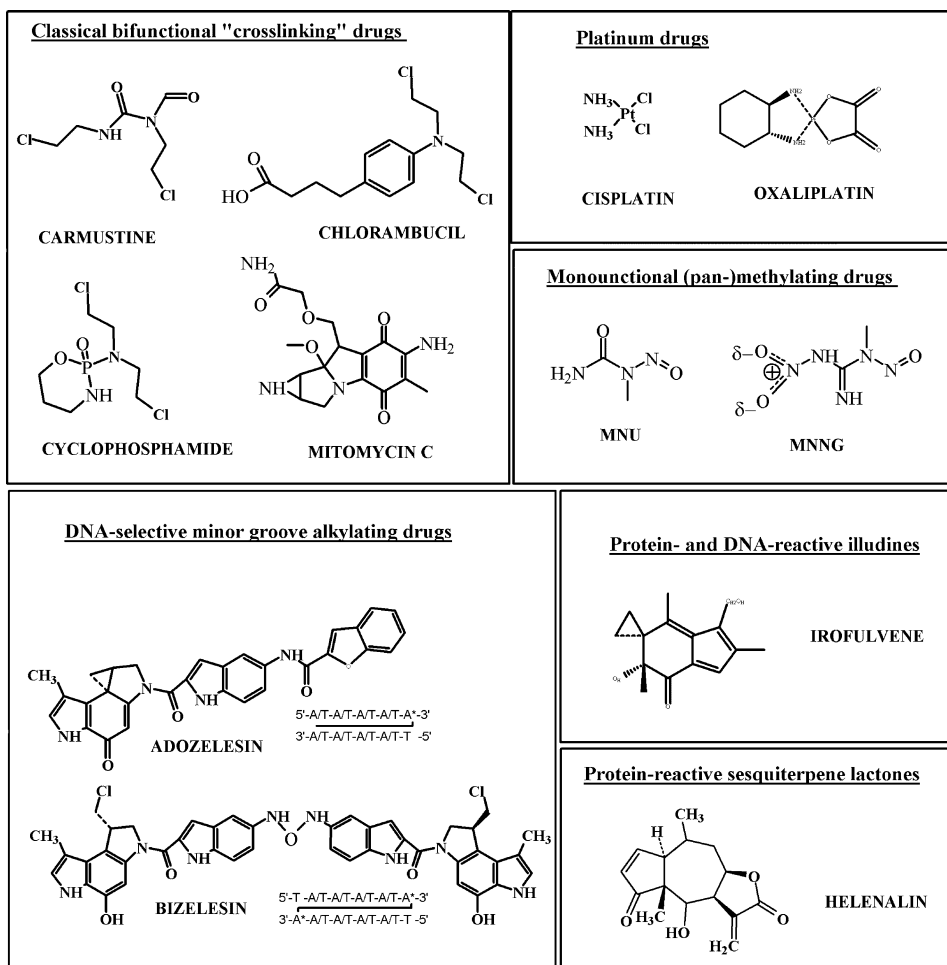


Fig. 1. The structures of compounds representing different classes of alkylating agents and platinum drugs.

Table 1
Alkylating Agents and Platinum Drugs and Their Targeting Preferences

<i>Type/clinical status</i>	<i>Specific Targeting Attributes^d</i>	<i>Examples</i>	<i>References</i>
Bifunctional alkylating agents <ul style="list-style-type: none"> • Broad targeting: Proteins +SMINs > > DNA +RNA • DNA crosslinks • Used in clinic 	DNA + Monoadducts > > inter-strand crosslinks \cong DNA-protein crosslinks Preference for N ⁷ G (up to 90% of DNA adducts), although O ⁶ G adducts thought important for some drugs Marginal sequence preference for G clusters Genome distribution: nearly random RNA + Proteins + + + + + Specificity: some binding preferences possible Small nucleophiles + + + + +	Nitrogen mustards: melphalan and chlorambucil Quinone antibiotics: mitomycin C Nitrosoureas: carmustine and CCNU Cyclophosphamides: cyclophosphamide and iphosphamide	17–22
Anticancer platinum drugs <ul style="list-style-type: none"> • Broad targeting: proteins +SMINs > > DNA +RNA • DNA crosslinks 	DNA + Intrastrand crosslinks + monoadducts > > inter-strand crosslinks \cong DNA-protein crosslinks. Marginal sequence preference for G clusters Genome distribution: nearly random	Cisplatin, oxaliplatin and carboplatin	9, 18, 23–33

(Continued)

Table 1
(Continued)

<i>Type/clinical status</i>	<i>Specific Targeting Attributes^a</i>	<i>Examples</i>	<i>References</i>
<ul style="list-style-type: none"> Used in clinic 	<p>RNA + Proteins +++++ Specificity: some binding preferences possible (thioredoxin reductase) Small nucleophiles^b +++++</p>		
<p>Monofunctional alkylating (methylating) agents</p> <ul style="list-style-type: none"> Broad targeting: proteins +SMNs > > DNA +RNA Carcinogenic Some clinical utility 	<p>DNA +</p> <p>Monoadducts at various sites, activity attributed to the formation of O⁶G adducts Sequence specificity: none Genome distribution: random</p> <p>RNA + Proteins +++++ Specificity: unlikely Small nucleophiles^a +++++</p> <p>DNA +++++</p>	<p>Methylnitrosourea, methylnitrosoguanidine, methylmethanesulfonate, temozolomide and dacarbazine</p>	<p>4,34–36</p>
<p>Minor groove alkylating drugs</p> <ul style="list-style-type: none"> DNA selective 	<p>Selective reactivity: only with double-stranded DNA (at N³A)</p>	<p>CPIs: bizelesin, adozelesin and duocarmycins Non-covalent minor groove binders with alkylating moieties: tallimustine</p>	<p>37–45</p>
<ul style="list-style-type: none"> Highly cytotoxic 	<p>Sequence-specific monoadducts (adozelesin, duocarmycin and tallimustine) or interstrand crosslinks (bizelesin). Strong non-covalent duplex stabilizing interaction</p>		

<ul style="list-style-type: none"> ● In clinical development 	<p>High sequence specificity for discrete AT-rich motifs (which depend on individual drugs)</p> <p>Genome distribution: some CPI drugs target defined genomic regions (AT islands)</p> <p>RNA –</p> <p>Proteins –</p> <p>Small nucleophiles –</p> <p>DNA + (monoadducts)</p> <p>RNA +</p> <p>Proteins ++++</p>	Irofulven and illudin S	46–52
<p>Illudines</p> <ul style="list-style-type: none"> ● Protein subset >>> ● DNA + RNA ● Promising anticancer properties (irofulven) ● In clinical development 	<p>Specificity: some selectivity (thioredoxin)</p> <p>Small nucleophiles +</p> <p>DNA – (helenalin)</p> <p>RNA –</p> <p>Proteins +++ (some selectivity, p65 subunit of NF-κB, glutaredoxin and thioredoxin)</p> <p>Small nucleophiles +++</p>		
<p>Sesquiterpene lactones</p> <ul style="list-style-type: none"> ● Protein subset/SMNs ● Numerous compounds from medicinal plants with anticancer activity ● Preclinical development 		Helenalin and parthenolide	53–62

CPI, cyclopropylindoline; SMN, small molecule nucleophile.

^a Symbols ('+' and '–') denote relative drug affinity to individual types of targets.

^b SMNs, such as cysteine and glutathione.

range of pathways and specific biomolecules have been implicated in pharmacological apoptosis (8), no universal markers have emerged thus far that would consistently predict apoptosis by AA/Pt drugs. Sensitivity or resistance to some drugs in specific cellular models can be attributed to differences in the formation and repair of DNA damage, but predictive generalizations are hard to make, as exceptions and discrepancies are common. Importantly, certain types of AA drugs are proapoptotic, even though they do not react with DNA at all. These seemingly inconsistent properties lead to several questions. Is DNA damage invariably the sole or decisive factor in apoptosis by AA/Pt drugs? If DNA damage plays a non-exclusive role, what other mechanisms may participate in cell death induced by AA/Pt drugs? Is the classic DNA damage-initiated and caspase-mediated apoptosis the only and/or always essential route for AA/Pt drugs? Finally, the ultimate goal of all cancer strategies is to eradicate cancer cells without causing excessive harm to normal cells. Can then normal cells be spared from apoptosis by AA/Pt drugs? What factors may predict such differential responses?

Complete answers to these pressing questions may not be available at present, despite the vast literature on apoptotic responses to AA/Pt drugs. Generalizations are particularly difficult, given the variability of responses. Therefore, it would be a daunting task to comprehensively review all aspects of apoptosis induction by AA/Pt drugs. Several key areas, such as the roles of DNA damage, repair mechanisms and DNA damage-related apoptotic signaling, are already the topics of recent in-depth review articles (9–13). It seems worthwhile, however, to critically evaluate those aspects of lethal effects of AA/Pt drugs that are potentially important yet have received less attention and/or are a potential source of confusion or misinterpretations. This chapter attempts to streamline some of such understudied issues by focusing on the targeting diversity of various types of AA/Pt drugs and, in particular, underscoring non-DNA targets as factors contributing to apoptosis.

2. TARGETING PROFILES OF AA AND PT DRUGS

Cellular targets obviously represent the most fundamental level in the action of any drug and are the origin of all the downstream responses, including apoptosis. Still, the recognition of the primary cellular targets of AA/Pt drugs and, consequently, the interpretations of downstream signaling induced by these compounds are often subject to oversimplifications. AA/Pt anticancer drugs differ profoundly in their structures, chemistry and biological properties (Fig. 1). Whereas DNA is undoubtedly an important target for many AA/Pt drugs, referring to such drugs as “DNA-reactive agents” may be misleading without additional qualifiers. All AA/Pt drugs share electrophilic nature that makes them inherently reactive toward a spectrum of cellular nucleophiles rather than toward a single type of biomolecules. Irrespective of how important DNA reactivity might be, it reflects only one side of the multifaceted and complex molecular pharmacology of these compounds. Electrophilic drugs tend to react much more readily with sulfhydryl groups than with nucleophilic groups in DNA [the general order of reactivity with nucleophiles is thiols \gg amino groups $>$ phosphates $>$ hydroxyls (14)]. Therefore, typically 80–90% of the total macromolecular adducts of conventional AA/Pt compounds in the cell are formed with proteins (some remarkable exceptions are discussed in Section 2.3.). In addition to protein targeting, most AA/Pt drugs show substantial reactivity with small molecule nucleophiles (SMNs) such as

glutathione (GSH). Consistent with multiple targets, these drugs elicit profoundly complex networks of cellular responses (15).

With researchers' attention focused on DNA damage, these "other" reactivities are too often ignored in the interpretation of cell responses to AA/Pt drugs or dismissed by labeling these agents as "non-specific DNA alkylators." However, the viewpoint that an ideal antitumor drug would have only one target is at variance with the fact that AA/Pt drugs are among the most efficacious and beneficial mainstream drugs in the clinic, whereas various narrowly targeted strategies are plagued by the insufficient ability to eradicate cancer cells (16). An alternative perspective is to properly recognize AA/Pt drugs as poly-targeted agents and make every effort to better understand their complex molecular pharmacology. Examples of AA/Pt drugs of diverse targeting profiles are listed in Table 1 and discussed in the subsequent sections.

2.1. Multiple Cellular Targets of Conventional Bifunctional AA and Pt Drugs

2.1.1. DNA BINDING—ASSORTED LESIONS OF LOW-SEQUENCE SPECIFICITY

Clinically relevant conventional bifunctional AA/Pt agents share several key mechanistic characteristics despite differences in their chemistry. Both bifunctional AA and Pt drugs form irreversible (covalent) adducts with nuclear DNA, reacting mainly with N7 position in guanines (17,18). The biological activity of bifunctional AA drugs is thought to depend mainly on the formation of bi-functional adducts, specifically interstrand crosslinks. Interstrand crosslinks are also formed by Pt drugs, although intrastrand crosslinks represent the prevailing type of Pt-derived bifunctional lesion.

Interstrand crosslinks are regarded as one of the most lethal types of DNA damage. These lesions cannot be readily by-passed by replication/transcription machinery, and their removal requires complex repair mechanisms (10). Hence, drugs such as nitrogen mustards, mitomycins, nitrosoureas and Pt compounds are often referred to as "DNA-crosslinking agents." However, these drugs also induce a spectrum of non-crosslinked DNA lesions. For example, interstrand crosslinks correspond to only 1–10% of all DNA adducts formed by bifunctional AA drugs and 5–20% of Pt–DNA adducts (17,18,29). Another type of bifunctional lesion formed by both AA and Pt drugs, DNA–protein crosslinks, comprise a similar fraction of total adducts. Monoadducts, which constitute a majority of DNA lesions formed by these nominally "crosslinking" agents, are, in principle, less lethal and easier to repair (18,64). Thus, monoalkylating compounds are expected to be inferior to their bifunctional, crosslink-capable counterparts. Still, there are exceptions to this rule that cannot be readily explained in terms of DNA targeting. For instance, the crosslinking mitomycin C is not more cytotoxic than its non-crosslinking (monoalkylating) derivative (65). Low lethality of monoadducts makes these lesions highly mutagenic. Accordingly, monoadducts in normal tissues, formed in large numbers also by bifunctional AA/Pt drugs, are linked to secondary, treatment-induced tumors (17,66,67).

Conventional AA/Pt drugs lack the ability to target specific genes or defined functional regions. Reflecting the preference for G clusters, GC-rich regions in cellular DNA are somewhat more affected by drugs such as cisplatin or oxaliplatin than AT-rich regions (30). However, G clusters or crosslinking motifs GC and CG are ubiquitous

(39). Therefore, at the genomic level, DNA adducts by the conventional AA/Pt drugs are nearly randomly distributed (30,39,68).

2.1.2. RNA—AN IGNORED UNKNOWN

Little is known about RNA adducts formed by “DNA-reactive” AA/Pt drugs. Levels of RNA adducts are comparable (23,69) or even several times higher than the levels of DNA adducts (70). RNA alkylation, for example, by nitrogen mustard, interferes with protein synthesis on the adducted RNA template (71) and may elicit RNA repair processes (36). Although the significance of drug–RNA adducts remains virtually unknown, it is reasonable to expect that even a partial and “non-specific” interference with the functions of various classes of RNA would contribute to cell responses, synergizing with the consequences of DNA damage. For example, drug effects on RNA functions might affect transcriptional responses. Yet, changes in transcription of specific genes in cells exposed to AA/Pt drugs are routinely interpreted solely in terms of responses to DNA damage.

2.1.3. PROTEINS AND SMNs—LIKELY CONTRIBUTING TARGETS

The ability of bifunctional AA drugs and Pt agents to react with proteins and with SMNs is well known (14,17,18,23). Although AA agents bind to sulfhydryls in cysteine residues, Pt drugs are also reactive with methionine residues (25–28,72,73). Drugs of the cyclophosphamide group can form adducts with proteins and SMNs through their principal active form (phosphoramidate mustard) and also through acrolein that is released as a by-product of drug activation (22,74).

Bifunctional AA/Pt drugs react extensively with proteins (75–78). For instance, as much as 90% of macromolecular adducts of Pt drugs are formed with cellular proteins (23), while the nitrogen mustard drug chlorambucil binds profoundly to proteins in a variety of cellular structures (79). Unfortunately, systematic investigations of protein targeting in the mechanistic context are rather uncommon. This bias is illustrated by the stark contrast between approximately a dozen of reports devoted to protein reactivity of Pt drugs and many thousands of publications on DNA damage and DNA adducts formed by these agents. Precisely, the opposite bias is evident in the reactivity of AA/Pt drug—in the presence of both DNA and thiol compounds, cisplatin reacts almost exclusively with biological thiols, whereas the formation of Pt–DNA adducts is marginal (24). Moreover, other studies have ruled out the possibility that Pt–protein adducts might form transiently, serving as a “reservoir” of drug for DNA binding (25,80). Various drugs, including cisplatin, may have an even greater affinity to SMNs than to proteins (25,80). Other drugs may favor proteins. For example, the hydrophobic cyclohexane moiety of oxaliplatin may facilitate oxaliplatin docking in hydrophobic pockets of targeted proteins (29,32). Protein targeting by oxaliplatin is further enhanced, relative to cisplatin, by a greater reactivity of oxaliplatin with sulfhydryls and methionine residues (72,81).

Various bifunctional AA and Pt drugs probably react with a multitude of proteins. Solid evidence exists that at least some of the affected proteins are highly relevant cancer targets. Notable examples include the inhibition of the activity of important redox-regulating proteins: thioredoxin reductase (TrxR), GSH reductase, glutaredoxin (Grx) and, to lesser degree, Trx by bifunctional AAs (such as carmustine) and Pt drugs (82–87).

2.2. *Omni-Reactive and Non-Specific Monofunctional Alkylating Drugs*

Methylating agents represent an extreme case of non-specific targeting by AA drugs. Lethal effects of compounds such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and methyl methanesulfonate are often attributed to the massive formation of O⁶ guanine adducts, although N-methylations by such drugs tend to outnumber O-methylations (4,34–36,88). Although both types of methylations are proapoptotic, these unremarkable DNA lesions are relatively readily repaired, profoundly less lethal and more mutagenic than bifunctional DNA lesions. Also, methylating compounds broadly affect proteins and SMNs (89). It is puzzling that such non-specific agents might still be able to differentiate cancer from normal cells, as implied by their clinical utility.

2.3. *Selective DNA Targeting by Sequence-Specific Minor Groove Binding DNA Alkylators*

The only known class of AA drugs that are truly and inherently selective for DNA comprises some agents that combine the alkylation of DNA bases with a tight fit in the minor groove allowing for strong non-covalent interaction. These unique agents include cyclopropylindolines (CPI), such as bizelesin and adozelesin, and their subgroup of cyclopropylbenzoindolines (CBI) (Fig. 1, Table 1) (37,40,41,90). DNA alkylation by CPI/CBI drugs (at N3 position of adenine residues) is preceded by the induced fit-activation that absolutely requires double-stranded DNA (38). Because of the need for this unique mode of in situ activation, CPI/CBI drugs do not react with single-stranded nucleic acids (DNA or RNA), proteins and SMNs.

CPI/CBI drugs are capable of remarkably high sequence specificity in their interactions with DNA. For example, bizelesin binds to DNA occupying 6 bp sites with a very high preference for T(A/T)₄A (Fig. 1). CPI drugs with a single reactive center (e.g., adozelesin) form monoadducts with DNA, whereas those with two centers (bizelesin) form interstrand crosslinks (40,42). However, the combination of covalent monoadducts and non-covalent groove interaction (e.g., by adozelesin) affects DNA duplex in a way that is functionally equivalent to the effects of a covalent inter-strand crosslink. Thus, adozelesin–DNA monoadducts can be regarded as covalent/non-covalent crosslinks.

The CPI–DNA adducts fitting tightly in the minor groove induce relatively little distortion to DNA structure, unlike bulky and distortive N⁷-G adducts of classical AA/Pt drugs. Consequently, DNA adducts formed by CPI compounds (crosslinks and monoadducts alike) are inefficiently recognized by repair mechanisms (91). Finally, the remarkable sequence specificity of some of the CPI drugs translates into their ability to preferentially target regions of repetitive AT-rich DNA in the genome (termed AT islands) (40,41,92,93). CPI drugs are among the most potent anticancer compounds ever discovered inhibiting cell growth in the low picomolar concentrations. Yet, puzzlingly, these compounds are poorly apoptotic (94,95).

Like CPI/CBI drugs, various other sequence-specific alkylating drugs combine an alkylating “warhead,” such as a nitrogen mustard, with a backbone of non-covalent minor groove binders (distamycin, netropsin, bisbenzimidazole or poly-amides capable of “reading” DNA sequence) [for review, see refs 96–98]. Tallimustine, a benzoyl nitrogen mustard derivative of distamycin A, is one such compound that, unlike conventional untethered poly-targeted nitrogen mustards, seems to react only with DNA (68,99).

Collectively, alkylating drugs capable of composite covalent/non-covalent interaction in the DNA minor groove offer the potential for sequence-specific and region-specific DNA damage combined with non-reactivity toward nucleophiles other than DNA. Therefore, minor groove binding AA drugs are qualitatively different from conventional AA and Pt drugs.

2.4. Nucleic Acids and Protein Targeting by Novel “Dual-Action” Alkylating Drugs

Another category of novel alkylating drugs comprises sesquiterpenoids of the illudins family (100–102). Irofulven (hydroxymethylacylfulvene) is a semi-synthetic illudin with demonstrated clinical efficacy (103–106) and broad activity against solid tumors. Like the conventional AAs, irofulven reacts with nucleic acids, forming under pharmacologically relevant conditions several million DNA adducts/cell, which probably reflects monoalkylation events (47,49,50). However, the levels of irofulven adducts to proteins exceed 4-fold to 10-fold the level of DNA adducts (47,107).

Irofulven binds to a range of cellular proteins but in a distinctly non-random fashion (107). The key redox-regulating protein, cytoplasmic Trx1, was identified as the most adducted protein in cytoplasmic extracts from cells exposed to [¹⁴C]irofulven (107). Irofulven binding to the purified Trx1 and its partner TrxR1 have also been demonstrated (107). In reaction with Trx, the drug alkylates only one of five cysteine residues (107). In addition, irofulven has decisively greater affinity for protein thiols, such as those in Trx1, over SMNs (107), unlike other electrophilic agents that tend to react more readily with SMNs. Targeting and inactivation of Trx and other redox-regulating proteins seem to play major roles in the potent apoptotic properties of irofulven and other dual-action drugs (as discussed in Sections 5. and 6.).

2.5. Protein Targeting by DNA Unreactive (Single Action) Alkylating Drugs

A distinct targeting pattern is exhibited by sesquiterpene lactones (SLs), natural AAs that have been repeatedly identified as biologically active components of various medicinal plants (108,109). SLs with promising antitumor activity, such as helenalin and parthenolide, resemble illudins in their reactivity toward thiols and in their potent proapoptotic properties. However, these SLs fundamentally differ not only from the illudins but also from all the conventional clinical AAs, in that helenalin and parthenolide do not react with DNA.

Although SLs react with many cellular proteins, studies on parthenolide and helenalin have centered in recent years on their ability to inhibit nuclear factor (NF)- κ B, an important transcriptional regulator implicated in survival pathways and inflammatory processes. These SLs, however, are not specific NF- κ B inhibitors (60,110). For example, helenalin inhibits the activities of multiple enzymes in each of the following categories: energy metabolism, enzymes of DNA synthesis and redox-regulating proteins (Trx, Grx, glutathione-S-transferase and several mixed function oxidases) (56,57,111). Although SLs rapidly react also with SMNs (notably GSH) (57,58), this reaction is reversible so that a fraction of drug molecules is always available for reaction with protein targets (58).

3. GENERAL ATTRIBUTES OF CELL DEATH INDUCED BY AA AND PT DRUGS

Apoptosis reflects not a single feature that can be measured and quantified in a universal way but rather a multifaceted phenomenon that is defined based on a variety of biochemical markers and morphological changes characterized by a specific spatio-temporal order (6,112–114). It is thus not easy to identify a universal set of attributes that would (i) adequately and uniformly define cell death in response to AA/Pt drugs and (ii) would allow for prediction of such responses. Poorly understood poly-targeting diversity of specific AA/Pt drugs blurs our understanding of these apoptotic responses. The interpretations are further complicated by the profound dependence of responses to AA/Pt drugs on treatment conditions and intricate temporal and causative relationships among various molecular markers of the apoptotic process. Even in the same cell population, individual apoptotic markers peak at different times and show significant variability in their magnitudes (115,116). Even the underlying mechanistic nature of specific events that serve as apoptotic markers is subject to change over time. Another layer of complexity is added by the diversity of biological models that respond very differently to the same insult. Therefore, attempts to generalize disparate literature observations by cross-comparing data extracted from separate studies are difficult, at best, and are further complicated by fundamental conceptual differences among researchers on what constitutes apoptosis.

A widespread notion equates apoptosis with a caspase-mediated cell death involving either the intrinsic pathway mediated by mitochondrial dysfunction or the extrinsic pathway mediated by death receptors, or the interplay of both routes (117,118). However, inhibition of the caspase pathway frequently fails to protect cells from death, resulting merely in a phenotypic shift. It is well established that some agents, in addition to caspase-mediated pathways, activate parallel caspase-independent death routes (112,119,120). The term “caspase-independent apoptosis” is often used to denote specifically cell death commencing by the leakage from mitochondria of apoptosis-inducing factor (AIF) and endonuclease G, although various caspase-independent mechanisms of cell demise are likely to exist (119).

Both caspase-independent and caspase-mediated death pathways tend to result in early reorganization of phospholipids in the cell membrane (119), which offers a characteristic and widely used marker of early apoptosis. The caspase-mediated route ultimately leads to extensive and progressive cleavage of nuclear chromatin producing a range of fragments (~2–50 kbp, sometimes oligonucleosomal length) (121). By contrast, AIF-mediated apoptosis is associated only with infrequent long-range DNA cleavage (120,122). In both caspase-mediated and AIF-mediated routes, the loss of cell membrane integrity is a late event, which is one of the characteristic attributes of apoptosis as opposed to rapid loss of membrane integrity in necrosis.

For the purpose of this chapter, the term apoptosis is used in a broad sense as an irreversible cascade of cell de-organization initiated “from inside” and resulting in cell demise. This definition emphasizes the following universal aspects of apoptosis: (i) self-accelerating de-organization and degradation of cellular components signifies the irreversible phase when the cell becomes destined to die, (ii) caspase cascade may coexist with (or be supplanted by) other types of degradative processes and (iii) the irreversible degradation commences when affected cells still maintain basic morphological structures and remain metabolically active.

This broad definition fits not only both caspase-dependent and caspase-independent routes but also other morphologically identifiable manifestations of cell death that might escape the classical strict differentiation as either apoptosis or necrosis, such as apoptosis-like necrosis or necrosis-like apoptosis (112,119). It needs to be underscored that for anticancer drugs, diverse manifestations of cell death (i) stem from the common insult, (ii) are often present concurrently, and (iii) cumulatively contribute to the irreversible eradication of cancer cells. The last attribute is by far the most relevant in the pharmacological context.

4. IS THE TARGETING OF DNA ALONE ALWAYS SUFFICIENT FOR APOPTOSIS BY AA?

The subsequent sections address the characteristics of apoptotic responses associated with specific targeting profiles of AA/Pt drugs. Targeting DNA will be compared and contrasted to single-action protein targeting and to dual actions on DNA and proteins.

4.1. The Classical Model of DNA Damage-Induced Apoptosis

In principle, DNA adducts interfering with DNA template properties are sufficient for the inhibition of cell proliferation, albeit not necessarily for cell death (2). In addition to direct effects on DNA function, however, DNA lesions initiate a cascade of responses that involve DNA damage recognition and repair systems, chromatin remodeling, cell cycle checkpoints, transcriptional reprogramming and downstream signaling through routes that are often redundant, highly branched, interdependent and cell type specific (recently reviewed in refs 10,12 and 36). In this web of responses, DNA lesions that remain unrepaired (or incompletely repaired) shift the balance toward pathways that lead to apoptotic cell death (10,12,88).

Our understanding of the role of DNA damage in apoptosis stems largely from the investigations on ionizing radiation (IR). In the IR-based model, unrepaired DNA damage initiates signaling in which a pivotal role is played by tumor suppressor p53. Transcriptional transactivation by p53 promotes death signaling, which often involves the apoptosis signal-regulating kinase 1 (ASK1)/MAPK/c-Jun N-terminal kinase (JNK) pathway (11). Non-transcriptional effects of p53 are thought to mediate the activation of death receptor pathways (123). Importantly, wild-type p53 participates in the repair of DNA damage, which may enhance survival. Although mutations or inactivation of p53 are, in principle, expected to attenuate apoptotic responses to IR-induced DNA damage, this is not always the case (124,125). The complex roles of p53 in sensitivity/resistance to IR (126,127) underscore how elusive are the connections between DNA damage and apoptosis even for IR, which is not only the most extensively investigated DNA damaging agent but is also mechanistically incomparably simpler than multifaceted AA/Pt drugs.

DNA damage by IR as well as by drugs that act as relatively “pure” DNA-damaging agents (such as topoisomerase poison etoposide) results in a shift in the balance of proapoptotic and antiapoptotic members of the Bcl-2 family, mitochondrial dysfunction (but no early release of AIF) and the activation of the initiator caspase-9 and the executioner caspase-3 (61,128–131). Consequently, cell death commences essentially through the caspase-dependent apoptosis (132), even though some AIF release may occur as a secondary effect caused by caspase activation (133).

The IR-based model of apoptosis is, in part, applicable to cell death induced by DNA-damaging AA/Pt drugs, albeit with two important caveats. First, at the clinically relevant IR doses that are sufficient to induce potent “reproductive cell death,” IR is a relatively poor inducer of apoptosis (134–136). In addition, apoptotic effects caused by the clinically relevant IR doses reflect mainly post-mitotic “secondary” apoptosis that is delayed by several days and triggered by mitotic catastrophe, rather than by direct apoptotic signaling from residual DNA damage (134,137,138). By contrast, apoptosis induced by various AA/Pt drugs is characterized by much more extensive magnitude and a markedly faster progression. Moreover, unlike the post-mitotic effects of IR, drug effects may reflect direct, pre-mitotic apoptosis (139). The second caveat stems from the complicated targeting profiles of AA/Pt drugs. Although biological effects of IR are mainly derived from DNA damage, the poly-targeted nature of many AA/Pt drugs makes it difficult to unambiguously isolate (experimentally as well as conceptually) the responses to DNA damage from the contribution of drug effects on other targets.

4.2. Low Apoptotic Potency of Single-Action DNA-Selective Alkylating Drugs

Agents that solely target DNA (such as bizelesin and adozelesin) are poorly apoptotic despite potent inhibition of cell proliferation in the picomolar range of concentrations (68,94). For example, bizelesin-inhibited acute lymphoblastic leukemia CEM cells remain irreversibly arrested in G2 but show apoptosis only at drug concentrations exceeding by two orders of magnitude cell growth inhibitory concentrations (91,140,141). Only at $100 \times \text{IC}_{80}$, apoptotic DNA fragmentation became noticeable, albeit still at a relatively low level (140). The less-specific adozelesin, but not bizelesin, induced modest apoptotic responses (albeit still at high drug concentrations) in colon carcinoma HCT116 cells (142). Interestingly, p21-deficient HCT116 cells responding to either adozelesin or bizelesin showed a markedly weaker G2/M block accompanied by enhanced apoptotic indices compared with wild-type cells (142). As the apoptotic responses in p21-deficient HCT116 cells were delayed (by 48 h), it is possible that they corresponded not to direct drug effects but rather to “generic” secondary apoptosis triggered by the mitotic catastrophe, like post-mitotic apoptosis in response to the clinically relevant doses of IR.

A plausible explanation for the poor apoptosis in response to bizelesin is that the extremely lethal DNA lesions induced by this drug are simply too infrequent (estimated at $< 10/\text{cell}$ at cell growth inhibitory concentrations) (39,94) to elicit significant apoptotic signaling. Somewhat more noticeable apoptosis by adozelesin parallels a markedly higher number of DNA adducts formed by this drug (94). Data for other DNA-selective alkylating minor groove binders lend support to the possibility that apoptosis may be inversely proportional to the lethality of DNA lesions. A modest apoptosis only at supra-lethal concentrations was observed for duocarmycin A, a CBI compound that is reminiscent of adozelesin in terms of cytotoxic potency (143). By contrast, substantial apoptosis was observed at pharmacologically relevant levels of an achiral-*seco* CBI analog that acts at micromolar rather than picomolar concentrations (45) and most likely forms substantially more DNA adducts than equitoxic levels of adozelesin.

Furthermore, tallimustine, another alkylating minor groove binder, forms two orders of magnitude more DNA adducts than bizelesin at equitoxic concentrations (68).

Accordingly, unlike bizelesin, tallimustine does promote some apoptosis at pharmacologically relevant concentrations, but these effects are still modest compared to equitoxic concentrations of conventional AA drugs that form orders of magnitude more adducts than tallimustine (144,145). On the other hand, a tallimustine congener, brostallicin, which is nominally less “specific” because it has additional targets besides DNA, is markedly more apoptotic (M. Brogini, personal communication).

4.2.1. CONCLUSION

The mechanisms of cell death promoted by pharmacologically relevant levels of AA/Pt drugs are probably much more complex than the general model of DNA damage-induced apoptosis that is derived largely from responses to supra-lethal levels of IR. Solely targeting DNA may not result in significant apoptosis, when relatively few DNA lesions of high lethality are produced. A similar idea has been proposed for IR that DNA lesions must exceed certain threshold level for the activation of DNA damage signaling and apoptotic responses (146). This threshold of DNA damage is likely to be modulated by the cellular milieu and drug effects on other cellular targets.

5. PROTEIN DAMAGE IN CELL DEATH—THE PROTEOME AS A COLLECTIVE APOPTOTIC TARGET

Extensive evidence from studies using various protein-reactive agents leaves no doubt that protein damage is sufficient to produce substantial apoptosis in the absence of DNA adducts or any other apoptotic stimulus (141,147–151). As discussed above (Section 2.5.), protein binding of sulfhydryl-reactive AA drugs, such as SLs (helenalin and parthenolide), functionally inactivates the adducted molecules (56,60,108,110,111). It does not mean that the activities of those specific proteins in the cell are, or need to be, completely abrogated. As adducts formed by a majority of protein alkylators are distributed among many proteins, only a proportion of any specific protein type is likely to become directly inactivated. It is essential, however, to realize that all the drug-protein adducts together shift the equilibrium between the reduced sulfhydryl forms of cellular proteins (Prot-SH) and the oxidized disulfide forms (Prot-S-S). Re-equilibration of Prot-SH/Prot-S-S balance across the proteome also affects those protein molecules that are not directly adducted. Both direct inactivation and all of the oxidative changes in the entire proteome collectively affect the redox state, and their consequences are amplified by the redox-sensing systems.

5.1. Redox Distortion—Achilles Heel of Cancer Cells

Understanding the mechanisms of cellular redox regulation may help one to appreciate how important the distortion of this regulation could be for apoptotic responses to AA/Pt drugs in cancer cells and their potential to spare normal cells. One anticipated uniform consequence of protein targeting by AA/Pt drugs (either single action or poly-targeted) is oxidative distortion of redox homeostasis. Aberrant redox status of the cell is one of the critical determinants of cell death (152,153). Normally functioning cells maintain reductive conditions that are manifested by the abundance of reducing equivalents and the proper reduced status of cellular proteins (154,155). In cancer cells, however, adaptive metabolic changes to a high glucose-utilizing phenotype (the

“Warburg effect”—the well-known hallmark of malignant transition) (156) result in a shift toward a more oxidized state (154,157). Vast literature corroborates the abnormal redox environment and increased sensitivity to oxidative stress of human cancers compared with normal tissues (158–160).

Whereas oxidative stress is often associated with reactive oxygen species (ROS) generation, ROS formation is only one of the possible symptoms of abnormal redox status. Oxidative distortion of the sulfhydryl-disulfide balance at the protein level and depletion of NADPH, the primary cellular source of reducing equivalents, may not trigger immediate ROS generation. Not only is abnormal Prot-SH/Prot-SS balance alone proapoptotic, but it can also facilitate and enhance death responses to DNA damage.

5.2. Apoptotic Effects of Single-Action Protein-Reactive AAs

Single-action protein targeting alkylators, such as helenalin and parthenolide, are potent apoptotic stimuli. Pharmacologically relevant levels of these agents are known to trigger massive apoptosis as a direct result of the decrease in the ratio of Prot-SH to Prot-S-S (i.e., the pro-oxidative distortion of the proteome) (148,151,161). As these drugs react also with SMNs, the effects on the proteome are enhanced by the distortion of the redox balance at the level of small molecules. The latter effects reflect the depletion of cellular GSH and the generation of ROS (54,161–164). Apoptotic effects of parthenolide involve the dissipation of mitochondrial membrane potential ($\Delta\Psi_m$) (54). The resulting cell death is associated with the activation of caspase-7, caspase-8 and caspase-9. In parthenolide-treated colorectal cancer cells, caspase-8 activation preceded cleavage of Bid, a proapoptotic member of the Bcl-2 family, Bax translocation to the mitochondria and release of cytochrome c to cytoplasm (165). Apoptotic effects of parthenolide are abrogated by an antioxidant *N*-acetyl cysteine (NAC) and potentiated by a treatment promoting oxidative stress (164). Rapid induction of apoptosis by parthenolide in leukemic cells involves an atypical pattern of responses (53,166). Specifically, although some cells showed activation of caspases followed by a rather rapid loss of plasma membrane integrity, other cells (observed concurrently) underwent a caspase-independent death, assessed as atypical necrosis (53,166). Atypical apoptosis involving rapid loss of plasma membrane integrity in response to parthenolide was also observed in breast cancer cells (167). These effects were linked to targeting of the plasma membrane proteins by parthenolide (166). Interestingly, although parthenolide induces potent apoptosis in leukemic cells, normal hematopoietic cells are relatively resistant (164). This differential apoptosis was mainly attributed to the increased oxidative state of cancer cells.

Parthenolide-induced activation of JNK was found to lead to massive apoptosis in cancer cell lines resistant to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and TNF- α (167,168). These effects involved phosphorylation of Bid and increased Bid cleavage (167). In addition, parthenolide was able to promote apoptosis in antiestrogen-resistant breast cancer cells (derived from MCF-7 cells) (169) and to sensitize breast cancer cells to paclitaxel (170). In part, these effects were attributed to suppressed NF- κ B activation (168–170). However, NF- κ B inhibition alone does not sufficiently explain apoptotic potency of parthenolide, for example, in cells with inactive NF- κ B (53,164,166). Collectively, several lines of evidence implicates

distorted redox status as a major factor in the antiproliferative and apoptotic effects of parthenolide (54,164).

Redox distortion plays a role also in proapoptotic effects of helenalin (162,171,172). The key event in helenalin-induced apoptosis is mitochondrial dysfunction, including $\Delta\Psi_m$ collapse and the release of cytochrome c (60–62,151) followed by the activation of caspase-3 and caspase-8, phosphatidylserine externalization and the “classical” apoptotic nuclear morphology. Both phosphatidylserine translocation and apoptotic DNA fragmentation in helenalin-treated leukemic cells were abrogated by the pan-caspase inhibitor Z-VAD-fmk [benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone] (62). However, apoptotic responses induced by helenalin were not attenuated by the overexpression of the antiapoptotic proteins Bcl-x(L) or Bcl-2 (61,62). In addition, caspase inactivation had no effect on the cytotoxic effects of helenalin in prostate cancer cells (Woynarowska and Woynarowski, unpublished data). These results suggest that cell death caused by helenalin has Bcl-2-independent and caspase-independent components.

Bcl-2-independent mitochondrial dysfunction and caspase-independent apoptosis might well be a hallmark response to protein-alkylators in general. Bax/Bcl-2-independent $\Delta\Psi_m$ collapse and the release of AIF are relatively well-understood consequences of the oxidation of proteins that control the integrity of the inner mitochondrial membrane (120,122,133,150). AIF translocates to the nucleus, where it promotes a long-range disruption of the nuclear chromatin. The AIF-mediated route represents an “alternative” apoptotic pathway that coexists with the Bax-mediated mitochondrial dysfunction in responses to agents that oxidize critical thiol groups in mitochondrial membrane proteins. AIF-mediated caspase-independent apoptosis is a characteristic feature of cell death induced by the protein oxidant diamide (122). As discussed below (Section 6.3.), Bcl-2-independent and caspase-independent effects are implicated in cellular responses to protein-reactive AA/Pt drugs that also target DNA (dual-action agents).

5.2.1. CONCLUSION

Reactions of single-action AAs with proteins distort protein redox balance, which leads to potent apoptotic effects. These effects may be enhanced by the oxidative distortion at the level of small molecules and ROS generation. Unlike apoptosis originating from DNA damage, apoptosis reflecting protein damage/ROS generation is marginally affected by antiapoptotic members of the Bcl-2 family. Apoptosis resulting from targeting proteins (or, more precisely, the proteome as a collective target) may involve caspase-independent routes, in addition to caspase-dependent components.

5.3. At the Crossroads of Apoptotic Signaling—the Trx System

Whether or not oxidative insult (such as exposure to thiol-reactive AA/Pt drugs) results in apoptosis depends on the compensatory capacity of the endogenous redox buffers. The redox status of the cell is controlled by two major interacting systems—the GSH system and the Trx system. Although the GSH system buffers oxidative changes affecting small molecules, the independently regulated Trx system controls the redox status at the protein level (173–175). The Trx system is universally and in multiple ways involved in cellular responses to protein-damaging agents and in modulation of responses to DNA damage (Fig. 2).

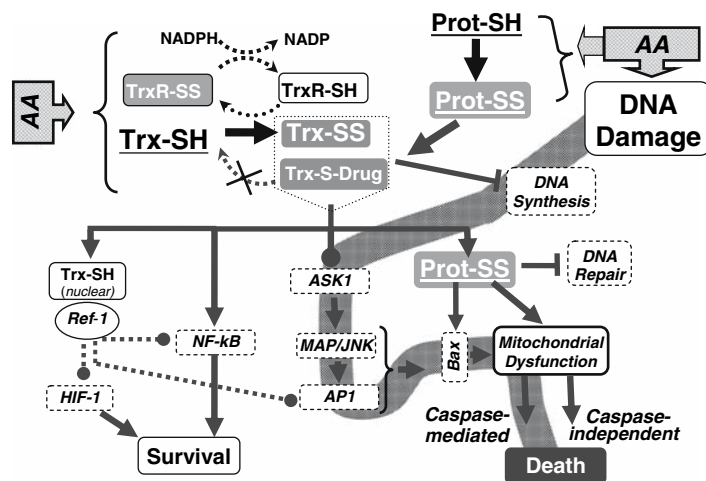


Fig. 2. Distortion of protein redox balance and targeting of the thioredoxin system in the interplay of death and survival responses to protein-damaging and DNA-damaging agents. The thioredoxin (Trx) system acts as a sensor and “nano-switch” regulating protein redox status. Depletion of reduced Trx (Trx-SH) by oxidation (reversible) and by drug adduction (irreversible) affects the global protein thiol-disulfide status. Reciprocally, a pro-oxidative shift in global protein redox balance (such as that caused by massive protein adduction by poly-targeted AA/Pt drugs) affects the status of the Trx system. The pathways of protein damage processing and the resulting signaling are marked with black arrows. Protein damage directly leads to apoptosis through caspase-dependent and independent routes, but also facilitates the execution of apoptosis and enhances death responses originating from DNA damage (the latter responses are symbolized by the gray ribbon).

Catalytically active (reduced) Trx acts as electron donor to numerous proteins that require the reduced sulfhydryl groups for their proper folding and function (176) restoring the reduced status of oxidatively inactivated proteins. Trx also plays an essential role in the repair of drug-adducted proteins by removing drug molecules in a trans-alkylation reaction. In either case, having re-activated another protein, Trx itself becomes inactivated. Oxidized Trx can be regenerated by the NADPH-dependent flavoenzyme @ thioredoxin reductase (TrxR) (177). As the regeneration reaction consumes NADPH, its efficiency depends on the global balance of cellular redox equivalents. Thus, under conditions of oxidative stress, a proportion of oxidized (inactive) Trx molecules increases. Moreover, either Trx or TrxR can be irreversibly inactivated by direct reaction with AA/Pt drugs.

The Trx system has been proposed to act as a molecular “nano-switch” (178). The “on” or “off” states, determined by the redox status of Trx/TrxR redox couple, are critically linked to proliferation, differentiation and apoptosis. Both the global non-specific protein damage/oxidation and specific targeting of Trx and/or TrxR proteins compromise the redox status and, thereby, the vital functions of the Trx system (Fig. 2). Even slight changes in the available reduced Trx (Trx-SH) are rapidly amplified in a cascade of responses.

Trx-SH is essential for the maintenance/restoration of the proper reduced status of many functionally critical proteins (173). Therefore, even partial dysfunction of the Trx system has detrimental consequences that can propagate into more severe oxidative changes (Fig. 2). Irreversible cell degradation commences when such oxidative changes

progress far enough to disrupt the regulation of the redox status of mitochondrial proteins. Thus, oxidation of proteins that control the integrity of the inner mitochondrial membrane leads to the collapse of mitochondrial membrane potential ($\Delta\Psi_m$) and the leakage of apoptogenic components (174,178). The released factors activate not only the caspase cascade but also caspase-independent death route. As Trx-SH acts as a negative regulator of ASK1, oxidized Trx facilitates the activation of ASK1/JNK/MAPK death signaling pathways (which play important roles in transducing DNA damage signaling, Fig. 2), leading to mitochondrial dysfunction and caspase-mediated apoptosis (179,180).

Distortion to the Trx redox status is magnified by the cascades of redox-dependent transcriptional reprogramming (177,181–183). Trx oxidation in cytoplasm may initially promote the nuclear translocation of such redox-dependent effectors as NF- κ B and AP-1. However, the transactivation of these effectors [as well as hypoxia-inducible factor-1 (HIF-1)] to the transcriptionally competent forms in the nucleus depends on reduced redox effector factor-1 (Ref-1), whose reduction in turn requires reduced nuclear Trx (Fig. 2). As Ref-1 also functions as a basic site endonuclease (APE) in base excision repair (88), its oxidized status may enhance cell death by adversely affecting the repair of DNA damage. Moreover, Trx/Ref-1 couple is also involved in the redox-mediated control of p53 protein conformation and p53-dependent signaling (184,185). Thus, even though redox distortion may initiate pro-survival signaling, this signaling is likely to be futile under conditions of persistent redox imbalance and impeded Trx activity.

Tumor cells that function under abnormal oxidative conditions may need to accelerate the turnover of Trx and TrxR to maintain the redox integrity of their proteomes. Both Trx and TrxR proteins are highly inducible under stress conditions (173,186). Accordingly, increased aggressiveness and radioresistance and chemoresistance of a large proportion of clinical tumors are attributed to elevated levels of Trx and/or TrxR (187–189). The increased dependence of cancer cells on the Trx system, however, constitutes also their Achilles heel by opening a window of opportunity for agents targeting Trx and other redox-regulating proteins (190).

5.3.1. CONCLUSION

The state of the Trx system and redox-dependent signaling in cancer cells could be critical for cell responses to all AA/Pt drugs that react with proteins. The oxidative shift in protein sulfhydryl/disulfide balance elicits potent apoptotic signaling and fundamentally changes cellular milieu. The distortion affects several vital pathways of signal transduction, including those involved in responses to DNA damage.

6. ENHANCED APOPTOSIS BY COMBINED TARGETING OF DNA AND PROTEIN REDOX STATUS

If DNA damage alone and protein damage alone are proapoptotic, it is reasonable to expect that a co-induction of both types of damage would lead to cumulative apoptotic effects (141). Can the “dual action” amount to more than the sum of its components in terms of apoptosis induction? What is more important—DNA damage or protein damage? These questions have not yet been fully answered, as it is inherently difficult to dissect the interplay between signal transduction initiated by damage to nuclear DNA and protein damage-activated redox signaling. It may well be that some

AA drugs promote apoptosis mainly through protein damage, with DNA damage (if induced) providing an additional boost. A reversed situation is also plausible (e.g., for Pt drugs) that DNA damage can be essential, although protein damage would still substantially enhance lethal consequences of DNA lesions. In either scenario, poly-targeting, including proapoptotic consequences of protein damage, may help equalize apoptotic responses across diverse biological models.

As illustrated by the promising properties of irifolven and oxaliplatin, both variants of dual action may offer pharmacological advantages, as both drugs consistently induce apoptosis in tumor cells, including those models that are resistant to the classical DNA damage-promoted apoptosis. Moreover, irifolven and oxaliplatin serve as a paradigm that apoptosis induced by dual-action drugs may spare normal cells.

6.1. Irifolven—Interplay Between Redox Distortion from Protein Damage and Nuclear Signaling from DNA Damage

Irifolven at concentrations promoting significant apoptosis in cancer cells forms approximately 10^8 drug–protein adducts/cell, a significant fraction of which affect Trx (107). Although irifolven also induces numerous DNA lesions, they are less frequent than drug–protein adducts (~10-fold difference in prostate cancer cells) and do not include crosslinks that typify highly lethal DNA damage. Thus, the balance of lethal effects of irifolven might be skewed toward a significant contribution from protein damage and, in particular, from targeted redox-regulating proteins.

6.1.1. DISTORTION OF REDOX HOMEOSTASIS BY IROFULVEN

Consistent with a role of protein damage and Trx targeting, irifolven distorts protein redox homeostasis (107). As found in prostate cancer cells, early redox-mediated responses include transient induction of Trx expression and DNA binding activation of redox-regulated transcription factors NF- κ B and AP-1. Importantly, however, even at the peak of Trx protein level, *Trx activity* remains below the level observed in untreated cells (107). This inhibition of Trx activity progressed markedly with time and was followed by symptoms of a global pro-oxidative distortion of redox homeostasis, that is, elevated protein carbonyls and decreased protein sulfhydryls. In addition to Trx, irifolven inhibited, albeit to a lesser degree, intracellular TrxR and another redox-regulating protein, glutathione S-transferase (GST) (107,191). Irifolven cytotoxicity and apoptosis are inversely correlated with basal levels of Trx protein in prostate cancer LNCaP cells and its two genetically related sublines LNCaP-Pro5 and LNCaP-LN3 (48) (Woynarowska and co-workers, unpublished results).

Noteworthy, ROS generation was modest and delayed relative to changes in Trx expression and its activity (107,140). In addition, several ROS scavengers tested were unable to block apoptotic effects of irifolven (Woynarowska and Woynarowski, unpublished data). Thus, ROS formation seems to reflect a consequence rather than a cause in irifolven-induced apoptosis, in agreement with other data suggesting that irifolven reacts more readily with proteins than SMNs, unlike SLs, helenalin and parthenolide. Accordingly, although cytotoxicity of ROS-inducing SLs depends on GSH levels, GSH depletion has marginal effect on irifolven cytotoxicity (192) (Woynarowska, unpublished data).

6.1.2. OXIDATIVELY DISTORTED ENVIRONMENT AND DNA DAMAGE SIGNALING — CAVEATS AND OPPORTUNITIES

Irofulven is consistently cytotoxic and is a potent inducer of apoptosis in cellular models that are deficient in specific aspects of the classical DNA damage signaling (48,49,193,194). Likewise, forced overexpression of Bcl-2 has little effect on irofulven-induced caspase-mediated DNA fragmentation and virtually no effect on the decrease in cell viability and growth inhibition (61). Thus, DNA damage and resulting signaling might not be a decisive factor in responses to irofulven. Nevertheless, irofulven–DNA adducts are probably not insignificant. By interfering with DNA functions and activating partially non-overlapping apoptotic routes, DNA lesions may accelerate cell death and/or increase the proportion of irreversibly inactivated cells. DNA damage located in transcribed genes seems important for cell survival after exposure to irofulven, as impediments to transcription-coupled repair machinery sensitizes cells to the drug (195,196). By contrast, the integrity of the global repair is not vital (195,196), which also suggests that a large proportion of irofulven-induced DNA lesions may be relatively inconsequential.

Importantly, DNA damage signaling promoted by drugs such as irofulven is complicated by overlapping effects on the proteome. Responses that are usually presumed to originate from DNA damage may be profoundly modulated by the oxidatively distorted environment. Possible contribution of protein redox distortion needs to be considered in such aspects as (i) the activities of DNA damage recognition and processing proteins, (ii) the status of DNA damage signaling pathways, and (iii) cell-cycle redistribution (Fig. 2). For instance, compromised redox homeostasis is known to impede DNA damage recognition and repair by oxidative inactivation of cysteine-rich proteins involved in these processes (173,197). In fact, one of such proteins, ataxia telangiectasia mutated (ATM), which is a major transducer in responses to DNA damage, has been demonstrated to be involved in sensing/modulating intracellular redox homeostasis (198). Moreover, inactivation of Trx may cause the S-phase arrest by inhibiting the regeneration of ribonucleotide reductase (173).

Even though some of the DNA damage recognition/repair proteins may not be vital for the repair of irofulven–DNA adducts, the possibility that the drug may compromise their function (either directly by formation of drug adducts or indirectly by global redox change) is relevant to the combinations of irofulven with other DNA-damaging agents, including the demonstrated potentiation of IR effects (136). Studies on irofulven/IR combination in brain tumor cells implicated drug inhibitory effects on DNA-protein kinase (DNA-PK) (199), one of the vital proteins in the recognition and repair of IR-induced DNA double-strand breaks. As irofulven is not a DNA strand scission agent, DNA-PK status has marginal effect on the cytotoxicity and apoptotic responses to the drug alone in glioma MO59K cells harboring wild-type DNA-PK versus its isogenic subline MO59J with a mutated DNA-PK. By contrast, the radiosensitization conferred by irofulven is markedly more pronounced in the inherently radioresistant MO59K cells than in MO59J cells (199). Given that the drug inhibits DNA-PK activity in cell-free system, irofulven effects on DNA-PK may, at least partly, contribute to the greater radiosensitization in MO59K cells.

Another potential benefit of redox distortion by irofulven pertains to targeting hypoxic cells. In general, the activation of the Trx/Ref-1-dependent HIF-1 pathway is a frequent response of hypoxic cells that leads to chemoresistance and radioresistance.

Consistent with the ability to impede Trx function, however, irifolven inhibited HIF-1 transactivation and downstream HIF-1-mediated signaling in hypoxic cells (191). Accordingly, irifolven retained fully its lethal potency against prostate cancer and glioma cells under hypoxia.

Given the pivotal significance of redox signaling to both DNA repair and apoptosis, it is conceivable that the levels, localization and the oxidation status of redox-regulating factors (such as Trx and/or Ref-1) might prove useful for predicting the overall death/survival outcomes in cells exposed to dual-action drugs. Further investigations with irifolven and other drugs are highly warranted to better understand therapeutic opportunities created by the environment with oxidatively distorted protein redox status.

6.2. Oxaliplatin and Other Pt Drugs—Apoptosis Promoted by Lethal DNA Lesions, but Enhanced by Protein Targeting?

6.2.1. COMPLICATED CONNECTIONS BETWEEN DNA DAMAGE SIGNALING AND APOPTOSIS

Apoptosis-inducing levels of Pt drugs result in fewer DNA adducts compared with irifolven ($\sim 4 \times 10^4$ /cell for oxaliplatin), but the majority of these lesions are intra- and inter-strand DNA crosslinks as well as DNA-protein crosslinks (29,200). In general, DNA damage produced by Pt drugs is believed to be a primary determinant of their lethal effects (9,201). It is also clear that apoptosis is an important aspect of antiproliferative effects of cisplatin and other Pt drugs. In some models, the causative relationship between DNA damage and apoptosis induction is reasonably well established. For example, apoptotic responses to cisplatin are known to be particularly intense in testicular cancer cells that tend to have inherently low ability to repair DNA damage induced by Pt drugs (202,203). In other models, however, the kinetics and magnitude of apoptotic effects relative to DNA damage may vary widely.

One particularly ambiguous and controversial aspect is the role of p53 that is expected to mediate responses to DNA damage by Pt drugs. Given a multitude of disparate outcomes, the still widespread notion that Pt drugs exert apoptosis in a manner dependent on wild-type p53 is an oversimplification. Even the distinct sensitivity of malignant testicular cells to cisplatin is not correlated with p53 status and reflects both p53-dependent and p53-independent pathways (204,205). Mutated p53 may actually enhance cisplatin apoptosis (206). In addition, cisplatin remains apoptotic in p53-negative hepatoma cells (207).

Also oxaliplatin is capable of promoting similar levels of apoptotic responses in cancer cells differing in their p53 status (200). Even though oxaliplatin is more growth inhibitory in some cells with wild-type p53 than in some cells with aberrant p53 (200), the p53 status in a panel of 30 colorectal cancer cell lines fails to correlate with the sensitivity of these cell lines to oxaliplatin (208). Although oxaliplatin was more apoptotic in p53+/+ colon carcinoma HCT116 than in its p53-/- subline (208), another study concluded that p53 signaling only marginally contributes to oxaliplatin-induced apoptosis in HCT116 cells (209). Furthermore, oxaliplatin was more lethal against colon carcinoma and breast cancer cells with mutated p53 than against their respective isogenic counterparts with wild-type p53 (210). Finally, both cisplatin and oxaliplatin are able to promote an efficient response of p53 negative tumors *in vivo* (211).

In addition to the controversy surrounding the role of p53 as a major mediator of apoptotic signaling from DNA damage, relationship between DNA damage induced by Pt drugs and apoptosis is not always unambiguous. For example, differential sensitivity to the induction of apoptosis by cisplatin in proliferating and quiescent cells was independent of the levels of drug accumulation and DNA adduct formation (212). Lethality of DNA adducts formed by different Pt drugs is not uniform. Oxaliplatin needs to form significantly fewer DNA lesions than cisplatin for equitoxic effects. This difference is not attributable to altered repair of Pt–DNA lesions (29,200,213). Moreover, direct comparison in a panel of several colon cell lines demonstrated that Pt–DNA adducts formation and repair were correlated with cisplatin but not with oxaliplatin cytotoxicity (214).

More direct evidence suggesting that factors other than DNA damage at least modulate apoptotic responses to Pt agents is mounting. For example, experiments using enucleated cells (cytoplasts), which eliminate nuclear signaling, demonstrate that Pt drugs initiate apoptotic pathways that are independent of damage to nuclear DNA. In both nucleated cells and cytoplasts, cisplatin was shown to activate endoplasmic reticulum-specific caspase-12 and, subsequently, caspase-3 (215). Likewise, enucleation did not prevent oxaliplatin-induced mitochondrial dysfunction (209). Experiments with cytoplasts demonstrated that apoptotic resistance to oxaliplatin can be conferred not only by the nuclear compartment (which would be consistent with the prevailing significance of DNA damage) but also by cytoplasmic compartment (209). In contrast to Pt drugs, topoisomerase poison etoposide fails to promote apoptotic signaling in cytoplasts, as expected for an agent that acts through damage to nuclear DNA (215).

6.2.2. INTERFERENCE OF Pt DRUGS WITH THE ANTIAPOPTOTIC TRXR

In addition to damage to nuclear DNA, protein reactivity of Pt drugs is one of the plausible factors that may contribute to apoptosis induction at several levels. Cisplatin macromolecule binding data (23) suggest that there are approximately 10^6 protein adducts/cell at apoptosis-inducing cisplatin concentrations. A higher number of protein adducts is expected for oxaliplatin, which is more reactive with cysteine and methionine residues than cisplatin (72,81). Thus, apoptosis induction by Pt drugs is accompanied by protein damage, albeit most likely not as massive as for dual action agents such as irifolven.

It remains unclear how selective Pt drugs are in affecting cellular proteins. Moreover, the potential apoptotic consequences of protein damage by Pt drugs are by and large under-investigated, although there is one important exception. Namely, studies by several groups have implicated the interference with redox regulation and the antiapoptotic Trx system in the effects of Pt drugs (83,84,216–220). Cisplatin and other Pt complexes have been demonstrated in cell-free and cellular models to inactivate redox-regulating proteins, in particular TrxR (83,84,87,216). Recent data directly demonstrate that the inactivation of TrxR by oxaliplatin results from drug covalent adducts to this protein (87). Oxaliplatin binding to TrxR was several-fold greater than binding to Trx under the same conditions. Consistent with TrxR being a preferred target, oxaliplatin is a more potent inhibitor of the enzymatic activity of purified and cellular TrxR than Trx (87). The preference of Pt complexes (TrxR>Trx) is inverted relative to that of irifolven (Trx>TrxR), which illustrates the potential of chemically distinct drugs to differentiate among members of the same class of redox-regulating proteins.

Consistent with the role of TrxR targeting, attenuation of intracellular TrxR activity in cisplatin-treated cells correlates with the decrease in cell viability (85). In addition, downregulation of TrxR expression sensitizes cells to cisplatin (85), whereas forced overexpression of TrxR, but not the catalytically inactive TrxR mutant, confers resistance to oxaliplatin (87). In addition to the effects of Pt drugs on cytoplasmic form of TrxR (TrxR1), a link was suggested between the inhibition of mitochondrial TrxR2 by cisplatin and drug-induced dissipation of the mitochondrial membrane potential (221). Even though Pt drugs prefer TrxR, targeting of Trx may not be insignificant. In various types of cancer cells, elevated Trx (cellular or secreted) was associated with resistance to cisplatin (219,220,222,223). A Trx inhibitor 1-methyl-propyl-2-imidazolozyl disulfide was shown to increase cisplatin-induced apoptosis and enhance growth inhibition (224). In some models, however, Trx is not critical for cisplatin activity (225,226).

6.2.3. CONCLUSION

Oxaliplatin typifies a dual-action drug for which DNA damage is most likely essential, whereas protein damage could be secondary. Still, the inhibition of TrxR is probably an important factor in apoptosis induced by oxaliplatin and various other Pt drugs.

6.3. Convergence of DNA Damage and Protein Damage Signaling in Mitochondrial Dysfunction

6.3.1. CASPASE-DEPENDENT AND CASPASE-INDEPENDENT CELL DEATH INDUCED BY IROFULVEN

Apoptotic signals resulting from both DNA damage and protein damage are predicted to converge at the level of mitochondrial dysfunction (Fig. 2). Recent investigations in our laboratory have confirmed that mitochondrial dysfunction indeed plays a central role in irifolven-induced apoptosis and have comprehensively characterized its nature and downstream consequences in prostate cancer cells (116). In conjunction with data on irifolven responses obtained in other cancer models, these studies have allowed us to delineate irifolven effects leading to cell death and to suggest how the revealed overlapping pathways can be related to the initial drug-induced insult.

Irifolven promotes the “classical” Bax/Bcl-2-mediated mitochondrial dysfunction. The observed early (6–12 h) effects involve translocation of Bax to mitochondria, loss of mitochondrial membrane potential ($\Delta\Psi_m$) and cytochrome c release (116). Downstream from mitochondrial dysfunction, irifolven activates the initiator caspase-9 and executioner caspases (61,116,227). Activated caspases provide a positive feedback loop that enhances cytochrome c release and facilitates $\Delta\Psi_m$ collapse at later times. Noteworthy, caspase-3, which serves as a major executioner for various apoptotic stimuli, is dispensable in irifolven-induced apoptosis. Although irifolven activates caspase-3, other caspases are processed more extensively, and caspase-3 inhibition has marginal effect on the subsequent apoptotic DNA fragmentation (116,227). Moreover, MCF-7 breast cancer cells, which are generally resistant to apoptosis because of caspase-3 deficiency, are as sensitive to irifolven as caspase-3-proficient breast cancer cells MDA-MB-231 (227).

The initial manifestations of apoptosis by irifolven are accompanied by caspase-dependent DNA fragmentation, phospholipid remodeling and chromatin condensation (48,49,61,116,227). General and specific caspase inhibitors abrogate irifolven-induced apoptotic DNA fragmentation with the following order of potency against their caspase targets: pan-caspase \geq casp-9 > casp-8/casp-6 > casp-2 > casp-3/casp-7 > casp-1/casp-4 (116). The potent inhibitory effect of the caspase-9 inhibitor corroborates the initiator role of this caspase.

A study with synchronized cells demonstrated that irifolven-induced apoptosis is pre-mitotic (139). The drug arrested the S-phase progression and blocked cell entry from G1 into S. In either case, apoptotic cells were directly recruited from blocked cell population, indicating that neither cells traverse through mitosis nor active DNA synthesis is needed for irifolven-induced cell death (139). These attributes could explain the ability of the drug to efficiently kill non-proliferating (contact inhibited) cancer cells (191).

Progression of irifolven-induced apoptosis results in compromised cell membrane integrity, which is typically observed 24–36 h later than early manifestations of apoptosis (61,116,227). A significant contribution of apoptosis to the antiproliferative effects of irifolven is further corroborated by (i) formation of sub-G1 particles, (ii) net cell loss in short-term cytotoxicity assays, and (iii) a long-term inhibition of clonogenic survival (48,136,227). In addition to several prostate cancer cells, a similar pattern of responses to irifolven was observed in other types of cancer cells, including leukemic, breast, ovarian, colon and brain tumor cells.

It must be highlighted that the classical Bax/Bcl-2-mediated caspase-dependent pathway reflects only one facet of irifolven-induced apoptosis. As mentioned above (Section 5.), protein-reactive agents may cause mitochondrial dysfunction bypassing the antiapoptotic regulation by Bcl-2 or Bcl-X_L. In such cases, mitochondrial permeability transition is initiated by changes in redox-sensing mitochondrial membrane proteins and results in the release of AIF (120,122,133,150,228). Several observations demonstrate that Bcl-2-independent effects play a role in responses to irifolven and contribute to the caspase-independent cell death (116).

The timing of irifolven-induced loss of $\Delta\Psi_m$ relative to cytochrome c release suggests that the inner membrane depolarization is initially independent of Bax/Bcl-2-regulated outer mitochondrial membrane permeability changes that lead to cytochrome c release (116). Accordingly, the early $\Delta\Psi_m$ collapse is not inhibitable by forced overexpression of Bcl-2 (61). Moreover, forced Bcl-2 overexpression had marginal to weak inhibitory effect on irifolven-induced caspase-mediated DNA fragmentation (61,139). Neither early phospholipids remodeling nor the inhibition of cell proliferation were affected by Bcl-2 overexpression. The relevance of Bcl-2-independent effects to cell death is further substantiated by the lack of correlation between Bcl-2 gene expression and irifolven cytotoxicity in the NIH panel of 60 cells lines and drug apoptosis in several of these cell lines (61). Collectively, these data show that Bax/Bcl-2 imbalance mediates only a minor portion of irifolven-induced apoptotic effects.

Consistent with the observed caspase-independent death, irifolven binds to a subset of mitochondrial proteins and promotes the release of AIF (in leukemic CEM cells, Liang and Woynarowska, unpublished data). Moreover, preventing the reactivity of irifolven with the mitochondrial membrane proteins partially antagonizes the apoptotic and antiproliferative effects of irifolven in prostate cancer cells (116). In contrast,

blocking the caspase cascade, which largely abrogates DNA fragmentation, only minimally protects against the loss of cell viability and does not affect cell growth inhibition (116).

6.3.1.1. Conclusion. The fate of irifolven-treated cells is probably determined by a complex interplay of signaling routes originating from drug effects on its diverse cellular targets. These signals are integrated at the mitochondrial level and proceed along two coexisting routes: caspase dependent and caspase independent. This redundancy of death modes could be essential for the ability of irifolven to efficiently kill cancer cells, including those cell types that tend to be resistant to other apoptosis inducers.

6.3.2. DEATH ROUTES PROMOTED BY CISPLATIN AND OXALIPLATIN

Mechanistic attributes of apoptosis induced by Pt drugs show many similarities with irifolven. One potential difference is that in addition to the prominent role of mitochondrial dysfunction, apoptosis by Pt drugs may also involve, at least in some models, the death receptor-mediated apoptotic pathway. The activation of both pathways was implicated, in particular, in the rapid and efficient apoptosis of testicular cancer cells treated with cisplatin (203). Likewise, oxaliplatin increased CD95 receptor and CD95 ligand levels in colon carcinoma cells (229). A role for the death receptor pathway is consistent with the activation by oxaliplatin and cisplatin of caspase-8 (in addition to the caspase-9 and caspase-3 that are characteristic of the mitochondrial dysfunction pathway) (229,230). However, the extent to which apoptosis by Pt drugs depends on the death receptor pathway remains controversial. For example, even in Fas-competent models, exogenously added anti-Fas/FasL reagents may fail to attenuate drug-induced apoptosis (231,232). Moreover, caspase-8 activation by Pt drugs does not always reflect the death receptor pathway but can also be a by-product of activation of other caspases. Cisplatin activates caspase-8 in Hep3B cells that are Fas deficient (207). The activation of caspase-8 by cisplatin in other models was also suggested to proceed in a mitochondria-dependent and Fas-independent manner (233).

Mitochondrial effects of Pt drugs involve proapoptotic and antiapoptotic proteins of the Bcl-2 family. Bax upregulation is an early event in cisplatin-induced apoptosis in testicular tumor cells (234). Oxaliplatin-induced apoptosis in colorectal carcinoma HCT116 cells involves Bax translocation to mitochondria, which precedes cytochrome c release (208). Apoptotic effects of oxaliplatin in these cells are enhanced by downregulation of Bcl-X_L (235). Bax/Bak-dependent mitochondrial apoptosis was implicated in oxaliplatin resistance (208,209). Bax-mediated mitochondrial dysfunction and the release of cytochrome c along with another caspase activator DIABLO characterized the responses of hepatoma cells to cisplatin (207). Cytochrome c release was also observed in cisplatin-treated cervical carcinoma and squamous carcinoma cells (236,237). Correspondingly, forced overexpression of Bcl-2 conferred partial protection from cisplatin and oxaliplatin in glioma cells and cervical carcinoma cells (61,238). It needs to be noted, however, that a significant portion of caspase-dependent DNA fragmentation induced by both cisplatin and oxaliplatin in cervical carcinoma cells was resistant to Bcl-2 overexpression (61). Interestingly, cisplatin-activated caspases were shown to promote a proapoptotic Bcl-2 cleavage to a Bax-like fragment in

melanoma cells, suggesting a positive feedback by which caspase activation may enhance mitochondrial dysfunction (239).

In addition to caspase-8, the initiator caspase-9 and executioner caspase-3 are consistently found activated in responses of various models to cisplatin and oxaliplatin (200,207,208,229,230,236,237,240). Reconstitution of caspase-3 sensitized caspase-3-deficient MCF-7 cells to cisplatin action (241–243). Caspase activation by Pt drugs is typically accompanied by the cleavage of poly(ADP-ribose) polymerase (PARP).

Caspase-mediated apoptosis is clearly not the only death pathway activated by Pt drugs. In Fanconi anemia lymphoblasts, low concentration of cisplatin caused chromatin condensation and phosphatidylserine externalization in the absence of significant caspase activation (albeit caspase-3, caspase-8 and caspase-9 were activated at higher drug levels) (230). The activation by cisplatin of caspase-9 and caspase-3 in ovarian cancer cells was blocked, as expected, by the overexpression of crosslinked inhibitor of apoptosis protein (XIAP) (240). Yet, this inhibition had no effect on the magnitude of cisplatin-induced apoptotic cell morphology. Likewise, in cisplatin-treated melanoma cells, the inhibition of caspase-3 and caspase-7 abrogated neither the apoptotic morphology nor the proteolytic PARP degradation (244). Pretreatment with the pan-caspase inhibitor Z-VAD-fmk inhibited cisplatin-induced phosphatidylserine externalization in some epithelial cancer cell lines but had no effect in L1210 cells (245). Likewise, pretreatment with Z-VAD-fmk afforded only partial decrease in cisplatin-induced apoptotic morphology in hepatocellular carcinoma cells (207). Finally, inhibition of caspases had no effect on the lethality of oxaliplatin in LNCaP-Pro5 prostate cancer cells (87).

6.3.2.1. Conclusion. Mechanistic attributes underlying apoptosis by Pt drugs are consistent with a contributing role of protein damage. Mitochondrial dysfunction induced by Pt drugs appears to be partly Bcl-2 independent. In addition to caspase-dependent apoptosis, Pt drugs promote caspase-independent cell death. Caspase-independent component seems sufficient for cell inactivation caused by oxaliplatin, which is reminiscent of analogous effect promoted by irifolven.

6.4. Differential Apoptosis in Cancer Versus Normal Cells

As discussed in Section 5.2., redox-regulating systems may constitute the Achilles heel of cancer cells that need to cope with the challenges of a more oxidative state than normal cells have. If redox distortion plays a role in the effects of dual-action AA/Pt drugs, it is then reasonable to expect that apoptosis in cancer cells may be facilitated compared to responses in normal cells. Clinically effective AA/Pt drugs obviously have a degree of selectivity *in vivo*. Yet, a question remains whether inherent differences exist in basic responses to dual-action drugs in tumor versus normal cells. Somewhat surprisingly, until recent years, drug effects were rarely investigated in normal cell lines in a side-by-side comparison to tumor cell lines. Nonetheless, such comparative data for irifolven and, to a lesser extent, for oxaliplatin are available. These data suggest that both drugs can indeed preferentially induce apoptosis in cancer cells.

6.4.1. NON-LETHAL EFFECTS OF IROFULVEN IN NORMAL CELLS—BUFFERING OF REDOX CHANGES?

Studies in our laboratories have extensively compared effects of irofulven in various types of cancer cells (including prostate, colon, breast, brain, cervical and leukemic cancer cell lines) against various normal cell lines (prostate, breast, renal and mammary epithelial cells, colon mucosa, fibroblasts and endothelial cells) (48,136,141,191,227,246). Collectively, these studies demonstrate that the responses of normal cells to irofulven are qualitatively different from the responses of tumor cells.

These differences are manifested at several levels. An equivalent “load” of drug macromolecule adducts is 3-fold to 4-fold less growth inhibitory in all tested normal cell lines (48). Most importantly, however, growth-inhibited normal cells remain viable and capable of resuming proliferation after the removal of the drug, whereas cancer cells are irreversibly inactivated (48,136,227). In contrast to the profound apoptosis in tumor cells, marginal or very low levels of apoptosis are detected in normal cells. These qualitatively dissimilar responses are consistently observed for all tumor and normal cell lines examined, a range of treatment conditions and multiple apoptotic endpoints (48,136,227). Normal cells remain non-apoptotic and fully viable after a prolonged (4 days) exposure to drug concentrations exceeding by 1–2 orders of magnitude levels that are apoptotic and lethal in cancer cells after 12–24 h (136). Furthermore, contact-inhibited normal cells remain fully viable under treatment conditions that completely inactivate contact-inhibited prostate cancer cells (191). Hence, irofulven is cytotoxic (lethal) in proliferating and non-proliferating tumor cells but merely reversibly cytostatic and essentially non-lethal in normal cells.

This resistance of normal cells to apoptosis reflects different responses to irofulven-induced protein damage and DNA damage, not altered drug accumulation or reduced binding to macromolecules (48,107). Levels of irofulven macromolecular adducts that are similar or higher than those resulting in profound apoptosis in cancer cells are non-apoptotic in normal cells such as normal prostate epithelial cells (PrECs) and normal colon mucosa NCM-460 cells. Microarray analysis revealed that in response to irofulven, NCM-460 and PrEC cells rapidly upregulate several genes pertinent to apoptosis and cell-cycle regulation (246). The significantly upregulated genes included transcriptional activator E2F, several damage recognition genes and cyclins and antiapoptotic survivin. In contrast to the normal cells, prostate cancer LNCaP-Pro5 cells extensively downregulated multiple antiapoptotic, pro-proliferative and DNA damage recognition genes. Proteome profiling by 2D gel analysis revealed corresponding patterns (246). Normal cells displayed multiple upregulated proteins and no discernible downregulation, whereas the pattern for tumor cells indicated downregulation and degradation of multiple proteins. These data suggest that normal cells, but not cancer cells, are able to neutralize the irofulven-induced insult by a sustained corrective response, probably involving buffering of redox changes. In contrast to progressive inhibition of the activity of Trx, TrxR and GST, and the global distortion of protein redox state in sensitive cancer cells, in normal cells, even higher levels of irofulven caused only a transient and modest decrease in Trx activity with no inhibition of TrxR and GST (107,191). After a few hours, normal cells were able to restore the pre-insult activity of Trx as well as the global protein redox balance. These results suggest that a more efficient protein redox regulation is a likely major factor in the ability of normal cells to avoid apoptosis in response to irofulven.

The buffering of oxidative changes in protein redox status is mainly governed by the cytoplasmic compartment, in which most of cellular Trx activity and the activities of other redox-regulating proteins are located. The role of the cytoplasmic compartment in apoptotic resistance of normal cells to irifolven is supported by the experiments with apoptosis mimicking cell-free systems consisting of isolated nuclei and cytosolic extracts. Incubation of nuclei from apoptosis-resistant normal cells with cytosols from irifolven-treated cancer cells resulted in characteristic apoptotic changes: partial chromatin condensation, nuclei breakage and the release of small chromatin fragments (247). In striking contrast, cytosols from irifolven-treated normal cells had no effect on the morphology of nuclei from either tumor or normal cells. These findings demonstrate that nuclei in normal cells remain sensitive to irifolven-induced apoptosis. However, apoptotic signals in these cells are attenuated at the cytoplasmic level.

6.4.2. OXALIPLATIN—A GREATER SELECTIVITY FOR CANCER CELLS THAN CISPLATIN?

Relevant data on oxaliplatin effects in normal cells are considerably less extensive than those for irifolven. Still, normal prostate cells (PrEC) and normal fibroblasts WI-38 were found to be remarkably resistant to oxaliplatin (200). Both PrEC and WI-38 cells remained viable under conditions resulting in potent apoptosis induction in several tumor cell lines. Oxaliplatin is thus another dual-action drug that appears to have the ability to differentially kill cancer cells while sparing normal cells.

Not all the Pt drugs may be equally selective for cancer cells in their apoptotic effects. Cisplatin was reported to be apoptotic in normal WI-38 cells (248). Likewise, consistent with the known renal toxicity and drug-related hearing losses, cisplatin induces significant apoptosis in normal renal cells and normal auditory cells (245,249). It is plausible that the superior toxicological profile of oxaliplatin might in part reflect oxaliplatin's potential to better differentiate between tumor cells and normal cells in apoptosis induction.

6.4.2.1. Conclusion. Normal cells can neutralize the adverse consequences of high levels of irifolven macromolecular adducts that are lethal to cancer cells. The Trx system and a greater capacity of normal cells to maintain protein redox homeostasis are likely factors in these differential apoptotic responses. Also, oxaliplatin seems able to differentially kill cancer cells while sparing normal cells. Further exploration of redox regulation as the molecular basis underlying differential apoptosis should help to optimize the use of current AA/Pt drugs and facilitate the design of better agents.

7. CONCLUDING REMARKS

AA/Pt drugs remain among the most useful clinical anticancer agents. Yet, they are often perceived as “non-specific” agents that must be inherently inferior to newer strategies aimed at defined molecular targets. Reviewed literature suggests an alternative viewpoint—that the ability of AA/Pt drugs to affect multiple cellular targets underlies their apoptotic effects and that this poly-targeting could, in fact, be a crucial attribute of various clinically active AA/Pt drugs. Moreover, a potent lethal component provided by such drugs could be essential to complement narrowly targeted strategies (16). It thus seems highly relevant to thoroughly explore and understand the connection

between the poly-targeted nature of these agents and their desirable proapoptotic properties.

Regrettably, the pleiotropic reactivity of AA/Pt drugs with many cellular biomolecules remains understudied and conceptually underdeveloped, being overshadowed by the assumption that DNA damage is by far the main, if not the only, biologically important effect. Unrepaired damage to nuclear DNA undoubtedly plays a critical role in cell inactivation by many AA/Pt drugs. However, DNA-specific alkylators (which do not react with other targets) tend to be less proapoptotic than protein-specific AA agents (which do not react with DNA) and dual-action drugs (that react with both DNA and proteins). Accordingly, there is a growing appreciation of targets other than DNA as factors that markedly contribute to cell death induced by AA/Pt drugs.

Promoting cell death at multiple levels accelerates the process and reduces dependence on known adverse factors that might limit apoptosis by narrowly targeted agents. The paradigm of dual-action drugs such as irifolven and oxaliplatin suggests that the combination of DNA damage and protein targeting elicits cumulative apoptotic effects that involve the co-activation of caspase-mediated and caspase-independent routes. Consequently, irifolven and oxaliplatin are consistently apoptotic not only in generally sensitive cell types but also in various apoptosis-resistant models. The idea that drug effects on multiple targets cumulate in enhanced apoptosis is pertinent to many other AA/Pt drugs that react with DNA and proteins. In essence, a poly-targeted agent can be viewed as a virtual drug combination (which may be pharmacologically advantageous over real combinations of multiple narrowly acting drugs).

The concept of poly-targeting affecting the cell homeostasis, rather than a unique biomolecular target, underscores the need to revise the still common perception of pharmacological apoptosis as an execution of a hardwired teleological “program,” in which cell responses rigidly follow specific pathways. It is not surprising that in the conceptual frame of “programmed” death, networks of cell responses to poly-targeted AA/Pt drugs are “shockingly complex” (15) and difficult to generalize in predictive ways. However, pharmacological apoptosis can be viewed, more simply, just as cell demise mediated by a spontaneous, entropy-driven breakdown of cellular structures and organization.

The fundamental principle of life is struggle for entropy to maintain order and organization. According to the second law of thermodynamics, the sum of entropy of all the biochemical processes in the cell is always positive (250). As information fluxes are formally equivalent to negative entropy (250,251), drug-induced cellular responses (“signaling”) distort the entropic steady state in the unfavorable direction. This “entropy penalty” must be compensated either by net entropy flowing into the system (through exchanges of energy and materials with the environment) or by cell de-organization, whichever is feasible and thermodynamically most advantageous for given states of the metabolome and environment (250,251). Pharmacological apoptosis can thus be perceived as a “lost struggle for entropy,” in which spontaneous cell degradation compensates for negative entropy change caused by cell responses to an overwhelming drug insult. De-compartmentalization, such as leakage of mitochondrial components, and proteolytic/nucleolytic cascades generate entropy. Likewise, hypercondensation of nuclear chromatin, one of the most characteristic morphological hallmarks of apoptosis, has been proposed to be driven by favorable changes in conformational entropy (252).

The entropic perspective implies cell de-organization along the thermodynamic “path of the least resistance” rather than along fixed, predefined pathways. Accordingly, drug-induced apoptosis shows multiple and context-dependent manifestations. When one route (e.g., caspase cascade) is not available or becomes inhibited, cell de-organization may shift to alternative routes. This implies that some specific effectors/pathways that are important in some models may become marginalized in others. Another implication of practical significance is that the global state of the metabolome (reflecting the thermodynamic status of the cell) may represent the ultimate determinant of the “point of no return” and predictor of death/survival outcomes, prevailing over the status of any specific proapoptotic or antiapoptotic effector molecule.

Disruption of cell redox homeostasis by poly-targeted AA/Pt drugs exemplifies a scenario in which the global status of the cell affects a multitude of specific effectors and signaling pathways. Pro-oxidative changes in the protein redox status directly promote apoptosis and facilitate apoptotic signaling from other stimuli. AA/Pt drugs can distort protein redox status by a combination of (i) their total adducts in the entire proteome (serving as a collective target) and (ii) inactivation of the key proteins that regulate protein redox homeostasis. An increasing body of evidence demonstrates that various AA/Pt drugs interfere with the functions of the antiapoptotic Trx system that plays pivotal roles in the global regulation of cell redox status. Moreover, the state of redox imbalance profoundly affects various cellular functions, including DNA synthesis, DNA damage repair/signaling and transcriptional responses, as various proteins involved in these processes are redox sensitive. Protein redox status may be one of the decisive factors determining the death or life cellular switch and overriding some specific pathways and apoptotic effectors. An attractive possibility has emerged that, unlike cancer cells, normal cells may be able to avoid apoptosis in response to poly-targeted AA/Pt drugs because of their less strained redox regulation systems and, consequently, a greater capacity to buffer oxidative distortion.

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REFERENCES

1. Nelson SM, Ferguson LR, Denny WA. DNA and the chromosome – varied targets for chemotherapy. *Cell Chromosome* 2004; 3:2.
2. Hurley LH. DNA and its associated processes as targets for cancer therapy. *Nat Rev Cancer* 2002; 2:188–200.

3. Fuertes MA, Castillab J, Alonso C, Perez JM. Cisplatin biochemical mechanism of action: from cytotoxicity to induction of cell death through interconnections between apoptotic and necrotic pathways. *Curr Med Chem* 2003; 10:257–66.
4. Meyers M, Hwang A, Wagner MW, Boothman DA. Role of DNA mismatch repair in apoptotic responses to therapeutic agents. *Environ Mol Mutagen* 2004; 44:249–64.
5. Panasci L, Xu ZY, Bello V, Aloyz R. The role of DNA repair in nitrogen mustard drug resistance. *Anticancer Drugs* 2002; 13:211–20.
6. Blagosklonny MV. Cell death beyond apoptosis. *Leukemia* 2000; 14:1502–8.
7. Kaufmann SH, Earnshaw WC. Induction of apoptosis by cancer chemotherapy. *Exp Cell Res* 2000; 256:42–9.
8. Pommier Y, Sordet O, Antony S, Hayward RL, Kohn KW. Apoptosis defects and chemotherapy resistance: molecular interaction maps and networks. *Oncogene* 2004; 23:2934–49.
9. Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. *Nat Rev Drug Discov* 2005; 4:307–20.
10. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem* 2004; 73:39–85.
11. Norbury CJ, Zhitovitsky B. DNA damage-induced apoptosis. *Oncogene* 2004; 23:2797–808.
12. Berardi P, Russell M, El-Osta A, Riabowol K. Functional links between transcription, DNA repair and apoptosis. *Cell Mol Life Sci* 2004; 61:2173–80.
13. Boland MP. DNA damage signalling and NF-kappaB: implications for survival and death in mammalian cells. *Biochem Soc Trans* 2001; 29:674–8.
14. Ehrenberg L, Osterman-Golkar S. Alkylation of macromolecules for detecting mutagenic agents. *Teratog Carcinog Mutagen* 1980; 1:105–27.
15. Begley TJ, Samson LD. Network responses to DNA damaging agents. *DNA Repair (Amst)* 2004; 3:1123–32.
16. Blagosklonny MV. Prospective strategies to enforce selectively cell death in cancer cells. *Oncogene* 2004; 23:2967–75.
17. Sanderson BJ, Shield AJ. Mutagenic damage to mammalian cells by therapeutic alkylating agents. *Mutat Res* 1996; 355:41–57.
18. Lawley PD, Phillips DH. DNA adducts from chemotherapeutic agents. *Mutat Res* 1996; 355:13–40.
19. Sartorelli AC, Hodnick WF, Belcourt MF, et al. Mitomycin C: a prototype bioreductive agent. *Oncol Res* 1994; 6:501–8.
20. Tomasz M, Palom Y. The mitomycin bioreductive antitumor agents: cross-linking and alkylation of DNA as the molecular basis of their activity. *Pharmacol Ther* 1997; 76:73–87.
21. Szilagyi Z, Fenselau C. Molecular dynamics simulation of metallothionein-drug complexes. *Drug Metab Dispos* 2000; 28:174–9.
22. Cai Y, Wu MH, Ludeman SM, Grdina DJ, Dolan ME. Role of O6-alkylguanine-DNA alkyltransferase in protecting against cyclophosphamide-induced toxicity and mutagenicity. *Cancer Res* 1999; 59:3059–63.
23. Akaboshi M, Kawai K, Maki H, Akuta K, Ujeno Y, Miyahara T. The number of platinum atoms binding to DNA, RNA and protein molecules of HeLa cells treated with cisplatin at its mean lethal concentration. *Jpn J Cancer Res* 1992; 83:522–6.
24. Volckova E, Dudones LP, Bose RN. HPLC determination of binding of cisplatin to DNA in the presence of biological thiols: implications of dominant platinum-thiol binding to its anticancer action. *Pharm Res* 2002; 19:124–31.
25. Peleg-Shulman T, Najajreh Y, Gibson D. Interactions of cisplatin and transplatin with proteins. Comparison of binding kinetics, binding sites and reactivity of the Pt-protein adducts of cisplatin and transplatin towards biological nucleophiles. *J Inorg Biochem* 2002; 91:306–11.
26. Kung A, Strickmann DB, Galanski M, Keppler BK. Comparison of the binding behavior of oxaliplatin, cisplatin and analogues to 5'-GMP in the presence of sulfur-containing molecules by means of capillary electrophoresis and electrospray mass spectrometry. *J Inorg Biochem* 2001; 86:691–8.
27. el-Khateeb M, Appleton TG, Gahan LR, Charles BG, Berners-Price SJ, Bolton AM. Reactions of cisplatin hydrolytes with methionine, cysteine, and plasma ultrafiltrate studied by a combination of HPLC and NMR techniques. *J Inorg Biochem* 1999; 77:13–21.
28. Ivanov AI, Christodoulou J, Parkinson JA, et al. Cisplatin binding sites on human albumin. *J Biol Chem* 1998; 273:14721–30.

29. Woynarowski JM, Faivre S, Herzig MCS, et al. Oxaliplatin-induced damage of cellular DNA. *Mol Pharmacol* 2000; 58:920–7.
30. Woynarowski JM, Chapman WG, Napier C, Herzig M, Juniewicz P. Sequence- and region-specificity of oxaliplatin adducts in naked and cellular DNA. *Mol Pharmacol* 1998; 54:770–7.
31. O'Neill CF, Koberle B, Masters JRW, Kelland LR. Gene-specific repair of Pt/DNA lesions and induction of apoptosis by the oral platinum drug JM216 in three human ovarian carcinoma cell lines sensitive and resistant to cisplatin. *Br J Cancer* 1999; 81:1294–303.
32. Raymond E, Faivre S, Chaney S, Woynarowski J, Cvitkovic E. Cellular and molecular pharmacology of oxaliplatin. *Mol Cancer Ther* 2002; 1:227–35.
33. Almeida GM, Duarte TL, Steward WP, Jones GD. Detection of oxaliplatin-induced DNA crosslinks *in vitro* and in cancer patients using the alkaline comet assay. *DNA Repair (Amst)* 2006; 5:219–25.
34. Gerson SL. Clinical relevance of MGMT in the treatment of cancer. *J Clin Oncol* 2002; 20:2388–99.
35. Liu L, Gerson SL. Therapeutic impact of methoxyamine: blocking repair of abasic sites in the base excision repair pathway. *Curr Opin Investig Drugs* 2004; 5:623–7.
36. Drablos F, Feyzi E, Aas PA, et al. Alkylation damage in DNA and RNA—repair mechanisms and medical significance. *DNA Repair (Amst)* 2004; 3:1389–407.
37. Tse WC, Boger DL. Sequence-selective DNA recognition: natural products and nature's lessons. *Chem Biol* 2004; 11:1607–17.
38. Wolkenberg SE, Boger DL. Mechanisms of *in situ* activation for DNA-targeting antitumor agents. *Chem Rev* 2002; 102:2477–95.
39. Woynarowski JM. Region-specific lesions by DNA-reactive antitumor drugs. In Jones GB, Chapman EJ, eds. *Advances in DNA Sequences Specific Agents*, Vol. 4. Elsevier Press Inc., Amsterdam, The Netherlands, 2002:1–27.
40. Woynarowski JM, Trevino AV, Rodriguez KA, Hardies SC, Benham CJ. AT-rich islands in genomic DNA as a novel target for AT-specific DNA-reactive antitumor drugs. *J Biol Chem* 2001; 276:40555–66.
41. Woynarowski JM. Targeting critical regions in genomic DNA with AT-specific anticancer drugs. *Biochim Biophys Acta* 2002; 1587:300–8.
42. Woynarowski JM, Chapman WG, Napier C, Herzig MCS. Induction of AT-specific DNA-interstrand crosslinks by bizelesin in genomic and simian virus 40 DNA. *Biochim Biophys Acta* 1999; 1444:201–17.
43. Cozzi P. The discovery of a new potential anticancer drug: a case history. *Farmaco* 2003; 58:213–20.
44. Marchini S, Brogini M, Sessa C, D'Incalci M. Development of distamycin-related DNA binding anticancer drugs. *Expert Opin Investig Drugs* 2001; 10:1703–14.
45. Kupchinsky S, Centioni S, Howard T, et al. A novel class of achiral seco-analogs of CC-1065 and the duocarmycins: design, synthesis, DNA binding, and anticancer properties. *Bioorg Med Chem* 2004; 12:6221–36.
46. McMorris TC, Yu J, Estes LA, Kelner MJ. Reaction of antitumor hydroxymethylacylfulvene (HMAF) with thiols. *Tetrahedron* 1997; 53:14579–90.
47. Herzig MCS, Arnett B, MacDonald JR, Woynarowski JM. Drug uptake and cellular targets of hydroxymethylacylfulvene (HMAF). *Biochem Pharmacol* 1999; 58:217–25.
48. Woynarowska BA, Woynarowski JM, Herzig MC, Roberts K, Higdon AL, MacDonald JR. Differential cytotoxicity and induction of apoptosis in tumor and normal cells by hydroxymethylacylfulvene (HMAF). *Biochem Pharmacol* 2000; 59:1217–26.
49. Woynarowski JM, Napier C, Koester SK, et al. Effects on DNA integrity and apoptosis induction by a novel antitumor sesquiterpene drug, 6-hydroxymethylacylfulvene (HMAF, MGI 114). *Biochem Pharmacol* 1997; 54:1181–93.
50. Kelner MJ, McMorris TC, Montoya MA, et al. Characterization of MGI 114 (HMAF) histiospecific toxicity in human tumor cell lines. *Cancer Chemother Pharmacol* 1999; 44:235–40.
51. McMorris TC, Elayadi AN, Yu J, Hu Y, Kelner MJ. Metabolism of antitumor hydroxymethylacylfulvene by rat liver cytosol. *Drug Metab Dispos* 1999; 27:983–5.
52. Dick RA, Yu X, Kensler TW. NADPH alkenal/one oxidoreductase activity determines sensitivity of cancer cells to the chemotherapeutic alkylating agent irifolven. *Clin Cancer Res* 2004; 10:1492–9.
53. Pozarowski P, Halicka DH, Darzynkiewicz Z. NF-kappaB inhibitor sesquiterpene parthenolide induces concurrently atypical apoptosis and cell necrosis: difficulties in identification of dead cells in such cultures. *Cytometry A* 2003; 54:118–24.

54. Wen J, You KR, Lee SY, Song CH, Kim DG. Oxidative stress-mediated apoptosis. The anticancer effect of the sesquiterpene lactone parthenolide. *J Biol Chem* 2002; 277:38954–64.
55. Garcia-Pineres AJ, Castro V, Mora G, et al. Cysteine 38 in p65/NF-kappaB plays a crucial role in DNA binding inhibition by sesquiterpene lactones. *J Biol Chem* 2001; 276:39713–20.
56. Hall IH, Williams WL, Jr., Grippo AA, Lee KH, Holbrook DJ, Chaney SG. Inhibition of nucleic acid synthesis in P-388 lymphocytic leukemia cells in culture by sesquiterpene lactones. *Anticancer Res* 1988; 8:33–42.
57. Schmidt TJ. Glutathione adducts of helenalin and 11 alpha,13-dihydrohelenalin acetate inhibit glutathione S-transferase from horse liver. *Planta Med* 2000; 66:106–9.
58. Schmidt TJ, Lyss G, Pahl HL, Merfort I. Helenanolide type sesquiterpene lactones. Part 5: the role of glutathione addition under physiological conditions. *Bioorg Med Chem* 1999; 7:2849–55.
59. Schmidt TJ. Helenanolide-type sesquiterpene lactones—III. Rates and stereochemistry in the reaction of helenalin and related helenanolides with sulfhydryl containing biomolecules. *Bioorg Med Chem* 1997; 5:645–53.
60. Gertsch J, Sticher O, Schmidt T, Heilmann J. Influence of helenanolide-type sesquiterpene lactones on gene transcription profiles in Jurkat T cells and human peripheral blood cells: anti-inflammatory and cytotoxic effects. *Biochem Pharmacol* 2003; 66:2141–53.
61. Herzig MCS, Trevino AV, Liang H, et al. Apoptosis induction by the dual-action DNA- and protein-reactive antitumor drug irifolven is largely Bcl-2-independent. *Biochem Pharmacol* 2003; 65:503–13.
62. Dirsch VM, Stuppner H, Vollmar AM. Helenalin triggers a CD95 death receptor-independent apoptosis that is not affected by overexpression of Bcl-x(L) or Bcl-2. *Cancer Res* 2001; 61:5817–23.
63. Lyss G, Knorre A, Schmidt TJ, Pahl HL, Merfort I. The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-kappaB by directly targeting p65. *J Biol Chem* 1998; 273:33508–16.
64. Tilby MJ, McCartney H, Gould KA, et al. A monofunctional derivative of melphalan: preparation, DNA alkylation products, and determination of the specificity of monoclonal antibodies that recognize melphalan-DNA adducts. *Chem Res Toxicol* 1998; 11:1162–8.
65. Kim SY, Rockwell S. Cytotoxic potential of monoalkylation products between mitomycins and DNA: studies of decarbamoyl mitomycin C in wild-type and repair-deficient cell lines. *Oncol Res* 1995; 7:39–47.
66. Giles FJ, Koeffler HP. Secondary myelodysplastic syndromes and leukemias. *Curr Opin Hematol* 1994; 1:256–60.
67. Boffetta P, Kaldor JM. Secondary malignancies following cancer chemotherapy. *Acta Oncol* 1994; 33:591–8.
68. Herzig MC, Trevino AV, Arnett B, Woynarowski JM. Tallimustine lesions in cellular DNA are AT sequence-specific but not region-specific. *Biochemistry* 1999; 38:14045–55.
69. McCartney H, Martin AM, Middleton PG, Tilby MJ. Antibody recognition of melphalan adducts characterized using immobilized DNA: enhanced alkylation of G-Rich regions in cells compared to *in vitro*. *Chem Res Toxicol* 2001; 14:71–81.
70. Wang M, Hecht SS. A cyclic N7,C-8 guanine adduct of N-nitrosopyrrolidine (NPYR): formation in nucleic acids and excretion in the urine of NPYR-treated rats. *Chem Res Toxicol* 1997; 10:772–8.
71. Masta A, Gray PJ, Phillips DR. Nitrogen mustard inhibits transcription and translation in a cell free system. *Nucleic Acids Res* 1995; 23:3508–15.
72. Heudi O, Mercier-Jobard S, Cailleux A, Allain P. Mechanisms of reaction of L-methionine with carboplatin and oxaliplatin in different media: a comparison with cisplatin. *Biopharm Drug Dispos* 1999; 20:107–16.
73. Manka S, Becker F, Hohage O, Sheldrick WS. Cisplatin-mediated selective hydrolytic cleavage of methionine-containing peptides with neighboring serine or histidine residues. *J Inorg Biochem* 2004; 98:1947–56.
74. Cai Y, Ludeman SM, Wilson LR, Chung AB, Dolan ME. Effect of O6-benzylguanine on nitrogen mustard-induced toxicity, apoptosis, and mutagenicity in Chinese hamster ovary cells. *Mol Cancer Ther* 2001; 1:21–8.
75. Luo FR, Wyrick SD, Chaney SG. Biotransformations of oxaliplatin in rat blood *in vitro*. *J Biochem Mol Toxicol* 1999; 13:159–69.
76. Pendyala L, Creaven PJ. *In vitro* cytotoxicity, protein binding, red blood cell partitioning, and biotransformation of oxaliplatin. *Cancer Res* 1993; 53:5970–6.

77. Lof K, Hovinen J, Reinikainen P, Vilpo LM, Seppala E, Vilpo JA. Kinetics of chlorambucil *in vitro*: effects of fluid matrix, human gastric juice, plasma proteins and red cells. *Chem Biol Interact* 1997; 103:187–98.
78. Granath F, Ehrenberg L, Tornqvist M. Degree of alkylation of macromolecules *in vivo* from variable exposure. *Mutat Res* 1992; 284:297–306.
79. Begleiter A, Mowat M, Israels LG, Johnston JB. Chlorambucil in chronic lymphocytic leukemia: mechanism of action. *Leuk Lymphoma* 1996; 23:187–201.
80. Peleg-Shulman T, Gibson D. Cisplatin-protein adducts are efficiently removed by glutathione but not by 5'-guanosine monophosphate. *J Am Chem Soc* 2001; 123:3171–2.
81. Hagrman D, Goodisman J, Souid AK. Kinetic study on the reactions of platinum drugs with glutathione. *J Pharmacol Exp Ther* 2003.
82. Biaglow JE. The thioredoxin reductase/thioredoxin system: novel redox targets for cancer therapy. *Cancer Biol Ther* 2005; 4:6–13.
83. Arner ES, Nakamura H, Sasada T, Yodoi J, Holmgren A, Spyrou G. Analysis of the inhibition of mammalian thioredoxin, thioredoxin reductase, and glutaredoxin by cis-diamminedichloroplatinum (II) and its major metabolite, the glutathione-platinum complex. *Free Radic Biol Med* 2001; 31:1170–8.
84. Becker K, Gromer S, Schirmer RH, Mueller S. Thioredoxin reductase as a pathophysiological factor and drug target. *Eur J Biochem* 2000; 267:6118–25.
85. Sasada T, Nakamura H, Ueda S, et al. Possible involvement of thioredoxin reductase as well as thioredoxin in cellular sensitivity to cis-diamminedichloroplatinum (II). *Free Radic Biol Med* 1999; 27:504–14.
86. Wells WW, Rocque PA, Xu DP, Yang Y, Deits TL. Interactions of platinum complexes with thioltransferase(glutaredoxin), *in vitro*. *Biochem Biophys Res Commun* 1991; 180:735–41.
87. Woynarowska BA, Vallejo MA, Losiewicz MD, Woynarowski JM. Targeting of redox-controlling proteins of the thioredoxin family by oxaliplatin. *Proc Am Assoc Cancer Res* 2005; 46:550.
88. Kaina B, Ochs K, Grosch S, et al. BER, MGMT, and MMR in defense against alkylation-induced genotoxicity and apoptosis. *Prog Nucleic Acid Res Mol Biol* 2001; 68:41–54.
89. Shertzer HG, Sainsbury M, Berger ML. Importance of protein thiols during N-methyl-N'-nitro-N-nitrosoguanidine toxicity in primary rat hepatocytes. *Toxicol Appl Pharmacol* 1990; 105:19–25.
90. Weiland KL, Dooley TP. *In vitro* and *in vivo* DNA bonding by the CC-1065 analogue U-73975. *Biochemistry* 1991; 30:7559–65.
91. Jackson J, Trevino AV, Herzig MCS, Woynarowski JM. Differential cytotoxicity of bizelesin in model tumor and normal cells parallels differences in abundance and nuclear organization of AT-rich fragile sites. *Proc Am Assoc Cancer Res* 2002; 43:1092.
92. Jackson JA, Trevino AV, Herzig MC, Herman TS, Woynarowski JM. Matrix attachment region (MAR) properties and abnormal expansion of AT island minisatellites in FRA16B fragile sites in leukemic CEM cells. *Nucleic Acids Res* 2003; 31:6354–64.
93. Woynarowski JM. AT islands - their nature and potential for anticancer strategies. *Curr Cancer Drug Targets* 2004; 4:219–34.
94. Woynarowski JM, Napier C, Trevino AV, Arnett B. Region-specific DNA damage by AT-specific DNA-reactive drugs is predicted by drug binding specificity. *Biochemistry* 2000; 39:9917–27.
95. Herzig MC, Rodriguez KA, Trevino AV, et al. The genome factor in region-specific DNA damage: the DNA-reactive drug U-78779 prefers mixed A/T-G/C sequences at the nucleotide level but is region-specific for long pure AT islands at the genomic level. *Biochemistry* 2002; 41:1545–55.
96. Denny WA. DNA minor groove alkylating agents. *Curr Med Chem* 2001; 8:533–44.
97. Wemmer DE. Designed sequence-specific minor groove ligands. *Annu Rev Biophys Biomol Struct* 2000; 29:439–61.
98. Baraldi PG, Nunez Mdel C, Espinosa A, Romagnoli R. Distamycin A as stem of DNA minor groove alkylating agents. *Curr Top Med Chem* 2004; 4:231–9.
99. Brogini M, Erba E, Ponti M, et al. Selective DNA interaction of the novel distamycin derivative FCE 24517. *Cancer Res* 1991; 51:199–204.
100. Kelner MJ, McMorris TC, Taetle R. Preclinical evaluation of illudins as anticancer agents: basis for selective cytotoxicity. *J Natl Cancer Inst* 1990; 82:1562–5.
101. Kelner MJ, McMorris TC, Taetle R. *In vitro* and *in vivo* studies on the anticancer activity of dehydroilludin M. *Anticancer Res* 1995; 15:873–8.

102. Kelner MJ, McMorris TC, Montoya MA, et al. Characterization of cellular accumulation and toxicity of illudin S in sensitive and nonsensitive tumor cells. *Cancer Chemother Pharmacol* 1997; 40:65–71.
103. Kelner MJ, McMorris TC, Estes LA, et al. Efficacy of MGI 114 (HMAF) against the MRP+metastatic MV522 lung carcinoma xenograft. *Anticancer Drugs* 2000; 11:217–24.
104. Kelner MJ, McMorris TC, Rojas RJ, Trani NA, Estes L. Enhanced antitumor activity of irifolven in combination with thiotepa or mitomycin C. *Cancer Chemother Pharmacol* 2002; 49:412–8.
105. Kelner MJ, McMorris TC, Rojas RJ, et al. Enhanced antitumor activity of irifolven in combination with antimetabolic agents. *Invest New Drugs* 2002; 20:271–9.
106. Senzer N, Arsenau J, Richards D, Berman B, MacDonald JR, Smith S. Irifolven demonstrates clinical activity against metastatic hormone-refractory prostate cancer in a phase 2 single-agent trial. *Am J Clin Oncol* 2005; 28:36–42.
107. Woynarowska BA, Woynarowski JM, Liang H, et al. Irifolven binding and inactivation of purified and cellular redox-controlling proteins. *Proc Am Assoc Cancer Res* 2004; 45:Abstract 1510.
108. Grippo AA, Hall IH, Kiyokawa H, Muraoka O, Shen YC, Lee KH. The cytotoxicity of helenalin, its mono and difunctional esters, and related sesquiterpene lactones in murine and human tumor cells. *Drug Des Discov* 1992; 8:191–206.
109. Lee KH. Novel antitumor agents from higher plants. *Med Res Rev* 1999; 19:569–96.
110. Garcia-Pineros AJ, Lindenmeyer MT, Merfort I. Role of cysteine residues of p65/NF-kappaB on the inhibition by the sesquiterpene lactone parthenolide and N-ethyl maleimide, and on its transactivating potential. *Life Sci* 2004; 75:841–56.
111. Chapman DE, Holbrook DJ, Chaney SG, Hall IH, Lee KH. *In vitro* inhibition of mouse hepatic mixed-function oxidase enzymes by helenalin and alantolactone. *Biochem Pharmacol* 1989; 38:3913–23.
112. Broker LE, Kruyt FA, Giaccone G. Cell death independent of caspases: a review. *Clin Cancer Res* 2005; 11:3155–62.
113. Blagosklonny MV. Apoptosis, proliferation, differentiation: in search of the order. *Semin Cancer Biol* 2003; 13:97–105.
114. Rodriguez M, Schaper J. Apoptosis: measurement and technical issues. *J Mol Cell Cardiol* 2005; 38:15–20.
115. Kravtsov VD, Daniel TO, Koury MJ. Comparative analysis of different methodological approaches to the *in vitro* study of drug-induced apoptosis. *Am J Pathol* 1999; 155:1327–39.
116. Liang H, Salinas RA, Leal BZ, et al. Caspase-mediated apoptosis and caspase-independent cell death induced by irifolven in prostate cancer cells. *Mol Cancer Ther* 2004; 3:1385–96.
117. Debatin KM. Apoptosis pathways in cancer and cancer therapy. *Cancer Immunol Immunother* 2004; 53:153–9.
118. Henry-Mowatt J, Dive C, Martinou JC, James D. Role of mitochondrial membrane permeabilization in apoptosis and cancer. *Oncogene* 2004; 23:2850–60.
119. Kroemer G, Martin SJ. Caspase-independent cell death. *Nat Med* 2005; 11:725–30.
120. Cregan SP, Dawson VL, Slack RS. Role of AIF in caspase-dependent and caspase-independent cell death. *Oncogene* 2004; 23:2785–96.
121. Walker PR, Sikorska M. New aspects of the mechanism of DNA fragmentation in apoptosis. *Biochem Cell Biol* 1997; 75:287–99.
122. Costantini P, Belzacq AS, Vieira HL, et al. Oxidation of a critical thiol residue of the adenine nucleotide translocator enforces Bcl-2-independent permeability transition pore opening and apoptosis. *Oncogene* 2000; 19:307–14.
123. Cain K, Bratton SB, Cohen GM. The Apaf-1 apoptosome: a large caspase-activating complex. *Biochimie* 2002; 84:203–14.
124. Kyprianou N, Rock S. Radiation-induced apoptosis of human prostate cancer cells is independent of mutant p53 overexpression. *Anticancer Res* 1998; 18:897–905.
125. Butt AJ, Firth SM, King MA, Baxter RC. Insulin-like growth factor-binding protein-3 modulates expression of Bax and Bcl-2 and potentiates p53-independent radiation-induced apoptosis in human breast cancer cells. *J Biol Chem* 2000; 275:39174–81.
126. Strobel T, Swanson L, Korsmeyer S, Cannistra SA. Radiation-induced apoptosis is not enhanced by expression of either p53 or BAX in SW626 ovarian cancer cells. *Oncogene* 1997; 14:2753–8.
127. Yu J, Zhang L. The transcriptional targets of p53 in apoptosis control. *Biochem Biophys Res Commun* 2005; 331:851–8.

128. Kyprianou N, King ED, Bradbury D, Rhee JG. bcl-2 over-expression delays radiation-induced apoptosis without affecting the clonogenic survival of human prostate cancer cells. *Int J Cancer* 1997; 70:341–8.
129. Elliott MJ, Murphy KM, Stribinskiene L, et al. Bcl-2 inhibits early apoptotic events and reveals post-mitotic multinucleation without affecting cell cycle arrest in human epithelial tumor cells exposed to etoposide. *Cancer Chemother Pharmacol* 1999; 44:1–11.
130. Yang XH, Edgerton S, Thor AD. Reconstitution of caspase-3 sensitizes MCF-7 breast cancer cells to radiation therapy. *Int J Oncol* 2005; 26:1675–80.
131. Essmann F, Engels IH, Totzke G, Schulze-Osthoff K, Janicke RU. Apoptosis resistance of MCF-7 breast carcinoma cells to ionizing radiation is independent of p53 and cell cycle control but caused by the lack of caspase-3 and a caffeine-inhibitable event. *Cancer Res* 2004; 64:7065–72.
132. Joseph B, Marchetti P, Formstecher P, Kroemer G, Lewensohn R, Zhivotovsky B. Mitochondrial dysfunction is an essential step for killing of non-small cell lung carcinomas resistant to conventional treatment. *Oncogene* 2002; 21:65–77.
133. Cande C, Vahsen N, Garrido C, Kroemer G. Apoptosis-inducing factor (AIF): caspase-independent after all. *Cell Death Differ* 2004; 11:591–5.
134. Stuschke M, Sak A, Wurm R, et al. Radiation-induced apoptosis in human non-small-cell lung cancer cell lines is secondary to cell-cycle progression beyond the G2-phase checkpoint. *Int J Radiat Biol* 2002; 78:807–19.
135. Dewey WC, Ling CC, Meyn RE. Radiation-induced apoptosis: relevance to radiotherapy. *Int J Radiat Oncol Biol Phys* 1995; 33:781–96.
136. Woynarowska BA, Roberts K, Woynarowski JM, MacDonald JR, Herman TS. Targeting apoptosis by hydroxymethylacetylfulvene in combination with gamma radiation in prostate tumor cells. *Radiat Res* 2000; 154:429–38.
137. Roninson IB, Broude EV, Chang BD. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist Updat* 2001; 4:303–13.
138. Shinomiya N. New concepts in radiation-induced apoptosis: ‘premitotic apoptosis’ and ‘postmitotic apoptosis’. *J Cell Mol Med* 2001; 5:240–53.
139. Woynarowski JM, Woynarowska BA, Trevino AV, et al. Cell cycle effects and induction of premitotic apoptosis by irrofulven in synchronized cancer cells. *Cancer Biol Ther* 2004; 3:1137–42.
140. Woynarowski JM, Koester S, Woynarowska B, et al. Is drug-induced DNA damage sufficient for apoptosis induction? *Proc Am Assoc Cancer Res* 1999; 40:736.
141. Woynarowska BA, Woynarowski JM. Preferential targeting of apoptosis in tumor versus normal cells. *Biochim Biophys Acta* 2002; 1587:309–17.
142. Cao PR, McHugh MM, Melendy T, Beerman T. The DNA minor groove-alkylating cyclopropylpyrroloindole drugs adozelesin and bizelesin induce different DNA damage response pathways in human colon carcinoma HCT116 cells. *Mol Cancer Ther* 2003; 2:651–9.
143. Tada-Oikawa S, Oikawa S, Kawanishi M, Yamada M, Kawanishi S. Generation of hydrogen peroxide precedes loss of mitochondrial membrane potential during DNA alkylation-induced apoptosis. *FEBS Lett* 1999; 442:65–9.
144. Bianchi N, Chiarabelli C, Borgatti M, Mischiati C, Fibach E, Gambari R. Accumulation of gamma-globin mRNA and induction of erythroid differentiation after treatment of human leukaemic K562 cells with tallimustine. *Br J Haematol* 2001; 113:951–61.
145. Yamori T, Matsunaga A, Sato S, et al. Potent antitumor activity of MS-247, a novel DNA minor groove binder, evaluated by an *in vitro* and *in vivo* human cancer cell line panel. *Cancer Res* 1999; 59:4042–9.
146. Collis SJ, Schwaninger JM, Ntambi AJ, Keller TW, Nelson WG, Dillehay LE, Dewese TL. Evasion of early cellular response mechanisms following low level radiation-induced DNA damage. *J Biol Chem* 2004; 279:49624–49632.
147. Iwase H, Sakurada K, Ikegaya H, et al. Thiol-oxidizing agent diamide and acidic pH enhance lipid peroxidation of rat heart mitochondria and cardiolipin-cytochrome c complex. *IUBMB Life* 2001; 51:39–43.
148. Coffey RN, Watson RW, Hegarty NJ, et al. Thiol-mediated apoptosis in prostate carcinoma cells. *Cancer* 2000; 88:2092–104.
149. Kushnareva YE, Sokolove PM. Prooxidants open both the mitochondrial permeability transition pore and a low-conductance channel in the inner mitochondrial membrane. *Arch Biochem Biophys* 2000; 376:377–88.

150. Pallis M, Grundy M, Turzanski J, Kofler R, Russell N. Mitochondrial membrane sensitivity to depolarization in acute myeloblastic leukemia is associated with spontaneous *in vitro* apoptosis, wild-type TP53, and vicinal thiol/disulfide status. *Blood* 2001; 98:405–13.
151. Dirsch VM, Stuppner H, Vollmar AM. Cytotoxic sesquiterpene lactones mediate their death-inducing effect in leukemia T cells by triggering apoptosis. *Planta Med* 2001; 67:557–9.
152. Chandra J, Samali A, Orrenius S. Triggering and modulation of apoptosis by oxidative stress. *Free Radic Biol Med* 2000; 29:323–33.
153. Hirota K, Nakamura H, Masutani H, Yodoi J. Thioredoxin superfamily and thioredoxin-inducing agents. *Ann N Y Acad Sci* 2002; 957:189–99.
154. Hochachka PW, Rupert JL, Goldenberg L, Gleave M, Kozlowski P. Going malignant: the hypoxia-cancer connection in the prostate. *Bioessays* 2002; 24:749–57.
155. Higuchi M, Manna SK, Sasaki R, Aggarwal BB. Regulation of the activation of nuclear factor kappaB by mitochondrial respiratory function: evidence for the reactive oxygen species-dependent and -independent pathways. *Antioxid Redox Signal* 2002; 4:945–55.
156. Boros LG, Lee WN, Go VL. A metabolic hypothesis of cell growth and death in pancreatic cancer. *Pancreas* 2002; 24:26–33.
157. Cook JA, Gius D, Wink DA, Krishna MC, Russo A, Mitchell JB. Oxidative stress, redox, and the tumor microenvironment. *Semin Radiat Oncol* 2004; 14:259–66.
158. Manoharan S, Kolanjiappan K, Kayalvizhi M, Sethupathy S. Lipid peroxidation and antioxidant status in cervical cancer patients. *J Biochem Mol Biol Biophys* 2002; 6:225–7.
159. Kumaraguruparan R, Kabalimoorthy J, Nagini S. Correlation of tissue lipid peroxidation and antioxidants with clinical stage and menopausal status in patients with adenocarcinoma of the breast. *Clin Biochem* 2005; 38:154–8.
160. Hileman EO, Liu J, Albitar M, Keating MJ, Huang P. Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity. *Cancer Chemother Pharmacol* 2004;53:209–19.
161. Zhang S, Ong CN, Shen HM. Critical roles of intracellular thiols and calcium in parthenolide-induced apoptosis in human colorectal cancer cells. *Cancer Lett* 2004; 208:143–53.
162. Merrill JC, Kim HL, Safe S, Murray CA, Hayes MA. Role of glutathione in the toxicity of the sesquiterpene lactones hymenoxon and helenalin. *J Toxicol Environ Health* 1988; 23:159–69.
163. Jodynis-Liebert J, Murias M, Bloszyk E. Effect of several sesquiterpene lactones on lipid peroxidation and glutathione level. *Planta Med* 1999; 65:320–4.
164. Guzman ML, Rossi RM, Karnischky L, et al. The sesquiterpene lactone parthenolide induces apoptosis of human acute myelogenous leukemia stem and progenitor cells. *Blood* 2005;105:4163–9.
165. Zhang S, Ong CN, Shen HM. Involvement of proapoptotic Bcl-2 family members in parthenolide-induced mitochondrial dysfunction and apoptosis. *Cancer Lett* 2004; 211:175–88.
166. Pozarowski P, Halicka DH, Darzynkiewicz Z. Cell cycle effects and caspase-dependent and independent death of HL-60 and Jurkat cells treated with the inhibitor of NF-kappaB parthenolide. *Cell Cycle* 2003; 2:377–83.
167. Nakshatri H, Rice SE, Bhat-Nakshatri P. Antitumor agent parthenolide reverses resistance of breast cancer cells to tumor necrosis factor-related apoptosis-inducing ligand through sustained activation of c-Jun N-terminal kinase. *Oncogene* 2004; 23:7330–44.
168. Zhang S, Lin ZN, Yang CF, Shi X, Ong CN, Shen HM. Suppressed NF-kappaB and sustained JNK activation contribute to the sensitization effect of parthenolide to TNF-alpha-induced apoptosis in human cancer cells. *Carcinogenesis* 2004; 25:2191–9.
169. Riggins RB, Zwart A, Nehra R, Clarke R. The nuclear factor kappa B inhibitor parthenolide restores ICI 182,780 (Faslodex; fulvestrant)-induced apoptosis in antiestrogen-resistant breast cancer cells. *Mol Cancer Ther* 2005; 4:33–41.
170. Patel NM, Nozaki S, Shortle NH, et al. Paclitaxel sensitivity of breast cancer cells with constitutively active NF-kappaB is enhanced by I kappa B alpha super-repressor and parthenolide. *Oncogene* 2000; 19:4159–69.
171. Jordan J, d'Arcy Doherty M, Cohen GM. Effects of glutathione depletion on the cytotoxicity of agents toward a human colonic tumour cell line. *Br J Cancer* 1987; 55:627–31.
172. Heilmann J, Wasescha MR, Schmidt TJ. The influence of glutathione and cysteine levels on the cytotoxicity of helenanolide type sesquiterpene lactones against KB cells. *Bioorg Med Chem* 2001; 9:2189–94.
173. Watson WH, Yang X, Choi YE, Jones DP, Kehrer JP. Thioredoxin and its role in toxicology. *Toxicol Sci* 2004; 78:3–14.

174. Nkabyo YS, Ziegler TR, Gu LH, Watson WH, Jones DP. Glutathione and thioredoxin redox during differentiation in human colon epithelial (Caco-2) cells. *Am J Physiol Gastrointest Liver Physiol* 2002; 283:G1352–9.
175. Trotter EW, Grant CM. Non-reciprocal regulation of the redox state of the glutathione-glutaredoxin and thioredoxin systems. *EMBO Rep* 2003; 4:184–8.
176. Davis W, Jr., Ronai Z, Tew KD. Cellular thiols and reactive oxygen species in drug-induced apoptosis. *J Pharmacol Exp Ther* 2001; 296:1–6.
177. Nordberg J, Arner ES. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* 2001; 31:1287–312.
178. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 2001; 30:1191–212.
179. Saitoh M, Nishitoh H, Fujii M, et al. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* 1998; 17:2596–606.
180. Liu H, Nishitoh H, Ichijo H, Kyriakis JM. Activation of apoptosis signal-regulating kinase 1 (ASK1) by tumor necrosis factor receptor-associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin. *Mol Cell Biol* 2000; 20:2198–208.
181. Karimpour S, Lou J, Lin LL, et al. Thioredoxin reductase regulates AP-1 activity as well as thioredoxin nuclear localization via active cysteines in response to ionizing radiation. *Oncogene* 2002; 21:6317–27.
182. Welsh SJ, Powis G. Hypoxia inducible factor as a cancer drug target. *Curr Cancer Drug Targets* 2003; 3:391–405.
183. Sen CK. Cellular thiols and redox-regulated signal transduction. *Curr Top Cell Regul* 2000; 36:1–30.
184. Ueno M, Masutani H, Arai RJ, et al. Thioredoxin-dependent redox regulation of p53-mediated p21 activation. *J Biol Chem* 1999; 274:35809–15.
185. Hainaut P, Mann K. Zinc binding and redox control of p53 structure and function. *Antioxid Redox Signal* 2001; 3:611–23.
186. Kondo N, Ishii Y, Kwon YW, et al. Redox-sensing release of human thioredoxin from T lymphocytes with negative feedback loops. *J Immunol* 2004; 172:442–8.
187. Lincoln DT, Ali Emadi EM, Tonissen KF, Clarke FM. The thioredoxin-thioredoxin reductase system: over-expression in human cancer. *Anticancer Res* 2003; 23:2425–33.
188. Gromer S, Urig S, Becker K. The thioredoxin system—from science to clinic. *Med Res Rev* 2004; 24:40–89.
189. Powis G, Mustacich D, Coon A. The role of the redox protein thioredoxin in cell growth and cancer. *Free Radic Biol Med* 2000; 29:312–22.
190. Biaglow JE, Miller RA. The thioredoxin reductase/thioredoxin system: novel redox targets for cancer therapy. *Cancer Biol Ther* 2005; 4:6–13.
191. Woynarowska BA, Alvarez A, Woynarowski JM. The potential of irifulven to overcome chemoresistance of hypoxic cells. *Proc Am Assoc Cancer Res* 2005; 46:779.
192. Thomas JP, Wartman MB, Bailey HH. *In vitro* cytotoxicity of HMAF with and without buthionine sulfoximine against drug-resistant tumor cell lines with elevated glutathione. *Proc Am Assoc Cancer Res* 1999; 40:297.
193. Herzig MC, Liang H, Johnson AE, Woynarowska B, Woynarowski JM. Irifulven induces apoptosis in breast cancer cells regardless of caspase-3 status. *Breast Cancer Res Treat* 2002; 71:133–43.
194. Izbicka E, Davidson K, Lawrence R, Cote R, MacDonald JR, Von Hoff DD. Cytotoxic effects of MGI 114 are independent of tumor p53 or p21 expression. *Anticancer Res* 1999; 19:1299–307.
195. Jaspers NG, Raams A, Kelner MJ, et al. Anti-tumour compounds illudin S and Irifulven induce DNA lesions ignored by global repair and exclusively processed by transcription- and replication-coupled repair pathways. *DNA Repair (Amst)* 2002; 1:1027–38.
196. Koepfel F, Poindessous V, Lazar V, Raymond E, Sarasin A, Larsen AK. Irifulven cytotoxicity depends on transcription-coupled nucleotide excision repair and is correlated with XPG expression in solid tumor cells. *Clin Cancer Res* 2004; 10:5604–13.
197. Ayene IS, Koch CJ, Tuttle SW, Stamato TD, Perez ML, Biaglow JE. Oxidation of cellular thiols by hydroxyethyl-disulphide inhibits DNA double-strand-break rejoining in G6PD deficient mammalian cells. *Int J Radiat Biol* 2000; 76:1523–31.
198. Barzilai A, Yamamoto K. DNA damage responses to oxidative stress. *DNA Repair (Amst)* 2004; 3:1109–15.

199. Liang H, Leal B, Baluna R, Keovongxay P, Salinas RA, Woynarowska BA. Growth inhibition and apoptosis by irifolven in combination with γ -radiation in model brain tumor cells. *Proc Am Assoc Cancer Res* 2002; 43:130.
200. Faivre S, Chan D, Woynarowska B, Woynarowski JM. DNA strand breaks and apoptosis induced by oxaliplatin in cancer cells. *Biochemical Pharmacol* 2003; 66:225–37.
201. Barry MA, Behnke CA, Eastman A. Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. *Biochem Pharmacol* 1990; 40:2353–62.
202. Welsh C, Day R, McGurk C, Masters JR, Wood RD, Koberle B. Reduced levels of XPA, ERCC1 and XPF DNA repair proteins in testis tumor cell lines. *Int J Cancer* 2004; 110:352–61.
203. Spierings DC, de Vries EG, Vellenga E, de Jong S. The attractive Achilles heel of germ cell tumours: an inherent sensitivity to apoptosis-inducing stimuli. *J Pathol* 2003; 200:137–48.
204. Schweyer S, Soruri A, Meschter O, et al. Cisplatin-induced apoptosis in human malignant testicular germ cell lines depends on MEK/ERK activation. *Br J Cancer* 2004; 91:589–98.
205. Burger H, Nooter K, Boersma AW, Kortland CJ, Stoter G. Lack of correlation between cisplatin-induced apoptosis, p53 status and expression of Bcl-2 family proteins in testicular germ cell tumour cell lines. *Int J Cancer* 1997; 73:592–9.
206. Lai MD, Lin WC, Sun YM, Chang FL. Phosphorylated and hypoacetylated mutant p53 enhances cisplatin-induced apoptosis through caspase-9 pathway in the absence of transcriptional activation or translation. *Int J Mol Med* 2005; 15:725–34.
207. Kim JS, Lee JM, Chwae YJ, et al. Cisplatin-induced apoptosis in Hep3B cells: mitochondria-dependent and -independent pathways. *Biochem Pharmacol* 2004; 67:1459–68.
208. Arango D, Wilson AJ, Shi Q, et al. Molecular mechanisms of action and prediction of response to oxaliplatin in colorectal cancer cells. *Br J Cancer* 2004; 91:1931–46.
209. Gourdiere I, Crabbe L, Andreau K, Pau B, Kroemer G. Oxaliplatin-induced mitochondrial apoptotic response of colon carcinoma cells does not require nuclear DNA. *Oncogene* 2004; 23:7449–57.
210. Seo YR, Chen EI, Smith ML. Sensitivity of p53-deficient cells to oxaliplatin and thio-TEPA (N, N', N'' triethylenethiophosphoramidate). *Breast Cancer Res Treat* 2002; 72:255–63.
211. Petit T, Bearss DJ, Troyer DA, Munoz RM, Windle JJ. p53-independent response to cisplatin and oxaliplatin in MMTV-ras mouse salivary tumors. *Mol Cancer Ther* 2003; 2:165–71.
212. Evans DL, Tilby M, Dive C. Differential sensitivity to the induction of apoptosis by cisplatin in proliferating and quiescent immature rat thymocytes is independent of the levels of drug accumulation and DNA adduct formation. *Cancer Res* 1994; 54:1596–603.
213. Hector S, Bolanowska-Higdon W, Zdanowicz J, Hitt S, Pendyala L. *In vitro* studies on the mechanism of oxaliplatin resistance. *Cancer Chemother Pharmacol* 2001; 48:398–406.
214. Arnould S, Hennebelle I, Canal P, Bugat R, Guichard S. Cellular determinants of oxaliplatin sensitivity in colon cancer cell lines. *Eur J Cancer* 2003; 39:112–9.
215. Mandic A, Hanson J, Linder S, Shoshan MC. Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signaling. *J Biol Chem* 2003; 278:9100–6.
216. Becker K, Herold-Mende C, Park JJ, Lowe G, Schirmer RH. Human thioredoxin reductase is efficiently inhibited by (2, 2' : 6', 2'-terpyridine)platinum(II) complexes. Possible implications for a novel antitumor strategy. *J Med Chem* 2001; 44:2784–92.
217. Nakamura H, Bai J, Nishinaka Y, et al. Expression of thioredoxin and glutaredoxin, redox-regulating proteins, in pancreatic cancer. *Cancer Detect Prev* 2000; 24:53–60.
218. Suzuki Y, Kondo Y, Himeno S, Nemoto K, Akimoto M, Imura N. Role of antioxidant systems in human androgen-independent prostate cancer cells. *Prostate* 2000; 43:144–9.
219. Yokomizo A, Ono M, Nanri H, et al. Cellular levels of thioredoxin associated with drug sensitivity to cisplatin, mitomycin C, doxorubicin, and etoposide. *Cancer Res* 1995; 55:4293–6.
220. Yamada M, Tomida A, Yoshikawa H, Taketani Y, Tsuruo T. Increased expression of thioredoxin/adult T-cell leukemia-derived factor in cisplatin-resistant human cancer cell lines. *Clin Cancer Res* 1996; 2:427–32.
221. Bragadin M, Scutari G, Folda A, Bindoli A, Rigobello MP. Effect of metal complexes on thioredoxin reductase and the regulation of mitochondrial permeability conditions. *Ann N Y Acad Sci* 2004; 1030:348–54.
222. Sasada T, Iwata S, Sato N, et al. Redox control of resistance to cis-diamminedichloroplatinum (II) (CDDP): protective effect of human thioredoxin against CDDP-induced cytotoxicity. *J Clin Invest* 1996; 97:2268–76.

223. Kawahara N, Tanaka T, Yokomizo A, et al. Enhanced coexpression of thioredoxin and high mobility group protein 1 genes in human hepatocellular carcinoma and the possible association with decreased sensitivity to cisplatin. *Cancer Res* 1996; 56:5330–3.
224. Arnold NB, Ketterer K, Kleeff J, Friess H, Buchler MW, Korc M. Thioredoxin is downstream of Smad7 in a pathway that promotes growth and suppresses cisplatin-induced apoptosis in pancreatic cancer. *Cancer Res* 2004; 64:3599–606.
225. Yamada M, Tomida A, Yoshikawa H, Taketani Y, Tsuruo T. Overexpression of thioredoxin does not confer resistance to cisplatin in transfected human ovarian and colon cancer cell lines. *Cancer Chemother Pharmacol* 1997; 40:31–7.
226. Sasada T, Nakamura H, Ueda S, et al. Secretion of thioredoxin enhances cellular resistance to cis-diamminedichloroplatinum (II). *Antioxid Redox Signal* 2000; 2:695–705.
227. Herzig MCS, Liang H, Johnson AE, Woynarowska B, Woynarowski JM. Irofulven induces apoptosis in breast cancer cells regardless of caspase-3 status. *Breast Cancer Res Treat* 2002; 71:133–43.
228. Halestrap AP, Brenner C. The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death. *Curr Med Chem* 2003; 10:1507–25.
229. Marchetti P, Galla DA, Russo FP, et al. Apoptosis induced by oxaliplatin in human colon cancer HCT15 cell line. *Anticancer Res* 2004; 24:219–26.
230. Ferrer M, Izeboud T, Ferreira CG, Span SW, Giaccone G, Kruyt FA. Cisplatin triggers apoptotic or nonapoptotic cell death in Fanconi anemia lymphoblasts in a concentration-dependent manner. *Exp Cell Res* 2003; 286:381–95.
231. de Jong S, Timmer T, Heijenbrok FJ, de Vries EG. Death receptor ligands, in particular TRAIL, to overcome drug resistance. *Cancer Metastasis Rev* 2001; 20:51–6.
232. Poulaki V, Mitsiades CS, Mitsiades N. The role of Fas and FasL as mediators of anticancer chemotherapy. *Drug Resist Updat* 2001; 4:233–42.
233. Ferreira CG, Span SW, Peters GJ, Kruyt FA, Giaccone G. Chemotherapy triggers apoptosis in a caspase-8-dependent and mitochondria-controlled manner in the non-small cell lung cancer cell line NCI-H460. *Cancer Res* 2000; 60:7133–41.
234. Boersma AW, Nooter K, Burger H, Kortland CJ, Stoter G. Bax upregulation is an early event in cisplatin-induced apoptosis in human testicular germ-cell tumor cell line NT2, as quantitated by flow cytometry. *Cytometry* 1997; 27:275–82.
235. Hayward RL, Macpherson JS, Cummings J, Monia BP, Smyth JF, Jodrell DI. Enhanced oxaliplatin-induced apoptosis following antisense Bcl-x1 down-regulation is p53 and Bax dependent: genetic evidence for specificity of the antisense effect. *Mol Cancer Ther* 2004; 3:169–78.
236. Schloffer D, Horky M, Kotala V, Wesierska-Gadek J. Induction of cell cycle arrest and apoptosis in human cervix carcinoma cells during therapy by cisplatin. *Cancer Detect Prev* 2003; 27:481–93.
237. Azuma M, Tamatani T, Ashida Y, Takashima R, Harada K, Sato M. Cisplatin induces apoptosis in oral squamous carcinoma cells by the mitochondria-mediated but not the NF-kappaB-suppressed pathway. *Oral Oncol* 2003; 39:282–9.
238. Weller M, Malipiero U, Aguzzi A, Reed JC, Fontana A. Protooncogene bcl-2 gene transfer abrogates Fas/APO-1 antibody-mediated apoptosis of human malignant glioma cells and confers resistance to chemotherapeutic drugs and therapeutic irradiation. *J Clin Invest* 1995; 95:2633–43.
239. Del Bello B, Valentini MA, Zunino F, Comporti M, Maellaro E. Cleavage of Bcl-2 in oxidant- and cisplatin-induced apoptosis of human melanoma cells. *Oncogene* 2001; 20:4591–5.
240. Asselin E, Mills GB, Tsang BK. XIAP regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. *Cancer Res* 2001; 61:1862–8.
241. Wesierska-Gadek J, Gueorguieva M, Horky M. Dual action of cyclin-dependent kinase inhibitors: induction of cell cycle arrest and apoptosis. A comparison of the effects exerted by roscovitine and cisplatin. *Pol J Pharmacol* 2003; 55:895–902.
242. Polisenio L, Mariani L, Collecchi P, Piras A, Zaccaro L, Rainaldi G. Bcl2-negative MCF7 cells overexpress p53: implications for the cell cycle and sensitivity to cytotoxic drugs. *Cancer Chemother Pharmacol* 2002; 50:127–30.
243. Blanc C, Deveraux QL, Krajewski S, et al. Caspase-3 is essential for procaspase-9 processing and cisplatin-induced apoptosis of MCF-7 breast cancer cells. *Cancer Res* 2000; 60:4386–90.
244. Del Bello B, Valentini MA, Mangiavacchi P, Comporti M, Maellaro E. Role of caspases-3 and -7 in Apaf-1 proteolytic cleavage and degradation events during cisplatin-induced apoptosis in melanoma cells. *Exp Cell Res* 2004; 293:302–10.

245. Cummings BS, Kinsey GR, Bolchoz LJ, Schnellmann RG. Identification of caspase-independent apoptosis in epithelial and cancer cells. *J Pharmacol Exp Ther* 2004;310:126–134.
246. Woynarowska B, Woynarowski JM, Jackson JA, Moyer MP, Waters SJ. Dissimilar patterns of transcriptional and proteome responses produced by iriffulven in tumor (LNCaP-Pro5) and normal (NCM 460) cell lines. *Proc Am Assoc Cancer Res* 2003; 44:913.
247. Woynarowski JM, Woynarowska BA, Trevino AV, Moyer MP, Waters SJ. Cytoplasmic components of apoptosis by iriffulven revealed by cell-free apoptotic systems. *Proc Am Assoc Cancer Res* 2004; 45:Abstract 5185.
248. Yen HC, Nien CY, Majima HJ, et al. Increase of lipid peroxidation by cisplatin in WI38 cells but not in SV40-transformed WI38 cells. *J Biochem Mol Toxicol* 2003; 17:39–46.
249. Devarajan P, Savoca M, Castaneda MP, et al. Cisplatin-induced apoptosis in auditory cells: role of death receptor and mitochondrial pathways. *Hear Res* 2002; 174:45–54.
250. Schneider ED, Kay JJ. Life as a manifestation of the second law of thermodynamics. Mathematical and Computer Modelling. *Math Comput Model* 1992; 19:25–48.
251. Toussaint O, Remacle J, Dierick JF, et al. Approach of evolutionary theories of ageing, stress, senescence-like phenotypes, calorie restriction and hormesis from the view point of far-from-equilibrium thermodynamics. *Mech Ageing Dev* 2002; 123:937–46.
252. Burgoyne LA. The mechanisms of pyknosis: hypercondensation and death. *Exp Cell Res* 1999; 248:214–22.

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Contributions of Apoptosis and Senescence to Cytotoxicity Produced by Microtubule-Stabilizing Agents

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SUMMARY

The antitumor drug Taxol is recognized for its efficacy in the treatment of cancer and has become the prototype for a class of drugs known as the microtubule-stabilizing agents (MSAs). Despite years of investigation, a clear and detailed mechanism by which these compounds bring about tumor cell death remains elusive. Apoptosis, with its well-defined biochemical and cell-structural characteristics, has often been presented as the most important direct result of treatment with Taxol. It is now clear that the major response to the MSAs is far more complex and depends on the cell type, the specific drug being studied, and the concentration of drug to which the cell is exposed. Although apoptosis may occur at a downstream step in the response of cells to these agents, it has not been demonstrated that this effect is the direct result of the binding of Taxol to its known target, the microtubules, except in hematopoietic cancer cells. Other cell death processes that do follow directly from the binding of Taxol to the microtubules include caspase-independent cell death, with or without an apoptotic morphology, and a characteristic aberrant mitosis that is often referred to as mitotic catastrophe. These processes appear to produce a significant amount of cell death well after that which would be produced by direct caspase activation. In addition to cell killing, cytotoxic agents are known to elicit the stress-induced, terminal cell proliferation referred to as premature or accelerated senescence. This response had been thought to play a minor role in the actions of the MSAs, but recently, one such agent, discodermolide, was shown to elicit a powerful induction of accelerated senescence. The senescence phenotype appeared after the characteristic aberrant mitosis seen with these agents, and it was invariably followed by a delayed form of cell death. The powerful effects of Taxol and much of the promise perceived in newer MSAs appear to result from their complex and widespread effects on cell function and from many options they have for inhibiting uncontrolled tumor cell proliferation.

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1. INTRODUCTION

Taxol (paclitaxel) was the first microtubule-stabilizing agent (MSA) reported (1,2), and today, many other compounds with a similar mechanism of action have been described (3). Approved by the FDA for ovarian, breast, and small-cell lung cancer, it is used in the treatment of a wide variety of cancers. A second-generation taxane, Taxotere (docetaxol), is approved for metastatic- and anthracycline-resistant breast cancer (4). Other naturally occurring compounds, not structurally related to Taxol, have been found to produce comparable microtubule-stabilizing effects in cancer cell lines and inhibition of tumor cell growth, while improving upon some of the problems encountered with Taxol, such as those involving solubility and resistance. The epothilones, discodermolide, and laulimalide have been extensively studied in an effort to identify important structural correlates with regard to microtubule effects, drug resistance, and pharmacological properties and have also been incorporated into clinical trials (5–7). In addition, one of these agents, discodermolide, has demonstrated synergism when administered with Taxol to cell lines and to mice harboring tumor xenografts (8,9).

Many have attempted to uncover the mechanism(s) by which MSAs suppress tumor growth. The primary effects of Taxol have long been believed to result from its interference with mitotic spindle assembly and function, leading to the failure of accurate chromosome segregation (10). Yet, the path to tumor growth inhibition and cell death that follows MSA binding to the major target, microtubules, has not been adequately defined. Despite extensive experimentation that describes this cell death as being apoptotic, the story is more complex than what can be described as apoptotic cell death and varies with the specific drug, its concentration, and tumor type (11–13).

Given the importance to an organism of a cell's decision to live or die, it is intuitively reasonable that redundant mechanisms may have evolved to assure that more than one cell death option is available. Many have criticized the use of the term apoptosis as a catchall for cell death accompanied by caspase activation following treatment with cytotoxic agents and have demonstrated other, caspase independent, mechanisms of cell death (14–17). The actions of Taxol and the other MSAs also appear to support these observations in that many different cell signals are initiated after microtubule stabilization in different cell types, and these processes can initiate a delayed, downstream activation of caspases and/or other proteases, all of which may contribute to cell death (18,19).

2. CELL DEATH AFTER MICROTUBULE STABILIZATION

Certain effects on microtubule structure and function are well established as being direct actions of Taxol and the other MSAs. Most of these agents bind to β -tubulin that is in the α/β -tubulin dimer and promote microtubule stabilization, inhibit microtubule disassembly, and suppress the normal dynamic behavior of microtubules because of the inhibition of lengthening and shrinking at their polymer ends (20,21). This stabilization interferes with a host of diverse cell functions including the maintenance of cell structure, intracellular trafficking of proteins and vesicles by motor proteins and both the capture and the separation of sister chromatids during mitosis (22–25). As mitotic spindle microtubules are more dynamic than those assembled during interphase, the

binding of an MSA is particularly disruptive of cell division and the checkpoint processes that insure its fidelity (26). Depending on cell type and drug concentration, these initial effects can produce a number of secondary responses ranging from mitotic arrest leading to apoptosis to aberrant mitosis with multipolar spindles that yield aneuploid interphase cells. An impressive amount of delayed cell death may occur, sometimes involving caspase activity (27–29).

2.1. Apoptosis

As Taxol-induced cell death has been reported to be accompanied by some caspase activation, and/or to produce an apoptotic morphology, it has frequently been said to result from apoptosis. The assumption had been, for many years, that MSAs induce programmed cell death through inhibitory actions on the mitotic spindle (30–32). One of the weaknesses in this assumption is that in many of the studies cited, cancer cell lines were exposed to micromolar drug concentrations for prolonged periods of time. Under these conditions, cells can arrest in metaphase, prior to apoptosis, enter a G1 block, or be involved in endoreduplication (DNA proliferation uncoupled from cell division) (28). Although Taxol infusions in humans may expose a small population of cells within the tumor to a brief spike of a micromolar drug concentration, drug metabolism and clearance quickly reduce this concentration to the nanomolar range (33). Thus, long-term, high concentrations of Taxol cannot address the question of which cell growth-inhibitory mechanisms participate during drug treatment.

A second issue concerns the many studies that purport to assess cell killing of a specific cancer cell type by an MSA but consider only cell death that consists of the rapid appearance of classical apoptotic morphological characteristics. When Taxol or another MSA binds to microtubules at a more clinically relevant, nanomolar concentration, both non-transformed and cancer cells with wild-type p53 and p21 expression arrest in G1 and/or G2 of the cell cycle, protecting them from mitotic arrest. Cells that do not express either a functional p53 or a functional p21 are more sensitive to mitotic arrest and the associated caspase-dependent cell death with an apoptotic morphology (28,34). In either case, the cells that manage to exit from mitotic arrest and enter into the G1 phase display aneuploidy and multiple micronuclei and are often referred to as having undergone slippage (28,29). This leads to a poorly understood process that may play a major role in cell killing by these drugs and has been referred to as “slow death” (13). In ovarian cancer cells treated with nanomolar concentrations of Taxol, it was reported that there was almost no decrease in cell viability after 24 h and only a 10% decrease in cell number at 48 and 72 h (35). Although values for cell death were not obtained at later time points, these authors report that only a small percentage of the surviving cells were able to proliferate and form colonies. These findings support the idea that Taxol treatment acts through a mechanism leading to a delayed cell death. Such delayed cell killing was shown to occur despite the use of broad spectrum caspase inhibitors but was blocked by inhibitors of the lysosomal protease cathepsin B, suggesting that lysosomal permeabilization is affected by the binding of MSAs and may play a role in this delayed cell killing by Taxol (36).

Taxol has also been reported to initiate apoptosis by various cell-signaling pathways. One of the most actively investigated areas is the phosphorylation of Bcl-2 following c-Raf-1 or Ras activation (37–39) or mTOR activation (40). Although the inactivation of Bcl-2 undoubtedly contributes to Taxol-mediated cell killing, the subject

remains controversial given that the observed Taxol-mediated phosphorylation may reflect the increased percentage of cells blocked in mitosis (41,42). Taxol also directly modulates the levels of Bcl-2 family members in certain cancer cell models (43–46). The drug produces an aberrant activation of cyclin-dependent kinases with checkpoint protein activation that may play a role in the cell death resulting from microtubule stabilization (47,48). Depending on cell type, these agents have been shown to produce activation/suppression of one or more of the mitogen-activated protein kinases (MAPKs), which may also play a role in the cell killing observed (49–53).

Although many such studies can be cited as evidence of the ability of MSAs to produce cell death, this approach has demonstrated convincingly a prominent role for apoptosis only in the death of hematopoietic cancer cells (54–57). In solid tumors, such studies have shown caspase activation to be delayed, often occurring downstream of the commitment to cell death. This suggests that apoptosis is only one of the many possible mechanisms involved in Taxol-mediated cell death (12,13,18,58–61). Other studies have noted a lack of caspase activation prior to cell death resulting from Taxol administration (11,62) or unchanged cell killing after the inhibition of caspases produces a loss of apoptotic phenotype (63). However, cell death with a classically apoptotic morphology may also be produced by other proteases (19). Thus, although caspase activation has been shown to participate in cell killing by MSAs, and the treated cancer cells often present with a classical apoptotic morphology, apoptosis has not been demonstrated to be the major cell death pathway utilized by these agents.

2.2. Mitotic Catastrophe (Fig. 1)

Tumor cells grown in the presence of MSAs produce abnormal mitotic figures and yet often manage to exit this abnormal mitosis because of deficiencies in prophase checkpoint proteins. These cells undergo an apoptotic cell death or, more commonly, become enlarged, display multiple micronuclei, and undergo a delayed form of cell death. The term mitotic catastrophe has been used, by different investigators, to describe both the apoptotic cell death and the delayed death of the giant multinucleated cells (64,65). Still others have suggested that mitotic catastrophe is not a mechanism of cell death, such as apoptosis or necrosis, but a condition that results from either a failure to complete cell division or a fusion of cells after mitosis (66). In the scenario where mitotic catastrophe is defined and investigated as a special case of apoptosis, cells enter into and die in metaphase after DNA structure checkpoint inactivation, caspase activation, and mitochondrial damage. This pattern of cell killing has been demonstrated to require a functional spindle checkpoint in a series of experiments involving the disruption of kinetochore protein function and therefore the spindle checkpoint. When this occurred in p53-deficient cancer cells treated with DNA-damaging agents, they survived long enough to undergo an aberrant mitosis, form multinucleated cells, and undergo a delayed cell death (67). Although some caspase activation may accompany the death of multinucleated cells following Taxol treatment, the absence of both DNA ladder formation and DNA breaks detectable with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining argues for this pathway being distinct from apoptosis (68,69). Similarly, neither caspase inhibition nor Bcl-2 overexpression prevent aberrant mitosis or the formation of multinucleated cells, further supporting the contention that the resulting cell death is non-apoptotic (70–72).

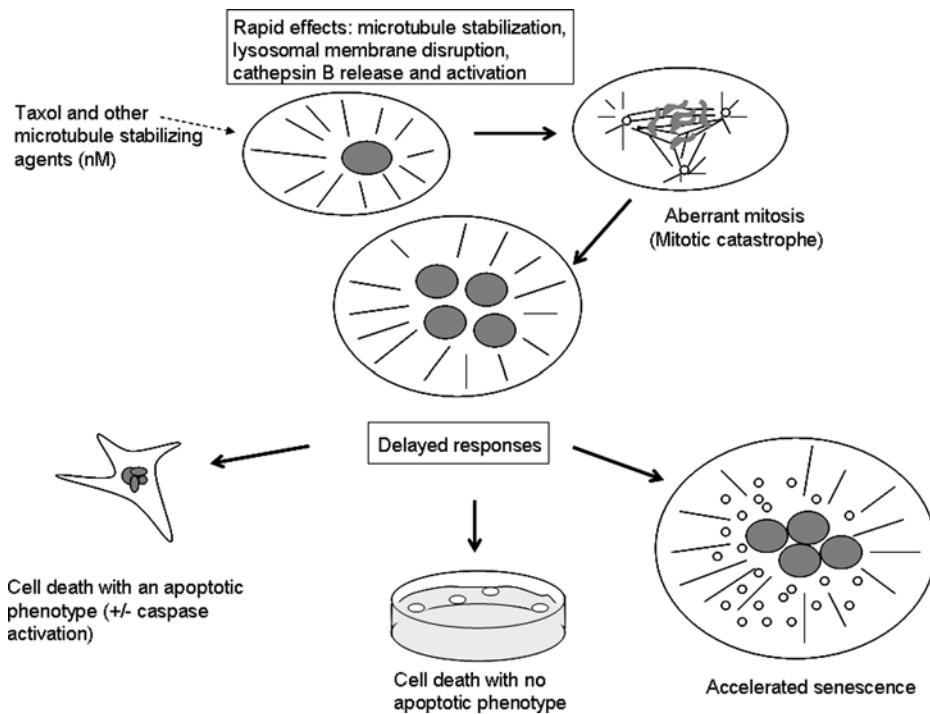


Fig. 1. The response of solid tumor cells to nanomolar concentrations of Taxol. Following Taxol exposure but prior to mitosis, microtubules are stabilized and lysosomal membranes disrupted. Owing to the latter effect, proteases, including cathepsin B, may be released and activated. After an aberrant mitotic division (mitotic catastrophe), cells enter G1 enlarged and multi/micronucleated. These cells later die with an apoptotic phenotype that may or may not be attributed to caspase activation, lose their ability to adhere to the plate, and die without any indication of apoptosis or present with an enlarged, flattened senescent phenotype (including an enhanced production of lysosomal vesicles) prior to cell death.

In their review on mitotic death, Erenpreisa and Cragg (73) describe and contrast rapid death occurring in sensitive cells within a few hours of genotoxic insult, with the delayed reproductive or “mitotic” death pathway seen in more resistant (p53-mutated) cancer cells. Some of the characteristics described for the latter include “a disruption of coupling between interphase apoptosis and the G1/S checkpoint, a delay during the G2 phase of the cell cycle, aberrant mitoses which can end in mitotic death, formation and disintegration of multinuclear giant cells” (which they say is often, but not always, called mitotic catastrophe), “delayed apoptosis and, sometimes despite all this, cell survival” (73). They describe how this uncoupling of apoptosis from the G1/S checkpoint and the push toward a G2 arrest, or a subsequent endoreduplication, can yield increasingly greater repair capacity, favoring cell survival over apoptosis. More specifically, related to Taxol and the MSAs, they cite reports that down-regulation of the anaphase-promoting complex activity by spindle damage may retard exit from mitosis and allow entry into the endocycle (74–76). From this perspective, the formation of multinucleated cells following treatment with MSAs may represent an attempt at repair and cell survival. Despite such attempts, the induction of aneuploidy following an aberrant mitosis creates chromosomal breakage and interchromosomal

concatenation, favoring increased genetic instability in tumor cells (77). The observation that concentrations of MSAs required to inhibit cell proliferation correspond more closely to those concentrations that produce aneuploidy than to those required to induce mitotic arrest, suggesting that delayed, mitotic death plays an important role in the mechanism of MSA action (29).

2.3. Caspase-Independent Programmed Cell Death

Although apoptosis is the best defined and most thoroughly investigated mechanism of programmed cell death, more recent work has begun to define an apoptosis-like programmed cell death that is an active, signaling-dependent process that is not necessarily driven by caspases but is distinct from both autophagic cell death and necrosis (78). Such a process may or may not produce cells with an apoptotic morphology and may be initiated by processes other than the activation of caspases. One such mechanism was observed in human neuroblastoma cells where Taxol induced a rapid, caspase-independent mitochondrial production of reactive oxygen species and cell killing prior to any caspase activation (79). Other proteases such as granzymes, lysosomal cathepsins, matrix metalloproteinases, and proteasomal proteases that have recently been presented as potential players in the cell death brought about by Taxol treatment have just begun to be investigated (19).

One lysosomal protease is now known to participate in the production of aberrant mitosis and killing of cancer cells by MSAs. Taxol, epothilone, and discodermolide were shown to induce lysosomal disruption and cathepsin B activation in non-small-cell lung carcinoma cells (36). The resulting aberrant mitosis and cell death were blocked by the inhibition of cathepsin B activation but not by inhibition of caspases or other lysosomal proteases. Although the underlying mechanism leading from the binding of an MSA to a microtubule to lysosomal membrane disruption is not certain, the authors suggested a possible role for signal transduction cascades. Oncogenic Ras in glioma and gastric cancer cell lines previously had been shown to produce lysosomal vacuoles and a genetically regulated, caspase-independent cell death mechanism (80). Taxol produces just such a prolonged Ras activation in certain cell types, including human esophageal squamous cancer cells (81). Discodermolide was seen to produce an even more powerful and sustained ERK activation than Taxol in A549 non-small-cell lung carcinoma cells (82). These A549 cells subsequently presented as multinucleated senescent cells with extensive cytoplasmic vacuoles and finally underwent a delayed cell death. These observations suggest that the MAPK signaling pathway may play a role in connecting the binding of MSAs to microtubules, with the lysosomal disruption/cathepsin B activation observed in the findings of Broker et al. (36).

3. ACCELERATED SENESCENCE

Premature or accelerated cell senescence occurs when radiation, DNA damage, oxidative damage, overexpression of mitogenic signals such as oncogene activation, or a host of other cell stressors produce a mitogen-resistant, terminal proliferation state in previously dividing cells (83–86). The resulting cells present with a phenotype much like that observed when repeated passaging of normal primary cells produces telomere shortening leading to replicative senescence (87,88). They display the characteristic enlarged, flattened, granular morphology, an interphase (either G1 or G2) cell-cycle

block, changes in protein expression levels and activities, including a β -galactosidase activity at pH 6 (89), but may or may not have the shortened telomeres seen in replicative senescence. The developing consensus is that this “stress-induced” senescence is an important fail-safe mechanism that must be bypassed for either tumorigenesis or resistance to chemotherapy to allow unchecked cell proliferation (90,91). The involvement of many signaling pathways in the production of this proliferation barrier and what was believed until recently to be the irreversible nature of senescence have caused it to be likened to apoptosis and have encouraged a search for a similarly well-defined mechanism (92).

The signaling mechanisms controlling the onset of accelerated senescence are complex and redundant, and this, in itself, suggests the physiological importance of this tumor suppressor mechanism. Stressors have been thought to set off various cell signals that can activate one or both the pathways considered critical for the induction and maintenance of this state (93). Activation of the p19ARF/p53/p21 and the p16INK4a/retinoblastoma (Rb) pathways leads to the dephosphorylation and activation of the Rb protein. The Rb protein associates with and silences the transactivation functions of several transcription factors, significantly the E2F proteins, and thereby down-regulates critical cell-cycle proteins (such as cyclins E and A), inhibits proliferation, and induces the many characteristic features of senescence (94). In many cell types, as in the case with replicative senescence, up-regulation of p21 initiates this process, and the p16/INK4a gene expression is activated at a later time point and maintains the senescence phenotype (95–97). Although inactivation of p53 can reverse the proliferation arrest established by shortened telomeres, inactivation of pRB is unable to overcome the proliferation barrier established by p16 and permit proliferation to resume (98). However, a small percentage of p53 null, p16-deficient H1299 carcinoma cells were observed to escape the senescence produced by any number of chemotherapeutic agents, including the small induction by Taxol. Depending on the drug used, between 5 and 90% of the cells displayed a senescence phenotype after 3 days and a small percentage of these were able to resume proliferation between day 18 and day 24 (99). These “senescence escape cells” overexpressed the cyclin-dependent kinase Cdc2/Cdk1, and these authors proposed that this up-regulation may constitute a mechanism responsible for tumor recurrence.

Although the idea that disruption of cytoskeletal function may initiate a stress response leading to accelerated senescence is intuitively satisfying, the actual mechanisms involved have only recently been explored. An overproduction of vimentin intermediate filaments was observed to accompany senescence in cultured human fibroblasts and promoted a senescent cell morphology in young fibroblasts (100). The stress produced by representative microtubule-binding agents (Taxol and vincristine) was also shown to make a contribution, albeit a weak one when compared with the effect produced by DNA-damaging agents, to the induction of accelerated senescence (69). Discodermolide was recently shown to produce a powerful senescence induction in various carcinoma cell lines (82). Given the similar microtubule-stabilizing actions of Taxol and discodermolide, the synergy they produced, as noted in the introduction, was surprising and difficult to explain (8). Although a relative difference in senescence induction provides one explanation for this synergy, the cause of this differential senescence induction remains unexplained. One of the differences observed between Taxol and discodermolide signaling was that the latter produced a far more powerful

and sustained ERK activation in the wild-type p53, p16 null A549 cells studied. Previous work had demonstrated that oncogenic Ras and Raf could induce senescence in IMR-90 lung fibroblasts through induction of p16 (85,86). More recently, Deng et al. (101) demonstrated that high-intensity signaling from the Ras pathway was required to induce senescence. Along with previous studies, this research implicated a strong and sustained MEK–ERK activation, leading to activation of p38, as central to the production of the senescence phenotype produced by oncogenic Ras and as playing a critical role in the common senescence signaling pathway produced by telomere shortening and other cell stressors (102–104). That this same mechanism might be triggered downstream of microtubule stabilization and mediate the accelerated senescence produced by discodermolide is of interest.

4. CONCLUSION

A quick perusal of the mechanisms by which MSAs might inhibit the proliferation of tumor cells suggests a reason for the proven effectiveness of Taxol and its value in the treatment of cancer. By interfering with the normal dynamic behavior of microtubules, which are critical to a host of physiological cell activities, these agents can potentially initiate multiple cell death mechanisms or interfere with cell proliferation by interfering in many processes. Clearly, classical apoptosis is important in Taxol-mediated killing of leukemia and lymphoma cells and may participate, to varying extents, in the cell death produced in solid tumors. Yet, cell death from MSAs is certainly not synonymous with apoptosis. The aberrant mitosis and delayed cell death produced by these agents, primarily through caspase-independent mechanisms, are now recognized as being critical measures of their efficacy. Accelerated senescence, as a naturally occurring blockade to uncontrolled cell proliferation, is another potentially useful program to be targeted in the development of ever more effective treatment. Given the powerful senescence induced by discodermolide, it may serve as a prototype for the development of microtubule-stabilizing drugs that can arrest the proliferation of cells in aggressive tumors. The especially attractive feature of accelerated senescence induced by discodermolide is that it appears to be preceded by aberrant mitosis and followed, ultimately, by a delayed cell death. The net effect is that rapid cell proliferation is arrested prior to cell death. The effective pairing of this course of action with a second type of agent, such as Taxol, that would begin the cell killing at an earlier time point could help to undermine any escape from senescence that might occur because of the inactivation of p53 or silencing of p16 that are so common in cancer cells.

REFERENCES

1. Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly *in vitro* by taxol. *Nature* 1979;277(5698):665–7.
2. Schiff PB, Horwitz SB. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc Natl Acad Sci USA* 1980;77(3):1561–5.
3. Orr GA, Verdier-Pinard P, McDaid H, Horwitz SB. Mechanisms of Taxol resistance related to microtubules. *Oncogene* 2003;22(47):7280–95.
4. Schwetz BA. From the food and drug administration. *JAMA* 2001;286(17):2085.
5. Stachel SJ, Biswas K, Danishefsky SJ. The epothilones, eleutherobins, and related types of molecules. *Curr Pharm Des* 2001;7(13):1277–90.
6. Mooberry SL, Randall-Hlubek DA, Leal RM, et al. Microtubule-stabilizing agents based on designed laulimalide analogues. *Proc Natl Acad Sci USA* 2004;101(23):8803–8.

7. Isbrucker RA, Gunasekera SP, Longley RE. Structure-activity relationship studies of discodermolide and its semisynthetic acetylated analogs on microtubule function and cytotoxicity. *Cancer Chemother Pharmacol* 2001;48(1):29–36.
8. Martello LA, McDaid HM, Regl DL, et al. Taxol and discodermolide represent a synergistic drug combination in human carcinoma cell lines. *Clin Cancer Res* 2000;6(5):1978–87.
9. Huang GS, Lopez-Barcons L, Freeze BS, et al. Potentiation of Taxol efficacy by Discodermolide in Ovarian Carcinoma Xenograft-Bearing Mice. *Clin Cancer Res* 2006;12(1):298–304.
10. Long BH, Fairchild CR. Paclitaxel inhibits progression of mitotic cells to G1 phase by interference with spindle formation without affecting other microtubule functions during anaphase and telephase. *Cancer Res* 1994;54(16):4355–61.
11. Ofir R, Seidman R, Rabinski T, et al. Taxol-induced apoptosis in human SKOV3 ovarian and MCF7 breast carcinoma cells is caspase-3 and caspase-9 independent. *Cell Death Differ* 2002;9(6):636–42.
12. Sun XM, MacFarlane M, Zhuang J, Wolf BB, Green DR, Cohen GM. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. *J Biol Chem* 1999;274(8):5053–60.
13. Blagosklonny MV, Robey R, Sheikh MS, Fojo T. Paclitaxel-induced FasL-independent apoptosis and slow (non-apoptotic) cell death. *Cancer Biol Ther* 2002;1(2):113–7.
14. Abend M. Reasons to reconsider the significance of apoptosis for cancer therapy. *Int J Radiat Biol* 2003;79(12):927–41.
15. Lockshin RA, Zakeri Z. Caspase-independent cell deaths. *Curr Opin Cell Biol* 2002;14(6):727–33.
16. Assuncao Guimaraes C, Linden R. Programmed cell deaths. Apoptosis and alternative deathstyles. *Eur J Biochem* 2004;271(9):1638–50.
17. Jaattela M. Multiple cell death pathways as regulators of tumour initiation and progression. *Oncogene* 2004;23(16):2746–56.
18. Broker LE, Huisman C, Ferreira CG, Rodriguez JA, Kruyt FA, Giaccone G. Late activation of apoptotic pathways plays a negligible role in mediating the cytotoxic effects of discodermolide and epothilone B in non-small cell lung cancer cells. *Cancer Res* 2002;62(14):4081–8.
19. Lockshin RA, Zakeri Z. Caspase-independent cell death? *Oncogene* 2004;23(16):2766–73.
20. Jordan MA, Toso RJ, Thrower D, Wilson L. Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. *Proc Natl Acad Sci USA* 1993;90(20):9552–6.
21. Honore S, Kamath K, Braguer D, Wilson L, Briand C, Jordan MA. Suppression of microtubule dynamics by discodermolide by a novel mechanism is associated with mitotic arrest and inhibition of tumor cell proliferation. *Mol Cancer Ther* 2003;2(12):1303–11.
22. Gloushankova NA, Lyubimova AV, Tint IS, Feder HH, Vasiliev JM, Gelfand IM. Role of the microtubular system in morphological organization of normal and oncogene-transfected epithelial cells. *Proc Natl Acad Sci USA* 1994;91(18):8597–601.
23. Giannakakou P, Sackett DL, Ward Y, Webster KR, Blagosklonny MV, Fojo T. p53 is associated with cellular microtubules and is transported to the nucleus by dynein. *Nat Cell Biol* 2000;2(10):709–17.
24. Kelling J, Sullivan K, Wilson L, Jordan MA. Suppression of centromere dynamics by Taxol in living osteosarcoma cells. *Cancer Res* 2003;63(11):2794–801.
25. Lamendola DE, Duan Z, Yusuf RZ, Seiden MV. Molecular description of evolving paclitaxel resistance in the SKOV-3 human ovarian carcinoma cell line. *Cancer Res* 2003;63(9):2200–5.
26. Desai A, Mitchison TJ. Microtubule polymerization dynamics. *Annu Rev Cell Dev Biol* 1997;13:83–117.
27. Torres K, Horwitz SB. Mechanisms of Taxol-induced cell death are concentration dependent. *Cancer Res* 1998;58(16):3620–6.
28. Giannakakou P, Robey R, Fojo T, Blagosklonny MV. Low concentrations of paclitaxel induce cell type-dependent p53, p21 and G1/G2 arrest instead of mitotic arrest: molecular determinants of paclitaxel-induced cytotoxicity. *Oncogene* 2001;20(29):3806–13.
29. Chen JG, Horwitz SB. Differential mitotic responses to microtubule-stabilizing and -destabilizing drugs. *Cancer Res* 2002;62(7):1935–8.
30. Woods CM, Zhu J, McQueney PA, Bollag D, Lazarides E. Taxol-induced mitotic block triggers rapid onset of a p53-independent apoptotic pathway. *Mol Med* 1995;1(5):506–26.
31. Wang TH, Wang HS, Soong YK. Paclitaxel-induced cell death: where the cell cycle and apoptosis come together. *Cancer* 2000;88(11):2619–28.
32. Mollinedo F, Gajate C. Microtubules, microtubule-interfering agents and apoptosis. *Apoptosis* 2003;8(5):413–50.

33. Huizing MT, Keung AC, Rosing H, et al. Pharmacokinetics of paclitaxel and metabolites in a randomized comparative study in platinum-pretreated ovarian cancer patients. *J Clin Oncol* 1993;11(11):2127–35.
34. Barboulet N, Chadebecq P, Baldin V, Vidal S, Valette A. Involvement of p21 in mitotic exit after paclitaxel treatment in MCF-7 breast adenocarcinoma cell line. *Oncogene* 1997;15(23):2867–75.
35. Seidman R, Gitelman I, Sagi O, Horwitz SB, Wolfson M. The role of ERK 1/2 and p38 MAP-kinase pathways in taxol-induced apoptosis in human ovarian carcinoma cells. *Exp Cell Res* 2001;268(1):84–92.
36. Broker LE, Huisman C, Span SW, Rodriguez JA, Kruyt FA, Giaccone G. Cathepsin B mediates caspase-independent cell death induced by microtubule stabilizing agents in non-small cell lung cancer cells. *Cancer Res* 2004;64(1):27–30.
37. Blagosklonny MV, Giannakakou P, el-Deiry WS, et al. Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death. *Cancer Res* 1997;57(1):130–5.
38. Lanzi C, Cassinelli G, Cuccuru G, et al. Cell cycle checkpoint efficiency and cellular response to paclitaxel in prostate cancer cells. *Prostate* 2001;48(4):254–64.
39. Davis JM, Navolanic PM, Weinstein-Oppenheim CR, et al. Raf-1 and Bcl-2 induce distinct and common pathways that contribute to breast cancer drug resistance. *Clin Cancer Res* 2003;9(3):1161–70.
40. Asnaghi L, Calastretti A, Bevilacqua A, et al. Bcl-2 phosphorylation and apoptosis activated by damaged microtubules require mTOR and are regulated by Akt. *Oncogene* 2004;23(34):5781–91.
41. Scatena CD, Stewart ZA, Mays D, et al. Mitotic phosphorylation of Bcl-2 during normal cell cycle progression and Taxol-induced growth arrest. *J Biol Chem* 1998;273(46):30777–84.
42. Laird AD, Morrison DK, Shalloway D. Characterization of Raf-1 activation in mitosis. *J Biol Chem* 1999;274(7):4430–9.
43. Liu QY, Stein CA. Taxol and estramustine-induced modulation of human prostate cancer cell apoptosis via alteration in bcl-xL and bak expression. *Clin Cancer Res* 1997;3(11):2039–46.
44. Jones NA, Turner J, McIlwrath AJ, Brown R, Dive C. Cisplatin- and paclitaxel-induced apoptosis of ovarian carcinoma cells and the relationship between bax and bak up-regulation and the functional status of p53. *Mol Pharmacol* 1998;53(5):819–26.
45. Haldar S, Chintapalli J, Croce CM. Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. *Cancer Res* 1996;56(6):1253–5.
46. Srivastava RK, Srivastava AR, Korsmeyer SJ, Nesterova M, Cho-Chung YS, Longo DL. Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cyclic AMP-dependent protein kinase. *Mol Cell Biol* 1998;18(6):3509–17.
47. Wang LG, Liu XM, Kreis W, Budman DR. The effect of antimicrotubule agents on signal transduction pathways of apoptosis: a review. *Cancer Chemother Pharmacol* 1999;44(5):355–61.
48. Huang TS, Shu CH, Chao Y, Chen SN, Chen LL. Activation of MAD 2 checkpoint protein and persistence of cyclin B1/CDC 2 activity associate with paclitaxel-induced apoptosis in human nasopharyngeal carcinoma cells. *Apoptosis* 2000;5(3):235–41.
49. Bacus SS, Gudkov AV, Lowe M, et al. Taxol-induced apoptosis depends on MAP kinase pathways (ERK and p38) and is independent of p53. *Oncogene* 2001;20(2):147–55.
50. Stone AA, Chambers TC. Microtubule inhibitors elicit differential effects on MAP kinase (JNK, ERK, and p38) signaling pathways in human KB-3 carcinoma cells. *Exp Cell Res* 2000;254(1):110–9.
51. Shtil AA, Mandelkar S, Yu R, et al. Differential regulation of mitogen-activated protein kinases by microtubule-binding agents in human breast cancer cells. *Oncogene* 1999;18(2):377–84.
52. Wang TH, Popp DM, Wang HS, et al. Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-Jun N-terminal kinase (JNK)-dependent and -independent pathways in ovarian cancer cells. *J Biol Chem* 1999;274(12):8208–16.
53. Yujiri T, Fanger GR, Garrington TP, Schlesinger TK, Gibson S, Johnson GL. MEK kinase 1 (MEKK1) transduces c-Jun NH2-terminal kinase activation in response to changes in the microtubule cytoskeleton. *J Biol Chem* 1999;274(18):12605–10.
54. Bhalla K, Ibrado AM, Tourkina E, Tang C, Mahoney ME, Huang Y. Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. *Leukemia* 1993;7(4):563–8.
55. Perkins C, Kim CN, Fang G, Bhalla KN. Overexpression of Apaf-1 promotes apoptosis of untreated and paclitaxel- or etoposide-treated HL-60 cells. *Cancer Res* 1998;58(20):4561–6.

56. von Haefen C, Wieder T, Essmann F, Schulze-Osthoff K, Dorken B, Daniel PT. Paclitaxel-induced apoptosis in BJAB cells proceeds via a death receptor-independent, caspases-3/8-driven mitochondrial amplification loop. *Oncogene* 2003;22(15):2236–47.
57. Park SJ, Wu CH, Gordon JD, Zhong X, Emami A, Safa AR. Taxol induces caspase-10-dependent apoptosis. *J Biol Chem* 2004;279(49):51057–67.
58. Goncalves A, Braguer D, Carles G, Andre N, Prevot C, Briand C. Caspase-8 activation independent of CD95/CD95-L interaction during paclitaxel-induced apoptosis in human colon cancer cells (HT29-D4). *Biochem Pharmacol* 2000;60(11):1579–84.
59. Blajeski AL, Kottke TJ, Kaufmann SH. A multistep model for paclitaxel-induced apoptosis in human breast cancer cell lines. *Exp Cell Res* 2001;270(2):277–88.
60. Brown JM, Wouters BG. Apoptosis: mediator or mode of cell killing by anticancer agents? *Drug Resist Updat* 2001;4(2):135–6.
61. Bhalla KN. Microtubule-targeted anticancer agents and apoptosis. *Oncogene* 2003;22(56):9075–86.
62. Huisman C, Ferreira CG, Broker LE, et al. Paclitaxel triggers cell death primarily via caspase-independent routes in the non-small cell lung cancer cell line NCI-H460. *Clin Cancer Res* 2002;8(2):596–606.
63. Tan G, Heqing L, Jiangbo C, et al. Apoptosis induced by low-dose paclitaxel is associated with p53 upregulation in nasopharyngeal carcinoma cells. *Int J Cancer* 2002;97(2):168–72.
64. Castedo M, Perfettini JL, Roumier T, et al. Mitotic catastrophe constitutes a special case of apoptosis whose suppression entails aneuploidy. *Oncogene* 2004;23(25):4362–70.
65. Roninson IB. Tumor senescence as a determinant of drug response in vivo. *Drug Resist Updat* 2002;5(5):204–8.
66. Chu K, Teele N, Dewey MW, Albright N, Dewey WC. Computerized video time lapse study of cell cycle delay and arrest, mitotic catastrophe, apoptosis and clonogenic survival in irradiated 14-3-3sigma and CDKN1A (p21) knockout cell lines. *Radiat Res* 2004;162(3):270–86.
67. Nitta M, Kobayashi O, Honda S, et al. Spindle checkpoint function is required for mitotic catastrophe induced by DNA-damaging agents. *Oncogene* 2004;23(39):6548–58.
68. He QY, Liang YY, Wang DS, Li DD. Characteristics of mitotic cell death induced by enediyne antibiotic lidamycin in human epithelial tumor cells. *Int J Oncol* 2002;20(2):261–6.
69. Chang BD, Broude EV, Dokmanovic M, et al. A senescence-like phenotype distinguishes tumor cells that undergo terminal proliferation arrest after exposure to anticancer agents. *Cancer Res* 1999;59(15):3761–7.
70. Lock RB, Stribinskiene L. Dual modes of death induced by etoposide in human epithelial tumor cells allow Bcl-2 to inhibit apoptosis without affecting clonogenic survival. *Cancer Res* 1996;56(17):4006–12.
71. Roninson IB, Broude EV, Chang BD. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist Updat* 2001;4(5):303–13.
72. Broker LE, Kruyt FA, Giaccone G. Cell death independent of caspases: a review. *Clin Cancer Res* 2005;11(9):3155–62.
73. Erenpreisa J, Cragg MS. Mitotic death: a mechanism of survival? A review. *Cancer Cell Int* 2001;1(1):1.
74. Sutou S, Arai Y. Possible mechanisms of endoreduplication induction. Membrane fixation and/or disruption of the cytoskeleton. *Exp Cell Res* 1975;92(1):15–22.
75. King RW, Deshaies RJ, Peters JM, Kirschner MW. How proteolysis drives the cell cycle. *Science* 1996;274(5293):1652–9.
76. Grafi G. Cell cycle regulation of DNA replication: the endoreduplication perspective. *Exp Cell Res* 1998;244(2):372–8.
77. Schimke RT, Kung A, Sherwood SS, Sheridan J, Sharma R. Life, death and genomic change in perturbed cell cycles. *Philos Trans R Soc Lond B Biol Sci* 1994;345(1313):311–7.
78. Jaattela M, Tschopp J. Caspase-independent cell death in T lymphocytes. *Nat Immunol* 2003;4(5):416–23.
79. Andre N, Carre M, Brousseau G, et al. Paclitaxel targets mitochondria upstream of caspase activation in intact human neuroblastoma cells. *FEBS Lett* 2002;532(1–2):256–60.
80. Chi S, Kitanaka C, Noguchi K, et al. Oncogenic Ras triggers cell suicide through the activation of a caspase-independent cell death program in human cancer cells. *Oncogene* 1999;18(13):2281–90.

81. Okano J, Rustgi AK. Paclitaxel induces prolonged activation of the Ras/MEK/ERK pathway independently of activating the programmed cell death machinery. *J Biol Chem* 2001;276(22):19555–64.
82. Klein LE, Freeze BS, Smith AB 3rd, Horwitz SB. The microtubule stabilizing agent discodermolide is a potent inducer of accelerated cell senescence. *Cell Cycle* 2005;4(3):501–7.
83. Chen QM, Tu VC, Liu J. Measurements of hydrogen peroxide induced premature senescence: senescence-associated beta-galactosidase and DNA synthesis index in human diploid fibroblasts with down-regulated p53 or Rb. *Biogerontology* 2000;1(4):335–9.
84. Lin AM, Chyi BY, Wu LY, Hwang LS, Ho LT. The antioxidative property of green tea against iron-induced oxidative stress in rat brain. *Chin J Physiol* 1998;41(4):189–94.
85. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 1997;88(5):593–602.
86. Zhu J, Woods D, McMahon M, Bishop JM. Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev* 1998;12(19):2997–3007.
87. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature* 1990;345(6274):458–60.
88. Campisi J. Replicative senescence: an old lives' tale? *Cell* 1996;84(4):497–500.
89. Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc Natl Acad Sci USA* 1995;92(20):9363–7.
90. Campisi J. Suppressing cancer: the importance of being senescent. *Science* 2005;309(5736):886–7.
91. Dimri GP. What has senescence got to do with cancer? *Cancer Cell* 2005;7(6):505–12.
92. Narita M, Lowe SW. Executing cell senescence. *Cell Cycle* 2004;3(3):244–6.
93. Campisi J. Cellular senescence as a tumor-suppressor mechanism. *Trends Cell Biol* 2001;11(11):S27–31.
94. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 1999;13(12):1501–12.
95. Itahana K, Dimri G, Campisi J. Regulation of cellular senescence by p53. *Eur J Biochem* 2001;268(10):2784–91.
96. Cheng M, Olivier P, Diehl JA, et al. The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J* 1999;18(6):1571–83.
97. Schwarze SR, Shi Y, Fu VX, Watson PA, Jarrard DF. Role of cyclin-dependent kinase inhibitors in the growth arrest at senescence in human prostate epithelial and uroepithelial cells. *Oncogene* 2001;20(57):8184–92.
98. Beausejour CM, Krtolica A, Galimi F, et al. Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J* 2003;22(16):4212–22.
99. Roberson RS, Kussick SJ, Vallieres E, Chen SY, Wu DY. Escape from therapy-induced accelerated cellular senescence in p53-null lung cancer cells and in human lung cancers. *Cancer Res* 2005;65(7):2795–803.
100. Nishio K, Inoue A, Qiao S, Kondo H, Mimura A. Senescence and cytoskeleton: overproduction of vimentin induces senescent-like morphology in human fibroblasts. *Histochem Cell Biol* 2001;116(4):321–7.
101. Deng Q, Liao R, Wu BL, Sun P. High intensity ras signaling induces premature senescence by activating p38 pathway in primary human fibroblasts. *J Biol Chem* 2004;279(2):1050–9.
102. Haq R, Brenton JD, Takahashi M, et al. Constitutive p38HOG mitogen-activated protein kinase activation induces permanent cell cycle arrest and senescence. *Cancer Res* 2002;62(17):5076–82.
103. Wang W, Chen JX, Liao R, et al. Sequential activation of the MEK-extracellular signal-regulated kinase and MKK3/6-p38 mitogen-activated protein kinase pathways mediates oncogenic ras-induced premature senescence. *Mol Cell Biol* 2002;22(10):3389–403.
104. Iwasa H, Han J, Ishikawa F. Mitogen-activated protein kinase p38 defines the common senescence-signalling pathway. *Genes Cells* 2003;8(2):131–44.

24

Tyrosine Kinase Inhibitors

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SUMMARY

The extraordinary success of imatinib for the treatment of chronic myeloid leukemia (CML), gastrointestinal stromal tumors and subgroups of patients with hypereosinophilic syndrome and chronic myelomonocytic leukemia has greatly stimulated the development of small molecule inhibitors for targeted therapy of malignant diseases. Nothing short of a major breakthrough, imatinib has undoubtedly set a precedent and provided proof of principle for a completely new concept in cancer therapy. Unfortunately, diseases other than CML may prove more resilient to targeted tyrosine kinase inhibition, and even in CML, acquired resistance is a significant clinical problem. This chapter will review the current status of tyrosine kinase inhibitors for therapy of malignant disease. Much space will be given to imatinib, as the experience gained from the development of this agent is applicable to other conditions. The emerging concept is that for the patient's maximum therapeutic benefit, disease classifications will have to integrate therapeutic targets, and this will have implications for clinical trial design.

Key Words: targeted therapy; BCR-ABL; FLT3; EGFR; tyrosine kinase inhibitors; imatinib; gefitinib; erlotinib; cancer; leukemia

1. TYROSINE KINASES IN MALIGNANT DISEASE

That protein phosphorylation plays a role in the regulation of protein function, that is, reversible phosphorylation regulates the activity of glycogen phosphorylase, was first discovered by Fischer and Krebs, almost 50 years ago. With the advent of more sophisticated molecular biology technology, it has become clear that protein phosphorylation is central to the regulation of metabolism in eukaryotic organisms. Protein kinases catalyze the transfer of phosphate from ATP to serine, threonine or tyrosine residues of proteins according to the basic reaction $\text{MgATP}^{1-} + \text{protein-OH} \rightarrow \text{protein-OPO}_3^{2-} + \text{MgADP} + \text{H}^+$. Large-scale sequencing revealed the existence of 518 human protein kinases, encompassing 1.7% of all human genes and representing the fifth largest protein family in the human genome (1). Protein kinases are grouped in eight families, according to structural features and their ability to phosphorylate serine/threonine or tyrosine residues (1). There are 90 recognized tyrosine kinases, 58

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of which are receptor tyrosine kinases and 32 non-receptor or cytoplasmic tyrosine kinases. In addition, 43 tyrosine kinase-like kinases have been identified.

Tyrosine kinases are key mediators of growth and survival signals, which is why their involvement in the pathogenesis of malignant conditions may be disproportionately high compared to serine/threonine kinases. Cytokine receptors, such as KIT or the platelet growth factor receptors (PDGFRs), are themselves tyrosine kinases. Signaling through other receptors such as the erythropoietin, interleukin-3 or granulocyte monocyte-colony stimulating factor (GM-CSF) receptors involves the recruitment of tyrosine kinases, such as JAK family kinases as early critical events after ligand binding. The activity of tyrosine kinases is counterbalanced by tyrosine phosphatases that remove the phosphate groups transferred by the kinases. There are 87 members in this family, including 53 classical tyrosine phosphatases, 33 dual specificity phosphatases and one low molecular weight phosphatase (2). Under physiological conditions, this equilibrium is very much in favor of non-phosphorylation, and it has been estimated that less than 1% of tyrosine residues are phosphorylated in a cell at any given time. This ascertains a constantly high level of responsiveness to signals such as growth factors, while at the same time limiting the duration of “dangerous” growth stimuli. Constitutive activation of a tyrosine kinase uproots the delicate balance and, in simplistic terms, leads to a state of permanent activation of growth and survival signals (Fig. 1). This endows the malignant cells with a growth advantage over their normal counterparts, whose proliferation is dependent on external signals. The majority of the known tyrosine kinases have been implicated in human malignancies, either directly or indirectly (Table 1) (3).

1.1. Kinase Activation by Mutation

Tyrosine kinase activation may occur as a result of several types of mutations. In receptor tyrosine kinases, dimerization is physiologically inhibited by intrinsic mechanisms that usually involve amino acid sequences in the juxtamembrane domain, the region of the receptor immediately adjacent to the inner leaflet of the cell membrane (Fig. 2). Binding of ligand overcomes the intrinsic inhibition of dimerization. Conversely, mutations that disrupt the mechanism that prevents spontaneous dimerization also activate the kinase. An example of this mechanism of action is the internal tandem duplications (ITDs) in the juxtamembrane domain of FLT3 (4). Point mutations can have the same effect, for example, in some gastrointestinal stromal tumors (GISTs) (5). Similar to receptor tyrosine kinases, cytoplasmic tyrosine kinases may also be activated by dimerization. However, dimerization is the result of chromosomal translocations that fuse unrelated sequences with the capacity to form dimers N-terminally to the kinase domain. Examples include BCR fused to ABL in the Philadelphia translocation (6), the hallmark of chronic myeloid leukemia (CML) or TEL-PDGFR in the (5;12)(q33;p13) reciprocal translocation that characterizes a subset of patients with chronic myelomonocytic leukemia (CMML) (7). It is thought that the unphysiological dimerization enables phosphorylation in trans. Both cytoplasmic and receptor tyrosine kinases may also be activated by point mutations, insertions or deletions in the kinase domain that disrupt autoinhibitory mechanisms. The same tyrosine kinase may be activated by both types of mutation, not infrequently in a tumor type-specific manner. For example, mutations of the juxtamembrane domain of KIT are common in GISTs, while point mutations affecting D816 in the activation loop of the kinase domain are typical of systemic mastocytosis (SM) (5,8). From the

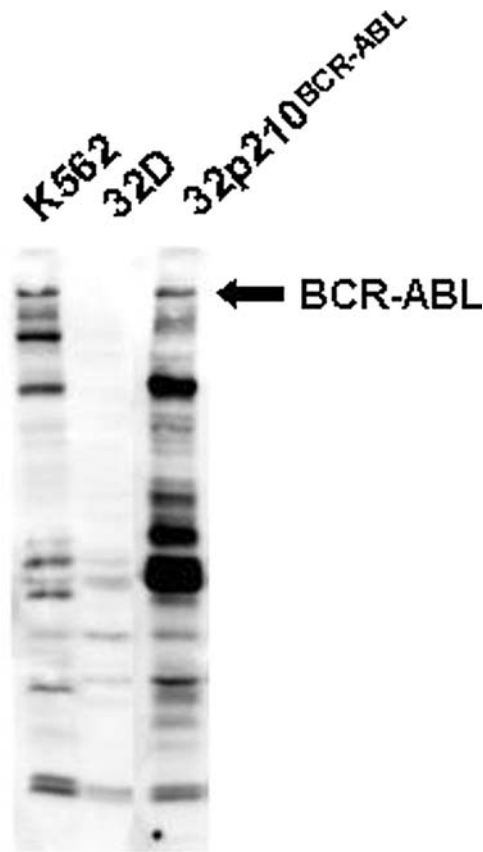


Fig. 1. Increased tyrosine phosphorylation of proteins in cells expressing an activated tyrosine kinase. Lysates from K562 cells, a chronic myeloid leukemia cell line, 32D cells, a murine myeloid cell line that grows in interleukin-3, and 32Dp210^{BCR-ABL} cells (32D cells transduced with BCR-ABL) were analysed for tyrosine phosphorylated proteins by Western blot analysis.

viewpoint of targeted therapy this is relevant, as mutations of the enzyme active site of the kinase may alter substrate binding, while this structure is not altered by abnormal dimerization. Thus, an inhibitor that binds the catalytic site of the unmutated kinase with high affinity may have much lower affinity if a mutation is present. For example, imatinib inhibits unmutated KIT in the nanomolar dose range but is practically inactive against the D816V activation loop mutant (9,10). In contrast, AP23464, an ABL/SRC inhibitor, is more active against the D816V mutant than wild-type KIT (11). Yet, another mechanism of kinase activation is mutations in the extracellular domain that alter ligand affinity. This has recently been shown for KIT exon 4 mutants, which show increased responsiveness to low concentrations of stem cell factor compared to wild-type KIT (12).

1.2. Activation by Increased Expression

In contrast to mutations, the significance of overexpression of the tyrosine kinases compared to phenotypically matched non-neoplastic cells is less well defined. For example, KIT is highly expressed on the blasts of many patients with acute myeloid

Table 1
Tyrosine Kinases Involved in Human Cancer

<i>Oncogenic alteration and target tissue/disease phenotype</i>	
Receptor tyrosine kinases	
EGFR/ErbB1	GOF point mutations, small deletions, insertions: NSCLC Overexpression (amplification): NSCLC, breast and ovarian cancer Extracellular domain deletions: glioblastoma multiforme
ERBB2/HER2/Neu	Overexpression (amplification): breast, ovarian, gastric, colon, lung cancer GOF point mutations: NSCLC
ERBB3/HER3	Overexpression: breast cancer
ERBB4/HER4	Overexpression: breast cancer, granulose cell tumors
IGF-1R	Overexpression: cervical and other carcinomas, sarcomas
PDGFR- α	Overexpression (amplification): glioma, glioblastoma, ovarian carcinoma Gene fusions: FLP1L1-PDGFR- α fusion [del(4q12)]: hypereosinophilic syndrome BCR-PDGFR- α [t(8;22)]: atypical CML GOF mutations: GISTs
PDGFR- β	Gene fusions: ETV6-PDGFR- β fusion [t(5;12)] and other fusions: CMML Overexpression: glioma
CSF-1R (FMS)	GOF point mutations, overexpression: acute and chronic myelomonocytic leukemia, monocytic tumors, malignant histiocytosis, endometrial cancer, glioma
KIT/SCFR	Overexpression: AML, SCLC, other carcinomas GOF point mutations and small deletions: GISTs, AML, SM, seminomas/dysgerminomas
FLK2/FLT3	GOF point mutations, internal tandem duplications: AML, myelodysplastic syndrome
FLT1/VEGFR1	Expression: tumor angiogenesis
FLT1/VEGFR2	Expression: tumor angiogenesis
FLT4/VEGFR3	Overexpression: tumor angiogenesis; vascular tumors (Kaposi's sarcoma, Hemangiosarcoma, lymphangiosarcoma)
FGFR1	Overexpression: various tumors Gene fusions: ZNF 198-FGFR1 [t(8;13)] and other fusions: myeloproliferative syndrome, T-cell lymphoma, AML Point mutations: autosomal skeletal disorders/dysplasias
FGFR2/K-SAM	Overexpression (amplification) and C-terminal truncation: gastric, breast, prostate cancer
FGFR3	Overexpression: IgH locus/MMSET translocation [t(4;14)] placing FGFR3 downstream of IgH/MMSET: multiple myeloma GOF point mutations: achondroplasia, thantophoric dysplasia, hypochondroplasia
FGFR4	Overexpression (amplification): breast, ovarian cancer
TRKA	Gene fusions: TPR-TRKA [t(1;1)], TFG-TRKA [t(1;3)]: Papillary thyroid carcinomas, neuroblastomas
TRKC	Gene fusion: ETV6-TRKC [t(12;15)]: congenital fibrosarcoma, AML

HGFR	Gene fusion: TPR-MET [t(1;7)]: Papillary thyroid carcinomas Overexpression: rhabdomyosarcoma, hepatocellular carcinoma GOF point mutations: renal carcinoma, SCLC
RON	Overexpression/increased kinase activity of splice variants: colon cancer, hepatocellular cancer
EPHA2	Overexpression: melanoma
EPHB2	Overexpression: gastric, esophageal and colon cancer
EPHB4	Overexpression: infiltrating ductal mammary carcinomas
AXL	Overexpression: AML
TEK/TIE2	Expression: tumor angiogenesis (endothelium)
RET	Gene fusions and inversions: H4-Ret (PTC1), R1 α -Ret (PTC2); ELE1-Ret (PTC3 & PTC4); RFG5-Ret (PTC5); HTIF1-Ret (PTC6); RFG7-Ret (PTC7); KTN1-Ret (PTC8); ELKS-Ret: papillary thyroid carcinomas GOF point mutations: multiple endocrine neoplasia type 2A+B: medullary thyroid carcinoma, parathyroid hyperplasia, pheochromocytoma, enteric mucosal ganglioneuromas; familial medullary thyroid carcinoma
ROS	Overexpression: glioblastomas, astrocytomas
Non-receptor tyrosine kinases	
ALK	Gene fusions: NPM-ALK [t(2;5)]; Ig λ -ALK PTK [t(2; 22)]; other fusions: anaplastic large cell lymphoma
SRC	C-terminal truncation (increased kinase activity): colon cancer Overexpression: breast and pancreatic cancers, neuroblastomas, others
FGR	C-terminal truncation and GOF point mutations: AML, chronic lymphocytic leukemia, EBV-associated lymphomas
YES	C-terminal truncation, GOF point mutations, overexpression: colon cancer, melanoma, other cancers
LCK	Overexpression due to gene fusion: TCR β -LCK [t(1; 7)]: T-cell ALL
ABL1	Gene fusions: BCR-ABL1 [t(9;22)]: CML, ALL, AML ETV6-ABL1 [t(9;12)]: myeloproliferative syndrome, ALL NUP214-ABL1: T-cell ALL
ABL2 (ARG)	Gene fusion: ETV6-ABL2 [(t(1;12)]: AML
JAK1	Overexpression: various leukemias
JAK2	Point mutation: V617F in myeloproliferative diseases Gene fusion: TEL-JAK2 [(t(9;12)]: T-cell childhood ALL, AML, atypical CML
JAK3	Point mutation: Megakaryocytic leukemia Overexpression: various leukemias and B-cell malignancies
FAK	Overexpression and/or altered tyrosine kinase activity: modulation of adhesion, invasion and metastasis of diverse malignancies
PYK2	Overexpression and/or altered tyrosine kinase activity: modulation of adhesion, invasion and metastasis of diverse malignancies
BRK	Overexpression: breast cancer
SYK	Downregulation (acts as a tumor suppressor): breast cancer

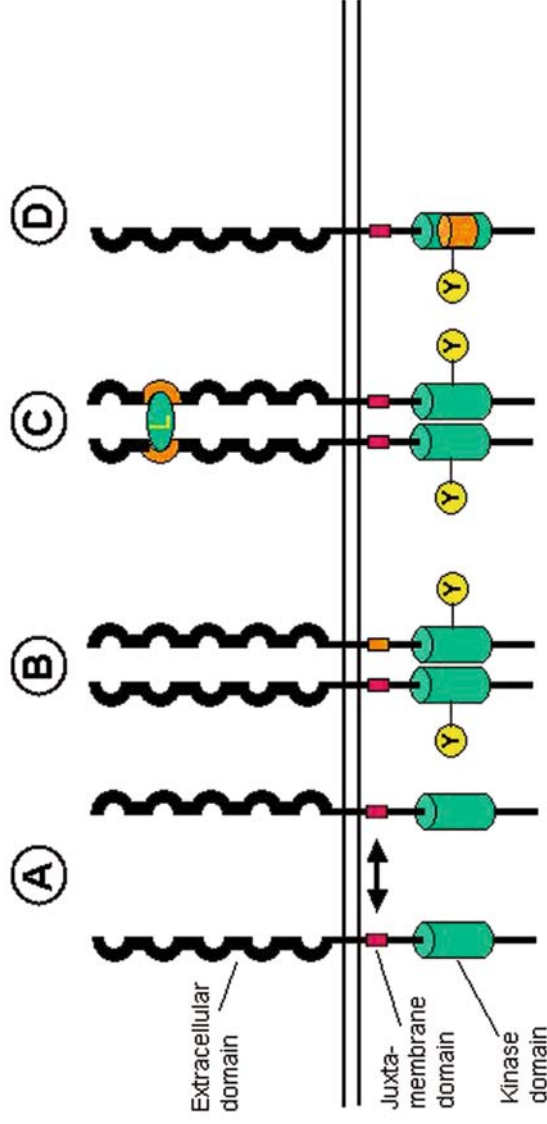


Fig. 2. Mutational activation of receptor tyrosine kinases. (A) Under physiological conditions, dimerization in the absence of ligand is prevented by a motif in the juxtamembrane domain (magenta). (B) Disruption of this mechanism by mutation leads to constitutive dimerization and kinase activation. (C) Mutations in the extracellular domain may increase affinity for ligand (L), leading to dimerization in the presence of low concentrations of ligand. (D) Mutations in the kinase may activate the kinase independently of dimerization.

leukemia (AML), but most of these patients do not respond to imatinib (13). However, there may be exceptions to this rule. For example, FLT3 mutations are found in approximately 30% of patients with AML (14). Although responses to specific FLT3 inhibitors tend to correlate with the presence of a mutation, activity was also seen in some patients with high surface expression of FLT3 (15,16). It is thought that a very high concentration of FLT3 molecules on the cell surface may lead to kinase activation by spontaneous dimerization in the absence of ligand. Enhanced signaling via the wild-type receptor may also occur as a result of increased expression of the ligand. For example, autocrine loops involving the PDGF/PDFGR system are operational in some glioblastoma cell lines, which provided the rationale for therapeutic trials of imatinib in patients with glioblastoma (17). It is not possible to predict with certainty how dependent a particular tumor is on signaling from a specific tyrosine kinase that is overexpressed. Generally, increased expression of a wild-type kinase that is caused by gene amplification is more likely to be critical to pathogenesis than simply high levels of expression in the absence of an underlying mutation. For example, responses to the epidermal growth factor receptor (EGFR) kinase inhibitor gefitinib in patients with non-small cell lung cancer (NSCLC) are not correlated with high expression of EGFR as measured by immunohistochemistry but with the presence of activating EGFR mutations (18,19) and gene amplification (20). Myeloma cells that overexpress FGFR3 as a result of t(4;14)(p16;q32) are responsive to treatment with FGFR3 inhibitors (21). This suggests that the mutant cell clones have been selected on the basis of the newly acquired genetic feature and are thus “addicted” to the respective growth and survival signals.

2. DEVELOPMENT OF IMATINIB

The development of imatinib (STI571, CGP57148), a specific inhibitor of the ABL tyrosine kinase, as a therapeutic agent for CML has become a paradigm for successful translational research and stimulated intense interest in the exploitation of tyrosine kinase inhibition for the treatment of malignant disorders. Although a rare disease, CML has been a pacemaker for important steps in oncology. The clinical presentation of a CML patient led Virchow (22) to coin the term leukemia, the Philadelphia chromosome was the first chromosomal abnormality consistently associated with a human malignancy (23) and t(9;22) the first reciprocal translocation (24), whose translocation partners, the BCR and ABL genes, were identified (25,26). From the perspective of targeted therapy, the most important discovery was that the chimeric BCR-ABL protein had constitutive tyrosine kinase activity that correlated with its ability to transform cells (27). With this in mind, attempts were initiated to develop inhibitors of ABL kinase activity for potential therapeutic use. In 1992, Anafi and colleagues (28,29) reported on a “tyrphostin,” a small molecule that inhibited the tyrosine kinase activity of BCR-ABL and suggested that it might be possible to design specific compounds for the treatment of ABL-associated human leukemias. In subsequent studies, the derivatives AG568, AG957 and AG1112 were identified as the most specific compounds. Despite their promising *in vitro* activity, the tyrphostins were never developed for clinical use.

Starting in the late 1980s, scientists at Ciba Geigy (now Novartis), under the direction of N. Lydon and A. Matter, initiated medicinal chemistry studies to identify compounds

with inhibitory activity against protein kinases. In one such project focusing on protein kinase C (PKC) as a target, a 2-phenylaminopyrimidine derivative was identified as a lead compound (30,31). Modifications of this compound greatly increased activity against tyrosine kinases, while all but eliminating activity against serine/threonine kinases and improved solubility, eventually leading to CGP57148, which was subsequently re-named STI571 and eventually imatinib (Gleevec, Glivec) (Fig. 3).

Imatinib has activity against ABL, the ABL homolog ARG (also referred to as ABL2), KIT and the PDGFRs in the micromolar dose range, whereas most other kinases, including SRC and serine/threonine kinases, are not inhibited at concentrations of up to 100 μM (32,33). Given the high level of sequence homology between the kinase domains of ABL and SRC, this selectivity was initially surprising but its structural basis became clear when the crystal structure of the ABL kinase domain in complex with an imatinib analog was solved (34). The catalytic domains of protein kinases have a rather uniform bi-lobar structure, with a smaller N-terminal lobe that consists predominantly of antiparallel beta-sheets and a larger C-terminal lobe that is predominantly α -helical. The amino acid residues critical for catalysis line the groove between the two lobes, and kinase activity is dependent on their mutual position. Equally important, access of the catalytic cleft to substrate and ATP is controlled by the activation loop of the kinase, a highly flexible structure whose conformation is regulated by tyrosine phosphorylation. While the activation loop conformations of active kinases are similar, they are quite distinct in the inactive states. Contrary to expectations, the crystal structure revealed that imatinib bound the kinase inactive form of ABL, with the activation loop in a closed position (Fig. 4). Given the much greater structural differences between inactive ABL and inactive SRC, this explained imatinib's high level of specificity. Thus, imatinib is not a straightforward ATP-competitive inhibitor but rather acts by stabilizing the kinase in a unique inactive conformation. The interaction with the catalytic site is tight, engaging no less than 19 amino acid residues in hydrogen bonds or hydrophobic interactions (35).

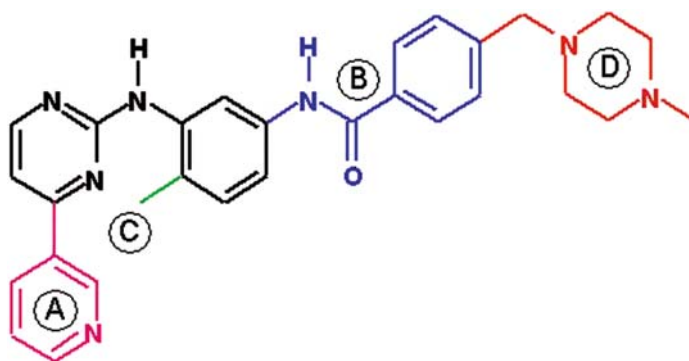


Fig. 3. Development of imatinib from a 2-phenylaminopyrimidine backbone (shown in black). (A) Activity in cellular assays was improved by introduction of a 3' pyridyl group (magenta) at the 3' position of the pyrimidine. (B) Activity against tyrosine kinases was further enhanced by addition of a benzamide group (blue) to the phenyl ring. (C) Attachment of a "flag-methyl" group (green) ortho to the diaminophenyl ring strongly reduced activity against protein kinase C. (D) Addition of an *N*-methylpiperazine (red) increased water solubility and oral bioavailability. Adapted from Deininger et al. (33) with permission.

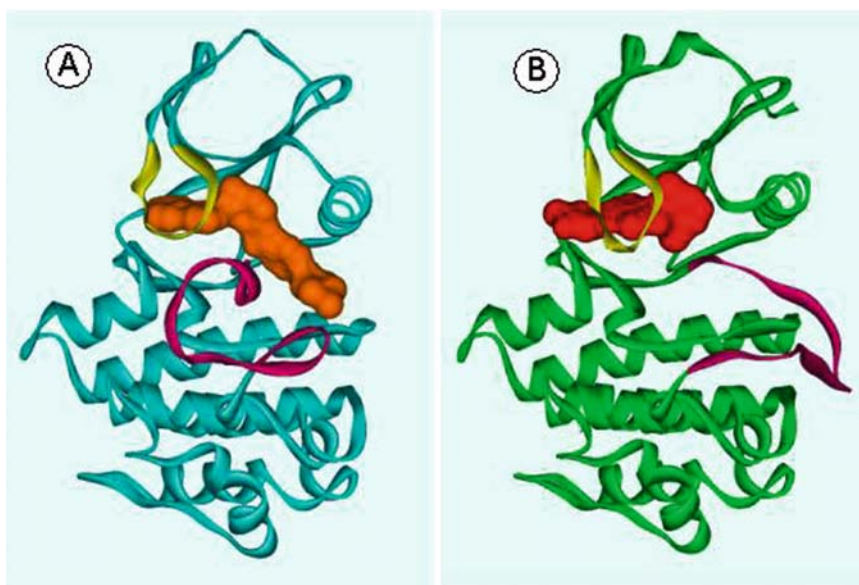


Fig. 4. (A) Conformation of ABL (blue) in complex with imatinib (orange), with the A-loop (magenta) in a “closed” conformation. (B) Conformation of ABL (green) in the PD180970 (red) complex with the a-loop (magenta) in an “open” conformation. The p-loop (yellow) folds down over the inhibitor in both cases. Adapted from Deininger et al. (33) with permission.

2.1. Imatinib for Therapy of CML

From the perspective of targeted therapy, CML is an almost ideal disease. There is a more than 90% consistency between typical morphology and the presence of BCR-ABL, a much more favorable situation than in most other types of malignancy (36). Only polycythemia vera has a similarly tight association with a specific mutation ($JAK2^{V617F}$) (37). Equally important, BCR-ABL is both necessary and sufficient for induction of CML. Transplantation of murine bone marrow cells expressing BCR-ABL into lethally irradiated recipient mice induces a myeloproliferative syndrome that resembles CML (38). Even more convincing, the reversibility of BCR-ABL-positive myeloproliferative disease was recently demonstrated in a transgenic mouse model that used an inducible expression system (39). Thus, the acquisition of BCR-ABL by a hematopoietic progenitor cell appears to be an early, probably the initiating event of CML, which may not be the case with tyrosine kinase mutations found in AML (40,41). Last, most CML patients are diagnosed in the chronic phase, a situation that may be compared with an adenoma and represents an early stage of leukemia evolution.

2.1.1. PHASE I STUDIES

Under the direction of Brian Druker, a phase I trial with imatinib was initiated in spring 1998. This study initially included patients with CML in late chronic phase but was later extended to patients in blast crisis, based on extremely promising efficacy data (42,43). In chronic phase, patients treated with at least 300 mg of imatinib daily, there was an almost 100% rate of complete hematological response (CHR, defined as normal blood counts and absence of splenomegaly or other signs of disease) and major

cytogenetic response rate (defined as the presence of the Philadelphia chromosome in less than 35% of metaphases). Based on the efficacy of doses > 300 mg daily, a decision was made to use 400 mg daily as the standard dose for the phase II studies. At this dose, median trough plasma levels were 1.43 μM , approximately 5-fold higher than the IC_{50} for *in vitro* inhibition of cell proliferation (32,44). Imatinib was generally well tolerated, with most non-hematological toxicities being grade 1 or grade 2. Compared to late chronic phase, results in blast crisis were less impressive and remissions were usually not maintained.

2.1.2. IMATINIB FOR NEWLY DIAGNOSED CML IN CHRONIC PHASE

Imatinib has become the first-line drug therapy for CML in all phases of disease. The rates of CHR and complete cytogenetic response (CCR) are highest in patients with early disease, that is, those with newly diagnosed CML in chronic phase. A randomized phase III comparison with cytarabine/interferon-alpha, the previous standard drug therapy, showed imatinib vastly superior with respect to rates of CHR and CCR and freedom from progression to accelerated phase and blast crisis (Table 2). Due to crossover design of the study and the much higher efficacy and tolerability of imatinib, the interferon/cytarabine arm collapsed, when imatinib was approved and many patients in the interferon/cytarabine arm withdrew consent. This meant that the original randomized trial essentially became an one-armed study. A recent update of the patients who received imatinib up-front showed a 98% cumulative rate of CHR and 87% cumulative rate of CCR (45). Freedom from progression to accelerated phase or blast crisis was 93% and overall survival 89%. Importantly, the annual rates of progression to accelerated phase or blast crisis have decreased with longer follow-up (Table 3), suggesting that a plateau may be reached eventually. Another crucial observation is that the clinical response to imatinib seems to override pre-therapeutic risk factors. Thus, patients who attain CCR or major molecular response [MMR, defined as a more than 3-log reduction of BCR-ABL mRNA compared to a standardized baseline (46)] have a very low risk of relapse or disease progression, irrespective of their individual pre-therapeutic risk. There is some evidence that higher doses of imatinib (800 mg) may be superior to the standard dose of 400 mg daily. As of now, this notion is not derived from randomized trials but rather from sequential studies performed at

Table 2
Responses to Imatinib vs. Interferon (IFN) Plus Cytarabine in Newly Diagnosed Chronic Myeloid Leukemia Patients in Chronic Phase

	CHR	MCR	CCR	Progression-free survival (14 months)
Imatinib (%) (n = 553)	95.3	85.2	73.8	92.1
IFN+Cytarabine (%) (n = 553)	55.5	22.1	8.5	73.5
P value	0.001	0.001	0.001	0.001

CCR, complete cytogenetic response; CHR, complete hematologic response; MCR, major cytogenetic response.

Median duration of follow-up is 19 months.

Table 3
**Progression Events in Newly Diagnosed Patients
 Treated with 400 mg Imatinib Daily Up-Front**

<i>Year</i>	<i>All progression events^a(%)</i>	<i>AP/BC (%)</i>
1	3.4	1.5
2	7.5	2.8
3	4.8	1.6
4	1.5	0.9
5	0.9	0.6

^aIncludes loss of complete hematologic response, loss of major or complete cytogenetic response and progression to accelerated phase (AP) or blast crisis (BC).

the MD Anderson Cancer Center. These trials showed higher rates of CCR, MMR complete molecular response (CMR, undetectability of BCR-ABL transcripts by RT-PCR) in the patients treated with 800 mg (47,48). It remains to be seen whether this will ultimately translate into a survival advantage, as the low-dose cohort appears to catch up with time in terms of rates of MMR and CMR (49). Nonetheless, a more recent update of the studies showed a trend toward reduced rates of early progression events with high doses of imatinib. Thus, the advantage of this approach may be to prevent early progression in some patients, presumably individuals with high Sokal risk. Unfortunately, being non-randomized, these studies do not meet vigorous quality standards, but the question will ultimately be resolved by several large multicenter trials that are under way in Europe and the United States. Additional evidence that higher doses may be more effective comes from the phase II studies in advanced CML, where 600 mg were superior to 400 mg, although again this was not a randomized comparison (50,51). Lastly, in some patients, dose escalation from 400 to 800 mg is able to overcome hematologic or cytogenetic refractoriness (52). Given that the choice of 400 mg as the standard dose was largely arbitrary [based on an almost 100% rate of CHR in patients with late chronic phase treated with 300 mg and the observation of cytogenetic responses at 400 mg (43)], this is not surprising. Overall, the issue of optimal dosing of targeted therapy has wider ramifications. There is currently no established test to monitor effective target inhibition in patients on imatinib, although the phosphorylation of CRKL, a rather specific substrate of BCR-ABL, is used in a research setting (43). This test is not very sensitive and it is likely that a substantial fraction of BCR-ABL kinase remains active in patients treated with standard doses of imatinib. Better alternatives may emerge in the form of FACS analysis of patient cells either on therapy or *in vitro* (53,54). It could be argued that instead of being based on a reliable and sensitive test, dosing should be based on the maximum tolerated dose (MTD), just like with conventional chemotherapy. There is some suspicion that in the setting of NSCLC, suboptimal dosing may have contributed to the failure of gefitinib, a EGFR kinase inhibitor (55,56). This is discussed in detail in Section 4.

2.1.3. IMATINIB FOR ADVANCED CML

Imatinib is effective in patients with CML in accelerated phase and blast crisis. However, the rates of CHR and CCR are much lower in these patients, and relapse after an initial response is common (Table 4). Thus, long-term imatinib monotherapy

Table 4
Responses to Imatinib in Advanced Phases of Chronic Myeloid Leukemia

	Overall hematologic response/CHR	Sustained hematologic responses (> 4 weeks)	MCR	CCR	Median survival
Myeloid blast crisis (%) (n = 229)	52/15	31	16	7	6.8 months
Ph-positive ALL ^a (%) (n = 56)	59/22	27	Not available	Not available	4.9 months
Accelerated phase (%) (n = 181)	82/53	69	24	17	Not reached
Chronic phase after failure of IFN	6	95	60	41	Not reached

ALL, acute lymphoblastic leukemia CCR, complete cytogenetic response; CHR, complete hematologic response; IFN, interferon; MCR, major cytogenetic response.

^a Includes lymphoid blast crisis of chronic myeloid leukemia only CHR reported.

in this group of patients is problematic, and more aggressive strategies are justified if long-term remission is the therapeutic goal. Most experts agree that allogeneic stem cell transplantation should be offered to patients with advanced CML, if this is an option (49,33). Fortunately, there is no evidence that imatinib therapy preceding an allogeneic transplant has a negative impact on transplant-related mortality (57).

2.1.4. SIDE EFFECTS OF IMATINIB

Perhaps as surprising as the excellent therapeutic efficacy of imatinib is the fact that side effects are usually minor. ABL knockout mice have a high rate of neonatal lethality, and the viable offspring suffers from impaired immune function, bone defects and an ill-defined wasting syndrome (58,59). Even more significantly, disruption of both ABL and ARG (ABL2) generates an embryonic lethal phenotype, due to a complete defect of neurulation (60). Mice null for PDGFR β have vascular defects and edema (61), and KIT is important for early hematopoietic development (62). While imatinib does have some typical side effects, such as fluid retention, joint and musculoskeletal pain and skin rashes, one would expect more significant side effects from prolonged inhibition of these kinases. One explanation for the relative lack of side effects is that the relevance of the target kinases during embryonal development (reflected by the knockout mice) may be much greater than during adult life. Another reason may be that the inhibition of kinase activity is incomplete, which though (probably) undesirable from the pharmacodynamic point of view may help to reduce undesired drug effects. An exception to the rule that side effects are mild is hematologic toxicity, which is frequently grade 3 or grade 4, particularly in patients with advanced disease. Evidently, one could argue that hematologic toxicity reflects effective suppression of leukemic hematopoiesis by imatinib and is thus not a side effect *sensu strictu* (63). Despite the generally benign profile of side effects, it remains possible that additional adverse effects may be detected as experience with imatinib grows or with longer follow-up. For example, severe congestive heart failure has recently been reported in a small series of patients (64). Although the overall incidence of this may be low, it is a reminder that imatinib is a potent agent that should be used by experienced physicians.

2.1.5. RESISTANCE

Primary resistance or refractoriness is frequent in blast crisis (50,51,65). The underlying mechanisms are largely unknown but may be related to drug transport mechanisms (66). Acquired resistance after an initial response is common in advanced disease, less frequent in late chronic phase and quite rare in newly diagnosed chronic phase (67,68). One key observation from the study of patients with acquired resistance to imatinib was that relapse is almost invariably accompanied by reactivation of BCR-ABL signaling. Thus, the transforming principle responsible for pathogenesis in the first place retains its central role at the time of relapse. Reactivation of BCR-ABL signaling may occur as a result of point mutations within the kinase domain of ABL (Fig. 5) or, less frequently, as a result of increased expression. Point mutations have been described in more than 40 different positions (46) and may impair drug binding by one of three mechanisms (69–76). Bulky substitutions in spatially confined areas may sterically clash with imatinib. The pivotal example is the exchange of isoleucine for threonine at position 315 (T315I), which in addition to causing sterical

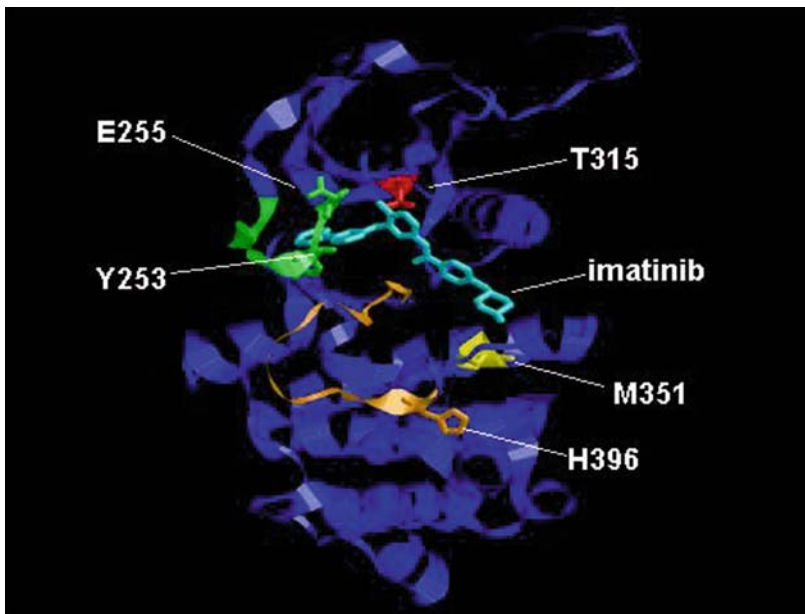


Fig. 5. Amino acids frequently mutated in patients with imatinib resistance. E255 and Y253 are localized in the ATP binding loop (p-loop, green), T315 (red) is the site of a hydrogen bond to imatinib, M351 (yellow) contacts the SH2 domain of the ABL in the autoinhibited conformation of the kinase, and H396 is localized in the activation loop (brown). Courtesy of Dr. Ian Griswold, Oregon Health & Science University.

hindrance abolishes a hydrogen bond between the pyrimidine nitrogen of imatinib and threonine 315. A second type of mutation prevents the conformational changes that are required for optimally accommodating imatinib. Examples include mutations in the ATP-binding loop (p-loop) of BCR-ABL. This structure undergoes extensive conformational changes on imatinib binding (34). Thus, mutations in the p-loop may prevent the structural changes required for drug binding. Lastly, activation loop mutations probably favor the active conformation of the kinase to which imatinib is unable to bind (77). The frequent M351T mutation may also belong to this category, as M351 is the site of an intramolecular kinase inhibitory interaction in ABL that may be abolished as a result of the mutation, favoring the kinase active state (33). The degree of resistance depends on the specific type of mutation. Some mutations retain almost wild-type drug sensitivity (78), while others like T315I are completely resistant. For the clinical management of patients with mutations, this has major implications, as dose escalation of imatinib is unlikely to benefit patients with highly resistant mutations but may recapture responses in the case of mutations that confer low or intermediate levels of resistance (79).

2.1.6. STRATEGIES TO OVERCOME IMATINIB RESISTANCE

A common strategy to overcome imatinib resistance is combining imatinib with conventional chemotherapeutic agents. The consensus reading from a large number of *in vitro* studies is that most combinations with conventional agents are additive or synergistic, as long as the resistant cells retain some sensitivity to imatinib (80).

Exceptions include the combination of imatinib with methotrexate or topotecan (81). In addition, imatinib has been combined with many other signal transduction inhibitors that target pathways known to be activated in BCR-ABL-positive cells. Several imatinib combinations are currently in phase I/II testing (reviewed in ref. (33)). However, the most promising development is alternative inhibitors of ABL that retain activity against the mutant ABL kinase. These alternative ABL inhibitors belong to different chemical classes. With the exception of AMN107 (nilotinib) and NS-187, they were originally developed as SRC inhibitors (Table 5) (82–88). Crystal structure analysis revealed that PD173955, a pyridopyrimidine initially developed as a Src inhibitor, binds the active conformation of the ABL kinase domain, with less stringent conformational requirements compared to imatinib (Fig. 4) (35). Similar binding modes have subsequently been demonstrated for other Src/Abl inhibitors, including dasatinib (BMS354825) (89), which has recently been approved by the FDA for the treatment of patients with imatinib failure. In contrast, nilotinib (AMN107) and NS-187, both derivatives of imatinib, bind the inactive conformation of ABL (87,88). Their increased potency is the result of improvements to the imatinib molecule. Thus, in AMN107, the piperazinyl group was replaced by a trifluorinated imidazole, resulting in the elimination of two energetically unfavorable hydrogen bonds, with more free binding energy available for the remaining molecule (90). The activity profiles of these alternative ABL inhibitors against the various kinase domain mutants are quite similar. Most mutants are covered, with the exception of T315I which is completely resistant. At a closer look however, differences become apparent that reflect the different binding modes. Thus, while in the case of nilotinib and NS-187 the relative differences between wild-type and mutant ABL are preserved, this is not the case for dasatinib. This suggests that while nilotinib and NS-187 “overpower” the mutants by their overall increased binding affinity, dasatinib exhibits an entirely different mode of binding.

There are very significant differences in bioavailability that prevented the clinical development of some of the compounds. All in all, four compounds are in clinical development. Dasatinib (Sprycel™) has recently been approved by the FDA, nilotinib (AMN107) is in advanced phase II testing, and NS-187 as well as SKI-606 are in phase studies. Both dasatinib and nilotinib have shown considerable clinical activity (Table 6) (91,92). Similar to imatinib, response rates are highest in patients with chronic phase and lowest in blast crisis. A direct comparison at this time is impossible, due to slightly different inclusion criteria and the different length of follow-up.

The Achilles heel of all the novel inhibitors in clinical development is that they are inactive against the T315I mutant. Thus, it is possible that this particular mutant will emerge as the “default mutant” in patients treated with compounds like dasatinib or nilotinib (93,94). This is suggested by *in vitro* data showing selection of T315I in the presence of nilotinib, dasatinib and both combined in BaF3 cells engineered to express BCR-ABL, while culture in the presence of imatinib produced a range of different mutations that rather faithfully represented the spectrum of mutations observed in patients with clinical resistance (95,96). Efforts are underway to develop inhibitors of T315I. One compound with anti-T315I activity termed ON012380 appears to act as a non-ATP-competitive inhibitor of BCR-ABL kinase activity. The profile of off-target effects of this compound is not yet clear, so it remains to be seen whether it will be a therapeutically viable compound (97). VX-680, an aurora kinase inhibitor, has moderate activity in the low micromolar dose range (77). This agent is in phase 1 trials for

Table 5
Alternative ABL Kinase Inhibitors with Activity Against Kinase Domain Mutants

<i>Compound</i>	<i>Class</i>	<i>IC₅₀ (cell proliferation) (nM)</i>					<i>References</i>
		<i>Wild-type BCR-ABL</i>	<i>E255K</i>	<i>M351T</i>	<i>H396P</i>	<i>T315I</i>	
PD180970	Pyridopyrimidine	25	140	45	15	840	82
SKI606	Pyridopyrimidine	~11	~30	ND	~10	>500	83
AP23848	Trisubstituted purine	14	94	24	8	9050	88
BMS-354825	2-amino-thiazole-5-carboxamide	0.087-1	Low nanomolar range			>1000	158,159
AMN107	2-phenylaminopyrimidine	20	150	31	ND	>10,000	88
NS-187	2-phenylaminopyrimidine	22	98	150	95	>10,000	87

ND, not detected.

Table 6
Results of Phase I trials of Nilotinib and Dasatinib

<i>Disease phase and response</i>	<i>Dasatinib</i>	<i>Nilotinib</i>
Chronic phase	<i>n</i> = 40 ^a	<i>n</i> = 12 ^b
Major hematologic response	37 (92%)	11 (92%)
Complete cytogenetic response	14 (35%)	6 (55%)
Accelerated phase	<i>n</i> = 11	<i>n</i> = 24
Major hematologic response	9 (82%)	24 (52%)
Complete cytogenetic response	2 (18%)	6 (13%)
Myeloid blast crisis	<i>n</i> = 23	<i>n</i> = 24
Major hematologic response	14 (61)	4 (17)
Complete cytogenetic response	6 (26)	1 (4)
Lymphoid blast crisis and Ph-positive ALL	<i>n</i> = 10	<i>n</i> = 9
Major hematologic response	8 (80%)	1 (11%)
Complete cytogenetic response	3 (30%)	1 (11%)

^aEight patients in chronic phase, two in accelerated phase and one each in myeloid and lymphoid blast crisis were intolerant of imatinib.

^bAdditional 10 patients had clonal cytogenetic evolution as the only feature defining accelerated phase. All five patients with active hematologic disease attained complete hematologic response and two patients attained complete cytogenetic response.

refractory leukemia. Arguably the most promising candidate is a series of compounds that have been generated with a crystallography-based approach. Compounds of this class including SGX393 are active against T315I in the nanomolar dose range (98). The structures have not been published yet, and no clinical trials have been initiated.

Will it be possible to cure CML with BCR-ABL kinase targeted therapy? The jury is out. Most with CCR remain positive by RT-PCR, in contrast to recipients of stem cell transplants (99,100), and recurrence of disease is the rule if imatinib is discontinued (49). The causes underlying the persistence of minimal residual disease are not well understood but may include mechanisms that are non-dependent on BCR-ABL kinase activity or not even on BCR-ABL (33,101). For example, it is conceivable that leukemic stem cells are able to utilize physiological signals, such as growth factors, to maintain viability in the face of BCR-ABL inhibition. This would obviously limit the potential of BCR-ABL kinase targeted therapy.

2.2. Imatinib for the Treatment of Other Hematologic Malignancies

Some 50% of patients with hypereosinophilic syndrome (HES) harbor a FIP1L1-PDGFR α fusion gene that is generated as a result on an interstitial deletion on chromosome 4 (102). These individuals respond extremely well to imatinib, even at doses that are ineffective in CML, consistent with the higher imatinib sensitivity of PDGFR α compared to ABL (88). Interestingly, it was the imatinib responsiveness of HES that directed the path to the identification of the underlying genetic lesion (103). CMML with translocations that activate PDGFR β is also extremely responsive to imatinib (104). Lastly, some patients with SM express juxtamembrane mutants of KIT that are imatinib sensitive, in contrast to the activation loop mutants that characterize the majority of SM patients (105,106).

Imatinib was tried in many other types of hematologic cancer, sometimes without clear biological rationale. Given the favorable profile of side effects, this could be done safely, but not unexpectedly the results were generally disappointing. For example, there were no significant responses in 23 patients with idiopathic myelofibrosis (IMF) (107). Similarly, only isolated patients with AML responded (13,108). An exception is polycythemia vera, where responses have been observed rather consistently in the form of reduced phlebotomy requirements, although usually only at high doses of imatinib (109). In some patients, this leads to a significant reduction but not elimination of the polycythemia vera cell clone as detected by quantitative PCR for the V617F mutation of JAK2 (110).

2.3. Imatinib for the Treatment of Gastrointestinal Stromal Tumors

GISTs are rare GI tract tumors that were previously classified as leiomyomas or leiomyosarcomas. As a result of inconsistent histopathological diagnosis, the incidence of GISTs is not exactly known but may be in the range of 2 per 10^5 population and thus slightly higher than the incidence of CML (111). GISTs are derived from the interstitial cells of Cajal, the pacemaker cells of the intestine, and are most frequently located in the stomach and small intestine. Activating mutations of KIT are found in 80–85% of patients, and activating mutations of PDGFR α in 7%. Studies in very small tumors revealed a high incidence of KIT mutations, which is a direct evidence that the acquisition of KIT mutations is an early event in the pathogenesis of GISTs (112). Several families with germline KIT mutations have been identified. Such individuals have diffuse interstitial cell hyperplasia and frequently develop multiple GISTs at an early age (5).

Mutations in the form of deletions, insertions, ITDs as well as point mutations occur most frequently (66.7%) in the juxtamembrane domain, which is thought to inhibit dimerization of the receptor in the absence of ligand. The mutations disrupt the autoinhibitory function of the juxtamembrane domain, leading to constitutive dimerization and activation of the kinase. Mutations in the extracellular domain, kinase domain I and kinase activation loop are less frequent (5). Expression of mutant KIT renders cytokine-dependent cell lines factor-independent, consistent with gain-of-function status.

In vitro studies showed that imatinib was active against a cell line juxtamembrane mutant KIT (113) and led the manufacturer to allow compassionate use of the drug in a patient with metastatic GIST (114). The impressive response seen in this individual quickly led to clinical trials, a multicenter study that compared 400 vs. 600 mg imatinib daily (115) and a phase I EORTC trial that used 400–1000 mg daily (116). Objective partial responses were seen in approximately half the patients and stable disease in another 25%. Responses were generally stable during the 6–9 month observation time of these trials. One crucial observation from these studies was that response rates depended on KIT mutation status. The partial response rate in patients with juxtamembrane mutations was 83.5%, but only 48.7% in patients with mutations in the extracellular domain and 0% in patients without mutation (5). This validated KIT as the target of imatinib in GISTs. Moreover, as the *in vitro* sensitivity to imatinib of extracellular domain and juxtamembrane domain mutants of KIT is not different, these data show that the specific type of mutation must influence the biology of the disease irrespective of its sensitivity to imatinib (5). Differential sensitivity to imatinib was also observed in the rare GIST patients with mutations of PDGFR α . Patients with

mutations in the juxtamembrane domain have imatinib responsive disease, while the disease in patients with activation loop mutations is imatinib resistant (117).

Unfortunately, as with imatinib in advanced phases of CML, GIST patients tend to relapse after an initial response. The mechanisms underlying resistance appear to be similar to those observed in CML patients, involving overexpression and mutations of KIT that impair drug binding. In addition, some patients with resistant disease exhibit downregulation of KIT expression, consistent with the activation of alternative signaling pathways, and there is “functional resistance” in some cases, with sensitive disease *in vitro* but resistance *in vivo* (5). Similar to imatinib-resistant CML, the most promising approach to overcome resistance in GIST is alternative inhibitors of KIT that retain activity against the kinase domain mutant protein (118).

2.4. Imatinib for the Treatment of Dermatofibrosarcoma Protuberans

Dermatofibrosarcoma protuberans is an infiltrative skin tumor that presently is treated with surgery. Although these tumors rarely metastasize, there is a high risk of local recurrence after surgery. The causal genetic lesion is a chromosomal rearrangement fusing the collagen type I α 1 (*COL1A1*) gene to the PDGF B-chain (*PDGF-B*) gene. The resulting *COL1A1/PDGF-B* fusion protein is processed to mature PDGF-BB, which in an autocrine loop stimulates tumor growth. Studies in cell lines showed that interruption of this autocrine loop by imatinib leads to apoptosis, and anti-tumor activity was also documented in a murine xenograft model (119). Clinical activity was demonstrated in several patients with metastatic disease (120).

3. FLT3 INHIBITORS FOR ACUTE MYELOID LEUKEMIA

3.1. Biological Basis

Approximately 30% of patients with AML have mutations of FLT3, a type III receptor tyrosine kinase expressed on early hematopoietic progenitor cells. FLT3 mutations are associated with monocytic or promyelocytic differentiation and high white cell counts and in most studies with an adverse prognosis. The most frequent type of mutation, found in approximately 25% of patients with AML, is ITDs within the juxtamembrane domain. These duplications encompass between 5 and 40 amino acids, undermine the ability of the juxtamembrane domain to prevent dimerization and result in constitutive activation of receptor signalling (4,14). Another 7% of AML patients exhibit mutations in the activation loop, typically in position D835 that is homologous to D816 in KIT (121).

FLT3 ITDs or activation loop mutants transform BaF3 cells independent of growth factor but do not induce AML in the standard transduction/transplantation model of murine leukemia. Rather, they lead to a myeloproliferative syndrome (122). However, in combination with PML-RARA, the fusion gene typical for acute promyelocytic leukemia (APL) induces AML with a promyelocytic phenotype, consistent with the high prevalence of FLT3 ITDs in patients with APL (123). These experiments show that the roles of BCR-ABL in CML and FLT3 mutations in AML are different. BCR-ABL alone is capable of inducing a CML-like myeloproliferative syndrome while the induction of AML by constitutively active FLT3 requires cooperating genetic lesions. As discussed in Section 3.2., this has consequences for the success of FLT3 targeted therapy of AML.

3.2. Clinical Trials

Several FLT3 inhibitors have entered clinical development (Table 7). The various compounds are structurally diverse and have a variety of other kinase targets, mainly other type III receptor tyrosine kinases, such as KIT or PDGFR. The IC_{50} for inhibition of FLT3 autophosphorylation *in vitro* ranges between 2 nM for CEP-701 and 528 nM for PKC412, a compound previously developed as a PKC inhibitor (124). Overall, the results of early clinical trials were quite variable. For example, MLN518 was tested in a phase I trial of 40 patients with refractory or relapsed AML, or myelodysplastic syndrome (MDS) who were not pre-screened for FLT3 mutations. Stable disease was

Table 7
FLT3 Inhibitors in Clinical Development

Compound	Class	Target	FLT3 IC_{50} (nM)	Clinical trials	References
PKC412	Benzoylstauosporine	PKC PDGFR KDR KIT FLT3 ABL	528	Phase 2: AML with/without FLT3-ITD	128
CEP-701	Indolocarbazole	FLT3 TRKA KDR PKC PDGFR EGFR	2–3	Phase 2: AML with FLT3-ITD	160
CT35318	Piperazinyl quinazoline	KIT PDGFR FLT3 FMS	170–220	Phase 1: AML/MDS with/without FLT3-ITD Phase 2: AML with FLT3-ITD	125,161
SU5416	Indolinone	FLT3 KDR KIT	250	Phase 2: refractory AML/ MDS/MPD Phase 2: refractory AML (c-KIT ⁺)	126,127
SU11248	Indolinone	FLT3 KDR PDGFR	10	Phase 1: AML	162

AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; MPD, myeloproliferative disease; PDGFR, platelet growth factor receptor; PKC, protein kinase C.

reported in two patients without evidence of FLT3 mutations and a partial response in one patient with a FLT3 ITD (125). Similarly, only one complete and seven partial responses were seen in 43 patients with advanced AML treated with SU5416 (126). This study had been conceived with VEGFR rather than FLT3 as the therapeutic target. Nonetheless, FLT3 ITDs were demonstrated in seven patients, but none of these patients responded to treatment. Another study tested SU5416 in patients with relapsed or refractory AML or MDS and reported only three partial responses in 55 patients (127). More promising results were obtained with PKC412 in a cohort of 20 relapsed or refractory AML patients with FLT mutations (128). In this trial, a reduction of peripheral blood blast counts by 50% or more was seen in 70% of patients. Half of these patients completely cleared blasts from the peripheral blood. Two patients achieved a complete response in the marrow (<5% blasts). Collateral studies demonstrated inhibition of FLT3 autophosphorylation in blasts from patients on treatment, an indication of efficient target inhibition. Another trial with an orally bioavailable FLT3 inhibitor, SU11248, reported responses in 4/4 patients with FLT3 mutations, including three partial responses and complete marrow response without platelet recovery. However, responses were generally short-lived. Altogether these data are reminiscent of the results of imatinib for CML in blast crisis, not surprising, given that mutant FLT3 is unable to induce an AML phenotype in the absence of cooperating mutations (122). In fact, it is likely that the acquisition of the tyrosine kinase mutation is the second rather than the first hit on the path to AML, and reversal of a late event would naturally not be capable of eradicating the disease. Consistent with this, it has been demonstrated that FLT3 mutations may be acquired at the time of relapse but may also “disappear” with disease recurrence after chemotherapy, when another clone with wild-type FLT3 becomes dominant (14,41). Interestingly, this sequence has also been shown for AML with mutations of the core binding factors such as AML1-ETO and CBFbeta-MYH11 in association with mutations of KIT. In patients in long-term remission to chemotherapy, AML1-ETO remains demonstrable, but not the activating mutation of KIT, suggesting that the latter is the secondary event (40,129).

Bearing all this in mind, it is clear that monotherapy with FLT3 inhibitors will not be the future. Rather, it will be crucial to integrate these compounds into conventional therapy regimens and/or allograft approaches. One concern is that drugs with a broad spectrum of activity against several kinases (FLT3, KIT, in some cases KDR) with important roles in early hematopoietic development may significantly increase the stem cell toxicity of conventional agents. Fortunately, studies in mice suggest that CT53518 does not adversely affect recovery from chemotherapy-induced myelosuppression or engraftment after bone marrow transplantation (15). MLN518 is currently being tested in combination with standard chemotherapy (cytarabine+daunorubicin) in a phase I study of newly diagnosed patients.

4. DEVELOPMENT OF GEFITINIB AND ERLOTINIB FOR NON-SMALL CELL LUNG CANCER

The EGFR is highly expressed on various types of epithelial tumors, including NSCLC (130). Based on this observation, EGFR was proposed as a drug target in NSCLC, and several murine and humanized murine antibodies were developed, the best

known of which is cetuximab (131). These antibodies target the extracellular domain of EGFR where they block the binding of ligand. In the early 1990s, a drug discovery program at AstraZeneca led to the identification of anilinoquinazoline derivatives as specific ATP-competitive inhibitors of the EGFR tyrosine kinase (132,133). Gefitinib (Iressa) was chosen as the compound for clinical development. Activity was demonstrated against a number of cell lines derived from NSCLC and ovarian, breast, colon, and head and neck tumors and in various xenograft models. Phase I trials in patients with many different types of refractory tumors showed mild toxicity (134–138), the MTD being 1000 mg/day. Importantly, target modulation, that is, inhibition of EGFR phosphorylation, was demonstrated in skin biopsies obtained during treatment (139). Antitumor activity was observed at doses greater than 150 mg/day, with no clear dose response above this level. As a consequence, similar to imatinib, the phase II trials did not use the MTD but rather settled at 250 and 500 mg gefitinib daily. Based on the promising phase I data, two large phase II trials (IDEAL-1 and IDEAL-2) were initiated in patients with NSCLC who had failed cis-platinum-based chemotherapy. Each of these trials compared 250 and 500 mg gefitinib daily in more than 200 patients (140,141). Disease control was seen in 42–54% of patients treated with 250 mg and 36–51% of patients treated with 500 mg gefitinib daily. One-year overall survival was approximately 30%, which compared very favorably with historical controls. Objective response rates were around 20% in the IDEAL-1 trial and 10% in the IDEAL-2 trial, a difference that may reflect the inclusion of patients with slightly more advanced disease in the IDEAL-2 study. Severe (grades 3 and 4) toxicity was more frequent with 500 mg compared to 250 mg (18 vs. 7% in the IDEAL-2 trial), which led to the choice of 250 mg daily for the phase III trials, which tested gefitinib plus gemcitabine/cis-platinum (INTACT-1 study) or gefitinib plus paclitaxel/carboplatinum (INTACT-2 study) (142,143) vs. chemotherapy alone or gefitinib vs. placebo and best supportive care in patients previously treated with chemotherapy (ISEL study, $n = 1692$) (55). Unexpectedly, the chemotherapy combination studies did not show an advantage over chemotherapy alone, although synergistic effects of the drugs under investigation had been shown *in vitro*. The precise reasons why the *in vitro* data did not translate into a clinical benefit are not known, but may include dose administration (continuous in the trial, but pulsed in animal studies), antagonistic effects and the fact that no three-drug chemotherapy regimen had shown superiority over the two-drug combinations (56). The ISEL trial showed a significantly higher objective response rate in the gefitinib group (8 vs. 1%, $p < 0.0001$), but failed to show an overall survival benefit (median survival 5.6 vs. 5.1 months, $p =$ not significant). However, significantly superior results were observed for patients who never smoked (8.9 vs. 6.2 months, $p = 0.012$) and in patients of Asian origin (9.5 vs. 5.5 months, $p = 0.01$) in pre-planned subgroup analysis.

The results of the ISEL trial became available one month after erlotinib (Tarceva™) had been approved by the FDA based on the results of a similar but smaller study (BR21; $n = 732$) (144). In contrast to the ISEL trial, the BR21 study showed a significant improvement of overall survival in the erlotinib group compared to placebo (6.7 vs. 4.7 months, $p < 0.001$) and a more substantial improvement for patients of Asian origin (12.3 vs. 5.5 months, $p < 0.001$). On the basis of the BR21 results, the FDA relabeled gefitinib for use in patients already receiving it and obtaining

a clinical benefit from it. It should be noted that erlotinib in combination with carboplatin/paclitaxel or cisplatin/gemcitabine was not superior to placebo in two studies (TALENT and TRIBUTE trials) with a design analogous to the IMPACT studies (145).

The reasons for the discrepancy between the ISEL and the BR21 trials have been the subject of much discussion. The profile of responders was similar in both studies and included female gender and adenocarcinoma histology (and Japanese ethnicity in the IDEAL-2 trial). Subsequent studies showed that the striking responses seen in a minority of patients were associated with the presence of mutations in the kinase domain of EGFR (18,19) or multiple gene copies of EGFR or high levels of protein (20). Various types of mutations were detected, including deletions, point mutations and in frame insertions. Compared to the wild-type EGFR, the mutant proteins exhibit more prolonged and stronger tyrosine phosphorylation in response to EGF. At the same time, there is increased sensitivity to gefitinib. This suggests that the mutations may increase the affinity to ATP but also to gefitinib. There are also differences in the signaling pathways activated by mutant compared to wild-type receptor, with the mutant receptor preferentially activating AKT (146). As the phase II trials had not shown an association between response and EGFR expression as analyzed by immunohistochemistry, EGFR status was not used as a selection criterion for either the ISEL or the BR21 trials. Retrospective EGFR copy number analysis in both trials showed a significant association between gene amplification and response (56). Mutation analysis for the ISEL trials has not yet been completed. As information on less than half of the patients is available, it is currently impossible to determine whether the inclusion of more patients with a favorable “molecular make-up” in the BR21 trial explains the superior results. A recent comparison of baseline characteristics showed equal distributions of major risk factors, including smoking history and previous chemotherapy. Fewer patients of Asian origin enrolled in the BR21 study (13 vs. 21% in the treatment arms), but more patients with a complete or partial response to previous chemotherapy (38 vs. 18%) (56). The latter could explain the failure of the ISEL study, as in an exploratory analysis of the BR21 trial a significant survival benefit for erlotinib was demonstrated in patients with a prior response to chemotherapy but not for those with stable or progressive disease as their best response (144). Another factor not related to patient selection may be the dose. Erlotinib was used at the MTD of 150 mg daily, while gefitinib was used at 250 mg daily, significantly below the MTD. These data are concordant with the lower rate of skin and gastrointestinal side effects in the ISEL trial, which suggest lower levels of systemic exposure. If a suboptimal dose was indeed the reason for the failure of the ISEL study, it would evidently be an “easy fix” for the drug.

Interestingly, in a recent study, mutations in the kinase domain of ERBB2 (also known as HER2 or Neu) were identified in 4% of NSCLC samples (10% in the adenocarcinoma subtype). These mutations (mainly one specific type of insertion) activate the kinase, consistent with their position within the kinase domain that is similar to the localization of activating EGFR mutations (147). Inhibition of this type of mutations has recently been shown for HKI-272, an orally available arylaminoquinoline (148).

Unfortunately, as with advanced CML and GISTs, acquired gefitinib resistance due to a point mutation in the kinase domain has recently been demonstrated in a

patient who relapsed after two years of gefitinib therapy (149). Notably, the mutation (T790M) is structurally homologous to T315I in ABL, the notorious imatinib-resistant mutant first described by Gorre et al. (150). It is currently unclear, however, whether point mutations are as important in the scenario of NSCLC as in BCR-ABL-positive leukemia, as at least in some reported cases, mutant alleles constitute only a small minority of all alleles (151). Regardless, irreversible inhibitors of EGFR and ERBB2 have shown promise in preclinical studies of T790M mutant EGFR and are currently in phase I trials (151,152).

5. PERSPECTIVE

The success of imatinib for the treatment of CML, HES and GISTs has sparked enormous interest in targeting tyrosine kinases for therapy of malignant disease. Large-scale sequencing projects suggest that tyrosine kinase mutations may be a widespread principle in carcinogenesis. For example, sequencing of the “tyrosine kinome” in colorectal cancer cell lines and tumor specimens revealed somatic mutations in 14 kinase genes. Although the localization of the mutations in critical regions of the kinases suggest that they may be activating, these data still require confirmation in biochemical assays (153). Additionally, even with this approach, tyrosine kinase mutations were detected in only one-third of the samples. This suggests that alternative mechanisms of kinase activation may be operational, such as amplification or activation by translocations that would be missed by the sequencing approach. The other possibility is that mutations may be present in serine/threonine kinases, lipid kinases or phosphatases. There are precedents for all of these. Mutations that lead to constitutive activation of BRAF are frequent in melanoma and pancreatic carcinoma and occur at lower rates in several other types of solid tumors (154). Mutations of the p110 subunit of the phosphatidylinositol 3' kinase have recently been detected in a variety of solid tumors (155), and sequencing of the human tyrosine phosphatome revealed mutations in several phosphatases (2).

Will it be possible to repeat the success of imatinib? It appears that this will largely depend on the genetics of the disease in question. One extreme of the spectrum may be diseases like polycythemia vera, where a rather uniform clinical entity exhibits a slowly progressive phenotype and is associated with an activating tyrosine kinase mutation (JAK2^{V617F}) in almost all cases (37,155,156). The prediction is that a JAK2 inhibitor would be at least as effective as imatinib in CML. The same should hold true for patients with JAK2^{V617F}-positive essential thrombocythemia and perhaps even for IMF, although the frequent chromosomal abnormalities present in IMF suggest that the pathogenesis may be more complex, involving mechanisms in addition to JAK2 activation (157). In contrast, it is quite clear that the pathogenesis of solid tumors is much more complex, leading to more potential targets but also to more redundancy. Nonetheless, the example of gefitinib shows that tyrosine kinase inhibitors can be effective even in very advanced cancers. To fully exploit the potential of tyrosine kinase and other specific signal transduction inhibitors, it will be crucial to get the right drug to the right patient. For this to be accomplished, future classifications of malignant disorders will have to use biochemical rather than morphological criteria as the basis for a personalized approach to therapy. The first steps have been taken but much work still remains to be done.

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REFERENCES

1. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science* 2002;298:1912–1934.
2. Wang Z, Shen D, Parsons DW et al. Mutational analysis of the tyrosine phosphatome in colorectal cancers. *Science* 2004;304:1164–1166.
3. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. *Nature* 2001;411:355–365.
4. Nakao M, Yokota S, Iwai T et al. Internal tandem duplication of the *flt3* gene found in acute myeloid leukemia. *Leukemia* 1996;10:1911–1918.
5. Corless CL, Fletcher JA, Heinrich MC. Biology of gastrointestinal stromal tumors. *J Clin Oncol* 2004;22:3813–3825.
6. Smith KM, Yacobi R, Van Etten RA. Autoinhibition of BCR-ABL through its SH3 domain. *Mol Cell* 2003;12:27–37.
7. Golub TR, Goga A, Barker GF et al. Oligomerization of the ABL tyrosine kinase by the Ets protein TEL in human leukemia. *Mol Cell Biol* 1996;16:4107–4116.
8. Reilly JT. Receptor tyrosine kinases in normal and malignant haematopoiesis. *Blood Rev* 2003;17:241–248.
9. Ma Y, Zeng S, Metcalfe DD et al. The c-KIT mutation causing human mastocytosis is resistant to STI571 and other KIT kinase inhibitors; kinases with enzymatic site mutations show different inhibitor sensitivity profiles than wild-type kinases and those with regulatory-type mutations. *Blood* 2002;99:1741–1744.
10. Heinrich MC, Griffith DJ, Druker BJ et al. Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor. *Blood* 2000;96:925–932.
11. Corbin AS, Demehri S, Griswold IJ et al. *In vitro* and *in vivo* activity of ATP-based kinase inhibitors AP23464 and AP23848 against activation loop mutants of Kit. *Blood* 2005;2004–2012.
12. Kohl TM, Schnittger S, Ellwart JW, Hiddemann W, Spiekermann K. KIT exon 8 mutations associated with core binding factor (CBF) - acute myeloid leukemia (AML) cause hyperactivation of the receptor in response to stem cell factor. *Blood* 2004;2004–2006.
13. Kindler T, Breitenbuecher F, Marx A et al. Efficacy and safety of imatinib in adult patients with c-kit-positive acute myeloid leukemia. *Blood* 2004;103:3644–3654.
14. Thiede C, Studel C, Mohr B et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood* 2002;99:4326–4335.
15. Griswold IJ, Shen LJ, LaRosee P et al. Effects of MLN518, a dual FLT3 and KIT inhibitor, on normal and malignant hematopoiesis. *Blood* 2004;104:2912–2918.
16. Armstrong SA, Kung AL, Mabon ME et al. Inhibition of FLT3 in MLL. Validation of a therapeutic target identified by gene expression based classification. *Cancer Cell* 2003;3:173–183.
17. Lokker NA, Sullivan CM, Hollenbach SJ, Israel MA, Giese NA. Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. *Cancer Res* 2002;62:3729–3735.
18. Lynch TJ, Bell DW, Sordella R et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to Gefitinib. *N Engl J Med* 2004;350:2129–2139.
19. Paez JG, Janne PA, Lee JC et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–1500.
20. Cappuzzo F, Hirsch FR, Rossi E et al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. *J Natl Cancer Inst* 2005;97:643–655.
21. Grand EK, Chase AJ, Heath C, Rahemtulla A, Cross NC. Targeting FGFR3 in multiple myeloma: inhibition of t(4;14)-positive cells by SU5402 and PD173074. *Leukemia* 2004;18:962–966.

22. Virchow R. Weisses Blut. *Frorieps Notizen*. 1845;36:151–156.
23. Nowell P, Hungerford D. A minute chromosome in human chronic granulocytic leukemia. *Science* 1960;132:1497.
24. Rowley JD. A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* 1973;243:290–293.
25. Bartram CR, de Klein A, Hagemeijer A et al. Translocation of c-abl oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. *Nature* 1983;306:277–280.
26. Groffen J, Stephenson JR, Heisterkamp N et al. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. *Cell* 1984;36:93–99.
27. Lugo TG, Pendergast AM, Muller AJ, Witte ON. Tyrosine kinase activity and transformation potency of BCR-ABL oncogene products. *Science* 1990;247:1079–1082.
28. Anafi M, Gazit A, Gilon C, Ben Neriah Y, Levitzki A. Selective interactions of transforming and normal abl proteins with ATP, tyrosine-copolymer substrates, and tyrophostins. *J Biol Chem* 1992;267:4518–4523.
29. Anafi M, Gazit A, Zehavi A, Ben Neriah Y, Levitzki A. Tyrophostin-induced inhibition of p210BCR-ABL tyrosine kinase activity induces K562 to differentiate. *Blood* 1993;82:3524–3529.
30. Zimmermann J, Buchdunger E, Mett H et al. Phenylamino-pyrimidine (PAP)-derivatives: a new class of potent and highly selective PDGF-receptor autophosphorylation inhibitors. *Bioorg Med Chem Lett* 1996;6:1221–1226.
31. Zimmermann J, Buchdunger E, Mett H, Meyer T, Lydon NB. Potent and selective inhibitors of the Abl kinase - phenylamino-pyrimidine (PAP) derivatives. *Bioorg Med Chem Lett* 1997;7:187–192.
32. Druker BJ, Tamura S, Buchdunger E et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of BCR-ABL positive cells. *Nat Med* 1996;2:561–566.
33. Deininger M, Buchdunger E, Druker BJ. The development of imatinib as a therapeutic agent for chronic myeloid leukemia. *Blood* 2005;105:2640–2653.
34. Schindler T, Bornmann W, Pellicena P et al. Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. *Science* 2000;289:1938–1942.
35. Nagar B, Bornmann WG, Pellicena P et al. Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). *Cancer Res* 2002;62:4236–4243.
36. Deininger MW, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. *Blood* 2000;96:3343–3356.
37. James C, Ugo V, Le Couedic JP et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature* 2005;434:1144–1148.
38. Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science* 1990;247:824–830.
39. Koschmieder S, Goettgens B, Zhang P et al. Inducible chronic phase of myeloid leukemia with expansion of hematopoietic stem cells in a transgenic model of BCR-ABL leukemogenesis. *Blood*. 2005;105:324–334.
40. Wang YY, Zhou GB, Yin T et al. AML1-ETO and C-KIT mutation/overexpression in t(8;21) leukemia: implication in stepwise leukemogenesis and response to Gleevec. *Proc Natl Acad Sci USA* 2005;102:1104–1109.
41. Kottaridis PD, Gale RE, Langabeer SE et al. Studies of FLT3 mutations in paired presentation and relapse samples from patients with acute myeloid leukemia: implications for the role of FLT3 mutations in leukemogenesis, minimal residual disease detection, and possible therapy with FLT3 inhibitors. *Blood* 2002;100:2393–2398.
42. Druker BJ, Sawyers CL, Kantarjian H et al. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N Engl J Med* 2001;344:1038–1042.
43. Druker BJ, Talpaz M, Resta DJ et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 2001;344:1031–1037.
44. Deininger M, Goldman JM, Lydon NB, Melo JV. The tyrosine kinase inhibitor CGP57148B selectively inhibits the growth of BCR-ABL positive cells. *Blood* 1997;90:3691–3698.
45. Simonsson B for the IRIS study group. Beneficial effects of cytogenetic and molecular response on longterm outcome in patients with newly diagnosed chronic myeloid leukemia in chronic phase (CML-CP) treated with imatinib (IM): updata from the IRIS study [abstract]. *Blood* 2005;106:52a.

46. Hughes TP, Deininger MW, Hochhaus A et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors - Review and recommendations for 'harmonizing' current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. *Blood* 2006;108:28–37.
47. Cortes J, Giles F, O'Brien S et al. Result of high-dose imatinib mesylate in patients with Philadelphia chromosome-positive chronic myeloid leukemia after failure of interferon- α . *Blood* 2003;102:83–86.
48. Kantarjian HM, Talpaz M, O'Brien S et al. Imatinib mesylate for Philadelphia chromosome-positive, chronic-phase myeloid leukemia after failure of interferon- α : follow-up results. *Clin Cancer Res* 2002;8:2177–2187.
49. Deininger MW. Management of early stage disease. *Hematology Am Soc Hematol Educ Program* 2005;174–182.
50. Talpaz M, Silver RT, Druker BJ et al. Imatinib induces durable hematologic and cytogenetic responses in patients with accelerated phase chronic myeloid leukemia: results of a phase 2 study. *Blood* 2002;99:1928–1937.
51. Sawyers CL, Hochhaus A, Feldman E et al. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood* 2002;99:353–3539.
52. Kantarjian HM, Talpaz M, O'Brien S et al. Dose escalation of imatinib mesylate can overcome resistance to standard-dose therapy in patients with chronic myelogenous leukemia. *Blood* 2003;101:473–475.
53. Schultheis B, Szydlo R, Mahon FX, Apperley JF, Melo JV. Analysis of total phosphotyrosine levels in CD34+ cells from CML patients to predict the response to imatinib mesylate treatment. *Blood* 2005;105:4893–4894.
54. White D, Saunders V, Lyons AB et al. In-vitro sensitivity to imatinib-induced inhibition of ABL kinase activity is predictive of molecular response in de-novo CML patients. *Blood* 2005;106:2520–2526.
55. Thatcher N, Chang A, Parikh P et al. Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). *Lancet* 2005;366:1527–1537.
56. Blackhall F, Ranson M, Thatcher N. Where next for gefitinib in patients with lung cancer? *Lancet Oncol* 2006;7:499–507.
57. Deininger M, Schleuning M, Greinix H et al. The effect of prior exposure to imatinib on transplant-related mortality. *Haematologica* 2006;91:452–459.
58. Tybulewicz VL, Crawford CE, Jackson PK, Bronson RT, Mulligan RC. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. *Cell* 1991;65:1153–1163.
59. Schwartzberg PL, Stall AM, Hardin JD et al. Mice homozygous for the ablm1 mutation show poor viability and depletion of selected B and T cell populations. *Cell* 1991;65:1165–1175.
60. Koleske AJ, Gifford AM, Scott ML et al. Essential roles for the Abl and Arg tyrosine kinases in neurulation. *Neuron* 1998;21:1259–1272.
61. Lindahl P, Johansson BR, Leveen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* 1997;277:242–245.
62. Lyman SD, Jacobsen SE. c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. *Blood* 1998;91:1101–1134.
63. Deininger MW, O'Brien SG, Ford JM, Druker BJ. Practical management of patients with chronic myeloid leukemia receiving imatinib. *J Clin Oncol* 2003;21:1637–1647.
64. Kerkela R, Grazette L, Yacobi R et al. Cardiotoxicity of the cancer therapeutic agent imatinib mesylate. *Nat Med* 2006;12:908–916.
65. Ottmann OG, Druker BJ, Sawyers CL et al. A phase 2 study of imatinib in patients with relapsed or refractory Philadelphia chromosome-positive acute lymphoid leukemias. *Blood* 2002;100:1965–1971.
66. Lange T, Gunther C, Kohler T et al. High levels of BAX, low levels of MRP-1, and high platelets are independent predictors of response to imatinib in myeloid blast crisis of CML. *Blood* 2003;101:2152–2155.
67. Kantarjian H, Sawyers C, Hochhaus A et al. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N Engl J Med*. 2002;346:645–652.

68. O'Brien SG, Guilhot F, Larson RA et al. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med* 2003;348:994–1004.
69. Gorre ME, Ellwood-Yen K, Chiosis G, Rosen N, Sawyers CL. BCR-ABL point mutants isolated from patients with imatinib mesylate-resistant chronic myeloid leukemia remain sensitive to inhibitors of the BCR-ABL chaperone heat shock protein 90. *Blood* 2002;100:3041–3044.
70. Shah NP, Nicoll JM, Nagar B et al. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell* 2002;2:117–125.
71. Al Ali HK, Heinrich MC, Lange T et al. High incidence of BCR-ABL kinase domain mutations and absence of mutations of the PDGFR and KIT activation loops in CML patients with secondary resistance to imatinib. *Hematol J* 2004;5:55–60.
72. Hochhaus A, Kreil S, Corbin AS et al. Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. *Leukemia* 2002;16:2190–2196.
73. Barthe C, Cony-Makhoul P, Melo JV, Mahon JR. Roots of clinical resistance to STI-571 cancer therapy. *Science* 2001;293:2163.
74. Branford S, Walsh CT, Rudzki Z et al. BCR-ABL kinase domain mutations in CML patients on imatinib: incidence is correlated with duration of CML and mutations in the P-loop may be indicative of a poor outcome [abstract]. *Blood* 2002;100:367a.
75. Branford S, Rudzki Z, Walsh S et al. Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. *Blood* 2003;102:276–283.
76. Hofmann WK, Jones LC, Lemp NA et al. Ph(+) acute lymphoblastic leukemia resistant to the tyrosine kinase inhibitor STI571 has a unique BCR-ABL gene mutation. *Blood* 2002;99:1860–1862.
77. Young MA, Shah NP, Chao LH et al. Structure of the kinase domain of an imatinib-resistant Abl mutant in complex with the aurora kinase inhibitor VX-680. *Cancer Res* 2006;66:1007–1014.
78. Corbin AS, Rosee PL, Stoffregen EP, Druker BJ, Deininger MW. Several BCR-ABL kinase domain mutants associated with imatinib mesylate resistance remain sensitive to imatinib. *Blood* 2003;101:4611–4614.
79. Nicolini FE, Corm S, Le QH et al. Mutation status and clinical outcome of 89 imatinib mesylate-resistant chronic myelogenous leukemia patients: a retrospective analysis from the French intergroup of CML (Fi(phi)-LMC GROUP). *Leukemia* 2006;20:1061–1066.
80. La Rosee P, Johnson K, Corbin AS et al. *In vitro* efficacy of combined treatment depends on the underlying mechanism of resistance in imatinib-resistant BCR-ABL-positive cell lines. *Blood* 2004;103:208–215.
81. Thiesing JT, Ohno-Jones S, Kolibaba KS, Druker BJ. Efficacy of STI571, an abl tyrosine kinase inhibitor, in conjunction with other antileukemic agents against BCR-ABL-positive cells. *Blood* 2000;96:3195–3199.
82. La Rosee P, Corbin AS, Stoffregen EP, Deininger MW, Druker BJ. Activity of the BCR-ABL kinase inhibitor PD180970 against clinically relevant BCR-ABL isoforms that cause resistance to imatinib mesylate (Gleevec, STI571). *Cancer Res* 2002;62:7149–7153.
83. von Bubnoff N, Veach DR, Miller WT et al. Inhibition of wild-type and mutant BCR-ABL by pyrido-pyrimidine-type small molecule kinase inhibitors. *Cancer Res* 2003;63:6395–6404.
84. Huron DR, Gorre ME, Kraker AJ et al. A novel pyridopyrimidine inhibitor of Abl kinase is a picomolar inhibitor of BCR-ABL-driven K562 cells and is effective against STI571-resistant BCR-ABL mutants. *Clin Cancer Res* 2003;9:1267–1273.
85. O'Hare T, Pollock R, Stoffregen EP et al. Inhibition of wild-type and mutant BCR-ABL by AP23464, a potent ATP-based oncogenic protein kinase inhibitor: Implications for CML. *Blood* 2004;104:2532–2539.
86. Shah N, Tran C, Lee FY, and Sawyers C. BMS-354825 is a novel orally bioavailable small molecule ABL tyrosine kinase inhibitor that successfully and safely inhibits the kinase activity of multiple imatinib-resistant BCR-ABL isoforms *in vitro* and *in vivo* [abstract]. AACR meeting 2004. 2004.
87. Kimura S, Naito H, Segawa H et al. NS-187, a potent and selective dual BCR-ABL/Lyn tyrosine kinase inhibitor, is a novel agent for imatinib-resistant leukemia. *Blood* 2005;106:3948–3954.
88. Weisberg E, Manley PW, Breitenstein W et al. Characterization of AMN107, a selective inhibitor of native and mutant BCR-ABL. *Cancer Cell* 2005;7:129–141.

89. Lombardo L, Yee F, Chen P et al. Discovery of *N*-(2-Chloro-6-methylphenyl)-2-(6-(4-(20hydroxyethyl)-piperazin-1-yl)-2-methylprimidin-4-ylamino) thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. *J Med Chem* 2004;47:6658–6661.
90. Weisberg E, Griffin JD. Mechanism of resistance to the ABL tyrosine kinase inhibitor STI571 in BCR/ABL-transformed hematopoietic cell lines. *Blood* 2000;95:3498–3505.
91. Talpaz M, Shah NP, Kantarjian H et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. *N Engl J Med* 2006;354:2531–2541.
92. Kantarjian H, Giles F, Wunderle L et al. Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL. *N Engl J Med* 2006;354:2542–2551.
93. O'Hare T, Walters DK, Deininger MWN, Druker BJ. AMN101: tightening the grip of imatinib. *Cancer Cell* 2005;7:117–119.
94. Deininger MW, Druker BJ. SRCircumventing imatinib resistance. *Cancer Cell* 2004;6:108–110.
95. von Bubnoff N, Manley PW, Mestan J et al. BCR-ABL resistance screening predicts a limited spectrum of point mutations to be associated with clinical resistance to the Abl kinase inhibitor nilotinib (AMN107). *Blood* 2006;108:1328–1333.
96. Bradeen HA, Eide CA, O'Hare T et al. Comparison of imatinib, dasatinib (BMS-354825), and nilotinib (AMN107) in an n-ethyl-n-nitrosourea (ENU)-based mutagenesis screen: high efficacy of drug combinations. *Blood* 2006;108:2332–2338.
97. Gumireddy K, Baker SJ, Cosenza SC et al. A non-ATP-competitive inhibitor of BCR-ABL overrides imatinib resistance. *Proc Natl Acad Sci USA* 2005;102:1992–1997.
98. Burley S. Application of FAST TM fragment-based lead discovery and structure-guided design to discovery of small molecule inhibitors of BCR-ABL tyrosine kinase active against the T3151 imatinib resistant mutant [abstract]. *Proc Am Assoc Canc Res* 2006;47:1139.
99. Lange T, Niederwieser DW, Deininger MW. Residual disease in chronic myeloid leukemia after induction of molecular remission. *N Engl J Med* 2003;349:1483–1484.
100. Lange T, Bumm T, Mueller M et al. Durability of molecular remission in chronic myeloid leukemia patients treated with imatinib vs allogeneic stem cell transplantation. *Leukemia* 2005;19:1262–1265.
101. Deininger MW, Holyoake TL. Can we afford to let sleeping dogs lie? *Blood* 2005;105:1840–1841.
102. Cools J, DeAngelo DJ, Gotlib J et al. A tyrosine kinase created by fusion of the PDGFRA and FIP1L1 genes as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome. *N Engl J Med* 2003;348:1201–1214.
103. Gleich GJ, Leiferman KM, Pardanani A, Tefferi A, Butterfield JH. Treatment of hypereosinophilic syndrome with imatinib mesilate. *Lancet* 2002;359:1577–1578.
104. Apperley JF, Gardembas M, Melo JV et al. Response to imatinib mesylate in patients with chronic myeloproliferative diseases with rearrangements of the platelet-derived growth factor receptor beta. *N Engl J Med* 2002;347:481–487.
105. Pardanani A, Elliott M, Reeder T et al. Imatinib for systemic mast-cell disease. *Lancet* 2003;362:535–536.
106. Pardanani A, Tefferi A. Imatinib targets other than bcr/abl and their clinical relevance in myeloid disorders. *Blood* 2004;104:1931–1939.
107. Tefferi A, Mesa RA, Gray LA et al. Phase 2 trial of imatinib mesylate in myelofibrosis with myeloid metaplasia. *Blood* 2002;99:3854–3856.
108. Kindler T, Breitenbuecher F, Marx A et al. Sustained complete hematologic remission after administration of the tyrosine kinase inhibitor imatinib mesylate in a patient with refractory, secondary AML. *Blood* 2003;101:2960–2962.
109. Silver RT. Imatinib mesylate (Gleevec(TM)) reduces phlebotomy requirements in polycythemia vera. *Leukemia* 2003;17:1186–1187.
110. Jones AV, Silver RT, Waghorn K et al. Minimal molecular response in polycythemia vera patients treated with imatinib or interferon alpha. *Blood* 2006;107:3339–3341.
111. Nilsson B, Bumming P, Meis-Kindblom JM et al. Gastrointestinal stromal tumors: the incidence, prevalence, clinical course, and prognostication in the preimatinib mesylate era. *Cancer* 2005;103:821–829.
112. Corless CL, McGreevey L, Haley A, Town A, Heinrich MC. KIT mutations are common in incidental gastrointestinal stromal tumors one centimeter or less in size. *Am J Pathol* 2002;160:1567–1572.

113. Tuveson DA, Willis NA, Jacks T et al. STI571 inactivation of the gastrointestinal stromal tumor c-KIT oncoprotein: biological and clinical implications. *Oncogene* 2001;20:5054–5058.
114. Joensuu H, Roberts PJ, Sarlomo-Rikala M et al. Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. *N Engl J Med* 2001;344:1052–1056.
115. Demetri GD, von Mehren M, Blanke CD et al. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med* 2002;347:472–480.
116. van Oosterom AT, Judson I, Verweij J et al. Safety and efficacy of imatinib (STI571) in metastatic gastrointestinal stromal tumours: a phase I study. *Lancet* 2001;358:1421–1423.
117. Heinrich MC, Corless CL, Duensing A et al. PDGFRA activating mutations in gastrointestinal stromal tumors. *Science* 2003;299:708–710.
118. Prenen H, Cools J, Mentens N et al. Efficacy of the kinase inhibitor SU11248 against gastrointestinal stromal tumor mutants refractory to imatinib mesylate. *Clin Cancer Res* 2006;12:2622–2627.
119. Sjoblom T, Shimizu A, O'Brien KP et al. Growth inhibition of dermatofibrosarcoma protuberans tumors by the platelet-derived growth factor receptor antagonist STI571 through induction of apoptosis. *Cancer Res* 2001;61:5778–5783.
120. Rubin BP, Schuetze SM, Eary JF et al. Molecular targeting of platelet-derived growth factor B by imatinib mesylate in a patient with metastatic dermatofibrosarcoma protuberans. *J Clin Oncol* 2002;20:3586–3591.
121. Yamamoto Y, Kiyoi H, Nakano Y et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood* 2001;97:2434–2439.
122. Kelly LM, Liu Q, Kutok JL et al. FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. *Blood* 2002;99:310–318.
123. Sohal J, Phan VT, Chan PV et al. A model of APL with FLT3 mutation is responsive to retinoic acid and a receptor tyrosine kinase inhibitor, SU11657. *Blood* 2003;101:3188–3197.
124. Wadleigh M, DeAngelo DJ, Griffin JD, Stone RM. After chronic myelogenous leukemia: tyrosine kinase inhibitors in other hematologic malignancies. *Blood* 2005;105:22–30.
125. Heinrich MC, Druker BJ, Curtin P et al. A “first in man” study of the safety and PK/PD of an oral Flt3 inhibitor (MLN518) in patients with AML or high risk myelodysplasia. *Blood* 2003;100:336a.
126. Fiedler W, Mesters R, Tinnefeld H et al. A phase 2 clinical study of SU5416 in patients with refractory acute myeloid leukemia. *Blood* 2003;102:2763–2767.
127. Giles FJ, Stopeck AT, Silverman LR et al. SU5416, a small molecule tyrosine kinase receptor inhibitor, has biologic activity in patients with refractory acute myeloid leukemia or myelodysplastic syndromes. *Blood* 2003;102:795–801.
128. Stone RM, DeAngelo DJ, Klimek V et al. Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. *Blood* 2005;105:54–60.
129. Miyamoto T, Nagafuji K, Akashi K et al. Persistence of multipotent progenitors expressing AML1/ETO transcripts in long-term remission patients with t(8;21) acute myelogenous leukemia. *Blood* 1996;87:4789–4796.
130. Rusch V, Baselga J, Cordon-Cardo C et al. Differential expression of the epidermal growth factor receptor and its ligands in primary non-small cell lung cancers and adjacent benign lung. *Cancer Res* 1993;53:2379–2385.
131. Goldstein NI, Prewett M, Zuklys K, Rockwell P, Mendelsohn J. Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. *Clin Cancer Res* 1995;1:1311–1318.
132. Ward WH, Cook PN, Slater AM et al. Epidermal growth factor receptor tyrosine kinase. Investigation of catalytic mechanism, structure-based searching and discovery of a potent inhibitor. *Biochem Pharmacol* 1994;48:659–666.
133. Wakeling AE, Guy SP, Woodburn JR et al. ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. *Cancer Res* 2002;62:5749–5754.
134. Swaisland H, Laight A, Stafford L et al. Pharmacokinetics and tolerability of the orally active selective epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 in healthy volunteers. *Clin Pharmacokinet* 2001;40:297–306.
135. Herbst RS, Maddox AM, Rothenberg ML et al. Selective oral epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 is generally well-tolerated and has activity in non-small-cell lung cancer and other solid tumors: results of a phase I trial. *J Clin Oncol* 2002;20:3815–3825.

136. Ranson M, Hammond LA, Ferry D et al. ZD1839, a selective oral epidermal growth factor receptor-tyrosine kinase inhibitor, is well tolerated and active in patients with solid, malignant tumors: results of a phase I trial. *J Clin Oncol* 2002;20:2240–2250.
137. Baselga J, Rischin D, Ranson M et al. Phase I safety, pharmacokinetic, and pharmacodynamic trial of ZD1839, a selective oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with five selected solid tumor types. *J Clin Oncol* 2002;20:4292–4302.
138. Nakagawa K, Tamura T, Negoro S et al. Phase I pharmacokinetic trial of the selective oral epidermal growth factor receptor tyrosine kinase inhibitor gefitinib ('Iressa', ZD1839) in Japanese patients with solid malignant tumors. *Ann Oncol* 2003;14:922–930.
139. Albanell J, Rojo F, Averbuch S et al. Pharmacodynamic studies of the epidermal growth factor receptor inhibitor ZD1839 in skin from cancer patients: histopathologic and molecular consequences of receptor inhibition. *J Clin Oncol* 2002;20:110–124.
140. Fukuoka M, Yano S, Giaccone G et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. *J Clin Oncol* 2003;21:2237–2246.
141. Kris MG, Natale RB, Herbst RS et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *JAMA* 2003;290:2149–2158.
142. Herbst RS, Giaccone G, Schiller JH et al. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial—INTACT 2. *J Clin Oncol* 2004;22:785–794.
143. Giaccone G, Herbst RS, Manegold C et al. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial—INTACT 1. *J Clin Oncol* 2004;22:777–784.
144. Shepherd FA, Rodrigues PJ, Ciuleanu T et al. Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* 2005;353:123–132.
145. Herbst RS, Prager D, Hermann R et al. TRIBUTE: a phase II trial of erlotinib hydrochloride (OSI-774) combined with carboplatin and paclitaxel chemotherapy in advanced non-small-cell lung cancer. *J Clin Oncol* 2005;23:5892–5899.
146. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science* 2004;305:1163–1167.
147. Stephens P, Hunter C, Bignell G et al. Lung cancer: intragenic ERBB2 kinase mutations in tumours. *Nature* 2004;431:525–526.
148. Shimamura T, Ji H, Minami Y et al. Non-small-cell lung cancer and Ba/F3 transformed cells harboring the ERBB2 G776insV_G/C mutation are sensitive to the dual-specific epidermal growth factor receptor and ERBB2 inhibitor HKI-272. *Cancer Res* 2006;66:6487–6491.
149. Kobayashi S, Boggon TJ, Dayaram T et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2005;352:786–792.
150. Gorre ME, Mohammed M, Ellwood K et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* 2001;293:876–880.
151. Kwak EL, Sordella R, Bell DW et al. Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. *Proc Natl Acad Sci USA* 2005;102:7665–7670.
152. Kobayashi S, Ji H, Yuza Y et al. An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor. *Cancer Res* 2005;65:7096–7101.
153. Bardelli A, Parsons DW, Silliman N et al. Mutational analysis of the tyrosine kinome in colorectal cancers. *Science* 2003;300:949.
154. Davies H, Bignell GR, Cox C et al. Mutations of the BRAF gene in human cancer. *Nature* 2002;417:949–954.
155. Samuels Y, Wang Z, Bardelli A et al. High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004;304:554.
156. Baxter EJ, Scott LM, Campbell PJ et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet* 2005;365:1054–1061.
157. Levine RL, Wadleigh M, Cools J et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell* 2005;7:387–397.
158. Mesa RA, Li CY, Ketterling RP et al. Leukemic transformation in myelofibrosis with myeloid metaplasia: a single institution experience with 91 cases. *Blood* 2005;105:973–977.
159. Shah NP, Tran C, Lee FY et al. Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science* 2004;305:399–401.

160. Lee FY, Lombardo L, Borzilleri R et al. BMS-354825 - a potent SRC/ABL kinase inhibitor possessing curative efficacy against imatinib sensitive and resistant human CML models *in vivo* [abstract]. AACR meeting 2004. 2004.
161. Smith BD, Levis M, Beran M et al. Single-agent CEP-701, a novel FLT3 inhibitor, shows biologic and clinical activity in patients with relapsed or refractory acute myeloid leukemia. *Blood* 2004;103:3669–3676.
162. Kelly LM, Yu JC, Boulton CL et al. CT53518, a novel selective FLT3 antagonist for the treatment of acute myelogenous leukemia (AML). *Cancer Cell* 2002;1:421–432.
163. Fiedler W, Serve H, Dohner H et al. A phase 1 study of SU11248 in the treatment of patients with refractory or resistant acute myeloid leukemia (AML) or not amenable to conventional therapy for the disease. *Blood* 2005;105:986-993.

VI

RECENT AND DEVELOPING CANCER THERAPIES

25

Monoclonal Antibodies in Lymphomas

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SUMMARY

Immunotherapy has markedly altered the treatment options available for patients with non-Hodgkin's lymphoma (NHL). Monoclonal antibodies have revolutionized the treatment of cancers in two respects. First, they represent a therapy with a different mechanism of action than chemotherapy. By providing an alternative type of attack, they may improve outcomes through better efficacy. Second, they represent a major step forward in improving the tolerability of cancer therapies. As a more targeted therapy, monoclonal antibodies enable patients to receive treatment that might not have otherwise been tolerated, potentially extending their lives and decreasing symptomatology. Although each monoclonal antibody was initially developed for one indication, their approvals have enabled exploration of other possible indications. We are only beginning to understand the breadth of diseases, malignant and non-malignant, that might benefit from treatment with these monoclonal antibodies, and how best to use them. Attempts to further improve outcomes in NHL are being explored, including the role of modifications of dose and schedule, chemotherapy combinations, and through the use of other biologics. Monoclonal antibodies represent the epitome of targeted therapy and have the potential to move forward the treatment of lymphoma more than any other development since the first use of multiagent chemotherapy.

Key Words: Monoclonal antibodies; immunotherapy; targeted therapy.

1. INTRODUCTION

The goal of cancer therapy is to eliminate the malignant cells without harming the patient. Current cancer treatment is dependent primarily upon three different modalities: surgery, chemotherapy, and radiation. Surgery and radiation therapy are effective at removing local disease while only damaging non-malignant tissue in the immediate

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vicinity. Thus, although surgery and radiation have no damaging effects on distant cells, they also do not treat malignant cells that have spread outside of the immediate area. Chemotherapy, on the contrary, penetrates throughout the body and is able to eliminate malignant cells that have spread beyond the local area. Unfortunately, chemotherapy will also damage normal tissues.

Although chemotherapy might demonstrate specificity by inhibiting a particular enzyme or damaging a particular protein, the targets of chemotherapeutic drugs are also typically found in normal tissue. This results in chemotherapy harming normal cells, with the primary impact generally expressed in rapidly dividing tissues, such as the bone marrow and gastrointestinal epithelium. The “selectivity” of chemotherapy is generally ascribed to the increased sensitivity of cancer cells to these agents. This increased sensitivity results, in part, from the malignant cells being under greater biologic stress because of their greater cell proliferation rate and energy demands, and because of less time and opportunity for repair prior to the next cell division. Although this strategy is somewhat effective, there is a great deal of damage to normal cells. This damage is seen as toxicities of therapy. These toxicities often result in painful and undesirable outcomes and limit the use of these conventional treatments.

If cancer treatments could be delivered specifically to the cancer cells, there would be the expectation of less associated toxicity. The therapy could also be delivered more safely and possibly in higher doses, potentially with greater effectiveness. Antibodies offer a means to target specific cells in the body, thereby limiting the toxicity to non-targeted tissues.

Antibodies are composed of two identical heavy chains and two identical light chains held together by disulfide bonds. Each antibody can bind two copies of its target antigen. The antigen binding domain is composed of the variable region of one light chain and one heavy chain. Each light chain possess one variable and one constant region. Each heavy chain possess one variable region and either 3 or 4 constant regions depending upon the immunoglobulin class. The effector functions of an antibody are mediated by the constant domains of the two heavy chains. (See Figure 1).

The specificity of antibody therapy results from features of both the antibody and the antigen. Through the processes of VDJ recombination, somatic hypermutation, and nucleotide base addition, the immune system is able to generate antibodies that possess extraordinary specificity. The second level of specificity is derived from the selection of an appropriate antigen whose expression is restricted to the targeted cells. The ideal target for a therapeutic antibody would be an antigen expressed only on malignant cells. Unfortunately, the identification of such malignancy-associated antigens, or tumor markers, has been difficult. The epitome of such selective expression would be the idiotype demonstrated by a particular lymphocyte clone. This target, created by the hypervariable region of the surface immunoglobulin of the lymphocyte, is unique to that one clone of lymphocytes. Currently, many investigators are working on developing anti-idiotype vaccines as treatments for lymphomas. This approach is difficult as it requires that a novel treatment be developed for each individual.

Lymphocyte differentiation is well characterized by the expression of a series of surface proteins termed “cluster of differentiation” (CD) antigens. Given the ontogenic relationship between lymphomas and normal lymphocytes, the antigens expressed on lymphomas are almost always expressed by their non-malignant counterparts. The damage that might result from targeting of an antigen on both normal and malignant

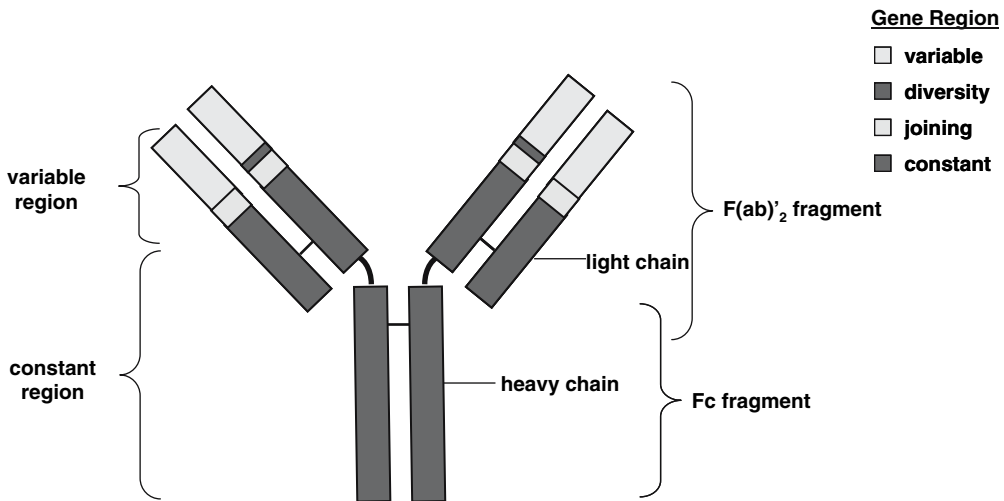


Fig. 1. Antibody structure. Each heavy chain is composed of variable region derived from the V, D, and J gene regions and a constant region derived from the C gene region. Each light chain possess a variable region derived from V and J gene regions and a constant region derived from the C gene region. The variable regions from both the light and heavy chains make up the antigen binding pocket. The F(ab)₂ fragment, derived from pepsin digestion, contains the antigen binding regions. The Fc fragment, derived from papain digestion, contains the immune effector functions.

lymphocytes is minimized by the ability to regenerate normal lymphocytes after therapy, limiting the duration and severity of immunosuppression.

The antigens that are targeted by monoclonal antibodies are cell surface-bound or soluble molecules. The features that make each of these types of antigens contributes to target differ. For cell surface-based targets, an ideal antigen would fulfill several criteria, including (i) expression of the antigen should be restricted so as to spare normal tissues; (ii) expression of the antigen on the tumor cell should be sufficiently intense in order to bind adequate numbers of antibodies; (iii) the antigen's cell surface expression should not be modulated in order that the antibody will remain on the surface once bound to the antigen (with the exception for toxin conjugates that require intracellular delivery); (iv) the antigen should be present in almost all cases of the malignancy; and (v) the antigen should not be present in a soluble or secreted form that would prevent the antibody from reaching the targeted cell or lead to soluble immune complexes.

For soluble targets, the ideal target would be a molecule that will have its activity neutralized once bound by antibody; also interference with its function will not impact upon other cells. The binding of an antibody to a soluble molecule would stereotactically inhibit its ability to bind to its receptor. Alternatively, the binding of the antibody might lead to increased clearance of the antigen, reducing its availability to activate its receptor. This latter mechanism of action requires antibodies of very high affinities and concentrations, as soluble molecules are typically meant to act over very short distances and at very low concentrations.

Antibodies eliminate cancer cells through several potential mechanisms: (i) complement-dependent cytotoxicity (CDC); (ii) antibody-dependent cellular cytotoxicity (ADCC); (iii) delivery of a toxin or radiation to the target cell; (iv) signaling

through the target antigen; and (v) deprivation of a necessary survival factor. Mechanisms (i–iii) are mediated by the Fc portion of the antibody. Mechanisms (iv and v) of action are mediated by the antigen-binding domain of the antibody. Currently, there are nine monoclonal antibodies approved for therapeutic oncology indications (Table 1). There are also many antibodies under clinical development whose targets include CD3, CD20, CD22, CD23, CD25, CD30, CD40, CD74, and CD80, vascular endothelial-derived growth factor, interleukin-1 (IL-1), IL-6, and tumor necrosis factor- α .

Several different processes are used to generate monoclonal antibodies, resulting in antibodies of varying degrees of similarity to human immunoglobulins. Monoclonal

Table 1
Nomenclature System for Monoclonal Antibodies

guidelines:

1. the suffix –mab is used for monoclonal antibodies and fragments.
2. infixes identify the product source and the disease or target class.
3. distinct syllable selected to create unique name selected as prefix
4. stems combined in the following sequence: prefix + target disease infix + product source infix + stem

target:

viral	-vir-
bacterial	-bac-
immune	-lim-
infectious	-les-
cardiovascular	-cir-
colon	-col-
melanoma	-mel-
mammary	-mar-
testis	-got-
ovary	-gov-
prostate	-pr(o)-
miscellaneous	-tum-

product source identifiers:

o	= mouse
a	= rat
e	= hamster
xi	= chimera
zu	= humanized
u	= human
i	= primate

stem (for all monoclonal antibodies):

-mab

examples:

murine: tositumomab: “-tum-o-mab”

chimeric: rituximab: “-tu(m)-xi-mab”

humanized: alemtuzumab: “-tu(m)-zu-mab”

human: ofatumumab: “-tum-u-mab”

antibodies are named based upon a system that has been adopted by the World Health Organization's Program on International Nonproprietary Names and the United States Adopted Names Council. Each antibody is named using a prefix, two infixes each representing: 1) the antibody target and 2) the species from which the antibody is derived, and a suffix. (See Table 2). The first monoclonal antibodies were generated in 1975 utilizing hybridoma technology and were murine in nature (1). To generate these monoclonal antibodies, mice were vaccinated with the target antigen and then splenectomized. The B lymphocytes from the spleen were fused with a murine myeloma cell line. The myeloma cell line provided a means for immortalizing the B lymphocytes from the mouse. Fused cell lines were then screened for production of the desired antibody, and the monoclonal antibodies were collected from the supernatant of the cultured hybridomas. Antibodies generated in this manner were fully murine in character.

One of the initial and more significant obstacles to producing monoclonal antibodies was the need for the vaccinated antigen to maintain its native conformation. Processing of antigens that destroyed relevant epitopes would not produce relevant antibodies. Later, when murine antibodies were investigated as therapeutic tools, the murine nature of the antibodies presented three additional problems. First, murine antibodies (or almost any non-human species) have shortened half-lives in human serum, as an antibody's half-life in serum is determined by its Fc sequence and the protection it affords from proteolysis. Second, murine antibodies are immunogenic and might lead to the development of antibodies by the human recipient against the murine antibodies, human anti-murine antibody (HAMA). These HAMAs would have the potential to neutralize the therapeutic antibodies by either interfering with the antibody binding its antigen or increasing its clearance from the serum. They could also lead to serious infusion reactions because of the formation of antibody-antigen complexes rapidly in the serum upon infusing. Third, and perhaps most importantly, murine antibodies are unable to trigger the immunologic functions performed by the Fc portion of antibodies, including CDC and ADCC. These are both potent activities of the immune system that would lead to elimination of the targeted tumor cells.

In an attempt to rectify some of these issues, chimeric monoclonal antibodies were generated. Chimeric monoclonal antibodies utilize the murine variable domains and human constant domains. These antibodies were generated by cloning the cDNA for the heavy and light variable chains from the murine antibody to the cDNA for the heavy and light chain constant regions of human immunoglobulin (2,3). These antibodies typically contain sequences that are 33% murine and 67% human. These chimeric antibodies are able to retain the specificity of the murine antibody, possess the effector functions of human immunoglobulins leading to CDC and ADCC, and lessen the likelihood for antibody production against the chimeric antibodies [human anti-chimeric antibodies (HACAs)].

Subsequent work generated what are termed "humanized" monoclonal antibodies. Humanized antibodies involve combining the hypervariable, complementarity-determining regions (CDRs) of a murine antibody, which fold to form the antigen-binding pocket, with human variable chain framework regions (4). The DNA coding the murine CDRs are sequenced and cloned into a human immunoglobulin gene of an antibody-producing cell line. These humanized antibodies contain 90–95% human and only 5–10% murine sequences. The only murine sequences are the amino acids that

Table 2
Approved mAbs for Oncology Indications (as of 1/1/07)

<i>Name (generic, trade)</i>	<i>Target</i>	<i>approval</i>	<i>source</i>	<i>conjugate</i>	<i>Indication</i>	<i>Mechanism of action</i>
rituximab (Rituxan)	CD20	11/26/97	chimeric	naked	CD20+ follicular lymphoma breast cancer	CDC, ADCC, induction of apoptosis by binding CD20 toxin conjugate (calicheamicin)
trastuzumab (Herceptin)	EGFR2/HER2	9/25/98	humanized	naked		inhibition of receptor activation by blocking ligand
gemtuzumab (Mylotarg)	CD33	5/17/00	humanized	calicheamicin	AML	inhibition of receptor activation by blocking ligand
alemtuzumab (Campath)	CD52	5/7/01	humanized	naked	CLL	CDC, ADCC
ibritumomab tiuxetan (Zevalin)	CD20	2/19/02	murine	RIT: yttrium-90	lymphoma	radioconjugate (yttrium-90)
tositumomab-I 131 (Bexxar)	CD20	6/27/03	murine	RIT: iodine 131	lymphoma	radioconjugate (iodine-131)
cetuximab (Erbix)	EGFR1/HER1	1/12/04	chimeric	naked	colorectal cancer	receptor blocking
bevacizumab (Avastin)	VEGF	2/26/04	humanized	naked	colorectal cancer	inhibition of receptor activation by blocking ligand
panitumumab (Vectibix)	EGFR	9/27/06	human	naked	colorectal cancer	inhibition of receptor activation by blocking ligand

comprise the CDR. The humanized antibodies afford all the Fc functions of human antibodies with minimal immunogenicity.

The newest technique produces “human” monoclonal antibodies. These antibodies are generated by two methods. The first method utilizes a transgenic mouse whose murine immunoglobulin genes have been replaced by human immunoglobulin genes (5,6). These mice will generate a series of human antibodies in response to an antigen challenge. Antibody-producing hybridomas are then generated. The second process, known as phage display, utilizes bacteriophage containing almost every possible nucleotide sequence to produce antibodies with different specificities (7). Colonies of bacteriophage are screened and then the sequences cloned.

The initial therapeutic use for monoclonal antibodies involved heavily treated patients with non-Hodgkin’s lymphomas (NHLs). These patients’ immune systems were quite impaired because of the underlying lymphoma or the immunosuppressant treatments delivered as lymphoma therapy. As a result, there was little concern for these patients developing a HAMA. This is best exemplified by the early studies of Bexxar in patients with follicular lymphoma. In the initial pivotal study, HAMAs developed in 8% of the patients from this heavily pretreated population. When Bexxar was studied as therapy for untreated patients with follicular lymphoma, the HAMA rate reached 63% (8,9). Both of these studies utilize Bexxar as a single agent. When Leonard et al. (10) administered three cycles of fludarabine prior to Bexxar in patients with previously untreated follicular lymphoma, HAMAs developed in only 6% of the patients. Although the fludarabine was used in this study to debulk the patients with the hope of making the Bexxar more effective and safer, it had the additional benefit of immunosuppressing the patients sufficiently to prevent the generation of HAMA responses. Since, there was little chance that these patients would have sufficient effector cell function to take advantage of the presence of an Fc receptor, the use of a murine antibody is not problematic. Additionally, owing to the lack of effector cells, the antibodies would have to possess alternate means for inducing apoptosis, either radioactivity or a toxin conjugate.

Subsequently, chimeric, humanized, and human antibodies were developed that allowed the range of antibody use to increase.

In the remaining parts of this chapter, data will be presented regarding specific antibodies utilized in the treatment of patients with lymphomas.

2. RITUXIMAB

Rituximab (IDEC-C2B8), the first monoclonal antibody to be approved for a cancer indication in the USA, received approval from the Food and Drug Administration (FDA) in November 1997. Rituximab is approved as treatment for patients with relapsed or refractory, low-grade or follicular, CD20-positive, B-cell NHL. Rituximab is an IgG1 kappa chimeric monoclonal antibody directed against the CD20 molecule, whose expression is restricted to B lymphocytes (11). The variable regions of rituximab are derived from the murine anti-CD20 antibody, 2B8 (12). Rituximab is composed of two heavy chains of 451 amino acids and two light chains of 213 amino acids, with an approximate molecular weight of 145 kDa. Rituximab is produced by Chinese Hamster Ovary (CHO) cells that contain the human/murine cDNA sequences for rituximab and secrete the antibody in large quantities into culture, where it is purified by affinity

and ion exchange chromatography. Rituximab binds to the CD20 antigen with a high affinity, 5×10^{-9} mol/L (12).

CD20 is expressed only on normal and malignant B cells (13,14). Its expression is first detectable at the pre-B-cell stage of lymphocyte development, and its expression remains present at high levels until the lymphoplasmacytoid stage, when it begins to become down-regulated. CD20 is not expressed on plasma cells, the most terminally differentiated B cell. This is of significance as rituximab will not target the earliest B-lymphocyte progenitors or plasma cells, allowing continuation of some antibody production and replenishment of normal B lymphocytes. The CD20 molecule is expressed in over 90% of B-cell lymphomas (11,13).

CD20 is believed to play a role in regulating the activation, proliferation, and differentiation of B cells through its regulation of transmembrane calcium conductance that is responsive to signaling through the B-cell receptor (15–17). CD20 possesses four transmembrane domains with its C-termini and N-termini both located within the cytoplasm. Initial experiments supported the idea that CD20 does not internalize (18), is tightly bound in the membrane, and not present in the plasma (13). Subsequent work demonstrated the presence of low amounts of circulating CD20 in the plasma in healthy controls and significantly elevated levels in patients with CLL (19,20). In the CLL patients, the level of circulating CD20 was identified as an independent prognostic indicator (20). In addition to its spectrum of expression profile, CD20 serves as a good therapeutic target, because it is a transmembrane protein whose presence is most likely required for B-lymphocyte survival. Thus, it is well anchored to the cell, and there is very low potential for the development of CD20-negative B cells through selection.

The exact mechanism of action for rituximab remains debated, but most likely depends upon a combination of (i) complement activation (Complement-dependent cell cytotoxicity, CDC) (12,21–23); (ii) ADCC (21,24,25); and (iii) antibody-mediated apoptosis involving signaling through the CD20 protein (26–31). In support of CDC are several observations. The Fc portion of rituximab binds human C1q effectively, initiating the complement cascade (12). Blocking CD55 and CD59, cellular-based inhibitors of complement, increased the sensitivity of lymphoma cells to rituximab, and levels of CD55 correlated with resistance to rituximab *in vitro* (21,23). Finally, cells cultured in complement-depleted media appeared to be less sensitive to the effects of rituximab (22). However, other investigators have not found a correlation between inhibitors of complement and sensitivity to rituximab (32). It is interesting to note that rituximab has anecdotally demonstrated some activity when used for intrathecal treatment, given that cerebrospinal fluid is not expected to have complement or cells to mediate ADCC (33,34).

The importance of ADCC has been demonstrated in a series of experiments by Clynes et al (24). They demonstrated that mice deficient in the activating Fc γ RIIIa receptor were unable to control transplanted tumors *in vivo* with rituximab treatment. In contrast, mice deficient in the inhibitory Fc γ RIIb receptor demonstrate more ADCC and better control with rituximab. In humans, the best evidence in support of a role for ADCC comes from studies of gene polymorphisms for two activating Fc receptors, Fc γ RIIIa and Fc γ RIIa (25,35). For Fc γ RIIIa, the polymorphism results in either a phenylalanine (F) or a valine (V) being present at amino acid position 158. Human IgG1 binds more strongly to V/V phenotype compared with the F/F or F/V phenotypes. As Fc γ RIIIa mediates ADCC involving natural killer (NK) cells and macrophages, it is

hypothesized that the Fc receptors with higher binding avidity would result in a better response to rituximab. The overall response rates at 2 and 12 months after rituximab therapy were 100 and 90% for the patients with V/V phenotype compared with 67 and 51% for patients with either F/V or F/F phenotypes. An additional study by Weng and Levy supported the finding by Cartron, et al. and also identified a polymorphism of amino acid 131 of the Fc γ RIIa receptor that correlated with outcome.

Although rituximab is not the physiologic ligand for CD20, its binding initiates a series of signaling cascades that often leads to apoptosis. Alas et al. (31) demonstrated that rituximab leads to a decrease in STAT3 activity, resulting in decreased Bcl-2 expression and apoptosis. Others have shown similar effects on signaling pathways involving anti-CD20 and anti-B-cell receptor-mediated apoptosis (27). Jazirehi et al. (28,29) demonstrated a decrease in Bcl-XL levels in response to rituximab and that this was mediated through inhibition of nuclear factor- κ B (NF- κ B).

The initial phase II study of rituximab as treatment for low-grade lymphomas demonstrated response rates of 46%, with 8% complete responses (CRs) and 38% partial responses (PRs) (36). The pivotal study utilized this same dosing scheme of 375 mg/m² four times weekly in a multicenter, open-label, single-arm study involving 166 patients with relapsed or refractory low-grade or follicular B-cell lymphomas (37). The overall response rate was 48% with 6% CR and 42% PR rates. The median duration of response was 11.2 months, with a range of 1.9–42.1+ months. Multivariate analysis of the data demonstrated a higher response rate in patients with International Working Formulation (IWF) histologic subtypes B, C, and D compared with IWF subtype A (58 vs. 12%), higher in patients whose largest lesion was <5 vs. >5 cm in greatest diameter (53 vs. 38%), and higher in patients with chemosensitive relapse as compared with chemoresistant relapse (53 vs. 36%).

Most patients demonstrated a rapid decrease in the number of peripheral blood B cells by day 4, which remained nearly undetectable until approximately 6 months post-treatment, followed by a gradual recovery. Mean serum immunoglobulin levels remained stable over the course of the study, with IgG and IgA levels remaining within the normal range but IgM levels falling slightly below the lower limits of normal. One out of 166 patients developed a HACA response at day 50, which was not associated with any clinical or laboratory abnormalities. Tumor response correlated with serum rituximab concentrations, with responding patients having a higher median serum concentration at all time points during treatment, but with the difference being statistically significant predose for infusions 2 and 4 and post-treatment infusion 4.

Given the tolerability and effectiveness of rituximab in the relapsed setting, Witsig et al. from the North Central Cancer Treatment Group studied rituximab in patients with untreated grade I follicular lymphomas. As might be expected with untreated patients, they demonstrated a higher overall response rate of 72%, with a 36% CR rate. The median time to progression was 2.2 years with only 56% of the patients progressing with a median follow-up of only 2.6 years (38). Rituximab thus may serve as a means to delay the exposure of patients to chemotherapy and its resulting toxicities. It will be even more important to determine the numbers of these patients who respond to retreatment with rituximab. When rituximab retreatment was investigated in patients with relapsed, low-grade NHL who had previously demonstrated a response of at least

6 months, Davis et al. (39) reported an overall response rate (ORR) of 40%, with 11% CRs and 30% PRs.

Although rituximab does demonstrate substantial activity with acceptable toxicity, the benefits of the standard dosing schedule are transient, with relapse inevitable. A number of investigators have attempted to improve outcomes through modifications of the dose and schedule. Although these studies have demonstrated some improvement in response rates and time to treatment progression, it is unclear whether they improve overall survival (37,39–44).

2.1. Chemoimmunotherapy Combinations for Indolent NHL

Another means for utilizing rituximab is in combination with chemotherapy. These chemoimmunotherapy regimens attempt to take advantage of an additive or even synergistic effect of combining chemotherapy with rituximab through concurrent or sequential administration. Two hypotheses are put forward to explain the rationale for combination chemoimmunotherapy. In the first theory, rituximab increases the sensitivity of the lymphoma cells to chemotherapy. This occurs by rituximab altering intracellular signaling pathways or by causing a “double hit”, resulting in the cells being more sensitive to chemotherapy. The second hypothesis is that the rituximab will eliminate those cancer cells that are not sensitive to the chemotherapy.

The first clinical trial to evaluate the safety and efficacy of combination chemoimmunotherapy was performed by Czuczman et al. (45) between April 1994 and March 1996. Forty patients, 38 of whom had received prior therapy, with low grade and follicular lymphoma received six cycles of CHOP chemotherapy at standard doses with two infusions of rituximab administered before the first cycle, single infusions before the third and fifth cycles, and two infusions after the sixth cycle. They demonstrated an ORR of 100% (87% CR) with a median time to progression of 82.3 months. Ten years after the study’s inception, 42% of the patients remained in remission.

Czuczman and colleagues also studied concurrent fludarabine and rituximab in patients with low-grade and follicular NHL (46). They treated 40 patients with six cycles of fludarabine (25 mg/m²/day for 5 days every 28 days) plus two infusions of rituximab prior to the first cycle, single infusions with the second, fourth, and sixth cycles, and two final infusions 4 weeks after the final fludarabine treatment. The ORR was 90%, with 80% CRs and median duration of response that had not been reached at 40+ months. Molecular remissions were achieved in 88% of the patients who were positive for the 14;18 translocation at enrollment.

The first randomized study comparing concurrent chemoimmunotherapy to chemotherapy alone in follicular lymphoma was reported by Marcus and colleagues (47). About 321 patients with previously untreated, stages III and IV follicular lymphoma were randomized to treatment with eight cycles of CVP chemotherapy every 21 days (notably at a perhaps attenuated cyclophosphamide dose of 750 mg/m²/cycle) with or without concurrent rituximab administered on day 1 of each cycle. Overall and CR rates were 81 and 41% in the R-CVP arm and were 57 and 10% in the CVP arm, respectively ($p < 0.0001$). Median time to progression was 32 vs. 15 months ($p < 0.0001$) for the two arms respectively. One important feature was that there was no evidence of increased toxicity when rituximab was added to CVP with the exception of infusion-related toxicities. Although these data are compelling, the reported follow-up was short and not reported for overall survival.

The German Low-grade Lymphoma Study Group compared treatment with fludarabine, cyclophosphamide, and mitoxantrone with and without rituximab as treatment for relapsed and refractory follicular and mantle cell lymphoma (48). The addition of rituximab increased the ORR to 79% (33% CRs and 45% PRs) from 58% (13% CRs and 45% PRs) and was associated with a significantly longer progression-free survival (16 vs. 10 months) and overall survival (not reached vs. 24 months).

Another interesting approach investigated has been the use of rituximab followed by short-duration chemotherapy (CVP or CHOP for 3 cycles) (49). The intention of this combination is to minimize chemotherapy-related toxicities by limiting the amount of chemotherapy necessary to achieve the necessary response. This type of approach is of particular importance when treating elderly patients or patients who might be expected to receive many repetitive cycles of chemotherapy, as seen in patients with follicular lymphomas. A trial of 86 patients showed an overall response rate of 93% (55% CR) with 4-year progression-free survival of 62%. The promising results in these initial studies are limited by the small sample size (and potential patient selection issues) and lack of a chemotherapy-only control group, though they have served as the basis for larger follow-up studies in broader settings.

Zinzani and coinvestigators investigated the use of chemotherapy followed by rituximab. They compared a regimen of fludarabine plus mitoxantrone (+/– rituximab) with CHOP (+/– rituximab) in 140 patients with untreated follicular lymphoma (50). This study added rituximab treatment after chemotherapy only in those patients with persistent clinical or molecular evidence of lymphoma. The FM arm demonstrated higher CR rates than the CHOP arm (68 vs. 42%), with 39% of the FM-treated and 19% of the CHOP-treated patients achieving molecular remissions after chemotherapy alone. Overall, rituximab improved the rate of molecular CR from 29 to 61% (71% in FM-treated and 51% in CHOP-treated patients). However, with a median follow-up of 19 months, there was no difference observed in progression-free or overall survival amongst any of the different treatment groups. When these data mature, an important analysis will be to determine whether there is a benefit to be gained by using rituximab to convert a clinical CR to a molecular CR in patients with follicular lymphoma.

Patients treated in the study by Zanzani et al. received four doses of rituximab after completing their chemotherapy. The next step in the use of rituximab was to investigate utilizing “maintenance” rituximab after chemotherapy. Two randomized, controlled studies investigating the use of maintenance rituximab after chemotherapy were undertaken by McLaughlin et al. and the Eastern Cooperative Oncology Group (ECOG). McLaughlin compared treatment with fludarabine, mitoxantrone, and dexamethasone (FND) plus concurrent rituximab followed by interferon- α (IFN- α) with FND followed by IFN- α plus rituximab in patients with follicular NHL. After a relatively short 30 months of follow-up, no significant differences in response rates or survival have been identified between the treatment groups (51).

In the study conducted by ECOG (E1496), untreated patients with indolent NHL were initially treated with CVP chemotherapy followed by randomization to either observation or maintenance rituximab administered as four weekly doses every 6 months for 2 years (52). Patients who received rituximab after CVP had significantly higher progression free survival (PFS) at 2 and 4 years after randomization (74 and 58%, respectively) compared with those who only received CVP (42 and 34%, respectively). This improvement in PFS with rituximab maintenance was seen in all tumor histologies,

in patients with bulky disease, and in patients with evidence of minimal residual disease following induction therapy. Although a difference in survival has not been observed after a median follow-up of 1.2 years, this trial provides the first randomized evidence that adding rituximab maintenance to chemotherapy in untreated patients who achieve a response or stable disease can prolong response duration.

The European Organization for Research and Treatment of Cancer (EORTC) conducted a randomized study of CHOP vs. concurrent CHOP-R followed by a secondary randomization to either observation or maintenance rituximab (375 mg/m² for one dose every 3 months) in patients with recurrent follicular lymphoma (53). The trial was stopped at the second planned interim analysis because of a significant difference in CR rate for CHOP-R vs. CHOP (30.4 vs. 18.1%). Additionally, rituximab maintenance has also demonstrated a longer PFS at 1 and 3 years compared with observation (80.2 and 67.7% vs. 54.9 and 31.2%, respectively).

2.2. Chemoimmunotherapy in Aggressive Lymphoma

Coiffier et al. (54) demonstrated in a phase II study of single agent rituximab in aggressive lymphomas an ORR of 31% (9% CRs and 22% PRs). Even with single agent rituximab fairing much worse in aggressive lymphomas than in indolent lymphomas, there still remains the potential benefit of combination chemotherapy and immunotherapy. A phase II study of rituximab given on day 1 combined with standard dose CHOP given on day 3 demonstrated an ORR of 94% (61% CRs and 33% PRs). With a minimum follow-up of 24 months, 88% of the patients remained alive and free of disease.

The Groupe d'Etude des Lymphomes de l'Adulte (GELA) conducted the first phase III, randomized study comparing eight cycles of CHOP with rituximab plus CHOP (R-CHOP) as treatment for diffuse large B-cell lymphoma (DLBCL) in previously untreated patients over the age of 60 years (55,56). In this study, patients randomized to receive R-CHOP received both the rituximab and CHOP chemotherapy on day 1 of the cycle. With a median follow-up of 5 years, R-CHOP produced statistically significant improvements compared with CHOP in terms of CR rate (76 vs. 63%), 5-year PFS (54 vs. 30%), and 5-year overall survival (OS) (58 vs. 45%).

More recently, the Mabthera International Trial (MinT) evaluated the addition of concurrent rituximab to a CHOP-like regimen in good-prognosis patients younger than 60 years of age (57). With median follow-up of 2 years, the addition of rituximab to chemotherapy resulted in higher CR rates (81 vs. 67%), longer 2-year time-to-treatment failure (TTF) rates (76 vs. 60%), and better 2-year OS (94 vs. 87%). Follow-up continues, and data on potential benefits of rituximab in patient subgroups (chemotherapy type, bulk of disease, use of radiation treatment, and prognosis) are anticipated.

The ECOG 4494 Trial investigated the use of CHOP plus rituximab in elderly patients with diffuse large B-cell lymphoma utilizing a 2 × 2 factorial design (58,59). Patients were first randomized to treatment with either CHOP chemotherapy with rituximab administered prior to cycle 1 and concurrently with cycles 3, 5, and 7 or CHOP alone. Responding patients were then randomized to either observation or maintenance rituximab administered as a 4-week course every 6 months for 2 years. The ORR was not different between the induction regimens, 79% for R-CHOP and 76% for CHOP alone. Weighted analysis was performed to enable each randomization

to be examined individually. The data demonstrated an improvement in TTF for R-CHOP compared with CHOP induction at 3 years (52 vs. 39%) and in overall survival at 3 years (67% vs. 58%). Likewise, maintenance rituximab prolonged TTF compared to observation but with no difference in overall survival. Overall, in the patients reaching the second randomization, the 2-year TTF was 77, 79, 74, and 45% for R-CHOP, R-CHOP+MR, CHOP+MR, and CHOP, respectively. These findings indicate that maintenance rituximab improved TTF in patients treated with CHOP alone [hazard ratio (HR) = .45, $p = .004$] but did not provide any additional benefit to patients treated with R-CHOP (HR = .93, $p = .81$). The primary findings of the study confirm the benefits of CHOP-R induction therapy over CHOP alone and also suggest that with R-CHOP induction regimen, maintenance rituximab therapy is unnecessary in DLBCL. It could thus be said that it does not matter when rituximab is administered as part of a CHOP induction regimen for DLBCL, so long as it is administered.

Current studies are evaluating the effects of rituximab in combination with other regimens (such as dose-adjusted EPOCH) in DLBCL and other lymphoma histologies with varying results (48,60,61). It is not clear that all aggressive lymphomas benefit from the addition of rituximab. In some studies involving HIV-associated aggressive lymphomas, an improvement in response rate is counter balanced by an increase risk of toxic death, leading to no improvement in overall survival (62). Several hypotheses have been generated to explain the increased toxicity, including hypogammaglobulinemia (63), prolongation of the neutropenia through cytokines (64), and autoantibody production (65). This prolonged neutropenia and infection-related deaths were not seen in the studies investigating the combination of rituximab and chemotherapy in non-HIV-associated lymphomas, suggesting that the deaths may have been the result of the combination of hypogammaglobulinemia and the CD4 cell lymphopenia seen in HIV patients. But other studies have refuted these findings (66–69).

As can be seen from these studies, response rates are increased, but at the risk of serious infections. These studies were not standardized with regard to infectious prophylaxis or the use of granulocyte colony-stimulating factors. Therefore, in the right patients with the correct precautions, there may be much to gain from the use of rituximab in combination with chemotherapy for aggressive lymphomas in HIV-positive patients.

3. ALEMTUZUMAB

Alemtuzumab (Campath-1H) is a genetically engineered humanized IgG1 monoclonal antibody directed against CD52. Alemtuzumab, named Campath after *Cambridge Pathology*, was first developed in 1983 as a rat monoclonal antibody that was very effective in reducing graft-versus-host disease following allogeneic bone marrow transplantation. The antigen-binding determinants of the alemtuzumab antibody were eventually cloned into human immunoglobulin-producing cells generating a humanized monoclonal antibody. As a humanized antibody, it was believed that alemtuzumab would be more effective at mediating Fc-mediated activities with decreased immunogenicity. Alemtuzumab was first used clinically in 1988 in a patient with refractory lymphoma (70). But it was not until 1991 that the CD52 antigen was cloned and recognized as the target for alemtuzumab.

CD52 is a non-modulating glycosylphosphatidylinositol-anchored glycoprotein that is expressed at high density on all lymphocytes and monocytes but not on granulocytes,

erythrocytes, platelets, and hematopoietic stem cells (71,72). CD52 is one of the most abundantly expressed membrane glycoproteins, with lymphocytes possessing almost 450,000 molecules per cell. CD52 is almost universally expressed on all lymphomas, with more intense expression seen on normal and malignant T cells compared with normal and malignant B cells, as well as low-grade lymphomas compared with aggressive lymphomas (73). Alemtuzumab was demonstrated to be more effective against blood and bone marrow disease compared with lymph node disease, as well as against indolent disease compared with aggressive disease (74). These data led to the initial development of alemtuzumab for use as treatment of patients with CLL. Alemtuzumab is able to fill a niche that rituximab could not because of the low intensity of CD20 expression present on CLL cells. Based on data generated in the pivotal study, Campath was approved by the FDA in 2000 for treatment of patients with CLL who have relapsed after fludarabine therapy (75).

As T-cell lymphomas do not express CD20, they represent another area where rituximab is not of value. Peripheral T-cell lymphomas represent 5–10% of NHLs and typically have a poorer prognosis than B-cell lymphomas (76). Anthracycline-containing chemotherapy regimens are expected to result in long-term survival in only approximately 30–40% of the patients (77,78). Early studies suggested that responses to alemtuzumab correlated with levels of CD52 expression, further supporting a role for alemtuzumab in T-cell lymphomas (79). Investigators began to utilize Campath in T-cell lymphomas in a manner analogous to that of rituximabs in B-cell lymphomas. Several phase II studies have been performed utilizing alemtuzumab as single-agent treatment delivered either by intravenous or by subcutaneous injection for either B-cell or T-cell lymphomas.

Three studies investigating alemtuzumab in patients with NHL demonstrated response rates of 16, 20, and 44% (80–82). When Lundin et al. analyzed their eight patients with mycosis fungoides separately, they identified an ORR of 50%, with two CRs. Enblad et al. (83) conducted a trial involving patients with chemotherapy refractory peripheral T-cell lymphomas and demonstrated an ORR of 36% (5 of 14), including three CRs. In their study, hematologic and infectious complications were considerable and resulted in the study closing prematurely with five deaths attributed in part to alemtuzumab. Given the refractory nature of these patients' disease, the authors concluded that alemtuzumab has considerable activity and may be sufficiently safe in less heavily treated patients. The same group tested alemtuzumab in 22 patients with advanced mycosis fungoides (84) and achieved an ORR of 55%, with 32% of the patients achieving a CR. In this study, 10 patients developed infections, resulting in one death because of pulmonary aspergillosis. Four patients developed CMV reactivation, with all four responding to IV ganciclovir. The infectious complications in this study appear more manageable, supporting a role for the further development of alemtuzumab as a treatment for lymphomas. Another approach to reduce the infectious complications was studied by Zinzani et al. (85) utilizing a dosing regimen of 10 mg (instead of 30 mg) administered intravenously three times a week for 4 weeks. In the 10 patients treated, there were six responses, with four PRs and two CRs. Only one infection was noted, which was CMV reactivation adequately treated with ganciclovir. As supportive therapies improve, such as, valganciclovir, and more clinical experience is obtained with alemtuzumab, higher response rates with safer outcomes could be expected.

3.1. Other Immunotherapies in Use in NHL

The use of other biologic agents either alone or in combination with rituximab is an appealing strategy to avoid the toxicity of chemotherapy or to enhance its efficacy. Immunostimulatory agents are under active investigation for this purpose. IL-2 administration expands and activates circulating NK cell populations, which potentially improves ADCC. As ADCC is an important mechanism of rituximab activity, IL-2 in combination with rituximab might have synergistic effects. Additionally, as previously discussed, less benefit is derived from rituximab use in patients with certain Fc receptor polymorphisms. In an attempt to investigate this combination, IL-2 was administered at escalating doses from 4.5 to 14 million units subcutaneously three times per week in conjunction with four weekly standard rituximab doses (86). In this phase I study, most patients responded. Higher circulating NK cell numbers and increased ADCC activity were seen with IL-2 therapy and correlated with clinical response. Eisenbeis and colleagues (87) have evaluated the combination of IL-2 plus rituximab in patients with rituximab-refractory indolent lymphoma. As this patient population was resistant to rituximab, any responses would be significant. Clinical responses were identified in five of 23 patients. When the Fc receptor polymorphisms of these patients were examined, it was noted that all of the responding patients analyzed were homozygous for phenylalanine polymorphism at position 158. This polymorphism is generally associated with less effective ADCC and less favorable outcome with rituximab. These findings are consistent with the hypothesis that IL-2 may be able to overcome rituximab resistance when it is related to relatively less effective ADCC because of Fc receptor polymorphism.

Immunostimulatory sequences of DNA in the form of CpG oligonucleotides have a wide range of immune effects including enhancement of antigen presentation and cytokine induction (supporting ADCC). Friedberg and colleagues (88) conducted a phase I trial with four doses of a CpG oligodeoxynucleotide (1018 ISS) with rituximab and noted clinical responses with minimal toxicity. Interestingly, correlative studies demonstrated that CpG administration was associated with a dose-related increase in expression of several IFN-inducible genes, a reassuring sign of biological effect. CpG administration is under active exploration both as a single agent and in combination with other therapies in lymphoma as well as other malignancies.

Other investigators have attempted to improve on rituximab therapy by creating new variations of CD20-directed antibodies. Preclinical studies have been conducted with a number of these agents that have been designed to either have human or humanized structure (other than murine or chimeric) to improve pharmacokinetics and reduce immunogenicity or to have enhanced complement-mediated cytotoxicity or ADCC (89,90). Such agents have moved into phase I clinical trials in some cases, and clinical responses have been observed. Whether the theoretical advantages to these antibody modifications translate into a clinical benefit for patients remains to be determined.

Alternative targets for monoclonal antibodies (particularly in B-cell malignancies) are also under evaluation. The CD22 antigen is widely expressed in normal and malignant B cells and plays a role in B-cell activation and trafficking (91). We conducted phase I trials of epratuzumab, a humanized anti-CD22 antibody, and demonstrated a favorable toxicity profile at doses of up to 1000 mg/m²/week administered for 4 weeks (92,93). Clinical responses were noted in follicular NHL and in diffuse

large B-cell lymphoma. In some cases, these have lasted for >1 year, and second responses (upon retreatment at progression) have been noted. Epratuzumab is currently under active investigation in combination with rituximab and CHOP-R chemotherapy, as well as in other B-cell malignancies and in autoimmune disease.

3.2. Radioimmunotherapy

Given the exquisite radiosensitivity of lymphomas, the attachment of a radioisotope to a monoclonal antibody can enhance the activity of the antibody through the addition of targeted radiation (94). The monoclonal antibody serves as the means to deliver the radiation directly to the intended target. When conventional radiation is delivered by external beam radiation, normal and malignant tissues are irradiated. For tumors that are not localized, avoiding the normal tissues becomes impossible. The antibody delivers the radioisotope into proximity of the intended target. When the radioisotope decays, the radiation is released to damage the target cell, typically by inducing DNA strand breaks, leading to apoptosis of the cell. The radiation may be delivered not only to the antibody-bound targeted cell but also to neighboring cells that are located within the path length of the radiation. This “cross-fire” effect results in therapeutic benefit if the neighboring cells are tumor cells not bound by antibody or toxicity if the neighboring cells are non-malignant cells. This is of particular concern with low-grade lymphomas as they frequently involve the bone marrow and hematopoietic stem cells that are very radiosensitive. Thus, myelosuppression is one of the dose-limiting toxicities for radioimmunotherapy (RIT).

Two such agents have been FDA approved, yttrium-90 ibritumomab tiuxetan (Zevalin, Biogen Idec) and iodine-131 tositumomab (Bexxar, Corixa, and Glaxo-SmithKline), for use in patients with CD20+ transformed or non-transformed follicular lymphoma whose disease is refractory to rituximab and has relapsed following chemotherapy. Both radioimmunoconjugates chelate their radioisotope to a murine antibody targeting the CD20 antigen. As the therapeutic modality is radiation, there is no need for a human Fc portion of the immunoglobulin to be present to elicit ADCC or CDC. Utilization of the murine antibody allowed for quicker and simpler therapeutic development of RIT, as well as afforded a means to limit infusional-related toxicities associated with humanized or chimeric antibodies.

One problem with the use of Zevalin and Bexxar antibodies is that they are murine antibodies, leading to the potential for developing HAMAs. These HAMAs may impair the effectiveness of the RIT or subsequent immunotherapy-utilizing murine antibodies or result in the development of infusion-related toxicities. Patients treated with RIT as part of the initial studies were at low risk for developing HAMAs due to their immune dysfunction resulting from the high number of prior chemotherapy regimens they received. The pivotal trial of I-131 tositumomab in low-grade NHL demonstrated a HAMA rate of only 8% (9). Patients enrolled on this study had a median of four prior chemotherapy regimens. In the phase I/II study of Y-90 ibritumomab tiuxetan, 2% of the patients developed HAMAs or HACAs (95,96). These data compare to a rate of 63% for the development of HAMAs when Kaminski studied I-131 tositumomab as initial therapy in patients with follicular lymphoma (8). Once again, it is unclear what the clinical implications of a HAMA are, but Kaminski reported that among the 23 patients in whom HAMA levels were more than five times the lowest level of detection, the 5-year rate of PFS was 35% compared with 70% for the remaining 53

patients. Additionally, 75% of the patients in the subgroup with high HAMA titers experienced grade 2 or higher fever, myalgias, arthralgias, or rash as compared with 12% of the other patients (8).

I-131 tositumomab and Y-90 ibritumomab tiuxetan utilize different radioisotopes. The I-131 beta emissions travel on average 0.4 mm or several cell diameters. The beta emissions from Y-90 are of higher energy, resulting in a longer path length compared with I-131 (5 mm). This translates into an optimal target diameter for Y-90 of 3.4 cm compared with 0.34 cm for I-131 (97). This higher beta energy may be an advantage where deeper penetration is desired but a disadvantage where it will result in greater toxicity. Additionally, I-131 emits gamma radiation that allows for imaging to be performed using external gamma cameras to determine dosimetry. As Y-90 does not emit gamma radiation, dosimetry needs to be performed utilizing an indium-111-labeled ibritumomab tiuxetan.

I-131 tositumomab and Y-90 ibritumomab tiuxetan are both individually dosed for each patient based on dosimetry calculations. For I-131 tositumomab, the dose is calculated to deliver an exposure of total-body radiation of 75 cGy; whereas for Y-90 ibritumomab tiuxetan, a dose of 0.4 mCi/kg (to a maximum of 32 mCi) is delivered (95,98). Both RITs were found to require dose reductions in patients who had platelet counts between 100 and 149,000/ μ L, as well as recommendations for not treating patients with >25% bone marrow involvement with lymphoma.

In its pivotal study, responses to I-131 tositumomab treatment were compared with the efficacy seen with the patient's last qualifying chemotherapy (LQC) regimen (9). This enabled refractory patients to be studied without needing to utilize a comparator arm, as these patients had already demonstrated refractoriness to chemotherapy. All patients treated on protocol had either not responded or a response of <6 months to their LQC. An ORR of 65%, with 20% CRs, was seen for I-131 tositumomab compared with 28% (with 3% CRs) after the LQC. The median response duration for those patients achieving a CR after I-131 tositumomab had not been reached after 47 months of follow-up compared with 6.1 months after LQC. Given the refractory nature of these patients' disease, these responses are of particular significance.

In the pivotal study for Y-90 ibritumomab tiuxetan, patients with low-grade, follicular, or transformed B-cell NHL were randomized to treatment with Y-90 ibritumomab tiuxetan or rituximab (99). The ORR and CR rate for the two groups were 80 and 30% for Y-90 ibritumomab tiuxetan and 56 and 16% for rituximab, respectively. Additionally, more durable responses (>6 months in length) were seen with Y-90 ibritumomab tiuxetan than rituximab.

In the study by Witzig et al. just discussed, the labeled and unlabeled antibodies differed not just in regard to the presence of the radioisotope but also in that Y-90 ibritumomab tiuxetan is a murine antibody, whereas rituximab is a chimeric antibody. Two studies were conducted utilizing the same antibody with and without an attached radioisotope that confirmed the importance of the radioisotope to the anti-CD20 therapy. In a study that compared I-131-labeled tositumomab to unlabeled tositumomab, Davis et al. demonstrated ORRs of 55 vs. 19%, with 33 vs. 8% CRs, for I-131-labeled tositumomab vs. unlabeled tositumomab (94). In a second study, Horning et al. studied I-131 tositumomab in patients who had previously received rituximab (100). The prior responses to rituximab demonstrated by the 40 patients in this study included 24 patients without any response, 11 patients with responses of < 6 months,

and five patients with responses >6 months. The ORR was 65%, with a CR rate of 38%, for all the patients in the study, and a progression-free survival for responders of 24.5 months. There was no correlation between the response to I-131 tositumomab and that of the rituximab, indicating that refractoriness to rituximab does not predict for a poor response to I-131 tositumomab.

In an attempt to maximize the benefits of RIT for patients, I-131 tositumomab was evaluated in indolent or transformed B-cell NHL at the first or second relapse. Earlier use of the agent demonstrated a response rate of 76%, with a CR rate of 49% (101). Median overall duration of remission was 1.3 years, and approximately one-fourth of the patients remained in a continuous remission ranging from 2.6+ to 5.2+ years after treatment. Kaminski and colleagues (8) assessed the utility of radioimmunotherapy as initial therapy for follicular lymphoma. Seventy-six patients with untreated, advanced stage follicular lymphoma were treated with a single course of I-131 tositumomab. The overall response rate was 95%, including 75% CRs. Eighty percent of the assessable patients achieved a molecular remission based on polymerase chain reaction assessment for BCL2 gene rearrangements. After a median follow-up of >5 years, estimated progression-free survival was 6.1 years. Treatment was well tolerated. As previously discussed, HAMA formation occurred in 63% of these patients, far greater than the 8% reported in the earlier studies of heavily pretreated patients.

These findings have led to further studies that will ultimately help to determine the utility of RIT as a component for initial therapy for indolent lymphoma. A phase II study conducted by the Southwest Oncology Group (SWOG) investigated CHOP followed by I-131 tositumomab as initial therapy in patients with follicular lymphoma (102). Preliminary results appear quite favorable, with an ORR 90%, a CR rate of 67%, and a 2-year progression-free survival of 81%. As a follow-up, the SWOG and Cancer and Leukemia Group B (CALGB) are currently conducting a randomized trial of CHOP plus concurrent rituximab vs. CHOP followed by I-131 tositumomab as first therapy for follicular NHL.

Another potential use studied for I-131 tositumomab has been in conjunction with autologous stem cell transplantation. These studies investigated I-131 tositumomab as a single agent at myeloablative doses of approximately 25 Gy in place of chemotherapy as part of an autologous stem cell transplant for patients with relapsed lymphoma (103,104). The dose-limiting toxicity for RIT is myelosuppression. By utilizing stem cell rescue to overcome this toxicity, the dose of radiation can be increased to the tolerance of the next most sensitive organ. This allows the radiation to be increased from 75 cGy to 25 Gy, an increase in radiation dose of more than 30-fold. This study consisted primarily of patients with low-grade lymphoma. The strategy was to utilize radiation to overcome the presence of any resistance to chemotherapy in previously treated patients. Eighteen of 21 patients (86%) responded, with a 76% CR rate and a 62% 2-year progression-free survival.

A second approach for utilizing RIT as part of stem cell transplants is in combination with chemotherapy. A phase I study of RIT in combination with BEAM (carmustine, etoposide, cytarabine, and melphalan) chemotherapy, was investigated in lymphoma patients refractory to chemotherapy. In this regimen, the RIT is being used to provide an additional, non-cross tolerant mode of therapy. Investigators were able to reach a dose of I-131 tositumomab of 75cGy (105). The 3-year overall survival and event-free survival for this study after a median follow-up of 38 months were 55 and 39%,

respectively. These patients would not have been considered eligible for standard autologous stem cell transplants given the chemorefractory nature of their disease, making any responses noteworthy.

One concern related to RIT is the potential exposure of hematopoietic stem cells in the bone marrow to radiation. As most lymphomas invade the bone marrow, there is significant opportunity for toxicity because of “cross-fire.” In the short term, this results in pancytopenia which in most cases, will resolve without complications. Long term, there is concern that the use of RIT will lead to myelodysplastic syndromes (MDS) and secondary acute myeloid leukemia (AML), both of which carry very poor prognoses. Patients with lymphomas who have been heavily pretreated with chemotherapy are at a significant risk for developing MDS or AML. When the data for I-131 tositumomab are analyzed in aggregate, the annualized incidence for the development of either AML or MDS is 1.6% per year (106). This rate is consistent with what would be expected for this group of patients treated only with chemotherapy, suggesting no increase risk of AML or MDS from the addition of RIT. To further support this, when the patients who underwent RIT as initial therapy, and thus had no chemotherapy exposure, were examined, no cases of AML or MDS were identified.

4. CONCLUSIONS

The addition of monoclonal antibodies to the armamentarium available for treatment of lymphomas has dramatically altered the care of lymphoma patients. Monoclonal antibodies represent effective therapies that are typically not cross-resistant with standard cytotoxic chemotherapies and often without the associated toxicities of chemotherapy. This has enabled treatment to be available for many patients who would otherwise be unable to receive treatment because of comorbidities or cytopenias. Additionally, delaying the exposure of patients to chemotherapy through the use of monoclonal antibodies helps patients avoid the accumulation of toxicities that can impair longevity and quality of life.

Monoclonal antibodies have been successfully placed into all stages of treatment algorithms for lymphomas. The first antibodies were limited by the choice of targets determined by the immune response generated by the vaccinated mouse. With molecular genetics and computer modeling of antigens, future antibodies will be able to target more effective epitopes and with higher affinities. These antibodies will also have new radioisotopes and novel toxin conjugates of which to take advantage.

It should also be hoped that as our knowledge regarding cell biology expands, additional cell surface molecules can be identified which can be manipulated by the binding of monoclonal antibodies. These antibodies could activate or inhibit, leading to turning on or off cellular processes to generate desired responses. These responses could include apoptosis, enhanced antigen presentation, cytokine production or inhibition of cytokine production, or any of a number of other possibilities. This would enable physicians to manipulate a patient’s own cells as a treatment of cancer.

With the improved tolerability and more targeted approach, monoclonal antibodies offer the potential to make indolent lymphomas, among others, truly chronic diseases. Repetitive dosing without the accumulation of toxicities would allow continued control of a disease until resistance develops. Future research needs to focus on means to circumvent the ability of lymphomas to resist treatment.

REFERENCES

1. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975, 256:495–497.
2. Morrison SL, Johnson MJ, Herzenberg LA, Oi VT. Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc Natl Acad Sci USA* 1984, 81:6851–6855.
3. Boulianne GL, Hozumi N, Shulman MJ. Production of functional chimaeric mouse/human antibody. *Nature* 1984, 312:643–646.
4. Jones PT, Dear PH, Foote J, Neuberger MS, Winter G. Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* 1986, 321:522–525.
5. Green LL. Antibody engineering via genetic engineering of the mouse: XenoMouse strains are a vehicle for the facile generation of therapeutic human monoclonal antibodies. *J Immunol Methods* 1999, 231:11–23.
6. Kellermann SA, Green LL. Antibody discovery: the use of transgenic mice to generate human monoclonal antibodies for therapeutics. *Curr Opin Biotechnol* 2002, 13:593–597.
7. Kretzschmar T, von Ruden T. Antibody discovery: phage display. *Curr Opin Biotechnol* 2002, 13:598–602.
8. Kaminski MS, Tuck M, Estes J, Kolstad A, Ross CW, Zasadny K, Regan D, Kison P, Fisher S, Kroll S, et al. 131I-tositumomab therapy as initial treatment for follicular lymphoma. *N Engl J Med* 2005, 352:441–449.
9. Kaminski MS, Zelenetz AD, Press OW, Saleh M, Leonard J, Fehrenbacher L, Lister TA, Stagg RJ, Tidmarsh GF, Kroll S, et al. Pivotal study of iodine I 131 tositumomab for chemotherapy-refractory low-grade or transformed low-grade B-cell non-Hodgkin's lymphomas. *J Clin Oncol* 2001, 19:3918–3928.
10. Leonard JP, Coleman M, Kostakoglu L, Chadburn A, Cesarman E, Furman RR, Schuster MW, Niesvizky R, Muss D, Fiore J, et al. Abbreviated chemotherapy with fludarabine followed by tositumomab and iodine I 131 tositumomab for untreated follicular lymphoma. *J Clin Oncol* 2005, 23:5696–5704.
11. Nadler LM, Ritz J, Hardy R, Pesando JM, Schlossman SF, Stashenko P. A unique cell surface antigen identifying lymphoid malignancies of B cell origin. *J Clin Invest* 1981, 67:134–140.
12. Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, Newman RA, Hanna N, Anderson DR. Depletion of B cells *in vivo* by a chimeric mouse human monoclonal antibody to CD20. *Blood* 1994, 83:435–445.
13. Anderson KC, Bates MP, Slaughenhaupt BL, Pinkus GS, Schlossman SF, Nadler LM. Expression of human B cell-associated antigens on leukemias and lymphomas: a model of human B cell differentiation. *Blood* 1984, 63:1424–1433.
14. Tedder TF, Streuli M, Schlossman SF, Saito H. Isolation and structure of a cDNA encoding the B1 (CD20) cell-surface antigen of human B lymphocytes. *Proc Natl Acad Sci USA* 1988, 85:208–212.
15. Tedder TF, Boyd AW, Freedman AS, Nadler LM, Schlossman SF. The B cell surface molecule B1 is functionally linked with B cell activation and differentiation. *J Immunol* 1985, 135:973–979.
16. Tedder TF, Engel P. CD20: a regulator of cell-cycle progression of B lymphocytes. *Immunol Today* 1994, 15:450–454.
17. Riley JK, Sliwkowski MX. CD20: a gene in search of a function. *Semin Oncol* 2000, 27:17–24.
18. Press OW, Appelbaum F, Ledbetter JA, Martin PJ, Zarling J, Kidd P, Thomas ED. Monoclonal antibody 1F5 (anti-CD20) serotherapy of human B cell lymphomas. *Blood* 1987, 69:584–591.
19. Giles FJ, Vose JM, Do KA, Johnson MM, Manshoury T, Bociek G, Bierman PJ, O'Brien SM, Keating MJ, Kantarjian HM, et al. Circulating CD20 and CD52 in patients with non-Hodgkin's lymphoma or Hodgkin's disease. *Br J Haematol* 2003, 123:850–857.
20. Manshoury T, Do KA, Wang X, Giles FJ, O'Brien SM, Saffer H, Thomas D, Jilani I, Kantarjian HM, Keating MJ, et al. Circulating CD20 is detectable in the plasma of patients with chronic lymphocytic leukemia and is of prognostic significance. *Blood* 2003, 101:2507–2513.
21. Golay J, Zaffaroni L, Vaccari T, Lazzari M, Borleri GM, Bernasconi S, Tedesco F, Rambaldi A, Introna M. Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab *in vitro*: CD55 and CD59 regulate complement-mediated cell lysis. *Blood* 2000, 95:3900–3908.

22. Manches O, Lui G, Chaperot L, Gressin R, Molens JP, Jacob MC, Sotto JJ, Leroux D, Bensa JC, Plumas J. *In vitro* mechanisms of action of rituximab on primary non-Hodgkin lymphomas. *Blood* 2003, 101:949–954.
23. Bellosillo B, Villamor N, Lopez-Guillermo A, Marce S, Esteve J, Campo E, Colomer D, Montserrat E. Complement-mediated cell death induced by rituximab in B-cell lymphoproliferative disorders is mediated *in vitro* by a caspase-independent mechanism involving the generation of reactive oxygen species. *Blood* 2001, 98:2771–2777.
24. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate *in vivo* cytotoxicity against tumor targets. *Nat Med* 2000, 6:443–446.
25. Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol* 2003, 21:3940–3947.
26. Shan D, Ledbetter JA, Press OW. Signaling events involved in anti-CD20-induced apoptosis of malignant human B cells. *Cancer Immunol Immunother* 2000, 48:673–683.
27. Mathas S, Rickers A, Bommert K, Dorken B, Mapara MY. Anti-CD20- and B-cell receptor-mediated apoptosis: evidence for shared intracellular signaling pathways. *Cancer Res* 2000, 60:7170–7176.
28. Jazirehi AR, Huerta-Yepez S, Cheng G, Bonavida B. Rituximab (chimeric anti-CD20 monoclonal antibody) inhibits the constitutive nuclear factor- κ B signaling pathway in non-Hodgkin's lymphoma B-cell lines: role in sensitization to chemotherapeutic drug-induced apoptosis. *Cancer Res* 2005, 65:264–276.
29. Jazirehi AR, Vega MI, Chatterjee D, Goodglick L, Bonavida B. Inhibition of the Raf-MEK1/2-ERK1/2 signaling pathway, Bcl-xL down-regulation, and chemosensitization of non-Hodgkin's lymphoma B cells by Rituximab. *Cancer Res* 2004, 64:7117–7126.
30. Byrd JC, Kitada S, Flinn IW, Aron JL, Pearson M, Lucas D, Reed JC. The mechanism of tumor cell clearance by rituximab *in vivo* in patients with B-cell chronic lymphocytic leukemia: evidence of caspase activation and apoptosis induction. *Blood* 2002, 99:1038–1043.
31. Alas S, Bonavida B. Rituximab inactivates signal transducer and activation of transcription 3 (STAT3) activity in B-Non-Hodgkin's lymphoma through inhibition of the interleukin 10 autocrine/paracrine loop and results in down-regulation of Bcl-2 and sensitization to cytotoxic drugs. *Cancer Res* 2001, 61:5137–5144.
32. Weng WK, Levy R. Expression of complement inhibitors CD46, CD55, and CD59 on tumor cells does not predict clinical outcome after rituximab treatment in follicular non-Hodgkin lymphoma. *Blood* 2001, 98:1352–1357.
33. Pels H, Schulz H, Manzke O, Hom E, Thall A, Engert A. Intraventricular and intravenous treatment of a patient with refractory primary CNS lymphoma using rituximab. *J Neurooncol* 2002, 59:213–216.
34. Rubenstein JL, Combs D, Rosenberg J, Levy A, McDermott M, Damon L, Ignoffo R, Aldape K, Shen A, Lee D, et al. Rituximab therapy for CNS lymphomas: targeting the leptomeningeal compartment. *Blood* 2003, 101:466–468.
35. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, Watier H. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor Fc γ RIIIa gene. *Blood* 2002, 99:754–758.
36. Maloney DG, Grillo-Lopez AJ, White CA, Bodkin D, Schilder RJ, Neidhart JA, Janakiraman N, Foon KA, Liles TM, Dallaire BK, et al. IDEC-C2B8 (Rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma. *Blood* 1997, 90:2188–2195.
37. McLaughlin P, Grillo-Lopez AJ, Link BK, Levy R, Czuczman MS, Williams ME, Heyman MR, Bence-Bruckler I, White CA, Cabanillas F, et al. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol* 1998, 16:2825–2833.
38. Witzig TE, Vukov AM, Habermann TM, Geyer S, Kurtin PJ, Friedenberg WR, White WL, Chalhah HI, Flynn PJ, Fitch TR, et al. Rituximab therapy for patients with newly diagnosed, advanced-stage, follicular grade I non-Hodgkin's lymphoma: a phase II trial in the North Central Cancer Treatment Group. *J Clin Oncol* 2005, 23:1103–1108.
39. Davis TA, Grillo-Lopez AJ, White CA, McLaughlin P, Czuczman MS, Link BK, Maloney DG, Weaver RL, Rosenberg J, Levy R. Rituximab anti-CD20 monoclonal antibody therapy in non-Hodgkin's lymphoma: safety and efficacy of re-treatment. *J Clin Oncol* 2000, 18:3135–3143.

40. Piro LD, White CA, Grillo-Lopez AJ, Janakiraman N, Saven A, Beck TM, Varns C, Shuey S, Czuczman M, Lynch JW, et al. Extended Rituximab (anti-CD20 monoclonal antibody) therapy for relapsed or refractory low-grade or follicular non-Hodgkin's lymphoma. *Ann Oncol* 1999, 10: 655–661.
41. Hainsworth JD, Litchy S, Burris HA, III, Scullin DC, Jr, Corso SW, Yardley DA, Morrissey L, Greco FA. Rituximab as first-line and maintenance therapy for patients with indolent non-Hodgkin's lymphoma. *J Clin Oncol* 2002, 20:4261–4267.
42. Ghielmini M, Schmitz SF, Cogliatti SB, Pichert G, Hummerjohann J, Waltzer U, Fey MF, Betticher DC, Martinelli G, Peccatori F, et al. Prolonged treatment with rituximab in patients with follicular lymphoma significantly increases event-free survival and response duration compared with the standard weekly $\times 4$ schedule. *Blood* 2004, 103:4416–4423.
43. Hainsworth JD, Litchy S, Shaffer DW, Lackey VL, Grimaldi M, Greco FA. Maximizing therapeutic benefit of rituximab: maintenance therapy versus re-treatment at progression in patients with indolent non-Hodgkin's lymphoma—a randomized phase II trial of the Minnie Pearl Cancer Research Network. *J Clin Oncol* 2005, 23:1088–1095.
44. Gordan LN, Grow WB, Pusateri A, Douglas V, Mendenhall NP, Lynch JW. Phase II trial of individualized rituximab dosing for patients with CD20-positive lymphoproliferative disorders. *J Clin Oncol* 2005, 23:1096–1102.
45. Czuczman MS, Weaver R, Alkuzweny B, Berlfein J, Grillo-Lopez AJ. Prolonged clinical and molecular remission in patients with low-grade or follicular non-Hodgkin's lymphoma treated with rituximab plus CHOP chemotherapy: 9-year follow-up. *J Clin Oncol* 2004, 22:4711–4716.
46. Czuczman MS, Koryzna A, Mohr A, Stewart C, Donohue K, Blumenson L, Bernstein ZP, McCarthy P, Alam A, Hernandez-Ilizaliturri F, et al. Rituximab in combination with fludarabine chemotherapy in low-grade or follicular lymphoma. *J Clin Oncol* 2005, 23:694–704.
47. Marcus R, Imrie K, Belch A, Cunningham D, Flores E, Catalano J, Solal-Celigny P, Offner F, Walewski J, Raposo J, et al. CVP chemotherapy plus rituximab compared with CVP as first-line treatment for advanced follicular lymphoma. *Blood* 2005, 105:1417–1423.
48. Forstpointner R, Dreyling M, Repp R, Hermann S, Hanel A, Metzner B, Pott C, Hartmann F, Rothmann F, Rohrberg R, et al. The addition of rituximab to a combination of fludarabine, cyclophosphamide, mitoxantrone (FCM) significantly increases the response rate and prolongs survival as compared with FCM alone in patients with relapsed and refractory follicular and mantle cell lymphomas: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. *Blood* 2004, 104:3064–3071.
49. Hainsworth JD, Litchy S, Morrissey LH, Andrews MB, Grimaldi M, McCarty M, Greco FA. Rituximab plus short-duration chemotherapy as first-line treatment for follicular non-Hodgkin's lymphoma: a phase II trial of the minnie pearl cancer research network. *J Clin Oncol* 2005, 23: 1500–1506.
50. Zinzani PL, Pulsoni A, Perrotti A, Soverini S, Zaja F, De Renzo A, Storti S, Lauta VM, Guardigni L, Gentilini P, et al. Fludarabine plus mitoxantrone with and without rituximab versus CHOP with and without rituximab as front-line treatment for patients with follicular lymphoma. *J Clin Oncol* 2004, 22:2654–2661.
51. McLaughlin P, Rodriguez MA, Hagemester FB, Romaguera J, Sarris AH, Younes A, Dang NH, Goy A, Samaniego F, Hess M. Stage IV indolent lymphoma: A randomized study of concurrent vs. sequential use of FND chemotherapy (fludarabine, mitoxantrone, dexamethasone) and rituximab (R) monoclonal antibody therapy, with interferon maintenance. *Proc Am Soc Clin Oncol* 2003, 22:564 (abstract 2269).
52. Hochster HS, Weller E, Ryan T, Habermann TM, Gascoyne R, Frankel SR, Horning SJ. Results of E1496: A phase III trial of CVP with or without maintenance rituximab in advanced indolent lymphoma (NHL). *Proc Am Soc Clin Oncol* 2004, 23:556 (abstract 6502).
53. Van Oers MHJ, Van Glabbeke M, Teodorovic I, Rozewicz C, Klasa R, Marcus RE, Wolf M, Kimby E, Hagenbeek A. Chimeric anti-CD20 monoclonal antibody (rituximab; mabtheraa) in remission induction and maintenance treatment of relapsed/resistant follicular non-Hodgkin's lymphoma : a phase III Randomized intergroup clinical trial. *Blood* 2004, 104:169a (abstract 586).
54. Coiffier B, Haioun C, Ketterer N, Engert A, Tilly H, Ma D, Johnson P, Lister A, Feuring-Buske M, Radford JA, et al. Rituximab (anti-CD20 monoclonal antibody) for the treatment of patients with relapsing or refractory aggressive lymphoma: a multicenter phase II study. *Blood* 1998, 92:1927–1932.

55. Coiffier B, Lepage E, Briere J, Herbrecht R, Tilly H, Bouabdallah R, Morel P, Van Den Neste E, Salles G, Gaulard P, et al. CHOP Chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. *N Engl J Med* 2002, 346:235–242.
56. Feugier P, Van Hoof A, Sebban C, Solal-Celigny P, Bouabdallah R, Ferme C, Christian B, Lepage E, Tilly H, Morschhauser F, et al. Long-term results of the R-CHOP study in the treatment of elderly patients with diffuse Large B-Cell lymphoma: a study by the Groupe d'Etude des Lymphomes de l'Adulte. *J Clin Oncol* 2005, 23:4117–4126.
57. Pfreundschuh M, Truemper L, Gill D, Osterborg A, Pettengell R, Trneny M, Imrie K, Walewski J, Zinzani P-L, Loeffler M. First analysis of the completed Mabthera international (Mint) trial in young patients with low-risk diffuse large B-cell lymphoma (DLBCL): addition of rituximab to a CHOP-like regimen significantly improves outcome of all patients with the identification of a very favorable subgroup with IPI = 0 and no bulky disease. *Blood* 2004, 104:48a (abstract 157).
58. Habermann TM, Weller EA, Morrison VA, Cassileth PA, Cohn JB, Dakhil SR, Gascoyne RD, Woda B, Fisher RI, Peterson BA, et al. Phase III trial of rituximab-CHOP (R-CHOP) vs. CHOP with a second randomization to maintenance rituximab (MR) or observation in patients 60 years of age and older with diffuse large B-cell lymphoma (DLBCL). *Blood* 2003, 102:(abstract 8).
59. Habermann TM, Weller E, Morrison VA, Cassileth PA, Cohn J, Dakhil S, Gascoyne RD, Woda B, Fisher R, Peterson BA, et al. Rituximab-CHOP versus CHOP with or without maintenance rituximab in patients 60 years of age or older with diffuse large B-cell lymphoma (DLBCL). *Blood* 2004, 104:(abstract 127).
60. Ghilmini M, Schmitz SF, Cogliatti S, Bertoni F, Waltzer U, Fey MF, Betticher DC, Schefer H, Pichert G, Stahel R, et al. Effect of single-agent rituximab given at the standard schedule or as prolonged treatment in patients with mantle cell lymphoma: a study of the Swiss Group for Clinical Cancer Research (SAKK). *J Clin Oncol* 2005, 23:705–711.
61. Lenz G, Dreyling M, Hoster E, Wormann B, Duhren U, Metzner B, Eimermacher H, Neubauer A, Wandt H, Steinhauer H, et al. Immunochemotherapy with rituximab and cyclophosphamide, doxorubicin, vincristine, and prednisone significantly improves response and time to treatment failure, but not long-term outcome in patients with previously untreated mantle cell lymphoma: results of a prospective randomized trial of the German Low Grade Lymphoma Study Group (GLSG). *J Clin Oncol* 2005, 23:1984–1992.
62. Kaplan LD, Lee JY, Ambinder RF, Sparano JA, Cesarman E, Chadburn A, Levine AM, Scadden DT. Rituximab does not improve clinical outcome in a randomized phase III trial of CHOP with or without rituximab in patients with HIV-associated non-Hodgkin's lymphoma: AIDS-malignancies consortium trial 010. *Blood* 2005.
63. Miles SA, McGratten M. Persistent panhypogammaglobulinemia after CHOP-rituximab for HIV-related lymphoma. *J Clin Oncol* 2005, 23:247–248.
64. Dunleavy K, Hakim F, Kim HK, Janik JE, Grant N, Nakayama T, White T, Wright G, Kwak L, Gress R, et al. B-cell recovery following rituximab-based therapy is associated with perturbations in stromal derived factor-1 and granulocyte homeostasis. *Blood* 2005, 106:795–802.
65. Voog E, Morschhauser F, Solal-Celigny P. Neutropenia in patients treated with rituximab. *N Engl J Med* 2003, 348:2691–2694 (discussion 2691–2694).
66. Boue F, Gabarre J, Gisselbrecht C, Reynes J, Plantier I, Morlat P, Milpied N, Raphael M, Lancar R, Costagliola D. CHOP chemotherapy plus rituximab in HIV patients with high grade lymphoma—results of an ANRS trial. *Blood* 2002, 100:470a (abstract 1824).
67. Spina M, Tirelli U. HIV-related non-Hodgkin's lymphoma (HIV-NHL) in the era of highly active antiretroviral therapy (HAART): some still unanswered questions for clinical management. *Ann Oncol* 2004, 15:993–995.
68. Spina M, Jaeger U, Sparano JA, Talamini R, Simonelli C, Michieli M, Rossi G, Nigra E, Berretta M, Cattaneo C, et al. Rituximab plus infusional cyclophosphamide, doxorubicin, and etoposide in HIV-associated non-Hodgkin lymphoma: pooled results from 3 phase 2 trials. *Blood* 2005, 105:1891–1897.
69. Sparano JA, Lee S, Chen MG, Nazeer T, Einzig A, Ambinder RF, Henry DH, Manalo J, Li T, Von Roenn JH. Phase II trial of infusional cyclophosphamide, doxorubicin, and etoposide in patients with HIV-associated non-Hodgkin's lymphoma: an Eastern Cooperative Oncology Group Trial (E1494). *J Clin Oncol* 2004, 22:1491–1500.
70. Hale G, Dyer MJ, Clark MR, Phillips JM, Marcus R, Riechmann L, Winter G, Waldmann H. Remission induction in non-Hodgkin lymphoma with reshaped human monoclonal antibody CAMPATH-1H. *Lancet* 1988, 2:1394–1399.

71. Xia MQ, Hale G, Lively MR, Ferguson MA, Campbell D, Packman L, Waldmann H. Structure of the CAMPATH-1 antigen, a glycosylphosphatidylinositol-anchored glycoprotein which is an exceptionally good target for complement lysis. *Biochem J* 1993, 293 (Pt 3):633–640.
72. Hale G, Xia MQ, Tighe HP, Dyer MJ, Waldmann H. The CAMPATH-1 antigen (CDw52). *Tissue Antigens* 1990, 35:118–127.
73. Salisbury JR, Rapson NT, Codd JD, Rogers MV, Nethersell AB. Immunohistochemical analysis of CDw52 antigen expression in non-Hodgkin's lymphomas. *J Clin Pathol* 1994, 47:313–317.
74. Dyer MJ, Hale G, Hayhoe FG, Waldmann H. Effects of CAMPATH-1 antibodies *in vivo* in patients with lymphoid malignancies: influence of antibody isotype. *Blood* 1989, 73:1431–1439.
75. Keating MJ, Flinn I, Jain V, Binet J-L, Hillmen P, Byrd J, Albitar M, Brettman L, Santabarbara P, Wacker B, et al. Therapeutic role of alemtuzumab (Campath-1H) in patients who have failed fludarabine: results of a large international study. *Blood* 2002, 99:3554–3561.
76. Harris NL, Jaffe ES, Stein H, Vardiman JW (Ed): *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon: IARC Press; 2001.
77. Gisselbrecht C, Gaulard P, Lepage E, Coiffier B, Briere J, Haioun C, Cazals-Hatem D, Bosly A, Xerri L, Tilly H, et al. Prognostic significance of T-cell phenotype in aggressive non-Hodgkin's lymphomas. Groupe d'Etudes des Lymphomes de l'Adulte (GELA). *Blood* 1998, 92:76–82.
78. Melnyk A, Rodriguez A, Pugh WC, Cabannillas F. Evaluation of the revised European-American lymphoma classification confirms the clinical relevance of immunophenotype in 560 cases of aggressive non-Hodgkin's lymphoma. *Blood* 1997, 89:4514–4520.
79. Ginaldi L, De Martinis M, Matutes E, Farahat N, Morilla R, Dyer MJ, Catovsky D. Levels of expression of CD52 in normal and leukemic B and T cells: correlation with *in vivo* therapeutic responses to Campath-1H. *Leuk Res* 1998, 22:185–191.
80. Khorana A, Bunn P, McLaughlin P, Vose J, Stewart C, Czuczman MS. A phase II multicenter study of CAMPATH-1H antibody in previously treated patients with nonbulky non-Hodgkin's lymphoma. *Leuk Lymphoma* 2001, 41:77–87.
81. Uppenkamp M, Engert A, Diehl V, Bunjes D, Huhn D, Brittinger G. Monoclonal antibody therapy with CAMPATH-1H in patients with relapsed high- and low-grade non-Hodgkin's lymphomas: a multicenter phase I/II study. *Ann Hematol* 2002, 81:26–32.
82. Lundin J, Osterborg A, Brittinger G, Crowther D, Dombret H, Engert A, Epenetos A, Gisselbrecht C, Huhn D, Jaeger U, et al. CAMPATH-1H monoclonal antibody in therapy for previously treated low-grade non-Hodgkin's lymphomas: a phase II multicenter study. European Study Group of CAMPATH-1H treatment in low-grade non-Hodgkin's lymphoma. *J Clin Oncol* 1998, 16:3257–3263.
83. Enblad G, Hagberg H, Erlanson M, Lundin J, MacDonald AP, Repp R, Schetelig J, Seipelt G, Osterborg A. A pilot study of alemtuzumab (anti-CD52 monoclonal antibody) therapy for patients with relapsed or chemotherapy-refractory peripheral T-cell lymphomas. *Blood* 2004, 103:2920–2924.
84. Lundin J, Hagberg H, Repp R, Cavallin-Stahl E, Freden S, Juliusson G, Rosenblad E, Tjonnfjord G, Wiklund T, Osterborg A. Phase 2 study of alemtuzumab (anti-CD52 monoclonal antibody) in patients with advanced mycosis fungoides/Sezary syndrome. *Blood* 2003, 101:4267–4272.
85. Zinzani PL, Alinari L, Tani M, Fina M, Pileri S, Baccarani M. Preliminary observations of a phase II study of reduced-dose alemtuzumab treatment in patients with pretreated T-cell lymphoma. *Haematologica* 2005, 90:702–703.
86. Gluck WL, Hurst D, Yuen A, Levine AM, Dayton MA, Gockerman JP, Lucas J, Denis-Mize K, Tong B, Navis D, et al. Phase I studies of interleukin (IL)-2 and rituximab in B-cell non-Hodgkin's lymphoma: IL-2 mediated natural killer cell expansion correlations with clinical response. *Clin Cancer Res* 2004, 10:2253–2264.
87. Eisenbeis CF, Leonard J, Rosenblatt J, Khan KD, Porcu P, Milan S, Wilson SE, Hurst D, Caligiuri MA, Piro L. Conversion of antibody-resistant cancer patients to antibody-sensitive: investigation of Fc receptor polymorphisms and response to IL-2 and rituximab treatment in rituximab-refractory NHL patients. *Proc Am Soc Clin Oncol* 2004, 22:171 (abstract 2534).
88. Friedberg JW, Kim H, McCauley M, Hessel EM, Sims P, Fisher DC, Nadler LM, Coffman RL, Freedman AS. Combination immunotherapy with a CpG oligonucleotide (1018 ISS) and rituximab in patients with non-Hodgkin lymphoma: increased interferon-alpha/beta-inducible gene expression, without significant toxicity. *Blood* 2005, 105:489–495.

89. Stein R, Qu Z, Chen S, Rosario A, Shi V, Hayes M, Horak ID, Hansen HJ, Goldenberg DM. Characterization of a new humanized anti-CD20 monoclonal antibody, IMMU-106, and its use in combination with the humanized anti-CD22 antibody, epratuzumab, for the therapy of non-Hodgkin's lymphoma. *Clin Cancer Res* 2004, 10:2868–2878.
90. Teeling JL, French RR, Cragg MS, van den Brakel J, Pluyter M, Huang H, Chan C, Parren PW, Hack CE, Dechant M, et al. Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. *Blood* 2004, 104:1793–1800.
91. Tedder TF, Tuscano J, Sato S, Kehr JH. CD22, a B lymphocyte-specific adhesion molecule that regulates antigen receptor signaling. *Annu Rev Immunol* 1997, 15:481–504.
92. Leonard JP, Coleman M, Ketas JC, Chadburn A, Ely S, Furman RR, Wegener WA, Hansen HJ, Ziccardi H, Eschenberg M, et al. Phase I/II trial of epratuzumab (humanized anti-CD22 antibody) in indolent non-Hodgkin's lymphoma. *J Clin Oncol* 2003, 21:3051–3059.
93. Leonard JP, Coleman M, Ketas JC, Chadburn A, Furman R, Schuster MW, Feldman EJ, Ashe M, Schuster SJ, Wegener WA, et al. Epratuzumab, a humanized anti-CD22 antibody, in aggressive non-Hodgkin's lymphoma: phase I/II clinical trial results. *Clin Cancer Res* 2004, 10:5327–5334.
94. Davis TA, Kaminski MS, Leonard JP, Hsu FJ, Wilkinson M, Zelenetz A, Wahl RL, Kroll S, Coleman M, Goris M, et al. The radioisotope contributes significantly to the activity of radioimmunotherapy. *Clin Cancer Res* 2004, 10:7792–7798.
95. Witzig TE, White CA, Wiseman GA, Gordon LI, Emmanouilides C, Raubitschek A, Janakiraman N, Gutheil J, Schilder RJ, Spies S, et al. Phase I/II trial of IDEC-Y2B8 radioimmunotherapy for treatment of relapsed or refractory CD20(+) B-cell non-Hodgkin's lymphoma. *J Clin Oncol* 1999, 17:3793–3803.
96. Gordon LI, Molina A, Witzig T, Emmanouilides C, Raubitschek A, Darif M, Schilder RJ, Wiseman G, White CA. Durable responses after ibritumomab tiuxetan radioimmunotherapy for CD20+ B-cell lymphoma: long-term follow-up of a phase 1/2 study. *Blood* 2004, 103:4429–4431.
97. Gordon LI, Witzig TE, Wiseman GA, Flinn IW, Spies SS, Silverman DH, Emmanouilides C, Cripe L, Saleh M, Czuczman MS, et al. Yttrium 90 ibritumomab tiuxetan radioimmunotherapy for relapsed or refractory low-grade non-Hodgkin's lymphoma. *Semin Oncol* 2002, 29:87–92.
98. Knox SJ, Goris ML, Trisler K, Negrin R, Davis T, Liles TM, Grillo-Lopez A, Chinn P, Varns C, Ning SC, et al. Yttrium-90-labeled anti-CD20 monoclonal antibody therapy of recurrent B-cell lymphoma. *Clin Cancer Res* 1996, 2:457–470.
99. Witzig TE, Gordon LI, Cabanillas F, Czuczman MS, Emmanouilides C, Joyce R, Pohlman BL, Bartlett NL, Wiseman GA, Padre N, et al. Randomized controlled trial of yttrium-90-labeled ibritumomab tiuxetan radioimmunotherapy versus rituximab immunotherapy for patients with relapsed or refractory low-grade, follicular, or transformed B-cell non-Hodgkin's lymphoma. *J Clin Oncol* 2002, 20:2453–2463.
100. Horning SJ, Younes A, Jain V, Kroll S, Lucas J, Podaloff D, Goris M. Efficacy and safety of tositumomab and iodine-131 tositumomab (Bexxar) in B-cell lymphoma, progressive after rituximab. *J Clin Oncol* 2005, 23:712–719.
101. Davies AJ, Rohatiner AZ, Howell S, Britton KE, Owens SE, Micallef IN, Deakin DP, Carrington BM, Lawrance JA, Vinnicombe S, et al. Tositumomab and iodine I 131 tositumomab for recurrent indolent and transformed B-cell non-Hodgkin's lymphoma. *J Clin Oncol* 2004, 22:1469–1479.
102. Press OW, Unger JM, Brazier RM, Maloney DG, Miller TP, LeBlanc M, Gaynor ER, Rivkin SE, Fisher RI. A phase 2 trial of CHOP chemotherapy followed by tositumomab/iodine I 131 tositumomab for previously untreated follicular non-Hodgkin lymphoma: Southwest Oncology Group Protocol S9911. *Blood* 2003, 102:1606–1612.
103. Press OW, Eary JF, Appelbaum FR, Martin PJ, Nelp WB, Glenn S, Fisher DR, Porter B, Matthews DC, Gooley T, et al. Phase II trial of 131I-B1 (anti-CD20) antibody therapy with autologous stem cell transplantation for relapsed B cell lymphomas. *Lancet* 1995, 346:336–340.
104. Liu SY, Eary JF, Petersdorf SH, Martin PJ, Maloney DG, Appelbaum FR, Matthews DC, Bush SA, Durack LD, Fisher DR, et al. Follow-up of relapsed B-cell lymphoma patients treated with iodine-131-labeled anti-CD20 antibody and autologous stem-cell rescue. *J Clin Oncol* 1998, 16:3270–3278.

105. Vose JM, Bierman PJ, Enke C, Hankins J, Bociek G, Lynch JC, Armitage JO. Phase I trial of iodine-131 tositumomab with high-dose chemotherapy and autologous stem-cell transplantation for relapsed non-Hodgkin's lymphoma. *J Clin Oncol* 2005, 23:461–467.
106. Bennett JM, Kaminski MS, Leonard JP, Vose JM, Zelenetz AD, Knox SJ, Horning S, Press OW, Radford JA, Kroll SM, et al. Assessment of treatment-related myelodysplastic syndromes and acute myeloid leukemia in patients with non-Hodgkin lymphoma treated with tositumomab and iodine I131 tositumomab. *Blood* 2005, 105:4576–4582.

26

Role of Apoptosis in Anti-Angiogenic Cancer Therapies

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SUMMARY

Angiogenesis, the growth of new blood vessels from the existing vasculature, is necessary for normal growth and development and in the adult during wound healing and the reproductive cycles. In most adult tissues, however, the vasculature is maintained in a quiescent state by the balanced presence of both angiogenic inducers and inhibitors in the tissue milieu. For progressive growth and metastasis, cancer cells must shift this balance to favor angiogenic induction. When inducers predominate, vascular endothelial cells (VECs) become activated, proliferating and migrating toward the source of the angiogenic inducer. In the activated VECs, distinct cell signaling pathways are initiated compared with that of quiescent VECs, providing the specificity of anti-angiogenic therapies to the tumor vasculature. VEC apoptosis has been well documented in regressing vessels, and it has been shown that, in addition to activating the VECs, some inducers such as vascular endothelial growth factor also up-regulate Fas expression, thus sensitizing the cell to apoptotic stimuli. Endogenous angiogenesis inhibitors, such as thrombospondin-1 (TSP-1) and pigment epithelium-derived factor (PEDF), stimulate signaling cascades within the VECs and also induce the expression of Fas ligand in activated VECs. Therefore, when inhibitors predominate, the apoptotic cascade is initiated. Depleting the supply of angiogenic inducers can also induce apoptosis, and thus, anti-angiogenic therapies can target the inducer supply or directly target the VECs. Although clinical studies are promising, most to date suggest that anti-angiogenic therapies may prove to be most effective when used in combination with traditional therapies.

Key Words: Angiogenesis; apoptosis; thrombospondin-1; endothelial cell; anti-angiogenic.

1. INTRODUCTION: ANGIOGENESIS IN TUMOR GROWTH

Angiogenesis, the growth of new blood vessels from the existing vasculature, is a normal and necessary process during growth and development. It also occurs in the adult during wound healing and the reproductive cycles. However, in most normal adult

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tissues, the vasculature is maintained in a quiescent state by the balanced presence of both pro-angiogenic and anti-angiogenic molecules in the tissue milieu (1). Vascular endothelial cells (VECs) have a very low turn over time, estimated anywhere from 1000 days (2,3) to 7 years (4). Judah Folkman first proposed in 1971 that tumor growth is dependent on the induction of angiogenesis (5). Prior to angiogenic induction, tumors remain in a dormant stage, not growing beyond a few millimeters in diameter. Folkman and colleagues (6,7) demonstrated that this is due to a balance between tumor cell proliferation and apoptotic rates. In this non-angiogenic environment, tumor cell apoptosis is triggered by hypoxia, cell starvation, and a build-up of cellular waste products. The same paradigm applies to micrometastases, which remain dormant until a pro-angiogenic environment arises (7,8). Tumors shift the balance to favor angiogenic induction by increasing expression of angiogenic inducers, decreasing expression of angiogenic inhibitors, or a combination of both (9). These changes in expression result from the same genetic and epigenetic changes that drive tumor progression, such as oncogene expression, loss of tumor suppressor gene expression, and tissue hypoxia (9,10). For example, Bouck and colleagues demonstrated that in fibroblasts progressing toward malignancy, loss of the p53 tumor suppressor gene resulted in decreased expression of thrombospondin-1 (TSP-1), a potent angiogenesis inhibitor, and increased expression of vascular endothelial growth factor (VEGF), a potent angiogenesis inducer, resulting in a pro-angiogenic phenotype (11,12). Figure 1 illustrates this change in angiogenic mediator balance.

The angiogenic balance can also be altered indirectly through tumor cell stimulation of the stromal cells to produce pro-angiogenic factors and/or to decrease inhibitor production (13). A large number of angiogenic inducers and inhibitors have now been identified, and they span a variety of molecule types, including growth factors, cytokines, hormones, and lipids (9,14). Table 1 lists some of the most well-studied endogenous angiogenic regulators.

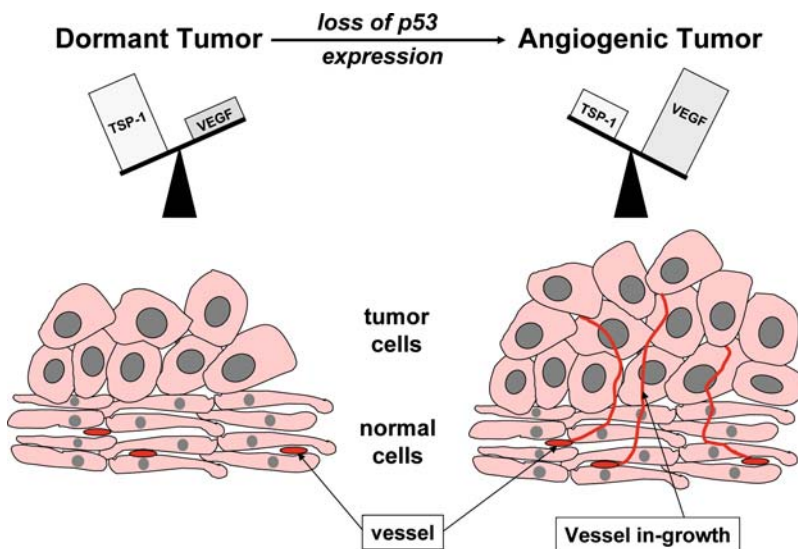


Fig. 1. Shift in balance of angiogenic mediators to favor angiogenic induction in tumors. Model based on data presented in refs. 11,12.

Table 1
Examples of Endogenous Angiogenic Mediators

Pro-angiogenic factors	Anti-angiogenic factors
Angiogenin	Angiostatin isoforms (fragments of plasminogen)
Epidermal growth factor	Endostatin (fragment of collagen XVIII)
Interleukin-8	Interferon- α
Hepatocyte growth factor	Interleukin-1, interleukin-6, and interleukin-12
Fibroblast growth factor-1 (acidic FGF)	Pigment epithelium-derived factor
Fibroblast growth factor-2 (basic FGF)	Platelet factor-4
Platelet-derived growth factor	Thrombospondin-1
Proliferin	Thrombospondin-2
Prostaglandins E ₁ and E ₂	Tissue inhibitor of metalloproteinase-2 and
Vascular endothelial growth factor	metalloproteinase-3
	1,25-Dihydroxy-vitamin D ₃

Reviewed in refs. 9,14.

When a sufficient number of cells develop a pro-angiogenic phenotype, vessels grow into the tumor, supplying nutrients and oxygen and removing wastes. Subsequent to vascularization, the tumor cell apoptotic rate decreases significantly, whereas the proliferative rate persists, resulting in net tumor growth (7). In addition, this neovascularization can also further stimulate tumor growth by providing the tumor with paracrine growth factors secreted by the VECs themselves, such as basic fibroblast growth factor (bFGF), interleukin-6, insulin-like growth factor, and platelet-derived growth factor, and also factors released by macrophages or other blood cells delivered to the tumor through the vasculature (13,15,16). Furthermore, these vessels provide an avenue for tumor cells to metastasize to distant locations. Thus, the transition to a pro-angiogenic phenotype provides significant advantages for tumor growth and metastases.

2. ANGIOGENIC INDUCTION AND THE TUMOR VASCULATURE

The formation of new vessels requires several steps, including extracellular matrix degradation, migration, proliferation, and tube formation. When the balance of angiogenic mediators shifts to favor angiogenic induction, VECs become activated, resulting in changes in gene and protein expression patterns. The specific signal transduction pathways activated will be discussed in detail in Sections 5 and 6 below. Proteases released by the activated VECs degrade the basement membrane, allowing migration toward the angiogenic inducer source. VEC proliferation subsequently ensues, followed by lumen formation (17). In addition, coculture experiments of tumor cells (U87 human glioma cells) with one type of VEC, human umbilical vein endothelial cells, have demonstrated coordinated increases in both expression of specific ligands and their receptors in the endothelial cells in response to the tumor cell secretions, creating autocrine stimulation of the VECs (18), thus further promoting angiogenesis.

The tumor vessels formed are markedly different than the vessels in normal tissues and organs. They are associated with vascular leakage and the presence of inflammatory cells (17,19). In addition, they often do not have associated pericytes and have reduced basement membrane formation, likely contributing to their leakiness (17). Pericytes and

the basement membrane structures are thought to provide maturation signals as well as survival factors and anti-apoptotic signals to the VECs, thus maintaining the quiescent vasculature (20). For example, anchorage of VECs to the basement membrane results in increased expression of focal adhesion kinase (FAK), anti-apoptotic bcl-2, as well as repression of p53 and pro-apoptotic bax (20,21). Furthermore, the tumor vessels are constantly undergoing remodeling and expansion in response to the angiogenic signals produced by the tumor. Thus, the tumor vasculature and the associated activated VECs differ significantly from the normal vasculature and quiescent VECs, histologically and in gene and protein expression profiles. This provides the specificity of anti-angiogenic therapies for the activated VECs associated with the tumor vessels. Achieving a better understanding of these differences, at the histological and cellular and molecular levels, is one of the major hurdles in developing specific and effective therapies targeting the tumor vasculature.

3. TARGETING THE VASCULATURE IN CANCER THERAPY

The advent of anti-angiogenic therapies has added a new option for cancer treatment, supplementing the traditional approaches of surgery, chemotherapy, and radiation therapy. It is important to note that angiogenic therapies (both pro- and anti-angiogenic agents) have potential therapeutic benefits in other diseases as well. The focus here, however, is solely on their role as anti-cancer agents. In principal, anti-angiogenic cancer therapies offer several advantages over the traditional cytotoxic chemotherapies and radiation therapies. First, they are not mutagenic, so secondary tumors are unlikely (9,22). Second, they target the tumor vasculature specifically; therefore, they do not cause the side effects associated with traditional chemotherapies, such as nausea, hair loss, and bone marrow suppression (9,22). Although, this does not preclude the occurrence of other types of side effects, expected or unexpected. Third, they can act synergistically with current chemotherapies, radiation and gene therapies (23–27). Fourth, they can be easily delivered through the circulation to their target cells (9,22). Last, as they do not target the tumor cell, but instead the genetically stable VECs, the tumor cells are unlikely to develop resistance to therapy (28,29). However, in this last advantage also lies a disadvantage. As the VECs, and not the tumor cells, are the target, these therapies do not kill the tumor cells directly and are therefore not curative. With these therapies, theoretically, the tumor would be reduced to avascular size limits. Thus, to overcome this issue, two approaches have been taken. One approach is to view anti-angiogenic therapies as chronic, long-term therapies. Another approach is to combine anti-angiogenic therapies with cytotoxic therapies that do directly target the tumor cell.

Traditionally, chemotherapies are given on a maximum tolerated dose (MTD) schedule over a short period of time with extended breaks between treatments to allow recovery of normal tissues. Most data to date suggest that anti-angiogenic therapies are more effective when administered on a more frequent, or metronomic, low dose schedule (30). Folkman prefers the term “anti-angiogenic schedule” to describe this type of dosing to emphasize that the VECs are the target of the treatment rather than the tumor cells, as metronomic implies only a regular interval of dose delivery; however, the term metronomic is most often used. The MTD schedule appears to allow for recovery of the tumor vessels between treatments and thus does not effectively suppress vessel growth. The continuous presence of the anti-angiogenic agent

prevents vascular re-growth, therefore, suppressing tumor growth more efficiently. For example, continuous delivery of endostatin, an endogenous angiogenesis inhibitor, through osmotic pump implantation had significantly greater anti-tumor effect than the same dose given once a day as a bolus injection, and only the osmotic pump delivery achieved tumor regression (31). As anti-angiogenic agents have fewer side effects in general, especially when used on lower, metronomic dosing schedules, it is feasible that anti-angiogenic agents could be developed as long-term, chronic therapies rather than acute therapies, such as done for the treatment of high blood pressure or high cholesterol.

In support of this hypothesis, some chemotherapeutic drugs have been found to have anti-angiogenic activity, when used on metronomic dosing schedules. Kerbel and colleagues (32) called such chemotherapeutic drugs “accidental” anti-angiogenic drugs. For example, vinblastine and cyclophosphamide (CPP) were more efficacious when administered on a low, metronomic dose schedule as compared with a MTD schedule in mouse tumor models with significantly diminished toxicity, although complete tumor regression was not achieved (33–35). It was later determined that the effect of CPP, with metronomic dosing, was due to increased TSP-1 expression (36). TSP-1 is known to induce VECs apoptosis (37), as will be discussed in detail in Section 6 below. Subsequently, other small molecule drugs have been shown to induce expression of anti-angiogenic proteins, including celecoxib, doxycycline, and rosiglitazone (38). In addition, many more have been shown to have anti-angiogenic activity, including thalidomide, tamoxifen, captopril, D-penicillamine, *N*-acetylcysteine, and dexamethasone; however, the mechanisms of these are less clear (39). Using chemotherapeutic agents on metronomic dosing schedules offers several advantages over traditional dosing schedules in that side effects are greatly reduced and tumor resistance is less likely. Although many of these agents are very effective in eradicating tumors in mice, whether or not the approach of using chemotherapeutic agents with anti-angiogenic activity or pure anti-angiogenic agents on metronomic dosing schedules is effective in cancer patients is still being evaluated.

Anti-angiogenic agents have been in clinical trials for many years. To date, however, many such agents, when used alone, even on metronomic scheduling, have not shown significant efficacy in the majority of patients or take an extended period of time to achieve significant tumor regression, even up to 1 year or more (40). In animal models, even the chemotherapies used on metronomic schedules do not appear to achieve complete tumor regression when used as single agents. However, when these treatments are combined with a second agent, metronomic chemotherapies do achieve significant or complete tumor regression. Teicher and colleagues (23–26) were the first to pioneer this combined treatment modality and produced several convincing studies demonstrating synergy between various anti-angiogenic therapies and various cytotoxic therapies in mouse models. Her first study, reported in 1992, demonstrated synergy in treatments combining an anti-angiogenic steroid with one of several different chemotherapies or radiation therapy (26). Studies by other researchers have supported her work. One caveat, however, is that when anti-angiogenic agents were used with chemotherapies on a MTD schedule, the combination produced some serious side effects (41,42). Therefore, combining anti-angiogenic therapies with chemotherapies that have demonstrated anti-angiogenic function and efficacy on a low dose, metronomic schedule, rather than a MTD schedule, may prove to be the safest and most

effective approach. Supporting this approach, in a neuroblastoma model, the combination of vinblastine, on a low, metronomic dosing schedule, with DC101, an antibody against the VEGF receptor, was more effective in achieving tumor regression than either agents alone (35). Similarly, metronomic CPP treatment was more effective against CPP-resistant lung and breast tumor models when TNP-470, an anti-angiogenic agent, was added to the CPP treatment, with the combined treatment completely eradicating the tumors (43). In addition, we and others have shown that treatment with an angiostatin isoform, angiostatin4.5, is synergistic with radiation therapy (44,45) and chemotherapies (46,47) in achieving tumor regression in mouse models. Based on these animal studies, it appears that combining anti-angiogenic agents with traditional cytotoxic therapies may be the most promising treatment avenue, and this is being actively pursued in clinical trials, as discussed in Section 4 below.

4. EVIDENCE FOR ENDOTHELIAL CELL APOPTOSIS AS A PRIMARY EVENT IN ANTI-ANGIOGENIC THERAPY

To inhibit angiogenesis, an anti-angiogenic agent could target the VECs at any of the steps necessary for neovascularization, such as extracellular matrix degradation, migration, proliferation, or tube formation. For example, platelet factor-4 inhibits endothelial cell proliferation (48), whereas tissue inhibitor of matrix metalloproteinases-2 inhibits extracellular matrix degradation, tube formation, and VEC proliferation (49). However, in many tissues that have active tissue remodeling, such as in reproductive tissue cycles, wound healing and tumor growth, VEC apoptosis can be readily detected. This is in contrast to normal tissues where the VECs are normally long lived, with very low proliferation and apoptotic rates. Thus, it seems likely that both proliferation and apoptosis of VECs are necessary for active remodeling of the vasculature. Therefore, it is reasonable that an angiogenesis inhibitor functions by actively promoting apoptosis of VECs. *In vitro* studies have shown that many endogenous angiogenesis inhibitors do indeed induce apoptosis, including TSP-1 (37), pigment epithelium-derived factor (PEDF) (50), prolactin (51), angiostatin isoforms (52,53), endostatin (54), tumstatin (55), and canstatin (56). Other anti-angiogenic agents and/or chemotherapeutic agents have also been shown to induce VEC apoptosis, such as 2-methoxyestradiol (57), and doxorubicin (58).

Many studies have demonstrated VEC apoptosis *in vivo* using animal tumor models, and in the prostate, in particular, the vasculature has been intensely studied for its involvement in prostatic growth regulation. In castrated rats, which have an atrophied prostate, testosterone treatment increases VEGF expression and stimulates angiogenesis prior to prostatic re-growth, and androgen ablation therapy also appears to target the vasculature (59,60). In both the normal prostate and prostate tumor models, androgen ablation therapy increases VEC apoptosis, reduces blood flow, and results in vascular regression, and the VEC apoptosis precedes the epithelial cell apoptosis and prostate tissue regression (61,62). In both rodent and human prostate tissues, in response to androgen ablation therapy in the normal and tumor epithelial cells as well as stromal cells, VEGF expression is decreased (63,64) and the expression of the angiogenesis inhibitors PEDF (65) and TSP-1 is increased (J. A. Doll and S. E. Crawford, unpublished observation). The changes in expression of VEGF, TSP-1, and PEDF, in response to androgen withdrawal, likely shift the angiogenic mediator balance thus triggering

VEC apoptosis and vascular regression. These studies support a critical role for angiogenic regulation in normal and malignant prostate growth and emphasize the role of VEC apoptosis in growth regulation.

In animal tumor models, VEC apoptosis has also been documented following treatments with anti-angiogenic agents. For example, in subcutaneous prostate tumors in nude mice, we have shown that VEC apoptosis occurs following PEDF treatment by coimmunostaining tumor tissue for factor VIII-related antigen, an endothelial cell marker, and TUNEL (65). Browder and colleagues (33) showed that CPP, when used on a low dose metronomic schedule, induced VEC apoptosis *in vivo* in a neuroblastoma tumor model. The authors further demonstrate that the VEC apoptosis precedes the tumor cell apoptosis by four days (33). These observations and additional studies showing that VEC apoptosis precedes tumor cell apoptosis, following anti-angiogenic therapy supports a key role for VEC apoptosis in the anti-tumor activity of anti-angiogenic cancer therapies.

5. THE ACTIVATED VEC: PRIMED FOR APOPTOSIS

It is well established that endothelial cell survival depends on the balance of both stimulatory and inhibitory signals in the extracellular milieu. When a predominance of inducers is present, the VECs become activated, with subsequent changes in gene and protein expression profiles. There are many angiogenic inducers now known (Table 1); however, of these, VEGF seems to be up-regulated in a wide range of tumor types and thus has been the most intensely studied in tumor biology (21,66). Therefore, here we will focus on the pathways stimulated by VEGF in the VECs. It is important to note, however, that pathways stimulated by other growth factors and molecules overlap with those stimulated by VEGF. VEGF is a member of the VEGF family of related proteins. It is the original member and is denoted as VEGF-A or simply VEGF. It is produced as several isoforms resulting from alternative splicing, with the most abundant ones being VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆. Of these, VEGF₁₆₅ is the most prevalent isoform and is over-expressed in many tumor types (21,66).

There are several VEGF receptors on VECs, including receptors 1 and 2 (VEGFR-1 and VEGFR-2) and neuropilin-1. VEGFR-1 (also called flt-1) primarily plays a regulatory role in VECs, as a “decoy” receptor, rather than a signal transduction role (67,68). Neuropilin-1 has signaling activity in other cell types; however, in VECs, intracellular signaling has not been demonstrated, but it may function to stabilize the VEGF/VEGFR-2 complex (21,69). VEGFR-2 (also called flk-1 or Kinase insert domain receptor (KDR)) is expressed on the surface of most VECs and is primarily responsible for VEGF signal transduction. It is a transmembrane protein with seven immunoglobulin-like extracellular domains, a single transmembrane region and an intracellular tyrosine kinase domain, interrupted by a kinase insert domain (21). VEGF binding to VEGFR-2 stimulates receptor dimerization and auto-phosphorylation of tyrosine residues in the cytoplasmic tail, which then triggers the phosphorylation of SH2-domain containing proteins (69,70). This initiates multiple signaling cascades within the VECs ultimately supporting survival, mitogenesis, migration, and tube formation (66). Cascades leading to vascular permeability are also initiated but will not be discussed here.

Survival and proliferation cascades are initiated through the phosphatidylinositol-3 kinase (PI3K)/Akt pathway and Ras pathway, whereas migration is initiated through

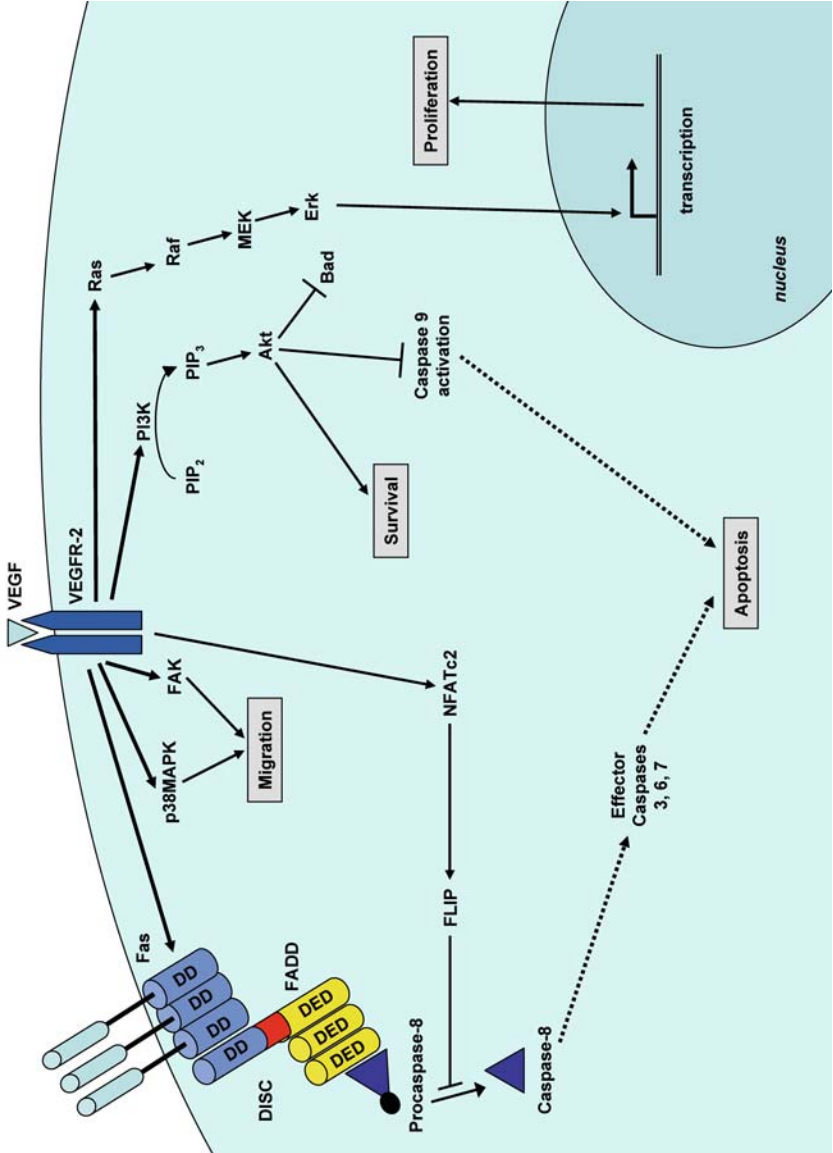


Fig. 2. Model of vascular endothelial growth factor (VEGF)-initiated signaling pathways in vascular endothelial cells (VECs). Solid arrows indicate pathways activated by VEGF signaling. Dashed arrows indicate pathways that become inactivated. Many of the pathways depicted involve additional factors that are not illustrated here for simplicity. Abbreviations not in text are as follows: DD, death domain; DED, death effector domain; FADD, Fas-associated death domain; and DISC, death-inducing signaling complex. See text for references.

the p38 mitogen-activated protein kinase (p38MAPK) and FAK pathways (66,69). VEGF also directly inhibits apoptosis through induction of expression of anti-apoptotic proteins such as bcl-2, survivin and A1, and repression of expression of pro-apoptotic proteins such as Bad, Bax, and caspase-9 (70,71). In addition, through the PI3K/Akt pathway, VEGF also stimulates expression of the anti-apoptotic Flice-like inhibitor protein (FLIP) (72). FLIP inhibits the activation of procaspase-8, and cells expressing elevated levels of FLIP are resistant to Fas-mediated apoptosis. Several studies have investigated the regulation of FLIP expression. One study demonstrated that elevated FLIP levels resulted from inactivation of the FOXO3a transcription factor, which suppresses FLIP expression; however, this study used serum stimulation of the VECs (73). Another study showed that VEGF treatment activated NFATc2, another transcription factor (74). NFATc2 has been shown to induce FLIP expression in VECs (75). These VEGF-stimulated signaling cascades ultimately result in VEC survival and the necessary steps for angiogenesis, including degradation of the extracellular matrix, VEC proliferation, migration, and tube formation. Figure 2 presents a basic schematic of the signaling cascades initiated by VEGF binding to VEGFR-2 on the VECs.

Interestingly, in addition to the pro-angiogenic/pro-survival cascades initiated, VEGF also induces expression of a protein that primes the pro-apoptotic cascades. VEC treatment with VEGF results in increased localization of the Fas (CD95) protein, a death receptor, to the cell surface (72,76). This simultaneous stimulation of pro-survival pathways and the priming of apoptotic pathways poise the activated VECs to be extremely sensitive to apoptotic signals. In addition, as discussed in Section 2 above, the tumor vasculature, which lacks associated pericytes and basement membrane structure, does not receive the normal pro-survival signals provided by these structures (20). Quiescent vessels, (with intact pericytes, basement membrane structures and that are not activated by VEGF) do not express Fas at high levels. Therefore, the quiescent VECs are resistant to the effects of circulating angiogenesis inhibitors, whereas the activated VECs are sensitized to respond to apoptotic stimuli. Therefore, if the balance shifts such that angiogenesis inhibitors predominate within the tumor microenvironment, apoptosis will be initiated in the activated VECs. The differences between quiescent and activated VECs provide the specificity that anti-angiogenic therapies have for activated VECs associated with the tumor vasculature.

6. APOPTOTIC INDUCTION IN ACTIVATED VECs

VEC apoptosis is essential during normal development and in the adult during phases of reproductive cycles and wound healing. As in other cell types, VEC apoptosis can occur through either the extrinsic (death receptor) or the intrinsic (mitochondrial) pathway. In addition to signals received by these pathways, Duval and colleagues (20) delineated four other essential signaling pathways that can regulate the fate of VECs, including paracrine, autocrine and endocrine factors (i.e. survival factor withdrawal), cell adhesion, blood flow and sheer stress, and the transcriptome and glycome. These pathways reflect the complex environment of the VECs. In respect to endogenous anti-angiogenic agents, the most studied of these pathways are the death receptor and mitochondrial-associated signaling pathways. For many endogenous inhibitors, including angiostatin, endostatin, TSP-1, and PEDF, caspase activation is necessary

for induction of VEC apoptosis (76). Of these, TSP-1 and PEDF have been the most intensely studied, with intracellular pathways more defined; therefore, these will be the primary focus here.

TSP-1 binds to the CD36 receptor on the VEC surface (77,78). PEDF also binds to the VEC surface; however, the surface receptor has not yet been identified. Following receptor binding, both TSP-1 and PEDF treatment stimulates an increase in Fas ligand (FasL/CD95 ligand) expression (76). For a TSP-1 peptide, called DI-TSP, this up-regulation has also been shown *in vivo* in a mouse tumor model (79). As VEGF stimulates Fas expression, the activated VEC is primed to respond to the FasL. PEDF treatment has also been shown to decrease FLIP expression through PEDF-stimulated inhibition of NFATc2 (75). FLIP, as discussed in Section 5 above, inhibits activation of procaspase-8; therefore, decreases in FLIP are permissive to apoptosis. The PEDF-induced FLIP repression is dependent on c-Jun NH₂-terminal kinase (JNK) activity as a JNK inhibitor blocks the FLIP repression (75). This repression is via JNK-1/2 stimulating NFATc2 nuclear export and JNK-2 retaining NFATc2 in the cytoplasm (75). Decreased FLIP activity, as well as activation of Fas by the FasL, results in activated caspase-8 and initiation of the apoptotic cascade. These data, and the VEGF data presented above, suggest that the levels of Fas/FasL and FLIP may represent two check points in VECs that determine survival or apoptosis. Figure 3 illustrates how these two inhibitors affect the signaling pathways in activated VECs, as is currently understood.

Among the other endogenous angiogenesis inhibitors, the mechanism of action of angiostatin has also been intensely studied. However, its biology is more complex as angiostatin, a kringle-containing fragment of plasminogen, exists as several isoforms. Plasminogen has five kringle domains, and the angiostatin isoforms, generated by proteolysis, vary in their kringle domain content. Angiostatin isoforms can inhibit VEC migration, tube formation, and proliferation (80–84) and can also induce apoptosis of VECs (52,53). And, for the angiostatin isoform containing kringles 1–3, caspase activation is necessary for the anti-angiogenic activity (76). Using angiostatin4.5 (AS4.5), an angiostatin isoform containing kringles 1–4 and 85% of kringle 5, we and others identified specific activation of caspase-8, caspase-9 and caspase-3, which is necessary for apoptotic induction (85,86). This suggests that both the intrinsic and extrinsic apoptotic pathways are activated by AS4.5 and may explain why AS4.5 is one of the most potent angiostatin isoforms and angiogenesis inhibitors identified to date. Chen and colleagues showed that treatment of VECs with an angiostatin isoform containing kringles 1–4 induced p53-mediated, Bax-mediated and t-Bid-mediated release of cytochrome c into the cytosol, thus initiating the intrinsic (mitochondrial) apoptotic pathway (87). This treatment also up-regulated FasL mRNA, decreased FLIP, and activated caspase-3, thus also initiating the extrinsic apoptotic pathway (87).

Another study using an angiostatin isoform containing kringles 1–3 showed down-regulation of cdk5, a cell cycle protein that is up-regulated in response to bFGF treatment (88). In a study using just the kringle 5 domain, Gao et al. (89) showed that treatment of human retinal capillary endothelial cells down-regulated VEGF expression and up-regulated PEDF expression, thus tipping the autocrine loop toward apoptosis. This held even under hypoxic conditions (89), which normally increases VEGF (66) and decreases PEDF (65). These studies indicate that angiostatin isoforms may have multiple effects on VECs or that the different isoforms inhibit angiogenesis by different mechanisms. The latter seems more likely as one study found that kringle 5 treatment

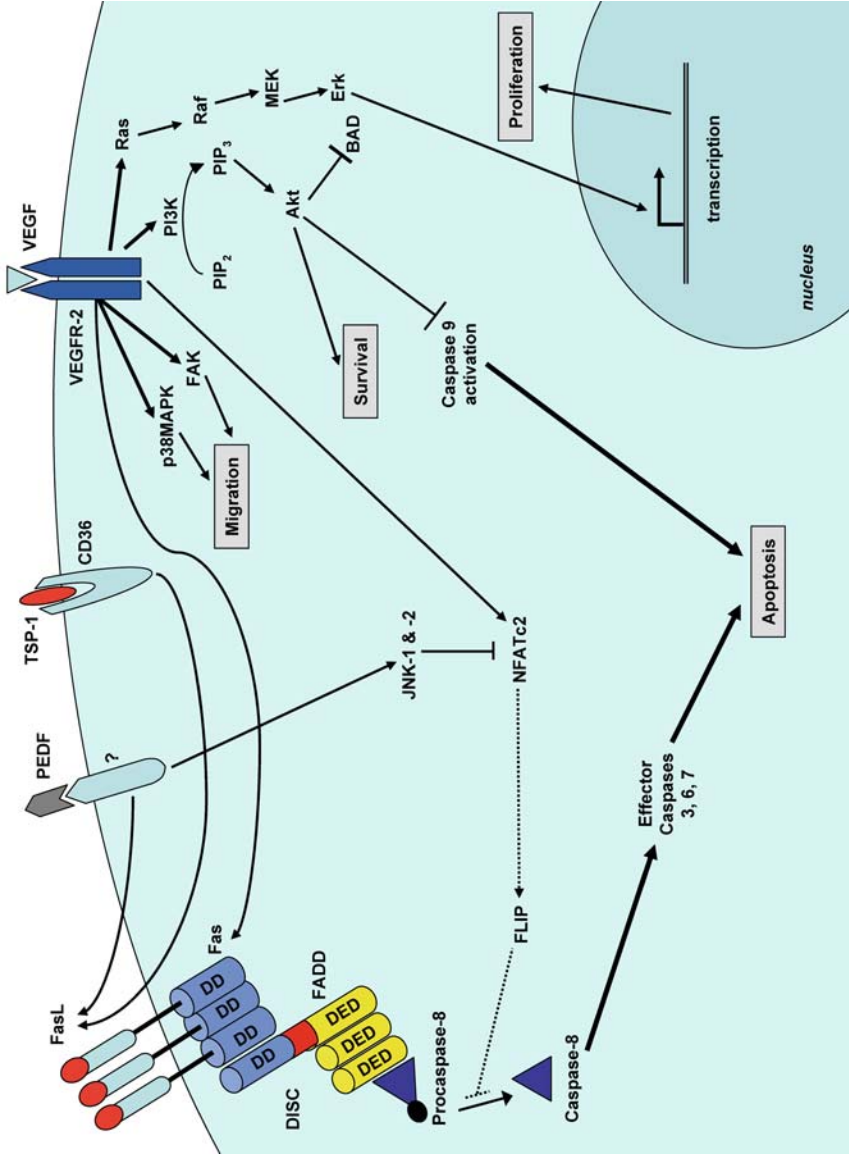


Fig. 3. Model of thrombospondin-1 (TSP-1)-induced and pigment epithelium-derived factor (PEDF)-induced vascular endothelial cell (VEC) apoptosis. Solid arrows indicate pathways stimulated by VEGF, TSP-1, or PEDF. Dashed arrows indicate pathways that become inactivated. Many of the pathways depicted involve additional factors that are not illustrated here for simplicity. Abbreviations not in text are as follows: DD, death domain; DED, death effector domain; FADD, Fas-associated death domain; and DISC, Fas-associated death complex. See text for references.

acted synergistically with AS4.5 treatment (90). Thus, by elucidating the mechanism of action of the various angiogenesis inhibitors, we could more effectively design anti-angiogenic combination therapies, combining inhibitors with different mechanisms of action, therefore, potentially enhancing anti-angiogenic and anti-tumor activity.

7. CLINICAL TRIALS: DIRECT AND INDIRECT ANTI-ANGIOGENIC AGENTS

Many of the molecules regulating angiogenesis have now been identified, and we have begun to identify the unique properties of the tumor vasculature which distinguishes it from the normal vessels. As our basic understanding of tumor angiogenesis progresses, so does our ability to develop effective anti-angiogenic agents clinically, and a large number of anti-angiogenic agents are currently in clinical trials, as monotherapies and in combination with other anti-angiogenic or anti-cancer therapies. These agents vary in their molecular and cellular target(s) and are classified in different ways. The system used here classifies them as direct and indirect agents (40,91). As a comprehensive review of all of these agents is beyond the scope of this review, more detailed information can be found in the reviews cited herein as well as on the NCI clinical trials website (<http://www.cancer.gov>).

7.1. Direct Angiogenesis Inhibitors

Direct angiogenesis inhibitors act to prevent the VECs from responding to pro-angiogenic stimuli or to directly induce VEC apoptosis. To prevent the VECs from responding to pro-angiogenic molecules, several different approaches have been taken, including sequestering the inducer in the tumor microenvironment, inhibition of binding of the inducer to its cognate receptor on the VECs, and inhibition of receptor signaling. Sequestration of inducers has been achieved using soluble receptors (decoy receptors) and inducer-binding antibodies. As VEGF is a potent angiogenic inducer and has been shown to be over expressed in many tumor types, it is the target for many agents of this type. Bevacuzimab (Avastin; Genentech, South San Francisco, CA, USA), a humanized anti-VEGF antibody, has recently gained FDA approval for treatment of some types of cancer. Bevacuzimab was first approved for use in colorectal cancer, and a recent phase III study combining Bevacuzimab with cytotoxic therapy showed improved survival in patients with metastatic colorectal cancer (92,93). Additional studies in non-small-cell lung cancer and metastatic breast cancer are in progress (94,95). The VEGF-trap (Regeneron, Tarrytown, NY, USA), also in clinical trials, is a soluble VEGF decoy receptor. It binds to VEGF, preventing binding to the VECs, and it is reported to have higher VEGF binding affinity than the monoclonal VEGF antibodies (96). Many additional drugs targeting VEGF are also in development.

One theoretical disadvantage of many first generation agents of this class is that they target a single angiogenic inducer. Many tumors secrete multiple inducers, and cells within a given tumor can produce different angiogenic inducers; therefore, inhibiting one inducer may only select for the sub-population of tumor cells expressing different inducers or may not be effective against tumors that secrete multiple angiogenic inducers. Using these drugs in combination with other types of angiogenic inhibitors or with low dose (metronomic) chemotherapy, as do the Bevacuzimab studies mentioned above, should overcome this limitation (30,33,35). Developing agents that inhibit multiple inducers

would also compensate for this shortcoming, as do some of the indirect agents, as discussed in Section 7.2 below. A more direct approach would be to develop agents that directly induce apoptosis of VECs as do many of the endogenous inhibitors identified thus far, including endostatin, angiostatin isoforms, TSP-1 and PEDF, as discussed in detail in Section 6 above. An advantage of this approach is that it would be effective against the activity of multiple inducers and thus multiple tumor types. Although the clinical development of these agents has lagged behind anti-inducer therapies because of the technical limitations of developing recombinant proteins or small molecule mimetics, some progress has been made. Of the endogenous inhibitors, a peptide of TSP-1, ABT-510 (Abbott Laboratories, Abbott Park, IL, USA), endostatin, and angiostatin isoforms have been tested in clinical trials. In addition, a trial of a drug cocktail to produce angiostatin^{4.5} *in vivo* from a patient's own plasminogen is also in progress (97).

7.2. Indirect Angiogenesis Inhibitors

Indirect angiogenesis inhibitors target tumor cells to block the expression and/or secretion of pro-angiogenic proteins that stimulate tumor-associated VECs or to increase expression of angiogenesis inhibitors. These agents appear to hold much promise as they can potentially act to change the expression pattern of multiple angiogenic mediators. One of the most thoroughly studied of this class of inhibitors is Iressa (Gefitinib/ZD1839; Astrazeneca, Wilmington, DE, USA). Iressa is a small molecule inhibitor that targets the tyrosine kinase activity of the epidermal growth factor receptor (EGFR), a member of the ErbB family of cell surface receptors. Although this drug, as many in its class, was developed to directly inhibit the tumor cell, it has also been found to inhibit angiogenesis as blocking this signaling pathway inhibits tumor cell synthesis of pro-angiogenic VEGF, bFGF, transforming growth factor- α , and interleukin-8 (98,99). This decrease in angiogenic inducer expression would result in a shift to an anti-angiogenic environment. Erbitux (IMC225; Imclone, New York, NY, USA), Tarceva (Erlotinib/OSI774; OSI Pharmaceuticals, Melville, NY, USA), and several other agents in clinical development also target the EGFR (40).

Many indirect agents currently in development target several signaling pathways and thus are sometimes labeled multiple target agents. For example, SU11248 (Pfizer, New York, NY, USA) inhibits the signaling activity of both the VEGF and PDGF receptors, and SU6668 (Pfizer, New York, NY, USA) targets these two receptors as well as the bFGF receptor. The advantage of this type of agent is that it may be effective against tumors with different types of mutations. In addition, some of these indirect agents can also act on the tumor-associated VECs if the targeted receptor is expressed in the VECs. Thus, the drug could potentially have a greater effect. For example, when VEGF binds to the VEGFR on the VECs, receptor signaling stimulates the release and/or production of several growth factors, which contributes to the pro-angiogenic stimuli present in the tumor microenvironment. If the receptor signaling is inhibited in the VECs, this autocrine loop will be shut off, further inhibiting angiogenesis.

8. CONCLUSIONS AND FUTURE POSSIBILITIES

Folkman first began championing his hypothesis that tumor growth was dependent on angiogenesis in 1971, and with the FDA approval of Avastin in 2004, anti-angiogenic therapies are finally a proven tool in the treatment of cancer. Through this long journey,

many of the mediators of this process have been identified and their mechanisms of action on the VECs elucidated. As our understanding evolves, so will our ability to design more effective anti-angiogenic agents. One of the challenges in trials of anti-angiogenic therapies has been in appropriate evaluation of efficacy at each stage of the clinical trials. With traditional therapies, phase I trials have focused on determining the MTD. However, as we have learned thus far with anti-angiogenic therapies, the MTD is not the optimal means of delivering these agents. Thus, we have had to re-evaluate the means by which we judge the efficacy of these drugs in our search for the optimal biologic dose. Looking for tumor mass or tumor marker responses, while appropriate in phase II and III trials, is not a fair measure in phase I dose determining studies, and evaluating microvessel density or angiogenic mediator expression in tumor biopsies is not practical. Urine and serum markers as well as accumulation of angiogenic mediators in platelets are being intensely studied in the hopes of identifying markers that are predictive of efficacy of anti-angiogenic agents. Identification of such cancer biomarkers will not only aid in the clinical development of anti-angiogenic agents and other novel drug therapies but also may provide additional means by which to detect cancer before it becomes symptomatic.

Another shift occurring in the treatment of cancer is in the design of therapies. Historically, cancer treatments were based on the tissue of origin of the tumor, with all patients with the same tumor type receiving the same therapy. Later treatments considered the histology of the tumor, pathological grade, and stage classifications. Around the turn of the century came a significant change to the traditional approaches. The successful development of Herceptin, an EGFR tyrosine kinase inhibitor, for therapy against ErbB2-positive breast cancer, gave birth to a new era in cancer drug design, tailoring therapies to the genetic profile of a given patient's tumor. Drugs that specifically target a signaling pathway known to be mutated in cancers are also called molecular targeting agents. Using genomic and/or proteomic screening, tumors could be profiled for genetic mutations and protein expression patterns, thus identifying the specific changes in angiogenic mediator expression. With this information, a tumor could be effectively treated with agents specifically targeting the identified culprits. Such designer therapies are the future of all cancer therapies and should prove to be much more effective than traditional approaches.

REFERENCES

1. Carmeliet, P. (2003). Angiogenesis in health and disease. *Nat Med* **9**, 653–60.
2. Denekamp, J. (1993). Review article: angiogenesis, neovascular proliferation and vascular pathophysiology as targets for cancer therapy. *Br J Radiol* **66**, 181–96.
3. Hobson, B. & Denekamp, J. (1984). Endothelial proliferation in tumours and normal tissues: continuous labelling studies. *Br J Cancer* **49**, 405–13.
4. Li, V. W., Folkherth, R. D., Watanabe, H., Yu, C., Rupnick, M., Barnes, P., Scott, R. M., Black, P. M., Sallan, S. E. & Folkman, J. (1994). Microvessel count and cerebrospinal fluid basic fibroblast growth factor in children with brain tumours. *Lancet* **344**, 82–6.
5. Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. *N Engl J Med* **285**, 1182–6.
6. Achilles, E. G., Fernandez, A., Allred, E. N., Kisker, O., Udagawa, T., Beecken, W. D., Flynn, E. & Folkman, J. (2001). Heterogeneity of angiogenic activity in a human liposarcoma: a proposed mechanism for “no take” of human tumors in mice. *J Natl Cancer Inst* **93**, 1075–81.
7. Holmgren, L., O'Reilly, M. S. & Folkman, J. (1995). Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat Med* **1**, 149–53.

8. Gimbrone, M. A., Jr., Leapman, S. B., Cotran, R. S. & Folkman, J. (1972). Tumor dormancy *in vivo* by prevention of neovascularization. *J Exp Med* **136**, 261–76.
9. Bouck, N. (1996). P53 and angiogenesis. *Biochim Biophys Acta* **1287**, 63–6.
10. Pugh, C. W. & Ratcliffe, P. J. (2003). Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med* **9**, 677–84.
11. Dameron, K. M., Volpert, O. V., Tainsky, M. A. & Bouck, N. (1994). Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science* **265**, 1582–4.
12. Volpert, O. V., Dameron, K. M. & Bouck, N. (1997). Sequential development of an angiogenic phenotype by human fibroblasts progressing to tumorigenicity. *Oncogene* **14**, 1495–502.
13. Folkman, J. (2003). Angiogenesis and apoptosis. *Semin Cancer Biol* **13**, 159–67.
14. Sridhar, S. S. & Shepherd, F. A. (2003). Targeting angiogenesis: a review of angiogenesis inhibitors in the treatment of lung cancer. *Lung Cancer* **42**(Suppl 1), S81–91.
15. Folkman, J. (1995). Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. *N Engl J Med* **333**, 1757–63.
16. Rak, J. W., St Croix, B. D. & Kerbel, R. S. (1995). Consequences of angiogenesis for tumor progression, metastasis and cancer therapy. *Anticancer Drugs* **6**, 3–18.
17. Blood, C. H. & Zetter, B. R. (1990). Tumor interactions with the vasculature: angiogenesis and tumor metastasis. *Biochim Biophys Acta* **1032**, 89–118.
18. Khodarev, N. N., Yu, J., Labay, E., Darga, T., Brown, C. K., Mauceri, H. J., Yassari, R., Gupta, N. & Weichselbaum, R. R. (2003). Tumour-endothelium interactions in co-culture: coordinated changes of gene expression profiles and phenotypic properties of endothelial cells. *J Cell Sci* **116**, 1013–22.
19. Dvorak, H. F., Galli, S. J. & Dvorak, A. M. (1986). Cellular and vascular manifestations of cell-mediated immunity. *Hum Pathol* **17**, 122–37.
20. Duval, H., Harris, M., Li, J., Johnson, N. & Print, C. (2003). New insights into the function and regulation of endothelial cell apoptosis. *Angiogenesis* **6**, 171–83.
21. Ferrara, N. (2001). Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol* **280**, C1358–66.
22. Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* **1**, 27–31.
23. Teicher, B. A., Holden, S. A., Ara, G. & Northey, D. (1993). Response of the FSaII fibrosarcoma to antiangiogenic modulators plus cytotoxic agents. *Anticancer Res* **13**, 2101–6.
24. Teicher, B. A., Holden, S. A., Ara, G., Sotomayor, E. A., Huang, Z. D., Chen, Y. N. & Brem, H. (1994). Potentiation of cytotoxic cancer therapies by TNP-470 alone and with other anti-angiogenic agents. *Int J Cancer* **57**, 920–5.
25. Teicher, B. A., Holden, S. A., Ara, G., Korbut, T. & Menon, K. (1996). Comparison of several antiangiogenic regimens alone and with cytotoxic therapies in the Lewis lung carcinoma. *Cancer Chemother Pharmacol* **38**, 169–77.
26. Teicher, B. A., Sotomayor, E. A. & Huang, Z. D. (1992). Antiangiogenic agents potentiate cytotoxic cancer therapies against primary and metastatic disease. *Cancer Res* **52**, 6702–4.
27. Wilczynska, U., Kucharska, A., Szary, J. & Szala, S. (2001). Combined delivery of an antiangiogenic protein (angiostatin) and an immunomodulatory gene (interleukin-12) in the treatment of murine cancer. *Acta Biochim Pol* **48**, 1077–84.
28. Boehm, T., Folkman, J., Browder, T. & O'Reilly, M. S. (1997). Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature* **390**, 404–7.
29. Kerbel, R. S. (1997). A cancer therapy resistant to resistance. *Nature* **390**, 335–6.
30. Hanahan, D., Bergers, G. & Bergsland, E. (2000). Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. *J Clin Invest* **105**, 1045–7.
31. Kisker, O., Becker, C. M., Prox, D., Fannon, M., D'Amato, R., Flynn, E., Fogler, W. E., Sim, B. K., Allred, E. N., Pirie-Shepherd, S. R. & Folkman, J. (2001). Continuous administration of endostatin by intraperitoneally implanted osmotic pump improves the efficacy and potency of therapy in a mouse xenograft tumor model. *Cancer Res* **61**, 7669–74.
32. Kerbel, R. S., Vilorio-Petit, A., Klement, G. & Rak, J. (2000). 'Accidental' anti-angiogenic drugs. Anti-oncogene directed signal transduction inhibitors and conventional chemotherapeutic agents as examples. *Eur J Cancer* **36**, 1248–57.

33. Browder, T., Butterfield, C. E., Kraling, B. M., Shi, B., Marshall, B., O'Reilly, M. S. & Folkman, J. (2000). Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res* **60**, 1878–86.
34. Emmenegger, U., Man, S., Shaked, Y., Francia, G., Wong, J. W., Hicklin, D. J. & Kerbel, R. S. (2004). A comparative analysis of low-dose metronomic cyclophosphamide reveals absent or low-grade toxicity on tissues highly sensitive to the toxic effects of maximum tolerated dose regimens. *Cancer Res* **64**, 3994–4000.
35. Klement, G., Baruchel, S., Rak, J., Man, S., Clark, K., Hicklin, D. J., Bohlen, P. & Kerbel, R. S. (2000). Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J Clin Invest* **105**, R15–24.
36. Bocci, G., Francia, G., Man, S., Lawler, J. & Kerbel, R. S. (2003). Thrombospondin 1, a mediator of the antiangiogenic effects of low-dose metronomic chemotherapy. *Proc Natl Acad Sci USA* **100**, 12917–22.
37. Jimenez, B., Volpert, O. V., Crawford, S. E., Febbraio, M., Silverstein, R. L. & Bouck, N. (2000). Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat Med* **6**, 41–8.
38. Folkman, J. (2004). Endogenous angiogenesis inhibitors. *Apms* **112**, 496–507.
39. Gately, S. & Kerbel, R. (2001). Antiangiogenic scheduling of lower dose cancer chemotherapy. *Cancer J* **7**, 427–36.
40. Kerbel, R. & Folkman, J. (2002). Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* **2**, 727–39.
41. Kuenen, B. C., Levi, M., Meijers, J. C., van Hinsbergh, V. W., Berkhof, J., Kakkar, A. K., Hoekman, K. & Pinedo, H. M. (2003). Potential role of platelets in endothelial damage observed during treatment with cisplatin, gemcitabine, and the angiogenesis inhibitor SU5416. *J Clin Oncol* **21**, 2192–8.
42. Marx, G. M., Steer, C. B., Harper, P., Pavlakis, N., Rixe, O. & Khayat, D. (2002). Unexpected serious toxicity with chemotherapy and antiangiogenic combinations: time to take stock! *J Clin Oncol* **20**, 1446–8.
43. Browder, T., Folkman, J. & Pirie-Shepherd, S. (2000). The hemostatic system as a regulator of angiogenesis. *J Biol Chem* **275**, 1521–4.
44. Gorski, D. H., Mauceri, H. J., Salloum, R. M., Gately, S., Hellman, S., Beckett, M. A., Sukhatme, V. P., Soff, G. A., Kufe, D. W. & Weichselbaum, R. R. (1998). Potentiation of the antitumor effect of ionizing radiation by brief concomitant exposures to angiostatin. *Cancer Res* **58**, 5686–9.
45. Mauceri, H. J., Hanna, N. N., Beckett, M. A., Gorski, D. H., Staba, M. J., Stellato, K. A., Bigelow, K., Heimann, R., Gately, S., Dhanabal, M., Soff, G. A., Sukhatme, V. P., Kufe, D. W. & Weichselbaum, R. R. (1998). Combined effects of angiostatin and ionizing radiation in antitumor therapy. *Nature* **394**, 287–91.
46. Galaup, A., Opolon, P., Bouquet, C., Li, H., Opolon, D., Bissery, M. C., Tursz, T., Perricaudet, M. & Griscelli, F. (2003). Combined effects of docetaxel and angiostatin gene therapy in prostate tumor model. *Mol Ther* **7**, 731–40.
47. Mauceri, H. J., Seetharam, S., Beckett, M. A., Schumm, L. P., Koons, A., Gupta, V. K., Park, J. O., Manan, A., Lee, J. Y., Montag, A. G., Kufe, D. W. & Weichselbaum, R. R. (2002). Angiostatin potentiates cyclophosphamide treatment of metastatic disease. *Cancer Chemother Pharmacol* **50**, 412–8.
48. Gengrinovitch, S., Greenberg, S. M., Cohen, T., Gitay-Goren, H., Rockwell, P., Maione, T. E., Levi, B. Z. & Neufeld, G. (1995). Platelet factor-4 inhibits the mitogenic activity of VEGF121 and VEGF165 using several concurrent mechanisms. *J Biol Chem* **270**, 15059–65.
49. Stetler-Stevenson, W. G. & Seo, D. W. (2005). TIMP-2: an endogenous inhibitor of angiogenesis. *Trends Mol Med* **11**, 97–103.
50. Stellmach, V., Crawford, S. E., Zhou, W. & Bouck, N. (2001). Prevention of ischemia-induced retinopathy by the natural ocular antiangiogenic agent pigment epithelium-derived factor. *Proc Natl Acad Sci USA* **98**, 2593–7.
51. Martini, J. F., Piot, C., Humeau, L. M., Struman, I., Martial, J. A. & Weiner, R. I. (2000). The antiangiogenic factor 16K PRL induces programmed cell death in endothelial cells by caspase activation. *Mol Endocrinol* **14**, 1536–49.

52. Claesson-Welsh, L., Welsh, M., Ito, N., Anand-Apte, B., Soker, S., Zetter, B., O'Reilly, M. & Folkman, J. (1998). Angiostatin induces endothelial cell apoptosis and activation of focal adhesion kinase independently of the integrin-binding motif RGD. *Proc Natl Acad Sci USA* **95**, 5579–83.
53. Lucas, R., Holmgren, L., Garcia, I., Jimenez, B., Mandriota, S. J., Borlat, F., Sim, B. K., Wu, Z., Grau, G. E., Shing, Y., Soff, G. A., Bouck, N. & Pepper, M. S. (1998). Multiple forms of angiostatin induce apoptosis in endothelial cells. *Blood* **92**, 4730–41.
54. Dhanabal, M., Ramchandran, R., Waterman, M. J., Lu, H., Knebelmann, B., Segal, M. & Sukhatme, V. P. (1999). Endostatin induces endothelial cell apoptosis. *J Biol Chem* **274**, 11721–6.
55. Maeshima, Y., Manfredi, M., Reimer, C., Holthaus, K. A., Hopfer, H., Chandamuri, B. R., Kharbanda, S. & Kalluri, R. (2001). Identification of the anti-angiogenic site within vascular basement membrane-derived tumstatin. *J Biol Chem* **276**, 15240–8.
56. Kamphaus, G. D., Colorado, P. C., Panka, D. J., Hopfer, H., Ramchandran, R., Torre, A., Maeshima, Y., Mier, J. W., Sukhatme, V. P. & Kalluri, R. (2000). Canstatin, a novel matrix-derived inhibitor of angiogenesis and tumor growth. *J Biol Chem* **275**, 1209–15.
57. Yue, T. L., Wang, X., Loudon, C. S., Gupta, S., Pillarisetti, K., Gu, J. L., Hart, T. K., Lysko, P. G. & Feuerstein, G. Z. (1997). 2-Methoxyestradiol, an endogenous estrogen metabolite, induces apoptosis in endothelial cells and inhibits angiogenesis: possible role for stress-activated protein kinase signaling pathway and Fas expression. *Mol Pharmacol* **51**, 951–62.
58. Lorenzo, E., Ruiz-Ruiz, C., Quesada, A. J., Hernandez, G., Rodriguez, A., Lopez-Rivas, A. & Redondo, J. M. (2002). Doxorubicin induces apoptosis and CD95 gene expression in human primary endothelial cells through a p53-dependent mechanism. *J Biol Chem* **277**, 10883–92.
59. Franck-Lissbrant, I., Haggstrom, S., Damber, J. E. & Bergh, A. (1998). Testosterone stimulates angiogenesis and vascular regrowth in the ventral prostate in castrated adult rats. *Endocrinology* **139**, 451–6.
60. Lissbrant, I. F., Lissbrant, E., Damber, J. E. & Bergh, A. (2001). Blood vessels are regulators of growth, diagnostic markers and therapeutic targets in prostate cancer. *Scand J Urol Nephrol* **35**, 437–52.
61. Jain, R. K., Safabakhsh, N., Sckell, A., Chen, Y., Jiang, P., Benjamin, L., Yuan, F. & Keshet, E. (1998). Endothelial cell death, angiogenesis, and microvascular function after castration in an androgen-dependent tumor: role of vascular endothelial growth factor. *Proc Natl Acad Sci USA* **95**, 10820–5.
62. Shabsigh, A., Chang, D. T., Heitjan, D. F., Kiss, A., Olsson, C. A., Puchner, P. J. & Buttyan, R. (1998). Rapid reduction in blood flow to the rat ventral prostate gland after castration: preliminary evidence that androgens influence prostate size by regulating blood flow to the prostate gland and prostatic endothelial cell survival. *Prostate* **36**, 201–6.
63. Joseph, I. B. & Isaacs, J. T. (1997). Potentiation of the antiangiogenic ability of linomide by androgen ablation involves down-regulation of vascular endothelial growth factor in human androgen-responsive prostatic cancers. *Cancer Res* **57**, 1054–7.
64. Joseph, I. B., Nelson, J. B., Denmeade, S. R. & Isaacs, J. T. (1997). Androgens regulate vascular endothelial growth factor content in normal and malignant prostatic tissue. *Clin Cancer Res* **3**, 2507–11.
65. Doll, J. A., Stellmach, V. M., Bouck, N. P., Bergh, A. R., Lee, C., Abramson, L. P., Cornwell, M. L., Pins, M. R., Borensztajn, J. & Crawford, S. E. (2003). Pigment epithelium-derived factor regulates the vasculature and mass of the prostate and pancreas. *Nat Med* **9**, 774–80.
66. Hicklin, D. J. & Ellis, L. M. (2005). Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol* **23**, 1011–27.
67. Zeng, H., Dvorak, H. F. & Mukhopadhyay, D. (2001). Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) receptor-1 down-modulates VPF/VEGF receptor-2-mediated endothelial cell proliferation, but not migration, through phosphatidylinositol 3-kinase-dependent pathways. *J Biol Chem* **276**, 26969–79.
68. Zeng, H., Sanyal, S. & Mukhopadhyay, D. (2001). Tyrosine residues 951 and 1059 of vascular endothelial growth factor receptor-2 (KDR) are essential for vascular permeability factor/vascular endothelial growth factor-induced endothelium migration and proliferation, respectively. *J Biol Chem* **276**, 32714–9.
69. Cross, M. J., Dixelius, J., Matsumoto, T. & Claesson-Welsh, L. (2003). VEGF-receptor signal transduction. *Trends Biochem Sci* **28**, 488–94.
70. Munoz-Chapuli, R., Quesada, A. R. & Angel Medina, M. (2004). Angiogenesis and signal transduction in endothelial cells. *Cell Mol Life Sci* **61**, 2224–43.
71. Nor, J. E. & Polverini, P. J. (1999). Role of endothelial cell survival and death signals in angiogenesis. *Angiogenesis* **3**, 101–16.

72. Suhara, T., Mano, T., Oliveira, B. E. & Walsh, K. (2001). Phosphatidylinositol 3-kinase/Akt signaling controls endothelial cell sensitivity to Fas-mediated apoptosis via regulation of FLICE-inhibitory protein (FLIP). *Circ Res* **89**, 13–9.
73. Skurk, C., Maatz, H., Kim, H. S., Yang, J., Abid, M. R., Aird, W. C. & Walsh, K. (2004). The Akt-regulated forkhead transcription factor FOXO3a controls endothelial cell viability through modulation of the caspase-8 inhibitor FLIP. *J Biol Chem* **279**, 1513–25.
74. Hernandez, G. L., Volpert, O. V., Iniguez, M. A., Lorenzo, E., Martinez-Martinez, S., Grau, R., Fresno, M. & Redondo, J. M. (2001). Selective inhibition of vascular endothelial growth factor-mediated angiogenesis by cyclosporin A: roles of the nuclear factor of activated T cells and cyclooxygenase 2. *J Exp Med* **193**, 607–20.
75. Zaichuk, T. A., Shroff, E. H., Emmanuel, R., Filleur, S., Nelius, T. & Volpert, O. V. (2004). Nuclear factor of activated T cells balances angiogenesis activation and inhibition. *J Exp Med* **199**, 1513–22.
76. Volpert, O. V., Zaichuk, T., Zhou, W., Reiher, F., Ferguson, T. A., Stuart, P. M., Amin, M. & Bouck, N. P. (2002). Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor. *Nat Med* **8**, 349–57.
77. Dawson, D. W., Volpert, O. V., Gillis, P., Crawford, S. E., Xu, H., Benedict, W. & Bouck, N. P. (1999). Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. *Science* **285**, 245–8.
78. Guo, N. H., Krutzsch, H. C., Inman, J. K., Shannon, C. S. & Roberts, D. D. (1997). Antiproliferative and antitumor activities of D-reverse peptides derived from the second type-1 repeat of thrombospondin-1. *J Pept Res* **50**, 210–21.
79. Quesada, A. J., Nelius, T., Yap, R., Zaichuk, T. A., Alfranca, A., Filleur, S., Volpert, O. V. & Redondo, J. M. (2005). *In vivo* upregulation of CD95 and CD95L causes synergistic inhibition of angiogenesis by TSP1 peptide and metronomic doxorubicin treatment. *Cell Death Differ* **12**, 649–58.
80. Gately, S., Twardowski, P., Stack, M. S., Cundiff, D. L., Grella, D., Castellino, F. J., Enghild, J., Kwaan, H. C., Lee, F., Kramer, R. A., Volpert, O., Bouck, N. & Soff, G. A. (1997). The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. *Proc Natl Acad Sci USA* **94**, 10868–72.
81. Gately, S., Twardowski, P., Stack, M. S., Patrick, M., Boggio, L., Cundiff, D. L., Schnaper, H. W., Madison, L., Volpert, O., Bouck, N., Enghild, J., Kwaan, H. C. & Soff, G. A. (1996). Human prostate carcinoma cells express enzymatic activity that converts human plasminogen to the angiogenesis inhibitor, angiostatin. *Cancer Res* **56**, 4887–90.
82. Ji, W. R., Barrientos, L. G., Llinas, M., Gray, H., Villarreal, X., DeFord, M. E., Castellino, F. J., Kramer, R. A. & Trail, P. A. (1998). Selective inhibition by kringle 5 of human plasminogen on endothelial cell migration, an important process in angiogenesis. *Biochem Biophys Res Commun* **247**, 414–9.
83. Ji, W. R., Castellino, F. J., Chang, Y., Deford, M. E., Gray, H., Villarreal, X., Kondri, M. E., Marti, D. N., Llinas, M., Schaller, J., Kramer, R. A. & Trail, P. A. (1998). Characterization of kringle domains of angiostatin as antagonists of endothelial cell migration, an important process in angiogenesis. *FASEB J* **12**, 1731–8.
84. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H. & Folkman, J. (1994). Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* **79**, 315–28.
85. Hanford, H. A., Wong, C. A., Kassan, H., Cundiff, D. L., Chandel, N., Underwood, S., Mitchell, C. A. & Soff, G. A. (2003). Angiostatin(4,5)-mediated apoptosis of vascular endothelial cells. *Cancer Res* **63**, 4275–80.
86. Veitonmaki, N., Cao, R., Wu, L. H., Moser, T. L., Li, B., Pizzo, S. V., Zhivotovsky, B. & Cao, Y. (2004). Endothelial cell surface ATP synthase-triggered caspase-apoptotic pathway is essential for k1-5-induced antiangiogenesis. *Cancer Res* **64**, 3679–86.
87. Chen, Y. H., Wu, H. L., Chen, C. K., Huang, Y. H., Yang, B. C. & Wu, L. W. (2003). Angiostatin antagonizes the action of VEGF-A in human endothelial cells via two distinct pathways. *Biochem Biophys Res Commun* **310**, 804–10.
88. Sharma, M. R., Tuszyński, G. P. & Sharma, M. C. (2004). Angiostatin-induced inhibition of endothelial cell proliferation/apoptosis is associated with the down-regulation of cell cycle regulatory protein cdk5. *J Cell Biochem* **91**, 398–409.
89. Gao, G., Li, Y., Fant, J., Crosson, C. E., Becerra, S. P. & Ma, J. X. (2002). Difference in ischemic regulation of vascular endothelial growth factor and pigment epithelium-derived factor in brown

- norway and sprague dawley rats contributing to different susceptibilities to retinal neovascularization. *Diabetes* **51**, 1218–25.
90. Cao, R., Wu, H. L., Veitonmaki, N., Linden, P., Farnebo, J., Shi, G. Y. & Cao, Y. (1999). Suppression of angiogenesis and tumor growth by the inhibitor K1-5 generated by plasmin-mediated proteolysis. *Proc Natl Acad Sci USA* **96**, 5728–33.
 91. Davis, D. W., McConkey, D. J., Zhang, W. & Herbst, R. S. (2003). Antiangiogenic tumor therapy. *Biotechniques* **34**, 1048–50, 1052, 1054 passim.
 92. Hurwitz, H. (2004). Integrating the anti-VEGF-A humanized monoclonal antibody bevacizumab with chemotherapy in advanced colorectal cancer. *Clin Colorectal Cancer* **4**(Suppl 2), S62–8.
 93. Penland, S. K. & Goldberg, R. M. (2004). Combining anti-VEGF approaches with oxaliplatin in advanced colorectal cancer. *Clin Colorectal Cancer* **4**(Suppl 2), S74–80.
 94. Herbst, R. S., Johnson, D. H., Mininberg, E., Carbone, D. P., Henderson, T., Kim, E. S., Blumenstein, G., Jr., Lee, J. J., Liu, D. D., Truong, M. T., Hong, W. K., Tran, H., Tsao, A., Xie, D., Ramies, D. A., Mass, R., Seshagiri, S., Eberhard, D. A., Kelley, S. K. & Sandler, A. (2005). Phase III trial evaluating the anti-vascular endothelial growth factor monoclonal antibody bevacizumab in combination with the HER-1/epidermal growth factor receptor tyrosine kinase inhibitor erlotinib for patients with recurrent non-small-cell lung cancer. *J Clin Oncol* **23**, 2544–55.
 95. Miller, K. D., Chap, L. I., Holmes, F. A., Cobleigh, M. A., Marcom, P. K., Fehrenbacher, L., Dickler, M., Overmoyer, B. A., Reimann, J. D., Sing, A. P., Langmuir, V. & Rugo, H. S. (2005). Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. *J Clin Oncol* **23**, 792–9.
 96. Konner, J. & Dupont, J. (2004). Use of soluble recombinant decoy receptor vascular endothelial growth factor trap (VEGF Trap) to inhibit vascular endothelial growth factor activity. *Clin Colorectal Cancer* **4**(Suppl 2), S81–5.
 97. Soff, G. A., Wang, H., Cundiff, D. L., Jiang, K., Martone, B., Rademaker, A. W., Doll, J. A. & Kuzel, T. M. (2005). *In vivo* generation of angiostatin isoforms by administration of a plasminogen activator and a free sulfhydryl donor: a phase I study of an angiostatic cocktail of tissue plasminogen activator and mesna. *Clin Cancer Res* **11**, 6218–25.
 98. Ciardiello, F., Caputo, R., Bianco, R., Damiano, V., Fontanini, G., Cuccato, S., De Placido, S., Bianco, A. R. & Tortora, G. (2001). Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin Cancer Res* **7**, 1459–65.
 99. Kerbel, R. S., Yu, J., Tran, J., Man, S., Vilorio-Petit, A., Klement, G., Coomber, B. L. & Rak, J. (2001). Possible mechanisms of acquired resistance to anti-angiogenic drugs: implications for the use of combination therapy approaches. *Cancer Metastasis Rev* **20**, 79–86.

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Photodynamic Therapy-Induced Apoptosis

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SUMMARY

Photodynamic therapy (PDT) is a novel treatment for cancer and certain non-malignant conditions, which employs a photosensitive drug followed by light in the visible range to produce an oxidative stress and cell death in the targeted tissue. The photosensitizers (PSs) are most commonly porphyrins or related hydrophobic macrocycles that localize in one or more intracellular membranes and that are activated by long-wavelength (red) visible light. Singlet molecular oxygen and other reactive oxygen species are the primary damaging species produced by PDT, and these oxidize cellular substrates, including lipids and proteins of the membranes in which the PS resides. PDT is an efficient inducer of apoptosis; this has been demonstrated in many cell types and with many different PSs. Once induced, apoptosis follows recognized paths but most commonly the intrinsic (mitochondrial) pathway, mediated by activated caspases and resulting in DNA fragmentation and morphological apoptosis. The aspects of apoptosis that are unique to PDT are the molecular targets, the types of initial cellular damage, and the immediate consequences of that damage. Many PSs target mitochondria, resulting in changes in the permeability transition pore complex, the apoptotic proteins Bcl-2 and Bcl-xL, and/or phospholipids, especially cardiolipin. Some PSs also target the endoplasmic reticulum (ER), damaging calcium pumps and resulting in the efflux of stored calcium into the cytosol and subsequently mitochondria. With PSs that localize in lysosomes, photoactivation damages the lysosomal membrane, causing release of cathepsins and other factors that can activate apoptosis mediators such as Bid that in turn promote mitochondrial apoptosis. Although the plasma membrane is not commonly a direct target of most PSs, it can be secondarily affected by the release of ligands of membrane receptors, such as Fas ligand. Evidence supporting the roles of each of the organelles in PDT-induced apoptosis are reviewed, and a model is proposed in which mitochondrial damage by PDT directly activates apoptosis and damage to the ER and/or the lysosome promotes the central mitochondrial pathway of apoptosis.

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1. PHOTODYNAMIC THERAPY: AN INTRODUCTION

Photodynamic therapy (PDT) is a novel treatment for cancer and certain non-malignant conditions, which employs a photosensitive drug followed by light in the visible range to produce an oxidative stress and cell death in the targeted tissue (1–4). The US Food and Drug Administration (FDA) has approved PDT with the photosensitizer (PS) Photofrin® for treatment of advanced esophageal and early and late lung cancers (5). Approvals for several other cancers exist in other countries, and clinical trials for bladder, brain, skin, head-and-neck, gastrointestinal, genitourinary, and other cancers are ongoing (5). PDT is also FDA approved for the treatment of certain non-cancerous conditions: age-related macular degeneration using the PS Verteporfin®, actinic keratosis using Levulan®, and Barrett’s esophagus using Photofrin® (5). Numerous other PSs are being tested in pre-clinical models and in clinical trials (5).

PDT is a three-component treatment (1–3). The PS is delivered to the patient or animal (intravenously or topically) or cell culture (by addition to the culture medium), and after a suitable time to allow for accumulation of the dye in the desired compartment, the tissue or cell culture is exposed to non-thermal *visible light* of a wavelength absorbed by the PS. The third component of PDT is *molecular oxygen*. Upon absorption of a photon, the PS is activated to an excited triplet state (Fig. 1). In what is known as type II photochemistry, the triplet transfers energy to ground-state molecular oxygen ($^3\text{O}_2$), generating singlet oxygen ($^1\text{O}_2$), a non-radical but highly reactive form of oxygen thought to be the major cytotoxic mediator of PDT (6,7). The ground-state PS is regenerated and is then available to accept another photon. In type I photochemistry, the triplet PS (^3PS) can also undergo electron transfer (i.e., an oxidation–reduction reaction) with nearby cellular constituents, generating free radicals (6,7).

One of the advantages of PDT relative to other cancer therapies is the extreme specificity it offers in the localization of the photodynamic effect; most PSs accumulate somewhat preferentially in malignant or other abnormal tissue in comparison to the surrounding normal tissues, for reasons that still remain largely obscure, and the light can be precisely focused onto a selected region. Furthermore, because of the strong oxidative stress produced, the PDT response is unhindered by the usual modes of resistance to conventional cancer treatments. In addition, it can be used in combination with conventional treatments. And finally, but perhaps most importantly, both the PS and the light are inert by themselves, precluding most types of systemic toxicities (1–3).

Limitations of PDT also exist. Despite attempts to elicit an immune or vaccine response with PDT (8), at present it remains a local treatment. The PSs can distribute in a tumor unevenly, allowing some regions to escape photodynamic damage. The PSs can remain in the skin for up to several weeks, making the patient sensitive to sunlight; in practice, this is a problem only for Photofrin®. Finally, the penetration of photoactivating light through human tissue increases with wavelength and is most efficient for red and infrared light. The energy of the photon decreases with increasing wavelength, such that above approximately 800–850 nm, formation of the triplet PS state is inefficient. These factors confine the practical wavelength range for PDT to

Production of Singlet Oxygen by PDT: Type II Photochemistry

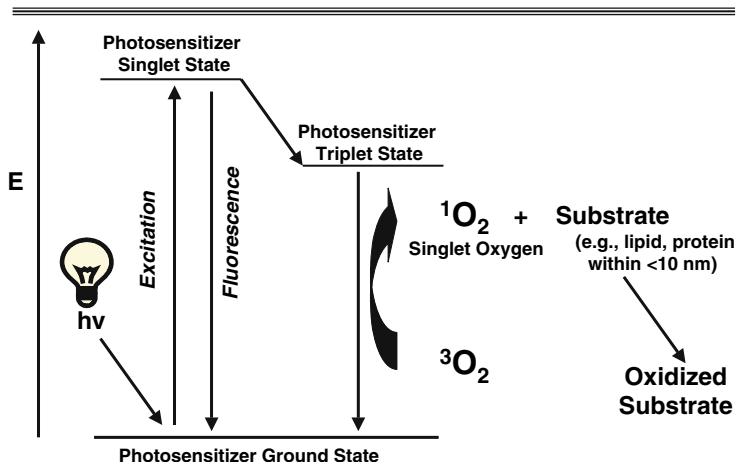


Fig. 1. A simplified energy diagram showing the conversion of light energy to cellular oxidative stress. The ground-state photosensitizer absorbs a photon and is energized to an excited singlet state, from which it can release energy as fluorescence or undergo intersystem crossing to the triplet state. The triplet photosensitizer transfers its energy to ground-state molecular oxygen (a triplet) to generate singlet molecular oxygen and the ground-state photosensitizer. Singlet oxygen reacts with nearby cellular targets. Because in cells, the photosensitizers are membrane localized, singlet oxygen formed in the membranes will oxidize unsaturated membrane lipids and proteins.

approximately 600–800 nm. Because Photofrin® and other simple porphyrins absorb poorly in this region ($\epsilon \sim 5000$, $\lambda_{\text{max}} \sim 630$ nm), many newer PSs have been developed with higher extinction coefficients at wavelengths above 650 nm (3,6,9,10).

In both animals and human patients, PDT can result in a complete tumor response within a few days. Three types of mechanisms are reported to contribute to the rapid PDT response *in vivo* (1–3,6). First, PDT can directly damage and kill the malignant cells of the tumor, by either apoptosis or a non-apoptotic mechanism. Second, PDT may produce profound changes in the tumor vasculature, including blood flow stasis, vascular collapse, and/or vascular leakage, that can result in indirect killing of the malignant cells. And third, PDT can promote release of cytokines and other inflammatory mediators from treated cells that induce an inflammatory response and recruit additional host cells to the tumor. The contribution of each mechanism to the overall tumor response depends upon the PS, the tumor, and the treatment parameters [e.g., the dose of PS and the amount (fluence) of light]. One critical parameter is the fluence rate, that is, the rate at which photons impinge on the tissue. It has long been known that the delivery of oxygen *in vivo* to the treatment site can be a limiting factor in PDT. Molecular oxygen is consumed in the photodynamic process to generate singlet oxygen, which is further fixed in oxidized substrates. High light fluence rates can deplete the targeted tissue of oxygen, limiting the impact of further photoirradiation. Slowing the rate of light delivery can spare the available oxygen, reduce vascular blockage allowing more oxygen to be delivered through the circulation, and produce a greater overall response (more tumor cures) at a lower total light dose. Lower fluence rate irradiation also produces a higher level of apoptosis in the treated tumors (11).

2. CHEMICAL AND BIOCHEMICAL PROPERTIES OF THE PHOTOSENSITIZERS USED IN PDT

Most PSs are porphyrins or related macrocycles, such as benzoporphyrins, chlorins, pheophorbides, purpurins, or phthalocyanines, whose hydrophobic ring systems tend to localize them to one or more intracellular membrane systems (3,4,6,9) (Fig. 2). Many analogs of the parental macrocycles have been produced, with varying hydrophilicity/hydrophobicity, pharmacokinetics, and affinity for cellular-binding and tissue-binding sites, through modifications of the ring systems and/or addition of substituents either on or axial to the ring (3,9). Some PSs are not porphyrins; for example, hypericin, a PS derived from St. John's Wort, which is under investigation in Europe and Singapore (10). Structures for a selection of the more widely studied PSs can be found in Fig. 2. Finally, some PDT protocols employ the heme biosynthetic precursor 5-aminolevulinic acid (ALA) to generate the natural PS protoporphyrin IX (PPIX), the penultimate intermediate of the heme biosynthetic pathway (12,13). Upon administration of ALA to cells or patients (either systemically, orally, or topically), PPIX is generated over the first 2–4 h, reaches a maximum level, and then is lost, either by metabolic conversion to heme through the introduction of iron catalyzed by the enzyme ferrochelatase or by diffusion out of the cell. Although PPIX remains at the site of its formation in the mitochondria, it is an efficient PS and can also be used for fluorescence detection of tumors, which are often more efficient in the generation of

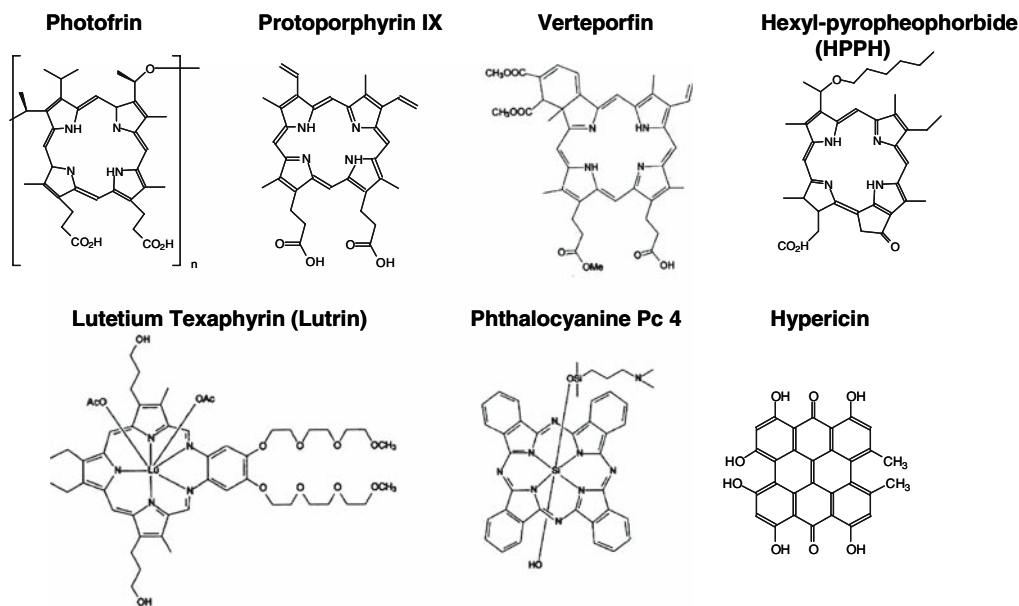


Fig. 2. Examples of photosensitizers used for photodynamic therapy. Photofrin is the first-generation photosensitizer now approved in the USA for treatment of lung and esophageal cancer and Barrett's esophagus and for numerous indications worldwide. As Photofrin is a complex mixture of porphyrin oligomers, a monomeric unit is shown. Protoporphyrin IX is the natural photosensitizer metabolically synthesized from exogenously applied 5-aminolevulinic acid or its esters. Verteporfin is Food and Drug Administration-approved for the treatment of age-related macular degeneration. All of the other photosensitizers shown here are in clinical trials.

PPIX than are normal tissues (12,13). Various esters of ALA have also been employed to enhance cell uptake of ALA (14).

3. PDT-INDUCED APOPTOSIS

The literature on this topic has grown markedly, since the first report of the induction of apoptosis by a PS and light (15). Several reviews have appeared, the most recent of which (3,4,6,16–19) have provided a comprehensive view of the knowledge to date. There are now a plethora of examples of the induction of apoptosis by the photoactivation of many different PSs in numerous *in vitro* and *in vivo* systems. This chapter will summarize our current understanding of the apoptosis pathways triggered by PDT and then focus on recent mechanistic insights. The reader is referred to the previous reviews for lists of cell and tumor types studied and pathways modulated with particular PSs. These reviews also provide a compilation of the many signaling pathways that are activated by PDT, some of which can modulate the extent or timing of apoptosis. The purpose of this chapter is to emphasize what is unique about PDT, most particularly the initial damages, and to distinguish mechanisms of PDT from those that are common to other cell stresses.

To make these comparisons, we will reserve the term “molecular target” to indicate molecules that are directly altered by the reactive oxygen species (ROS) that PDT generates. In most cases, the ROS is singlet oxygen, but as a result of oxidation–reduction reactions, other ROS can be formed, especially superoxide anion ($O_2^- \bullet$). Thus, a pathway that is triggered as a result of photooxidation of a target is a response but not itself a target. The preferred targets of singlet oxygen are unsaturated fatty acids and cholesterol of membranes, certain amino acids within proteins (cysteine, histidine, methionine, tyrosine, and tryptophan), and guanine bases within nucleic acids (20). However, because of its high reactivity, 1O_2 reacts within very short range (< 10 nm) of its site of formation, which must be at the site of binding of the PS (21). As mentioned above, owing to the hydrophobicity of their ring systems, the PSs tend to localize in membranes, where the initial photodynamic targets are located. DNA damage, when it occurs at all, is not relevant to the killing of cells by PDT (6).

PDT is an efficient inducer of apoptosis (3,15) both in cultured cells and *in vivo*. As with other toxic agents, for example, hyperthermia, PDT causes necrosis at higher doses than those inducing apoptosis, indicating that one factor in determining the pathway for the final demise of the cell is the overall amount of damage. A transition from apoptosis to necrosis as the overall dose is raised has been found to occur with PDT sensitized by numerous PSs, for example, hypericin, dimethyl tetrahydroxyhelianthone, phthalocyanines, and methylene blue derivative (22–27). Another factor that can affect the final cell death mechanism is the site of localization of the PS. For some, variable incubation times can result in different localization patterns and culminate in different modes of cell death (28). Moreover, the initial binding site of some PSs may be different from its preferred damage site in PDT (29). In addition, different reactive species produced at the same sites can have markedly different cellular effects (30), and singlet oxygen produced by PDT at the plasma membrane can still induce apoptosis (31). Differential sensitivity to PDT occurs among different cell types, (32,33) and PDT-induced apoptosis can occur in any stage of the cell cycle (34,35) and even despite cell-cycle arrest (36).

PDT activates many of the same signaling pathways turned on by other types of stress. These include the p38 and SAPK/JNK family of MAP kinases; NF- κ B, fos, jun, and other components of transcription factors; and phospholipases, cyclooxygenases, and other lipid-metabolizing enzymes that generate lipid-derived signaling molecules (such as ceramide, phosphoinositides, prostaglandins, thromboxanes, and leukotrienes). The specific conditions of PSs, cells, and overall doses that elicit these responses have been reviewed in detail (3,16,17). Many of these can promote cell death or act in a protective mode, as is true for their activation by other stresses.

4. CELLULAR TARGETS OF PDT: MITOCHONDRIA

Investigations of a variety of PDT systems found that upon photoirradiation, PSs that are localized to mitochondria kill cells more efficiently than do those targeted to the plasma membrane or lysosomes (37). Hence, the mitochondrion has been considered the most sensitive target for directly triggering PDT-induced apoptosis (3,38). Photodynamic damage to this organelle results in generation of ROS within it, loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$), and release of pro-apoptotic factors (e.g., second mitochondrial activator of caspases (SMAC), apoptosis-inducing factor (AIF) and cytochrome c) from the mitochondrial intermembrane space into the cytosol. Subsequent activation of the caspase cascade results in expression of the characteristic features of morphological apoptosis (3,18,39).

As the initial damage produced by PDT with mitochondrion-targeted PSs is in that organelle, it is necessary to ask whether the full apoptosis pathway is essential for the cells to die or whether the mitochondrial damage is lethal irrespective of the pathway for elimination of the cell. We examined this issue first in a pair of MCF-7 human breast cancer cells (40). The parental cells lack procaspase-3, and the two derivative cell lines were created by C. Froehlich of Northwestern University by transfection of either human procaspase-3 or the empty vector. When treated with Pc 4-PDT, only the cells with the stably overexpressed transgene were capable of efficiently activating caspase-3 and caspase-9, cleaving poly(ADP-ribose) polymerase (PARP), fragmenting DNA, and condensing their chromatin to generate morphological apoptosis. However, cytochrome c release was equally sensitive in the two cell lines, and they were equally sensitive to Pc 4-PDT when assayed for survival by clonogenicity, indicating that the damage that initiates apoptosis at the sites of Pc 4 binding is lethal, and the later caspase-dependent steps are necessary for apoptosis but not for cell death (40).

Further analysis of critical steps was carried out with cells that are deficient in Bax, the pro-apoptotic Bcl-2 family member that migrates from the cytosol to the mitochondria during apoptosis to mediate cytochrome c release. In DU145 cells, which lack Bax because of a mutation, the cells are incapable of undergoing all the steps in apoptosis, beginning with the release of cytochrome c. [Although the cells express Bak, it appears not to substitute for Bax in this situation, in contrast to the case of murine embryonic fibroblasts (41).] Cells could be induced to undergo apoptosis by transient transfection of a plasmid containing Bax cDNA, and the level of apoptosis in the transfected cells was further increased by PDT. Bax-deficient DU145 cells were as sensitive as other (Bax-replete) cell lines to killing by PDT, as judged by clonogenic assay (42). These studies have led us to a model wherein Pc 4-PDT damages intracellular membranes, primarily mitochondria, producing lethal damage.

Then, apoptosis is a convenient mechanism for disposing of a dead cell, not the process that determines life or death of the cell. Although it is likely that these conclusions apply to other PSs with localization properties similar to Pc 4, the relevant experiments remain to be done. Recent reports have demonstrated that in addition to apoptosis, PDT also activates the cell death process of autophagy, as demonstrated by the generation of autophagosomes within the cytoplasm of treated cells and by the autophagy-associated conversion of the protein LC3-I to LC3-II (43–45). Notably, autophagy was observed in Bax-deficient cells and may provide a mechanism for the elimination of lethally irradiated cells that are unable to undergo apoptosis. It will be important in future work to determine the relationship between these two death pathways in response to PDT.

Inhibition of respiration and oxidative phosphorylation is also a common early response with mitochondrion-localized photosensitization (23,46,47). Photofrin-PDT inhibited electron transport components, including succinate dehydrogenase and cytochrome c oxidase, and disrupted the mitochondrial electrochemical gradient (48–51). Strikingly, the inhibition of respiration caused by Pc 4-PDT in L5178Y-R murine lymphoma cells could largely be restored by addition of exogenous cytochrome c to the permeabilized cells (47), supporting the notion that the inhibition of respiration in that situation primarily resulted from cytochrome c release rather than from damage to the major enzyme complexes of the electron transport chain.

Although it is clear that PDT damage to the mitochondrion can efficiently induce the intrinsic pathway of apoptosis in cells that express all of the requisite intermediates, the exact order of the known steps can vary. For example, after PDT with different PSs, cytochrome c release has been observed to occur simultaneously with the loss of $\Delta\Psi_m$ (52), delayed after loss of $\Delta\Psi_m$ (39), simultaneously or delayed depending on the dose (53), or in the absence of any changes in transmembrane potential (54). For example, in mouse lymphoma L5178Y-R cells treated with Pc 4-PDT, high fluences caused a rapid dissipation of membrane potential concurrent with cytochrome c release, whereas lower fluences produced cytochrome c release without any measurable loss of transmembrane potential (53). As a result of these disparate observations, the mechanism by which cytochrome c is released from mitochondria following PDT remains unclear, some data suggesting that it is due to opening of the permeability transition (PT) pore, swelling of the inner membrane, and lysis of the outer membrane, whereas other data are consistent with opening of Bax-dependent channels on the outer membrane (55).

4.1. The Permeability Transition Pore Complex

One model for the movement of solutes into and out of the mitochondrion envisions a large conductance channel known as the permeability transition pore complex (PTPC), formed by association of proteins of the outer [voltage-dependent anion channel (VDAC) and peripheral benzodiazepine receptor (PBR)] and inner [adenine nucleotide translocator (ANT) and cyclophilin D] mitochondrial membranes (56,57). The components of the PTPC have received much attention as potential targets of PDT. One such component is the 18-kDa PBR, which was suggested to be the critical molecular target for PPIX and related porphyrins (58–60) based on a strong correlation between a porphyrin's binding affinity to the PBR and cellular photocytotoxicity. Support for the PBR as a PDT target was obtained in studies of ALA/PPIX (61) and pyropheophorbide

ethers (62). In contrast, the phthalocyanine Pc 4 bound to the PBR only at concentrations much higher than those needed for efficient photosensitization (63). As well, two positional isomers of PPIX (PPIII and PPXIII) with similar uptake and mitochondrial binding and the same ability to photosensitize L1210 leukemia cells as PPIX (as measured by a clonogenic assay) had significantly lower binding affinity to the PBR than PPIX (64). Thus, the relationship between PBR binding and photosensitizing efficacy may be limited to certain PSs but not apply to others.

In isolated rat liver mitochondria, hematoporphyrin photosensitization strongly inhibited the 30-kDa ANT and PTPC opening (65). Verteporfin-PDT permeabilized ANT-containing proteoliposomes, a response that could be inhibited by the ANT ligands, ADP and ATP (66). Although intriguing, these observations do not prove that ANT is the cellular target of PDT with either PS. Among the proteins associated with the PTPC is hexokinase, which was observed to be released into the cytosol and its activity inhibited by hypericin-PDT (67). Unfortunately, inhibitors of the PTPC have provided contradictory results. The loss of $\Delta\Psi_m$ after hypericin-PDT was not prevented by cyclosporin A or bongkreic acid (68). Cyclosporin A produced a partial inhibition of PTPC opening in human epidermoid carcinoma A431 cells exposed to Pc 4-PDT, but cyclosporin A combined with trifluoperazine strongly inhibited PTPC opening and apoptosis, supporting a transient opening of the PTPC (39). The dissipation of $\Delta\Psi_m$ in isolated mitochondria exposed to PDT with Victoria Blue dyes was insensitive to cyclosporin A and was proposed to result from direct effects of the dyes on inner membrane permeability to protons rather than on the mitochondrial PT (69). Thus, the diverse responses suggest that damage to components of the PTPC may contribute to the permeabilization of the mitochondria, allowing release of pro-apoptotic factors, such as cytochrome c, SMAC, and AIF.

4.2. Photodamage to Bcl-2 and Bcl-xL

A unique feature of PDT with PSs that target mitochondria and endoplasmic reticulum (ER) is “photodamage” to Bcl-2 and Bcl-xL (25,70–72), anti-apoptotic proteins which reside in the outer mitochondrial membrane, ER, and nuclear membrane, where they contribute to the resistance of tumors to many types of cancer treatment. PDT-generated “photodamage” has been observed on western blots as loss of the native protein in a dose-dependent but not time-dependent manner. This photodamage occurs immediately upon photoirradiation, for cells treated in the cold, for cells missing caspase-3 or Bax, and for cells given a variety of protease inhibitors; therefore, the response is a result of photochemical damage, not proteolytic or other enzymatic reactions. Bcl-2 now seems to be a target of PDT with all PSs formerly considered to target mitochondria but not those that specifically target lysosomes. Bcl-2 photodamage has been observed in every human cancer cell line in which we have looked and in both endogenous and overexpressed Bcl-2. Photodamage was found irrespective of estrogen or androgen receptor status of the cells, p53 status, or other factors that can contribute to the resistance to various cancer treatments (3). Bcl-2 photodamage correlated well with the ability of Pc 4-PDT to kill cells (73), and the membrane-anchoring regions of Bcl-2 were found to be critical for this sensitivity (74). Studies of deletion and point mutants of Bcl-2 suggest that a secondary membrane-anchorage site of Bcl-2 formed by the α -5 and α -6 helices that occur between the BH1 and BH2 domains is essential to form the photosensitive target for PDT-induced photodamage; the results support the

notion that PDT initiates Bcl-2 complex formation with nearby proteins and/or lipids to form large aggregates (75). These two alpha helices have been proposed to form a secondary membrane-binding site that is important for pore formation (76). However, in one study, the $\alpha 5/\alpha 6$ region was found within the membrane only after apoptosis was initiated (77). Bcl-2 and Bcl-xL interact with VDAC, the major outer mitochondrial membrane component of the PTPC (78), yet VDAC is not photodamaged in the same manner as Bcl-2 and Bcl-xL (72).

One hypothesis for the importance of the $\alpha 5/\alpha 6$ region of Bcl-2 is that when the region is inserted in the membrane, amino acids that are preferred targets of $^1\text{O}_2$ and oxygen radicals are oxidized by PDT. However, when all photooxidizable amino acids were substituted with amino acids having similar hydrophobicity but reduced ability to interact with $^1\text{O}_2$, the mutants responded to Pc 4-PDT like wild-type Bcl-2, indicating that the most $^1\text{O}_2$ -sensitive amino acids in the $\alpha 5/\alpha 6$ region are not the primary targets (75). These data suggest that the $\alpha 5/\alpha 6$ region is important for targeting of Bcl-2 by Pc 4-PDT, but it is not directly photooxidized, implying that its role is to interact with other membrane components to form the photosensitive target.

Additional insights into the role of photodamage to Bcl-2 in the induction of PDT-induced apoptosis were provided by Kessel et al. (79). They found that the bile acid ursodeoxycholic acid (UDCA) greatly potentiated apoptosis after PDT sensitized by 9-capronyloxytetrakis (methoxyethyl) porphycene (CPO) or tin etiopurpurin (SnET2), PSs that tend to bind to multiple cytoplasmic membranes. UDCA can bind to mitochondrial membranes and protect cells from apoptosis induced by a number of cytotoxic agents, including more hydrophobic bile acids (80). In contrast, UDCA *enhanced* PDT-induced apoptosis through potentiation of mitochondrial membrane potential loss, cytochrome c release, and activation of caspase-3. UDCA did not enhance the intracellular accumulation of these PSs; hence, the authors suggest that UDCA acted to sensitize mitochondrial membranes to photodamage.

Castelli et al. (81) recently studied the mechanism by which UDCA augments PDT-induced apoptosis. UDCA potentiates apoptosis triggered by PDT, but only with PSs that cause photodamage to Bcl-2. UDCA also potentiates apoptosis with the non-peptide Bcl-2/Bcl-xL antagonist HA14-1, which binds in the hydrophobic pocket created by folding of the BH1, BH2, and BH3 domains of Bcl-2, the pocket which normally binds the BH3 domain of the pro-apoptotic proteins Bax or Bak. These observations suggest that the bile acid causes a conformational change in the membrane housing Bcl-2, which enhances the effects of both HA-14-1 and PDT. Using fluorescence polarization of fluorescein-tagged Bak-BH3 peptide (flu-Bak), they showed that HA14-1 competed with flu-Bak for its binding site on Bcl-2, and UDCA enhanced the binding affinity of HA14-1. Castelli et al. (81) also measured fluorescence resonance energy transfer (FRET) between Bcl-2 and the PS CPO in a liposomal system; UDCA enhanced the FRET signal, which was interpreted as showing that UDCA causes a conformational change in Bcl-2 that both increases its affinity for HA14-1 and interferes with its anti-apoptotic function. Thus, for the class of PSs that cause photodamage to Bcl-2 and Bcl-xL, UDCA may offer a means to promote the efficacy of PDT with minimal adverse side effects.

4.3. Photodamage to Cardiolipin

As mentioned in Section 1, the localization of hydrophobic PDT PSs in membranes ensures that singlet oxygen is generated within the membranes, sufficiently close to its preferred targets, cholesterol and the unsaturated fatty acids of phospholipids. The significance of lipid oxidation in PDT-induced cell death has been given much attention by Girotti and co-workers, with analysis of lipid hydroperoxides (LOOHs) by high-performance liquid chromatography. For example, as a consequence of PDT photosensitized by merocyanine 540, LOOHs generated in L1210 leukemia cells were predominantly those of cholesterol (ChOOH) and phosphatidylcholine (PCOOH) (82). These hydroperoxides were detected at a PDT dose that resulted in approximately 90% cell death, thus lending support to the notion that photoperoxidative damage to membrane lipids is important in overall cell killing. Kriska et al. (83) showed that ALA-PDT of COH-BR1 breast cancer cells induced apoptosis when PPIX was located in the mitochondria, but induced necrosis when PPIX had diffused to peripheral sites. Under apoptotic but not necrotic conditions, cytochrome c was released from the mitochondria into the cytosol, and caspase-3 was activated. Furthermore, a related cell line that stably overexpressed the selenium-dependent phospholipid hydroperoxide glutathione peroxidase (PHGPx) in mitochondria was resistant to the accumulation of LOOHs in the mitochondria and to apoptosis, as the enzyme reduced LOOH to LOH.

The most recent evidence suggests that a likely target for the initial lethal damage of PDT with some mitochondrion-bound PSs is cardiolipin (CL), the phospholipid found uniquely in the mitochondrial inner membrane and at the mitochondrial contact sites (84). CL is a dimeric phospholipid; each CL molecule contains two negatively charged phosphates and four fatty acids composed of 95% linoleic acid (C18:2) (85), making it highly sensitive to oxidation and an attractive candidate for initial singlet oxygen attack. CL is important both structurally and functionally to mitochondrial integrity. It forms the attachment site and is an integral component of many proteins that are crucial to the mitochondria, such as the respiratory complexes, cytochrome c, and components of the PTPC (86,87). Two populations of cytochrome c have been demonstrated in rat liver mitochondria, tightly and loosely bound pools, anchoring to the inner membrane by electrostatic and hydrophobic interactions (88). According to current models, cytochrome c release occurs when the oxidation of CL generates a soluble pool of the protein, which can then pass into the cytosol in a Bax-dependent process to initiate apoptosis. In cell-free assays, Nomura et al. (89) demonstrated that the binding affinity of cytochrome c for CL is markedly reduced upon peroxidation of CL (to CLOOH), but the binding could be restored if CLOOH were reduced to CLOH by the detoxifying enzymatic action of PHGPx. Similarly, photoperoxidation of CL in PPIX-sensitized liposomes could disrupt the binding of cytochrome c, which was reversible by treatment with GSH and PHGPx (90).

Continuing work with the PHGPx-overexpressing and parental lines of COH-BR1 cells, Kriska et al. (90) found that the presence of the enzyme in mitochondria made the cells resistant to ALA-PDT-induced apoptotic cell death but not to necrotic cell death. Of all the measured LOOHs, CLOOH accumulation was preferentially inhibited by PHGPx overexpression and only when the PS PPIX was localized in mitochondria. These data demonstrate that enzymatic reduction and detoxification of mitochondrial LOOHs confers resistance to PDT-induced apoptosis. Moreover, the data suggest a specific mechanism whereby PDT with mitochondrion-targeting PSs can initiate

apoptosis: photooxidation of CL, resulting in the loosening of cytochrome c anchorage to CL, followed by release of cytochrome c from mitochondria in a Bax-dependent process.

Additional evidence for CL as a target of PDT is based on the apparent high affinity for binding of certain PSs at or near CL. Release of a zinc phthalocyanine (ZnPc) from liposomes to rat liver mitochondria was reduced when the liposomes contained CL, implying a higher affinity of ZnPc for CL than for other phospholipids (91). Other studies used the fluorescent dye nonyl-acridine orange (NAO), a specific probe of CL. As assayed in phospholipid monolayers, the affinity of NAO for CL was 50 times greater than for other negatively charged phospholipids (phosphatidyl serine and phosphatidyl inositol), and there was virtually no interaction of NAO with zwitterionic phospholipids (phosphatidyl choline and phosphatidyl ethanolamine) (92). The high-affinity binding may result from insertion of the nonyl chain into the bilayer at the hydrophobic surface created by the four fatty acid chains (93). NAO has been used to characterize a number of mitochondrial properties with respect to CL, including imaging by confocal microscopy (94), measurements of mitochondrial mass (95), and quantification of CL in the leaflets of the mitochondrial inner membrane (96). NAO competitively inhibited the uptake of Photofrin into mitochondria, as judged by confocal microscopy, suggesting that these two species were competing for the same site at CL (97). However, in cells, the specificity of NAO for CL is limited to low (nanomolar) concentrations and requires that the mitochondrial membrane potential be intact (94). Morris et al. (98) provided direct evidence for the binding of a PS, Pc 4, to sites on or near CL based on FRET between NAO and Pc 4. Co-localization by confocal microscopy and the observed FRET confirm that both species reside in the same mitochondrial regions and suggest that CL is a likely initial target for the initiation of apoptosis by Pc 4-PDT. FRET has also been observed between NAO and PPIX, strengthening the evidence for CL as a phototarget of PDT with that PS (Morris et al., unpublished).

5. CELLULAR TARGETS OF PDT: LYSOSOMES

Whereas the emphasis of PDT research has been on the triggering of apoptosis by mitochondrion-localized photodamage, there is now mounting evidence that damage to other organelles can elicit apoptotic cell death. Kessel, Reiners, and their colleagues have identified a number of PSs that bind preferentially to lysosomes and upon photoactivation can induce apoptosis (99–101). Photoactivation of NPe6-loaded murine hepatoma 1c1c7 cells caused a delayed apoptotic response, marked by rapid destruction of the lysosomes, Bid cleavage to generate truncated Bid (tBid), cytochrome c release from mitochondria, and activation of caspase-3 and caspase-9 (100). They further determined that lysosomal extracts contained proteolytic activity that was capable of cleaving Bid (100), in agreement with a similar finding of Stoka et al. (102). Although cathepsins are known to reside in lysosomes and to be released upon lysosomal damage, inhibitors of cathepsins B, D, and L were unable to block apoptosis triggered by NPe6-sensitized PDT (100). To further define the role of lysosomal proteases in PDT-induced apoptosis, the aryl hydrocarbon receptor (AhR)-deficient cell line, Tao, derived from 1c1c7 cells, was shown to be relatively resistant to the induction of apoptosis by NPe6-PDT, as judged by a colony-forming assay, despite a slightly better uptake of the

PS into its lysosomes (101). When compared at equal levels of cell killing, apoptosis induction in Tao cells was considerably delayed relative to the induction in 1c1c7 cells, an observation that could be attributed to lower levels of cathepsins and other protease activities in the endosomal/lysosomal compartment of the cells. The authors proposed that AhR has a role in the processing and uptake of cathepsins and other proteases into lysosomes. To test this idea, and because 1c1c7 cells had been mutagenized to produce Tao cells, they compared cell lines in which the AhR level had been altered by molecular techniques. In every case tested, sensitivity to NPe6-PDT tracked with AhR level. Thus, cells with higher levels of AhR, such as 1c1c7 cells, had higher levels of lysosomal proteases available for release, more rapid and extensive cleavage of Bid, activation of caspase-3 and caspase-9, and more rapid and extensive development of morphological apoptosis.

One unique response has been reported for ceramide signaling after PDT. Separovic and co-workers found that ceramide levels increase in a variety of different cells with a PDT dose–response similar to that for induction of apoptosis (34,103). Evidence favors the hypothesis that elevated ceramide promotes apoptosis, as has been proposed for other cell stresses (104). When cells are exposed to ionizing radiation and certain chemotherapeutic drugs, the increase in cellular ceramide has been shown to be due to activation of lysosomal and other sphingomyelinases, enzymes that cleave sphingomyelin to generate ceramide and phosphorylcholine (104). In contrast, when PDT sensitized by Pc 4 is the triggering agent, either no change or a decrease in sphingomyelinase activity was observed. Instead, the enhanced level of ceramide following PDT results from inhibition of two enzymes that utilize ceramide for the synthesis of complex sphingolipids, sphingomyelin synthase (SPS), and glucosylceramide synthase (GCS) (105). Still, data on pyropheophorbide-a methyl ester (PPME)-sensitized PDT in human colon cancer HCT-116 cells suggest the involvement of lysosomal acid sphingomyelinase in the generation of ceramide in response to PDT, although in that case, a contribution of SPS and GCS was not investigated (106). Furthermore, lymphoblasts from Niemann–Pick patients, which are genetically devoid of acid sphingomyelinase, were unable to either synthesize ceramide or undergo apoptosis in response to PDT, suggesting an important role for acid sphingomyelinase in this system (107).

6. CELLULAR TARGETS OF PDT: ER

There have been a number of observations of apparent photodamage to certain proteins of the ER membranes, a phenomenon similar to that found with Bcl-2 and Bcl-xL, that is, loss of the native protein observed on western blots (Section 4.2.). The sarcoplasmic/ER calcium (SERCA) pump may be subject to photodamage from Verteporfin-sensitized PDT in HeLa cells (66), and several peptides, including calreticulin precursor, p58 microsomal protein, and protein disulfide isomerase, are lost from HL60 cells immediately upon a high dose of PDT sensitized by ALA-PPIX (108). With Pc 4, PDT causes photodamage to the SERCA pump, but not the related plasma membrane calcium pump, and to ERp57, but not ERp72 (unpublished observations). The photosensitivity of these ER proteins is in keeping with the binding of the PSs to ER membranes. Damage to these membrane constituents and likely others not yet identified may explain the strong ability of PDT with many PSs to stimulate the rapid efflux of ER Ca^{2+} into the cytosol (109).

Intracellular Ca^{2+} is maintained by Ca^{2+} transporters on the plasma membrane and the membranes of the organelles. Several of these Ca^{2+} transporters have thiol groups

that are required to be in the reduced state to function properly. Considering that PDT induces profound oxidative damage to the membrane proteins, it is not surprising that dysregulation of intracellular Ca^{2+} occurs during PDT. Indeed, several studies report an increase in cytosolic free Ca^{2+} in various cell types induced by PDT with a wide range of PSs (110–122). The increased cytosolic Ca^{2+} may be due to influx of Ca^{2+} from the extracellular space or release from the internal stores, such as ER, or from mitochondria. Also, the sources contributing to increased cytosolic Ca^{2+} may vary depending on the PS used, which reflects its subcellular targets. Whether the increase in cytosolic Ca^{2+} contributes to PDT-induced cell death or whether it is merely a consequence of irreversible photodamage to the cells is still unclear.

Under physiological conditions, cytosolic Ca^{2+} is approximately 100 nM compared with extracellular Ca^{2+} of 1 mM, ER Ca^{2+} of approximately 500 μM , and Golgi apparatus of approximately 300 μM . A wide range of PSs perturb intracellular Ca^{2+} regulation and increase intracellular Ca^{2+} in different cell types (111, 114, 115, 118, 121). The intracellular calcium chelator BAPTA-AM inhibits the release of cytochrome c, caspase activation, and apoptotic death of cancer cells photosensitized with pheophorbide or 2,4,5,7-tetrabromorhodamine 123 bromide, suggesting that Ca^{2+} plays a role in PDT-induced apoptosis (123, 124). Increased cytosolic Ca^{2+} after PDT may originate from the influx of Ca^{2+} through ion channels in the plasma membrane (115, 121), release of Ca^{2+} from sequestered internal stores (111, 114), including ER and Golgi (109, 111, 113, 114, 118, 125), or activation of ion transport/exchange mechanisms (117).

Low cytoplasmic Ca^{2+} concentrations are maintained by high-affinity Ca^{2+} -ATPases and lower affinity $\text{Na}^+/\text{Ca}^{2+}$ exchangers on the plasma membrane (126). Direct photodamage of the Ca^{2+} transporters would potentially result in Ca^{2+} overload, provided that the PS of interest binds to the plasma membrane. Low doses of tetrasulfonated aluminum phthalocyanine (AlPcS_4), when photoactivated, induce intracellular Ca^{2+} increase mainly because of Ca^{2+} influx, whereas higher doses cause Ca^{2+} release from internal stores (111). The PS Pc 4 does not bind to the plasma membrane, as assessed by confocal microscopy, suggesting that direct damage to the plasma membrane Ca^{2+} transporters is probably not contributing to Pc 4-PDT-induced killing (39). Plasma membrane Ca^{2+} ATPases contain several possible caspase-3 cleavage sites (127). A recent study by Bano et al. (128) reports that the plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger is cleaved by Ca^{2+} -activated proteases, calpains, in neurons during excitotoxicity resulting in Ca^{2+} overload. As the effector caspases are frequently activated during PDT, the inactivation of the plasma membrane Ca^{2+} -ATPases by caspases may contribute to the subsequent delayed increase in cytosolic Ca^{2+} , resulting in Ca^{2+} overload and Ca^{2+} -dependent necrotic cell death, which often occurs during PDT.

The ER is a major intracellular source of Ca^{2+} . The intracellular release of inositol trisphosphate (IP3) from the plasma membrane causes IP3-induced Ca^{2+} release from the ER to the cytosol through the IP3 receptors. Ca^{2+} is returned to the ER lumen through SERCA pumps. Coordinated regulation of Ca^{2+} release and uptake mechanisms maintains the ER free- Ca^{2+} pool at approximately 500 μM in physiological conditions. Any perturbations of the release and uptake mechanisms eventually affect cytosolic Ca^{2+} concentrations. In HeLa cells, PDT photosensitized by Verteporfin released Ca^{2+} from the ER, which was associated with a rapid caspase-independent inhibition of SERCA-2 (118). In rat liver microsomes, Ca^{2+} uptake was impaired after photosensitization with hematoporphyrin or protoporphyrin (129). The SERCA pump may be important in PDT-induced cell death with those PSs that

localize to the ER membranes, such as Pc 4 (39). In rat pancreatic acini, PDT with gadolinium porphyrin-like macrocycle B or AIPcS₄ induced cytosolic Ca²⁺ oscillations by triggering ER Ca²⁺ release through IP₃ receptors (113,114). Similarly, AIPc-PDT induced a rapid transient release of Ca²⁺ from the ER in mouse L5178Y cells (109). A phospholipase C inhibitor, U73122, blocked the transient increases in both IP₃ and cytosolic Ca²⁺ and subsequent fragmentation of nuclear DNA (109). In other experimental systems apart from PDT, the thiol-specific agent, thimerosal, causes increased intracellular Ca²⁺ in HeLa cells by sensitizing the IP₃ receptor to the basal level of IP₃ in the cell (130). Oxidized glutathione alters the binding properties of IP₃ to its receptor by revealing IP₃-binding sites (131). Cytochrome c released from mitochondria during apoptosis has been shown to bind to IP₃ receptors amplifying calcium-dependent apoptosis (132). Thus, with PSs such as Pc 4 that localize in mitochondria and ER (39), the IP₃ receptor may be a target for PDT-induced oxidative damage, causing dysregulation of intracellular Ca²⁺ and resulting in cell death. Another recent study shows that CPO localizes predominantly in the ER membranes in murine leukemia L1210 cells (133). Although CPO induced increased cytosolic Ca²⁺, the chelation of intracellular Ca²⁺ with BAPTA did not protect cells from apoptosis, suggesting that elevated cytosolic Ca²⁺ levels were not required for cell death in that case.

The mitochondrial matrix free Ca²⁺ concentration is maintained by a balance between uptake and efflux mechanisms. A negative membrane potential across the inner mitochondrial membrane provides the driving force for Ca²⁺ influx through a Ca²⁺ uniporter (134). Ca²⁺ efflux involves 3Na⁺/2Ca²⁺ and Na⁺/H⁺ exchange driven by the mitochondrial membrane potential and pH gradient (135–140). Initially, it was proposed that mitochondria with their large capacity for Ca²⁺ uptake might regulate cytosolic Ca²⁺ concentration. Our current understanding is rather that cytosolic Ca²⁺ regulates mitochondrial Ca²⁺. In pathological situations, when mitochondrial Ca²⁺ increases, Ca²⁺ efflux through 3Na⁺/2Ca²⁺ exchange becomes saturated. Such Ca²⁺ overloading may lead to the mitochondrial PT, causing Ca²⁺ to be released from mitochondria through the mitochondrial PT pores. Presumably, two Ca²⁺-binding sites exist on the matrix side of the PT pore, and Ca²⁺ binding to these sites induces PT pore opening (141). Indeed, in isolated mitochondria, increased matrix rather than extramitochondrial Ca²⁺ induces the PT, resulting in mitochondrial depolarization (142). Mitochondrial ROS formation occurred within minutes after Pc 4-PDT. This was followed by inner membrane permeabilization, mitochondrial depolarization and swelling, cytochrome c release, and apoptotic death (39). As increased mitochondrial matrix Ca²⁺ is required for the mitochondrial PT to occur, these results suggest that mitochondria are exposed to microdomains of high cytosolic Ca²⁺ that may exceed the concentrations of bulk cytosolic Ca²⁺. Microdomains may result from a close interaction between the mitochondria and the ER Ca²⁺ channels (143,144). Thus, the dynamic interplay between the ER and mitochondria can contribute to Ca²⁺-mediated mitochondrial dysfunction, which leads to release of proapoptotic proteins from mitochondria and activation of the apoptotic cascade. The weight of the evidence suggests that PDT with many different PSs can efficiently trigger apoptosis through dysregulation of calcium homeostasis.

7. CELLULAR TARGETS OF PDT: PLASMA MEMBRANE

The most common pathway for apoptosis in response to PDT usually involves the release of cytochrome c followed by activation of caspase-3 and caspase-9; however,

other pathways can contribute, especially those through caspase-8, in situations where the dominant pathway is suppressed or when the cells respond by secreting ligands of plasma membrane death receptors (3). The death receptor complexes can play a role in PDT-induced apoptosis, for example, expression and secretion of tumor necrosis factor- α (TNF- α) and FasL (145–149). Nevertheless, combined effects of death receptor-targeted therapies and PDT remain inconclusive; for example, whereas treatment with anti-Fas antibody enhanced apoptosis induced by Verteporfin-PDT (150), recombinant human TNF- α had no effect on the ability of Pc 4-PDT to induce apoptosis (151). In a study of murine embryonic fibroblasts from wild-type FADD or FADD knock-out mice, the role of these plasma membrane death receptors was further delineated (152). Both cell lines were equally sensitive to apoptosis (TUNEL-positive cells and caspase-3 activity) after Pc 4-PDT, but the FADD knock-out cells were resistant to the induction of apoptosis by TNF- α ; hence, Pc 4-PDT-induced apoptosis does not require functional FADD. Taken together, the role of plasma membrane death receptors in PDT-induced apoptosis appears limited.

8. A MODEL FOR ORGANELLE PARTICIPATION IN APOPTOSIS FOLLOWING PDT

A model for the induction of apoptosis by PDT is presented in Fig. 3. The contact sites where the inner and outer mitochondrial membranes interact may offer a convergence site for the critical proteins and lipids involved in PDT-induced cell death,

Direct and Indirect Activation by PDT of Mitochondrial Pathway of Apoptosis

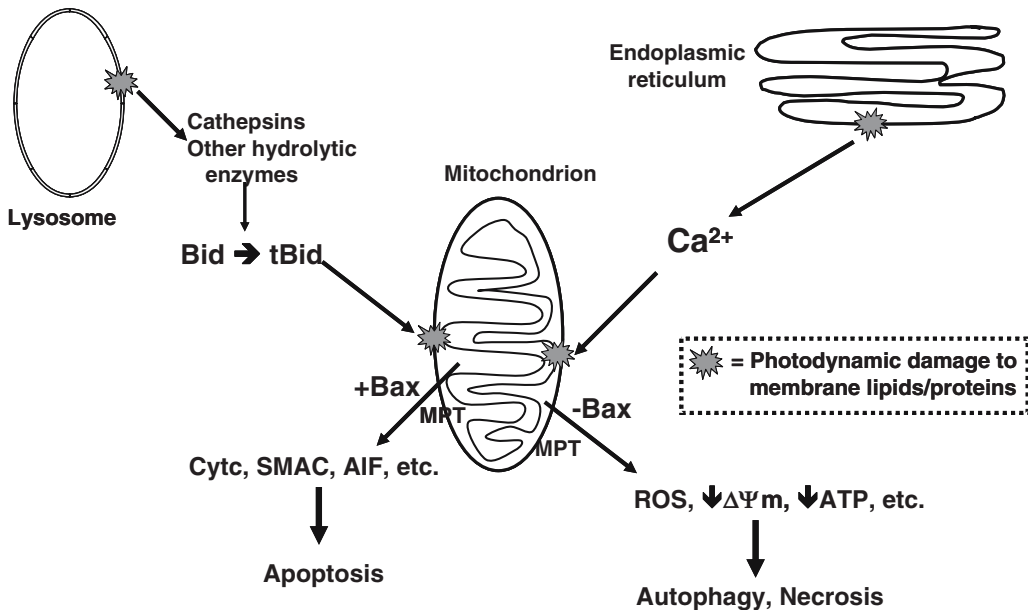


Fig. 3. A model for the induction of apoptosis by photodynamic therapy with photosensitizers localized in various subcellular compartments. Depending upon the photosensitizer, the relative level of damage to these organelles will vary.

especially with mitochondrion-targeting PSs. Enriched in both CL and Bcl-2/Bcl-xL, the contact sites make an attractive candidate for cytotoxic attack by singlet oxygen. As PSs typically bind to multiple organelles, we propose a model in which concomitant photodynamic damage to lysosomes or ER or both can promote and enhance mitochondrial pathways for cell death.

The pro-apoptotic Bcl-2 family member t-Bid is made available by photodynamic damage to lysosomes, and Ca^{2+} released from photodynamically damaged ER promotes mitochondrial swelling and release of cytochrome c. Depending on the level of damage, either apoptosis or necrosis can be initiated. In the absence of a complete pathway for apoptosis, autophagy may also contribute to the removal of lethally damaged cells. Further work will be required to evaluate this model and to provide the mechanistic details.

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REFERENCES

1. Dougherty TJ, Gomer CJ, Henderson BW, et al. Photodynamic therapy: review. *J Natl Cancer Inst* 1998; 90:889–902.
2. Oleinick NL, Evans HH. The photobiology of photodynamic therapy: cellular targets and mechanisms. *Radiat Res* 1998; 150:S146-S156.
3. Oleinick NL, Morris RL, Belichenko I. The role of apoptosis in response to photodynamic therapy: what, where, why, and how. *Photochem Photobiol Sci* 2002; 1:1–21.
4. Moan J, Peng Q. An outline of the hundred-year history of PDT. *Anticancer Res* 2003; 23:3591–600.
5. Dougherty TJ. An update on photodynamic therapy applications. *J Clin Laser Med Surg* 2002; 20:3–7.
6. Macdonald IJ, Dougherty TJ. Basic principles of photodynamic therapy. *J Porphyrins Phthalocyanines* 2001; 5:105–29.
7. Henderson BW, Dougherty TJ. How does photodynamic therapy work? *Photochem Photobiol* 1992; 55:145–57.
8. Gollnick SO, Vaughan L, Henderson BW. Generation of effective antitumor vaccines using photodynamic therapy. *Cancer Res* 2002; 62:1604–8.
9. Allen CM, Sharman WM, Van Lier JE. Current status of phthalocyanines in the photodynamic therapy of cancer. *J Porphyrins Phthalocyanines* 2001; 5:161–9.
10. Agostinis P, Vantighem A, Merlevede W, de Witte PA. Hypericin in cancer treatment: more light on the way. *Int J Biochem Cell Biol* 2002; 34:221–41.
11. Henderson BW, Gollnick SO, Snyder JW, et al. Choice of oxygen-conserving treatment regimen determines the inflammatory response and outcome of photodynamic therapy of tumors. *Cancer Res* 2004; 64:2120–6.
12. Kely CJ, Brown NJ, Reed MW, Ackroyd R. The use of 5-aminolaevulinic acid as a photosensitizer in photodynamic therapy and photodiagnosis. *Photochem Photobiol Sci* 2002; 1:158–68.
13. Peng Q, Warloe T, Berg K, et al. 5-Aminolevulinic acid-based photodynamic therapy. Clinical research and future challenges. *Cancer* 1997; 79:2282–308.
14. Lopez RF, Lange N, Guy R, Bentley MV. Photodynamic therapy of skin cancer: controlled drug delivery of 5-ALA and its esters. *Adv Drug Deliv Rev* 2004; 56:77–94.
15. Agarwal ML, Clay ME, Harvey EJ, Evans HH, Antunez AR, Oleinick NL. Photodynamic therapy induces rapid cell death by apoptosis in L5178Y mouse lymphoma cells. *Cancer Res* 1991; 51:5993–6.
16. Agostinis P, Buytaert E, Breysens H, Hendrickx N. Regulatory pathways in photodynamic therapy induced apoptosis. *Photochem Photobiol Sci* 2004; 3:721–9.

17. Almeida RD, Manadas BJ, Carvalho AP, Duarte CB. Intracellular signaling mechanisms in photodynamic therapy. *Biochim Biophys Acta* 2004; 1704:59–86.
18. Granville DJ, McManus BM, Hunt DW. Photodynamic therapy: shedding light on the biochemical pathways regulating porphyrin-mediated cell death. *Histol Histopathol* 2001; 16:309–17.
19. Girotti AW. Photosensitized oxidation of membrane lipids: reaction pathways, cytotoxic effects, and cytoprotective mechanisms. *J Photochem Photobiol B* 2001; 63:103–13.
20. Ochsner M. Photophysical and photobiological processes in the photodynamic therapy of tumours. *J Photochem Photobiol (Biol)* 1997; 39:1–18.
21. Moan J, Berg K. The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen. *Photochem Photobiol* 1991; 53:549–53.
22. He J, Oleinick NL. Cell death mechanisms vary with photodynamic therapy dose and photosensitizer. *Proc SPIE* 1995; 2371:92–6.
23. Noodt BB, Rodal GH, Wainwright M, et al. Apoptosis induction by different pathways with methylene blue derivative and light from mitochondrial sites in V79 cells. *Int J Cancer* 1998; 75:941–8.
24. Vantieghem A, Assefa Z, Vandenabeele P, et al. Hypericin-induced photosensitization of HeLa cells leads to apoptosis or necrosis. Involvement of cytochrome c and procaspase-3 activation in the mechanism of apoptosis. *FEBS Lett* 1998; 440:19–24.
25. Kim HR, Luo Y, Li G, Kessel D. Enhanced apoptotic response to photodynamic therapy after bcl-2 transfection. *Cancer Res* 1999; 59:3429–32.
26. Noodt BB, Berg K, Stokke T, Peng Q, Nesland JM. Different apoptotic pathways are induced from various intracellular sites by tetraphenylporphyrins and light. *Br J Cancer* 1999; 79:72–81.
27. Lavie G, Kaplinsky C, Toren A, et al. A photodynamic pathway to apoptosis and necrosis induced by dimethyl tetrahydroxyhelianthron and hypericin in leukaemic cells: possible relevance to photodynamic therapy. *Br J Cancer* 1999; 79:423–32.
28. Dellinger M. Apoptosis or necrosis following Photofrin photosensitization: influence of the incubation protocol. *Photochem Photobiol* 1996; 64:182–7.
29. Kessel D, Poretz RD. Sites of photodamage induced by photodynamic therapy with a chlorin e6 triacetoxymethyl ester (CAME). *Photochem Photobiol* 2000; 71:94–6.
30. Kochevar IE, Lynch MC, Zhuang S, Lambert CR. Singlet oxygen, but not oxidizing radicals, induces apoptosis in HL-60 cells. *Photochem Photobiol* 2000; 72:548–53.
31. Lin CP, Lynch MC, Kochevar IE. Reactive oxidizing species produced near the plasma membrane induce apoptosis in bovine aorta endothelial cells. *Exp Cell Res* 2000; 259:351–9.
32. He XY, Sikes RA, Thomsen S, Chung LWK, Jacques SL. Photodynamic therapy with Photofrin II induces programmed cell death in carcinoma cell lines. *Photochem Photobiol* 1994; 59:468–73.
33. Noodt BB, Berg K, Stokke T, Peng Q, Nesland JM. Apoptosis and necrosis induced with light and 5-aminolaevulinic acid-derived protoporphyrin IX. *Br J Cancer* 1996; 74:22–9.
34. Separovic D, He J, Oleinick NL. Ceramide generation in response to photodynamic treatment of L5178Y mouse lymphoma cells. *Cancer Res* 1997; 57:1717–21.
35. Xue L-Y, He J, Oleinick NL. Rapid tyrosine phosphorylation of HS1 in the response of mouse lymphoma L5178Y-R cells to photodynamic treatment sensitized by the phthalocyanine Pc 4. *Photochem Photobiol* 1997; 66:105–13.
36. Kessel D, Luo Y. Cells in cryptophycin-induced cell-cycle arrest are susceptible to apoptosis. *Cancer Lett* 2000; 151:25–9.
37. Kessel D, Luo Y. Photodynamic therapy: a mitochondrial inducer of apoptosis. *Cell Death Differ* 1999; 6:28–35.
38. Morgan J, Oseroff AR. Mitochondria-based photodynamic anti-cancer therapy. *Adv Drug Deliv Rev* 2001; 49:71–86.
39. Lam M, Oleinick NL, Nieminen AL. Photodynamic therapy-induced apoptosis in epidermoid carcinoma cells. Reactive oxygen species and mitochondrial inner membrane permeabilization. *J Biol Chem* 2001; 276:47379–86.
40. Xue L-Y, Chiu S-M, Oleinick NL. Photodynamic therapy-induced death of MCF-7 human breast cancer cells: a role for caspase-3 in the late steps of apoptosis but not for the critical lethal event. *Exp Cell Res* 2001; 263:145–55.
41. Wei MC, Zong WX, Cheng EH, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 2001; 292:727–30.

42. Chiu SM, Xue LY, Usuda J, Azizuddin K, Oleinick NL. Bax is essential for mitochondrion-mediated apoptosis but not for cell death caused by photodynamic therapy. *Br J Cancer* 2003; 89:1590–7.
43. Buytaert E, Callewaert G, Vandenhede JR, Agostinis P. Deficiency in apoptotic effectors Bax and Bak reveals an autophagic cell death pathway initiated by photodamage to the endoplasmic reticulum. *Autophagy* 2006; 2:238–40.
44. Buytaert E, Callewaert G, Hendrickx N, et al. Role of endoplasmic reticulum depletion and multidomain proapoptotic BAX and BAK proteins in shaping cell death after hypericin-mediated photodynamic therapy. *Faseb J* 2006; 20:756–8.
45. Kessel D, Vicente MG, Reiniers JJ Jr. Initiation of apoptosis and autophagy by photodynamic therapy. *Lasers Surg Med* 2006; 38:482–8.
46. Moreno G, Atlante A, Salet C, Santus R, Vinzens F. Photosensitivity of DNA replication and respiration to haematoporphyrin derivative (photofrin II) in mammalian CV-1 cells. *Int J Radiat Biol Relat Stud Phys Chem Med* 1987; 52:213–22.
47. Varnes ME, Chiu SM, Xue LY, Oleinick NL. Photodynamic therapy-induced apoptosis in lymphoma cells: translocation of cytochrome *c* causes inhibition of respiration as well as caspase activation. *Biochem Biophys Res Commun* 1999; 255:673–9.
48. Gibson SL, Hilf R. Interdependence of fluence, drug dose and oxygen on hematoporphyrin derivative induced photosensitization of tumor mitochondria. *Photochem Photobiol* 1985; 42:367–73.
49. Perlin DS, Murant RS, Gibson SL, Hilf R. Effects of photosensitization by hematoporphyrin derivative on mitochondrial adenosine triphosphatase-mediated proton transport and membrane integrity of R3230AC mammary adenocarcinoma. *Cancer Res* 1985; 45:653–8.
50. Atlante A, Moreno G, Parsarella S, Salet C. Hematoporphyrin derivative (PF II) photosensitization of isolated mitochondria: impairment of anion translocation. *Biochem Biophys Res Commun* 1986; 141:584–90.
51. Singh G, Jeeves WP, Wilson BC, Jang D. Mitochondrial photosensitization by Photofrin II. *Photochem Photobiol* 1987; 46:645–9.
52. Granville DJ, Carthy CM, Jiang H, Shore GC, McManus BM, Hunt DWC. Rapid cytochrome *c* release, activation of caspases 3, 6, 7 and 8 followed by Bap31 cleavage in HeLa cells treated with photodynamic therapy. *FEBS Lett* 1998; 437:5–10.
53. Chiu S-M, Oleinick NL. Dissociation of mitochondrial depolarization from cytochrome *c* release during apoptosis induced by photodynamic therapy. *Br J Cancer* 2001; 84:1099–106.
54. Carthy CM, Granville DJ, Jiang H, et al. Early release of mitochondrial cytochrome *c* and expression of mitochondrial epitope 7A6 with a porphyrin-derived photosensitizer: Bcl-2 and Bcl-xL overexpression do not prevent early mitochondrial events but still depress caspase activity. *Lab Invest* 1999; 79:953–65.
55. Dejean LM, Martinez-Caballero S, Guo L, et al. Oligomeric Bax is a component of the putative cytochrome *c* release channel MAC, mitochondrial apoptosis-induced channel. *Mol Biol Cell* 2005; 16:2424–32.
56. Halestrap AP, McStay GP, Clarke SJ. The permeability transition pore complex: another view. *Biochimie* 2002; 84:153–66.
57. Crompton M, Barksby E, Johnson N, Capano M. Mitochondrial intermembrane junctional complexes and their involvement in cell death. *Biochimie* 2002; 84:143–52.
58. Verma A, Nye J, Snyder SH. Porphyrins are endogenous ligands for the mitochondrial (peripheral-type) benzodiazepine receptor. *Mol Pharmacol* 1987; 84:2256–60.
59. Verma A, Snyder SH. Characterization of porphyrin interactions with peripheral type benzodiazepine receptors. *Mol Pharmacol* 1988; 34:800–5.
60. Verma A, Facchina SL, Hirsch DJ, et al. Photodynamic tumor therapy: mitochondrial benzodiazepine receptors as a therapeutic target. *Mol Med* 1998; 4:40–9.
61. Ratcliffe SL, Matthews EK. Modification of the photodynamic action of δ -aminolaevulinic acid (ALA) on rat preneoplasia cells by mitochondrial benzodiazepine receptor ligands. *Br J Cancer* 1995; 71:300–5.
62. Dougherty TJ, Sumlin AB, Greco WR, Weishaupt KR, Vaughan LA, Pandey RK. The role of the peripheral benzodiazepine receptor in photodynamic activity of certain pyropheophorbide ether photosensitizers: albumin site II as a surrogate marker for activity. *Photochem Photobiol* 2002; 76:91–7.

63. Morris RL, Varnes ME, Kenney ME, et al. The peripheral benzodiazepine receptor in photodynamic therapy with the phthalocyanine photosensitizer Pc 4. *Photochem Photobiol* 2002; 75:652–61.
64. Kessel D, Antolovich M, Smith KM. The role of the peripheral benzodiazepine receptor in the apoptotic response to photodynamic therapy. *Photochem Photobiol* 2001; 74:346–9.
65. Salet C, Moreno G, Ricchelli F, Bernardi P. Singlet oxygen produced by photodynamic action causes inactivation of the mitochondrial permeability transition pore. *J Biol Chem* 1997; 272:21938–43.
66. Belzacq AS, Jacotot E, Vieira HL, et al. Apoptosis induction by the photosensitizer verteporfin: identification of mitochondrial adenine nucleotide translocator as a critical target. *Cancer Res* 2001; 61:1260–4.
67. Miccoli L, Beurdeley-Thomas A, De Pinieux G, et al. Light-induced photoactivation of hypericin affects the energy metabolism of human glioma cells by inhibiting hexokinase bound to mitochondria. *Cancer Res* 1998; 58:5777–86.
68. Chaloupka R, Petit PX, Israel N, Sureau F. Over-expression of Bcl-2 does not protect cells from hypericin photo-induced mitochondrial membrane depolarization, but delays subsequent events in the apoptotic pathway. *FEBS Lett* 1999; 462:295–301.
69. Kowaltowski AJ, Turin J, Indig GL, Vercesi AE. Mitochondrial effects of triarylmethane dyes. *J Bioenerg Biomembr* 1999; 31:581–90.
70. Kessel D, Castelli M. Evidence that bcl-2 is the target of three photosensitizers that induce a rapid apoptotic response. *Photochem Photobiol* 2001; 74:318–22.
71. Xue L-Y, Chiu S-M, Oleinick NL. Photochemical destruction of the Bcl-2 oncoprotein during photodynamic therapy with the phthalocyanine photosensitizer Pc 4. *Oncogene* 2001; 20:3420–7.
72. Xue L-Y, Chiu S-M, Fiebig A, Andrews DW, Oleinick NL. Photodamage to multiple bcl-xL isoforms by photodynamic therapy with the phthalocyanine photosensitizer Pc 4. *Oncogene* 2003; 22:9197–204.
73. He J, Agarwal ML, Larkin HE, Friedman LR, Xue LY, Oleinick NL. The induction of partial resistance to photodynamic therapy by the protooncogene BCL-2. *Photochem Photobiol* 1996; 64:845–52.
74. Usuda J, Azizuddin K, Chiu SM, Oleinick NL. Association between the photodynamic loss of Bcl-2 and the sensitivity to apoptosis caused by phthalocyanine photodynamic therapy. *Photochem Photobiol* 2003; 78:1–8.
75. Usuda J, Chiu SM, Murphy ES, Lam M, Nieminen AL, Oleinick NL. Domain-dependent photo-damage to Bcl-2. A membrane anchorage region is needed to form the target of phthalocyanine photosensitization. *J Biol Chem* 2003; 278:2021–9.
76. Matsuyama S, Schendel SL, Xie Z, Reed JC. Cytoprotection by Bcl-2 requires the pore-forming alpha5 and alpha6 helices. *J Biol Chem* 1998; 273:30995–1001.
77. Kim PK, Annis MG, Dlugosz PJ, Leber B, Andrews DW. During apoptosis bcl-2 changes membrane topology at both the endoplasmic reticulum and mitochondria. *Mol Cell* 2004; 14:523–9.
78. Tsujimoto Y, Shimizu S. The voltage-dependent anion channel: an essential player in apoptosis. *Biochimie* 2002; 84:187–93.
79. Kessel D, Caruso JA, Reiners JJ Jr. Potentiation of photodynamic therapy by ursodeoxycholic acid. *Cancer Res* 2000; 60:6985–8.
80. Rodrigues CM, Steer CJ. The therapeutic effects of ursodeoxycholic acid as an anti-apoptotic agent. *Expert Opin Investig Drugs* 2001; 10:1243–53.
81. Castelli M, Reiners JJ, Kessel D. A mechanism for the proapoptotic activity of ursodeoxycholic acid: effects on Bcl-2 conformation. *Cell Death Differ* 2004; 11:906–14.
82. Bachowski GJ, Korytowski W, Girotti AW. Characterization of lipid hydroperoxides generated by photodynamic treatment of leukemia cells. *Lipids* 1994; 29:449–59.
83. Kriska T, Korytowski W, Girotti AW. Hyperresistance to photosensitized lipid peroxidation and apoptotic killing in 5-aminolevulinic-acid-treated tumor cells overexpressing mitochondrial GPX4. *Free Radic Biol Med* 2002; 33:1389–402.
84. Ardail D, Privat JP, Egret-Charlier M, Levrat C, Lerme F, Louisot P. Mitochondrial contact sites. Lipid composition and dynamics. *J Biol Chem* 1990; 265:18797–802.
85. Lesnefsky EJ, Slabe TJ, Stoll MS, Minkler PE, Hoppel CL. Myocardial ischemia selectively depletes cardiolipin in rabbit heart subsarcolemmal mitochondria. *Am J Physiol Heart Circ Physiol* 2001; 280:H2770–8.
86. McMillin JB, Dowhan W. Cardiolipin and apoptosis. *Biochim Biophys Acta* 2002; 1585:97–107.

87. Schlame M, Rua D, Greenberg ML. The biosynthesis and functional role of cardiolipin. *Prog Lipid Res* 2000; 39:257–88.
88. Ott M, Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S. Cytochrome c release from mitochondria proceeds by a two-step process. *Proc Natl Acad Sci USA* 2002; 99:1259–63.
89. Nomura K, Imai H, Koumura T, Kobayashi T, Nakagawa Y. Mitochondrial phospholipid hydroperoxide glutathione peroxidase inhibits the release of cytochrome c from mitochondria by suppressing the peroxidation of cardiolipin in hypoglycaemia-induced apoptosis. *Biochem J* 2000; 351:183–93.
90. Kriska T, Korytowski W, Girotti AW. Role of mitochondrial cardiolipin peroxidation in apoptotic photokilling of 5-aminolevulinic acid-treated tumor cells. *Arch Biochem Biophys* 2005; 433:435–46.
91. Ricchelli F, Nikolov P, Gobbo S, Jori G, Moreno G, Salet C. Interaction of phthalocyanines with lipid membranes: a spectroscopic and functional study on isolated rat liver mitochondria. *Biochim Biophys Acta* 1994; 1196:165–71.
92. Petit JM, Maftah A, Ratinaud MH, Julien R. 10N-nonyl acridine orange interacts with cardiolipin and allows the quantification of this phospholipid in isolated mitochondria. *Eur J Biochem* 1992; 209:267–73.
93. Mileykovskaya E, Dowhan W, Birke RL, Zheng D, Lutterodt L, Haines TH. Cardiolipin binds nonyl acridine orange by aggregating the dye at exposed hydrophobic domains on bilayer surfaces. *FEBS Lett* 2001; 507:187–90.
94. Jacobson J, Duchon MR, Heales SJ. Intracellular distribution of the fluorescent dye nonyl acridine orange responds to the mitochondrial membrane potential: implications for assays of cardiolipin and mitochondrial mass. *J Neurochem* 2002; 82:224–33.
95. Guidot DM. Endotoxin pretreatment *in vivo* increases the mitochondrial respiratory capacity in rat hepatocytes. *Arch Biochem Biophys* 1998; 354:9–17.
96. Garcia Fernandez M, Troiano L, Moretti L, et al. Changes in intramitochondrial cardiolipin distribution in apoptosis-resistant HCW-2 cells, derived from the human promyelocytic leukemia HL-60. *FEBS Lett* 2000; 478:290–4.
97. Wilson BC, Olivo M, Singh G. Subcellular localization of Photofrin and aminolevulinic acid and photodynamic cross-resistance *in vitro* in radiation-induced fibrosarcoma cells sensitive or resistant to photofrin-mediated photodynamic therapy. *Photochem Photobiol* 1997; 65:166–76.
98. Morris RL, Azizuddin K, Lam M, et al. Fluorescence resonance energy transfer reveals a binding site of a photosensitizer for photodynamic therapy. *Cancer Res* 2003; 63:5194–7.
99. Kessel D, Luo Y. Intracellular sites of photodamage as a factor in apoptotic cell death. *J Porphyrin Phthalocyanines* 2001; 5:181–4.
100. Reiners JJ Jr., Caruso JA, Mathieu P, Chelladurai B, Yin XM, Kessel D. Release of cytochrome c and activation of pro-caspase-9 following lysosomal photodamage involves Bid cleavage. *Cell Death Differ* 2002; 9:934–44.
101. Caruso JA, Mathieu PA, Joiakim A, et al. Differential susceptibilities of murine hepatoma 1c1c7 and Tao cells to the lysosomal photosensitizer NPe6: influence of aryl hydrocarbon receptor on lysosomal fragility and protease contents. *Mol Pharmacol* 2004; 65:1016–28.
102. Stoka V, Turk B, Schendel SL, et al. Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. *J Biol Chem* 2001; 276:3149–57.
103. Separovic D, Mann KJ, Oleinick NL. Association of ceramide accumulation with photodynamic treatment-induced cell death. *Photochem Photobiol* 1998; 68:101–9.
104. Haimovitz-Friedman A, Kolesnick RN, Fuks Z. Ceramide signaling in apoptosis. *Br Med Bull* 1997; 53:539–53.
105. Dolgachev V, Farooqui MS, Kulaeva OI, et al. De novo ceramide accumulation due to inhibition of its conversion to complex sphingolipids in apoptotic photosensitized cells. *J Biol Chem* 2004; 279:23238–49.
106. Matrroule JY, Bonizzi G, Morliere P, et al. Pyropheophorbide-a methyl ester-mediated photosensitization activates transcription factor NF-kappaB through the interleukin-1 receptor-dependent signaling pathway. *J Biol Chem* 1999; 274:2988–3000.
107. Separovic D, Pink JJ, Oleinick NA, et al. Niemann-Pick human lymphoblasts are resistant to phthalocyanine 4-photodynamic therapy-induced apoptosis. *Biochem Biophys Res Commun* 1999; 258:506–12.

108. Grebenova D, Halada P, Stulik J, Havlicek V, Hrkal Z. Protein changes in HL60 leukemia cells associated with 5-aminolevulinic acid-based photodynamic therapy. Early effects on endoplasmic reticulum chaperones. *Photochem Photobiol* 2000; 72:16–22.
109. Agarwal ML, Larkin HE, Zaidi SI, Mukhtar H, Oleinick NL. Phospholipase activation triggers apoptosis in photosensitized mouse lymphoma cells. *Cancer Res* 1993; 53:5897–902.
110. Tajiri H, Hayakawa A, Matsumoto Y, Yokoyama I, Yoshida S. Changes in intracellular Ca²⁺ concentrations related to PDT-induced apoptosis in photosensitized human cancer cells. *Cancer Lett* 1998; 128:205–10.
111. Hubmer A, Hermann A, Uberriegler K, Krammer B. Role of calcium in photodynamically induced cell damage of human fibroblasts. *Photochem Photobiol* 1996; 64:211–5.
112. Gederaas OA, Thorstensen K, Romslo I. The effect of brief illumination on intracellular free calcium concentration in cells with 5-aminolevulinic acid-induced protoporphyrin IX synthesis. *Scand J Clin Lab Invest* 1996; 56:583–9.
113. Cui ZJ, Kanno T. Photodynamic triggering of calcium oscillation in the isolated rat pancreatic acini. *J Physiol* 1997; 504 (Pt 1):47–55.
114. Cui ZJ, Habara Y, Wang DY, Kanno T. A novel aspect of photodynamic action: induction of recurrent spikes in cytosolic calcium concentration. *Photochem Photobiol* 1997; 65:382–6.
115. Penning LC, Rasch MH, Ben-Hur E, et al. A role for the transient increase of cytoplasmic free calcium in cell rescue after photodynamic treatment. *Biochim Biophys Acta* 1992; 1107:255–60.
116. Ben-Hur E, Dubbelman TM, Van Steveninck J. Phthalocyanine-induced photodynamic changes of cytoplasmic free calcium in Chinese hamster cells. *Photochem Photobiol* 1991; 54:163–6.
117. Specht KG, Rodgers MA. Plasma membrane depolarization and calcium influx during cell injury by photodynamic action. *Biochim Biophys Acta* 1991; 1070:60–8.
118. Granville DJ, Ruehlmann DO, Choy JC, et al. Bcl-2 increases emptying of endoplasmic reticulum Ca²⁺ stores during photodynamic therapy-induced apoptosis. *Cell Calcium* 2001; 30:343–50.
119. Ruck A, Heckelsmiller K, Kaufmann R, Grossman N, Haseroth E, Akgun N. Light-induced apoptosis involves a defined sequence of cytoplasmic and nuclear calcium release in ALPcS4-photosensitized rat bladder RR 1022 epithelial cells. *Photochem Photobiol* 2000; 72:210–6.
120. Yonuschot G. Early increase in intracellular calcium during photodynamic permeabilization. *Free Radic Biol Med* 1991; 11:307–17.
121. Joshi PG, Joshi K, Mishra S, Joshi NB. Ca²⁺ influx induced by photodynamic action in human cerebral glioma (U-87 MG) cells: possible involvement of a calcium channel. *Photochem Photobiol* 1994; 60:244–8.
122. An YP, Xiao R, Cui H, Cui ZJ. Selective activation by photodynamic action of cholecystokinin receptor in the freshly isolated rat pancreatic acini. *Br J Pharmacol* 2003; 139:872–80.
123. Inanami O, Yoshito A, Takahashi K, Hiraoka W, Kuwabara M. Effects of BAPTA-AM and forskolin on apoptosis and cytochrome c release in photosensitized Chinese hamster V79 cells. *Photochem Photobiol* 1999; 70:650–5.
124. Ogata M, Inanami O, Nakajima M, Nakajima T, Hiraoka W, Kuwabara M. Ca(2+)-dependent and caspase-3-independent apoptosis caused by damage in Golgi apparatus due to 2,4,5,7-tetrabromorhodamine 123 bromide-induced photodynamic effects. *Photochem Photobiol* 2003; 78:241–7.
125. Pinton P, Pozzan T, Rizzuto R. The Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca²⁺ store, with functional properties distinct from those of the endoplasmic reticulum. *Embo J* 1998; 17:5298–308.
126. Carafoli E, Santella L, Branca D, Brini M. Generation, control, and processing of cellular calcium signals. *Crit Rev Biochem Mol Biol* 2001; 36:107–260.
127. Schwab BL, Guerini D, Didszun C, et al. Cleavage of plasma membrane calcium pumps by caspases: a link between apoptosis and necrosis. *Cell Death Differ* 2002; 9:818–31.
128. Bano D, Young KW, Guerini CJ, et al. Cleavage of the plasma membrane Na⁺/Ca²⁺ exchanger in excitotoxicity. *Cell* 2005; 120:275–85.
129. Ricchelli F, Barbato P, Milani M, Gobbo S, Salet C, Moreno G. Photodynamic action of porphyrin on Ca²⁺ influx in endoplasmic reticulum: a comparison with mitochondria. *Biochem J* 1999; 338 (Pt 1):221–7.
130. Bootman MD, Taylor CW, Berridge MJ. The thiol reagent, thimerosal, evokes Ca²⁺ spikes in HeLa cells by sensitizing the inositol 1,4,5-trisphosphate receptor. *J Biol Chem* 1992; 267:25113–9.

131. Renard-Rooney DC, Joseph SK, Seitz MB, Thomas AP. Effect of oxidized glutathione and temperature on inositol 1,4,5-trisphosphate binding in permeabilized hepatocytes. *Biochem J* 1995; 310(Pt 1):185–92.
132. Boehning D, Patterson RL, Sedaghat L, Glebova NO, Kurosaki T, Snyder SH. Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis. *Nat Cell Biol* 2003; 5:1051–61.
133. Kessel D, Castelli M, Reiniers JJ. Ruthenium red-mediated suppression of Bcl-2 loss and Ca(2+) release initiated by photodamage to the endoplasmic reticulum: scavenging of reactive oxygen species. *Cell Death Differ* 2005; 12:502–11.
134. Gunter KK, Gunter TE. Transport of calcium by mitochondria. *J Bioenerg Biomembr* 1994; 26:471–85.
135. Crompton M, Kunzi M, Carafoli E. The calcium-induced and sodium-induced effluxes of calcium from heart mitochondria. Evidence for a sodium-calcium carrier. *Eur J Biochem* 1977; 79:549–58.
136. Crompton M, Moser R, Ludi H, Carafoli E. The interrelations between the transport of sodium and calcium in mitochondria of various mammalian tissues. *Eur J Biochem* 1978; 82:25–31.
137. Puskin JS, Gunter TE, Gunter KK, Russell PR. Evidence for more than one Ca²⁺ transport mechanism in mitochondria. *Biochemistry* 1976; 15:3834–42.
138. Fiskum G, Lehninger AL. Regulated release of Ca²⁺ from respiring mitochondria by Ca²⁺ /2H⁺ antiport. *J Biol Chem* 1979; 254:6236–9.
139. Jung DW, Baysal K, Brierley GP. The sodium-calcium antiport of heart mitochondria is not electroneutral. *J Biol Chem* 1995; 270:672–8.
140. Jung DW, Baysal K, Brierley GP. Properties of the Na(+)-Ca²⁺ antiport of heart mitochondria. *Ann N Y Acad Sci* 1996; 779:553–5.
141. Haworth RA, Hunter DR. The Ca²⁺-induced membrane transition in mitochondria. II. Nature of the Ca²⁺ trigger site. *Arch Biochem Biophys* 1979; 195:460–7.
142. Bernardi P, Broekemeier KM, Pfeiffer DR. Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane. *J Bioenerg Biomembr* 1994; 26:509–17.
143. Csordas G, Thomas AP, Hajnoczky G. Calcium signal transmission between ryanodine receptors and mitochondria in cardiac muscle. *Trends Cardiovasc Med* 2001; 11:269–75.
144. Rizzuto R, Duchen MR, Pozzan T. Flirting in little space: the ER/mitochondria Ca²⁺ liaison. *Sci STKE* 2004; 2004:re1.
145. Evans S, Matthews W, Perry R, Fraker D, Norton J, Pass HI. Effect of photodynamic therapy on tumor necrosis factor production by murine macrophages. *J Natl Cancer Inst* 1990; 82:34–9.
146. Kick G, Messer G, Plewig G, Kind P, Goetz AE. Strong and prolonged induction of c-jun and c-fos proto-oncogenes by photodynamic therapy. *Br J Cancer* 1996; 74:30–6.
147. Ahmad N, Gupta S, Feyes DK, Mukhtar H. Involvement of Fas (APO-1/CD-95) during photodynamic-therapy-mediated apoptosis in human epidermoid carcinoma A431 cells. *J Invest Dermatol* 2000; 115:1041–6.
148. Yslas I, Alvarez MG, Marty C, Mori G, Durantini EN, Rivarola V. Expression of Fas antigen and apoptosis caused by 5,10,15, 20-tetra(4-methoxyphenyl)porphyrin (TMP) on carcinoma cells: implication for photodynamic therapy. *Toxicology* 2000; 149:69–74.
149. Ali SM, Chee SK, Yuen GY, Olivo M. Hypericin induced death receptor-mediated apoptosis in photoactivated tumor cells. *Int J Mol Med* 2002; 9:601–16.
150. Jiang H, Granville DJ, McManus BM, Levy JG, Hunt DW. Selective depletion of a thymocyte subset *in vitro* with an immunomodulatory photosensitizer. *Clin Immunol* 1999; 91:178–87.
151. Azizuddin K, Kalka K, Chiu S, Ahmad N, Mukhtar H, Separovic D. Recombinant human tumor necrosis factor alpha does not potentiate cell killing after photodynamic therapy with a silicon phthalocyanine in A431 human epidermoid carcinoma cells. *Int J Oncol* 2001; 18:411–5.
152. Nagy B, Yeh W-C, Mak TW, Chiu S-M, Separovic D. FADD null mouse embryonic fibroblasts undergo apoptosis after photosensitization with the silicon phthalocyanine Pc 4. *Arch Biochem Biophys* 2001; 385:194–202.

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Modulation of TRAIL Signaling for Cancer Therapy

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SUMMARY

Apoptosis, the cell's intrinsic death program, is a key regulator of tissue homeostasis, and an imbalance between cell death and proliferation may result in tumor formation. Also, killing of cancer cells by cytotoxic therapies such as chemotherapy, γ -irradiation, or ligation of death receptors is predominantly mediated by triggering apoptosis in target cells. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily that induces apoptosis upon binding to its receptors. TRAIL is of special interest for cancer therapy, as TRAIL has been shown to predominantly kill cancer cells, while sparing normal cells. Importantly, combined treatment with TRAIL together with chemotherapy or γ -irradiation synergized to achieve antitumor activity in tumor cell lines and also in tumor models *in vivo*. However, failure to undergo apoptosis in response to TRAIL treatment may result in tumor resistance. Understanding the molecular events that regulate TRAIL-induced apoptosis and their deregulation in resistant forms of cancer may provide new opportunities for cancer therapy. Thus, novel strategies targeting tumor cell resistance will be based on further insights into the molecular mechanisms of cell death, for example, triggered by TRAIL.

Key Words: Apoptosis; TRAIL; drugs; cancer therapy.

1. INTRODUCTION

Apoptosis, a distinct, intrinsic cell death program, occurs in various physiological and pathological situations and has an important regulatory function in tissue homeostasis and immune regulation (1,2). Apoptosis is characterized by typical morphological and biochemical hallmarks including cell shrinkage, nuclear DNA fragmentation, and membrane blebbing (1). Various stimuli can trigger an apoptosis response, for example, withdrawal of growth factors, or stimulation of cell-surface receptors (1). Also, killing of tumor cells by diverse cytotoxic strategies such as

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anticancer drugs, γ -irradiation, or immunotherapy has been shown to involve induction of apoptosis in target cells (3–5). In addition, T cells or NK cells may release cytotoxic compounds such as granzyme B, which can directly initiate apoptosis effector pathways inside the cell (1). Proteolytic enzymes called caspases are important effector molecules of different forms of cell death (6). Apoptosis pathways are tightly controlled by a number of inhibitory and promoting factors (7). The antiapoptotic mechanisms regulating apoptotic cell death have also been implicated in conferring drug resistance to tumor cells (5). Importantly, combinations of anticancer agents together with death-inducing ligands have been shown to synergize in triggering apoptosis in cancer cells and may even overcome some forms of drug resistance (8). Further insights into the mechanisms controlling tumor cell death in response to death receptor ligation will provide a molecular basis for novel strategies targeting death pathways in apoptosis-resistant forms of cancer.

2. THE CORE APOPTOTIC MACHINERY

In most cases, anticancer therapies eventually result in the activation of caspases, a family of cysteine proteases that act as common death effector molecules in various forms of cell death (6). Caspases are synthesized as inactive proforms, and upon activation, they cleave next to aspartate residues. The fact that caspases can activate each other by cleavage at identical sequences results in the amplification of caspase activity through a protease cascade. Caspases cleave a number of different substrates in the cytoplasm or nucleus leading to many of the morphologic features of apoptotic cell death (1). For example, polynucleosomal DNA fragmentation is mediated by cleavage of inhibitor of caspase-activated DNase (ICAD), the inhibitor of the endonuclease CAD that cleaves DNA into the characteristic oligomeric fragments (9). Likewise, proteolysis of several cytoskeletal proteins such as actin or fodrin leads to loss of overall cell shape, whereas degradation of lamin results in nuclear shrinking (1).

Activation of caspases can be initiated from different angles, for example, at the plasma membrane upon ligation of death receptor (receptor pathway) or at the mitochondria (mitochondrial pathway) (8). Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TNF-related apoptosis-inducing ligand (TRAIL) receptors results in the activation of the initiator caspase-8, which can propagate the apoptosis signal by direct cleavage of downstream effector caspases such as caspase-3 (10). The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome c, apoptosis-inducing factor (AIF), Smac/Diablo, Omi/HtrA2, endonuclease G, caspase-2, or caspase-9 from the mitochondrial intermembrane space (11). The release of cytochrome c into the cytosol triggers caspase-3 activation through the formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex (12). Smac/Diablo and Omi/HtrA2 promote caspase activation through neutralizing the inhibitory effects to inhibitor of apoptosis proteins (IAPs) (11).

Links between the receptor and the mitochondrial pathway exist at different levels. Upon death, receptor triggering activation of caspase-8 may result in cleavage of Bid, a Bcl-2 family protein with a BH3 domain only, which in turn translocates to mitochondria to release cytochrome c thereby initiating a mitochondrial amplification loop (13). In addition, cleavage of caspase-6 downstream of mitochondria may feed back to the receptor pathway by cleaving caspase-8 (14).

3. TRAIL AND ITS RECEPTORS

TRAIL/Apo-2L was identified in 1995 based on its sequence homology to other members of the TNF superfamily (15,16). TRAIL is a type II transmembrane protein, the extracellular domain of which can be proteolytically cleaved from the cell surface. TRAIL is constitutively expressed in a wide range of tissues. Comprising five different receptors, the complexity of the TRAIL receptor system is unprecedented. TRAIL-R1 and TRAIL-R2, the two agonistic TRAIL receptors, contain a conserved cytoplasmic death domain motif, which enables them to engage the cell's apoptotic machinery upon ligand binding (17–21). TRAIL-R3 to TRAIL-R5 are antagonistic decoy receptors, which bind TRAIL, but do not transmit a death signal (22–26). TRAIL-R3 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface protein, which lacks a cytoplasmic tail, whereas TRAIL-R4 harbors a substantially truncated cytoplasmic death domain. In addition to these four membrane-associated receptors, osteoprotegerin is a soluble decoy receptor, which is involved in the regulation of osteoclastogenesis (27).

Similar to CD95L, TRAIL rapidly triggers apoptosis in many tumor cells (28–30). The TRAIL ligand and its receptors are of special interest for cancer therapy, as TRAIL has been shown to predominantly kill cancer cells while sparing normal cells. The underlying mechanisms for the differential sensitivity of malignant versus non-malignant cells for TRAIL have not exactly been defined. One possible mechanism of protection of normal tissues is thought to be based on the set of antagonistic decoy receptors, which compete with TRAIL-R1 and TRAIL-R2 for binding to TRAIL (31). However, screening of various tumor cell types and normal cells did not reveal a consistent association between TRAIL sensitivity and TRAIL receptor expression. Therefore, susceptibility for TRAIL-induced cytotoxicity has been suggested to be regulated intracellularly by distinct patterns of pro- and antiapoptotic molecules.

4. TRAIL SIGNALING

Similar to the CD95 system, ligation of the agonistic TRAIL receptors, TRAIL-R1 and TRAIL-R2, by TRAIL or agonistic antibodies results in receptor trimerization and clustering of the receptors' death domains (28,30,32–34). This leads to recruitment of adaptor molecules such as FADD through homophilic interaction mediated by the death domain. FADD in turn recruits caspase-8 to the activated TRAIL receptor complex. Oligomerization of caspase-8 at the activated TRAIL receptor complex drives its activation through self-cleavage. Caspase-8 then activates downstream effector caspases such as caspase-3. Also, activated caspase-8 can cleave Bid, which then translocates to mitochondria to induce cytochrome c release. In addition to caspase-8, caspase-10 is recruited to the TRAIL DISC (33). However, the importance of caspase-10 in the TRAIL DISC for apoptosis induction has been controversially discussed (35,36).

5. DEFECTIVE TRAIL SIGNALING IN CANCERS

Signaling by agonistic TRAIL receptors is regulated at various levels along the signaling pathway. Importantly, cancer cells have evolved numerous ways to evade induction of apoptosis triggered by the death ligand TRAIL, resulting in TRAIL resistance as outlined below.

5.1. *Trail Receptors*

For example, loss of expression of the agonistic TRAIL receptors, TRAIL-R1 and TRAIL-R2, may account for TRAIL resistance. Both receptors are located on chromosome 8p, a region of frequent loss of heterozygosity (LOH) in tumors (28). In a small percentage of cancers, for example, non-Hodgkin's lymphoma, colorectal, breast, head and neck, or lung carcinoma, deletions or mutations were found, which resulted in loss of both copies of TRAIL-R1 and TRAIL-R2 (37–41). In addition, loss of TRAIL-R1 or TRAIL-R2 expression may be caused by epigenetic alterations such as promotor hypermethylation, for example, in neuroblastoma (42).

5.2. *c-FLIP*

TRAIL signaling can also be negative by intracellular proteins that associate with the cytoplasmic domain of TRAIL receptors such as c-FLIP (43). c-FLIP has homology to caspase-8 and caspase-10 but lacks protease activity. By binding to FADD, c-FLIP prevents the interaction between the adaptor molecule FADD and procaspase-8. c-FLIP exists as a long (c-FLIP_L) and a short (c-FLIP_S) isoforms, both of which can inhibit death receptor-induced apoptosis. Interestingly, c-FLIP_S was identified in a screen for genes that could confer resistance to TRAIL-induced apoptosis (44). High c-FLIP expression has been detected in many tumors and has been correlated with resistance to TRAIL-induced apoptosis, for example, in melanoma. However, a consistent correlation between c-FLIP expression and TRAIL resistance has not always been found (45). Importantly, downregulation of FLIP expression by inhibition of protein synthesis or RNA translation, cytotoxic drugs, proteasome inhibitors, PPAR γ ligand, or siRNAI for c-FLIP restored sensitivity for TRAIL-induced apoptosis in several cell types (46–48).

5.3. *Bcl-2 Proteins*

Bcl-2 family proteins play an important role in the regulation of the mitochondrial pathway, as these proteins localize to intracellular membranes such as the mitochondrial membrane (13). They comprise both antiapoptotic members, such as Bcl-2 or Bcl-X_L, and proapoptotic molecules, such as Bax or Bid. Altered expression of Bcl-2 family proteins have been reported in various human cancers. Imbalances in the ratio of anti- and proapoptotic Bcl-2 proteins may favor tumor cell survival instead of cell death. Overexpression of Bcl-2 or Bcl-X_L blocked TRAIL-triggered apoptosis in many tumor cell lines, for example, prostate carcinoma, pancreatic carcinoma, neuroblastoma, or glioblastoma cells (49,50). In addition, gene ablation studies showed that Bax was absolutely required for TRAIL-induced apoptosis in colon carcinoma cells (44,51). However, overexpression of Bcl-2 or Bcl-X_L did not interfere with TRAIL-induced apoptosis in some cell types, for example, Jurkat or CEM T-cell leukemia cells (52). Thus, the contribution of the mitochondrial pathway to TRAIL-induced apoptosis may depend on the cell type.

5.4. *IAPs*

The family of endogenous caspase inhibitors IAPs are highly conserved throughout evolution and comprise the human analogs XIAP, cIAP1, cIAP2, survivin, and livin

(ML-IAP) (53). IAPs have been reported to directly inhibit active caspase-3 and caspase-7 and to block caspase-9 activation. In addition to regulation of apoptosis, IAP members such as survivin are involved in the regulation of mitosis (54). The activity of IAPs are controlled at various levels, for example, by the transcription factor NF κ B that has been reported to stimulate expression of cIAP1, cIAP, and XIAP (53). In addition, Smac/Diablo and Omi, which are released from mitochondria upon apoptosis induction, neutralize IAPs through binding to IAPs thereby displacing them from their caspase partners (11). Increased IAPs expression is detected in many tumors and has been correlated with adverse prognosis (45,53). Importantly, overexpression of XIAP blocked TRAIL-triggered apoptosis in several cellular systems (55,56).

5.5. NF κ B

The transcription factor NF κ B has been connected with multiple aspects of oncogenesis, including cell proliferation or inhibition of apoptosis (57). NF κ B is composed of hetero- or homodimers of NF κ B/Rel family of proteins, which mediate protein dimerization, nuclear import, and specific DNA binding. In most cell types, NF κ B is sequestered in the cytoplasm by its interaction with I κ B proteins and therefore remains inactive. Upon stimulation, I κ B becomes phosphorylated following activation of the IKK complex and is degraded through the proteasome thereby releasing NF κ B to translocate into the nucleus for transcription of target genes. NF κ B target genes include several antiapoptotic proteins, for example, cIAP1, cIAP2, TRAF1, TRAF2, Bfl-1/A1, Bcl-X_L, and FLIP. Interestingly, promoter activation of certain proapoptotic molecules of the TRAIL system including TRAIL-R1, TRAIL-R2, or TRAIL is also controlled by NF κ B consistent with reports that NF κ B can promote apoptosis under certain circumstances (58,59). NF κ B is constitutively active in certain tumor types such as Hodgkin's lymphoma or pancreatic carcinoma (57). Also, NF κ B activity is induced in response to various stimuli, for example, in response to cellular stress or anticancer agents. In addition, TRAIL can activate NF κ B, which is mediated by TRAIL-R1, TRAIL-R2, or TRAIL-R4 through a TRAF2-NIK-IKK α / β -dependent signaling cascade (18,60). Interestingly, TRAIL has been reported to activate NF κ B especially under conditions, when apoptosis is blocked (61,62). Inhibition of NF κ B signaling, for example, by proteasome inhibitors, which prevent I κ B α degradation, or by overexpression of non-degradable I κ B α mutants, has been reported to sensitize tumor cells for TRAIL-induced apoptosis, at least in some contexts (63).

6. TRAIL AND CANCER THERAPY

6.1. Safety of TRAIL Administration

The idea to specifically target death receptors to trigger apoptosis in tumor cells is attractive for cancer therapy as death receptors have a direct link to the cell's death machinery (10). In addition, apoptosis upon death receptor ligation has been reported to occur independent of the p53 tumor suppressor gene, which is deleted or inactivated in more than half of human tumors (64). However, the clinical application of CD95 ligand or TNF α is hampered by severe toxic side effects (30). Systemic administration of TNF α or CD95 ligand causes a severe inflammatory response syndrome or massive liver cell apoptosis. In contrast, TRAIL appears to be a relatively safe and

promising candidate for clinical application, particularly in its non-tagged, zinc-bound homotrimeric form (28). Studies in non-human primates such as chimpanzees and cynomolgus monkeys showed no toxicity upon intravenous infusion, even at high doses (65). In addition, no cytotoxic activity of TRAIL was reported on various normal human cells of different lineages including fibroblasts, endothelial cells, smooth muscle cells, epithelial cells, or astrocytes (66). However, some concerns about potential toxic side effects on human hepatocytes or brain tissue have also been raised (67,68). The loss of tumor selectivity may be related to the TRAIL preparations used in these studies. TRAIL preparations, which are antibody crosslinked or not optimized for Zn content, have been reported to form multimeric aggregates thereby overpassing the threshold of sensitivity of normal cells (28).

6.2. Antitumor Activity of TRAIL

Recombinant soluble TRAIL-induced apoptosis in a broad spectrum of cancer cell lines, including colon carcinoma, breast carcinoma, lung carcinoma, pancreas carcinoma, prostate carcinoma, renal carcinoma, thyroid carcinoma, malignant brain tumors, Ewing tumor, osteosarcoma, neuroblastoma, leukemia, and lymphoma (28,29). Also, TRAIL exhibited potent tumoricidal activity *in vivo* in several xenograft models of colon carcinoma, breast carcinoma, malignant glioma, or multiple myeloma. Furthermore, monoclonal antibodies that engage the TRAIL receptors, DR4 or DR5, also demonstrated potent antitumor activity against tumor cell lines and in preclinical cancer models (69,70).

6.3. Combination Therapy with TRAIL

Although these studies provided ample evidence of the potential of TRAIL for cancer therapy, many tumors remain refractory toward treatment with TRAIL, which has been related to the dominance of antiapoptotic signals. Importantly, numerous studies have shown that TRAIL synergized together with cytotoxic drugs or γ -irradiation to achieve antitumor activity in various cancers including malignant glioma, melanoma, leukemia, breast, colon, or prostate carcinoma (71–76). Remarkably, TRAIL and anticancer agents also cooperated to suppress tumor growth in different mouse models of human cancers. The molecular mechanisms, which account for this synergistic interaction, may include transcriptional upregulation of the agonistic TRAIL receptors, TRAIL-R1 and TRAIL-R2, which occurred in a p53-dependent or p53-independent manner (77,78). Of note, p53 has also been shown to transcriptionally activate the antagonistic TRAIL receptors, TRAIL-R3 and TRAIL-R4 (79). The synergistic interaction between cytotoxic drugs and TRAIL may also be mediated by downregulation of antiapoptotic proteins such as Bcl-2, Bcl-X_L, or FLIP upon drug treatment (80). In addition, anticancer agents may sensitize tumor cells for TRAIL treatment by upregulating proapoptotic molecules including caspases or FADD (81). Caspase-8 expression is frequently impaired by hypermethylation in several tumors including neuroblastoma, Ewing tumors, malignant brain tumors, or lung carcinoma (82,83). Importantly, restoration of caspase-8 expression by gene transfer or by demethylation treatment sensitized resistant tumor cells for TRAIL-induced apoptosis (83). Also, biological response modifiers such as IFN γ strongly enhanced the cytotoxic activity of TRAIL by upregulating caspase-8 expression in a STAT-1-dependent manner (84).

As TRAIL demonstrated synergistic interaction with anticancer agents or irradiation, TRAIL may be most effective in combination with conventional cancer treatments. In addition, small molecules may serve as molecular therapeutics to specifically target tumor cell resistance toward TRAIL in resistant forms of cancer. To this end, Smac agonists have recently been reported to potentiate the efficacy of TRAIL treatment by antagonizing the inhibitory effect of IAPs, which are overexpressed in many tumors (85). Importantly, Smac peptides synergized with TRAIL to eradicate malignant glioma in an orthotopic mouse model without any detectable toxicities to the normal brain tissue (85).

Aberrant architecture of the chromatin has been implicated in tumor formation and progression (86). Posttranslational modifications of histone proteins, in particular their acetylation status, control gene expression by regulating the accessibility of transcription factors to DNA (86). Histone acetylation is governed by the relative activities of histone acetyltransferases (HATs), which favor decompaction of the chromatin structure for efficient transcription, and histone deacetylases (HDACs), which remove acetyl groups from histone tails, thereby suppressing transcription (86). HDAC inhibitors (HDACIs) have been reported to cause growth arrest, differentiation, and/or apoptosis by altering the transcription of a small and finite number of expressed genes (87). Interestingly, HDACIs were also found to significantly enhance the efficacy of TRAIL in various human cancers including breast, colon, lung or prostate carcinoma, mesothelioma, melanoma, as well as acute or chronic leukemia (88–101). The molecular basis for the HDACI-mediated sensitization for TRAIL has been linked to upregulation of TRAIL-R1 and/or TRAIL-R2 expression in a SP1- or NF κ B-dependent manner (88–92,94,97,100). In addition, the cooperative interaction of HDACI and TRAIL has been reported to involve redistribution of agonistic TRAIL receptors to membrane lipid rafts (99) or increased efficacy of TRAIL-induced death-inducing signaling complex (DISC) formation (89,91). Moreover, increased mitochondrial damage (96,97), enhanced expression of proapoptotic molecules (96,97), or downregulation of antiapoptotic proteins, for example, Bcl-X_L, Bcl-2, Mcl-1, FLIP, XIAP, or survivin (89,90,95,97), have been identified to lower the threshold for apoptosis induction by TRAIL in the presence of HDACI. Although HDACI in combination with TRAIL have been described to exert no or little toxicity on non-transformed cells such as peripheral blood lymphocytes or human mammary epithelial cells (93,94,97), there is recent evidence that HDACI may revert the lack of toxicity of TRAIL on non-transformed cells, for example, human renal proximal tubule epithelial cells (100). Thus, the question whether HDACI sensitize for TRAIL-induced apoptosis in a cancer-selective fashion has not yet been answered.

Most studies investigating the TRAIL sensitivity of tumor cells have so far been performed *in vitro* (29). Despite many limitations of cell-culture experiments, *in vitro* studies may reflect some of the important features of tumor treatment *in vivo*. Importantly, experiments performed on primary cancer cells *ex vivo* in most cases yielded similar results. For example, combined treatment of primary cancer cells with TRAIL together with anticancer agents synergized in antitumor activity.

6.4. Tumor Surveillance

There is also mounting evidence for an important role of TRAIL in tumor surveillance, for example, from studies with TRAIL knockout mice (102,103). Although the

biology of the TRAIL system may differ significantly between mice and humans, as there is only one TRAIL receptor in mice, which is homologous to both TRAIL-R1 and TRAIL-R2, the phenotype of these knockout mice is informative with respect to the physiological function of TRAIL *in vivo*. Importantly, TRAIL-deficient mice were more susceptible to tumor metastasis than wild-type mice (103). These data are in accordance with studies showing an important role of NK cells, which constitutively express TRAIL, in the control of tumor metastasis (104,105). In addition, tumor formation induced by carcinogens was found to be enhanced in the presence of antagonistic TRAIL antibodies (104). Thus, TRAIL may play an essential role as innate effector molecule in immune surveillance during tumor formation and progression.

7. CONCLUSIONS

Over the decade key elements of the TRAIL signaling pathway have been identified, and numerous studies provided substantial insights into the molecular mechanisms regulating TRAIL-induced apoptosis. The death ligand TRAIL is of special interest for cancer therapy because of its differential toxicity toward transformed versus normal cells. Importantly, TRAIL strongly synergized together with chemotherapy or irradiation in antitumor activity, even against some resistant forms of cancer.

However, several points remain to be addressed in future studies. As the tumor selective toxicity of TRAIL is presently not fully understood, further preclinical safety testing is necessary to assess the potential toxicity of TRAIL or agonistic TRAIL receptor antibodies on non-malignant tissues. In addition, the possible toxicity of TRAIL on normal tissues under conditions of combined treatment with anticancer agents or irradiation remains to be determined. Also, the molecular mechanisms leading to activation of survival pathways such as NF κ B or AKT upon treatment with TRAIL and their significance for cancer therapy are only partially understood. Moreover, the promise of TRAIL alone or in combination protocols remains to be tested in clinical settings. Future studies on the role of TRAIL in individual tumors both *in vitro* and *in vivo* in tumor cells of patients under chemotherapy, for example, by DNA microarrays or proteomic studies, may provide the basis for “tailored” tumor therapy with TRAIL and may identify new targets for therapeutic interventions.

REFERENCES

1. Hengartner, M. O. The biochemistry of apoptosis. *Nature* 407(6805), 770–6. 2000.
2. Thompson, C. B. Apoptosis in the pathogenesis and treatment of disease. *Science* 267(5203), 1456–62. 1995.
3. Debatin, K. M., Poncet, D., and Kroemer, G. Chemotherapy: targeting the mitochondrial cell death pathway. *Oncogene* 21(57), 8786–803. 2002.
4. Lowe, S. W. and Lin, A. W. Apoptosis in cancer. *Carcinogenesis* 21(3), 485–95. 2000.
5. Johnstone, R. W., Ruefli, A. A., and Lowe, S. W. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 108(2), 153–64. 2002.
6. Thornberry, N. A. and Lazebnik, Y. Caspases: enemies within. *Science* 281(5381), 1312–6. 1998.
7. Igney, F. H. and Krammer, P. H. Immune escape of tumors: apoptosis resistance and tumor counterattack. *Journal of Leukocyte Biology* 71(6), 907–20. 2002.
8. Fulda, S. and Debatin, K. M. Death receptor signaling in cancer therapy. *Current Medicinal Chemistry. Anti-cancer Agents* 1(4), 253–62. 2003.
9. Nagata, S. Apoptotic DNA fragmentation. *Experimental Cell Research* 256(1), 12–8. 2000.

10. Ashkenazi, A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nature Reviews. Cancer* 2(6), 420–30. 2002.
11. van Loo, G., Saelens, X., van Gurp, M., MacFarlane, M., Martin, S. J., and Vandenabeele, P. The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. *Cell Death and Differentiation* 9(10), 1031–42. 2002.
12. Salvesen, G. S. and Renatus, M. Apoptosome: the seven-spoked death machine. *Developmental Cell* 2(3), 256–7. 2002.
13. Cory, S. and Adams, J. M. The Bcl2 family: regulators of the cellular life-or-death switch. *Nature Reviews. Cancer* 2(9), 647–56. 2002.
14. Cowling, V. and Downward, J. Caspase-6 is the direct activator of caspase-8 in the cytochrome c-induced apoptosis pathway: absolute requirement for removal of caspase-6 prodomain. *Cell Death and Differentiation* 9(10), 1046–56. 2002.
15. Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C. P., Nicholl, J. K., Sutherland, G. R., Smith, T. D., Rauch, C., Smith, C. A., and et al. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 3(6), 673–82. 1995.
16. Marsters, S. A., Pitti, R. M., Donahue, C. J., Ruppert, S., Bauer, K. D., and Ashkenazi, A. Activation of apoptosis by Apo-2 ligand is independent of FADD but blocked by CrmA. death and decoy receptors. *Current Biology* 6, 750–752. 1996.
17. Pan, G., Ni, J., Wei, Y. F., Yu, G., Gentz, R., and Dixit, V. M. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* 277(5327), 815–8. 1997.
18. Chaudhary, P. M., Eby, M., Jasmin, A., Bookwalter, A., Murray, J., and Hood, L. Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-kappaB pathway. *Immunity* 7(6), 821–30. 1997.
19. MacFarlane, M., Ahmad, M., Srinivasula, S. M., Fernandes-Alnemri, T., Cohen, G. M., and Alnemri, E. S. Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. *The Journal of Biological Chemistry* 272(41), 25417–20. 1997.
20. Walczak, H., Degli-Esposti, M. A., Johnson, R. S., Smolak, P. J., Waugh, J. Y., Boiani, N., Timour, M. S., Gerhart, M. J., Schooley, K. A., Smith, C. A., Goodwin, R. G., and Rauch, C. T. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *The EMBO Journal* 16(17), 5386–97. 1997.
21. Wu, G. S., Burns, T. F., McDonald, E. R., 3rd, Jiang, W., Meng, R., Krantz, I. D., Kao, G., Gan, D. D., Zhou, J. Y., Muschel, R., Hamilton, S. R., Spinner, N. B., Markowitz, S., Wu, G., and el-Deiry, W. S. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nature Genetics* 17(2), 141–3. 1997.
22. Degli-Esposti, M. A., Smolak, P. J., Walczak, H., Waugh, J., Huang, C. P., DuBose, R. F., Goodwin, R. G., and Smith, C. A. Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. *The Journal of Experimental Medicine* 186(7), 1165–70. 1997.
23. Pan, G., O'Rourke, K., Chinnaiyan, A. M., Gentz, R., Ebner, R., Ni, J., and Dixit, V. M. The receptor for the cytotoxic ligand TRAIL. *Science* 276(5309), 111–3. 1997.
24. Marsters, S. A., Sheridan, J. P., Pitti, R. M., Huang, A., Skubatch, M., Baldwin, D., Yuan, J., Gurney, A., Goddard, A. D., Godowski, P., and Ashkenazi, A. A novel receptor for Apo2L/TRAIL contains a truncated death domain. *Current Biology* 7(12), 1003–6. 1997.
25. Pan, G., Ni, J., Yu, G., Wei, Y. F., and Dixit, V. M. TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signalling. *FEBS Letters* 424(1–2), 41–5. 1998.
26. Degli-Esposti, M. A., Dougall, W. C., Smolak, P. J., Waugh, J. Y., Smith, C. A., and Goodwin, R. G. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity* 7(6), 813–20. 1997.
27. Emery, J. G., McDonnell, P., Burke, M. B., Deen, K. C., Lyn, S., Silverman, C., Dul, E., Appelbaum, E. R., Eichman, C., DiPrinzio, R., Dodds, R. A., James, I. E., Rosenberg, M., Lee, J. C., and Young, P. R. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *The Journal of Biological Chemistry* 273(23), 14363–7. 1998.
28. LeBlanc, H. N. and Ashkenazi, A. Apo-2L/TRAIL and its death and decoy receptors. *Cell Death and Differentiation* 10, 66–75. 2003.
29. Wajant, H., Pfizenmaier, K., and Scheurich, P. TNF-related apoptosis inducing ligand (TRAIL) and its receptors in tumor surveillance and cancer therapy. *Apoptosis* 7, 449–59. 2002.
30. Walczak, H. and Krammer, P. H. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Experimental Cell Research* 256(1), 58–66. 2000.

31. Ozoren, N. and El-Deiry, W. S. Cell surface death receptor signaling in normal and cancer cells. *Seminars in Cancer Biology* 13(2), 135–47. 2003.
32. Sprick, M. R., Weigand, M. A., Rieser, E., Rauch, C. T., Juo, P., Blenis, J., Krammer, P. H., and Walczak, H. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity* 12(6), 599–609. 2000.
33. Kischkel, F. C., Lawrence, D. A., Chuntharapai, A., Schow, P., Kim, K. J., and Ashkenazi, A. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity* 12(6), 611–20. 2000.
34. Bodmer, J. L., Holler, N., Reynard, S., Vinciguerra, P., Schneider, P., Juo, P., Blenis, J., and Tschopp, J. TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nature Cell Biology* 2(4), 241–3. 2000.
35. Kischkel, F. C., Lawrence, D. A., Tinel, A., LeBlanc, H., Virmani, A., Schow, P., Gazdar, A., Blenis, J., Arnott, D., and Ashkenazi, A. Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. *The Journal of Biological Chemistry* 276(49), 46639–46. 2001.
36. Sprick, M. R., Rieser, E., Stahl, H., Grosse-Wilde, A., Weigand, M. A., and Walczak, H. Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. *The EMBO Journal* 21(17), 4520–30. 2002.
37. Lee, S. H., Shin, M. S., Kim, H. S., Lee, H. K., Park, W. S., Kim, S. Y., Lee, J. H., Han, S. Y., Park, J. Y., Oh, R. R., Kang, C. S., Kim, K. M., Jang, J. J., Nam, S. W., Lee, J. Y., and Yoo, N. J. Somatic mutations of TRAIL-receptor 1 and TRAIL-receptor 2 genes in non-Hodgkin's lymphoma. *Oncogene* 20(3), 399–403. 2001.
38. Lee, S. H., Shin, M. S., Kim, H. S., Lee, H. K., Park, W. S., Kim, S. Y., Lee, J. H., Han, S. Y., Park, J. Y., Oh, R. R., Jang, J. J., Han, J. Y., Lee, J. Y., and Yoo, N. J. Alterations of the DR5/TRAIL receptor 2 gene in non-small cell lung cancers. *Cancer Research* 59(22), 5683–6. 1999.
39. Arai, T., Akiyama, Y., Okabe, S., Saito, K., Iwai, T., and Yuasa, Y. Genomic organization and mutation analyses of the DR5/TRAIL receptor 2 gene in colorectal carcinomas. *Cancer Letters* 133(2), 197–204. 1998.
40. Shin, M. S., Kim, H. S., Lee, S. H., Park, W. S., Kim, S. Y., Park, J. Y., Lee, J. H., Lee, S. K., Lee, S. N., Jung, S. S., Han, J. Y., Kim, H., Lee, J. Y., and Yoo, N. J. Mutations of tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and receptor 2 (TRAIL-R2) genes in metastatic breast cancers. *Cancer Research* 61(13), 4942–6. 2001.
41. Pai, S. I., Wu, G. S., Ozoren, N., Wu, L., Jen, J., Sidransky, D., and El-Deiry, W. S. Rare loss-of-function mutation of a death receptor gene in head and neck cancer. *Cancer Research* 58(16), 3513–8. 1998.
42. van Noesel, M. M., van Bezouw, S., Salomons, G. S., Voute, P. A., Pieters, R., Baylin, S. B., Herman, J. G., and Versteeg, R. Tumor-specific down-regulation of the tumor necrosis factor-related apoptosis-inducing ligand decoy receptors Dcr1 and Dcr2 is associated with dense promoter hypermethylation. *Cancer Research* 62(7), 2157–61. 2002.
43. Krueger, A., Baumann, S., Krammer, P. H., and Kirchhoff, S. FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. *Molecular and Cellular Biology* 21(24), 8247–54. 2001.
44. Burns, T. F. and El-Deiry, W. S. Identification of inhibitors of TRAIL-induced death (ITIDs) in the TRAIL sensitive colon carcinoma cell line SW480 using a genetic approach. *The Journal of Biological Chemistry* 276, 37879–86. 2001.
45. Hersey, P. and Zhang, X. D. How melanoma cells evade trail-induced apoptosis. *Nature Reviews. Cancer* 1(2), 142–50. 2001.
46. Fulda, S., Meyer, E., and Debatin, K. M. Metabolic inhibitors sensitize for CD95 (APO-1/Fas)-induced apoptosis by down-regulating Fas-associated death domain-like interleukin 1-converting enzyme inhibitory protein expression. *Cancer Research* 60(14), 3947–56. 2000.
47. Siegmund, D., Hadwiger, P., Pfizenmaier, K., Vornlocher, H. P., and Wajant, H. Selective inhibition of FLICE-like inhibitory protein expression with small interfering RNA oligonucleotides is sufficient to sensitize tumor cells for TRAIL-induced apoptosis. *Molecular Medicine* 8(11), 725–32. 2002.
48. Kim, Y., Suh, N., Sporn, M., and Reed, J. C. An inducible pathway for degradation of FLIP protein sensitizes tumor cells to TRAIL-induced apoptosis. *The Journal of Biological Chemistry* 277(25), 22320–9. 2002.

49. Munshi, A., Pappas, G., Honda, T., McDonnell, T. J., Younes, A., Li, Y., and Meyn, R. E. TRAIL (APO-2L) induces apoptosis in human prostate cancer cells that is inhibitable by Bcl-2. *Oncogene* 20(29), 3757–65. 2001.
50. Hinz, S., Trauzold, A., Boenicke, L., Sandberg, C., Beckmann, S., Bayer, E., Walczak, H., Kalthoff, H., and Ungefroren, H. Bcl-XL protects pancreatic adenocarcinoma cells against CD95- and TRAIL-receptor-mediated apoptosis. *Oncogene* 19(48), 5477–86. 2000.
51. Deng, Y., Lin, Y., and Wu, X. TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. *Genes & Development* 16, 33–45. 2002.
52. Walczak, H., Bouchon, A., Stahl, H., and Krammer, P. H. Tumor necrosis factor related apoptosis-inducing ligand retains its apoptosis-inducing capacity on Bcl-2 or Bcl-XL expressing chemotherapy-resistant tumor cells. *Cancer Research* 60, 3051–7. 2000.
53. Salvesen, G. S. and Duckett, C. S. IAP proteins: blocking the road to death's door. *Nature Reviews. Molecular Cell Biology* 3(6), 401–10. 2002.
54. Altieri, D. C. Validating survivin as a cancer therapeutic target. *Nature Reviews. Cancer* 3(1), 46–54. 2003.
55. Fulda, S., Meyer, E., and Debatin, K. M. Inhibition of TRAIL-induced apoptosis by Bcl-2 overexpression. *Oncogene* 21(15), 2283–94. 2002.
56. Ng, C. P. and Bonavida, B. X-linked inhibitor of apoptosis (XIAP) blocks Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis of prostate cancer cells in the presence of mitochondrial activation: sensitization by overexpression of second mitochondria-derived activator of caspase/direct IAP-binding protein with low pl (Smac/DIABLO). *Molecular Cancer Therapeutics* 1(12), 1051–8. 2002.
57. Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. NF-kappaB in cancer: from innocent bystander to major culprit. *Nature Reviews. Cancer* 2(4), 301–10. 2002.
58. Ravi, R., Bedi, G. C., Engstrom, L. W., Zeng, Q., Mookerjee, B., Gelinas, C., Fuchs, E. J., and Bedi, A. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-kappaB. *Nature Cell Biology* 3(4), 409–16. 2001.
59. Baetu, T. M., Kwon, H., Sharma, S., Grandvaux, N., and Hiscott, J. Disruption of NF-kappaB signaling reveals a novel role for NF-kappaB in the regulation of TNF-related apoptosis-inducing ligand expression. *Journal of Immunology* 167(6), 3164–73. 2001.
60. Schneider, P., Thome, M., Burns, K., Bodmer, J. L., Hofmann, K., Kataoka, T., Holler, N., and Tschopp, J. TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. *Immunity* 7(6), 831–6. 1997.
61. Harper, N., Farrow, S. N., Kaptein, A., Cohen, G. M., and MacFarlane, M. Modulation of tumor necrosis factor apoptosis-inducing ligand-induced NF-kappa B activation by inhibition of apical caspases. *The Journal of Biological Chemistry* 276(37), 34743–52. 2001.
62. Erhardt, H., Fulda, S., Schmid, I., Hiscott, J., Debatin, K. M., and Jeremias, I. TRAIL induced survival and proliferation in cancer cells resistant towards TRAIL-induced apoptosis mediated by NF-kappaB. *Oncogene* 22(25), 3842–52. 2003.
63. Jeremias, I., Kupatt, C., Baumann, B., Herr, I., Wirth, T., and Debatin, K. M. Inhibition of nuclear factor kappaB activation attenuates apoptosis resistance in lymphoid cells. *Blood* 91(12), 4624–31. 1998.
64. El-Deiry, W. S. Insights into cancer therapeutic design based on p53 and TRAIL receptor signaling. *Cell Death and Differentiation* 8(11), 1066–75. 2001.
65. Ashkenazi, A., Pai, R. C., Fong, S., Leung, S., Lawrence, D. A., Marsters, S. A., Blackie, C., Chang, L., McMurtrey, A. E., Hebert, A., DeForge, L., Koumenis, I. L., Lewis, D., Harris, L., Bussiere, J., Koeppen, H., Shahrokh, Z., and Schwall, R. H. Safety and antitumor activity of recombinant soluble Apo2 ligand. *The Journal of Clinical Investigation* 104(2), 155–62. 1999.
66. Lawrence, D., Shahrokh, Z., Marsters, S., Achilles, K., Shih, D., Mounho, B., Hillan, K., Totpal, K., DeForge, L., Schow, P., Hooley, J., Sherwood, S., Pai, R., Leung, S., Khan, L., Gliniak, B., Bussiere, J., Smith, C. A., Strom, S. S., Kelley, S., Fox, J. A., Thomas, D., and Ashkenazi, A. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. *Nature Medicine* 7(4), 383–5. 2001.
67. Jo, M., Kim, T. H., Seol, D. W., Espen, J. E., Dorko, K., Billiar, T. R., and Strom, S. C. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. *Nature Medicine* 6(5), 564–7. 2000.

68. Nitsch, R., Bechmann, I., Deisz, R. A., Haas, D., Lehmann, T. N., Wendling, U., and Zipp, F. Human brain-cell death induced by tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL). *Lancet* 356(9232), 827–8. 2000.
69. Ichikawa, K., Liu, W., Zhao, L., Wang, Z., Liu, D., Ohtsuka, T., Zhang, H., Mountz, J. D., Koopman, W. J., Kimberly, R. P., and Zhou, T. Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. *Nature Medicine* 7(8), 954–60. 2001.
70. Chuntharapai, A., Dodge, K., Grimmer, K., Schroeder, K., Marsters, S. A., Koeppen, H., Ashkenazi, A., and Kim, K. J. Isotype-dependent inhibition of tumor growth *in vivo* by monoclonal antibodies to death receptor 4. *Journal of Immunology* 166(8), 4891–8. 2001.
71. Rohn, T. A., Wagenknecht, B., Roth, W., Naumann, U., Gulbins, E., Krammer, P. H., Walczak, H., and Weller, M. CCNU-dependent potentiation of TRAIL/Apo2L-induced apoptosis in human glioma cells is p53-independent but may involve enhanced cytochrome c release. *Oncogene* 20(31), 4128–37. 2001.
72. Nagane, M., Pan, G., Weddle, J. J., Dixit, V. M., Cavenee, W. K., and Huang, H. J. Increased death receptor 5 expression by chemotherapeutic agents in human gliomas causes synergistic cytotoxicity with tumor necrosis factor-related apoptosis-inducing ligand *in vitro* and *in vivo*. *Cancer Research* 60(4), 847–53. 2000.
73. Gliniak, B. and Le, T. Tumor necrosis factor-related apoptosis-inducing ligand's antitumor activity *in vivo* is enhanced by the chemotherapeutic agent CPT-11. *Cancer Research* 59(24), 6153–8. 1999.
74. Keane, M. M., Rubinstein, Y., Cuello, M., Etenberg, S. A., Banerjee, P., Nau, M. M., and Lipkowitz, S. Inhibition of NF-kappaB activity enhances TRAIL mediated apoptosis in breast cancer cell lines. *Breast Cancer Research and Treatment* 64(2), 211–9. 2000.
75. Belka, C., Schmid, B., Marini, P., Durand, E., Rudner, J., Faltin, H., Bamberg, M., Schulze-Osthoff, K., and Budach, W. Sensitization of resistant lymphoma cells to irradiation-induced apoptosis by the death ligand TRAIL. *Oncogene* 20(17), 2190–6. 2001.
76. Chinnaiyan, A. M., Prasad, U., Shankar, S., Hamstra, D. A., Shanaiah, M., Chenevert, T. L., Ross, B. D., and Rehemtulla, A. Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. *Proceedings of the National Academy of Sciences of the United States of America* 97(4), 1754–9. 2000.
77. Meng, R. D. and El-Deiry, W. S. p53-independent upregulation of KILLER/DR5 TRAIL receptor expression by glucocorticoids and interferon-gamma. *Experimental Cell Research* 262(2), 54–69. 2001.
78. Takimoto, R. and El-Deiry, W. S. Wild-type p53 transactivates the KILLER/DR5 gene through an intronic sequence-specific DNA-binding site. *Oncogene* 19(14), 1735–43. 2000.
79. Meng, R. D., McDonald, E. R., 3rd, Sheikh, M. S., Fornace, A. J., Jr., and El-Deiry, W. S. The TRAIL decoy receptor TRUNDD (DcR2, TRAIL-R4) is induced by adenovirus-p53 overexpression and can delay TRAIL-, p53-, and KILLER/DR5-dependent colon cancer apoptosis. *Molecular Therapy: the Journal of the American Society of Gene Therapy* 1(2), 130–44. 2000.
80. Olsson, A., Diaz, T., and Aguilar-Santelises, M. Sensitization to TRAIL-induced apoptosis and modulation of FLICE-inhibitory protein in B chronic lymphocytic leukemia by actinomycin D. *Leukemia* 15, 1868–77. 2001.
81. Micheau, O., Hammann, A., Solary, E., and Dimanche-boitrel, M. T. Stat-1-independent upregulation of FADD and procaspase-3 and -8 in cancer cells treated with cytotoxic drugs. *Biochemical and Biophysical Research Communications* 256(3), 603–7. 1999.
82. Teitz, T., Wei, T., Valentine, M. B., Vanin, E. F., Grenet, J., Valentine, V. A., Behm, F. G., Look, A. T., Lahti, J. M., and Kidd, V. J. Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN [comment]. *Nature Medicine* 6(5), 529–35. 2000.
83. Fulda, S., Kufer, M. U., Meyer, E., van Valen, F., Dockhorn-Dworniczak, B., and Debatin, K. M. Sensitization for death receptor- or drug-induced apoptosis by re-expression of caspase-8 through demethylation or gene transfer. *Oncogene* 20(41), 5865–77. 2001.
84. Fulda, S. and Debatin, K. M. IFNgamma sensitizes for apoptosis by upregulating caspase-8 expression through the Stat1 pathway. *Oncogene* 21(15), 2295–308. 2002.
85. Fulda, S., Wick, W., Weller, M., and Debatin, K. M. Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma *in vivo*. *Nature Medicine* 8(8), 808–15. 2002.
86. Jenuwein, T. and Allis, C. D. Translating the histone code. *Science* 293, 1074–1080. 2001.

87. Marks, P., Rifkind, R. A., Richon, V. M., Breslow, R., Miller, T., and Kelly, W. K. Histone deacetylases and cancer: causes and therapies. *Nature Reviews. Cancer* 1, 194–202. 2001.
88. Chopin, V., Slomianny, C., Hondermarck, H., and Le Bourhis, X. Synergistic induction of apoptosis in breast cancer cells by cotreatment with butyrate and TNF-alpha, TRAIL, or anti-Fas agonist antibody involves enhancement of death receptors' signaling and requires P21waf1. *Experimental Cell Research* 298, 560–73. 2004.
89. Guo, F., Sigua, C., Tao, J., Bali, P., George, P., Li, Y., Wittmann, S., Moscinski, L., Atadja, P., and Bhalla, K. Cotreatment with histone deacetylase inhibitor LAQ824 enhances Apo-2L/tumor necrosis factor-related apoptosis inducing ligand-induced death inducing signaling complex activity and apoptosis of human acute leukemia cells. *Cancer Research* 64, 2580–9. 2004.
90. Hernandez, A., Thomas, R., Smith, F., Sandberg, J., Kim, S., Chung, D. H., and Evers, B. M. Butyrate sensitizes human colon cancer cells to TRAIL-mediated apoptosis. *Surgery* 130, 265–72. 2001.
91. Inoue, S., MacFarlane, M., Harper, N., Wheat, L. M., Dyer, M. J., and Cohen, G. M. Histone deacetylase inhibitors potentiate TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in lymphoid malignancies. *Cell Death and Differentiation* 11, S193–206. 2004.
92. Kim, Y. H., Park, J. W., Lee, J. Y., and Kwon, T. K. Sodium butyrate sensitizes TRAIL-mediated apoptosis by induction of transcription from the DR5 gene promoter through Sp1 sites in colon cancer cells. *Carcinogenesis* 25, 1813–20. 2004.
93. MacFarlane, M., Inoue, S., Kohlhaas, S. L., Majid, A., Harper, N., Kennedy, D. B. J., Dyer, M. J. S., and Cohen, G. M. Chronic lymphocytic leukemic cells exhibit apoptotic signaling via TRAIL-R1. *Cell Death and Differentiation* 12, 773–82. 2005.
94. Nakata, S., Yoshida, T., Horinaka, M., Shiraishi, T., Wakada, M., and Sakai, T. Histone deacetylase inhibitors upregulate death receptor 5/TRAIL-R2 and sensitize apoptosis induced by TRAIL/APO2-L in human malignant tumor cells. *Oncogene* 23, 6261–71. 2004.
95. Neuzil, J., Swettenham, E., and Gellert, N. Sensitization of mesothelioma to TRAIL apoptosis by inhibition of histone deacetylase: role of Bcl-xL down-regulation. *Biochemical and Biophysical Research Communications* 314, 186–91. 2004.
96. Rosato, R. R., Almenara, J. A., Dai, Y., and Grant, S. Simultaneous activation of the intrinsic and extrinsic pathways by histone deacetylase (HDAC) inhibitors and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) synergistically induces mitochondrial damage and apoptosis in human leukemia cells. *Molecular Cancer Therapeutics* 2, 1273–84. 2003.
97. Singh, T. R., Shankar, S., and Srivastava, R. K. HDAC inhibitors enhance the apoptosis-inducing potential of TRAIL in breast carcinoma. *Oncogene* 24, 4609–23. 2005.
98. Sonnemann, J., Gange, J., Kumar, K. S., Muller, C., Bader, P., and Beck, J. F. Histone deacetylase inhibitors interact synergistically with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to induce apoptosis in carcinoma cell lines. *Investigational New Drugs* 23, 99–109. 2005.
99. VanOosten, R. L., Moore, J. M., Ludwig, A. T., and Griffith, T. S. Depsipeptide (FR901228) enhances the cytotoxic activity of TRAIL by redistributing TRAIL receptor to membrane lipid rafts. *Molecular Therapy: the Journal of the American Society of Gene Therapy* 11, 542–52. 2005.
100. Vanoosten, R. L., Moore, J. M., Karacay, B., and Griffith, T. S. Histone deacetylase inhibitors modulate renal cell carcinoma sensitivity to TRAIL/Apo-2L-induced apoptosis by enhancing TRAIL-R2 expression. *Cancer Biology & Therapy* 4(10), 1104–12. 2005.
101. Zhang, X. D., Gillespie, S. K., Borrow, J. M., and Hersey, P. The histone deacetylase inhibitor suberic bishydroxamate: a potential sensitizer of melanoma to TNF-related apoptosis-inducing ligand (TRAIL) induced apoptosis. *Biochemical Pharmacology* 66, 1537–45. 2003.
102. Smyth, M. J., Takeda, K., Hayakawa, Y., Peschon, J. J., van den Brink, M. R., and Yagita, H. Nature's TRAIL—on a path to cancer immunotherapy. *Immunity* 18(1), 1–6. 2003.
103. Cretney, E., Takeda, K., Yagita, H., Glaccum, M., Peschon, J. J., and Smyth, M. J. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. *Journal of Immunology* 168(3), 1356–61. 2002.
104. Takeda, K., Hayakawa, Y., Smyth, M. J., Kayagaki, N., Yamaguchi, N., Kakuta, S., Iwakura, Y., Yagita, H., and Okumura, K. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nature Medicine* 7(1), 94–100. 2001.
105. Smyth, M. J., Cretney, E., Takeda, K., Wiltrott, R. H., Sedger, L. M., Kayagaki, N., Yagita, H., and Okumura, K. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. *The Journal of Experimental Medicine* 193(6), 661–70. 2001.

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