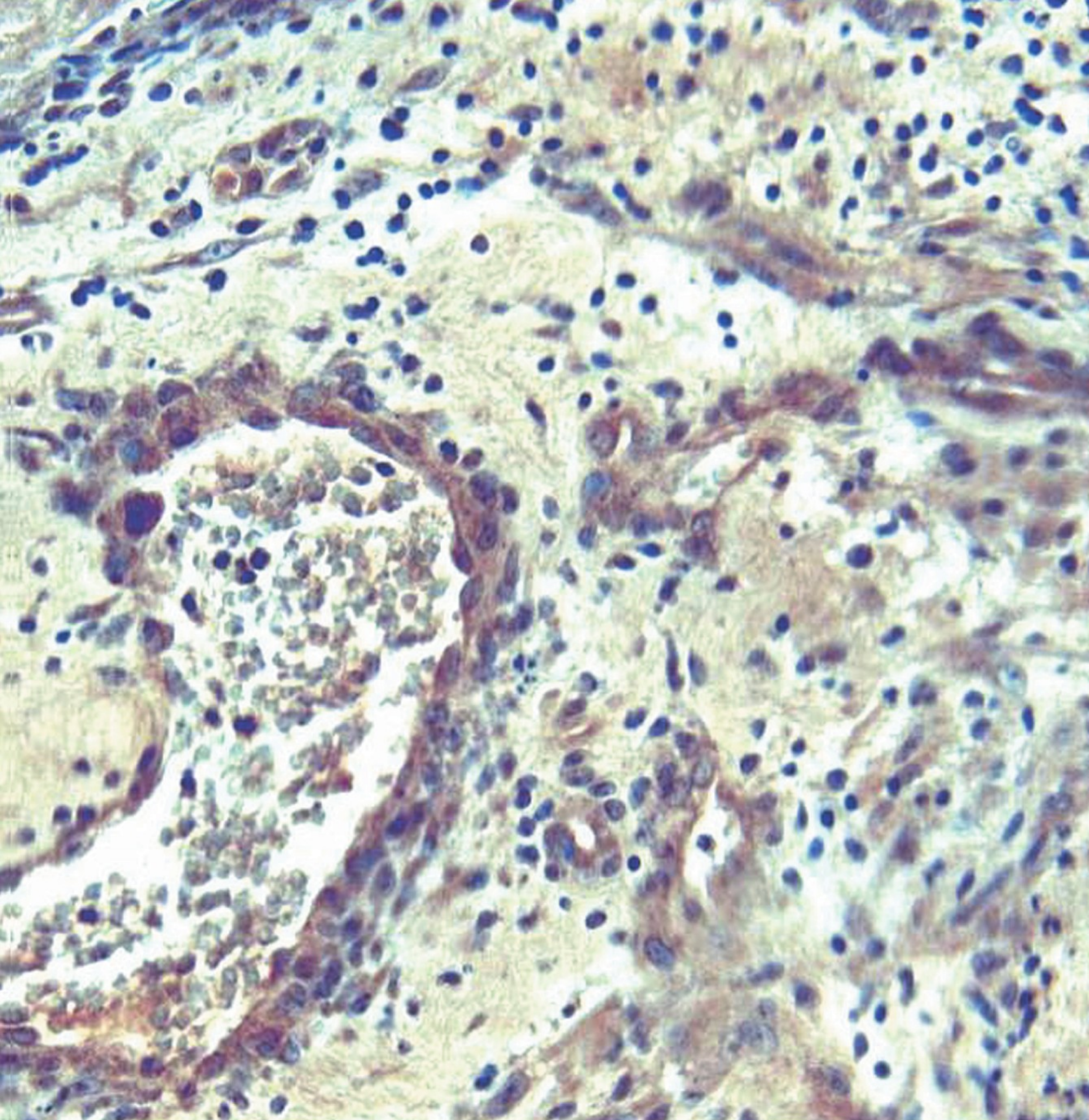

APOPTOTIC PATHWAYS AS TARGETS FOR NOVEL THERAPIES IN CANCER AND OTHER DISEASES

Edited by

MAREK LOS and SPENCER B. GIBSON

Apoptotic Pathways as Targets for Novel Therapies in Cancer and Other Diseases

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We would like to dedicate this publication to our wives (Wioletta and Erika) for their continued support and love. We would also like to thank all our laboratory personnel whom without their support and dedication this publication will not be possible.

PREFACE

This book is mainly aimed towards students and researchers investigating new strategies to be used to combat cancer, stroke, myocardial infarction, or various autoimmune diseases. It should assist in the transfer of knowledge as well as shaping new ideas between different fields of pharmacologic research. Due to its compactness and partitioning into small, self-explanatory units, it may serve policy makers well when seeking a compact overview of recent developments of new therapeutics in selected medical fields. The format of this book may also be of benefit to educated patients and their families willing (or seeking) to learn more about current and upcoming clinical trials involving specific groups of diseases. Thus, readers who do not work in the field of programmed cell death, students, and those with proficiency in selected subjects will be able to find quickly the information they are looking for. The sizable index and tabular summary of the strategies being pursued by the pharmacologic industry are meant to assist fast location of specific information.

To achieve the goal we were assisted by experts from various areas of biology and molecular medicine known for their excellence. These experts conducted research involving clinical and pharmacologic aspects of the apoptotic process. At this point we would like to thank them for their creative involvement and patience while handling our queries and emendations. We would also like to extend our thanks to the publishing team for their assistance and rapidity in publication.

Recent deciphering of human and a number of other mammalian genomes, in concert with the tremendous progress in our understanding of intracellular signaling pathways, fuels the current development of new pharmacologic strategies in an array of research fields. Apoptotic cell death is indispensable for the development and homeostasis of complex organisms, as well as for immune defense and immunoregulation. Therefore, it is not surprising that the vast majority of tested strategies used to combat cancer, stroke, myocardial infarction and even some autoimmune diseases, target apoptotic pathways. The key developments in this field was the discovery of membrane receptors that when triggered, induce cell death and the identification *c*ysteine-dependent ***asp***art***ases*** (*caspases*) as important downstream signaling molecules. Both findings helped to elucidate important questions in various areas of biology, especially the morpho-, organogenseis, oncogenesis and the regulation of the immune system. Caspase family

proteases participate in the key signaling and executioner events of apoptosis (see chapters I-III, IV-VI, IX, X and XV). Thus, together with the battery of kinases that regulate cell growth and cell death processes (portrayed mainly in chapter VIII), and an array of transcription factors and their regulators (chapters VII, XII and XIV), they are among the "hottest" targets for pharmacologic intervention in an array of pathologies. The prototype of the caspase family, Interleukin-1β converting enzyme (ICE, now termed caspase-1), had been originally identified as a cytokine maturating enzyme, provides a clear link between apoptotic processes and the regulation of the immune system. This link is discussed mainly in chapter IX, particularly how both processes can be targeted to treat autoimmune diseases.

Some chapters possess a more tutorial/supportive role. The first chapter serves as an introduction. It compactly covers most of the research being performed in the apoptotic field. Therefore, even readers unfamiliar to the subject can obtain a solid foundation and become accustomed with the basic terms used in further parts of the book. Special attention is given to the usage of antibodies (chapter XI) or RNA-interference (chapter XIII) as therapeutic approaches. However, not only are novel targets discussed, but also tumor specific drug targeting, the *"magic bullet approach"* has been illustrated in this book (chapter XVI). The last two chapters follow conceptually along the same line, but from the opposite direction. They broadly discuss old and new aspects and strategies that (may) achieve specific toxicity of therapeutic interventions when applied to fight cancer. These two chapters not only tackle the important aspects of adverse effects, but also largely substantiate the current strategies and the obstacles faced in cancer therapy. Finally chapter XV provides basic information and "cooking prescriptions" for the detection of apoptosis and the monitoring of drug sensitivity both *in vitro* and *in vivo*.

We hope that this efficiently written compendium will be of use to researchers from various fields of biology, pharmacology and medicine, helping to familiarize or broaden the knowledge of drug targets offered by programmed cell death in diverse biological processes.

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ABBREVIATIONS

53BP2	bcl-2 binding protein
5-FC	5-fluorocytosine
5-FU	5-fluorouracil
7-AAD	7-amino-actinomycin D
a.a.	aminoacids
AA	arachidonic acid
ABL	abelson kinase
Ac	Acetyl
ADEPT	antibody directed enzyme prodrug therapy
AES	amino-terminal enhancer of split
AFC	7-amino-4-trifluoromethylcoumarin
AICD	activation induced cell death
AIF	apoptosis inducing factor
AKT	protein kinase B
ALL	acute lymphocytic leukemia
ALLnL	N -acetyl-leucyl-leucyl norlucinal
ALS	amylotrophic lateral sclerosis
ALT	alanine aminotransferase
AMC	7-amino-4-methylcoumarin
AML	acute myeloid leukemia
ANP	atrial natriuretic peptide
ANT	adenosine nucleotide translocation
Apaf-1	apoptotic protease activating factor-1
APC	antigen presenting cell
Ara-c	1-β-D-arabinofuranosyl-cytosine-5-fluorouracil
ARC	apoptosis repressor with casapase recruitment domain
AS	splice acceptor site
ASK	apoptosis signaling kinase 1
ASO	antisense oligonucleotides
ATM	alaxia-telangiectasia mutant
ATP	adenosine triphosphate
ATR	rad3-related
Bap31	B cell antigen receptor-associated protein 31
Bcl-2	B-cell lymphoma gene 2

BCR	breakpoint cluster region
BH	bcl-2 homology
BH3	bcl-2 homology domain 3
BH3I	BH3 inhibitors
BHK	baby hamster kidney
Bid	BH3 domain-only death agonist protein
BIR	baculovirus inhibition of apoptosis protein repeat / baculoviral IAP repeat
Boc-D-fmk	t-Butyloxycarbonyl-aspartate-fluoromethylketone
BrP	branch-point domain
C.elegans	*Caenorhabditis elegans*
CAD	caspase-activated DNase
cAMP	cyclic AMP
CAPE	caffeine acid phenylether ester
CARD	caspase recruitment domain
CARP	caspase-8 and -10 associated RING protein
Caspase	cysteinyl aspartate-specific protease / cysteinyl aspartase
CBP	CREB-binding protein
CCKB	cholecystokinin b
CD	cytosine deaminase
CD40L	CD40 ligand
CD95	Custer of differentiation antigen 95
CDK	cyclin-dependent kinase
Cdki	cyclin-dependent kinase inhibitor
cDNA	complementary DNA
Ced-	cell death defective-
CFU-c	colony forming unit-culture
CHO	chinese hamster ovary
CI/WR	cold ischemia/warm reperfusion
c-IAP	cellular inhibition of apoptosis protein
CII	collagen type II
CLL	chronic lymphocytic leukemia
-cmk	choro-methylketone
CML	chronic myelogenous leukemia
COX-2	cyclooxygenase-2
CRF	corticotropin releasing factor
CrmA	cytokine Response Modifier A
CTL	cytotoxic T-lymphocyte
Cul	cullin
DAPK	death associated protein kinase
dATP	deoxyadenosine triphosphate
DC	dendritic cells
DcR	decoy receptor
DD	death domain
DED	death effector domain
DFF-45	DNA fragmentation factor 45 kDa subunit

DIABLO	direct IAP binding protein with low pI (Smac)
Dicer	RNase III-familial endoribonuclease
DISC	death inducing signaling complex
DLC1	dynein light chain 1
DN	dominant negative
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNM	dominant negative mutant
DOTATOC	$DOTA^0$-D-Phe^1-Tyr^3-octreotide
DPPE	N,N diethyl-2-[4-(phenylmethyl) phenoxy] ethanamine
DR	death receptor
D-RNA	hybrids of messenger RNA and complementary DNA
D-RNAi	cDNA-mRNA hybrid-induced RNA interference
DRP	DAPK related protein
DS	splice donor site
dsRNA	double-stranded RNA
DTT	dithiothreitol
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
E6-AP	E6-associated protein
EAE	experimental induced auto-immune encephalomyelitis
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF2-α	eukaryotic initiation factor 2-α
ELAM-1	endothelial leukocyte adhesion molecule-1
EndoG	endonuclease G
EPO	erythropoietin
ER	endoplasmatic reticulum
ERK	extracellular signal-regulated kinase
FADD	fas receptor associated death domain
FDG	2-[^{18}F]-2-deoxy-D-glucose
FGF	fibroblast Growth Factor
FGFR	fibroblast growth factor receptor
FLICE	FADD-like Interleukin-1β converting enzyme
FLIP	FLICE-inhibitory protein
-fmk	-fluoromethylketone
FP	fluorescence polarization
GAP	GTPase activating protein
GDP	guanosine diphosphate
GF	growth factor
GFP	green fluorescent protein
GLUT 1	glucose transporter 1
GML	GPI-anchored molecule-like protein
GNRP	guanyl nucleotide-release protein

GRP	gastrin releasing poly-peptide
GTP	guanosine triphosphate
HDAC	histone deacetylases
HECT	homologous to E6AP carboxy terminus
HER	human epidermal growth factor receptor
HGF	hepatocycte Growth Factor
HGFA	HGF activator
HHV	human herpesvirus
HIV	human immunodeficiency virus
HPC	hematopoietic progenitor cells
HSF	heat shock factor
HSP	heat shock protein
HSV-tk	herpes simplex virus thymidine kinase
HUVECs	human umbilical vein endothelial cells
IκBα	inhibitor of NF-κB
IAP	inhibition of apoptosis protein
ICAD	inhibitor of caspase-activated DNase
ICAM	intercellular adhesion molecule
ICE	interleukin-1β converting enzyme
IFN	interferon
IGF	insulin-like growth factor
IGFBP	IGF-binding protein
IKK	inhibitor of NF-κB kinase
IKKAP1	IKK-associated protein 1
IL	interleukin
IL-1β	interleukin-1β
IRS-1	IGF receptor substrates
ITGb1	integrin β1 gene
JNK	c-Jun N-terminal kinase
kDa	kilodalton
KIP	kinase inhibitor protein
LHRH	luteinizing hormone releasing hormone
LHRH	luteinizing hormone releasing hormone
LPA	lysophosphatidic acid
LPS	lipopolysaccharide
LT	lymphotoxin
LTα	lympholoxin
mAb	monoclonal antibodies
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
Mdm	murine double minute
MDR	multiple drug resistance
MEF	mouse embryonic fibroblast
MEKK	mitogen-activated protein kinase kinase kinase
MELAS	mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes

MHC	major histocompatibility complex
miRNA	microRNA
MKP	mitogen activated protein kinase phosphatase
MM	multiple myeloma
MMP	matrix metalloproteinase
M-MP	mitochondrial membrane permeablization
MMR	mis-match repair
MPC	multicatalytic proteinase complex
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
MRP	multidrug resistance associated protein
NAD^+	nicotine amide dinucleotide
NAIP	neuronal apoptosis inhibitory protein
NEMO	NF-κB essential modulator
NF-κB	nuclear factor κB
NGF	nerve growth factor
NHL	non-Hodgkin's-lymphoma
NIK	NF-kB-inducing kinase
nM	nanomolar
NMD	non-sense mRNA degradation
NMDA	N-methyl-D-aspartic acid
NMR	nuclear magnetic resonance
NO	nitric oxide
NOS	nitric oxide synthase
NRADD	neurotrophin receptor like death domain
NSAID	non-steroidal anti-inflammatory agent
NSCLC	non-small cell lung cancer
NT	neurotensin
OA	osteoarthritis
ODN	oligodeoxynucleotides
OMM	outer mitochondrial membrane
p53AIP1	p53-related apoptosis inducing protein 1
PA	proteasome activator
PAF	platelet activating factor
Par-4	prostate apoptosis response-4
PARP-1	poly(ADP-ribose) polymerase-1
PBMC	peripheral blood mononuclear cells
PBR	peripheral benzodiazepine receptor
PBS	phosphate buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction
PDGF	platelet-derived Growth Factor
PEG	polyethyleneglycol
PET	positron emission tomography
P-gp	P-glycoprotein
PH	pleckstrin homology

PI	propidium iodide
PI3K	phosphatidylinositol-3 kinase
PIDD	p53-induced protein with a DD
Pin-1	cis-trans peptidyl-prolyl-isomerase
PIP	3'-phosphatidylinositol phosphate
PKC	protein kinase C
PKR	DsRNA-activated protein kinase
PLA2	phospholipase A2
PLAD	preligand binding assembly domian
pM	picomolar
PMA	phorbol 12-myristate 13-acetate
PML	promyelocytic leukemia
pNA	para-nitroanilide
Pol-II	type-II RNA polymerases
Pol-III	type-III RNA polymerases
PPAR	peroxisome proliferator activator receptor
PPT	poly-pyrimidine tract
pre-mRNA	precursor messenger RNA
PS	phosphatidylserine
PTGS	posttranscriptional gene silencing
PTK	protein tyrosine kinase
PTPC	permeability transition pore complex
PyBHK	polyoma virus transformed BHK
RA	rheumatoid arthritis
RAI	rel A associated inhibitor
RAIDD	RIP associated Ich-1/CED homologous protein with death domain
Rb	retinoblastoma
RdRp	RNA-directed RNA polymerases
rGFP	recombinant red fluorescent protein
RING	really interesting new gene
RIP	receptor Interacting Protein
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNA-PCR	RNA-polymerase cycling reaction
Roc	regulator of cullins
ROI	reactive oxygen intermediate
ROS	reactive oxygen species
RSK	90 kDa ribosomal S6 protein kinase
RT	reverse transcriptase
RTK	receptor tyrosine kinase
rTPA	recombinant tissue plasminogen activator
SAHA	suberoylanilide hydoxamic acid
SCID	severe combined immunodeficiency
SCLC	small cell lung cancer
SCW	streptococcal cell wall

SDF	stromal cell derived factor
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
serpin	serine protease inhibitor
SF	synovial fibroblast
SH2	src Homology 2
shRNA	small hairpin RNA
siRNA	short interfering RNA
siRNA	small interfering RNA
Skp	S-phase kinase-associated protein
SLE	systemic lupus ethryematosus
SMA	spinal muscular atrophy
Smac	second mitochondria-derived activator of caspases (DIABLO)
SOS	son of sevenless
SpRNAi	splicing-competent artificial intron
ssRNA	shape-shifting RNA
STAT	Signal transducer and activator of transcription
SVMO	small ubiquitin-like modifier
TBI	traumatic brain injury
tBid	truncated Bid
TC-A	tetrocarcin A
TFD	transcription factor decoy
TGF-β	transforming growth factor beta
TNF	tumor necrosis factor-α
TNF-R	tumor necrosis factor-receptor
Top-I	topoisomerase I
TRADD	tumor necrosis factor-receptor associated death domain
TRAF	TNF-receptor-associated factors
TRAIL	TNF related apoptosis inducing ligand
Ub	ubiquitin
Ubc	ubiquitin conjugating enzyme
UMP	ubiquitin-mediated proteolysis
UPP	ubiquitin-proteasome pathway
UV	ultraviolet
VCAM	vascular cell adhesion molecule
VDAC	voltage dependent anion channel
VEGF	Vascular Endothelial Growth Factor
VEGFR	vascular endothelial growth factor receptor
VIP	vasoactive intestinal peptide
VSMC	vascular smooth muscle cell
XAF1	XIAP-associated factor 1
XIAP	X-linked inhibitor of apoptosis protein
z	Benzyloxycarbonyl
Z-LLL	carbobenzoxyl-leucinyl-leucynil-leucynal
Z-LLnV	carbobenzoxyl-leucinyl-leucynil-norvalinal
Zn	zinc

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APOPTOTIC PATHWAYS AND THEIR REGULATION

Chapter I

Greet Brouckaert[1,2], Michael Kalai[2], Xavier Saelens[1], and Peter Vandenabeele[1*]

1. SUMMARY

Many studies have led to the identification of molecules involved in the signaling to cell death and especially to apoptosis. This cell death program is characterized by distinct morphological changes occurring both in the cytoplasm and the nucleus, including membrane blebbing, cytoplasm and chromatin condensation, DNA degradation and inhibition of protein translation. Apoptosis signaling can be initiated either at the cell surface through a receptor-induced signaling pathway, or from within the cell itself via the release of proapoptotic factors such as cytochrome *c* from triggered mitochondria. Stress occurring in other organelles, including the ER, nucleus and lysosomes, is also capable of initiating specific apoptotic pathways. The main executioners of the apoptotic pathways are proteases of the caspase family that function in a tightly regulated proteolytic cascade leading to the disintegration of the cell. Furthermore, apoptosis is regulated by a family of Bcl-2 like proteins, some of which promote cell death, while others are anti-apoptotic. Apoptosis in homeostasis and pathology is connected with phagocytosis. Several apoptotic pathways can be considered as packaging phenomena that allow silent, non-inflammatory removal of dying cells. The central role of apoptosis in homeostasis and cell renewal is also illustrated by the fact that anti-apoptotic mechanisms are crucial in tumorigenesis and therapeutic resistance. Recovery of an apoptotic response in these tumour cells or induction of an alternative cell death pathway, such as necrosis or autophagy, is very relevant for defining new anti-cancer treatments.

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2. APOPTOTIC PATHWAYS AND THEIR REGULATION

2.1. The Apoptotic Process

Apoptosis is the major cell death pathway used to remove unneeded and harmful cells in a clean and orderly manner during embryonic development, tissue homeostasis and immune regulation (Cohen, 1991; Ellis, et al., 1991). It is also the preferred way for removing cancer cells. During the apoptotic cell death programme, a group of cystein-dependent aspartate-specific proteases, designated caspases, are activated (Enari, et al., 1996; Hasegawa, et al., 1996; Lamkanfi, et al., 2002; Van de Craen, et al., 1997). Caspases are the main executioners of apoptotic cell death. They are synthesized as inactive zymogens consisting of a prodomain of variable length, followed by a large (p20) and a small (p10) catalytic subunit. So far, 11 human caspases have been identified. Zymogens of long prodomain initiator caspases such as caspases-1, -2, -5, -8, -9 and –10, exist within the cell as monomers. These caspases are activated mainly through conformational changes and autoprocessing induced upon their recruitment to large molecular platforms, such as the death-inducing signaling complex (DISC) (Peter & Krammer, 2003), the apoptosome (Acehan, et al., 2002), PIDDosome (Tinel & Tschopp, 2004) and the inflammasome (Martinon, et al., 2002). Short prodomain caspases such as caspase-3, -6 and –7 are activated by other caspases by proteolytic cleavage at specific Asp residues located between the prodomain, p20 and p10 subunits (Salvesen & Abrams, 2004) (Fig. 1). Following cleavage, the resulting mature caspase forms a tetramere consisting of two p20/p10 heterodimers. Caspases cleave a variety of intracellular proteins, including structural elements of the cytoskeleton and the nucleus, apoptosis regulators, and proteins involved in signal transduction or in DNA replication and repair (Fischer, et al., 2003; Lamkanfi, 2002). This disables the cell's survival and repair mechanisms, and most of the macromolecular DNA (Fischer, et al., 2003), RNA (Fischer, et al., 2003) and protein (Saelens, et al., 2001) synthesis pathways are shut down.

Figure 1. Intrinsic and extrinsic apoptotic pathways. Diverse proapoptotic stimuli lead to permeabilization of the outer mitochondrial membrane and release of apoptogenic factors from the intermembrane space, initiating the intrinsic apoptotic pathway. These stimuli include radiation-induced nuclear DNA damage leading to transcriptional upregulation of proaptotic genes, ceramide synthesis, and activation of pro-apoptotic Bcl-2 family members. Proteins of the BH3-only family act as sentinels for stresses induced at various subcellular locations, and convert these into mitochondrial membrane damage in a Bax/Bak dependent way (reviewed in Festjens et al., 2004). Cytocolic cytochrome *c* (cyt c), one of the apoptogenic mitochondrial proteins, promotes the formation of the Apaf-1-containing apoptosome. Omi/HtrA2 and Smac/DIABLO promote caspase activation by releasing caspase-9 and active caspases-3 and –7 from the inhibition by XIAP, and c-IAP1 and -2. AIF and Endonuclease G are also released from the mitochondria, translocate to the nucleus and co-operatively initiate DNA degradation. Nuclear DNA degradation in an autonomous way is also performed by Caspase activated DNase (CAD). The extrinsic apoptotic pathways are initiated by ligand-mediated clustering of death domain receptors. For example, binding of Fas ligand to its receptor leads to recruitment of FADD and pro-caspase-8, and signals to apoptosis via a caspase cascade and/or a mitochondrial amplification loop propagated by the cleavage of Bid. Binding of TNF to TNF Receptor I initiates the recruitment of TRADD, RIP1 and TRAF2 resulting in the formation of Complex I, leading to NF-κB activation. Internalization of Complex I is accompanied by the release of the TRADD/RIP1/TRAF2 complex that is now able to recruit FADD and pro-caspase-8, forming Complex II. Complex II signals to apoptosis if pro-caspase-8 homodimers are engaged, or to cell survival depending on sufficient levels of c-FLIP$_L$ expression.

irradiation
Sphingo-
myelinase
Sphingomyelin
Ceramide
P73
P53
P63
(Bax, Bak, PUMA, Noxa, ...)
Transcription
DNA damage
EndoG
AIF
CAD
AIF
EndoG
Smac
Omi
Bak
Bax
Cyt c
ATP
WD40
CARD
Apaf-1
CARD
p20
p10
pro-caspase-9
Omi
Smac
Apoptosome
XIAP, cIAP1, -2
Bcl-2
tBid
Other BH-3 only
Anoikis
growth factor depletion
DNA damage
T-cell receptor stimulation
ER stress
FasL
FAS
FADD
DED
pro-caspase-8
Bid
type II
caspase-8
type I
Caspase-3, -6, -7
APOPTOSIS
ICAD
CAD
COMPLEX II
TRAF2
FADD
pro-caspase-8
TNF
TNF-R1
endocytosis
TRADD
RIP1
NF-κI
SURVIVAL

During apoptosis, the permeability of mitochondrial membranes changes, leading to the release of proapoptotic factors such as cytochrome *c*, Smac/DIABLO, Omi/HtrA2 and AIF from the intermembrane space (Kroemer & Reed, 2000; Saelens, et al., 2004; van Loo, et al., 2002b) (Fig. 1). Cytoplasm and chromatin condense, and organelles shrink (Wyllie, et al., 1980). Due to the depolymerization or cleavage of actin, cytokeratins, lamins and other cytoskeletal proteins, the cytoskeleton collapses (Bursch, et al., 2000; Hengartner, 2000). As a result, cells detach from the extracellular matrix, loose intercellular contacts and round up. Early in the apoptotic process the cellular membrane looses its aminophospholipid asymmetry (Denecker, et al., 2000). Phosphatidylserine (PS) flips from the inner membrane leaflet to the cell surface, and functions as a recognition signal for the phagocytes or surrounding cells that express the PS receptor (Bruckheimer & Schroit, 1996; Fadok, et al., 1992; Martin, et al., 1995). The disassembled cellular contents are concentrated into blebs formed by the plasma membrane, which are shed from the dying cell as apoptotic bodies. These 'bite-size' packages of cellular particles presenting PS are then easily recognized and engulfed by phagocytes or surrounding cells.

The most notable nuclear events occurring during apoptosis are DNA degradation, chromatin condensation and disappearance of the nuclear membrane. In most cells, apoptosis involves both the activation of mechanisms leading to DNA degradation and the inactivation of those required for DNA repair. DNA degradation is initiated by caspase-3-mediated cleavage of the inhibitor of caspase-activated DNase (ICAD), releasing CAD, which degrades DNA into oligonucleosomal fragments (Enari, et al., 1998). Additional DNA degradation occurs due to the release from the mitochondria of AIF and endonuclease G, which constitute a mitochondria-initiated apoptotic DNA degradation pathway that is conserved between *C. elegans* and mammals (Wang, et al., 2002) (Fig. 1). AIF carries both mitochondrial and nuclear localization signals. Upon the release from mitochondria in response to apoptogenic stimuli, AIF translocates to the nucleus and causes large-scale DNA fragmentation and peripheral chromatin condensation, distinct from the typical oligonucleosomal CAD related DNA laddering (Susin, et al., 2000; Susin, et al., 1999). Released endonuclease G also translocates to the nucleus, where it cooperates with exonucleases and DNase I to generate internucleosomal DNA fragments (Widlak, et al., 2001). Caspases cleave cytosolic Helicard, a helicase with 2 N-terminal CARD domains, separating the helicase from the CARD domains. The helicase-containing fragment translocates to the nucleus and accelerates DNA degradation (Kovacsovics, et al., 2002). Moreover, during phagocytosis of early apoptotic cells, phagosomal DNaseII contributes to the breakdown of apoptotic nuclear material (McIlroy, et al., 2000). In parallel, detection and repair of DNA damage are prevented, e.g. by the cleavage and inactivation of poly (ADP-ribose) polymerase-1 (PARP-1) by caspase-3 (Tewari, et al., 1995). Targeting of proteins of the nuclear pores (NUP153), inner nuclear membrane (LBR and Lap2) and the nuclear lamina (lamin B) by caspases leads to disassembly of the nuclear membrane (Buendia, et al., 1999; Duband-Goulet, et al., 1998; Gotzmann, et al., 2000). Complete condensation of DNA during apoptosis results from cleavage of lamin A by caspase-6 and Acinus by caspase-3 (Sahara, et al., 1999).

2.2. Extrinsic Activation of the Apoptotic Cell Death Pathway

Apoptosis can be triggered at the cell surface through a receptor-induced signaling pathway (extrinsic pathway), or from within the cell itself via the release of apoptogenic factors, such as cytochrome *c* from triggered mitochondria (intrinsic pathway) (Li, et al., 1997). Both pathways ultimately converge at proteolytic activation of the downstream effector caspases. A typical example of an extrinsic pathway is the activation of a death receptor by the binding of its specific ligand. Death receptors typically consist of an extracellular region containing varying numbers of cystein-rich domains (CRDs) required for ligand binding, and an intracellular region with a death domain (DD). They include tumor necrosis factor-receptor 1 (TNF-R1), Fas, death receptor (DR) 3 (APO-3/TRAMP), DR4 (TRAIL-R1), DR5 (TRAIL-R2/TRICK2), and DR6 (Chen & Goeddel, 2002; Itoh & Nagata, 1993; Sheikh & Fornace, 2000). Some of these receptors, including TNF-R1, Fas and TRAIL-R1, contain a preligand binding assembly domain (PLAD) that is located within the first N-terminal cysteine-rich domain. PLAD is involved in the homotrimeric preassociation of the receptors on the cell surface (Papoff, et al., 1999; Siegel, et al., 2000). Upon binding, death ligands reinforce the clustering of their death receptors, which is essential for apoptotic signaling (Beyaert, et al., 2002; Daniel, et al., 2001; Sheikh & Fornace, 2000). Death ligands include tumor necrosis factor α (TNF), Fas-ligand (FasL), Apo3-ligand, lymphotoxin (LTα) and TRAIL (TNF-related apoptosis inducing ligand/Apo-2 ligand) (Chen & Goeddel, 2002; Sheikh & Fornace, 2000). The initial apoptotic signal (ligand binding) is transduced by the death domain through homophilic interactions that recruit adaptor molecules also containing a DD, such as TRADD or FADD (Aravind, et al., 1999; Hofmann, 1999) (Fig. 1). FADD also contains a death effector domain (DED), which recruits apoptotic initiator caspases containing DED, such as pro-caspase-8 in mice and pro-caspase-8 and -10 in humans (Muzio, et al., 1996). Other death fold domains include the caspase recruitment domain (CARD) and PYRIN (PyD) (Lamkanfi, et al., 2002). All of these domains share a common structural scaffold that is crucial for mediating protein-protein interactions during the signaling towards either apoptosis or inflammation (Vaughn, et al., 1999). These domains provide a means for recruitment of caspases into protein complexes that allow conformational change and proximity-induced activation (Boatright, et al., 2003; Muzio, et al., 1998; Salvesen & Dixit, 1999; Stennicke, et al., 1999).

2.2.1. Fas-induced Apoptosis

Binding of Fas-ligand or agonistic antibodies to the homotrimeric Fas receptors leads to the intracellular aggregation of the death domains and thus the formation of a platform for the recruitment of FADD (Chinnaiyan, et al., 1996; Hill, et al., 2004; Kischkel, et al., 1995) (Fig. 1). Recruitment of pro-caspase-8 to FADD through its DED domain (Thomas, et al., 2002) results in the formation of a protein complex called DISC (death inducing signaling complex) (Kischkel, et al., 1995). Other DED-containing proteins such as caspase-10, Flice inhibitory protein (FLIP) and Daxx can also be recruited to the DISC (Boldin, et al., 1996; Peter & Krammer, 2003). The Fas receptor-ligand complex is then internalized and directed towards an endosomal pathway (Algeciras-Schimnich, et al., 2002).

In the Fas receptor-induced apoptotic pathway, two different scenarios are possible (Fig. 1), depending on the efficiency of DISC formation, the amount of active caspase-8, and on the sensitivity to cell death inhibition by Bcl-2 overexpression (Algeciras-Schimnich, et al., 2002; Scaffidi, et al., 1998). In the 'type I' pathway, active caspase-8 is released from the DISC within seconds of triggering in quantities sufficient to allow direct activation of downstream executioner caspases such as caspase-3 (Stennicke, et al., 1998). The 'type II' pathway leads to mitochondrial events that amplify the apoptotic signal, the central event being the cleavage of the BH3 domain-containing proapoptotic Bcl-2 family member Bid by caspase-8. Cleavage of Bid induces translocation of truncated Bid (tBid) from the cytosol to the mitochondria, and activates the mitochondrial apoptotic pathway (Li, et al., 1998). As a result, mitochondrial cytochrome *c* is released, and the apoptosome, an oligomeric complex consisting of cytochrome *c*/Apaf-1/caspase-9, is formed, reinforcing further downstream caspase activation (Esposti, 2002). The type II pathway is sensitive to cell death inhibition by Bcl-2 or Bcl-x_L overexpression (Scaffidi, et al., 1998).

2.2.2. TNF-induced Apoptosis

Clustering and trimerization of TNF-R1 receptors creates a high affinity binding site for TRADD, and is the first essential step in TNF signal transduction (Hsu, et al., 1996). The ensuing proapoptotic signaling pathway requires the formation of two distinct signaling complexes (Micheau & Tschopp, 2003) (Fig. 1). The TNF-R-bound TRADD recruits the DD-containing Ser/Thr kinase RIP1 (Ashkenazi & Dixit, 1998; Chen & Goeddel, 2002) and forms the basis of the first complex, called complex I. This plasma membrane-bound complex contains, among others, c-IAP1, TRAF2/5 and the IκB kinase (IKK) α/β complex, and activates the NF-κB, p38 MAPK and JNK pathways (Baud & Karin, 2001; Chen & Goeddel, 2002; Zhang, et al., 2000a). Within one to a few hours after formation, the entire complex I is internalized by endocytosis (Higuchi & Aggarwal, 1994; Jones, et al., 1999; Tsujimoto, et al., 1985). While the TNF-R1/TNF receptor ligand hexameric complex is enclosed in the endocytic vesicle and degraded, the remaining complex dissociates and is released into the cytoplasm. The DDs of TRADD or RIP1 are then free to recruit FADD, and subsequently pro-caspases-8 and/or –10, resulting in formation of complex II. Pro-caspase-8 undergoes conformational changes to form the active caspase-8 heterotetramer. Activated caspase-8 cleaves RIP1 in the complex, and is released to initiate both type I and type II apoptotic pathways.

2.2.3. TRAIL-induced Apoptosis

TRAIL-induced apoptosis is mediated by two receptors, DR4 (TRAIL-R1) (Pan, et al., 1997b; Schneider, et al., 1997) and DR5 (TRAIL-R2/TRICK2) (Pan, et al., 1997a; Screaton, et al., 1997; Walczak, et al., 1997). Interest in TRAIL initially arose from its capacity to selectively induce apoptosis in most transformed cells (Pitti, et al., 1996; Wiley, et al., 1995). Adenovirus mediated TRAIL-expression in liver hepatoma cells overcomes the resistance of these cells to recombinant soluble TRAIL. However, this approach also revealed toxicity of transduced TRAIL for primary hepatocytes (Armeanu,

et al., 2003). Recombinant TRAIL ligand was reported to instruct tumor cells to commit suicide regardless of their p53 status and their resistance or sensitivity to chemotherapeutics (Mitsiades, et al., 2001). Therefore, this ligand has become a focus of interest as a potential anticancer agent, and is awaiting clinical trials. The exact mechanism for TRAIL resistance in normal cells is not fully understood, but several candidate mechanisms have been proposed. Normal tissues show increased resistance to TRAIL due to the presence of two decoy receptors, DcR1/TRAIL-R3/LIT (Degli-Esposti, et al., 1997) and DcR2/TRAIL-R4/TRUNDD (Pan, et al., 1998). These decoy receptors are highly homologous to the extracellular domains of DR4 and DR5. Since DcR1 lacks the transmembrane and death domains, and DcR2 has a truncated, nonfunctional death domain, neither is capable of transmitting an apoptotic signal. TRAIL binds these decoy receptors with high affinity, and is sequestered away from the signaling receptors (Pan, et al., 1998; Sheridan, et al., 1997). Aberrant promoter methylation and resultant silencing of tumor suppressor genes play an important role in the pathogenesis of many tumor types. Interestingly, the promoters of DcR1 and DcR2 genes are frequently silenced by methylation in various tumor types (Shivapurkar, et al., 2004).

TRAIL forms a homotrimer that binds three receptors, and the TRAIL receptor/ligand complex is internalized through the endosomal pathway (Zhang, et al., 2000b). The composition of the TRAIL DISC appears to be more similar to that of Fas than to that of TNF-R1, as FADD is recruited but TRADD is not (Bodmer, et al., 2002; Kischkel, et al., 2000; Sprick, et al., 2000). Upon recruitment to the DISC, caspase-8 is activated. Caspase-10, which is not present in mice, was also shown to interact with FADD through homotypic association with its DED, and to be activated in the DISC (Kischkel, et al., 2001; Sprick, et al., 2002; Wang, et al., 2001). Caspase-10 can function independently of caspase-8 in initiating Fas and TRAIL receptor-mediated apoptosis (Kischkel, et al., 2001; Wang, et al., 2001). Nevertheless, the substrate specificity of caspase-10 appears to be different from that of caspase-8, e.g. RIP1, is preferentially cleaved by caspase-8 but not by caspase-10, implying different roles in death receptor signaling (Wang, et al., 2001).

2.3. The Intrinsic Cell Death Pathway

In the intrinsic pathway, the signal leading to cell death typically originates from within the cell itself. It is the cell's ultimate response to a diverse range of stress conditions, such as DNA damage induced by irradiation or chemotherapeutics, ER stress, heat-shock, hypoxia, growth factor withdrawal, loss of interactions with the extracellular matrix or chemical toxins. Central to this pathway are the mitochondria, as the members of the Bcl-2 family and proteins released from the mitochondria are the major players (Festjens, et al., 2004; Saelens, et al., 2004; van Loo, et al., 2002a).

2.3.1. Central Role of Mitochondria in the Regulation of Apoptotic Pathways

Proteins of the Bcl-2 family govern the release of cytochrome *c* and other proapoptotic factors from the mitochondria, by inducing or preventing permeabilization of the outer mitochondrial membrane. The Bcl-2 family includes both pro- and antiapoptotic members that were classified into three classes, according to the Bcl-2

homology (BH) domains they contain. Four of these domains have been characterized so far: BH1, BH2, BH3 and BH4 (Bouillet & Strasser, 2002; Liu, et al., 2003). The short (9-16 amino acid) BH3 domain is found in all of the members of the family. This domain is the minimal requirement for the proapoptotic function, and is essential for heterodimerization of different members (Kelekar & Thompson, 1998).

Proteins that are pro-survival and antiapoptotic, such as Bcl-2 and its closest structural relative Bcl-x_L, contain three or four BH domains. Proapoptotic family members contain either only the BH3 domain ('BH3-only' proteins), such as Bad, Bid, Noxa and PUMA, or two or three BH domains, such as the multidomain members Bax and Bak (Festjens, et al., 2004; Huang & Strasser, 2000). A C-terminal transmembrane domain targets these proteins to the cytoplasmic side of the mitochondria, endoplasmic reticulum and nucleus (Chen-Levy & Cleary, 1990; Lithgow, et al., 1994). Members of the family can form heterodimers through interaction between the amphipathic BH3 α-helix of the proapoptotic proteins and the hydrophobic groove on the antiapoptotic multidomain members created by the α-helices in the BH1, BH2 and BH3 regions (Adams & Cory, 2001; Petros, et al., 2004). BH3-only proteins are activated by post-translational modifications that include dephosphorylation (Zha, et al., 1996) and cleavage (Gross, et al., 1999a; Li, et al., 1998; Luo, et al., 1998), and translocate to the mitochondria. Bid, for example, is specifically cleaved by a variety of proteases to form tBid, and its mitochondrial translocation is enhanced by N-terminal myristoylation and the presence of cardiolipin in the mitochondrial membrane (Lutter, et al., 2000; Zha, et al., 2000). Bcl-2 or Bcl-x_L can prevent tBid-induced release of cytochrome *c*, but not its cleavage or translocation (Gross, et al., 1999b).

The exact mechanism by which mitochondrial proteins are released is still not completely clear (overview in (Festjens, et al., 2004)). Most likely, the BH3-only proteins cooperate with multidomain proapoptotic Bcl-2 family members. Bak is an integral protein of the mitochondrial outer membrane, whereas Bax translocates from the cytosol to the mitochondria upon activation. Interaction with the BH3 domains of BH3-only proteins (Letai, et al., 2002) such as tBid (Wei, et al., 2000) and Bim (Cheng, et al., 2001) induces Bak and Bax to undergo conformational changes, enabling them to multimerize to form a pore in the outer mitochondrial membrane (OMM) (Antonsson, et al., 2000; Antonsson, et al., 2001). Pore formation permeabilizes the mitochondrial membrane and leads to release of mitochondrial proteins, such as cytochrome *c*, Smac/DIABLO, HtrA2/Omi, endonuclease G and AIF (Fig. 1) (Antonsson, et al., 2000; Van Loo, et al., 2002; van Loo, et al., 2001; Wei, et al., 2000).

Alternatively, BH3-only proteins and/or Bax can also interact with the permeability transition pore (PTP), a large intrinsic mitochondrial protein complex (Zamzami & Kroemer, 2001). The PTP consists of proteins such as ANT (adenine nucleotide exchanger), located in the inner mitochondrial membrane (IMM), and VDAC (voltage dependent anion channel), found in the OMM. The PTP is located at sites of contact between the outer and inner mitochondrial membranes, and opening of the pore would induce loss of the mitochondrial membrane potential ($\Delta\psi_m$), swelling of the mitochondrial matrix and rupture of the OMM, resulting in the release of cytochrome *c* and other proteins from the intermembrane space (Sugiyama, et al., 2002; Zamzami, et al., 2000).

Once cytochrome *c* is released from the mitochondria, it participates in the formation of a high molecular weight proapoptotic complex, named the apoptosome, consisting of cytochrome *c* and Apaf-1, which specifically binds to and activates caspase-9 (Cain, et al., 1999; Li, et al., 1997; Zou, et al., 1997) (Fig. 1). Binding of cytochrome *c* induces Apaf-1 to change from a closed to an open conformation, which is then stabilized by binding of ATP on the nucleotide binding domain (Li, et al., 1997). This induces heptamerization of Apaf-1, providing a platform for recruitment of pro-caspase-9 through the N-terminal CARD domains present in both molecules (Acehan, et al., 2002; Li, et al., 1997). As a result, caspase-9 is activated (Stennicke, et al., 1999). The process does not require proteolysis, because uncleavable mutant forms of caspase-9 become enzymatically active upon integration into the apoptosome. Indeed, the long interdomain loop of pro-caspase-9 may allow the zymogen to adopt an active conformation (Acehan, et al., 2002). Caspase-3 is also recruited to this complex and is proteolytically activated by caspase-9. Caspase-3 in turn can cleave Apaf-1 at the CARD H1 helix (Bratton, et al., 2001; Lauber, et al., 2001), thereby disrupting the CARD-CARD interaction between caspase-9 and Apaf-1, and releasing caspase-9 from the apoptosome. Since the activity of caspase-9 is dependent on binding to Apaf-1, this results in inactivation of caspase-9 activity (Lauber, et al., 2001). On the other hand, caspase-3 can cleave caspase-9 in the apoptosome at DQLD(330). This cleavage results in an 8-fold increase of the apoptosome activity compared with caspase-9 autoprocessed at PEPD(315) (Zou, et al., 2003).

Besides cytochrome *c* and Apaf-1, the apoptosome may also contain XIAP, a member of the Inhibitor of Apoptosis Protein family. XIAP can interact with both caspase-9 within the apoptosome and with active caspase-3, and therefore efficiently blocks both caspase-activation and the ensuing proteolytic cascade (Fig. 1). XIAP can be inhibited by competitors, released from the mitochondria, that contain IAP-binding motif. One of these mitochondrial XIAP antagonists is Smac/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI). In the cytosol this protein promotes caspase-activation by selectively binding to IAPs via its IAP-binding motif, and promoting their auto-ubiquitination and consequent degradation (Du, et al., 2000; Verhagen, et al., 2000; Yang & Du, 2004). In addition, Omi/HtrA2, a serine protease, is released from the mitochondrial intermembrane space, and not only interacts with cytosolic IAP proteins, but irreversibly inactivates them by proteolytic cleavage (Hegde, et al., 2002; Martins, et al., 2002; Suzuki, et al., 2001; Yang, et al., 2003). Besides interacting with IAPs, Omi/HtrA2 is also an inducer/accelerator of cell death by virtue of its serine protease catalytic domain (Verhagen, et al., 2002).

2.4. Initiation of Apoptotic Cell Death Pathways from Organelles other than Mitochondria

2.4.1. Nuclear Damage (p53, p63, p73)

Irradiation, UV light, hyperoxia and many other exogenous and endogenous genotoxic agents may cause extensive DNA damage. In order to prevent the proliferation of cells with excess DNA damage and the possibility of carcinogenic transformation, it is critical that these events are recognized within the cell, and that signaling pathways are activated to either arrest growth and repair DNA, or to induce apoptosis. For this purpose,

the cell contains an extensive signal transduction network involving many factors, including several sensors for DNA damage, such as Ataxia-Telangiectasia mutated (ATM), ATM- and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK), that mediate a complex series of cellular responses (Sampath, et al., 2003; Sordet, et al., 2003). These kinases phosphorylate a large number of substrates, including several downstream kinases, such as c-Abl and the checkpoint protein Chk2 (Sordet, et al., 2003). This kinase cascade leads to the activation of transcription factors, which in turn regulate the expression of genes involved in DNA repair, cell cycle arrest and apoptosis. For example, c-Abl can induce apoptosis by inhibiting cell survival pathways (e.g. PI3 kinase) and activating cell death pathways (e.g. JNK, p53 and p73). In addition to its role in cell cycle arrest and/or DNA repair, Chk2 can induce apoptosis by phosphorylation/activation of the promyelocytic leukemia (PML) protein and the transcription factor and tumor suppressor protein p53. p53 and its two homologues, p63 and p73, and their numerous splice variants and isoforms, create a whole network of proteins regulating checkpoint control, transcription of target genes and programmed cell death (Dietz, et al., 2002). All these DNA damage sensors can activate checkpoint pathways that arrest cell cycle progression and signal to DNA repair. If, however, the damage caused is too overwhelming for these survival mechanisms, the DNA damage sensors activate signaling pathways leading to apoptosis (Sampath, et al., 2003). The exact mechanism leading to either outcome is only partially understood, but is known to involve general factors, such as p53 expression levels, type of stress signal and cell type, as well as co-activators such as Myc and the presence of p63, p73 and the E1A-binding p300 nucleoprotein (Balint & Vousden, 2001; Iyer, et al., 2004). p63 and p73 cooperate with p53 to upregulate the expression of several members of the Bcl-2 family, such as Bax, PUMA and Noxa. PUMA causes mitochondrial translocation of Bax and subsequent cytochrome *c* release (Melino, et al., 2004). In addition, transcription-independent, proapoptotic activities of p53 have been described. Upon activation, p53 accumulates in the cytosol and partially localizes to mitochondria (Marchenko, et al., 2000), where it interacts with the proapoptotic Bcl-2 family members Bax and Bak, leading to their oligomerization (Leu, et al., 2004). Release of cytochrome *c* and Smac/DIABLO from mitochondria then results in caspase activation and apoptosis (Chipuk, et al., 2004; Henry, et al., 2002; hLeu, et al., 2004). Since the proapoptotic functions of p53 may be critical for tumor suppression, restoration or stabilization of p53 is potentially interesting for anti-cancer therapy (Lane & Lain, 2002; Shen & White, 2001).

Other than activation of DNA damage sensors, radiation can induce ceramide generation, both at the plasma membrane by activating acid sphingomyelinase and enzymatic hydrolysis of sphingomyelin, and in mitochondria by inducing *de novo* synthesis of ceramide (Fig. 1). Ceramide then acts as a second messenger and initiates an apoptotic response. In some cells and tissues, this occurs through activation of Bax and release of mitochondrial cytochrome *c* (Kolesnick & Fuks, 2003). Another DNA damage-induced pathway has been suggested for regulating apoptosis induced by various stress stimuli, including those occurring due to exposure to genotoxins or irradiation. This suggested pathway involves the formation of a large protein complex called the PIDDosome, that recruits and activates the initiator caspase-2 (Lin, et al., 2000; Tinel & Tschopp, 2004). The molecular composition of the complex is not yet fully understood, but it contains the death domain-containing protein PIDD (p53-induced protein with a

DD), caspase-2 and the adaptor protein RAIDD. Caspase-2 interacts via its CARD domain with the adaptor molecules RAIDD and ARC (Apoptosis Repressor with Caspase Recruitment Domain) (Dowds & Sabban, 2001; Duan & Dixit, 1997; Koseki, et al., 1998). RAIDD interacts specifically with caspase-2, but not with any of the other CARD-containing initiator caspases, and forms perinuclear aggregates suggested to facilitate apoptosis by caspase-2 activation (Jabado, et al., 2004). Caspase-2 acts upstream of mitochondria, induces Bid cleavage, Bax translocation to mitochondria, and subsequent release of cytochrome *c* and Smac/DIABLO (Guo, et al., 2002; Lassus, et al., 2002; Robertson, et al., 2002), leading to apoptosome complex formation and downstream caspase activation (Guo, et al., 2002).

2.4.2. ER Stress and ER-dependent Apoptotic Pathways

Recent studies indicate that the endoplasmic reticulum (ER) can also sense and transduce apoptotic signals. Various types of stress can lead to changes in the internal ER environment, interfere with normal protein folding, and induce a complex response known as the unfolded protein response (Breckenridge, et al., 2003a; Paschen, 2003; Rao, et al., 2004). Abnormally folded proteins are susceptible to aggregation and accumulation in cells, and may ultimately lead to cell death. Several transducer proteins, including PERK, ATF6 and IRE1, detect ER stress and control both survival and death pathways. The initial response is promotion of cell survival, and includes inhibition of most but not all translation initiation events, transcription based induction of the expression of ER chaperones, and ER-associated degradation. If the stress persists, the response terminates in apoptosis. In addition to its role in protein production, the ER is the main intracellular Ca^{2+} store. Disturbing the Ca^{2+} equilibrium in the cell can also induce a typical ER stress response. Interestingly, localization of Bcl-2, Bax and Bak to the ER affects the cellular Ca^{2+} balance. Overexpression of Bcl-2 or deficiency in Bax and Bak reduces the concentration of Ca^{2+} in the ER and protects cells from apoptotic stimuli that provoke cell death by inducing a Ca^{2+} influx from the ER to the cytosol (Rudner, et al., 2002).

The main regulator of the activation of PERK, ATF6 and IRE1 is an ER chaperone designated BiP/GRP78 (Bertolotti, et al., 2000; Shen, et al., 2002). Under normal conditions, BiP/GRP78 is bound to a specific lumenal domain of PERK, ATF6 and IRE1, and prevents their activation (Fig. 2). During ER stress, BiP/GRP78 is released from these transducers and binds to unfolded proteins instead. The release of BiP/GRP78 from ATF6 permits its transport to the Golgi compartment for regulated intramembrane proteolysis, and allows PERK and IRE1 homodimerization and activation. PERK is an ER transmembrane resident protein kinase similar to PKR (Harding, et al., 1999). PERK induces phosphorylation of the eukaryotic initiation factor 2α (eIF2α) in response to ER stress, resulting in a steep drop in overall initiation of translation. This reversible decrease in *de novo* protein synthesis serves initially as a life saving mechanism by avoiding loading the ER lumen with incoming, newly synthesized proteins. However, the translation of selective mRNAs having a lower requirement for eIF2, such as the mRNA

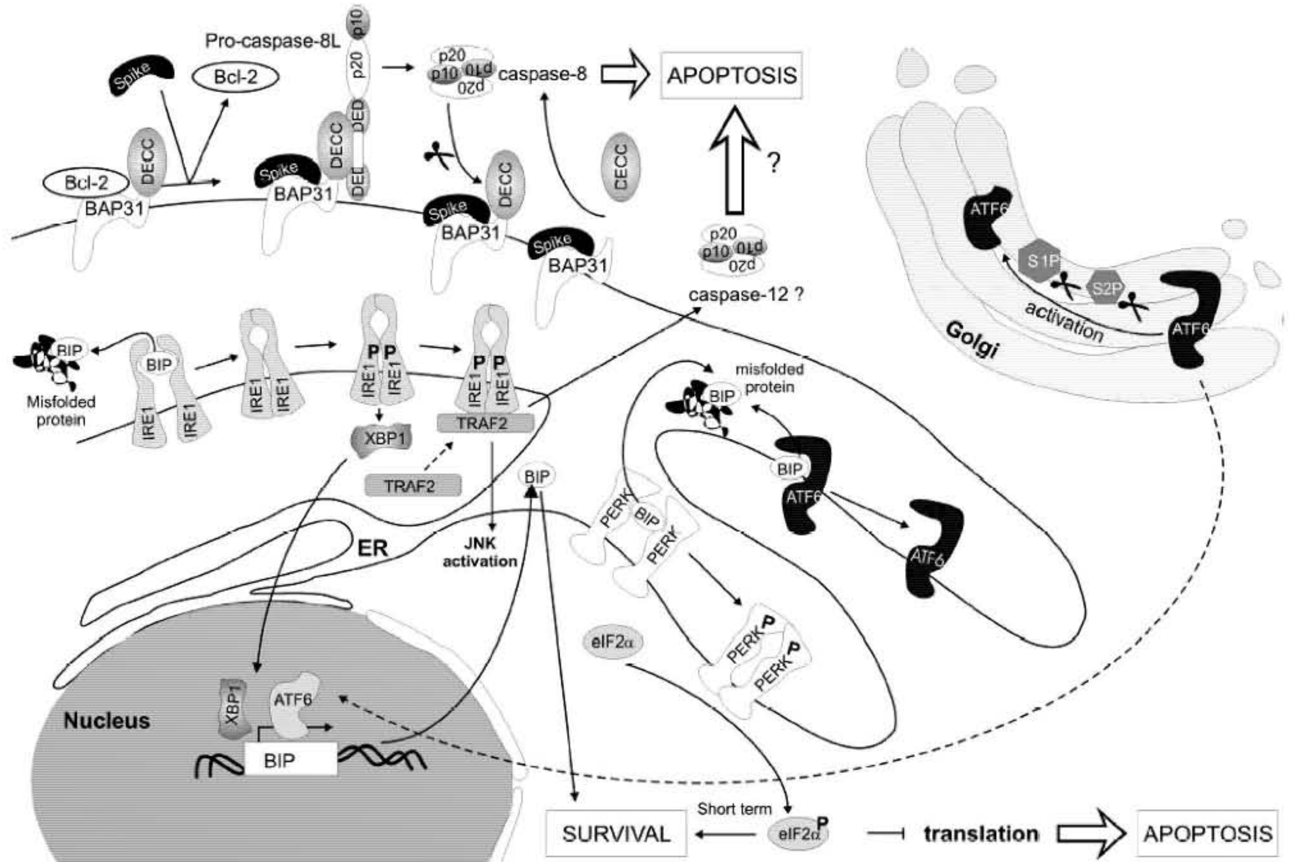
Pro-caspase-8L
Spike
Bcl-2
DECC
BAP31
DED
p20
p10
caspase-8
APOPTOSIS
?
caspase-12 ?
Golgi
ATF6
S1P
S2P
activation
BIP
Misfolded protein
IRE1
P
XBP1
TRAF2
JNK activation
ER
misfolded protein
PERK
eIF2α
Nucleus
SURVIVAL
Short term
translation

Figure 2. ER stress induced signaling to cell survival or apoptosis. Unfolded proteins accumulating in the ER-lumen lead to the release of the ER chaperone BIP from PERK, ATF6 and IRE1, causing their activation and initiating the pro-survival signaling typical for the ER stress response. PERK-induced phosphorylation of eIF2α leads to overall inhibition of translation. ATF6 first translocates to the Golgi, where it is activated by proteolytic processing. Active ATF6 migrates to the nucleus and induces ER chaperone expression. Dimerization and autophosphorylation of IRE1 activates its endonuclease activity, leading to XBP1 mRNA splicing and expression. XBP1 also acts as a transcription factor that induces expression of ER chaperone genes. Persisting ER stress causes apoptosis through prolonged translational arrest and IRE1 mediated caspase-activation. Alternatively, Spike binds to Bap31 and displaces the antiapoptotic Bcl-2, leading to the recruitment of pro-caspase-8L, a splice variant of caspase-8 that contains a larger prodomain. In the resulting Spike/Bap31/pro-caspase-8L complex, caspase-8 is activated and leads to apoptosis.

of activating transcription factor ATF4, is enhanced when PERK is active (Fernandez, et al., 2002; Harding, et al., 2000). ATF4 induces the transcription of GADD34, which in turn recruits protein phosphatase 1 to dephosphorylate eIF2α-P, and thus reverses the translational attenuation (Ma & Hendershot, 2003). ATF6 is a transmembrane transcription factor that activates the transcription of ER molecular chaperones (Shen, et al., 2002). Under conditions of ER stress, ATF6 translocates to the Golgi, where it is cleaved and activated by S1P and S2P proteases (Ye, et al., 2000). Active ATF6 is rapidly transferred to the nucleus and induces the expression of several ER chaperones, including BiP/GRP78, the Ca^{2+} binding chaperone calreticulin, and the transcription factor XBP1 (Hong, et al., 2004; Yoshida, et al., 1998; Yoshida, et al., 2001). These chaperones help in protein refolding, re-establishing the ER- Ca^{2+} stores, and alleviating the stress.

In mammals, two IRE1 genes have been identified, IRE1α and IRE1β (Bertolotti, et al., 2001; Liu, et al., 2000). These are ER transmembrane glycoproteins that contain both kinase and RNase activities in the cytoplasmic domain (Urano, et al., 2000). IRE1-signaling combines promotion of protein refolding with RNA degradation and attenuation of translation. ER stress leads to IRE1 autophosphorylation and the subsequent activation of its RNase activity. Active IRE1α and IRE1β remove a sequence of 26 bases from the coding region of XBP1 mRNA (Yoshida, et al., 2001). The resulting mature mRNA is translated into the XBP1 protein, an active transcription factor specific for ER stress genes such as BiP/GRP78. Therefore, the activation of both ATF6 and IRE1 are required for the production of XBP1 and lead to a full blown ER stress response (Lee, et al., 2002). In addition, the active IRE1β nuclease can also induce translational repression through 28S ribosomal RNA cleavage in response to ER stress (Iwawaki, et al., 2001).

When ER stress persists, the cell reaches a point of no return and dies. Prolonged PERK activation and inhibition of translation leads to cell cycle arrest during the G1 phase (Brewer & Diehl, 2000). In addition, the PERK/ATF4 pathway induces the expression of the proapoptotic-CHOP/GADD153 transcription factor (Harding, et al., 2000). CHOP/GADD153-induced apoptosis is mediated mainly by translocation of BAX from the cytosol to the mitochondria (Gotoh, et al., 2004). Prolonged activation of IRE1 leads to apoptosis. IRE1 can activate apoptosis-signaling kinase 1 (ASK1) via the recruitment of Jun N-terminal inhibitory kinase (JIK) and TRAF2 (Nishitoh, et al., 2002). It has been suggested that ASK1 then activates JNK and mitochondria/Apaf-1-dependent caspases. Another proposed apoptotic pathway involves the activation of pro-caspase-12, a caspase considered to be an ER-associated proximal effector of apoptosis (Nakagawa, et al., 2000). Two mechanisms of caspase-12 activation at the ER have been proposed so

far. In the first, it has been suggested that pro-caspase-12 remains inactive by bound TRAF2. The recruitment of TRAF2 to IRE1 results in the release of caspase-12 and activation of the protease, which in turn activates downstream caspases and leads to apoptosis (Yoneda, et al., 2001). The alternative suggestion is that pro-caspase-12 is processed and activated by calpain, a Cys-protease activated by Ca^{2+} released from the damaged ER (Nakagawa & Yuan, 2000). However, although caspase-12 is processed and activated in ER-stress induced apoptosis in murine cells, the enzyme is not absolutely essential for the process (Jimbo, et al., 2003; Kalai, et al., 2003). On the other hand, cells lacking caspase-8 or caspase-9 are highly resistant to ER stress-induced apoptosis (Jimbo, et al., 2003). Moreover, recent studies showed that the gene coding for the human homologue of murine *caspase-12* codes for a variant of the protein incapable of proteolytic activity (Fischer, et al., 2002; Lamkanfi, et al., 2004; Saleh, et al., 2004). However, functional replacement of caspase-12 by caspase-4 has been suggested (Hitomi, et al., 2004).

One of the mechanisms that could explain caspase-8 activation at the ER involves a recently discovered ER resident potential initiator of apoptosis, designated neurotrophin receptor like death domain protein (NRADD) (Wang, et al., 2003b). This protein has transmembrane and cytoplasmic regions highly homologous to death receptors. The induction of apoptosis by NRADD is dependent on the activation of caspase-8, but does not require the mitochondrial components of the death program. Interestingly, the N-glycosylated, NH_2-terminal domain of the protein is short and unique. Deletion of this domain produces a dominant-negative form of NRADD that protects cells from ER stress-induced apoptosis, suggesting that the lumenal part of the protein is required for inducing the proapoptotic signal.

In addition to propagating death-inducing stress signals, the ER contributes to apoptosis initiated by cell surface death receptors and to pathways resulting from DNA damage (Breckenridge, et al., 2003a). Mobilization of ER calcium stores can sensitize mitochondria to direct proapoptotic stimuli and promote the activation of cytoplasmic death pathways. Indeed, another pre-apoptotic complex that may play an important role in apoptosis assembles on the cytosolic face of the ER membrane and recruits pro-caspase-8. The central protein in this complex is B-cell receptor-associated protein 31 (Bap31), a 28 kDa polytopic integral ER protein (Breckenridge, et al., 2002; Ng, et al., 1997). Bap31 is anchored in the ER-membrane via three transmembrane domains with its NH_2-terminus in the ER-lumen and the COOH-end in the cytosol. The latter part contains predicted overlapping leucine zipper and weak DED homology regions, flanked by identical caspase cleavage sites (Breckenridge, et al., 2003b; Granville, et al., 1998; Ng, et al., 1997). Together with a homologous protein named BAP29, Bap31 is included in a high molecular weight complex involved in the retention of membrane bound IgD in the ER (Adachi, et al., 1996; Schamel, et al., 2003). In normal conditions, Bap31 is bound to pro-caspase-8L, an alternative form of caspase-8 with a longer prodomain than the previously described pro-caspase-8, and to Bcl-2 or Bcl-x_L (Breckenridge, et al., 2002; Mund, et al., 2003; Ng, et al., 1997; Ng & Shore, 1998) (Fig. 2). These antiapoptotic Bcl–2 family members can be displaced by Spike, a BH3 only protein that does not interact with any other Bcl-2 family member and is thus thought to specifically target the ER Bap31-complex (Mund, et al., 2003). Indeed, overexpression of Spike leads to caspase-8 activation and apoptosis. Bax is not recruited to the complex but can prevent

Bcl-2 from being recruited to Bap31 and thus promotes caspase-8 activation (Ng, et al., 1997). Active caspase-8, but not caspase-3, cleaves Bap31, generating a proapoptotic p20 Bap31-fragment (Breckenridge, et al., 2003b; Granville, et al., 1998; Ng, et al., 1997). This fragment is generated in various apoptotic conditions in which caspase-8 is activated, including those mediated by Fas or ER-stress, and induces mitochondrial fission through ER calcium signals that enhance the release of cytochrome *c* into the cytosol. The ER also plays a role in p53- and p73-dependent apoptosis induced by DNA damage. Indeed, both proteins can induce the expression of Scotin, which is localized to the ER and the nuclear membrane (Bourdon, et al., 2002; Terrinoni, et al., 2004). Expression of Scotin induces a typical ER-stress apoptotic cell death in a caspase-dependent manner, while inhibition of endogenous Scotin expression leads to resistance to p53-dependent apoptosis.

2.4.3. Lysosomes

A variety of signals, such as oxidative stress, growth factor withdrawal, the lipid second messenger sphingosine, and lysosomotropic toxins such as hydroxychloroquine, the quinolone antibiotics ciprofloxacin and norfloxacin, can all cause lysosomal destabilization and rupture (Brunk & Svensson, 1999; Roberg, et al., 1999). Depending on whether the rupture is moderate or extensive, cell death ensues through apoptosis and necrosis, respectively (Brunk & Svensson, 1999). Although evidence has indicated that mitochondria are indispensable for cell death initiated by lysosomal destabilization (Boya, et al., 2003a), and recent studies have clearly established the link between lysosomal and mitochondrial membrane permeabilization, the exact molecular cascade operating between the two organelles still remains to be established. Upon membrane destabilization, there is a decrease in the lysosomal pH gradient, and lysosomal cathepsins are released, thereby triggering activation of Bax and/or Bak and mitochondrial membrane permeabilization (M-MP) (Boya, et al., 2003a; Boya, et al., 2003b). Indeed, cathepsin D is an upstream trigger of Bax activation, and cathepsins B, H, L, S and K have been shown to cleave and activate Bid *in vitro* (Bidere, et al., 2003; Jaattela, et al., 2004). Cathepsins were also shown to be important mediators of sphingosine-induced apoptosis, working upstream of the caspase cascade and mitochondrial membrane-potential changes (Kagedal, et al., 2001).

2.4.4. Cytoskeleton

Cytoskeletal integrity is of great importance for many cellular functions, such as cell attachment to the extracellular matrix (ECM) in anchorage dependent cells, transport of cell organelles, cell maintenance and cell division. Loss of cell attachment to the ECM and other forms of cytoskeletal perturbation lead to apoptosis. Integrins are central regulators of the actin cytoskeleton, mediating anchorage to the ECM and the signaling required for cell survival. Release of integrin contacts leads to depolymerization of actin filaments and subsequent apoptosis (Frisch & Ruoslahti, 1997; Frisch & Screaton, 2001). The mitochondria-associated protein Bit1 and the BH3-only proteins Bim and Bmf guard the integrity of the cytoskeleton, and transduce death signals upon perturbation of microtubule dynamics by various cytoskeleton disrupting or destabilizing drugs, or by

cell detachment. Bim is a BH3-only Bcl-2 family member which is downregulated by integrins and EGF receptor in order to prevent anoikis, but strongly induced after cell detachment (Reginato, et al., 2003). Alternative splicing gives rise to three isoforms, Bim_S, Bim_L and Bim_{EL}. Bim_{EL} and Bim_L have been shown to bind to LC8, a component of the microtubule dynein motor complex (Puthalakath, et al., 1999), and the binding of Bim and Bmf to the cytoskeleton was suggested to keep them inactive (Coultas & Strasser, 2003). However, in normal resting T cells, most of Bim is found on the surface of the mitochondria, and is kept inactive due to an association with Bcl-2 or Bcl-x_L, not with the microtubules (Zhu, et al., 2004). Moreover, integrins signal Bcl-2 upregulation upon cell attachment and antiapoptotic proteins such as Bcl-2 can protect cells against anoikis (Frisch, et al., 1996; Matter & Ruoslahti, 2001; Zhang, et al., 1995). Bax is also activated very shortly after loss of integrin-mediated adhesion to ECM, and its conformational change induces cytochrome *c* release and caspase activation (Wang, et al., 2003a). Bit1 is normally located at the outer mitochondrial membrane, but it is released into the cytosol upon loss of integrin ligation. In the cytosol it forms a complex with the transcriptional coregulator 'amino-terminal enhancer of split' (AES), and induces apoptosis through a caspase-independent mechanism that has not yet been elucidated (Jan, et al., 2004). Integrin-mediated cell attachment reduces Bit1-AES complex formation and inhibits apoptosis. Microtubule damaging agents, such as paclitaxel (Taxol), are well-known in clinical cancer chemotherapy as potent promotors of apoptosis in cancer cells through inhibition of microtubule dynamic instability (Bhalla, 2003; von Haefen, et al., 2003). Disruption of microtubules also induces p53, further arresting cells in the G2/M phase of the cell cycle. If the cells do not overcome this G2/M stop, the mitochondrial pathway of apoptosis is triggered (Ganansia-Leymarie, et al., 2003).

3. ANTIAPOPTOTIC PATHWAYS

Cells possess a number of inhibitory mechanisms to keep the components of the apoptotic machinery in check, protecting them from unwanted or untimely triggering of cell death. Many molecules work at different levels to fulfill this task, amongst which are the antiapoptotic members of the Bcl-2 family, c-FLIP, and the inhibitors of apoptosis (IAP) family. Not surprisingly, many tumor cells use these inhibitory mechanisms to their advantage, and frequently show elevated expression levels of such antiapoptotic proteins (Altieri, 2003b; LaCasse, et al., 1998). Caspases are kept inactive by inhibitor of apoptosis proteins (IAPs), a family of proteins all of which contain at least one baculovirus IAP repeat (BIR) domain that is essential for their antiapoptotic function. XIAP contains 3 BIR domains and is the member of the family most potent in suppressing apoptosis. In addition to preventing the activity of caspases-3 and -7, XIAP is capable of inhibiting caspase-9 (Deveraux, et al., 1998; Deveraux, et al., 1997; Roy, et al., 1997). The mechanisms used by XIAP for inhibition of caspase-3 and caspase-9 are different. XIAP inhibits caspase-3 through its 'hook' and 'sinker' regions, which precede the BIR2 domain and interact with the catalytic groove of caspase-3. Inhibition of caspase-9, on the other hand, occurs through binding of XIAP's BIR3 domain to the IAP binding motif of caspase-9 (Salvesen & Duckett, 2002). In addition, some IAPs also have

a RING domain, suggested to lead to ubiquitinylation and degradation of both themselves and their selected targets (Salvesen & Duckett, 2002). Once the appropriate apoptotic stimulus is received, IAPs may undergo caspase-mediated cleavage (Clem, et al., 2001; Deveraux, et al., 1999), autoubiquitination and degradation (Yang, et al., 2000), or sequestration by released apoptogenic mitochondrial proteins such as Smac/DIABLO or Omi/HtrA2 (Salvesen & Duckett, 2002). Survivin, a IAP family member lacking the RING domain, is implicated in both control of apoptosis and regulation of cell division. Interestingly, the protein is aberrantly expressed in cancer but undetectable in normal, differentiated adult tissues (Altieri, 2003a; Li, 2003).

Caspase-inhibition by IAPs is limited to caspase-9, -3 and –7, and has no influence on caspase-8 and caspase-10, the DED caspases of the extrinsic pathway. Caspases-8 and -10-associated RING proteins (CARPs) are a family of apoptosis inhibitors that bind to and inhibit DED-containing caspases (McDonald & El-Deiry, 2004). They contain a phospholipid binding FYVE domain that is suggested to localize them to the plasma membrane, and an IAP-like RING domain with E3 ubiquitin ligase activity that contributes to the ubiquitin-mediated proteolytic degradation of caspase-8 and -10. CARP inhibition is negatively regulated by caspase-mediated cleavage during apoptosis (McDonald & El-Deiry, 2004).

Another antiapoptotic protein is FLIP, a protease-deficient pro-caspase-8-like protein, expressed either as a long ($FLIP_L$) or as a short ($FLIP_S$) splice form. While $FLIP_S$ contains two tandem DED repeats, $FLIP_L$ has an extra (inactive) protease-like domain, homologous to caspase-8, but lacking the key active sites. In death domain receptor-induced signaling, $FLIP_L$, if present, is recruited to the receptor complex. Upon binding to FADD, pro-caspase-8 preferentially interacts with $FLIP_L$, since it has higher affinity for $FLIP_L$ than for pro-caspase-10 or other pro-caspase-8 molecules (Irmler, et al., 1997; Krueger, et al., 2001; Wang, et al., 2001). c-$FLIP_L$ then induces partial pro-caspase-8 activation, but it prevents caspase-8 from being released from the DISC, because cleavage between the DED and the caspase-unit within caspase-8 and $FLIP_L$ does not occur (Irmler, et al., 1997; Micheau, et al., 2002). As a result, further signaling from caspase-8 to the downstream caspases is blocked, and the whole complex is directed for proteasome degradation, resulting in cell survival instead of apoptosis. While $FLIP_L$ allows partial activation of pro-caspase-8, $FLIP_S$ completely inhibits the cleavage and activation of caspase-8. FLIP expression is upregulated by NF-κB, a transcription factor that regulates the expression of various genes involved in cell growth and differentiation, immune response, and inhibition of apoptosis. NF-κB is normally located in the cytoplasm, where it is kept inactive through binding to IκB. Upon activation of IKKα/β, IκB is phosphorylated and degraded. As a consequence, NF-κB is released and translocates to the nucleus, where it activates gene expression (Mayo and Baldwin, 2000). Amongst the genes targeted by NF-κB are many antiapoptotic proteins, such as c–IAP1, c-IAP2, XIAP, TRAF1, TRAF2, TRAIL decoy receptors, Bcl-x_L and FLIP (Mayo & Baldwin, 2000; Zou, et al., 2004). Loss of NF-κB regulation results in deregulated growth, and is often observed in cancer, where it confers resistance to cytotoxic therapies (Deng, et al., 2002; Romieu-Mourez, et al., 2002). NF-κB can be activated in response to a whole range of stimuli, including cellular stress and anticancer agents, and in addition it is constitutively active in certain tumor types (Mayo & Baldwin,

2000). For example, in TNF receptor signaling, NF-κB is activated upon formation of complex I (Fig. 1). However, the decision on whether TNF signals cell survival or death is made at the level of the cytosolic complex II, and takes place following recruitment of FADD and pro-caspase-8. Complex II initiates apoptosis on condition that the NF-κB signal from complex I did not succeed in inducing high enough expression levels of antiapoptotic proteins such as $FLIP_L$ (Fig. 2). Indeed, in apoptosis-resistant cells, complex II typically contains increased amounts of $FLIP_L$ and IAP1, while in sensitive cells, the complex is devoid of $FLIP_L$ (Kreuz, et al., 2001; Micheau, et al., 2001).

Another pathway utilized by many cell types for inhibition of apoptosis and cellular survival is the phosphatidylinositol 3-kinase (PI3K)-AKT pathway. Activated AKT modulates the function of numerous substrates involved in the regulation of cell survival, cell cycle progression, and cellular growth. Survival signals such as growth factors can activate the PI3K/AKT pathway by phosphorylation of AKT, and thus induce NF-κB-responsive gene expression, providing protection from apoptosis through subsequent induction of antiapoptotic genes such as Bcl-x_L (Hatano & Brenner, 2001). PI3K/AKT signaling pathway components are frequently altered in human cancers. For example, certain cancer cells show elevated AKT activity, resulting in upregulated $FLIP_S$, which allows the cells to escape from apoptosis (Nam, et al., 2003; Nesterov, et al., 2001; Suhara, et al., 2001). Moreover, altered Bcl-2 family member expression plays an important role in tumorigenesis and resistance to anti-cancer therapy. Typically, proapoptotic Bax is decreased with tumour progression, whereas antiapoptotic Bcl-x_L and Mcl-1 appear to increase with progression (Agarwal & Naresh, 2002; Bush & Li, 2003; Spets, et al., 2002). Similarly, p53 targets many genes involved in the mitochondrial apoptotic pathway, e.g. p53 is a downregulator of Bcl-2 and a promoter of Bax. Therefore, dominant negative mutants of p53 are antiapoptotic and oncogenic (Galmarini, et al., 2002).

4. NONAPOPTOTIC FORMS OF CELL DEATH

4.1. Necrosis

When a cell for one or another reason can not die by apoptosis, it may respond by another type of cellular death called necrosis. This cell death program may occur following diverse traumatisms including hyperthermia, ischemia, physical injury or viral infection or appear as a result of the exposure to cytotoxic cytokines or substances (Denecker, et al., 2001; Kitanaka & Kuchino, 1999; Proskuryakov, et al., 2003). Necrosis is characterized by distinct morphological changes such as swelling of the cell and its organelles including most often the mitochondria. The swelling is thought to occur due to the absence of the regulation of internal pressures and results from the loss of membrane integrity. The process most often terminates with rapture of the cell and spilling of the cellular contents to the neighboring tissues and is usually associated with a strong inflammatory process (Li, et al., 2001). The molecular mechanisms leading to necrosis are still poorly understood. Nevertheless, the process apparently involves a certain degree of programming and several of the molecular events controlling it have already been discovered (Kitanaka & Kuchino, 1999; Proskuryakov, et al., 2003). Necrosis occurring

during embryonic development of the interdigital cells can replace apoptosis in correctly shaping the fingers in the paws of Apaf-1 deficient mice (Chautan, et al., 1999). Necrotic cell death was shown to require the presence and probably the kinase activity of RIP1 (Holler, et al., 2000; Kalai, et al., 2002; Vanden Berghe, et al., 2004a; Vanden Berghe, et al., 2003; Vanden Berghe, et al., 2004b) and involves mitochondrial ROS production (Denecker, et al., 2001; Goossens, et al., 1999; Kalai, et al., 2002). In contrast to apoptosis caspases are not only dispensable for necrosis but may actually prevent the process (Kalai, et al., 2002; Vercammen, et al., 1998a; Vercammen, et al., 1998b).

4.2. Autophagy

Another caspase independent cell death pathway is called autophagy (to eat oneself) (Gozuacik & Kimchi, 2004; Levine & Klionsky, 2004). The primary function of autophagy is the recycling of proteins and cellular organelles. Autophagy often occurs during the lifetime of a cell, when certain molecules denature and aggregate, or organelles age and deteriorate. In this process, a double membrane autophagic vacuole, called autophagosome, is formed and assures successively the sequestration then the digestion of a portion of the cellular cytoplasm, thus recycling different components. The origin of the autophagosomal membranes is still controversial; either the lysosomes engulf and digest a part of their own cellular material, or a vacuole initiated in the ER encircles a damaged zone and then fuses with the primary lysosome (Dunn, 1990; Kim & Klionsky, 2000). Autophagy is evolutionarily conserved, and probably originated as a survival mechanism to allow a cell to overcome periods of nutrient scarcity. However, the process can also be activated by various environmental stresses, and may result in the total destruction and disappearance of the cell (Gozuacik & Kimchi, 2004; Levine & Klionsky, 2004). Some of the proteins involved in autophagy and in autophagic cell death have been identified. These include: ubiquitin-like genes called *ATG* genes (Yu, et al., 2004), death associated protein kinase (DAPk), DAPk related protein (DRP-1) (Inbal, et al., 2002), and several cystein proteases called autophagins (Marino, et al., 2003). Interestingly, a recent report suggested that RIP1 is also required for autophagic cell death induced by exposure of cells to the pan-caspase inhibitor benzyloxycarbonyl-Valyl-Alanyl-Aspartic-acid (O-methyl)- fluoromethylketone (z-VAD-fmk) (Yu, et al., 2004). The molecular link between RIP1 kinase and the activation of the ATG proteins remains to be determined.

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MECHANISMS OF ANTICANCER DRUG ACTION

Chapter II

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1. SUMMARY

Activation of the endogenous suicide program is one of the major mechanisms by that cancer cells are eliminated by antitumor agents. Though the primary site of action might differ, the basic outcome of most anticancer drugs is usually the same - induction of the apoptotic program in the cancer cell. Conversely, defects within the apoptotic signaling machinery convey resistance of tumors to chemotherapy. In this review we will summarize the current knowledge of the apoptosis signaling in tumorigenesis, the molecular mechanisms of DNA-damaging anticancer drugs and drug resistance of tumor cells. Understanding of the molecular events that regulate the apoptotic machinery induced by anticancer drugs and the measures that the tumor cell takes to avoid apoptosis might help to develop new therapeutic strategies and drug development.

2. INTRODUCTION

Theoretically, a cancer cell is nothing but a cell propagating its anarchistic death defying principle of unlimited self dissemination that - if cellular safeguard mechanisms or the immune system fail to keep under control - is finally terminated by the demise of the whole organism itself. The basic objective of chemotherapy is therefore to exploit the different activities that distinguish malignant tumor cells from non-malignant cells in order to specifically eliminate the 'anarchistic dropouts'. An ideal anticancer drug would accompolish to specifically target the malignant cancer cell and leave the rest of the non-malignant cells in our body unaffected. Due to detrimental side effects on non-malignant tissues and the virtue of some tumors not to respond to the respective chemotherapy or to

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tissues and the virtue of some tumors not to respond to the respective chemotherapy or to develop resistance during chemotherapy this ideal concept is unfortunately rarely achieved. However, research of the last two decades improved our knowledge of tumor biology and enabled the identification of new targets for chemotherapy. Thus, in addition to uncontrolled cell proliferation, it is well established now that cancer cells also need to induce their supply with oxygen and nutrients by the induction of angiogenesis of surrounding blood vessels, extravasation and metastasis by releasing matrix metalloproteases for extracellular matrix degradation and so on. Whereas, the 1980's were dominated by research on the mechanisms of uncontrolled proliferation the 1990's also brought another feature of cancer cells into focus - that is the counterpart of mitosis i.e. apoptosis. Thus, it turned out that tumor cells not only propagate their anarchistic desire for unlimited proliferation but they also inactivate external and internal triggers of the cellular suicide program.

Anticancer drugs are used as therapeutic agents since 1940 with more or less success. Per definition a chemotherapeutic agent is any drug that contributes to tumor destruction. For decades the major feature of chemotherapeutic agents had been the induction of DNA-damage. This kind of therapy basically targets cells with a high proliferation rate that can not evade the consequences of DNA-damage by cell cycle arrest or DNA-repair. Consequently, other benign but highly proliferating tissues such as bone marrow, mucosal membranes, hair follicles and cells of the gastrointestinal and central nervous system are also affected. Recently, due to the rapidly expanding knowledge in tumor biology a new generation of anticancer drugs evolved that might contribute to the collection of weapons against cancer (Nygren & Larsson, 2003). This new generation of antitumor agents utilizes genuine features of cancer cells as targets for chemotherapy. Hence, these new approaches target cancer specific antigens by antibodies conjugated with cytotoxic drugs (e.g. imunoconjugates or antibody dependent prodrug therapy), target receptor tyrosine kinases for growth factors (such as EGF, PDGF, TGF-α), inhibit intracellular protein kinases (such as PKC, PKA), the proteasome or histone deacetylases (HDACs) (Nygren & Larsson, 2003). The new generation of anticancer drugs and new concepts in chemotherapy will be reviewed in detail by the other authors in the accompanying chapters herein. The focus of this review will therefore be to elucidate the apoptotic mechanisms of DNA-damaging anticancer drugs.

3. THE MAJOR APOPTOSIS SIGNALING PATHWAYS

The endogenous suicide program can be activated by a variety of stimuli including death receptor ligation, chemotherapeutic drugs, reactive oxygen species (ROS), withdrawal of growth factors, inhibitors of protein synthesis, kinase inhibitors (e.g. staurosporine), UV- or γ-irradiation. As pleiotropic these stimuli might be, they all lead to the final demise of the cell, characterized by shrinkage, chromatin condensation, DNA-fragmentation, membrane blebbing and the proteolytic activation of intracellular proteases, known as caspases (Schulze-Bergkamen & Krammer, 2004; Schulze-Osthoff et al., 1998). Caspases are the central executioners of programmed cell death and comprise a family of at least 14 different members in mammalian cells. They are cysteine proteases which cleave their substrates after a specific aspartate residue (Cryns & Yuan,

1998; Degterev et al., 2003; Los et al., 1999). Caspases are synthesized as catalytically inactive proenzymes and need to be activated by proteolytic cleavage. They consist of a prodomain followed by a region that forms the two subunits with the catalytic domain (Thornberry & Lazebnik, 1998). The apoptotic program is instigated by initiator caspases that in turn trigger an amplifying cascade of effector caspases. Effector caspases, such as caspase-3, -6 and -7, contain only a small prodomain and cleave diverse cellular substrates including gelsolin, fodrin, poly(ADP-ribose)polymerase-1 (PARP-1), ICAD/DFF45 and others (Fischer et al., 2003). Initiator caspases contain a long prodomain and exert regulatory roles by activating downstream effector caspases. Activation of initiator caspases is mediated by binding of adapter molecules to protein interaction motifs in their prodomains. Two general types of interaction have been identified (Martin et al., 1998; Muzio et al., 1998; Srinivasula et al., 1998). Pro-caspase-8 and -10 each contain two tandem death effector domains (DEDs), while pro-caspase-1, –2, -4 and -9 comprise a caspase recruitment domain (CARD). In each case, the pro-caspases bind to adapter molecules containing similar domains leading to caspase oligomerization and subsequent activation.

In principle there are two major signaling pathways that activate the endogenous proteolytic death machinery: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. Whereas the intrinsic apoptosis pathway is conserved from *C. elegans*, fruit flies to mammals, signaling by death receptors seems to be a prerequisite of the mammalian system. The death receptor pathway is activated by ligation of death receptors (such as CD95/Fas/Apo-1, TNF-R1/CD120a, TRAIL-R1/DR4/Apo-2, TRAIL-R2/DR5/Killer or TRAMP/DR3/Apo-3) with their respective ligand (CD95L/FasL/Apo-1L, TNF, TRAIL, TWEAK/Apo-3L). All death receptors represent a subfamily within the superfamily of TNF-receptors and are characterized by the possession of an intracellular death domain (DD). Amongst the different death receptors the signaling pathway of CD95 is the best characterized so far.

Trimerization of CD95 by its natural ligand CD95L or agonistic antibodies recruits the adapter molecule FADD to the receptor via mutual interaction of their DDs. FADD in turn recruits pro-caspase-8 (FLICE) through interaction between the DEDs of FADD and pro-caspase-8. Upon formation of this death-inducing signaling complex (DISC) pro-caspase-8 is autoproteolytically activated (Fig. 1). Apoptosis mediated by death receptors can be disrupted by so-called FLICE-inhibitory proteins (FLIPs). Mammalian cells express cellular FLIP (c-FLIP) as two splice variants: c-$FLIP_S$ consisting of two DED motifs, and c-$FLIP_L$ which additionally contains a catalytically inactive caspase domain (Goltsev et al., 1997; Irmler et al., 1997; Srinivasula et al., 1997). Since c-FLIP associates with FADD or caspase-8, it was suggested to inhibit death receptor-mediated apoptosis by displacing DED-containing caspases from the DISC (Hu et al., 1997; Irmler et al., 1997; Srinivasula et al., 1997).

The intrinsic mitochondrial death pathway is initiated at the mitochondrion by the release of cytochrome *c*. The release of cytochrome *c* can be initiated either through death receptor-mediated activation of the Bcl-2 related protein Bid (Green, 2000; Li et al., 1998; Luo et al., 1998) or independently of this pathway by other pro-apoptotic Bcl-2 members such as Bax, Bak, Bad, Bim, Bmf, Hrk, Nbk, Bnip3, Puma or Noxa (Adams

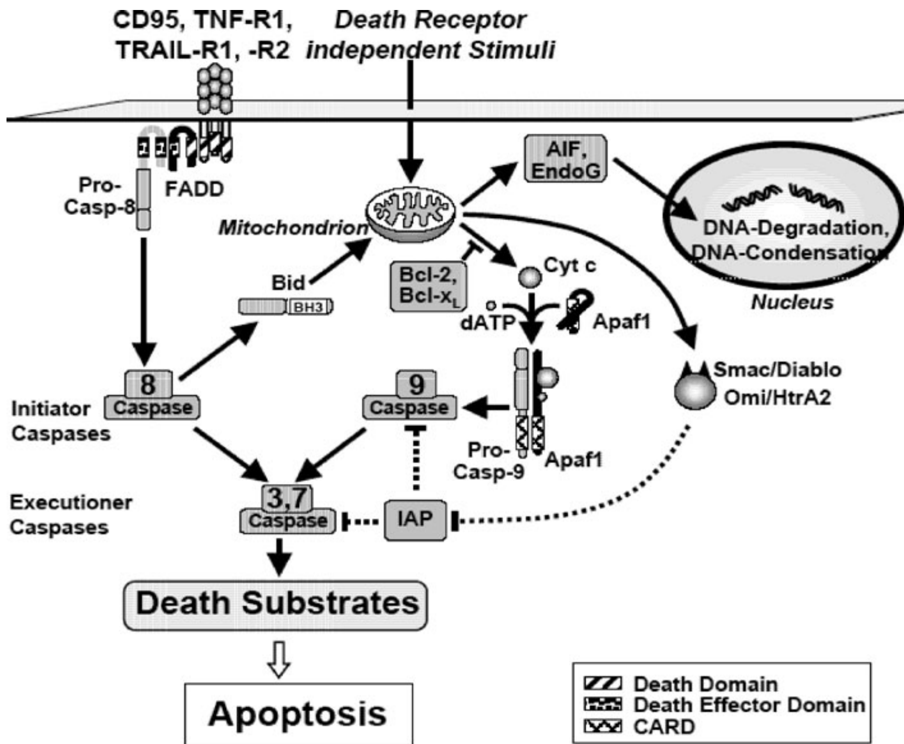

Figure 1: The two major signaling pathways of apoptosis. The death receptor pathway *(left)* is initiated upon receptor ligation, resulting in the recruitment of the adapter protein FADD through interaction between the death domains (DD) of both proteins. The death effector domain (DED) of FADD in turn recruits pro-caspase-8 which undergoes autoproteolytic activation at the receptor complex (DISC). The mitochondrial death pathway *(right)*, which is triggered by many apoptotic stimuli and pro-apoptotic Bcl-2 members, involves the release of cytochrome *c* into the cytosol. Cytochrome *c*, after hydrolysis of (d)ATP, binds to Apaf-1 and in turn allows binding of pro-caspase-9 through CARD/CARD interaction. Pro-caspase-9 is subsequently activated in this high molecular weight complex (apoptosome). Expression of anti-apoptotic Bcl-2 proteins (Bcl-2 or Bcl-x_L) inhibit the release of pro-apoptotic factors from the mitochondrion and thus the activation of the mitochondrial cytochrome *c*/Apaf-1/caspase-9 pathway. Activation of both pathways via initiator caspase-8 or -9 leads to the activation of effector-caspases (caspase-3, -6 and -7) that after cleavage of vital death substrates induce the final demise of the cell. Because caspase-8 cleaves Bid and generates a truncated, pro-apoptotic BH3-containing fragment that induces cytochrome *c* release, both pathways cross-communicate. Beside cytochrome *c* there are also other factors released from the mitochondrion such as Smac/DIABLO, Omi/HtrA2, apoptosis inducing factor (AIF), and endonuclease G (EndoG). AIF and EndoG together with the caspase-activated DNase (CAD) contribute to DNA-fragmentation and DNA-condensation. Cytolic Smac and Omi both neutralize the inhibitory effect of the inhibitor of apoptosis proteins (IAPs) on the activation of caspase-3, -7, and -9 and thereby contribute to caspase activation.

& Cory, 1998; Daniel et al., 2003; Kluck et al., 1997; Puthalakath & Strasser, 2002; Yang et al., 1997). Conversely, anti-apoptotic members of the Bcl-2 family such as Bcl-2, Bcl–x_L, Bcl-w, A1 and Mcl-1 block the mitochondrial release of cytochrome *c* and the subsequent activation of caspases and apoptosis (Adams & Cory, 1998; Daniel et al.,

2003; Kluck et al., 1997; Puthalakath & Strasser, 2002; Yang et al., 1997). In the cytosol, cytochrome *c* binds to the mammalian CED-4 homologue Apaf-1 and stimulates its subsequent (d)ATP dependent oligomerization and recruitment of pro-caspase-9 via a mutual interaction of their CARDs (Li et al., 1997). In this multimeric high molecular weight complex of ~700 kDa - also called apoptosome - pro-caspase-9 is activated and can now cleave and activate downstream effector caspases such as caspase-3 and -7 (Cain et al., 1999; Hu et al., 1999), (Fig. 1). Thus, caspase-9 constitutes the central initiator caspase for the mitochondrial pathway as caspase-8 represents it for the death receptor pathway. In analogy, the adapter protein Apaf-1 plays a similar role for the activation of the mitochondrial pathway as FADD does in the death receptor pathway. Activation of both pathways via initiator caspase-8 or -9 triggers an amplifying cascade of executioner caspases (as caspase-3, -6 and -7) that after cleavage of vital death substrates leads to the final demise of the cell (Fig. 1).

Beside the relocation of cytochrome *c* there are also other apoptotic factors released from mitochondria during apoptosis, such as Smac/DIABLO, Omi/HtrA2, apoptosis inducing factor (AIF), endonuclease G (EndoG), caspase-2 or caspase-9 (Du et al., 2000; Hegde et al., 2002; Kroemer & Reed, 2000; Martinou & Green, 2001; Martins et al., 2002; van Loo et al., 2002; Verhagen et al., 2000). Both Smac/DIABLO and Omi/HtrA2 induce apoptosis by neutralizing the inhibitory effect of the *inhibitor of apoptosis proteins* (IAPs) that have been reported to directly inhibit active caspase-3 and caspase-7 and to block caspase-9 activation (Deveraux & Reed, 1999; Goyal, 2001; Holcik & Korneluk, 2001; Reed & Bischoff, 2000). One important feature that differentiates Omi from Smac/DIABLO is its serine protease activity that might contribute to an atypical caspase independent cell death (Verhagen et al., 2000). AIF and EndoG induce chromatin condensation and large-scale DNA-fragmentation, and thus, contribute to the DNA-degradation mediated by caspase-activated DNase (CAD) (Enari et al., 1998; Li et al., 2001b; Liu et al., 1997; Susin et al., 1999), (Fig. 1).

In addition to the activation of the mitochondrial death pathway there exist at least two other intrinsic organelle specific apoptosis pathways within the cell: the endoplasmic reticulum (ER) specific stress pathway and the nuclear so-called PIDDosome. The ER-stress pathway is activated by accumulation of misfolded proteins or calcium signaling that subsequently induce apoptosis by the unfolded protein response and the ER-specific caspase-12 (Ferri & Kroemer, 2001; Nakagawa et al., 2000; Yoneda et al., 2001). Beside the high molecular weight complexes of death receptor signaling (DISC) and the mitochondrial death pathway (apoptosome) there also seems to exist a high molecular weight complex within the nucleus that is activated by genotoxic stress. This so-called PIDDosome consists of the death domain (DD) containing protein PIDD, the CARD and DD comprising adapter protein RAIDD and the CARD containing caspase-2 and is likely to be regulated in a p53-dependent manner in apoptosis induced by anticancer drugs (Lassus et al., 2002; Tinel & Tschopp, 2004).

4. MECHANISMS OF ANTICANCER DRUG INDUCED APOPTOSIS

The primary effect of classical anticancer therapy (i.e. anticancer drugs and γ-irradiation) is the generation of double-strand breaks within the DNA. DNA-damage, in

principle has three different effects: (*i*) DNA-repair, (*ii*) cell cycle arrest and (*iii*) as a final consequence - apoptosis (Norbury & Zhivotovsky, 2004; Rich et al., 2000). The importance of the latter lethal consequence for the survival of multicellular organisms could be appreciated by the fact that unicellular organism only activate cell cycle checkpoint and repair mechanims but lack the activation of the cell death program (Norbury & Zhivotovsky, 2004). The molecular signaling mechanisms how DNA-damaging anticancer drugs activate the endogenous apoptosis program has long been a controversial issue. Whereas initial reports claimed that anticancer drugs induce apoptosis via death receptors like CD95 (Beltinger et al., 1999; Dhein et al., 1995; Eichhorst et al., 2001; Eichhorst et al., 2000; Friesen et al., 1999; Friesen et al., 1996; Fulda et al., 1998a; Fulda et al., 2001; Fulda et al., 1998b; Fulda et al., 1997b; Fulda et al., 2000; Fulda et al., 1998c; Herr et al., 1997; Houghton et al., 1997; Kasibhatla et al., 1998; Los et al., 1997; Muller et al., 1997; Muller et al., 1998; Scaffidi et al., 1998), others supported the concept that chemotherapeutic agents induce apoptosis via the mitochondrial death pathway (Bantel et al., 1999; Boesen-de Cock et al., 1998; Eischen et al., 1997; Engels et al., 2000; Ferrari et al., 1998; Fuchs et al., 1997; Fulda et al., 1997a; Gamen et al., 1997; Glaser et al., 1999; Hakem et al., 1998; Kaufmann & Earnshaw, 2000; Kharbanda et al., 1997; Landowski et al., 1999; Los et al., 1999; Miyashita & Reed, 1993; Newton and Strasser, 2000; Strasser et al., 1995; Tolomeo et al., 1998; Varfolomeev et al., 1998; Villunger et al., 1997a; Wesselborg et al., 1999; Wieder et al., 2001 Glaser, et al., 2001; Yeh et al., 1998; Yoshida et al., 1998).

The concept that cytostatic agents induce apoptosis by death receptor signaling originated from the observation that anticancer drugs like doxorubicin induce the expression of CD95L (Friesen et al., 1996) and that leukemic T cell lines like Jurkat or CEM resistant to CD95 induced apoptosis were also cross-resistant to anticancer drug induced cell death (Friesen et al., 1996; Los et al., 1997). Blocking the interaction of CD95 with its ligand via antagonistic antibodies or soluble antagonistic CD95 also abrogated antitumor drug induced apoptosis (Friesen et al., 1996). Accordingly, an upregulation of CD95L was observed in many different tumor cell lines (such as hepatoma, leukemia, neuroblastoma, malignant brain tumors, colon, breast or lung cell carcinoma cells) in vitro and also in patients' derived primary tumor cells (Debatin, 2004; Debatin & Krammer, 2004; Schulze-Bergkamen & Krammer, 2004). In addition, AP-1, NF-κB and p53 binding sites were identified within the human CD95L promotor and activation of these transcription factors was shown to induce CD95L expression upon chemotherapy (Eichhorst et al., 2000; Herr et al., 1997; Kasibhatla et al., 1998; Muller et al., 1997; Muller et al., 1998). Beside CD95L the expression of the CD95 receptor and of TRAIL-R2/DR5 was also shown to be increased upon drug treatment (Fulda et al., 1998a; Fulda et al., 1998b; Fulda et al., 1998c; Wu et al., 1997a). In addition, pro-apoptotic components of the DISC like FADD and pro-caspase-8 were found to be upregulated and increasingly recruited to the DISC during treatment with anticancer drugs (Beltinger et al., 1999; Debatin & Krammer, 2004; Micheau et al., 1999a; Micheau et al., 1999b). Interestingly, similar to activation-induced cell death upon T-cell receptor stimulation, treatment with doxorubicin led to the expression of CD95L in leukemic T cells and subsequent apoptosis via CD95/CD95L interaction in a paracrine mode (Debatin & Krammer, 2004; Friesen et al., 1996; Fulda et al., 2000). Thus, these findings supported a concept that cytotoxic agents induce apoptosis via de novo synthesis of

CD95L and CD95 and subsequent activation of downstream located executioner caspases in a similar manner as activation-induced cell death in T cells (Dhein et al., 1995).

However, various other reports challenged this intriguing concept. Studies using CD95-, caspase-8- or FADD- deficient Jurkat cells or cell lines overexpressing dominant-negative mutants of FADD or c-FLIP could not observe any inhibition of anticancer drug induced apoptosis, whereas death receptor induced apoptosis was completely abolished. In addition, overexpression of the serpin CrmA (that inhibits caspase-1, -4 and -8) or addition of soluble antagonistic CD95 had no effect on anticancer drug induced apoptosis

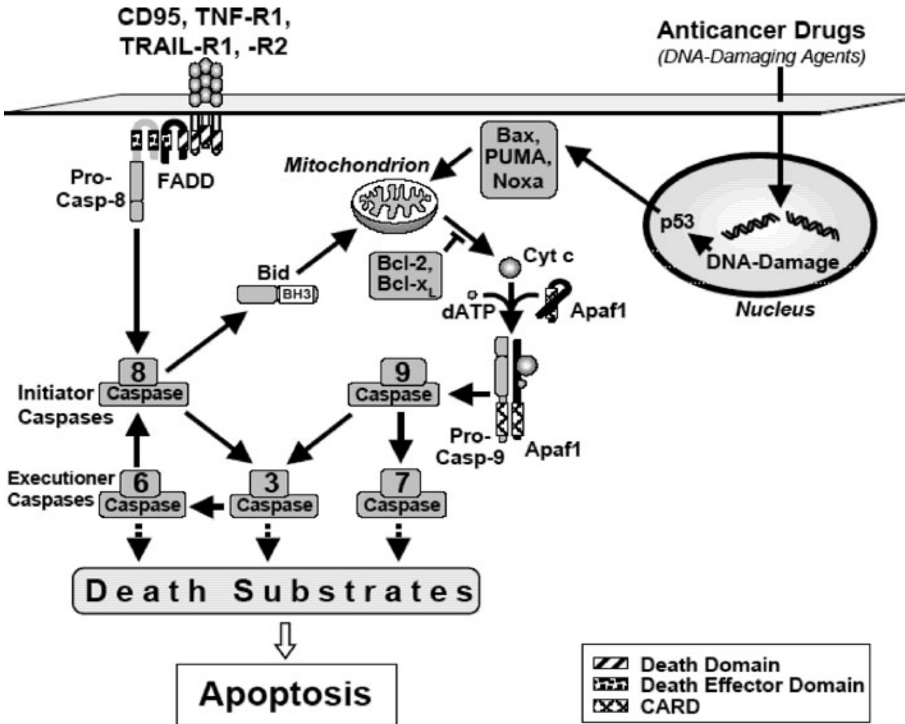

Figure 2: Signaling of DNA-damaging anticancer drugs (modified from Los et al., 1999). The major mechanism of most anticancer drugs is the induction of DNA-damage that leads to p53-mediated DNA-repair, cell cycle arrest or apoptosis. p53 induces apoptosis by the transcriptional expression of pro-apoptotic proteins (such as Bax, PUMA, Noxa, Apaf-1, PIDD, p53AIP1 Peg3/Pw1, CD95, TRAIL-R2, GML, P2XM, PAG608, or PIGs) and the donwregulation of anti-apoptotic proteins (like Bcl-2 or survivin). Besides its transcriptional activity p53 can also translocate to the mitochondria and directly trigger the mitochondrial death pathway via activation of Bax. Tumor cells comprise resistance to cytotoxic agents by downregulation the expression of pro-apoptotic proteins (like caspases, Bax, Apaf-1, or XAF1) or upregulation of anti-apoptotic factors (as Bcl-2, Bcl-x$_L$, or IAPs). Another possibility is the controversial model of the 'tumor counter attack', where tumor cells might protect themselves from the surveillance of the immune system via expression of CD95L on the cell surface and thus mediating the 'deadly kiss' to the immune cells.

though CD95 mediated cell death and caspase-8 activation was obstructed (Bantel et al., 1999; Engels et al., 2000; Glaser et al., 1999; Wesselborg et al., 1999; Wieder et al., 2001). Conversely, inhibition of the mitochondrial death pathway by depletion of intracellular ATP via oligomycin, overexpression of anti-apoptotic Bcl-2 or Bcl-x_L or of a dominant negative mutant of caspase-9 also blocked cytotoxic drug induced caspase activation and apoptosis (Bantel et al., 1999; Engels et al., 2000; Ferrari et al., 1998; Kharbanda et al., 1997; Miyashita & Reed, 1993; Newton & Strasser, 2000; Wesselborg et al., 1999). Interestingly, caspase-8 as the central initiator caspase of the death receptor pathway was also cleaved during anticancer drug induced apoptosis in CD95- or FADD-deficient Jurkat cells, Jurkat cells overexpressing c-FLIP and HeLa cells with enforcedexpression of a dominant negative mutant of FADD (Boesen-de Cock et al., 1998; Engels et al., 2000; Nencioni et al., 2003; Wesselborg et al., 1999). However, in caspase-3 deficient MCF7 cells antitumor drugs induced cleavage of caspase-9 but not of caspase-8, thus indicating that caspase-8 in anticancer drug induced activation of the mitochondrial death pathway is not functioning as an initiator caspase but is a target-caspase located downstream of caspase-9 > caspase-3 > caspase-6 and might contribute to an amplification loop via cleavage of Bid (Engels et al., 2000; Perkins et al., 2000; Slee et al., 1999a; Slee et al., 1999b; Slee et al., 2000; Tang et al., 2000), (Fig. 2).

However, the final proof that anticancer drugs induce apoptosis via the mitochondrial death pathway in the absence of death receptor signaling came from studies in knockout mice (Los et al., 1999). Thus, murine embryonic fibroblasts (MEF) from mice with targeted disruption of genes involved in death receptor signaling (such as FADD and caspase-8) showed no resistance to anticancer drug induced apoptosis whereas death receptor induced apoptosis was blocked (Varfolomeev et al., 1998; Yeh et al., 1998). Conversely, blocking of the mitochondrial death pathway using Apaf-1$^{-/-}$ thymocytes or caspase-9$^{-/-}$ stem cells conferred resistance to cytotoxic drugs whereas death receptor signaling remained intact (Hakem et al., 1998; Yoshida et al., 1998). Thus, it appears that the basic apoptotic mechanism of DNA-damaging anticancer drugs is the activation of the mitochondrial cytochrome *c*/Apaf-1/caspase-9 pathway (Debatin & Krammer, 2004; Kaufmann & Vaux, 2003; Los et al., 1999; Villunger & Strasser, 1998).

4.1. Role of p53 in Anticancer Drug Induced Apoptosis

If activation of the mitochondrial death pathway is the obvious consequence of DNA-damaging anticancer drugs the question remains what the molecular mechanisms are that link DNA-damage to the activation of the mitochondrial cytochrome *c*/Apaf-1/caspase-9 pathway. In this context, the key tumor suppressor protein p53 was the first determinant of susceptibility to DNA-damage induced apoptosis to be identified (Clarke et al., 1993; Lowe et al., 1994; Lowe et al., 1993a; Lowe et al., 1993b). Accordingly, p53 is the most frequently targeted genetic alteration in human cancers (Hollstein et al., 1991; Tokino & Nakamura, 2000; Vogelstein et al., 2000). Interestingly, unlike most other tumor suppressor genes that are inactivated by frameshift or nonsense mutations leading to disappearance of the gene product, almost 90% of p53 gene mutations are missense mutations leading to the expression of a stable protein that might display a dominant-negative role by heterooligomerization with wild-type p53 expressed by the second allele, or a specific gain of function of mutant p53 (Soussi, 2003; Soussi & Beroud, 2001).

Although most current anticancer drugs do not directly interact with p53, most of these agents (including DNA-damaging drugs, antimetabolites, microtubule-active drugs or inhibitors of the proteasome) cause an accumulation of wild type p53. Activation of p53 can induce growth arrest, apoptosis or senescence. The activity of p53 is mainly regulated by proteolytic degradation by the proteasome. DNA-damaging agents, microtubule-active drugs and transcription- or proteasome-inhibitors inhibit the degradation and, thus, stabilize p53 expression (Blagosklonny, 2002). DNA-damage afflicted by irradiation (γ- or UV-irradiation) or anticancer drugs (like doxorubicin, daunorubicin, dactinomycin (actinomycin D), etoposide, topotecan, mitomycin C, bleomycin, cisplatin, carboplatin, melphalan, chlorambucil, busulfan, cyclophosphamide or ifosfamide) induce the expression of wild type p53. As different the primary source for DNA-damage might be (DNA-intercalation, inhibition of topoisomerase I or II, DNA-crosslinking, alkylating agents) they all lead to DNA-strand breaks that seem to be the prime stimulus for p53 activation. Inside the cell p53 is usually bound to the negative regulator Mdm2 that subsequently targets the proteasomal degradation of p53. Sensors for DNA-damage like the kinases ATM (for ataxia teleangiectasia mutated) or Chk2 phosphorylate and stabilize p53 directly and inhibit Mdm2 mediated ubiquitination and subsequent proteasomal degradation of p53 (Johnstone et al., 2002; Khanna & Jackson, 2001; Rich et al., 2000).

Upon activation, p53 induces the expression of a variety of different apoptotic proteins such as components of the mitochondrial death pathway like Bax, PUMA, Noxa, Apaf-1, PIDD, p53AIP1, Peg3/Pw1, PERP (Attardi et al., 2000; Deng and Wu, 2000; Johnson et al., 2002; Lin et al., 2000; Miyashita & Reed, 1995; Moroni et al., 2001; Nakano & Vousden, 2001; Oda et al., 2000a; Oda et al., 2000b; Relaix et al., 2000; Schuler et al., 2003; Tinel & Tschopp, 2004; Villunger et al., 2003; Yu et al., 2001; Zhang et al., 2000) or of the death receptor pathway like CD95 and TRAIL-R2 (Bennett et al., 1998a; Wu et al., 1997a) and other proteins with apoptotic functions like GML, P2XM, or PAG608, or PIG 3 (Furuhata et al., 1996; Israeli et al., 1997; Polyak et al., 1997; Tokino et al., 1994; Urano et al., 1997). p53 mediated expression of the pro-apoptotic Bcl-2 proteins Bax, PUMA and Noxa leads to their translocation from the cytosol to the mitochondrion where they induce the permeabilization of the outer mitochondrial membrane, and thus, the release of pro-apoptotic factors such as cytochrome *c* (Ferri & Kroemer, 2001; Miyashita & Reed, 1995; Nakano & Vousden, 2001; Oda et al., 2000a; Schuler et al., 2003; Yu et al., 2001; Zhang et al., 2000). Expression of the p53-inducible protein Peg3/Pw1 that is unrelated to the Bcl-2 family induces the mitochondrial translocation of Bax through an yet unknown mechanism (Deng & Wu, 2000; Johnson et al., 2002; Relaix et al., 2000). Another Bcl-2 unrelated protein is the p53-regulated apoptosis-inducing protein 1 (p53AIP1) that is also localized at the mitochondrion where it induces the transition in mitochondrial membrane potential ($\Delta\Psi m$) and release of cytochrome *c* most likely by an interaction with Bcl-2 proteins at the mitochondrion (Matsuda et al., 2002; Oda et al., 2000b). Another p53 target that mediates p53 dependent apoptosis is PERP, a novel member of the PMP-22/gas3 family. PERP is a cellular transmembrane protein localized to the plasma-membrane, rather than to mitochondria, and therefore, stimulates apoptosis through a different mechanism from the BH3-containing proteins Bax, PUMA or Noxa (Attardi et al., 2000; Ihrie & Attardi, 2004; Ihrie et al., 2003; Reczek et al., 2003).

Another intriguing mechanism of DNA-damage induced apoptosis is the formation of the so-called PIDDosome. PIDD (p53-induced protein with a DD), was originally described as another death domain (DD) containing protein that is transcriptionally induced in a p53-dependent manner following exposure to DNA-damaging agents. Overexpression of PIDD induced apoptosis, whereas antisense inhibition of PIDD expression attenuated p53-mediated apoptosis (Lin et al., 2000). In addition to PIDD, the PIDDosome consists of the CARD and DD comprising adapter protein RAIDD and the CARD containing caspase-2 (Tinel & Tschopp, 2004). Caspase-2 has recently been described to be required for anticancer drug induced apoptosis. Inhibition of the expression of caspase-2 using small interfering RNA (siRNA) inhibited in a similar way antitumor drug induced apoptosis (using etoposide or cisplatin) as siRNA to Apaf-1. Interestingly, siRNA mediated inactivation of caspase-2 also inhibited the release of cytochrome *c*, Smac and the mitochondrial translocation of Bax whereas siRNA to Apaf-1 had no effect (Lassus et al., 2002; Robertson et al., 2002). This observation places caspase-2 upstream of the mitochondrial cytochrome *c*/Apaf-1/caspase-9 pathway. Tinel and Tschopp could now identify the nuclear complex responsible for caspase-2 activation showing that caspase-2 is activated within the PIDD-RAIDD-caspase-2 complex (the PIDDosome) upon genotoxic stress induced by etoposide or doxorubicin (Tinel & Tschopp, 2004). Thus, with the PIDDosome a new member of multimeric apoptogenic complexes (like the DISC and the apoptosome) goes on stage. Since in this scenario, caspase-2 activity is required for inducing Bid cleavage, Bax translocation to mitochondria and subsequent cytochrome *c* release this locates the PIDDosome (like the DISC) upstream of the cytochrome *c* activated apoptosome.

In addition, several other p53 inducible genes could be identified such as GML (GPI-anchored molecule-like protein) (Kagawa et al., 1997; Kimura et al., 1997; Tokino et al., 1994), the P2X purinoceptor P2XM (P2X specifically expressed in skeletal muscle) (Urano et al., 1997), the nuclear zinc finger protein PAG608 (Israeli et al., 1997), and PIG 3 (p53-regulated apoptosis-inducing protein 3) (Contente et al., 2002; Polyak et al., 1997; Venot et al., 1998) that all seem to be involved in anticancer drug induced apoptosis. As already mentioned p53-responsive elements have been identified in the first intron of the CD95 gene, as well as three putative p53-binding sites within the CD95 promotor, which showed homology with the p53 consensus binding site (Debatin & Krammer, 2004; Muller et al., 1997; Muller et al., 1998). Likewise TRAIL-R2/KILLER/DR5 is activated upon DNA-damage by wild-type p53 through an intronic sequence-specific DNA-binding site (Takimoto & El-Deiry, 2000; Wu et al., 1997a; Wu et al., 1997b). Though DNA-damaging anticancer drugs seem to induce the expression of CD95L, CD95 and TRAIL-R2, death receptor signaling obviously is not a prerequisite of anticancer drugs induced apoptosis (Kaufmann & Earnshaw, 2000). Next to the transcriptional activation of pro-apoptotic genes p53 has also been shown to be involved in the downregulation of anti-apoptotic genes such as Bcl-2, MAP4 and survivin (Haldar et al., 1994; Hoffman et al., 2002; Miyashita et al., 1994; Murphy et al., 1996; Slee et al., 2004).

Beside the transcriptional expression of pro-apoptotic proteins through direct interactions with both chromatin and regulators of transcription, p53 seems also to comprise transcriptional independent apoptotic features. Thus, p53 has recently been shown to translocate to the mitochondria and to directly activate the pro-apoptotic Bcl-2

protein Bax with subsequent mitochondrial membrane permeabilization, cytochrome *c* release and apoptosis induction. In addition, p53 can release pro-apoptotic Bcl-2 proteins sequestered by Bcl-x_L. Therefore, in the cytosol the versatile protein p53 apparently operates in a similar way as the pro-apoptotic Bcl-2 members (Chipuk & Green, 2003; Chipuk et al., 2004; Chipuk et al., 2003; Marchenko et al., 2000; Petros et al., 2004; Schuler et al., 2000).

Beside p53, there appears to be another chromatin-derived signal linking nuclear DNA-damage to mitochondria via the linker histone H1.2. H1.2 appears to be another cytochrome *c* releasing factor that appears in the cytosol after treatment with anticancer drugs or γ-irradiation (Konishi et al., 2003). While all nuclear histone H1 forms are released into the cytosol in a p53-dependent manner upon DNA-damage, only H1.2 is able to induce cytochrome *c* release from isolated mitochondria in a Bak dependent mode (Konishi et al., 2003; Yan & Shi, 2003). Another protein that translocates to mitochondria during apoptosis is the Peutz-Jegher gene product LKB1. LKB1 is a cytosolic and nuclear Ser/Thr kinase (mutated in Peutz-Jegher syndrome) that physically associates with p53 and regulates specific p53-dependent apoptosis pathways.

4.2. p53-independent Apoptotic Mechanisms of Anticancer Drugs

Though p53 is thought be a major element in the execution of anticancer drug induced apoptosis there obviously exist supplementary pathways that do not rely on p53. Thus, various different tumor cell lines - like CEM, Jurkat, MOLT4 (T-cells), Raji (B-cells), HEL (erythroleukemia) - display mutant p53 (Bull et al., 1998) but are still sensitive to anticancer drugs like daunorubicin, doxorubicin mitomycin C or etoposide (Herr et al., 2000; Wesselborg et al., 1999). This might be attributed to p63 and p73, two homologous genes of p53 that might compensate for the effect of p53. In this manner it could be demonstrated that ectopic expression of p73 could induce expression of p53AIP1 and apoptosis in p53-negative AsPC-1 cells (Rodicker & Putzer, 2003). In addition, it has been shown that p73 is essential for many anticancer drugs though its function can obviously be blocked by a particular category of p53 gain of function mutations (Bergamaschi et al., 2003; Irwin et al., 2003; Soussi, 2003). Interestingly, T lymphoma cells and activated T cells from p53 deficient mice were still sensitive to irradiation and chemotherapeutic agents but were apoptosis-resistant when the anti-apoptotic Bcl-2 protein was expressed. (Strasser et al., 1994). This indicates that the mitochondrial death pathway still plays a central role in anticancer drug induced apoptosis independent of p53. One candidate of p53-independent apoptosis induction is the pro-apoptotic nuclear orphan receptor transcription factor TR3/Nur77 (Li et al., 2000). TR3 is an immediate-early response gene and an orphan member of the steroid-thyroid hormone-retinoid receptor superfamily of transcription factors. Treatment of the human prostate cancer cells LNCaP with etoposide induced apoptosis that was blockable with antisense to TR3. In response to apoptotic stimuli such as etoposide, TR3 translocated from the nucleus to the mitochondria to induce cytochrome *c* release and apoptosis. Though being a nuclear localized transcription factor, it could be demonstrated that the mitochondrial targeting of TR3, but not its DNA-binding and transactivation was necessary for apoptosis induction (Li et al., 2000). Another factor that so far has not been shown to be p53-dependent is the XIAP-associated factor 1 (XAF1). XAF1 was isolated

based on its ability to bind to the anti-apoptotic protein XIAP. It was shown that XAF1 antagonized the apoptosis-protective effect of XIAP by redistribution of XIAP from the cytoplasm to the nucleus. Since the ubiquitously expressed protein is only present at low or undetectable levels in different cancer cell lines it is speculated that XAF1 might contribute to cancer resistance (Liston et al., 2001). Beside their ability to induce DNA-damage at least two cytotoxic drugs - etoposide and cisplatin - have been shown to induce cytochrome *c* release or caspase activation in the absence of DNA-damage using isolated mitochondria (Robertson et al., 2000) or enucleated cytoplasts (Mandic et al., 2003).

4.3. Mechanisms of Anticancer Drug Resistance

One simple way of counteracting the detrimental effect of cytotoxic agents is to simply pump the respective anticancer drugs out of the cell. The major drug transporters involved in drug resistance are the two MDR1 gene products P-glycoprotein and MRP (multidrug resistance-associated protein) that both belong to the ATP-binding cassette superfamily of transporter proteins (Cole et al., 1992; Palissot et al., 1998; Persidis, 1999). In addition, tumor cells specifically inactivate the apoptotic machinery in order to increase their survival. The most effective way to silence the endogenous suicide machinery would be to inactivate the apoptotic executioners i.e. the caspases within the cell. This principle has actually been realized in the human tumor cell line MCF7 derived from a breast carcinoma that lacks caspase-3 owing to the functional deletion of the CASP-3 gene that consequently no longer shows any DNA-fragmentation or morphological changes of apoptosis (Jänicke et al., 1998). Reconstitution of MCF7 cells with caspase-3 sensitized these cells to doxorubicin and etoposide induced apoptosis (Yang et al., 2001). In addition to caspase-3 other caspases like caspase-1, -4, -8 and -10 have been shown to be absent on mRNA level in different small cell lung carcinoma cell lines (Joseph et al., 1999). One mechanism utilized by tumor cells to downregulate the expression of pro-apoptotic proteins are epigenetic alterations such as hypermethylation of regulatory gene sequences. This has been shown for caspase-8 that is frequently inactivated by hypermethylation of regulatory sequences of the caspase-8 gene in a number of different tumor cells derived from neuroblastoma, malignant brain tumors, Ewing tumors, and small lung cell carcinoma both *in vitro* and *in vivo* in primary tumors (Debatin, 2004; Fulda et al., 2001; Teitz et al., 2000). Restoration of caspase-8 expression by methylation inhibitors or gene transfer sensitized tumor cells to death receptor or anticancer drug induced apoptosis (Fulda et al., 2001). Hypermethylation of regulatory gene sequences has also been described for XAF1 in human gastric adenocarcinoma and for Apaf-1 in human melanoma. Since the mitochondrial cytochrome *c*/Apaf-1/caspase-9 pathway represents an essential element in p53-induced apoptosis (Soengas et al., 1999) elimination of Apaf-1 seems to be an effective approach of tumor cells to evade the action of cytotoxic agents. However, the role of Apaf-1 and caspase-9 as tumor suppressors has been challenged since neither Apaf-1 nor caspase-9 were capable of suppressing c-myc-induced lymphomagenesis and embryo fibroblast transformation (Scott et al., 2004).

Another approach exploited by tumor cells is the downregulation of the expression of the pro-apoptotic protein Bax. Accordingly, reduced Bax expression has been

associated with poor responses to chemotherapy and shorter overall survival in breast and colorectal carcinoma (Guner et al., 2003; Sturm et al., 1999). Conversely, enhanced Bax levels correlated in esophageal squamous cell carcinoma with a good prognosis (Sturm et al., 2001). Likewise, cancer cells decrease the apoptotic effect of cytotoxic drugs by increasing the ratio of anti-apoptotic to pro-apoptotic Bcl-2 members by enhancing the expression anti-apoptotic Bcl-2 (Minn et al., 1995). Therefore, like reduced expression of Bax, overexpression of anti-apoptotic Bcl-2 family members confers anti-tumor drug resistance in a number of different tumors like ALL, AML, CLL, multiple myeloma, prostate carcinoma, malignant brain tumors, and neuroblastoma (Bargou et al., 1995; Campos et al., 1993; Debatin, 2004; Prokop et al., 2000; Reed, 1999). In addition to Bcl-2, also other anti-apoptotic proteins of the IAP family have been shown to be upregulated in tumors and to confer drug resistance (Adida et al., 1998; Adida et al., 2000; Li et al., 2001a; Tamm et al., 2000). Thus, XIAP, c-IAP1, c-IAP2 have been shown *in vitro* to inhibit apoptosis induced by a variety of different stimuli such as cisplatin, cytarabine, γ–irradiation, staurosporine or TRAIL (Datta et al., 2000; Suliman et al., 2001). Accordingly, the IAP-protein survivin that is usually undetectable in normal differentiated tissues is overexpressed in various different human cancers (such as neuroblastoma, melanoma, AML, breast, colon, lung and esophagus carcinoma) and is correlated with a poor prognosis (Adida et al., 1998; Adida et al., 2000; Altieri, 2001; Debatin, 2004; Li et al., 2001a; Salvesen & Duckett, 2002; Tamm et al., 2000).

Another intriguing mechanism of the tumor to evade the detrimental consequences of chemotherapy has been proposed by the observation that cytotoxic agents can induce the expression of CD95 and its ligand CD95L (Friesen et al., 1996). In addition, a high-constitutive CD95L expression has been found in distinct lineages of tumors, such as colon, lung, renal carcinoma, melanoma, hepatocellular carcinoma, astrocytoma and T- and B-cell-derived neoplasms (Hahne et al., 1996; Niehans et al., 1997; O'Connell et al., 1996; Saas et al., 1997; Shiraki et al., 1997; Strand et al., 1996; Tanaka et al., 1996). Though the anticancer drug induced expression of CD95L might not be causative for apoptosis induction mediated by cytotoxic agents it might have a pronounced effect on eliminating tumor-specific T-cells by an inverse 'deadly kiss'. In this so-called model of 'tumor counter attack', the tumor would gain a kind of 'immune privilege' by its virtue of displaying CD95L on its surface (Bellgrau et al., 1995; Ferguson et al., 2002; French & Tschopp, 2002; Green & Ferguson, 2001; Griffith et al., 1995; Griffith et al., 1996; Igney et al., 2000; Igney & Krammer, 2002; O'Connell et al., 1999; O'Connell et al., 1996; Restifo, 2001; Strand et al., 1996; Villunger & Strasser, 1999). Although, originally discovered on activated T-lymphocytes, various other non-lymphoid cells can express CD95L. A high constitutive expression is detected in Sertoli cells of the testis and epithelial cells of the anterior eye chamber (Bellgrau et al., 1995; Griffith et al., 1995; Griffith et al., 1996). This observation led to the proposal that CD95L is responsible for the maintenance of immune privilege, which characterizes the ability of certain organs to suppress graft rejection, even when transplanted in non-matched individuals (Bellgrau et al., 1995; Griffith et al., 1995). In case of tumor cells, CD95L mediated depletion of cytotoxic T-cells would enable the tumor cell to escape the host's immunosurveillance. Supporting the tumor counter attack model were reports that detected apoptotic tumor infiltrating lymphocytes *in situ* within the CD95L expressing tumor tissue (Bennett et al., 1998b; Hahne et al., 1996; O'Connell et al., 1996; Strand et al., 1996; Villunger et al.,

1997a; Villunger et al., 1997b). Conversely, it was also observed, that expression of CD95L by grafts or tumor cells targeted these cells for destruction by infiltrating neutrophils (Allison et al., 1997; Arai et al., 1997; Behrens et al., 2001; Kang et al., 1997a; Kang et al., 1997b). Thus, the CD95L mediated immune privilege of tumor cells and the hypothesis of tumor cells counter-attacking the immune system remains a controversial issue (Behrens et al., 2001; Debatin & Krammer, 2004; Igney et al., 2000; Restifo, 2000; Restifo, 2001).

4.4. Future Therapeutic Approaches Targeting the Apoptotic Program

The endogenous suicide program offers different therapeutic accesses for new drugs. Firstly, targeting of the tumor cell from the outside by triggering of death receptors. Unfortunately, targeting the prime candidate CD95 by agonistic antibodies lead to hypertension, liver failure and finally death. Similarly, TNF due to its toxic side effects is also a poor candidate for anticancer therapy. A very promising candidate will be TRAIL that has been shown to induce apoptosis in various different cancer cell lines, including carcinoma of the colon, lung, breast, pancreas, prostate, kidney, thyroid, malignant brain tumors, neuroblastoma, Ewing tumor, osteosarcoma, leukemia and lymphoma (Ashkenazi et al., 1999; LeBlanc & Ashkenazi, 2003; Walczak et al., 1999). Especially, TRAIL seems to display no toxicity in nonhuman primates such as chimpanzees, and cynomolgous monkeys (Ashkenazi et al., 1999) though the application form of the respective TRAIL construct seems to play an important role referring to toxicity on normal human hepatocytes (Ashkenazi et al., 1999; Ichikawa et al., 2001; Jo et al., 2000; Lawrence et al., 2001; LeBlanc & Ashkenazi, 2003; Nitsch et al., 2000; Walczak et al., 1999).

Another promising feature of TRAIL might be the treatment in combination with anticancer drugs or γ-irradiation. Thus, the combination of TRAIL with cytotoxic drugs or γ-irradiation has been shown to cooperate synergistically in order to overcome resistance to irradiation or anticancer drugs in various different cancers like melanoma, leukemia, breast, colon or prostate carcinoma and could even override Bcl-2 or Bcl-x_L mediated resistance to cytotoxic drugs and γ-irradiation (Ballestrero et al., 2004; Belka et al., 2001; Chinnaiyan et al., 2000; Gliniak & Le, 1999; Keane et al., 2000; Nagane et al., 2000; Rohn et al., 2001). In addition, TRAIL in combination with anticancer drugs also cooperated in suppression of tumor growth *in vivo* in different murine models of human cancers (Debatin & Krammer, 2004). A very promising approach is the combination of TRAIL with IAP antagonists including membrane permeable Smac-derived peptides that have been shown to increase the effect of TRAIL treatment by opposing the inhibitory effect of IAPs that are overexpressed in many tumors (Altieri, 2003a; Altieri, 2003b; Arnt et al., 2002; Fulda et al., 2002; Guo et al., 2002; Kanwar et al., 2001; Tamm et al., 2000; Tamm et al., 2003; Wajant et al., 2002; Xia et al., 2002; Yang et al., 2003). Notably, it could be demonstrated that Smac peptides synergized with TRAIL to eliminate malignant glioma in an orthotopic mouse model without affecting the normal brain tissue (Fulda et al., 2002).

Since a high expression of Bcl-2 is associated with poor response to chemotherapy *in vitro* and *in vivo* it has consequently been targeted by antisense approaches e.g. by G3139 (Genasense). G3139 is a phosphorothioate antisense oligonucleotide targeted to the

initiation codon region of the Bcl-2 mRNA that is currently being evaluated in several Phase II and Phase III clinical trials. This Bcl-2 antisense therapy is well tolerated and preclinical studies have shown that G3139 might increase the anti-tumor effect of several chemotherapeutic drugs (Banerjee, 2001; Chi et al., 2001; Cotter et al., 1994; Marcucci et al., 2003; Morris et al., 2002; Olie et al., 2002; Rudin et al., 2004; Rudin et al., 2002; Van De Donk et al., 2004; Waters et al., 2000; Webb et al., 1997; Ziegler et al., 1997).

Silencing of pro-apoptotic genes like Apaf-1, caspase-8 or XAF1 by hypermethylation has been shown to confer resistance to chemotherapeutic drugs (Byun et al., 2003; Soengas et al., 2001; Teitz et al., 2000). Therefore, reactivation of latent pro-apoptotic genes by demethylating agents and/or histone deacetylase (HDAC) inhibitors represents an intriguing concept and these agents are tested now in first clinical trials (Johnstone et al., 2002; Marks et al., 2001). However, in contrast to the specific targeting of pro-apoptotic factors these agents lack specificity and might cause detrimental side effects since other silenced genes like hTERT might be reactivated by HDAC inhibitors as well, and thus contribute to cellular immortalization and tumor progression (Marks et al., 2001; Paulsen & Ferguson-Smith, 2001; Takakura et al., 2001).

5. CONCLUSIONS

It would be a meager simplification to presume that inactivation of the endogenous suicide program represents the basic mechanism leading to cancerogenesis. However, the loss of function of pro-apoptotic regulators such as the tumor suppressor gene p53 or overexpression of anti-apoptotic proteins such as the oncogene Bcl-2 undoubtedly contribute to the survival and propagation of the tumor. Though, being a controversial issue over the last years it has become clear that the prime mechanism of cytotoxic agents seems to be the activation of the mitochondrial cytochrome *c*/Apaf-1/caspase-9 pathway and that the death receptor pathway appears to play a subsidiary role. One major feature of DNA-damaging anticancer drugs is the p53 mediated activation of the apoptotic machinery. This could be achieved by p53 mediated *de novo* synthesis of a variety of pro-apoptotic proteins (like Bax, PUMA, Noxa, Apaf-1, PIDD, p53AIP1 Peg3/Pwl, CD95, TRAIL-R2, GML, P2XM, PAG608, or PIGs). Beside its transcriptional activity, p53 can also directly activate the mitochondrial death pathway by mitochondrial translocation and subsequent activation of Bax. Though, p53 is a major executioner of anticancer drug induced cell death, cytotoxic agents can also activate the apoptotic program in the absence of functional p53. This might be addressed to the two other p53-family members p63 and p73, that might compensate for the effect of p53 or by other factors like XAF1 or TR3/Nur77, whose action has not been ascribed to p53 so far. Resistance to chemotherapy might be a prerequisite of a given tumor or acquired due to selection of cytotoxic drug resistant mutants during anticancer treatment. Thus, inactivation of pro-apoptotic factors like p53, Bax, Apaf-1, caspases or XAF1 by mutation or hypermethylation of respective regulatory gene sequences or overexpression of anti-apoptotic proteins like Bcl-2, Bcl-x_L, or IAPs confer resistance to activation of the apoptotic program by chemotherapeutic agents. Due to improvements of the knowledge in tumor biology new drug targets emerged that might reveal to be beneficial tools in cancer treatment. However, it is rather unlikely that the 'ideal' anticancer drug capable of

eliminating all different forms of tumors will ever exist. Most likely, as in HIV-treatment, the concerted action of drugs targeted at different features of the tumor (e.g. cell cycle, mitochondrial and death receptor pathway, angiogenesis, DNA-methylation, histone deacetylation, receptor tyrosine kinases, etc.) might in future enable a complete tumor eradication without remission.

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DEATH RECEPTOR PATHWAYS AS TARGETS FOR ANTICANCER THERAPY

Chapter III

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1. SUMMARY

Apoptosis, the cell's intrinsic program to death, plays a central role in physiological growth control and regulation of tissue homeostasis. Accordingly, tipping the balance between cell death and proliferation in favor of cell survival may result in tumor formation. Also, killing of cancer cells by cytotoxic therapies currently used for treatment of cancer, e.g. chemotherapy, γ-irradiation, immunotherapy or suicide gene therapy, largely depends on activation of apoptosis programs in cancer cells. To this end, death receptor signaling has been implied to contribute to the efficacy of cancer therapy. Failure to undergo apoptosis in response to anticancer therapy because of defects in death receptor pathways may therefore result in cancer resistance. Further insights into the mechanisms regulating apoptosis in response to anticancer therapy and how cancer cells evade cell death may provide novel opportunities for drug development. Thus, agents designed to selectively activate death receptor pathways may enhance the efficacy of conventional therapies and may even overcome some forms of cancer resistance.

2. INTRODUCTION

Killing of tumor cells by cytotoxic therapies, e.g. chemotherapy, γ-irradiation, immunotherapy or suicide gene therapy, is predominantly mediated by triggering apoptosis in cancer cells (Herr & Debatin, 2001). Apoptosis or programmed cell death is a distinct, intrinsic cell death program that occurs in various physiological and

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pathological situations (Hengartner, 2000). The underlying mechanisms for initiation of an apoptosis response upon cytotoxic therapy may depend on the individual stimulus and have not exactly been identified. However, damage to DNA or to other critical molecules is considered to be a common initial event which is then propagated by the cellular stress response (Rich et al., 2000). Multiple stress-inducible molecules, e.g. JNK, MAPK/ERK, NF-κB or ceramide have been implied in the regulation of apoptosis (Karin et al., 2002; Davis, 2000). Also, T cells or NK cells may contribute to tumor cell killing by releasing cytotoxic compounds such as granzyme B which directly activates downstream apoptosis effector mechanisms inside the cell (Hengartner, 2000). Apoptosis is characterized by typical morphological and biochemical hallmarks including cell shrinkage, nuclear DNA fragmentation and membrane blebbing (Hengartner, 2000). Proteolytic enzymes such as caspases are important effector molecules in apoptosis (Degterev et al., 2003). Because of the potential detrimental effects on cell survival in case of inappropriate activation of apoptosis programs, apoptosis pathways have to be tightly controlled. The anti-apoptotic mechanisms regulating cell death have also been implicated in conferring drug resistance to tumor cells (Igney & Krammer, 2002a). However, the concept that apoptosis represents the major mechanism by which cancer cells are eliminated may not universally apply and caspase-independent apoptosis or other modes of cell death have also to be considered as cell death response to cytotoxic therapy (Leist & Jaattela, 2001). Thus, a better understanding of these diverse modes of cell death in cancer therapy will provide a molecular basis for new strategies targeting death pathways in resistant forms of cancer.

3. APOPTOSIS SIGNALING PATHWAYS

In most cases, anticancer therapies eventually result in activation of caspases, a family of cysteine proteases that act as common death effector molecules in various forms of cell death (Degterev et al., 2003). Caspases are synthesized as inactive proforms and upon activation, they cleave next to aspartate residues (Degterev et al., 2003). The fact that caspases can activate each other by cleavage at identical sequences results in amplification of caspase activity through a protease cascade (Degterev et al., 2003). Caspases cleave a number of different substrates in the cytoplasm or nucleus leading to many of the morphologic features of apoptotic cell death (Degterev et al., 2003). For example, polynucleosomal DNA fragmentation is mediated by cleavage of ICAD (inhibitor of caspase-activated DNase), the inhibitor of the endonuclease CAD (caspase-activated DNase) that cleaves DNA into the characteristic oligomeric fragments (Nagata, 2000). Likewise, proteolysis of several cytoskeletal proteins such as actin or fodrin leads to loss of overall cell shape, while degradation of lamin results in nuclear shrinking (Hengartner, 2000).

Activation of caspases can be initiated from different angles, e.g. at the plasma membrane upon ligation of death receptor (receptor pathway) or at the mitochondria (mitochondrial pathway) (Hengartner, 2000). Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TRAIL receptors results in activation of the initiator caspase-8 which can propagate the apoptosis signal by direct cleavage of downstream effector caspases such as caspase-3 (Ashkenazi & Dixit, 1999). 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 (van Loo et al., 2002a). The release of cytochrome *c* into the cytosol triggers caspase-3 activation through formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex, while Smac/DIABLO and Omi/HtrA2 promote caspase activation through neutralizing the inhibitory effects to IAPs (van Loo et al., 2002a).

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 (van Loo et al., 2002a). In addition, cleavage of caspase-6 downstream of mitochondria may feed back to the receptor pathway by cleaving caspase-8 (Cowling & Downward, 2002).

4. DEATH RECEPTORS

Death receptors are members of the TNF receptor gene superfamily which consists of more than 20 proteins with a broad range of biological functions including regulation of cell death and survival, differentiation or immune regulation (Walczak & Krammer, 2000; Ashkenazi & Dixit, 1999; Ashkenazi & Dixit, 1998). Members of the TNF-receptor family share similar, cysteine-rich extracellular domains. In addition, death receptors are defined by a cytoplasmic domain of about 80 amino acids called „death domain“, which plays a crucial role in transmitting the death signal from the cell's surface to intracellular signaling pathways. The best-characterized death receptors comprise CD95 (APO-1/Fas), TNF receptor 1 (TNF-RI), TRAIL-R1 and TRAIL-R2, while the role of DR3 (TRAMP/Apo-3/ WSL-1/LARD) or DR6 has not exactly been defined (Walczak & Krammer, 2000; Ashkenazi & Dixit, 1999; Ashkenazi & Dixit, 1998). The p75 nerve growth factor (NGF) receptor also harbors a death domain through which cell death can be induced.

The corresponding ligands of the TNF superfamily comprise death receptor ligands such as CD95 ligand, TNF, lymphotoxin-α (the later two bind to TNF-RI), TRAIL and TWEAK, a ligand for DR3 (Walczak & Krammer, 2000; Ashkenazi & Dixit, 1999; Ashkenazi & Dixit, 1998). With the exception of lymphotoxin-α, all ligands are type II transmembrane proteins, which also exist as soluble molecules after cleavage by metalloproteases present in the microenvironment. Death receptors are activated upon oligomerization in response to ligand binding.

4.1. CD95

The CD95 receptor/CD95 ligand system is a key signal pathway involved in the regulation of apoptosis in several different cell types, e.g. in the immune system (Krammer, 2000) (Walczak & Krammer, 2000). CD95, a 48 kDa type I transmembrane receptor, is expressed on activated lymphocytes, on a variety of tissues of lymphoid or non-lymphoid origin, as well as on tumor cells. CD95 ligand, a 40 kDa type II transmembrane molecule, occurs in a membrane-bound and in a soluble form. CD95

ligand is produced by activated T cells and plays a crucial role in the regulation of the immune system by triggering autocrine suicide or paracrine death in lymphocytes or other target cells (Krammer, 2000). Furthermore, CD95L expression on cancer cells has been implicated in immune escape of tumors (Igney & Krammer, 2002b). By constitutive expression of death receptor ligands such as CD95L, tumors may adopt a killing mechanism from cytotoxic lymphocytes to delete the attacking anti-tumor T cells through induction of apoptosis via CD95/CD95L interaction. However, this model of tumor counterattack has also been challenged, since no study has so far conclusively demonstrated that tumor counterattack is a relevant immune escape mechanism in vivo (Igney & Krammer, 2002b).

4.2. TRAIL and its Receptors

TNF-related apoptosis-inducing ligand (TRAIL)/ Apo-2L was identified in 1995 based on its sequence homology to other members of the TNF superfamily (Wiley et al., 1995; Marsters et al., 1996). 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 (Pan et al., 1997; Chaudhary et al., 1997; MacFarlane et al., 1997; Walczak et al., 1997; Wu et al., 1997). TRAIL-R3 to R-5 are antagonistic decoy receptors, which bind TRAIL, but don't transmit a death signal (Degli-Esposti et al., 1997b; Pan et al., 1997; Marsters et al., 1997; Pan et al., 1998; Degli-Esposti et al., 1997a). TRAIL-R3 is a glycosyl-phosphatidylinositol GPI-anchored cell surface protein, which lacks a cytoplasmic tail, while 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 regulation of osteoclastogenesis (Emery et al., 1998).

4.3. Signaling through CD95 or TRAIL Receptors

Ligation of death receptors such as CD95 or the agonistic TRAIL receptors TRAIL-R1 and TRAIL-R2 by their cognate ligands or agonistic antibodies results in receptor trimerization, clustering of the receptors' death domains and recruitment of adaptor molecules such as FADD through homophilic interaction mediated by the death domain (Kischkel et al., 1995; Barnhart et al., 2003). FADD in turn recruits caspase-8 to the activated CD95 receptor to form the CD95 death-inducing signaling complex (DISC). Oligomerization of caspase-8 upon DISC formation drives its activation through self-cleavage. Caspase-8 then activates downstream effector caspases such as caspase-3. Besides caspase-8, caspase-10 is recruited to the TRAIL DISC (Kischkel et al., 2001). However, the importance of caspase-10 in the TRAIL DISC for apoptosis induction has been controversially discussed (Kischkel et al., 2001; Sprick et al., 2002). In addition to activation at the DISC caspase-8 can also be activated downstream of mitochondria, e.g. by caspase-6, depending on the cell type and/or apoptotic stimulus (Cowling & Downward, 2002). For the CD95 signaling pathway 2 distinct prototypic cell types have

been identified (Scaffidi et al., 1998). In type I cells, caspase-8 is activated upon death receptor ligation at the DISC in quantities sufficient to directly activate downstream effector caspases such as caspase-3 (Scaffidi et al., 1998). In type II cells, however, the amount of active caspase-8 generated at the DISC is insufficient to activate caspase-3 (Scaffidi et al., 1998). In these cells a mitochondrial amplification loop is required for full activation of caspases involving Bid, which translocates to mitochondria upon cleavage by caspase-8 to trigger the release of apoptogenic proteins such as cytochrome *c* from mitochondria into the cytosol (Scaffidi et al., 1998). Accordingly, CD95-induced apoptosis is blocked by overexpression of Bcl-2 or Bcl-x_L, which inhibit mitochondrial alterations only in type II, but not in type I cells (Scaffidi et al., 1998). Also, a similar cell type dependent organization (type I and type II) of the TRAIL signaling pathway has been described (Fulda et al., 2001).

4.4. TNF-R1 Signaling

Although TNF can signal through two different cell surface receptors, TNF-R1 and TNFR2, TNF-R1 initiates the majority of its biological functions (Grell et al., 1999). In principal, the interaction of TNF with TNF-R1 can activate several different signaling pathways, depending on the specific adaptor molecules recruited to the activated receptor. Ligation of TNF-RI by TNF results in receptor trimerization and release of the inhibitory protein SODD from the intracellular domain of TNFR1. This in turn enables the recruitment of the adaptor protein TRADD, which serves as a common platform adaptor for several signaling molecules that mediate the different biological functions of TNF. For example, FADD couples the TNF-RI-TRADD complex to apoptosis via recruitment and activation of caspase-8. Alternatively, TNFRI can engage via TRADD the adaptor molecule TRAF2 that initiates activation of the MAPK/JNK signaling cascade. This results in activation of the transcription factor c-jun, which is involved in survival signaling. Binding of RIP to TNF-RI via TRADD stimulates another survival pathway through activation of NF-κB. RIP is critical for the recruitment of the IKK complex, which comprises the two catalytic subunits IKKα and IKKß and the regulatory subunit IKKγ, resulting in degradation of IκB and NF-κB activation. NF-κB in turn suppresses apoptosis through transcriptional upregulation of anti-apoptotic molecules such as c-IAP1, c-IAP2, TRAF1, TRAF2 or FLIP.

5. SIGNALING THROUGH DEATH RECEPTORS IN CANCER THERAPY

5.1. The CD95 System and Cancer Therapy

The CD95 system has been implicated in chemotherapy-induced tumor cell death in a number of studies (Friesen et al., 1996; Fulda et al., 2001; Muller et al., 1997; Muller et al., 1998) (Houghton et al., 1997; Reap et al., 1997). To this end, treatment with anticancer drugs triggered an increase in CD95L expression which stimulated the receptor pathway in an autocrine or paracrine manner by binding to its receptor CD95. In support of this concept, upregulation of CD95L was observed in many different tumor cell lines, e.g. leukemia, neuroblastoma, malignant brain tumors, hepatoma, colon, breast

or small lung cell carcinoma cells in vitro and also ex vivo in primary, patients' derived tumor cells. Various anticancer agents with different primary intracellular targets have been used in these studies including DNA-damaging agents such as doxorubicin, etoposide, cisplatin or bleomycin. The CD95 receptor/ligand system has also been implicated in thymineless death in colon carcinoma cells following treatment with 5-FU (Houghton et al., 1997). In an ex vivo colon carcinoma cell model, CD95/CD95L interactions were found to link DNA damage induced by thymineless stress to the apoptotic machinery of colon carcinoma cells (Houghton et al., 1997). Activation of the transcription factors AP-1 and NF-κB was shown to mediate the increase in CD95L transcription and mRNA levels in response to chemotherapy (Kasibhatla et al., 1998). AP-1 and NF-κB binding sites were identified in the human CD95L promotor, which respond to DNA damage or inhibition of DNA metabolism by upregulating NF-κB activity (Kasibhatla et al., 1998). In addition to CD95L, CD95 expression on the cell's surface increased upon drug treatment, in particular in cells harboring wild-type p53 (Muller et al., 1997). p53-responsive elements have been identified in the first intron of the CD95 gene, as well as three putative p53-binding sites within the CD95 promotor, which showed limited homology with the p53 consensus binding site (Muller et al., 1998). Moreover, antagonistic CD95L antibodies, soluble antagonistic CD95 receptors or DN-FADD were found to reduce drug-induced apoptosis under certain circumstances. In addition to upregulation of CD95L and CD95, anticancer agents have been reported to activate the CD95 pathway by modulating expression and recruitment of pro- or anti-apoptotic components of the CD95 DISC to activated receptors. Upregulation of FADD and pro-caspase-8 was found upon treatment with doxorubicin, cisplatin or mitomycin C in colon carcinoma cells (Micheau et al., 1999). Also, increased recruitment of FADD and pro-caspase-8 to the CD95 receptor to form the CD95 DISC was observed in certain tumor cells upon drug treatment in a CD95L-dependent or CD95L-independent manner (Fulda et al., 2001). These findings indicate that in cells with an inducible CD95 receptor/ligand system, drug-induced apoptosis may involve CD95L-initiated DISC formation and activation of downstream effector programs similar to activation-induced cell death (AICD) in T-cells.

Despite the reproducibility of these findings in different model systems, other reports challenged the model that death receptor signaling is involved in drug-mediated cell death (Eischen et al., 1997; Petak & Houghton, 2001; Villunger et al., 1997; Engels et al., 2000). To that end, antagonistic antibodies against CD95L or CD95 did not confer protection against apoptosis induced by cytotoxic drugs in other cell lines. Although splenocytes from lpr mice showed decreased sensitivity to γ-irradiation, thymocytes of these mice did not show increased proliferation upon γ-irradiation or cytotoxic drugs (Reap et al., 1997). Enforced expression of FLIP, DN-FADD or the serpin crmA did not inhibit drug-induced apoptosis, although it inhibited caspase-8 activation (Kataoka et al., 1998). Also, targeted disruption of genes involved in death receptor signaling conferred no protection against cytotoxic drug treatment, at least in nontransformed cells. $FADD^{-/-}$ and $caspase\text{-}8^{-/-}$ fibroblasts were sensitive to cytotoxic drugs, while they remained resistant to death receptor stimulation (Yeh et al., 1998; LeBlanc et al., 2002).

The discrepancies in data may also be explained by differences in the inhibitory reagents used to block CD95/CD95L interaction. The quality of CD95/CD95L blocking agents including anti-CD95 antibody, anti-CD95L antibody or soluble decoy CD95-Fc

receptor constructs may vary depending on their origin and preparation. Also, the lack of efficacy of these CD95/CD95L neutralizing agents may be due to inaccessibility of their proposed targets. Experiments with adenoviral delivery of a CD95L-GFP construct showed that CD95 and CD95L are stored intracellularly and colocalize to the same intracellular compartments, e.g. the Golgi and/or endoplasmatic reticulum (Hyer et al., 2000). An anti-CD95 blocking antibody did not inhibit CD95L-induced cell death suggesting that CD95L may trigger CD95 within the same intracellular compartment and that these two molecules may already interact prior to surface presentation (Hyer et al., 2000). Thus, CD95/CD95L neutralizing agents may under certain circumstances not even gain access to their targets prior to triggering of the CD95 pathway. Moreover, some studies that challenge an involvement of the CD95 system in chemotherapy of tumor cells are based on experiments performed in nontransformed cells, e.g. embryonic fibroblasts, but not in cancer cells. However, the mechanisms regulating apoptosis in non-malignant cells may vary considerably from those in malignant tumor cells, which is highlighted by the differential sensitivity of these cell types to various death stimuli.

Although a considerable amount of data support a role of the CD95 system in anticancer drug-induced apoptosis, at least under certain circumstances, most cytotoxic drugs are considered to primarily initiate cell death by triggering a cytochrome *c*/Apaf-1/caspase-9 dependent pathway linked to mitochondria. Collectively, these data point to a key role of the mitochondrial pathway in drug-induced apoptosis, while the CD95 system may amplify drug-induced apoptosis under certain conditions. Importantly, this amplification of the chemoresponse may have important clinical implications, since it may critically affect the time required for execution of the death program. However, the concept of mitochondria being the central initiator to integrate stress stimuli into an apoptotic response has very recently been challenged by showing that a functional apoptosome is dispensable for stress-induced apoptosis. Activation of caspases, e.g. caspase-2, in response to cellular stress was found to be required for mitochondrial permeabilization rather than vice versa (Lassus et al., 2002). In addition, Bcl-2 was reported to regulate a caspase activation program independently of the cytochrome *c*/caspase-9/Apaf-1-containing apoptosome (Marsden et al., 2002). These studies indicate that mitochondria may act as amplifiers, but not initiators of cell death in stress-induced apoptosis. Thus, key elements of apoptosis signaling pathways may yet have to be reconsidered.

5.2. TRAIL and Cancer Therapy

Similar to CD95L, TRAIL rapidly triggers apoptosis in many tumor cells (LeBlanc & Ashkenazi, 2003; Wajant et al., 2002; Walczak & Krammer, 2000). The TRAIL ligand and its receptors are of special interest for cancer therapy, since 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 (Ozoren & El-Deiry, 2003). However, screening of various different 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 anti-apoptotic molecules.

Whereas systemic administration of CD95 ligand or TNF is hampered by severe toxic side effects (Walczak & Krammer, 2000), TRAIL appears to be a relatively safe candidate for clinical application, particularly in its non-tagged, zinc-bound homotrimeric form (LeBlanc and Ashkenazi, 2003). Studies in nonhuman primates such as chimpanzees and cynomolgus monkeys showed no toxicity upon intravenous infusion, even at high doses (Ashkenazi et al., 1999). In addition, no cytotoxic activity of TRAIL was reported on a variety of normal human cells of different lineages including fibroblasts, endothelial cells, smooth muscle cells, epithelial cells or astrocytes (Lawrence et al., 2001). However, some concerns about potential toxic side effects on human hepatocytes or brain tissue have also been raised (Jo et al., 2000; Nitsch et al., 2000). 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 (LeBlanc & Ashkenazi, 2003). Importantly, recent evidence suggest that besides triggering apoptosis, TRAIL is able to induce survival and proliferation in cancer cells resistant towards TRAIL-induced apoptosis, which is mediated by the transcription factor NF-κB (Erhardt et al., 2003). Thus, the death–inducing ligand TRAIL might paradoxically promote tumor growth under certain conditions, e.g. in TRAIL resistant tumors.

There is also mounting evidence for an important role of TRAIL in tumor surveillance, e.g. from studies with TRAIL knockout mice (Smyth et al., 2003; Cretney et al., 2002). Although the biology of the TRAIL system may differ significantly between mice and humans, since there is only one TRAIL receptor in mice, which is homologous to both TRAIL-R1 and TRAIL-R2, the phenotype of these knockout mice are 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 (Cretney et al., 2002). These data are in accordance with studies showing an important role of NK cells, which constitutively express TRAIL, in the control of tumor metastasis (Takeda et al., 2001; Smyth et al., 2001). In addition, tumor formation induced by carcinogens was found to be enhanced in the presence of antagonistic TRAIL antibodies (Takeda et al., 2001). Thus, TRAIL may play an essential role as innate effector molecule in immune surveillance during tumor formation and progression.

6. DEFECTIVE DEATH RECEPTOR SIGNALING IN CANCER

Based on the concept that cytotoxic therapies primarily act by triggering apoptosis in cancer cells, defects in apoptosis programs may result in primary or acquired resistance. Drug resistance constitutes a major clinical problem in oncology. Patients, who present at tumor relapse usually have tumors, which are more resistant to therapy than their primary tumors. Signaling to cell death in response to death receptor stimulation can principally be inhibited by an increase in anti-apoptotic molecules or by a decrease in pro-apoptotic proteins. Tumor cells can acquire resistance by multiple mechanisms, which interfere with the death receptor pathway at various levels as outlined below.

6.1. Death Receptors and Death-Induced Signaling Complex (DISC)

Surface expression of death receptors may vary between different cell types and can be downregulated or absent in resistant tumor cells, which has been assumed to contribute to immune escape of tumor cells from negative growth control. Drug-resistant leukemia or neuroblastoma cells showed strong downregulation of CD95 expression suggesting that critical levels of CD95 expression may have an impact on drug sensitivity (Friesen et al., 1997; Fulda et al., 1998). Also, drug-resistant tumor cells were found to be deficient in upregulation of CD95L in response to treatment with cytotoxic drugs that was involved in drug response in chemosensitive tumor cells (Friesen et al., 1997). Mutations of the CD95 gene have been identified in a variety of hematological malignancies and solid tumors (Debatin et al., 2003). The incidence of CD95 mutations found in human tumors has been taken as evidence that the CD95 system exerts a tumor suppressor function. Moreover, soluble receptors such as soluble CD95 or decoy receptor 3 (DcR3), which act as decoy receptors by competitively binding CD95 ligand, can interfere with CD95-triggered apoptosis (Ashkenazi & Dixit, 1999). Increased serum levels of soluble CD95 lacking a transmembrane anchor, which is generated by alternative mRNA splicing products, were found in adult T cell leukemia as well as in a variety of nonhematopietic malignancies (Ugurel et al., 2001; Midis et al., 1996). DcR3 is a decoy receptor unrelated to the CD95 protein, which can bind to both CD95 ligand and to LIGHT, another TNF cytokine member. DcR3 was found to be genetically amplified or overexpressed in lung carcinoma, colon carcinoma or glioblastoma (Roth et al., 2001) (Pitti et al., 1998). TRAIL-R3, a decoy receptor for TRAIL, was found to be overexpressed in gastric carcinoma (Sheikh et al., 1999). Moreover, loss of expression of the agonistic TRAIL receptors TRAIL-R1 and R2 may account for TRAIL resistance. Both receptors are located on chromosome 8p, a region of frequent loss of heterozygosity (LOH) in tumors (LeBlanc and Ashkenazi, 2003). In a small percentage of cancers, e.g. 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 or R2 (Lee et al., 2001; Lee et al., 1999; Arai et al., 1998; Shin et al., 2001; Pai et al., 1998).

Impaired transmembrane expression of CD95 or TRAIL receptors may also be caused by epigenetic changes such as CpG-island hypermethylation of gene promoters (Baylin, 2002; van Noesel et al., 2003; Maecker et al., 2002). Also, alterations in chromatin structure, e.g. chromatin condensation because of histone deacetylation, may block transcription by preventing the access of transcription factors to the DNA (Marks et al., 2001). Interestingly, epigenetic changes in CD95 expression have been reported to determine immune escape and response to therapy (Maecker et al., 2002). In tumors with epigentically silenced CD95, restoration of CD95 expression by histone deacetylase inhibitors resulted in suppression of tumor growth and restoration of chemosensitivity in an NK cell-dependent manner (Maecker et al., 2002). In addition, oncogenes have been reported to repress CD95 transcription. To this end, oncogenic RAS was found to downregulate the expression of CD95 (Peli et al., 1999), and STAT3 and c-jun cooperated in transcriptionally repressing CD95 expression (Ivanov et al., 2001).

Signaling by death receptors can also be negatively regulated by proteins that associate with their cytoplasmatic domains, e.g. FLIP or phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes-15kDa (PED/PEA-15) (Krueger et al.,

2001; Barnhart et al., 2003). Two splice variants of FLIP, a long form ($FLIP_L$) and a short form ($FLIP_S$), have been identified in human cells, which have sequence homology to caspase-8 and caspase-10, but lack its catalytic site (Krueger et al., 2001). Consequently, the recruitment of FLIP to the DISC instead of pro-caspase-8 or –10 can block caspase activation (Krueger et al., 2001). High FLIP expression has been found in many tumor cells and has been correlated with resistance to CD95- and TRAIL-induced apoptosis (Fulda et al., 2000) (Zhang et al., 1999). In addition, FLIP expression was associated with tumor escape from T cell immunity and enhanced tumor progression in experimental studies in vivo pointing to a role of FLIP as a tumor-progression factor (Medema et al., 1999; Djerbi et al., 1999). Viral analogues of FLIP are encoded by several tumorigenic viruses, e.g. human herpesvirus-8 (HHV8), which has been implicated in the pathogenesis of Kaposi's sarcoma (Sturzl et al., 1999). Interestingly, high FLIP levels were detected in advanced Kaposi's sarcoma, while FLIP expression was low in early disease stages (Sturzl et al., 1999). However, the impact of FLIP on apoptosis sensitivity towards cytotoxic drugs may vary between cell types, since overexpression of FLIP did not confer protection against cytotoxic drugs in T cell leukemia cells, while FLIP antisense oligonucleotides sensitized osteosarcoma cells for cisplatin (Kataoka et al., 1998; Kinoshita et al., 2000). Interestingly, recent evidence suggest that $FLIP_L$ is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis (Chang et al., 2002). At physiologically relevant levels, $FLIP_L$ enhanced pro-caspase-8 processing in the CD95 DISC and apoptosis, while it inhibited apoptosis only at high ectopic expression levels (Chang et al., 2002). PEA-15 is another death effector domain (DED)-containing protein, which blocks CD95-, TRAIL- or TNF-triggered apoptosis in a receptor-proximal manner by disrupting FADD and caspase-8 interactions (Barnhart et al., 2003; Hao et al., 2001). PEA-15 has been implicated in tumor resistance, since enhanced expression of PEA-15 has been detected in TRAIL-resistant malignant glioma (Xiao et al., 2002). Furthermore, activation of protein kinase C (PKC) was found to negatively regulate recruitment of death domain containing molecules into their respective death receptor-associated signaling complex (Harper et al., 2003).

6.2. Caspases

Despite the key role of caspases for cell death execution, caspase mutations in tumors have only been identified at low frequency in some tumors, e.g. colorectal cancer or head and neck carcinoma (Mandruzzato et al., 1997; Kim et al., 2003). Instead, caspase expression and function appears to be frequently impaired by epigenetic mechanisms in cancer cells. To this end, caspase-8 expression was found to be inactivated by hypermethylation of regulatory sequences of the caspase-8 gene in a number of different tumor cells derived from neuroblastoma, malignant brain tumors, Ewing tumor and small lung cell carcinoma both *in vitro* and also *in vivo* in primary tumor samples (Teitz et al., 2000) (Fulda et al., 2001; Hopkins-Donaldson et al., 2003). Interestingly, co-methylation for caspase-8 and FLIP and also for the pairs of agonistic TRAIL receptors TRAIL-R1/TRAIL-R2 and antagonistic TRAIL receptors TRAIL-R3/TRAIL-R4 was recently observed in neuroblastoma suggesting that genes are not randomly targeted by methylation in cancer (van Noesel et al., 2003). Importantly, restoration of caspase-8 expression by gene transfer or by demethylation treatment

sensitized resistant tumor cells for death-receptor- or drug-induced apoptosis (Fulda et al., 2001). Enhanced transcription of caspase-8 was also found upon treatment with IFNγ(Fulda et al., 2001). Interferon-sensitive response element were subsequently identified in the caspase-8 promotor showing that IFNγ could directly activate caspase-8 transcription (Yang et al., 2003b; Banelli et al., 2002). Another level of transcriptional regulation of caspase expression in cancers is alternative splicing of caspases. For example, the gene encoding pro-caspase-9 can generate short isoform that act as dominant-negative variant to suppress apoptosis (Srinivasula et al., 1999).

6.3. IAPs

The family of endogenous caspase inhibitors "inhibitor of apoptosis proteins" (IAPs) are highly conserved throughout evolution and comprise the human analogues XIAP, c–IAP1, c-IAP2, survivin and livin (ML-IAP) (Salvesen & Duckett, 2002; Altieri, 2003b). IAPs have been reported to directly inhibit active caspase-3 and -7 and to block caspase-9 activation (Salvesen & Duckett, 2002). Inhibition of apoptosis by IAPs in response to cytotoxic therapy, e.g. chemotherapy treatment with TRAIL or after γ-irradiation has been suggested by several experimental studies. XIAP, c-IAP1, c-IAP2 and survivin are expressed at high levels in many tumors, *e.g.* leukemia, neuroblastoma, and several carcinoma, which has been correlated with adverse prognosis (Salvesen & Duckett, 2002). The c-IAP2 gene is affected by the t(11;18)(q21;q21) translocation, which occurrs in 50% of mucosa-associated lymphoid tissue (MALT) lymphoma suggesting that c-IAP2 is involved in the pathogenesis of this disease (Dierlamm et al., 1999). High expression of livin (ML-IAP) is characteristic for melanoma compared to low livin expression in normal melanocytes (Hersey & Zhang, 2001). Survivin is expressed at high levels in the majority of human cancers representing the fourth most common transcriptome of the human genome, but is not expressed in normal adult tissues indicating that survivin may contribute to the malignant phenotype of cancer cells (Altieri, 2003b; Velculescu et al., 1999). The role of survivin in regulation of apoptosis and proliferation is more complex compared to other IAP family proteins. For example, recent evidence suggest that direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin rather than binding to and inhibition of effector caspases (Song et al., 2003). Interestingly, the antiapoptotic function of survivin has also been related to inhibition of mitochondrial and AIF-dependent apoptotic pathways, protecting against both caspase-independent and -dependent apoptosis (Blanc-Brude et al., 2003) (Liu et al., 2004). In addition to regulation of apoptosis, survivin have been found to be involved in the regulation of mitosis (Altieri, 2003). IAPs are negatively regulated by caspase-mediated cleavage or by proteasomal degradation (Salvesen & Duckett, 2002). In addition, mitochondrial proteins, e.g. Smac/DIABLO or Omi/HtrA2, or nuclear proteins such as XIAP-associated factor 1 (XAF-1), which translocate into the cytosol upon induction of apoptosis, antagonize the antiapoptotic function of IAPs through binding to IAPs (van Loo et al., 2002b; Du et al., 2000; Liston et al., 2001). Interestingly, reduced expression of XAF-1 was found in a variety of human cancers (Fong et al., 2000). The XAF-1 gene resides on chromosome 17p13.2, a region frequently targeted by mutation or loss of heterocygeoucity in human cancers (Fong et al., 2000). In addition to mutational inactivation, epigenetic silencing by CpG-island promotor

hypermethylation of the XAF-1 gene was recently detected in advanced human gastric adenocarcinomas (Byun et al., 2003) indicating that XAF-1 may act as a tumor suppressor gene.

6.4. Bcl-2 Proteins

Bcl-2 family proteins play a pivotal role in the regulation of the mitochondrial pathway, since these proteins localize to intracellular membranes, in particular the mitochondrial membrane (Cory & Adams, 2002). They comprise both anti-apoptotic members, e.g. Bcl-2, Bcl-x_L, Mcl-1, as well as pro-apoptotic molecules such as Bax, Bak, Bad and BH3 domain only molecules that link the death receptor pathway to the mitochondrial pathway (Bid, Bim, Puma, Noxa) (Cory & Adams, 2002). Upon apoptosis induction proapoptotic Bcl-2 proteins with multidomains such as Bax translocate from the cytoplasm to the outer mitochondrial membrane, where they oligomerize to form a pore like structure thereby promoting cytochrome *c* release (Wei et al., 2001) (Cheng et al., 2001). This translocation to mitochondria can be triggered by Bcl-2 proteins which have a BH3 domain only. BH3 domain only proteins include Bid, which is activated by caspase-8-mediated cleavage, Bim, a microtubule-associated protein, or Noxa and PUMA, two p53-induced proteins (Cory & Adams, 2002). Recent evidence obtained from studies in mice with either noxa or puma disrupted indicate that Puma and Noxa are critical mediators of the apoptotic responses induced by p53 and diverse p53-independent cytotoxic insults, including cytokine deprivation and exposure to glucocorticoids, the kinase inhibitor staurosporine, or phorbol ester (Villunger et al., 2003). Interestingly, pharmacologic activation of p53 was recently reported to elicits Bax-dependent apoptosis in the absence of transcription (Milosevic et al., 2003). Bcl-2 or Bcl-x_L exert their anti-apoptotic function, at least in part, by sequestering BH3 domain only proteins in stable mitochondrial complexes, thereby preventing activation and translocation of Bax or Bak to mitochondria (Cory & Adams, 2002). In addition, Bcl-2 and Bcl-x_L block apoptosis by preventing cytochrome *c* release through a direct effect on mitochondrial channels such as the voltage-dependent anion channel (VDAC) or the permeability transition pore complex (PTPC) (Cheng et al., 2003).

Imbalances in the ratio of anti-and pro-apoptotic Bcl-2 proteins may tip the balance in favor of tumor cell survival instead of cell death and have been shown to drastically alter apoptosis in response to several stimuli in a number of experimental systems (Cory & Adams, 2002). Also, Bcl-2 can cooperate with oncogenes such as the c-myc oncogene in the process of multistep carcinogenesis by blocking c-myc-induced apoptosis (Evan & Vousden, 2001). Alterations in the expression of anti-apoptotic or pro-apoptotic members of the Bcl-2 family proteins have been described in various human cancers and may involve structural gene alterations. For example, a characteristic feature of follicular lymphoma is the t(14;18) translocation, which brings the Bcl-2 gene under the control of the immunglobulin heavy chain locus resulting in enhanced Bcl-2 expression (Cory & Adams, 2002). Single nucleotide substitution or frameshift mutations, that inactivate the Bax gene in mismatch repair (MMR)-deficient colon cancer or hematopoetic malignancies, have also been described (Cory & Adams, 2002). Lack of Bax expression in MMR-deficient colon cancer cells was associated with resistance to TRAIL because of deficient release of Smac/DIABLO from mitochondria (Deng et al., 2002). Accordingly,

restoration of Bax expression in MMR-deficient colon cancer cells also restored sensitivity for TRAIL-induced apoptosis (Deng et al., 2002). Besides genetic alterations, aberrant expression of Bcl-2 family proteins is mostly regulated at the transcriptional or posttranscriptional level. For example, expression of several antiapoptotic Bcl-2 family proteins, e.g. Bcl-2, Bcl-x_L, Mcl-1 or Bfl-1, is transcriptionally regulated by NF-κB (Karin et al., 2002). Interestingly, Bcl-x_L has recently been reported to be transcriptionally upregulated by Stat3 and NF-κB already in premalignant lesions in an in vivo model of pancreatic carcinoma indicating that impaired apoptosis may contribute to pancreatic carcinogenesis (Greten et al., 2002). It is tempting to speculate that Bcl-x_L may cooperate with oncogenic H-RAS in the development of pancreatic cancer by blocking RAS-induced apoptosis, similar to the oncogenic cooperation described for Bcl-2 and c-myc (Evan & Vousden, 2001).

6.5. Novel Regulators of Death Receptor Signaling

In addition to well-characterized genes, novel regulators of death receptor signaling have recently been identified by high throughput screening platforms. Using an RNA interference-based forward genetic screen, novel regulators of the TRAIL pathway including *MYC, JNK3* or *DOBI* (downstream of Bid) were identified (Aza-Blanc et al., 2003). Moreover, a recent analysis by expression profiling revealed fundamental differences in gene expression between type I and type II CD95 tumor class cells (Algeciras-Schimnich et al., 2003). Type I cell lines expressed mesenchymal-like genes and were especially responsive to growth inhibition by actin-binding compounds. In contrast, type II cell lines were characterized by epithelium-like markers and responded to tubulin-interacting compounds (Algeciras-Schimnich et al., 2003). Future functional genomics studies may provide further insight into the complexity of the signaling network regulating death receptor-mediated apoptosis in response to anticancer therapy.

7. DEATH RECEPTOR-TARGETED THERAPIES FOR CANCER

7.1. Targeting Death Receptors and DISC Formation

The idea to specifically target death receptors to trigger apoptosis in tumor cells is attractive for cancer therapy since death receptors have a direct link to the cell's death machinery (Ashkenazi, 2002). Also, apoptosis upon death receptor triggering is considered to occur independent of the p53 tumor suppressor gene, which is impaired in the majority of human tumors (El-Deiry, 2001). 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, osteasarcoma, neuroblastoma, leukemia and lymphoma (LeBlanc & Ashkenazi, 2003; Wajant et al., 2002). 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 TRAIL-R1 or TRAIL-R2 also demonstrated potent antitumor activity against tumor cell lines and in preclinical cancer

models. In addition to recombinant soluble TRAIL ligand, several gene therapy approaches have been developed to specifically target tumor cells. To this end, an adenoviral vector expressing the TRAIL gene from the hTERT promoter elicited high levels of TRAIL expression and apoptosis specifically in breast cancer cells, whereas only minimal transgene expression and toxicity was detected in normal human primary mammary epithelial cells (Lin et al., 2002). Intralesional administration of adenoviral TRAIL effectively suppressed the growth of human breast cancer xenografts resulting in long-term tumor-free survival of mice (Lin et al., 2002). The antitumor effect of an intratumoral administration of an adenoviral vector expressing TRAIL in an *in vivo* model of breast carcinoma was attributed to direct tumor cell killing as well as to a bystander effect through presentation of TRAIL by transduced normal cells (Lee et al., 2002).

Despite expression of both agonistic TRAIL receptors, many tumors remain resistant towards treatment with death-inducing ligands such as TRAIL, which has been related to the dominance of anti-apoptotic signals, e.g. those delivered by NF-κB, AKT or by inhibitor of apoptosis proteins (IAPs). Importantly, numerous studies have shown that TRAIL together with cytotoxic drugs or γ-irradiation strongly synergized to achieve antitumor activity in various cancers including malignant glioma, melanoma, leukemia, breast, colon or prostate carcinoma (Rohn et al., 2001; Nagane et al., 2000) (Gliniak & Le, 1999; Keane et al., 2000; Belka et al., 2001; Chinnaiyan et al., 2000). 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 has been reported to occurr in a p53-dependent or p53-independent manner (Meng & El-Deiry, 2001; Takimoto & El-Deiry, 2000). Recent evidence suggest that p53 is crucial for sensitization to TRAIL by chemotherapy through transcriptional upregulation of TRAIL-R2 in some tumors, e.g. mismatch repair-deficient colorectal cancer cells harboring Bax mutations (Wang & El-Deiry, 2003). Intriguingly, pre-exposure to chemotherapy restored TRAIL sensitivity through p53-mediated increase of TRAIL-R2 expression even in resistant colorectal carcinoma cells lacking Bax expression indicating that sequential combination of anticancer agents with TRAIL may overcome some forms of resistance (Wang & El-Deiry, 2003). In addition, chemotherapy has been reported to enhance DISC assembly upon TRAIL receptor triggering in colon carcinoma cells (Lacour et al., 2003).

Targeting elevated FLIP expression constitutes another approach to enhance death receptor signaling in cancers. Downregulation of FLIP expression by metabolic inhibitors such as actinomycin D sensitized various tumor cells for death receptor-induced apoptosis (Kinoshita et al., 2000). Recent evidence indicate that PPARγ ligands or proteasome inhibitors may selectively reduce FLIP expression, thereby sensitizing tumor cells for TRAIL treatment (Kim et al., 2002) (Sayers et al., 2003). Furthermore, inhibitors of protein kinase C (PKC) restored sensitivity to TRAIL-or CD95-induced apoptosis, as PKC activation negatively regulates recruitment of death domain containing molecules into their respective death receptor-associated signaling complex (Harper et al., 2003).

7.2. Bcl-2/Bcl-x_L Antagonists

The majority of cancer cells falls into the catagory of type II cells, which depend on an intact mitochondrial pathway for cell death execution upon death receptor triggering. Since overexpression of Bcl-2 related proteins, which occurs in more than half of human cancers, confers tumor cell resistance by blocking the mitochondrial pathway, attempts aimed at overcoming the cytoprotective effect of Bcl-2 may prove to be an effective strategy to restore sensitivity of cancer cells for death receptor triggering. To this end, nuclease-resistant Bcl-2 antisense oligonucleotides downregulating Bcl-2 mRNA are currently tested in phase II/III clinical trials for hematological malignancies or solid tumors, as single agent or in combination with chemotherapy (Chi et al., 2001). Moreover, Bcl-2/Bcl-x_L small molecule antagonists targeting the BH3 binding site on Bcl-2 or Bcl-x_L are under preclinical evaluation (Letai et al., 2002). Also, agonistic BH3 peptides have been designed that mimic BH3 only proteins in activating proapoptotic Bax and Bak proteins (Letai et al., 2002).

7.3. IAP Antagonists

There is mounting evidence that cancer cells have an intrinsic drive to apoptosis that is held in check by IAPs. To this end, high basal levels of caspase-3 and caspase-8 activities and active caspase-3 fragments in the absence of apoptosis were detected in various tumor cell lines and cancer tissues, but not in normal cells (Yang et al., 2003a). Tumor cells, but not normal cells also expressed high levels of IAPs (Yang et al., 2003a) suggesting that upregulated IAP expression counteracted the high basal caspase activity selectively in tumor cells. Thus, IAPs may serve as important molecular targets for the development of cancer-specific therapeutics. To this end, several strategies have been developed to target survivin, given the differential expression of survivin in cancers versus normal tissues and its role in preservation of tumor cell viability. For example, downregulation of survivin expression by antisense oligonucleotides, ribozymes, RNA interference or dominant-negative survivin variants resulted in suppression of tumor growth in several tumor models in vitro and also in vivo, alone and/or in combination with chemotherapy (Williams et al., 2003; Altieri, 2003a; Blanc-Brude et al., 2003; Grossman et al., 2001). Furthermore, pharmacological inhibition of survivin phosphorylation at mitosis using cyclin-dependent kinase inhibitor such as flavopiridol or purvanolol A strongly enhanced the cytotoxic effect of taxol both in vitro and in vivo (O'Connor et al., 2002; Wall et al., 2003). Moreover, cancer vaccination strategies showed that a strong antigen-specific immune response could be mounted against survivin-bearing tumor cells (Pisarev et al., 2003; Schmidt et al., 2003; Schmitz et al., 2000).

Furthermore, Smac peptides were found to promote caspase activation by antagonizing IAPs and sensitized various tumor cells in vitro for apoptosis induced by death-receptor ligation or cytotoxic drugs (Fulda et al., 2002). Importantly, Smac peptides strongly enhanced the antitumor activity of TRAIL in an intracranial malignant glioma xenograft model in vivo (Fulda et al., 2002). Complete eradication of established tumors and survival of mice was only achieved upon combined treatment with Smac peptides and TRAIL without detectable toxicity to normal brain tissue (Fulda et al.,

2002). In addition to Smac peptides, peptides interacting with XIAP, which were identified by screening of a phage library (Tamm et al., 2003), and non-peptidic small molecule antagonists of XIAP derived from a polyphenylurea library were subsequently described to exhibit antitumor activity (Schimmer et al., 2004). Thus, low molecular weight antagonists targeting IAPs such as Smac peptides may be promising candidates for cancer therapy by potentiating the efficacy of cytotoxic therapies selectively in cancer cells.

8. CONCLUSIONS

Signaling by death receptors has been shown to play a crucial role in physiolo-gical growth control, tissue homeostasis and surveillance of tumor formation. Also, tumor cell killing by anticancer therapy is primarily mediated by triggering apoptosis in cancer cells. Accordingly, defects in death receptor pathways may result in tumor cell resistance. Targeting defects in apoptosis programs may therefore restore sensitivity in resistant forms of cancer. Numerous studies over the last years have delineated various signaling pathways involved in the regulation of tumor formation and progression. However, further insight into the complex signaling network activated in response to anticancer therapy using cancer cell lines, primary tumor cells and animal model systems are needed to identify the best molecular targets for further evaluation. Also, studies on the role of apoptosis signaling molecules in clinical samples using DNA or proteomic arrays are clearly necessary to assess the impact of these molecular parameters on clinical outcome. Hopefully, these studies may eventually allow the identification of novel therapeutic targets thereby providing the basis for targeted, individual tumor therapy.

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CASPASES; MODULATORS OF APOPTOSIS AND CYTOKINE MATURATION – TARGETS FOR NOVEL THERAPIES

Chapter IV

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1. INTRODUCTION

Numerous cells are eliminated during development of metazoans, through a process variously referred to as programmed cell death, physiological cell death or apoptosis. This cell removal also occurs throughout adult life, balancing cell division and regulating the number of cells in organs and tissues. Diseases can result from dysregulation of apoptosis. Inappropriate survival of dangerous cells (for example cells which are precancerous, infected or have autoimmune specificities) may contribute to cancer, infection and autoimmune diseases. Conversely, removal of essential cells through excessive apoptosis plays a role in degenerative diseases, and can compromise the success of organ transplantation. Modulating the expression or activity of apoptotic pathway components is therefore a useful therapeutic approach to counter either unwanted survival or death of particular cells associated with certain human diseases.

Most apoptotic pathways converge with the proteolytic activation of members of a family of cysteine aspartyl-specific proteases, abbreviated "caspases" (Alnemri et al., 1996). Other members of this family regulate maturation of inflammatory cytokines. Because they play such a central role in the initiation and execution of cell death and / or maturation of inflammatory cytokines, and their biochemical activation mechanisms are well understood, caspases represent key targets for drug discovery and development.

This chapter discusses a number of approaches aimed at manipulating caspase activity, with the goal of treating diseases whose pathogeneses involve perturbations in

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apoptosis and/or inflammation. Thousands of articles and numerous reviews have previously presented *in vitro* evidence for the utility of caspase modulation in treating disease. This chapter focuses on the impact of manipulating caspase activity in animal models of human diseases and highlights considerations for developing therapeutic caspase inhibitors.

1.1. The Caspase Family

The first member of the caspase family, caspase-1, was cloned and characterized years before the coining of the "caspase" term. This enzyme, formerly designated interleukin-1β-converting enzyme (ICE), proteolytically generates the pro-inflammatory cytokine interleukin (IL)-1β from its precursor (Thornberry et al., 1992). It also similarly activates pro-IL-18. Subsequent demonstration of sequence and functional homology between ICE / caspase-1 and CED-3, a nematode protein required for developmental programmed cell death, was the first indication of a role for ICE-like enzymes in apoptosis. Although overexpression of caspase-1 induced apoptosis in mammalian tissue culture cells, data from subsequent experiments implied that its primary role is in cytokine maturation rather than apoptosis. Caspases have been cloned from evolutionarily diverse organisms, including mammals, insects and nematodes. Eleven human relatives have now been identified. Some of these, like caspase-1, have primary roles in cytokine processing. Others functionally resemble CED-3, acting in apoptotic signaling pathways.

Like the first identified caspase substrate (pro-IL-1β), almost all known cellular caspase substrates are cleaved on the carboxyl side of aspartate residues (termed the P1 site). Two instances of caspases cleaving after glutamate residues have also been reported (Ethell et al., 2001; Hawkins et al., 2000). Somewhat surprisingly, no systematic exploration of the ability of caspases to cleave non-aspartate P1 amino acids has been published. However, based on the cleavage sites of natural substrates, caspases are generally assumed to be relatively aspartate-specific proteases.

1.2. Caspase Activation

Caspases are translated as dormant precursors and are typically activated by proteolytic processing after aspartate residues, releasing an amino terminal prodomain, a large protease subunit and a small protease subunit. Two molecules of each of the subunits form the active enzyme. The processing can be performed by active caspases, leading to a caspase cascade in which initiator caspases proteolytically activate effector caspases. At the onset of an apoptotic response, interactions between the prodomains of upstream caspases and adaptor proteins promote autoactivation of initiator caspases. For example, as detailed in Chapter III, ligation of receptors by death ligands of the TNF-family triggers recruitment and aggregation of an adaptor molecule FADD and caspases-8 and -10. The caspases become activated as a result of this "induced proximity" (Salvesen & Dixit, 1999). As discussed in Chapter V, DNA damage (caused by irradiation or chemotherapeutic drugs) triggers release of cytochrome-*c* from the mitochondria to the cytosol. In concert with the co-factor dATP, cytosolic cytochrome *c* forms a complex with another adaptor molecule, Apaf-1, which recruits and activates

pro-caspase-9. The cytokine maturation enzymes caspases-1 and -5 are activated via their recruitment into a third complex, dubbed the "inflammasome", which contains Pycard and NALP-1 (Martinon et al., 2002).

1.3. Substrate Specificity

The three residues preceding the P1 aspartate (P4-P2) strongly influence the efficiency of substrate cleavage by different caspases. Comprehensive analyses of the substrate specificity of caspases has been performed using a positional scanning substrate combinatorial library (Thornberry et al., 1997). Based on substrate specificity data from this and other studies, human caspases-1 to -10 have been sub-classified into three groups. Group I comprises the cytokine processing caspases-1, -4 and -5, whose genes reside in a cluster on chromosome 11q22.3. The downstream apoptotic caspases-3 and -7 along with caspase-2, whose role is less well understood (Troy & Shelanski, 2003), constitute Group II. Group III encompasses the distal death ligand signaling caspases-8 and -10. Caspase-6, a downstream pro-apoptotic family member which has received relatively little research attention to date, also falls into Group III. Four other mammalian caspases have also been described. The murine caspase-11 is probably a homologue of human caspase-4 (Van de Craen et al., 1997). Human caspase-12 bears premature stop codons, unlike the murine homologue, and is likely to be inactive (Fischer et al., 2002). Caspase-13 is a bovine gene (Koenig et al., 2001). Elucidation of the substrate specificity profile of caspase-14 has not yet been published.

1.4. Endogenous Caspase Inhibitor Proteins

Most, if not all, healthy cells contain caspases in their inactive precursor form. Tightly controlled signal transduction pathways control their activation. Once activated, caspases are regulated by endogenous caspase inhibitors. These include members of the Inhibitor of Apoptosis (IAP) family, that inhibit caspases-3, -7 and -9 (Deveraux & Reed, 1999) and c-FLIP, an enzymatically inactive relative of caspase-8 that acts as a dominant negative inhibitor. Some viral genomes encode caspase inhibitors, limiting the ability of the host cell response to elude viral replication by undergoing apoptosis. Enforced expression of caspase inhibitor proteins, such as the IAPs (Deveraux et al., 1997), have been used in animal models of various diseases to determine the effect of modulating caspase activity (Table 1).

1.5. Peptidomimetic Caspase Inhibitors

Members of the first generation of caspase inhibitors were designed to reflect the cleavage sites of natural caspase substrates. These synthetic inhibitors can either block caspases indiscriminately or be relatively specific for particular family members, depending on the nature of the peptide used. Fusions to particular chemical groups convert the peptide from a substrate into a pseudo-substrate inhibitor. Fusion of the peptides to aldehyde, semicarbazone or thiomethylketone groups yields reversible inhibitors while addition of fluoromethylketone, chloromethylketone, fluoroacyloxyme thylketone or acyloxymethylketone groups generates irreversible inhibitors (Wu &Fritz, 1999) . Fluoromethylketone (fmk) or chloromethylketone (cmk) groups are often used in

Table 1. Efficacy of protein-based caspase inhibitors

Inhibitor	Efficacy in experimental models
NAIP Member of the Inhibitor of Apoptosis family. Used portion containing just the BIR domains, which inhibits caspases-3 and -7 but not -1 nor -8 [1]	**Effective**: Stroke [2]
XIAP / hILP / BIRC4 / MIHA Member of the Inhibitor of Apoptosis family which inhibits caspase-3, -7 and -9 [3]	**Effective**: Stroke [4,5], ocular hypertension [6], Optic nerve axotomy [7]
PTD-XIAP XIAP fused to the protein transduction domain of human immunodeficiency virus transactivator of TAT (cell membrane permeable) [8]	**Effective**: Stroke [8]
Caspase-1 dominant negative (Active site mutant C285G)	**Effective**: Huntington's Disease [9,10], stroke [11], traumatic brain injury [10], Parkinson's Disease [12]
Apaf-1 dominant negative caspase recruitment domain (amino acids of 1-97) of Apaf-1 tagged with enhanced green fluorescent protein (EGFP). Interferes with the ability of wild type Apaf-1 to active caspase-9, thus blocking the mitochondrial apoptosis pathways.	**Effective**: Parkinson's Disease [13] **Ineffective**: Parkinson's Disease [13]

[1] Maier et al. (2002), [2] Xu et al. (1997), [3] Deveraux et al. (1997), [4] Xu et al. (1999), [5] Du et al. (2001), [6] McKinnon et al. (2002), [7] Kugler et al. (2000), [8] Onteniente et al. (2003), [9] Ona et al. (1999), [10] Fink et al. (1999), [11] Friedlander et al. (1997), [12] Klevenyi et al. (1999), [13] Mochizuki et al. (2001)

the context of *in vivo* settings, as they facilitate cellular uptake of the inhibitor. Esterification of aspartic acid residues can also improve cellular uptake.

The peptide-based inhibitors vary substantially in their specificity for particular members of the caspase family (Table 2). The caspase inhibitor most commonly used in the laboratory is z-VAD-fmk. This molecule inhibits all caspases except caspase-2, but its second order inactivation rates vary from 2.5 nM (caspases-3 and 8) to 130 nM (caspase-4) (Garcia-Calvo et al., 1998). In addition, z-VAD-fmk can inhibit cathepsins (non-caspase lysosomal cysteine peptidases), which may interfere with its *in vivo* efficacy and safety (Schotte et al., 1999).

Tetrapeptide caspase inhibitors are more specific than the tri-peptide z-VAD-fmk inhibitor. Inhibitors based on the site at which caspase-3 cleaves poly-(ADP-ribose)-polymerase-1 (DEVD) preferentially inhibit caspase-3 (K_i of Ac-DEVD-CHO is 0.23 nM), with caspase-8 (Ki=0.92 nM) and caspase-7 (Ki=1.6 nM) inhibited less efficiently (Garcia-Calvo et al., 1998). Ac-YVAD-cmk and Ac-YVAD-CHO, which were designed to mimic the caspase-1 cleavage site of pro-IL-1β, strongly inhibit caspase-1. The other caspases require at least 214 times the concentration of Ac-YVAD-CHO for inhibition (Garcia-Calvo et al., 1998). However, despite this apparent specificity for caspase-1, Ac-YVAD-cmk can bind to, and presumably inhibit, cathepsin B (Wu & Fritz, 1999). Caspase-2 activity is only efficiently suppressed by peptide inhibitors incorporating at least five amino acids (Talanian et al., 1997) and the most commonly used caspase-2 inhibitor is Ac-VDVAD-fmk. It is important to note that this inhibitor is unlikely to be

Table 2. Efficacy of peptidyl caspase inhibitors

Inhibitor	Efficacy in experimental models
z-D-fmk (Boc-Asp-fluoromethylketone)	**Effective**: Heart attack [1], stroke [2]
z-D-dcb (benzyloxycarbonyl-Asp-dichlorobenzene) In cell line studies this inhibitor was only transiently active, and was reversibly inactivated by an unknown intracellular mechanism [3]	**Effective**: Stroke [4], severe pulmonary hypertension [5] **Ineffective**: Severe pulmonary hypertension [5]
OPH-001 / Quinoline-Val-Asp(OMe)-CH_2-OPH Irreversible inhibitor. Sensitivity to inhibition*: Casp3>Casp1. Cathepsin B and calpain not inhibited[6]	**Effective**: Acute tubular necrosis [6]
z-VD-fmk / MX1013 / CV1013 (benzyloxycarbonyl-Val-Asp-fluoromethylketone) Sensitivity to inhibition: Casp1=3=6=7=8=9[7], less potent than tripeptide or tetrapeptide inhibitors. More water soluble than z-VAD-fmk, cell permeable [7] Poor inhibitor of calpain 1, cathepsin B, cathepsin D, renin, thrombin, Factor Xa. [7]	**Effective**: Fas-mediated liver failure [7], stroke [7], heart attack [7], endotoxin-induced liver failure [8]
VE-13,045 (benzyloxycarbonyl-Val-Ala-Asp-(O-Et)-CH_2O-dichlorobenzoate). *In vivo* this compound is rapidly hydrolysed to give VE-16,084 [9] which is the bioactive molecule. Irreversible peptidyl caspase-1 inhibitor[9]	**Effective**: Acute inflammatory disease [9], collagen-induced arthritis [9], severe hemorrhagic pancreatitis [10]
z-VAD-dbc (benzyloxycarbonyl-Val-Ala-Asp-dichlorobenzene). Sensitivity to inhibition: Casp1 > Casp4 >> Casp3 > Casp7 [11]	**Effective**: Stroke [12]
z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone). Short half life in vivo [13]. Sensitivity to inhibition : Casp1 = Casp8 > Casp9 > Casp5 > Casp7 > Casp3 > Casp6 > Casp4 >> Casp2 [14]. Also inhibitsd cathepsins B and H [15]	**Effective**: Heart attack [1,16-18], muscle ischemia-reperfusion injury [19], stroke [20-23], traumatic brain injury [24-26], amyotrophic lateral sclerosis [27], Huntington's Disease [28], systemic lupus ethryematosus [29], autoimmune disease [30,31], autoimmune encephalomyelitis [13], peritonitis [32], sepsis [33], pneumococcal meningitis [34], pulmonary fibrosis [35], endotoxin-induced liver failure [36], Fas-mediated induced liver failure [37,38], endotoxin-induced myocardial dysfunctic [39,40], pulmonary fibrosis [35] **Ineffective**: Neuronal transplant [41], stroke [42] **Adverse effects**: Exacerbated TNF-mediated liver failure
Ac-YVAD-CHO (*N*-acetyl-Tyr-Val-Ala-Asp-aldehyde) Reversible inhibitor of caspase-1 only[11,14]	**Ineffective**: Fas-mediated liver disease [44], Heart attack - reduced apoptosis but not infarct size [45]
Ac-YVAD-cmk (*N*-acetyl-Tyr-Val-Ala-Asp-chloromethylketone). Probably caspase-1 specific [46,47], however, unlike Ac-YVAD-CHO, unpublished data suggests wider inhibition [48]. Also binds cathepsin-B [49].	**Effective**: Heart attack [50-52], cerebral inflammation [53], neuronal injury [41], traumatic brain injury [26], stroke [54], severe acute pancreatitis [55], Fas-mediated liver failure [37,56], endotoxin-induced liver failure [57,58], inflammatory pain hypersensitivity [59], neuronal transplantation [60], liver failure from low dose endotoxin with D-galactosamine [57] **Ineffective**: Septic shock (high dose endotoxin) [57]
Ac-YVAD-fmk (*N*-acetyl-Tyr-Val-Ala-Asp-fluoromethylketone) Probably caspase-1 specific[46,47].	**Ineffective**: Autoimmune disease [31], heart attack [18]

Table 2. (continued).

z-VDVAD-fmk (benzyloxycarbonyl -Val-Asp-Val-Ala-Asp-fluoromethylketone). Sensitivity to inhibition (based on peptide cleavage) probably Casp2=Casp3>Casp7 [46].	**Ineffective**: Vasospasm [61]
z-DEVD-fmk (benzyloxycarbonyl -Asp-Glu-Val-Asp-fluoromethylketone). Sensitivity to inhibition (based on peptide cleavage) is probably Casp3 = Casp7 > Casp1 > Casp4 > Casp6. No cleavage by Casp2 [46,47].	**Effective**: Vasospasm [61], traumatic brain injury [62,63], stroke [64,65], heart attack [18] **Ineffective**: Stroke [42,64], Huntington's Disease [28], did not restore function after traumatic brain injury [63]
Ac-DEVD-CHO (*N*-Acetyl-Asp-Glu-Val-Asp-aldehyde). Sensitivity to inhibition reported as: Casp3 > Casp8 > Casp7 >> Casp10 > Casp1 > Casp6 > Casp9 > Casp4 > Casp5 > Casp2 [14] or Casp3 >> Casp1 > Casp7 > Casp4 [11]	**Effective**: Myocardial infarction [45], autoimmune disease [30], fas-mediated liver failure [44,66], endotoxin-induced liver disease [66] **Ineffective**: Heart attack - reduced apoptosis but not infarct size [45]
Ac-DEVD-cmk (*N*-Acetyl-Tyr-Val-Ala-Asp-chloromethylketone). By analogy with Ac-DEVD-CHO, sensitivity: Casp3 > Casp7 > Casp8 >> Casp10 > Casp1 > Casp6 > Casp9 > Casp4 > Casp5 > Casp2 [14]	**Effective**: Neuronal transplant [41], endotoxin-induced myocardial dysfunction [39] **Ineffective**: Heart attack [50]
Ac-IETD-CHO (*N*-Acetyl-Ile-Glu-Thr-Asp-aldehyde). Sensitivity to inhibition : Casp8 > Casp6 > Casp1 > Casp10>Casp9>Casp3>Casp5>Casp4>Casp7>Casp2 [14]	**Effective**: Endotoxin-induced liver disease [66].
z-LEHD-fmk (benzyloxycarbonyl-Leu-Glu-OMe-His-Asp(OMe)-fluoromethylketone)	**Ineffective**: Heart attack [50]

*= less than two fold better inhibition, > less than ten-fold better inhibition, >> more than ten-fold better inhibition, [1] Huang et al. (2000), [2] Cheng et al. (1998), [3] Sakurada et al. (2002), [4] Himi et al. (1998), [5] Tarasevicience-Stewart et al. (2002), [6] Melnikov et al. (2002), [7] Yang et al. (2003b), [8] Jaeschke et al. (2000), [9] Ku et al. (1996), [10] Norman et al. (1997), [11] Margolin et al. (1997), [12] Loddick et al. (1996), [13] Furlan et al. (1999), [14] Garcia-Calvo et al. (1998), [15] Schotte et al. (1999), [16] Yaoita et al. (1998), [17] Dumont et al. (2001), [18] Chapman et al. (2002), [19] Iwata et al. (2002), [20] Hara et al. (1997), [21] Endres et al. (1998), [22] Ma et al. (1998), [23] Schulz et al. (1998), [24] Felderhoff-Mueser et al. (2002), [25] Bittigau et al. (2003), [26] Fink et al. (1999), [27] Li et al. (2000), [28] Chen et al. (2000), [29] Seery et al. (2001), [30] Saegusa et al. (2002), [31] Iwata et al. (2003), [32] Catalan et al. (2003), [33] Hotchkiss et al. (1999), [34] Braun et al. (1999), [35] Kuwano et al. (2001), [36] Valentino et al. (2003), [37] Rodriguez et al. (1996), [38] Kunstle et al. (1997), [39] Fauvel et al. (2001), [40] Neviere et al. (2001), [41] Hansson et al. (2000), [42] Li et al. (2000), [43] Cauwels et al. (2003), [44] Suzuki. (1998), [45] Okamura et al. (2000), [46] Talanian et al. (1997), [47] Van de Craen et al. (1999), [48] Livingston. (1997), [49] Gray et al. (2001), [50] Kovacs et al. (2001), [51] Wang et al. (2003), [52] Holly et al. (1999), [53] Yao et al. (1999), [54] Rabuffetti et al. (2000), [55] Paszkowski et al. (2002), [56] Rouquet et al. (1996), [57] Mignon et al. (1999), [58] Mathiak et al. (2000), [59] Samad et al. (2001), [60] Schierle et al. (1999), [61] Aoki et al. (2002), [62] Yakovlev et al. (1997), [63] Clark et al. (2000), [64] Gillardon et al. (1999), [65] Fink et al. (1998), [66] Bajt et al. (2001)

chromogenic substrate (AcVDVAD-pNA) is cleaved by caspase-3 almost as efficiently as by caspase-2 (Talanian et al., 1997; Troy & Shelanski, 2003).

1.6. Non-Peptidic Caspase Inhibitors

The majority of the *in vivo* proof-of-concept studies using caspase inhibitors have been performed using the first generation peptidyl compounds discussed above.

Table 3. Efficacy of non-peptidyl caspase inhibitors

Inhibitor	Efficacy in experimental models
M867 ((3*S*)-3-({2*S*)-2-[5-*tert*-butyl-3-{[4-methyl-1,2,5-oxadiazol-3-yl)methyl]amino}-2-oxopyrazin-1(2H)-yl]butanoyl}amino)-5-[methyl(pentyl)-amino]-4-oxopentanoic acid. Reversible inhibitor. Sensitivity to inhibition*: Casp3 > Casp7 >> Casp4 > Casp5 > Casp6 > Casp8 >> Casp10 [1]	**Effective**: Sepsis [1]
M920 / L-826,920. Sensitivity to inhibition: Casp3>1>7>8>4>6>5 (Casp2 and 9 not inhibited) [2]	**Effective**: Peritonitis [2]
M791 / L-826,791. Sensitivity to inhibition: Casp3>>7>>8. (1, 2, 4, 5, 6, 9 not inhibited) [2]	**Effective**: Peritonitis [2]
Minocycline Tetracycline derivative, lipophilic, penetrates the blood-brain barrier [3]. Currently used for treating rheumatoid arthritis [4].	**Effective**: Spinal cord injury [5], Huntington's Disease[6,7,12,13], amyotrophic lateral sclerosis [8-11], Parkinson's Disease[14] **Ineffective**: Huntington's Disease [15-17] **Adverse effects**: Exacerbated MPTP-induced damage to dopaminergic neuronal death [18].
MMPSI / (S)-(+)-5-[1[(2-methoxymethylpyrro-lidinyl) sulfoyl]isatin. Reversible inhibitor of caspases-3 and -7 [19]	**Effective**: Heart attack [20]
IDN-1529 (*N*-[(indole-2-carbonyl)-alaninyl]-3-amino-4-oxo-5-fluoropentanoic acid). Irreversible. Sensitivity to inhibition: Casp9 >> Casp2 > Casp8 > Casp1 > Casp6 > Casp3 [21]	**Effective**: Heart attack [22], septic shock [23]
IDN-1965 (*N*-[(1,3-dimethylindole-2-carbonyl)-valinyl]-3-amino-4-oxo-5-fluoropentanoic acid). Rapidly cleared by and targeted to the liver [24]. Broad spectrum irreversible caspase inhibitor [25]. Sensitivity to inhibition: Casp9 > Casp6 > Casp8 > Casp2 > Casp3 > Casp1 [21]	**Effective**: Heart attack [22], liver transplant [26], Fas-mediated liver injury [24,25] **Ineffective**: Septic shock [23]
IDN-6556, distributed in all tissues, highest concentration in liver. Inhibits caspases –1, -3, -6, -7, -8, -9 in low to sub-nanomolar range [27]. No non-specific binding or activity was seen against a "broad range" or receptors and enzymes [27]. "Peptide-like" [28] but no structure disclosed.	**Effective**: Fas-mediated liver injury [27,29], mild hepatic impairment (clinical trial) [30] **Adverse effects**: Phlebitis after intravenous administration to clinical trial patients [30].

*= less than two fold better inhibition, > less than ten-fold better inhibition, >> more than ten-fold better inhibition, [1] Methot et al. (2004), [2] Hotchkiss et al. (2000), [3] Klein & Cunha. (1995), [4] Stone et al. (2003), [5] Lee et al. (2003), [6] Chen et al. (2000), [7] Wang et al. (2003), [8] Zhu et al. (2002), [9] Kriz et al. (2002), [10] Van Den Bosch et al. (2002), [11] Zhang et al. (2003), [12] Hersch et al. (2003), [13] Denovan-Wright et al. (2002), [14] Du et al. (2001), [15] Diguet et al. (2003), [16] Smith et al. (2003), [17] Bonelli et al. (2003), [18] Yang et al. (2003), [19] Lee et al. (2000), [20] Chapman et al. (2002), [21] Wu & Fritz. (1999), [22] Dumont et al. (2001), [23] Grobmyer et al. (1999), [24] Mazur et al. (1998), [25] Hoglen et al. (2001), [26] Natori et al. (1999), [27] Hoglen et al. (2003), [28] Reed (2002), [29] Hoglen et al. (2004), [30] Saegusa et al. (2002)

Questions have been raised about the suitability of these molecules for clinical usage due to their relatively low efficacyand non-selectivity. However, these pre-clinical studies provide valuable insight into the potential for caspase inhibitors to treat human diseases. Pharmaceutical companies are developing caspase inhibitorswith more suitable clinical profiles, enhancing parameters such as oral bioavailability, membrane permeability and selectivity. (These and other drug design considerations are discussed in more detail in section 3.) Designing specific inhibitors would also help to delineate more precisely the individual roles of caspases in diseases. "Peptoid inhibitors" are made by chemically

modifying peptide-based inhibitors to improve oral availability, potency and / or stability. Small molecule screens using either intact cells, lysates or purified caspases are also increasingly being used to seek novel caspase inhibitors. A number of promising inhibitors with various specificities have been netted from such screens and subsequent refinement (Table 3). These include isatin sulfonamides, which are potent inhibitors but exhibit metabolic instability (Lee et al., 2000), and anilinoquinazolines (Scott et al., 2003).

This chapter focuses on data reported in peer reviewed publications, so many preliminary findings that are promising but currently unpublished have been omitted (but are in some cases readily available on the company websites). Companies with active programs for developing caspase inhibitors include GlaxoSmithKline, BASF, Idun Pharmaceuticals, Vertex Pharmaceuticals, Aventis Pharma, Immunex, Merck, AstraZeneca, Maxim Pharmaceuticals, Sunesis Pharmaceuticals and Apoxis.

2. INDICATIONS FOR CASPASE ACTIVITY MODULATION

As mentioned above, diseases can result from either insufficient or excessive cell death. Although this would imply that agents that either activate or inhibit caspases may represent useful therapeutics, to date attention has been focused on the development of caspase inhibitors. Strategies aimed at elevating caspase activity have so far tended to target upstream elements of the pathways, rather than directly activating pro-caspases, and these indirect caspase activating approaches are discussed in other chapters. Many diseases have been associated with excessive apoptosis and/or inflammation, and some of these have already been explored as potential targets for therapeutic application of caspase inhibitors. Caspase inhibition has been tested in clinical trials and/or animal models for the treatment of both acute and chronic diseases of the heart, liver and nervous system, as well as autoimmune and infectious diseases.

2.1. Liver Disease

Apoptosis has been implicated in a number of diseases of the liver, including Wilson's disease and hepatitis (both alcoholic and viral). In addition, donor tissue suffers significant levels of apoptosis during liver transplantation. Caspase inhibitors have been tested in animal models of liver disease and transplantation, and the first published clinical trial of a caspase inhibitor tested its application in liver disease.

2.1.1. Fas Mediated Liver Failure

A widely used animal model of liver disease involves the systemic administration of FasL (or antibodies which mimic its actions) to rodents. This treatment triggers Fas signaling in hepatocytes, and hence activation of caspase-8 and downstream caspases, causing massive hepatocyte apoptosis, cardiovascular shock and death (Ogasawara et al., 1993). Early studies demonstrated that treatment with pseudo-substrate caspase inhibitors was protective (Bajt et al., 2001; Kunstle et al., 1997; Rodriguez et al., 1996; Rouquet et al., 1996; Suzuki, 1998). Arguing for the importance of apoptosis rather than

inflammation in this model of liver injury, inhibitors with preferences for caspases-3 and -8 were also effective (Bajt et al., 2001). z-VD-fmk, a broad spectrum, irreversible dipeptide pseudosubstrate inhibitor, was protective against Fas-mediated liver disease (Yang et al., 2003b). The novel pan-caspase inhibitor IDN-1965 reduced the apoptosis induced by systemic administration of anti-Fas antibodies and improved liver function, as measured by a decrease in alanine aminotransferase (ALT) activity (Hoglen et al., 2001). IDN-1965 was effective when administered orally, and could be given up to one hour after Fas-mediated liver injury (Mazur et al., 1998). A second novel irreversible inhibitor, IDN-6556, efficiently inhibits caspases-1, -3, -7, -8 and -9, with IC_{50}s in the low to sub-nanomolar range (Hoglen et al., 2003). IDN-6556 was protective when given orally and preferentially distributes to the liver (Chen et al., 2003; Hoglen et al., 2004; Hoglen et al., 2003).

2.1.2. Liver Disease Clinical Trial

IDN-6556 was the first caspase inhibitor to complete a phase I clinical trial (Valentino et al., 2003). In that study, normal controls or patients with impaired liver function (due either to hepatitis C infection, alcoholic liver disease, fatty liver disease or of unknown diagnosis) were treated. Despite the efficacy of oral administration in the animal study (Hoglen et al., 2004), IDN-6556 was administered intravenously to the human subjects. The drug's safety was determined to be acceptable, but as seen in rats given IDN-6556 (Hoglen et al., 2003), phlebitis (vein inflammation) was a quite common dose-dependent side-effect (Valentino et al., 2003). Statistically significant improvements in the liver function of the hepatically impaired patients were observed during the period of administration of IDN-6556, but this improvement was not maintained following drug withdrawal. This compound is currently in phase II trials for liver disease.

2.1.3. LPS and Galactosamine

In a commonly used animal model of TNF associated liver injury, a mixture of galactosamine and endotoxin are administered intraperitoneally to mice, causing parenchymal apoptosis with neutrophil recruitment. As discussed below (section 2.7), co-administration of galactosamine sensitizes animals to the lethal effects of LPS, leading to TNF production and liver failure, in contrast to the systemic effects reminiscent of septic shock provoked by high dose LPS. z-VD-fmk, a cell-permeable irreversible pan-caspase inhibitor, rescued animals from galactosamine/LPS lethality, suppressing the processing and activation of caspases-3 and -8 and the resultant apoptosis (Jaeschke et al., 2000). IDN-6556 could also reduce apoptosis and caspase activity, improve liver function in mice and rats given galactosamine and LPS. This protection was observed both in rodents that received IDN-6556 simultaneously with the apoptotic stimulus or within a few hours of the toxic treatment. Ac-YVAD-cmk completely abolished the mortality associated with LPS/galactosamine treatment, but curiously it did not affect IL-1β production (Mathiak et al., 2000; Mignon et al., 1999). Caspase-1 mediated IL-18 processing may therefore by important in this model. More perplexing, given the inability of Ac-YVAD-cmk to directly inhibit caspase-3, was the finding that caspase-3-like activity in liver cells

from LPS/galactosamine treated mice was significantly reduced in animals given Ac-YVAD-cmk, but caspase-1-like activity was unaffected (Mignon et al., 1999).

2.1.4. Liver Transplantation

In addition to applications in treating liver diseases, caspase inhibition may improve the outcome of liver transplantation. Considerable cell death occurs as preserved donor liver tissue is warmed and reperfused prior to transplantation (Lemasters et al., 1995). TNF has been implicated in this damage (Rudiger & Clavien, 2002) which, interestingly, primarily involves sinusoidal epithelial cells rather than hepatocytes (Caldwell-Kenkel et al., 1989; Ikeda et al., 1992). Using a rodent model of cold ischemia/warm reperfusion (CI/WR), Natori *et al* demonstrated that the caspase-3 processing and activity increased following experimentally induced CI/WR (Natori et al., 1999). Administration of IDN-1965 reduced caspase-3 activation, and rats that received IDN-1965 survived an average of 30 hours post-transplant; a considerable improvement over control animals, which died after nine hours (Natori et al., 1999).

2.1.5. TNF

Despite these promising data, caspase inhibition is evidently unable to prevent all forms of liver damage. z-DEVD-fmk, which preferentially inhibits caspase-3, did not protect rats suffering from liver injury associated with hemorrhagic shock and resuscitation (Mauriz et al., 2003). More disconcerting was the finding that, rather than protecting mice from liver disease stimulated by TNF, z-VAD-fmk hastened TNF-mediated death. Signalling through one of the two TNF receptors (TNF-R1) was implicated, as the lethality of human TNF (which can ligate murine TNFR1 but not TNF-R2) was similarly exacerbated by z-VAD-fmk. The cathepsin B inhibitor E64 and inhibitors relatively specific for particular caspases (z-DEVD-fmk, z-IETD-fmk, z-LEHD-fmk and z-VDVAD-fmk) had no such deleterious effects, in fact providing partial protection. The increase in TNF lethality by z-VAD-fmk was attributed to the generation of reactive oxygen species and phospholipase A2 (PLA2) (Cauwels et al., 2003).

2.2. Neurological Diseases

Many neurological diseases involve excessive apoptosis, and the ability of caspase inhibitors to reduce this cell death and slow disease progression has been examined. The animal studies described below illustrate that caspase inhibitors can reduce neuronal damage by directly inhibiting apoptosis (mainly by blocking caspase-3) and through reducing production of inflammatory cytokines IL-1β and IL-18 (via caspase-1 inhibition). Mature IL-1β is involved in neuronal cell death (Friedlander et al., 1996; Troy et al., 1996) and caspase-1 signaling has been implicated in the progression of both acute and chronic neurological diseases including Huntington's and Parkinson's Diseases, amylotrophic lateral sclerosis (ALS) and stroke. It is important to note that clinical administration of caspase inhibitors to patients with neurological diseases will often require overcoming the blood-brain barrier. While many of the studies summarized

below document promising results from animal models, often the drugs were injected directly into the brain - an unfavorable route of administration in a clinical setting.

2.2.1. Minocycline

Minocycline is an antibiotic for which a number of potential mechanisms of action have been proposed, including inhibiting caspase independent cell death, downregulation of expression of caspases-1 and -3 (Wang et al., 2003b) and / or direct caspase inhibition (Lee et al., 2003). Conflicting outcomes were reported regarding the efficacy of minocycline in animal models of neuronal disease including Huntington's Disease (Bonelli et al., 2003; Chen et al., 2000; Denovan-Wright et al., 2002; Diguet et al., 2003; Hersch et al., 2003; Lee et al., 2003; Smith et al., 2003; Thomas et al., 2003; Wang et al., 2003b) and Parkinson's Disease (Du et al., 2001; Thomas & Le, 2004; Yang et al., 2003a). Administration enhanced survival in models of ALS (Kriz et al., 2002; Van Den Bosch et al., 2002; Zhang et al., 2003; Zhu et al., 2002). It also enhanced recovery following spinal cord injury through decreasing apoptosis in rats (Lee et al., 2003). Minocycline is a drug with a proven safety record and effectively penetrates the blood brain barrier. It is currently in phase III clinical trials for ALS, and phases I and II for Huntington's disease. Further studies will hopefully clarify its mechanism of action and the diseases for which it will be clinically useful.

2.2.2. Huntington's Disease

Patients with the neurodegenerative Huntington's Disease express a mutated Huntingtin protein. Transgenic R6/2 mice, which are used as a model for the disease, express exon 1 of human *huntingtin* bearing a polyglutamine repeat. R6/2 mice crossed with transgenic mice expressing a dominant negative mutant of caspase-1 in the brain exhibited delayed symptoms and increased survival time (Ona et al., 1999). Further studies using these mice demonstrated that mutant *huntingtin* induces intracellular toxicity which upregulates and activates caspase-1. As the disease progresses, caspase-3 is upregulated, suggesting that blocking both caspases is required for effective neuroprotection. z-VAD-fmk treated mice lived 25% longer than controls. Co-administration of Ac-YVAD-cmk and Ac-DEVD-fmk increased survival but each of the inhibitors were ineffectual alone. Daily intraperitoneal provision of minocyclin also inhibited caspase-1 and -3 mRNA upregulation and increased survival by 14% (Chen et al., 2000; Ona et al., 1999).

2.2.3. Amylotrophic Lateral Sclerosis

Blocking caspases with z-VAD-fmk delayed the onset of ALS in a transgenic mouse model (Li et al., 2000b). In this study Li *et al* also reported that z-VAD-fmk treatment affected expression levels of caspases-1 and -3. Thus z-VAD-fmk mediated neuroprotection may be due to both suppressing apoptosis and inhibiting activation of the cytokine IL-1β.

2.2.4. Parkinson's Disease

Anti-caspase therapies have also been explored to treat the cell death that underlies the pathology of Parkinson's Disease. Administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mimics some of the characteristics of Parkinson's Disease. Enforced expression of a dominant negative Apaf-1 construct, using an adeno-associated virus, reduced nigrostriatial degeneration triggered by MPTP treatment, implicating the mitochondrial apoptotic pathway and caspase-9 activation in the disease process (Mochizuki et al., 2001). Similar virally induced expression of a protein designed to act as a dominant negative inhibitor of caspase-1 did not affect MPTP induced neurotoxicity (Mochizuki et al., 2001). In contrast, transgenic expression of the caspase-1 dominant negative protein could reduce MPTP toxicity (Klevenyi et al., 1999). This discrepancy may reflect differences in the cell types expressing the inhibitor, as the virus predominantly infected neurons, whereas the transgene was expressed in glial and neuronal cells. Mice deficient for caspase-11, the likely murine homologue of the human caspase-4, were resistant to MPTP induced neurological symptoms (Furuya et al., 2004), providing additional evidence for the importance of inflammatory cytokines in this model of Parkinson's Disease.

2.2.5. Neuronal Transplantation

Neuronal transplants may be clinically useful in treating Parkinson's Disease and other neuronal disorders. As with the liver transplants discussed above, substantial levels of apoptosis accompany neuronal grafts. Only 5-10 % of grafted dopaminergic neurons survive transplant (reviewed by Brundin et al., 2000). In a rat model of Parkinson's Disease, lesions were created in the ascending mesostriatal dopaminergic pathway. Exposure of donor ventral mesencephalic cells to the caspase-1 inhibitor Ac-YVAD-cmk, prior to implantation, decreased apoptosis of transplanted cells from 90% to 70% and eased Parkinsonian symptoms (Schierle et al., 1999). Subsequent experiments indicated that the caspase-3-preferring inhibitor Ac-DEVD-cmk was similarly effective at protecting donor cells from apoptosis. Surprisingly however, the more broad anti-caspase agent z-VAD-fmk was ineffectual (Hansson et al., 2000). Co-treatment with Ac-YVAD-cmk and a lipid peroxidation inhibitor, the lazaroid tirilazad mesylate, (Freedox, Pharmacia Upjohn, approved for clinical use in the treatment of vasospasm) afforded additional protection: three times the co-treated neurons survived compared to untreated controls (Hansson et al., 2000). Augmenting neuronal survival following grafting is an attractive therapy, as it would potentially reduce the amount of donor tissue required. Such an *ex vivo* application for caspase inhibitors may also bypass many potential safety issues associated with *in vivo* administration.

2.2.6. Stroke

Caspase inhibitors have been evaluated in animal models of stroke, where neuronal apoptosis has been implicated in both the core and penumbral regions. Due to the limited therapeutic window for stroke treatments, neuroprotective strategies are better focused on interfering with the secondary apoptotic expansion of the primary lesion that occurs in

the penumbral regions. Mitochondrial pathways have been implicated in this cell death (Benchoua et al., 2001). Pan-caspase inhibition afforded substantial protection against ishemia-induced neuronal cell death (Himi et al., 1998; Yang et al., 2003b), in some cases even when given hours post-ischemia (Endres et al., 1998; Felderhoff-Mueser et al., 2002; Loddick et al., 1996). Pre-treatment or post-treatment with z-DEVD-fmk also limited the apoptosis associated with cerebral ischemia (Endres et al., 1998; Fink et al., 1998). These protective effects of caspase inhibition were further augmented by co-treatment with an NMDA inhibitor (Ma et al., 1998; Schulz et al., 1998). Consistent with apoptotic caspases being important in brain ischemia, overexpression of the Inhibitor of apoptosis proteins (IAPs) XIAP or a portion of NAIP that inhibits caspases (Maier et al., 2002) rescued neurons from ischemia-mediated death (Xu et al., 1999; Xu et al., 1997). XIAP also limited the resultant functional deficiency (Xu et al., 1999). A novel method of delivering caspase inhibitors to the brain was explored by Onteniente *et al.* The protein transduction domain of the HIV transactivator of TAT protein assists attached proteins to cross the plasma membrane. A fusion protein in which this domain was linked to XIAP was given to mice following cerebral ischemia, substantially decreasing infarct volumes (Onteniente et al., 2003). As discussed below, mere inhibition of apoptosis may not provide functional improvement. Illustrating this point, z-DEVD-fmk protected hippocampal neuronal apoptosis following ischemia, but the surviving neurons were non-functional in long term potentiation assays (Gillardon et al., 1999).

2.2.7. Traumatic Brain Injury

Traumatic brain injury (TBI) is a major cause of death and morbidity, especially in children under the age of six, whose brains appear to be particularly vunerable. Three main animal models are used to recapitulate the key features of TBI: the fluid percussion, controlled cortical impact and weight drop models (Bittigau et al., 2003). Brain tissue in animals subjected to these procedures starts to undergo apoptosis within hours of the injury, and peaks around 24 hours post-injury (Bittigau et al., 2003). Enforced expression of a dominant negative caspase-1 mutant (Fink et al., 1999; Friedlander et al., 1997; Hara et al., 1997), caspase-1 gene deletion (Friedlander et al., 1997) or administration of the caspase-1 inhibitor Ac-YVAD-cmk (Rabuffetti et al., 2000) were protective in rodent brain trauma models, as was z-VAD-fmk (Bittigau et al., 2003; Felderhoff-Mueser et al., 2002). This implies that inflammatory cytokines are important in traumatic brain injury, however apoptosis may also play a role, as the caspase-3-preferring inhibitor zDEVD-fmk could also ameliorate damage caused by brain injury (Yakovlev et al., 1997). Importantly, this treatment also improved neurological function (Yakovlev et al., 1997). Another study assayed the effects on rat neurological function of cortical impact followed by mild hypoxia. Immediate post-trauma administration of z-DEVD-fmk reduced the neuronal death substantially, but the performance of injured rats treated with the caspase inhibitor in a number of behavioural tests was impaired to a similar extent to that of controls (Clark et al., 2000).

2.2.8. Glaucoma

Glaucoma stems from apoptosis of retinal ganglion cells. McKinnon et al. used an adeno-associated viral vector to express BIR4/XIAP in rat eyes, then experimentally induced glaucoma. Axonal survival following the rise in intraocular pressure varied considerably, but more axons survived from the eyes overexpressing the IAP than control eyes (McKinnon et al., 2002). XIAP could also suppress apoptosis following optic nerve axotomy in a rat model (Kugler et al., 2000).

2.2.9. Vasospasm

Endothelial cell apoptosis following cerebral vasospasm and subarachnoid haemorrhage was studied in a dog model (Aoki et al., 2002). Two caspase inhibitors were tested for efficacy in this model. One was z-VDVAD-fmk (the only efficient peptidyl inhibitor of caspase-2, but which probably inhibits caspase-3 almost as efficiently) and the other was the caspase-3-favoring inhibitor z-DEVD-fmk (Talanian et al., 1997). Both inhibitors blocked the endothelial apoptosis, but only z-DEVD-fmk had a marginal impact on the cerebral vasospasm (Aoki et al., 2002).

2.3. Cardiac / Vasculature Disease

2.3.1. Heart Attack

As for other organ systems mentioned above, apoptosis due to ischemia and reperfusion is an important contributing factor in many types of heart disease. Caspase inhibition by the di-peptide inhibitor z-VD-fmk halved the damage sustained by the hearts of experimental animals during acute myocardial infarction (Yang et al., 2003b). z–VAD-fmk was also effective in a similar experimental model (Chapman et al., 2002; Yaoita et al., 1998), significantly reducing the number of apoptotic cells and slightly diminishing the gross infarct size, even when administered after the onset of ischemia but before reperfusion (Huang et al., 2000). Ac-YVAD-fmk was effective in some studies but not others (Kovacs et al., 2001; Wang et al., 2003a). Ac-DEVD-cmk had little (Chapman et al., 2002) or no effect on infarct size (Kovacs et al., 2001), as did z-LEHD-fmk (Kovacs et al., 2001). Suprisingly, given the inefficient cellular uptake of aldehyde-conjugated peptides, one group reported that Ac-YVAD-CHO and Ac-DEVD-CHO decreased cardiac apoptosis following ischemia/reperfusion but could not reduce the infarct size (Okamura et al., 2000). A mouse model was developed which allows real-time visualization of cardiac apoptosis following ischemia/reperfusion. Using this technology, substantial staining with Annexin-V (which binds to the phosphotidyl serine on the outer leaflet of the plasma membrane in apoptotic cells) was detected once reperfusion commenced in the injured area of the heart (Dumont et al., 2001). This reperfusion-associated apoptosis was almost halved by pre-injection of IDN-1965 (relatively specific for caspases -6, -8 and -9) into the carotid artery. Pre-administration of z-VAD-fmk rescued two thirds of the cells and IDN-1529 (a pan-caspase inhibitor with a preference for caspase-9) protected 80% (Dumont et al., 2001).

2.3.2. Peripheral Ischemia

Arterial injuries, induced in the hind limbs of mice by prolonged ischemia followed by reperfusion, were reduced by administration of z-VAD-fmk prior to arterial clamping (Iwata et al., 2002). The injury caused by this arterial clamping substantially impaired hind leg function in the mice, however mice that received z-VAD-fmk prior to the onset of ischemia had almost normal hind leg function (Iwata et al., 2002).

2.3.3. Hypertension

Caspase inhibition was tested in a rat model of severe pulmonary hypertension, in which blockade of vascular endothelial growth factor receptors (VEGFR) combined with chronic hypoxia induces endothelial lung cell apoptosis (Taraseviciene-Stewart et al., 2002). Mice were given repeated intraperitoneal injections of the caspase inhibitor z-VAD-fmk during the hypoxic period of three weeks. Treatment with z-VAD-fmk abolished the effects of VEGFR blockade: z-VAD-fmk treated animals subjected to hypoxia and VEGFR blockage had similar pulmonary arterial pressure and ventricular mass to hypoxic animals not treated with the VEGFR antagonists. Caspase inhibition in this model was as effective as treatment with the bradykinin inhibitor B9430 (Taraseviciene-Stewart et al., 2002).

2.4. Immune System Disorders

2.4.1. Auto-immune Diseases

A complex process of lymphocyte differentiation and selection normally eliminates by apoptosis cells that recognize self-epitopes. Autoimmune disease results from the unwanted survival of these auto-reactive immune cells. Because caspases are critical for the removal of the autoreactive cells, one might imagine that therapeutic activation of caspases at the critical point during lymphocyte differentiation would be a useful avenue to pursue. However, once autoreactive cells have been generated and a disease has developed, it is not clear whether disease symptoms would be reduced through preventing generation of additional autoreactive cells. In fact, as the autoreactive immune cells cause problems by inducing apoptosis in their target cells, caspase inhibition has been explored as a strategy for easing symptoms associated with auto-immune disease. Clearly, the risk with such an approach would be that, unless the caspase inhibition is exquisitely targeted, survival of auto-immune cells may be boosted in individuals who already have a predisposition to the retention of auto-immune lymphocytes, potentially exacerbating the underlying problem. Despite this theoretical concern, a few researchers have reported promising outcomes of inhibiting caspases in animal models of autoimmune disease.

One system in which caspase inhibition has been tested is a mouse model of Sjorgen's syndrome, a condition caused by lymphocytic infiltration into the salivary and lacrimal glands. Caspase-mediated cleavage of α-fodrin creates a neo-epitope that acts as an auto-antigen. Repeated intravenous injection of z-VAD-fmk or Ac-DEVD-CHO

alleviated symptoms to a significant extent, as measured by a short term assay of saliva and tear production (Saegusa et al., 2002).

Transgenic mice expressing IFNγ in the suprabasal layers of the epidermis develop an autoimmune inflammatory skin disease resembling systemic lupus ethryematosus (SLE). Mice that had already started to develop symptoms were treated with z-VAD-fmk. The skin disease progressed despite caspase inhibition, and no clear decrease in autoantibody production was observed, but the kidney disease which develops in these transgenic animals was ameliorated (Seery et al., 2001).

Caspase-1 is a crucial component of immune mediated inflammation due to its pivotal role in the cellular export of IL-1β and IL-18, as mentioned in the introduction. Caspase-1 expression increased in a model of experimentally induced auto-immune encephalomyelitis (EAE; a model of multiple sclerosis) in which mice were immunized with spinal cord homogenate or myelin oligodendrocyte glycoprotein. Consistent with this notion, prior treatment with z-VAD-fmk lessened the development of symptoms, but it was not useful in treating the disease if given post-immunization (Furlan et al., 1999), suggesting that caspases are involved in the early stages of the disease leading to immune mediated demylination. Inhibiting caspase-1 activity may therefore be an attractive target for acute relapsing multiple sclerosis.

2.4.2. Asthma

Asthma is caused by an allergic response that leads to airway inflammation. This disease can be modelled in a mice in which animals are sensitized to the antigen OVA, and subsequently challenged immunologically with OVA peptides. Rather than prolonging the lifespan of T lymphocytes or eosinophils and aggravating asthma in this model, prior introduction of z-VAD-fmk had a protective effect, decreasing the infiltration of airways, mucus hypersecretion, edema and Th2 cytokine release (Iwata et al., 2003). Interestingly, Ac-YVAD-fmk had no such effect (Iwata et al., 2003). The inefficacy of Ac-YVAD-fmk implied that the activity of z-VAD-fmk most likely related to its ability to inhibit apoptotic caspases rather than caspase-1. The exact mechanism underlying this effect of z-VAD-fmk is somewhat obscure, but could involve caspase-3 dependent maturation of IL-16 (Zhang et al., 1998), which has been associated with airway hypersensitivity. Alternatively, suppression of caspase mediated differentiation of T cells (Schwerk & Schulze-Osthoff, 2003; Walsh et al., 2003) may play a part.

2.5. Arthritis

Rheumatoid arthritis is characterized by immune-mediated breakdown of joint cartilage. IL-1 is an important factor in the pathogenesis of this disease, and blockade of IL-1 receptors has been explored as a potentially valuable therapy. Prophylactic administration of VE-13,045, an irreversible peptidyl caspase-1 inhibitor, slowed symptoms of arthritis resulting from intradermal collagen injections, and reduced arthritic disease by 60% (Ku et al., 1996). In a therapeutic model, treatment with VE-13,045 ten days after the collagen booster was given (after the onset of observable symptoms) slowed the progression of the inflammation, prevented additional infiltration of immune cells and suppressed further cartilage erosion (Ku et al., 1996).

Vertex Pharmaceuticals completed a Phase IIa clinical trial with Pralnacasan, an inhibitor of caspase-1 for rheumatoid arthritis. Administered orally, Pralcacasan significantly reduced joint symptoms and inflammation. However it was withdrawn from clinical trials due to liver abnormalities in long-term animal studies.

Osteoarthritis is a degenerative joint disease is caused by the degradation of cartilage through chondrocyte apoptosis. The specific cause of the degeneration in this form of arthritis is unclear. "Wear-and-tear" seems to play a part, as the frequency of osteoarthritis increases with age and people who play vigorous sports tend to be disproportionally afflicted. The impact of caspase inhibition on osteoarthritis was evaluated in spontaneous and collagen-induced mouse models of the disease. Pralnacasan reduced joint damage in both models (Rudolphi et al., 2003).

2.6. Kidney Disease

Caspases have been shown to be mediators of necrotic renal cell injury *in vivo* (Ortiz et al., 2003), and administration of z-VAD-fmk prevented apoptosis, inflammation and tissue damage in an ischemia/reperfusion mouse model (Daemen et al., 1999). A newly developed broad-spectrum caspase inhibitor, OPH-001, was tested in a mouse model in which ischemic-induced acute renal failure was experimentally triggered by surgical clamping of the renal pedicles (Melnikov et al., 2002). Caspase-1 was previously implicated in ischemia-induced renal injury, as caspase-1 deficient mice do not undergo renal failure following this procedure (Melnikov et al., 2001). Administration of OPH-001 to wild-type mice an hour prior to clamping substantially prevented the onset of renal failure, as monitored by serum creatinine and blood urea nitrogen levels. Agonistic antibodies against IL-18 also conferred partial protection. While OPH-001 attenuated apoptosis, its predominant effect was on proximal tubule necrosis. Histological examination indicated that only 11-25% of tubules were necrotic in OPH-001 treated animals, compared to 46-75% of tubules in control animals. The substantial neutrophil infiltration accompanying ischemia was abolished by pretreatment with OPH-001.

2.7. Septic Shock and Infection

2.7.1. Sepsis

Given the importance of caspase-1 in the generation of the inflammatory cytokines, modulating its activity has been an attractive approach to controlling the many diseases in which these cytokines have been implicated, including infection (Rosenwasser, 1998; Sugawara, 2000). Early studies using knockout mice indicated that caspase-1 is required for septic shock induced by high doses of the bacterial cell wall component lipopolysaccharide (LPS) (Kuida et al., 1995; Li et al., 1995). Caspase-1 inhibition also provided protection in a central sepsis model (Yao et al., 1999). Anorexia results when LPS is administered intracranially. In contrast, mice deficient for caspase-1 or treated with Ac-YVAD-cmk had normal appetites following intracranial LPS administration (Yao et al., 1999). A new inhibitor (M867) with a preference for caspase-3 (and to a lesser extent caspase-7) was tested in a rodent model in which sepsis was triggered by cecal ligation and perforation. M867 dramatically improved survival following cecal

puncture (Hotchkiss et al., 2000), and substantially decreased thymocyte death when given intraveneously for 24 hours after sepsis was induced (Methot et al., 2004). Importantly, these studies defined the critical window during which caspase inhibition would be effective: lymphocyte survival during the first 24 hours following sepsis was crucial for animals to survive this treatment.

Administration of LPS is commonly used to experimentally induce hemodynamic and metabolic symptoms associated with septic shock. This treatment, which is lethal in sufficient doses, involves the generation of inflammatory cytokines, and the symptoms can be prevented by co-administration of caspase-1 inhibitors, such as Pralnacasan (Ku et al., 2002; Siegmund & Zeitz, 2003) and VE-13,045 (Ku et al., 1996). IDN-1529, whose preferred target is caspase-9 but which also inhibits caspase-1 quite well, promoted survival in this model, even when given after the onset of symptoms (Grobmyer et al., 1999). Consistent with the notion that caspase-1 activity is required for the lethal effects of high dose LPS, IDN-1965 (a pan-caspase inhibitor that inhibits caspase-1 23-fold less well than IDN-1529), was ineffectual (Grobmyer et al., 1999). Treatment with galactosamine dramatically sensitizes animals to LPS: lethal doses are reduced by 2,500 fold by co-treatment (Galanos et al., 1979). This co-treatment protocol has been used by many groups as a model for sepsis, however questions have been raised regarding its relevance to human sepsis (Leist et al., 1995; Mignon et al., 1999). It has been postulated that the lethality associated with cotreatment with galactosamine and LPS is due to TNF mediated liver injury rather than IL-1β related sepsis involving multiple organs. The impact of caspase inhibitors on symptoms triggered by co-treatment with galactosamine and LPS is discussed in section 2.1.

2.7.2. Peritonitis

Inhibition of caspase-1 was reported to hasten the resolution of peritonitis triggered in mice by intraperitoneal injection of *Staphylococcus aureus* (Catalan et al., 2003), modelling a major clinical complication of peritoneal dialysis. z-VAD-fmk treatment produced a slight improvement in bacterial clearance and a dramatic decrease in apoptosis of peritoneal neutrophils (Catalan et al., 2003).

2.7.3. Pulmonary Fibrosis

z-VAD-fmk was also effective in a mouse model of pulmonary fibrosis, a disease thought to result from alveolitis. In this study, symptoms of pulmonary fibrosis were triggered in mice by administration of bleomycin, and these symptoms were alleviated by inhalation of z-VAD-fmk (Kuwano et al., 2001).

2.7.4. Pancreatitis

Inhibition of caspase-1 afforded significant protection again acute pancreatitis, induced in rats by sodium taurocholate. The death rate from pancreatitis in this system was reduced by administration of Ac-YVAD-cmk (Norman et al., 1997; Rau et al., 2001), even when given after disease induction (Paszkowski et al., 2002).

2.7.5. Colitis

Colitis was induced in mice by feeding them 3.5% dextran sulfate in drinking water for 10 days (Loher et al., 2004). This treatment triggered IL-18 production, and subsequent IFNγ generation by splenocytes. Intraperitoneal Pralnacasan significantly reduced IL-18, IFNγ production and improved symptoms such as body weight and stool consistency (Loher et al., 2004). Oral administration was less effective (Loher et al., 2004).

3. CONSIDERATIONS FOR THERAPEUTIC CASPASE INHIBITOR DESIGN

The animal studies and single human clinical trial cited above yielded generally encouraging results, and it is likely that many caspase inhibitors will be developed and tested in clinical trials in the near future. It is therefore useful to discuss the considerations associated with developing and using caspase inhibitors clinically.

3.1. Limitations of Available Animal Models

The initial stages of drug development exploit animal models that permit validation of the predicted activity of novel drugs, but these often do not replicate human disease. While these proof-of-concept studies are essential, meaningful data regarding likely clinical efficacy of caspase inhibitors will only be yielded if the apoptotic pathway stimulated in the model accurately reflects that triggered in patients. This will be particularly true for inhibitors of specific caspases. Unfortunately many of the animal models currently available are not yet fully characterized at the molecular level, so while they may mimic the symptoms of human disease, the components of the apoptotic pathways may differ from those in patients' cells. For this reason, an enhanced understanding of the molecular pathways responsible for the apoptosis associated with particular diseases will be extremely valuable in choosing and developing appropriate animal models for testing candidate drugs.

3.2. Inhibiting Apoptosis versus Restoring or Retaining Function

A pertinent question is whether inhibiting apoptosis equates with restoring cellular viability and function. In many of the studies described above, drugs were deemed effective if they reduced apoptosis. However, *in vitro* at least, inhibiting caspase activity has sometimes been shown to merely shift the mode of cell death from apoptosis to necrosis (Hartmann et al., 2001; Lemaire et al., 1998) This may also be relevant *in vivo* - in one myocardial infarction model, caspase inhibition decreased apoptosis of heart cells but did not reduce infarct size (Okamura et al., 2000). Even if the cells rescued from apoptosis do survive, they may be substantially damaged and incapable of carrying out their normal physiological roles. This appears not to be the case in most liver injury models, where caspase inhibition either rescued the animals from lethality or improved their liver function as measured by transaminase levels. In some neuronal and cardiac models, caspase inhibition also resulted in retention of cellular function (Friedlander et

al., 1997; Himi et al., 1998; Mochizuki et al., 2001; Neviere et al., 2001; Ona et al., 1999; Schierle et al., 1999). However, other data indicate that apoptosis inhibition may not be suffficient to relieve clinically relevant symptoms in neuronal conditions (Clark et al., 2000; Gillardon et al., 1999) and, *in vitro*, neurons in which apoptosis was suppressed through caspase inhibition were functionally impaired (von Coelln et al., 2001). Future studies should therefore encompass measures of function, in addition to assaying cellular survival.

3.3. Therapeutic Caspase Inhibitor Design

Ideally, caspase inhibitors would possess the required pharmacokinetic properties to target the disease and caspase(s) of interest. Altering such properties of caspase inhibitors as specificity, stability, reversibility, solubility and perhaps even targeting specific tissues, imposes significant hurdles to successful drug design. Due to lesser requirements for stability, specificity and toxicity, companies are mainly focusing drug development programs on acute indications. Indeed broad spectrum caspase inhibition may be desirable to achieve effective therapy in acute diseases due to the amplifying nature of the caspase cascade. However, for the treatment of chronic disease (such as Capase-1 inhibition to delay the progression of Huntington's disease), selective inhibition will be paramount so that side effects are minimized, as discussed below. Drug stability is also an important issue, as the drug half-life requirements will be different for acute versus chronic diseases. Targeting particular tissues, especially in the cases of chronic diseases, may minimize adverse affects. As an example, IDN-6556 targets and is rapidly cleared by the liver, making it an ideal drug for liver disease. In contrast, targeting caspase inhibitors to the brain for stroke or neurodegenerative disorders would prove more difficult, and would require improvements in solubility for blood barrier penetration. Non-peptidyl inhibitors may be favored for diseases of the brain, as peptide-based inhibitors typically access the brain very poorly (Robertson et al., 2000). Providing an example of an experimental strategy adopted to overcome this problem, Onteniente et al., fused caspase inhibitors to the HIV-1 protein transduction domain protein, conferring membrane permeability (Onteniente et al., 2003). One particularly promising application of caspase inhibitors, which is likely to be safe, is the prevention of apoptosis in donor tissue, such as for liver transplants. In this application, targeting the caspase inhibitor to the donor cells is straightforward as the administration can be performed *in vitro*, sparing the recipient's cells from any side effects of the drug. An additional benefit may be that only short term treatment would be required.

When comparing reversible and irreversible inhibitors, which bind to caspases with similar affinities, irreversible inhibitors provide better protection from apoptosis at a given dose. To illustrate this point, Wu and Fritz calculated that IC_{50} values could be 42-137 fold better for irreversible inhibitors than reversible inhibitors of equivalent binding affinity (Wu & Fritz, 1999). This would argue that development of irreversible inhibitors for clinical use should be favored, as lower doses would be required to effect a given response. However it should be noted that this greater potency associated with irreversible inhibition may be deleterious if the inhibitor was not totally specific, as irreversible inactivation of molecules other than the target caspases could lead to adverse effects.

3.4. Administration

Route and timing of administration are important considerations. As for most drugs, oral administration of caspase inhibitors would be preferable, especially for long term administration for chronic diseases. For acute indications, injections may be more acceptable. The timing of drug administration also varies for different indications. It becomes critical for diseases such as stroke, where there is a therapeutic window to reduce apoptosis in the penumbral region. In many of the successful animal trials summarized above, caspase inhibitors were administered prior to or simultaneous with disease onset. Future studies should focus on whether candidate drugs are effective when given at a time reflecting feasible clinical administration (at the very least, after symptoms are detectable).

3.5. Avoiding Foreseeable Adverse Effects

Given the central and diverse roles played by apoptosis, one could envisage a number of adverse scenarios resulting from indiscriminate modulation of apoptotic pathways. Global suppression of IL-1β and IL-18 maturation could also provoke unwanted effects. Although less well understood, caspases may play roles in proliferation and differentation (Schwerk & Schulze-Osthoff, 2003), the inhibition of which may also lead to undesirable side-effects.

It should be noted that the studies reported to date and summarized here have only assessed the short-term impact of caspase inhibition. One theoretical long-term risk of caspase inhibitor administration would be cancer development. Inhibition of apoptotic pathways at other points has been conclusively demonstrated to contribute to oncogenesis (Igney & Krammer, 2002) and blocking upstream steps in apoptosis pathways, by enforcing Bcl-2 expression or knocking-out Apaf-1, increased clonigenic survival (Haraguchi et al., 2000; Vaux et al., 1992). In contrast, inhibition of apoptosis pathways at distal points, as would be achieved by caspase inhibition, appears to have a qualitatively different effect. Cells lacking caspase-9 merely exhibited a short term apoptosis resistance, but the surviving cells did not proliferate to form colonies (Ekert et al, 2004, in press). In total, these data tend to suggest that caspase inhibition would be unlikely to contribute to oncogenesis, however long-term animal studies addressing this issue would be warranted.

The possibility of deleterious ramifications caused by long term caspase inhibition, apart from cancer development, should also be considered. Vertex and Alventis announced, in a November 2003 press release, that phase IIb trials of the caspase-1 inhibitor Pralnacasan in rheumatoid arthritis patients had been discontinued when a toxicology study revealed that animals exposed to high doses for nine months developed liver abnormalities. Publication of the details of this animal toxicology study and others investigating the long term effects of various caspase inhibitors should assist in designing new drugs and treatment protocols to minimize the risks associated with therapeutic caspase inhibition.

Caspase inhibitor-based therapy for chronic viral conditions such as viral hepatitis could be quite risky. In evolutionary terms, apoptosis can be used to limit viral spread and replication (Cuconati & White, 2002). If caspase inhibitors are to be used for treating

viral hepatitis, for example, it would be wise to carefully assess the impact of the drug on viral load in medium and long term in animal models before embarking on clinical trials. The use of untargeted caspase inhibitors to prevent auto-immune cellular destruction may also be counterproductive, as it could limit the apoptotic removal of autoimmune cells.

4. CONCLUSIONS

Dysregulated apoptosis contributes to a multitude of human diseases, and drugs that modulate caspase activity may have many clinical applications in the future. Although the development of caspase inhibitors for clinical use has a long way to go, many promising results have already been gleaned from animal studies. As the compounds used in many of these studies are not optimal for *in vivo* caspase inhibition, new inhibitors with improved cell penetration and pharmacokinetic properties could well yield better results. In time, agents designed to enhance caspase activity, rather than inhibit it, will probably also be developed for therapeutic application. With due attention to the potential risks outlined above, therapeutic modulation of caspase activity is likely to offer significant health benefits for patients with a number of debilitating or fatal conditions.

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THE INTRINSIC (MITOCHONDRIAL) DEATH PATHWAY AND NEW CANCER THERAPEUTICS: BCL-2 FAMILY IN FOCUS

Chapter V

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1. INTRODUCTION

Apoptosis is a genetically programmed form of cell death by which multi-cellular organisms maintain tissue homeostasis during growth and development (Jacobson et al., 1997). The execution of apoptosis is generally tightly regulated through complex interactions between apoptosis-promoting and apoptosis-opposing molecules. However, aberrant regulation of apoptosis is seen in, and contributes to, a myriad of patho-physiologic conditions such as malignancy, neurologic disorders and autoimmune diseases (MacGibbon et al., 1997; Eguchi, 2001; DeVries et al., 2001; Johnstone et al., 2002; Shangary & Johnson, 2003).

Apoptosis ensures timely cellular suicide through two autonomous, but interconnected pathways: The intrinsic pathway, characterized by disruption of mitochondrial integrity, and the extrinsic pathway, characterized by death receptor/death ligand signaling. Members of the Bcl-2 protein family play a critical role in regulating the intrinsic, mitochondrial cell death pathway. Research performed over the past 15 years has shown that defective apoptosis due to abnormal expression or function of Bcl-2 family members is a characteristic feature of many malignancies. Overexpression of anti-apoptotic proteins, such as Bcl-2 and Bcl-x_L, not only promotes tumorigenesis, but subsequently confers resistance to traditional treatment modalities (Johnstone et al., 2002; Shangary & Johnson, 2003). Thus, there is tremendous need to develop novel anti-cancer agents which directly target apoptosis regulatory proteins. This review will focus on the regulation of the intrinsic apoptosis pathway by Bcl-2 family members, and will attempt

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to clarify the past, present and future of anti-cancer therapeutics targeting this cell death pathway.

2. MITOCHONDRIAL EVENTS ASSOCIATED WITH THE INTRINSIC APOPTOTIC PATHWAY

The intrinsic pathway of apoptosis is activated in response to cell death signals such as chemotherapy drugs, UV, DNA damage, growth factor withdrawal, and p53 and c-myc activation (Zong et al., 2001; Wei et al., 2001). These diverse apoptotic stimuli activate intracellular signals that converge at the level of the mitochondria, resulting in the loss of mitochondrial membrane potential and the release of apoptogenic proteins (Fig. 1). The precise mechanisms responsible for the release of apoptogenic factors into the cytosol are not well understood, but recent reports suggest an initial pore-dependent efflux of apoptogenic factors followed by a more lengthy remodeling of mitochondrial architecture and loss of membrane potential (Scorrano et al., 2002; Kuwana et al., 2002). The apoptogenic factors known to be released from the mitochondria include cytochrome *c* (Yang et al., 1997; Kluck et al., 1997; Liu et al., 1996), apoptosis-inducing factor (AIF) (Susin et al., 1999), and Smac/DIABLO (Du et al., 2000; Verhagen et al., 2000). Of these, cytochrome *c* is perhaps the best characterized. Once released into the cytosol, cytochrome *c* binds to Apaf-1 protein, inducing conformational changes which allow association with pro-caspase-9 (Li et al., 1997; Zou et al., 1999; Yoshida et al., 1998). Formation of this 'apoptosome' complex results in the activation of caspase-9, which

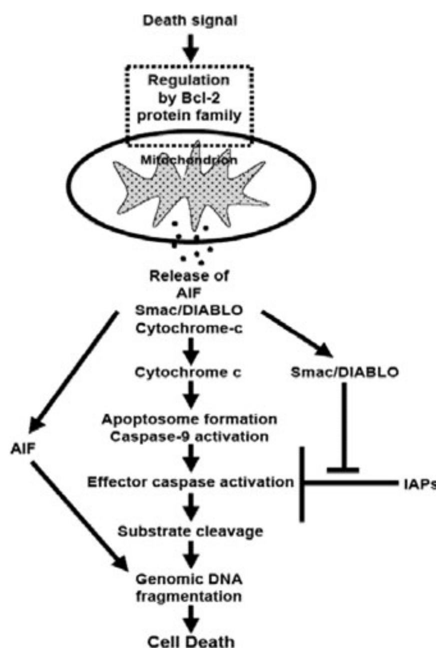

Figure 1. The intrinsic, mitochondrial-mediated cell death pathway.

then cleaves and activates the executioner caspases, caspase-3 and -7. Ultimately, activation of this caspase cascade leads to the fragmentation of genomic DNA and changes in cellular morphology that are characteristic of apoptotic death.

Although mitochondrial release of cytochrome *c* initiates a well amplified cascade, post-mitochondrial regulatory controls do exist. Cytosolic IAPs (inhibitors of apoptosis proteins), including c-IAP1, c-IAP2 and XIAP, have been shown to bind to inhibit caspases, and have been reported to be overexpressed in several tumor types (Tamm et al., 2000; Holcik et al., 2000). Smac/DIABLO normally acts to inhibit the activities of IAPs, such as XIAP, by displacing the inhibitor protein from caspase enzymes (Verhagen & Vaux, 2002). Additionally, apoptotic stimuli result in the mitochondrial release of AIF, which migrates to the nucleus and induces genomic DNA fragmentation (Susin et al., 1999).

3. THE INTRINSIC MITOCHONDRIAL PATHWAY OF CELL DEATH: REGULATION BY THE BCL-2 PROTEIN FAMILY

Members of the Bcl-2 protein family act as important regulators and participants in the intrinsic mitochondrial pathway. The release of apoptogenic proteins from the mitochondria is inhibited by anti-apoptotic Bcl-2 family members (e.g. Bcl-2 and Bcl-x_L)

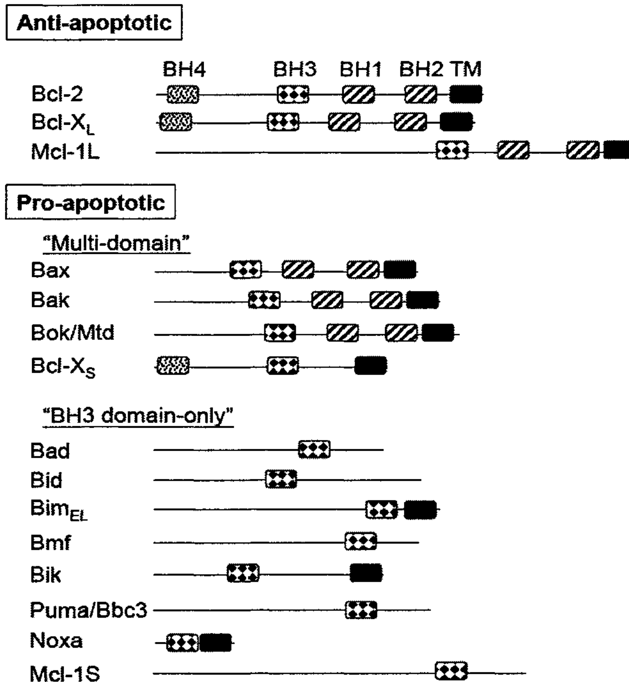

Figure 2. The Bcl-2 protein family. The figure shows schematic representations of anti-apoptotic and pro-apoptotic members of the Bcl-2 protein family. The following structural domains are shown: transmembrane (TM) domains (solid black), BH1 and BH2 (striped), BH3 (checkered), and BH4 domains (striped).

and promoted by pro-apoptotic members (e.g. Bax, Bak and Bid). However, these regulatory activities are modulated by protein/protein interactions between the Bcl-2 family members. Knowledge regarding the structural composition of Bcl-2 family proteins has contributed significantly to our understanding of the protein/protein interactions occurring in this family.

The Bcl-2 family consists of multiple proteins that share sequence homologies in regions termed Bcl-2 Homology domains (BH domains; Fig. 2). Anti-apoptotic family members share all four of the known Bcl-2 homology domains, BH1, BH2, BH3 and BH4, and include Bcl-2 (Tsujimoto & Croce, 1986), Bcl-x_L (Boise et al., 1993), Mcl-1 (Kozopas et al., 1993), Bfl-1 (Choi et al., 1995), and Bcl-w (Gibson et al., 1996). The pro-apoptotic Bcl-2 family members are functionally and structurally divided into multi-domain and 'BH3 domain-only' proteins. Multi-domain pro-apoptotic members are structurally similar to anti-apoptotic Bcl-2, and also contain BH1-3 domains. Currently identified members of the multi-domain pro-apoptotic subgroup include Bax (Oltvai et al., 1993), Bak (Chittenden et al., 1995b), Bok/Mtd (Hsu et al., 1997a; Inohara et al., 1998), and Bcl-x_S (Boise et al., 1993). Included among the 'BH3 domain-only' proteins are Bad (Yang et al., 1995), Bid (Wang et al., 1996), Bim/Bod (O'Connor et al., 1998), Bmf (Puthalakath et al., 2001), Bik/Nbk (Boyd et al., 1995), PUMA/Bbc3 (Nakano & Vousden, 2001; Yu et al., 2001), Noxa (Oda et al., 2000), and Mcl-1_S (Bae et al., 2000).

3.1. Anti-Apoptotic Members Exemplified by Bcl-2 and Bcl-x_L

Bcl-2, the prototypic anti-apoptotic protein, was initially discovered as an overexpressed oncoprotein in human follicular lymphomas harboring a t(14;18) translocation (Tsujimoto & Croce, 1986; Tsujimoto et al., 1984). The majority of anti-apoptotic proteins, including Bcl-2 and Bcl-x_L, contain carboxyl-terminal transmembrane domains which dictate their subcellular localization to organellar membranes, primarily the outer-mitochondrial membrane (OMM). In addition to the OMM, Bcl-2 and Bcl-x_L have also been found to be localized to endoplasmic reticulum (ER) and nuclear membranes (Conus et al., 2000; Hsu et al., 1997b). The localization of Bcl-2 and Bcl-x_L at the OMM enables both proteins to inhibit cytochrome *c* release in cells treated with an apoptotic stimulus.

Anti-apoptotic Bcl-2 family members characteristically contain BH1, BH2 and BH3 domains, and these domains fold together to form a hydrophobic binding pocket, termed the BH3 binding pocket (Aritomi et al., 1997; Muchmore et al., 1996). The BH3 binding pocket has been shown to be integral for the anti-death activity of these proteins (Yin et al., 1994). Additionally, early studies determined that anti-apoptotic proteins, such as Bcl-2, easily formed heterodimers with pro-apoptotic proteins such as Bax, and that this heterodimerization was dependent on an intact BH3 binding pocket in the anti-apoptotic protein (Yin et al., 1994; Zhang et al., 2000; Sedlak et al., 1995; Wang et al., 1998). An intact BH3 domain in the pro-apoptotic protein also was required for heterodimerization (Chittenden et al., 1995a; Simonen et al., 1997). The Bcl-2/Bax Rheostat Model, proposed by Oltvai and colleagues, in 1993, predicted that the ratio of anti- to pro-apoptotic proteins within a cell determines whether a cell will live or die in response to an apoptotic stimulus (Oltvai et al., 1993). This model rested on observations that pro-apoptotic proteins are neutralized by heterodimerization with anti-apoptotic members,

and vice versa. Supporting evidence for this model included reports that loss of function mutants of Bcl-2 and Bcl-x_L were unable to bind to pro-apoptotic Bax and Bak (Yin et al., 1994; Sedlak et al., 1995).

3.2. Multi-Domain Pro-Apoptotic Members Exemplified By Bax and Bak

Pro-apoptotic members of the Bcl-2 family are functionally and structurally divided into two subgroups: multi-domain proteins and 'BH3 domain-only' proteins. The most extensively characterized multi-domain pro-apoptotic proteins are Bax and Bak. Landmark studies in a murine Bax/Bak double knock-out model revealed that the expression of either Bax or Bak was essential for cell death induced by etoposide, staurosporine, or UV radiation (Zong et al., 2001; Wei et al., 2001).

Under normal conditions, Bax occupies a cytosolic distribution. However, in response to death stimuli, cytosolic Bax translocates to the mitochondria (Hsu et al., 1997b). In addition, conformational changes occur which reveal a mitochondrial targeting transmembrane domain, facilitating association with the OMM (Desagher et al., 1999)

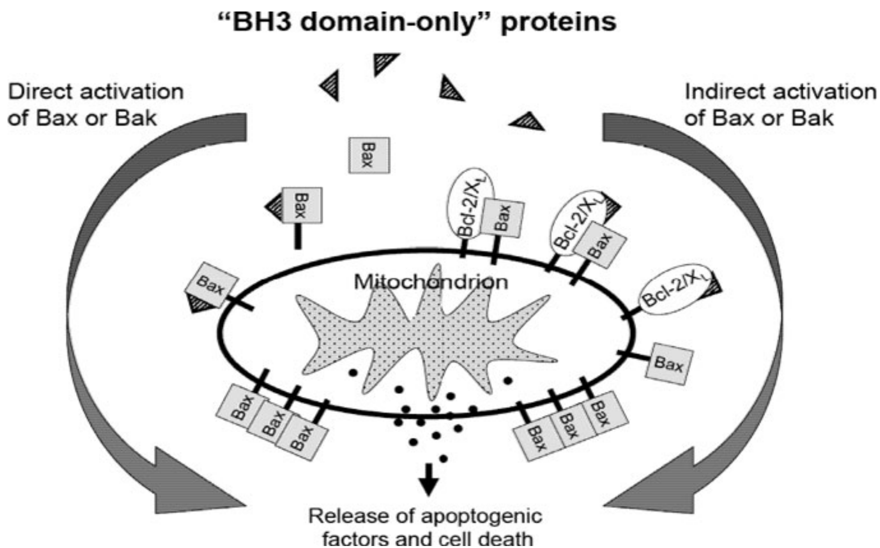

Figure 3. Apoptosis induction by "BH3 domain-only" proteins. The figure depicts two models for Bax or Bak activation by "BH3 domain-only" proteins (represented as triangles). In the first model, "BH3 domain-only" proteins (e.g. Bid and Bim) bind directly to Bax or Bak, inducing oligomerization of Bax or Bak in the mitochondrial membrane. The oligomerization of Bax or Bak then leads to the release of apoptogenic factors into the cytosol. In the second model, "BH3 domain-only" proteins (eg. Bad) bind to anti-apoptotic Bcl-2 or Bcl-X_L, displacing bound pro-apoptotic proteins. The released pro-apoptotic proteins may be Bax or Bak themselves, or may be other "BH3 domain-only" proteins that subsequently bind to Bax or Bak. The release of pro-apoptotic proteins from Bcl-2 or Bcl-X_L leads to oligomerization of Bax or Bak in the outer mitochondrial membrane, followed by the release of apoptogenic factors into the cytosol.

(Nechushtan et al., 1999; Suzuki et al., 2000). Once positioned at the mitochondria, Bax can bind to anti-apoptotic proteins such as Bcl-2, which serves to neutralize Bax pro-apoptotic activity. Alternatively, if Bcl-2 is in low abundance, or is inactivated by another mechanism, Bax can undergo oligomerization in the OMM, which promotes Bax pro-apoptotic activity (Antonsson et al., 2001; Mikhailov et al., 2001).

In contrast to Bax, Bak normally resides in the OMM, where it may be bound and neutralized by Bcl-2 or other anti-apoptotic proteins (Griffiths et al., 1999; Mahajan et al., 1998). The activation of Bak pro-apoptotic activity in response to death stimuli is characterized by oligomerization in the OMM (Wei et al., 2000). The activation and oligomerization of Bax and/or Bak leads to disruption of mitochondrial integrity and the release of apoptogenic factors (Fig. 3). Based on the structural resemblance between Bcl-2-related proteins and bacterial toxins that form pores in biologic membranes, it was suggested that Bax and Bak promote the release of apoptogenic proteins through pore formation (Petros et al., 2004; Aritomi et al., 1997; Muchmore et al., 1996). Indeed, accumulating evidence suggests that multimers of Bax and Bak form pores that are permeable to cytochrome *c* (Kuwana et al., 2002; Saito et al., 2000; Korsmeyer et al., 2000).

The premature or non-specific activation of Bax and Bak is inhibited by negative regulatory systems. Mitochondrial VDAC2 protein has been shown to associate with, and stabilize Bak as an inactive monomer in the OMM (Cheng et al., 2003), and cytosolic protein 14-3-3 has recently been shown to function as a similar negative regulator of Bax (Nomura et al., 2003). The interactions of Bax and Bak with their respective negative regulators are disrupted in response to overwhelming cell death signals.

3.3. 'BH3 Domain-Only' Pro-Apoptotic Members Of The Bcl-2 Family

'BH3 domain-only' members of the Bcl-2 family are a structurally diverse group, sharing homology only at the BH3 domain. This essential BH3 domain confers variable ability to heterodimerize with Bcl-2 family members that contain a BH3 binding pocket consisting of BH1-3 domains (i.e. Bcl-2, Bcl-x_L, Bax and Bak). Bid was the first member of the 'BH3 domain-only' subgroup to be identified (Wang et al., 1996), and is unique for its ability to link the extrinsic and intrinsic apoptotic pathways (Li et al., 1998; Luo et al., 1998). Several studies have shown that the 'BH3 domain-only' proteins require an intact BH3 domain as well as expression of Bax or Bak to induce cellular apoptosis (Wei et al., 2001; Zong et al., 2001). The 'BH3 domain-only' proteins reside upstream in the intrinsic apoptotic pathway, acting as sensors of cellular damage and transducing signals to the mitochondria that cause activation of multi-domain Bax or Bak and/or inhibition of anti-apoptotic Bcl-2 or Bcl-x_L (Fig. 3), (Oda et al., 2000; Nakano & Vousden, 2001; Yu et al., 2001; Jeffers et al., 2003; Putcha et al., 2001; Puthalakath et al., 1999; Puthalakath et al., 2001).

3.4. Integration of Bcl-2 Family Members in the Regulation of Apoptosis

Current evidence suggests that the conversion of an apoptotic stimulus to an intracellular cell death signal is mediated by 'BH3 domain-only' members of the Bcl-2 family (Luo et al., 1998; Puthalakath et al., 2001; Putcha et al., 2001). 'BH3 domain-

only' proteins are subject to numerous mechanisms of activation including, but not limited to dephosphorylation, caspase-mediated cleavage and disruption of protein/protein interactions. Two basic models have been proposed to account for the pro-apoptotic action of 'BH3 domain-only' proteins (Fig. 3) (Letai et al., 2002). In the first model, 'BH3 domain-only' proteins directly bind and activate multi-domain pro-apoptotic Bax or Bak, leading to Bax or Bak oligomerization and breech of mitochondrial integrity (Eskes et al., 2000; Marani et al., 2002; Liu et al., 2003; Letai et al., 2002). In the second model, there is an indirect initiation of the apoptotic cascade through inhibitory interactions of the 'BH3 domain-only' protein with Bcl-2 or Bcl-x_L. According to this model, interaction of the 'BH3 domain-only' protein with Bcl-2 or Bcl-x_L causes release and activation of sequestered pro-apoptotic Bax or Bak (Cheng et al., 2001; Yang et al., 1995; Letai et al., 2002). Alternatively, binding of the 'BH3 domain-only' protein to Bcl-2 or Bcl-x_L may cause release of a different, bound 'BH3 domain-only' protein, which then goes on to bind and activate Bax or Bak.

Structural variations among 'BH3 domain-only' proteins confer variable binding affinity to different Bcl-2 family members. Fluorescence polarization (FP) assays have uncovered significant differences in the abilities of BH3 peptides derived from various 'BH3 domain-only' proteins to bind to Bcl-2 and Bcl-x_L (Letai et al., 2002). In addition, sequences outside of the BH3 domain may influence binding affinities (Woo et al., 2003). Overall, the relative binding selectivity of 'BH3 domain-only' proteins to multi-domain Bcl-2 family members may influence whether a given 'BH3 domain-only' protein promotes apoptosis by directly interacting with Bax or Bak, or indirectly, by interacting with anti-apoptotic Bcl-2 or Bcl-x_L (Fig. 3). For example, Wei and colleagues found that Bid can directly bind Bak, inducing Bak oligomerization and cytochrome *c* release (Wei et al., 2000). By contrast, Letai and collaborators found that a peptide derived from the BH3 domain of Bad failed to directly activate Bak. Instead the Bad BH3 peptide sensitized cells to apoptosis by displacing pro-apoptotic proteins that were bound to Bcl-2 (Letai et al., 2002).

3.5. Transcriptional Regulation

Certain Bcl-2 family members are subject to finely tuned transcriptional regulation. Early studies identified Bax as a p53-regulated target (Miyashita & Reed, 1995; Thornborrow et al., 2002). More recently, the 'BH3 domain-only' proteins PUMA and Noxa have been shown to be induced by p53 (Oda et al., 2000; Nakano & Vousden, 2001; Yu et al., 2001; Han et al., 2001). Both Noxa and PUMA are critical mediators of cell death induced by DNA damaging agents (Villunger et al., 2003; Jeffers et al., 2003). PUMA is also important for apoptosis induced by p53-independent signals such as cytokine withdrawal, staurosporine, PMA and dexamethasone (Villunger et al., 2003; Jeffers et al., 2003). Due to the presence of p53 mutations in a large number of cancers, and the modulation of PUMA, NOXA and Bax by p53 expression, these Bcl-2 family members may hold prognostic and therapeutic significance in a variety of cancers. Additional studies have shown that the 'BH3 domain-only' protein Bim is transcriptionally regulated in growth factor-deprived neuronal cells via a JNK-dependent pathway (Putcha et al., 2001; Whitfield et al., 2001; Harris & Johnson, 2001), and in cytokine-deprived hematopoietic cells through a pathway dependent on forkhead

transcription factor (Dijkers et al., 2000; Shinjyo et al., 2001). Other genes, including *bcl–x* (Boise et al., 1993), *bfl-1/a1* (Lin et al., 1993; Zong et al., 1999), and *bcl-2* (von Freeden-Jeffry et al., 1997) are subject to transcriptional regulation in certain settings.

3.6. Post-Translational Modifications

Post-translational modifications are also critical in regulating the function of Bcl-2 family proteins. The two best characterized examples involve the 'BH3 domain-only' proteins, Bid and Bad. Bid is activated following engagement of cell surface death receptors by their cognate death ligands (Li et al., 1998; Luo et al., 1998). Death receptor activation results in the activation of caspase-8, which then is able to cleave full-length Bid (FL-Bid) to produce a 15 kDa fragment termed truncated Bid (tBid). Following cleavage, the tBid proteolytic fragment migrates to the mitochondria and activates the intrinsic apoptotic machinery through direct binding to, and activation of, Bax or Bak (Li et al., 1998; Eskes et al., 2000; Wei et al., 2000). N-Myristoylation of the tBid fragment has been found to facilitate translocation to the mitochondria (Zha et al., 2000). Additional evidence suggests that phosphorylation of FL-Bid by cellular kinases can inhibit caspase cleavage and tBid formation (Desagher et al., 1999).

In the case of Bad, survival signals such as IL-3 promote phosphorylation at specific serine residues. This results in binding to, and sequestration by, the ubiquitous cytosolic protein 14-3-3, thereby nullifying any pro-apoptotic activity. Phosphorylation of Bad has been demonstrated by a number of kinases including p90RSK, PKB/AKT, PKA, and the MAPK/ERK kinases (Zha et al., 1996b; Tan et al., 1999; Harada et al., 1999; Scheid et al., 1999). On the contrary, phosphorylation of Bik at T33 and S35 promotes the pro-apoptotic function of this protein (Verma et al., 2001).

Bim and Bmf are 'BH3 domain-only' proteins that are uniquely sequestered at two distinct cytoskeletal structures. The Bim isoforms Bim_{EL} and Bim_{L} are associated with microtubulin-associated dynein motor complex through interaction with an 8-kDa dynein light chain 1 (DLC1 or LC8). In response to UV- and γ-irradition, or treatment with taxol or staurosporine, DLC1-bound Bim dissociates from the dynein motor complex, translocates to mitochondria and binds to Bcl-2 (Puthalakath et al., 1999). The pro-apoptotic activity of Bim_{EL} can be further enhanced following phosphorylation of S65 by mitochondrial c-JNKs (Putcha et al., 2003). Bmf is sequestered by myosin V actin motor complex via interaction with DLC2. Death signals such as detachment from substratum or treatment with actin depolymerizing agents lead to detachment and activation of Bmf (Puthalakath et al., 2001).

Several studies have reported phosphorylation of Bcl-2, with consequent regulation of the anti-apoptotic function of this molecule. In addition, Deverman and colleagues have reported that treatment of cells with DNA damaging agents, including cisplatin and etoposide, resulted in deamidation of Bcl-x_L at asparagine residues 52 and 66, and loss of anti-apoptotic activity (Deverman et al., 2002). Clearly, further efforts are needed to identify the full range of post-translational modifications that impinge on the Bcl-2 protein family, and the impact these modifications have on the regulation of cellular apoptosis.

4. DYSREGULATION OF THE BCL-2 FAMILY AND CANCER

In view of the important role that Bcl-2 family members play in the regulation of apoptosis, it is perhaps not surprising that impairment in their function or expression has profound consequences on cellular development and leads to pathological conditions. Aberrant expression or function of Bcl-2 family members has been frequently observed in a variety of human malignancies. Below we describe what is known about dysregulation of Bcl-2 in human cancers. While much is known in this regard, less is known about the involvement of other Bcl-2 family members, and considerable work lies ahead in this area.

Overexpression of Bcl-2 has been observed in both hematologic and solid tumor malignancies. With respect to hematologic cancers, Bcl-2 is overexpressed in non-Hodgkin's lymphomas (NHL), chronic lymphocytic leukemias (CLL), multiple myeloma, and acute myeloid leukemias (AML). NHL is a malignancy of B or T cells and is comprised primarily of patients with either follicular lymphoma or diffuse large B-cell lymphoma. Over 85% of follicular lymphomas and 20% of diffuse large B-cell lymphomas display a t(14;18)(q32;21) chromosomal translocation (Crisan, 1996; Yunis et al., 1982; Ngan et al., 1988; Levine et al., 1985; Hill et al., 1996). This translocation places the *bcl-2* gene from chromosome 18 under the control of immunoglobulin heavy chain (IgH) promoter on chromosome 14, resulting in overexpression of Bcl-2. Elevated Bcl-2 levels have been reported in follicular lymphomas of diverse histologic grades and are associated with chemoresistance (Ngan et al., 1988; Gaulard et al., 1992; Gribben et al., 1993; Lorsbach et al., 2002). Moreover, the t(14;18)(q32;21) translocation is used as marker of clinical response in B-cell NHL and detection of this translocation correlated with disease relapse (Gribben et al., 1993; Hill et al., 1996).

Chronic lymphocytic leukemia (CLL) involves failed apoptosis and immune dysfunction resulting in the accumulation of $CD5^+$ B-cells. Bcl-2 is overexpressed in a majority of CLL samples, although only a minority of these cases exhibit the t(14;18) translocation (Adachi et al., 1990). Promoter hypomethylation has been cited as a cause of Bcl-2 overexpression in the absence of *bcl-2* gene rearrangement (Hanada et al., 1993). High levels of Bcl-2 and/or high Bcl-2/Bax ratios correlate with disease progression, poor clinical outcome, and chemoresistance in CLL (Hanada et al., 1993; Pepper et al., 1996; Aguilar-Santelises et al., 1996; Marschitz et al., 2000; Consoli et al., 2002; Robertson et al., 1996; Faderl et al., 2002; Stamatoullas et al., 2000; Pepper et al., 1998). Additionally, overexpression of Mcl-1 (Kitada et al., 1998) and a high Mcl-1/Bax ratio (Bannerji et al., 2003) are directly related to chemoresistance and inversely related to complete remission in B-CLL patients.

High levels of Bcl-2 (Campos et al., 1993; Karakas et al., 1998) and high Bcl-2/Bax ratios (Ong et al., 2000) are also frequently found in AML, where they are associated with chemoresistance and failure to achieve complete remission (Ong et al., 2000; Campos et al., 1993; Bincoletto et al., 1999; Karakas et al., 1998; Maung et al., 1994). High Bcl-x_L (Pallis et al., 1997), low Bcl-x_S (Yamaguchi et al., 2002) and/or a high Bcl-x_L/Bcl-x_S ratio (Deng et al., 1998) also correlates with poor prognosis or relapse in AML patients. Mcl-1 has also been implicated in the relapse of AML (Kaufmann et al., 1998).

Multiple myeloma (MM) is a disease in which slowly proliferating malignant plasma cells accumulate in the bone marrow. High expression of Bcl-2 in MM patients is linked

to resistance to interferon therapy (Harada et al., 1998; Sangfelt et al., 1995). Treatment of myeloma cells with dexamethasone and etoposide, traditionally used in MM therapy, was found to upregulate Bcl-2, rendering the MM cells resistant to future treatment (Gazitt et al., 1998; Tu et al., 1996). While Bcl-2 is present during early stages of MM, Bcl-x_L is expressed at later stages of the disease (Harada et al., 1998). The response of MM patients to chemotherapy is much lower in patients that overexpress Bcl-x_L (Tu et al., 1996). Mcl-1 also provides an important survival signal for MM cells (Zhang et al., 2002).

An altered expression profile of Bcl-2 family proteins is also found in non-hematologic malignancies such as malignant melanoma, prostate, breast, lung, and colon cancer, and in some cases is correlated with prognosis. In malignant melanoma, Bcl-2 expression levels have been found to increase with the severity of the disease and overexpression in metastatic melanoma is associated with shorter patient survival (Leiter et al., 2000; Grover & Wilson, 1996). Similarly, Bcl-x_L levels progressively increase during progression of primary malignant melanoma to metastatic disease (Leiter et al., 2000; Tang et al., 1998). Mcl-1 has been found to be overexpressed in primary as well as metastatic melanoma and is associated with chemoresistance (Tang et al., 1998).

Prostate cancer typically progresses from a hormone-dependent stage to a poorer prognosis hormone-independent stage. Treatment options for hormone-refractory prostate cancer include chemotherapy and radiotherapy, with limited success. One of the molecular events occurring during advancement to hormone-refractory status is an increase in Bcl-2 levels (McDonnell et al., 1992), and expression of Bcl-2 is a useful molecular marker of clinical outcome in prostate cancer (Bauer et al., 1996; Kaur et al., 2004; Pollack et al., 2003).

Overexpression of Bcl-2, in combination with reduced Bax, has been associated with lower apoptotic potential in breast carcinomas (Wu et al., 2000). Breast cancer patients that are responsive to chemotherapy showed decreased Bcl-2 in their tumors, while non-responders exhibited stable levels of Bcl-2 (Buchholz et al., 2003). However, other studies have indicated that Bcl-2 expression predicts a favorable prognosis in breast cancer (Hellemans et al., 1995; Yang et al., 2003b). As compared to Bcl-2, which is expressed in lower grade breast cancers, Bcl-x_L is expressed at high levels in invasive breast cancer. Overexpression of Bcl-x_L has been linked to decreased patient survival in this disease (Olopade et al., 1997).

Bcl-2 is also expressed in small-cell lung cancer (SCLC), (Ikegaki et al., 1994; Jiang et al., 1995) and non-small-cell lung cancers (NSCLC), (Groeger et al., 2004). In NSCLC, it remains controversial whether Bcl-2 expression is a positive or negative indicator of survival (Groeger et al., 2004; Gregorc et al., 2003; Martin et al., 2003; Fontanini et al., 1995; Pezzella et al., 1993). In SCLC, Bcl-2 has been reported to be overexpressed in 90% of patient samples, although the significance of this overexpression has not been determined (Jiang et al., 1995). Thus, despite a large number of studies, confusion remains over the significance of Bcl-2 overexpression in lung cancer (Martin et al., 2003).

Bcl-2 has also been implicated in ovarian cancer (Mano et al., 1999; Marx et al., 1997), neuroblastoma (Gallo et al., 2003), bladder transitional cell carcinoma (Pollack et al., 1997) and colorectal cancers (Sinicrope et al., 1995). In head and neck cancers, Bcl-2 and Bcl-x_L are overexpressed, although overexpression of Bcl-x_L is more common (Pena

et al., 1999). Bcl-x_L expression in pancreatic cancer is associated with decreased patient survival and poor prognosis (Friess et al., 1998; Miyamoto et al., 1999).

The studies cited above clearly indicate that anti-apoptotic Bcl-2 family members, particularly Bcl-2, Bcl-x_L, and Mcl-1 are involved in the development and progression of several forms of cancer, as well as the resistance of tumors to conventional chemotherapy and radiotherapy regimens. Additional studies have described cancer-associated mutations that result in functional inactivation of pro-apoptotic Bax (Meijerink et al., 1998; Packham, 1998). Thus, members of the Bcl-2 protein family represent attractive targets for novel anti-cancer agents. Further information on the expression profiles of these targets in various cancers and detailed knowledge regarding their molecular mechanisms of action will continue to facilitate the development of new cancer therapeutics. In the remaining sections, we highlight the progress that has been made in the development and application of novel agents that selectively target members of Bcl-2 protein family.

5. TARGETING THE BCL-2 PROTEIN FAMILY FOR ANTI-CANCER THERAPEUTICS

Anti-apoptotic Bcl-2 family members that are dysregulated in human cancers are being targeted by the design and discovery of novel agents which impair the expression or function of the target protein. A variety of targeting agents are being developed and tested, and can broadly be divided into three major categories: (*i*) antisense molecules, (*ii*) peptides, and (*iii*) small organic molecules. Antisense-based approaches were the first to be developed, but more recently, approaches using peptides and small organic molecules have capitalized on new structural and mechanistic knowledge, and are making considerable progress.

5.1. Antisense

Antisense-based targeting relies on a single strand of oligodeoxynucleotides (ODN) complementary to a specific sequence in the mRNA coding for the target protein. The first report describing antisense to Bcl-2 demonstrated a death-promoting activity of TI-AS, a 20-mer Bcl-2 antisense (5'-CAGCGTGCGCCATCCTTCCC-3') targeting the translation initiation site in Bcl-2 mRNA (Reed et al., 1990). Phosphorothioate modification was found to enhance the death-inducing activity of the TI-AS antisense molecule (Reed et al., 1990). *Ex vivo* treatment of AML blasts with TI-AS resulted in decreased Bcl-2 levels and enhanced sensitivity to chemotherapy drugs (Keith et al., 1995) (Konopleva et al., 2000; Campos et al., 1994). A slightly different Bcl-2 antisense molecule (5'-GTTCTCCCAGCG TGCGCCAT-3'), also targeting the Bcl-2 translation initiation site, was shown to downregulate Bcl-2 in DoHH2 lymphoma cells carrying a t(14;18) translocation (Cotter et al., 1994). Pre-treatment of the lymphoma cells with Bcl-2 antisense prior to inoculation into SCID mice prevented tumor development (Cotter et al., 1994).

Ziegler and coworkers screened a panel of thirteen antisense molecules targeting different regions of the Bcl-2 mRNA and identified ODN 2009 (20-mer; codons 141-147)

to be the most potent at reducing Bcl-2 protein levels (Ziegler et al., 1997). ODN 2009 was found to induce cell death in SW2 lung cancer cells, and enhance chemosensitivity in CLL cell lines and patient samples (Vu et al., 2000; Pepper et al., 1999).

An antisense molecule targeting codons 1-6 of Bcl-2 mRNA, better known as G3139 or Genasense (Genta Inc.; http://www.genta.com) or oblimersen sodium (Aventis Inc.; http://www.aventisoncology.com), has been the Bcl-2 antisense most extensively utilized in human clinical trials of cancer. Reported initially by Kitada colleagues, the G3139 antisense molecule (5'-TCTCCCAGCGTGCGCCAT-3') caused reduction of Bcl-2 levels in a t(14;18)-bearing cell line, and enhanced the sensitivity of lymphoma cells to cytosine-arabinoside (ara-C) and methotrexate (Kitada et al., 1994). In the first clinical trial of Bcl-2 antisense, G3139 was used as single agent in a phase I trial of 9 relapsing non-Hodgkin's lymphoma (NHL) patients that were positive for Bcl-2 (Webb et al., 1997). This study found that 2 of the 5 evaluable patients showed decreased Bcl-2 levels, 1 patient showed a complete response, 3 had stable disease and 5 had progressive disease. No adverse side effects were observed with the exception of an inflammatory response in one patient, a side effect normally associated with the phosphorothioate backbone (Webb et al., 1997). In a different phase I trial, 21 NHL patients who had previously been treated with chemotherapy were given the G3139 antisense. A reduction in Bcl-2 levels was seen in 7 of 16 evaluated patients. Additionally, 1 patient showed complete response, 2 had partial responses, 9 had stable disease, and 9 had progressive disease (Waters et al., 2000).

Aside from use as a single agent, the efficacy of G3139 has also been tested in combination with chemotherapy drugs in a SCID mouse model of lymphoma (Klasa et al., 2000). G3139 was found to be effective in combination with cyclophosphamide at reducing tumor burden (Klasa et al., 2000). G3139 in combination with 2 chemotherapy agents and G-CSF was tested in a phase I clinical study of 17 AML and 3 ALL patients that were either refractory to previous treatment or in relapse (Marcucci et al., 2003). Bcl-2 levels were lowered in 9 of 12 (7 AML and 2 ALL) assessable patients. Additionally, 9 of the 20 patients demonstrated a clinical response; 6 patients (5 AML, 1 ALL) went into complete remission and 3 (2 AML and 1 ALL) into incomplete remission (Marcucci et al., 2003).

G3139 has also proven to be effective in solid tumor malignancies. In a mouse model (Jansen et al., 1998) and in phase I-II human trials (Jansen et al., 2000) of malignant melanoma, G3139 decreased Bcl-2 levels and increased chemosenstivity; 6 of 14 patients showed minor to complete response. In a mouse xenograft model of human breast cancer, G3139 in combination with doxorubicin suppressed the growth of established breast tumors (Lopes de Menezes et al., 2000). In xenograft models of multi-drug resistant multidrug resistant (P-glycoprotein$^+$) breast carcinoma, G3139 in combination with PSC833 (P-gp$^+$ inhibitor) and doxorubicin effectively reduced the tumor growth (Lopes de Menezes et al., 2003).

Treatment of the Shionogi mouse model of androgen-dependent prostate cancer, or treatment of mice harboring androgen-dependent human prostate cancer, with G3139 as a single agent or in combination with paclitaxel caused regression of established tumors and delayed the appearance of hormone-independent tumors (Miayake et al., 2000; Miyake et al., 1999; Gleave et al., 1999; Leung et al., 2001). In human trials of hormone-refractory prostate cancer, G3139 has been used either as a single agent (Morris et al.,

2002) or in combination with mitoxantrone (Chi et al., 2001) or docetaxel (Tolcher, 2001). Additionally, a pilot trial of G3139 and paclitaxel was also conducted in chemorefractory SCLC patients (Rudin et al., 2002). In all of these trials, G3139 was generally well-tolerated with minor side effects that included fatigue, anorexia, and temporary hematological toxicities. Additionally, G3139, in concert with antisense against protein kinase A, showed anti-tumor activity in a mouse model of human colon cancer (Tortora et al., 2001), and in combination with cisplatin enhanced the survival of SCID mice bearing human gastric cancer (Wacheck et al., 2001).

Due to the presence of two potential CpG motifs in G3139, it has been argued that the anti-tumor activity of this molecule may be due to immune stimulation. However, generation of an immune-silent version of G3139 (G4232) by methylating the cytosine residue in the CpG motifs failed to diminish the anti-tumor activity in a SCID mouse model of human melanoma (Wacheck et al., 2002). Currently, peptide nucleic acid derivatives of G3139 are being designed to improve affinity, specificity, and biological activity (Gallazzi et al., 2003).

To date, antisense directed against Bcl-x_L has been applied in only limited fashion. Bcl-x_L antisense (ISIS 15999) sensitized CEM cells, an ALL cell line, to chemotherapy (Broome et al., 2002), and Bcl-x_L antisense treatment of BCR-ABL-overexpressing HL60 cells restored the sensitivity to staurosporine (Amarante-Mendes et al., 1998). Treatment of MCF7 breast cancer cells with antisense to Bcl-x_L downregulated Bcl-x_L and induced apoptosis (Simoes-Wust et al., 2000). A 20-mer antisense (ODN 4259) lowered the levels of Bcl-x_L in both SCLC and NSCLC, but was able to induce apoptosis only in NSCLC (Leech et al., 2000). Antisense to Bcl-x_L also sensitized prostate cancer cell lines to chemotherapy drugs (Lebedeva et al., 2000) and the PI3 kinase inhibitor LY294002 (Yang et al., 2003a). Additionally, Bcl-x_L antisense sensitized colorectal cancer cells to irinotecan (Hayward et al., 2003), gliobalstoma cells to paclitaxel (Guensberg et al., 2002), and squamous cell carcinoma cells to carboplatin (Itoh et al., 2002).

Some tumors simultaneously express Bcl-2 and Bcl-x_L, and forced downregulation of one molecule can lead to upregulation of the other. Thus, simultaneous downregulation of both molecules, using a cocktail of two different antisense molecules or a single bispecific antisense molecule, may be advantageous. Miyake and collaborators used a cocktail of Bcl-2 and Bcl-x_L antisense molecules along with taxol in the Shionogi mouse tumor model to delay the emergence of androgen-independent prostate cancer (Miyake et al., 2000). Treatment of lung cancer cell lines with a bispecific antisense molecule (ODN 4625) caused downregulation of Bcl-2 and Bcl-x_L, and induced apoptosis (Zangemeister-Wittke et al., 2000). This bispecific antisense (ODN 4625) also reduced Bcl-2 and Bcl-x_L expression levels *in vivo* in xenograft models of breast and colon cancer, and slowed the growth of tumors (Gautschi et al., 2001). Additionally, this antisense sensitized breast cancer cell lines to chemotherapy (Simoes-Wust et al., 2002), induced apoptosis in human melanoma cell lines and patient samples (Olie et al., 2002; Strasberg Rieber et al., 2001), and inhibited tumor angiogenesis (Del Bufalo et al., 2003).

In a markedly different approach, antisense has been used to alter pre-mRNA splicing. In the case of *bcl-x* gene expression, splicing determines whether an anti-apoptotic (Bcl-x_L) or a pro-apoptotic (Bcl-x_S) gene product is generated (Fig. 2). Taylor and coworkers designed a 20-mer antisense molecule (ISIS 22783) that targets and inhibits a key splicing site in *Bcl-x* pre-mRNA (Taylor et al., 1999). The ISIS 22783

molecule allows the splicing to occur in favor of pro-apoptotic Bcl-x_S. Treatment of lung cancer cells with ISIS 22783 resulted in decreased Bcl-x_L and a proportional increase of Bcl-x_S expression, and sensitized the cells to chemotherapy and UV radiation (Taylor et al., 1999). Mercante and collaborators employed a similar 18-nucleotide molecule (5'Bcl–x AS) that also shifted the expression pattern in favor of pro-apoptotic Bcl-x_S, and found that this antisense promoted cell death and chemosensitivity in prostate and breast cancer cell lines (Mercatante et al., 2001; Mercatante et al., 2002).

Recently, downregulation of gene expression using small interfering RNA (siRNA) technology has become popular. Wacheck and colleagues showed that Bcl-2 siRNA sensitized melanoma cells to chemotherapy (Wacheck et al., 2003). Jiang and Milner showed that Bcl-x_L siRNA induced apoptosis in a colorectal cancer line in p53-independent manner (Jiang & Milner, 2003). Future studies will undoubtedly focus on siRNA-based strategies as a means of targeting Bcl-2 family members in human cancers.

5.2. BH3 Peptides

Detailed knowledge regarding the mechanisms of action of Bcl-2 family members has facilitated the design of peptide-based agents that inhibit the function of Bcl-2 and Bcl-x_L. As discussed earlier, the function of Bcl-2 family members is regulated by homo- and hetero-dimerization interactions. Heterodimerization between anti- and pro-apoptotic proteins serves to neutralize the activities of the interacting molecules, and a high ratio of anti-apoptotic to pro-apoptotic proteins leads to cell survival even in the presence of death-inducing stimuli (Oltvai et al., 1993). Thus, an idea emerged that agents capable of disrupting heterodimerization interactions would liberate pro-apoptotic proteins and promote apoptosis. Efforts to rationally devise such agents have relied on our current understanding of the structural domains that are important for heterodimerization. Structure/function analyses have determined that heterodimerization of Bcl-2 with Bax or Bak requires all four BH domains in Bcl-2 (Hanada et al., 1995; Hunter et al., 1996; Hirotani et al., 1999; Yin et al., 1994), while Bax or Bak only need to contribute a functional BH3 domain (Zha et al., 1996a; Chittenden et al., 1995a). Also, the BH3 domains of Bax and Bak were found to be sufficient for the pro-apoptotic functions of these proteins (Simonen et al., 1997; Chittenden et al., 1995a). Moreover, replacement of the BH3 domain in Bcl-2 with a Bax BH3 domain converted Bcl-2 into an inducer of cell death (Hunter & Parslow, 1996). In "BH3 domain-only" molecules the BH3 domain is sufficient for binding to Bcl-2 and Bcl-x_L, and for pro-apoptotic activity, further stressing the importance of this region (O'Connor et al., 1998; Puthalakath et al., 2001; Yu et al., 2001; Zha et al., 1997). Structural studies of Bcl-x_L (Aritomi et al., 1997; Muchmore et al., 1996), and later Bcl-2 (Petros et al., 2001), revealed that folding of the BH1-3 domains in an anti-apoptotic protein creates a hydrophobic pocket that serves as the site where the BH3 domain of pro-apoptotic proteins dock. Thus, these studies suggested that short peptides based on BH3 domains in pro-apoptotic proteins might be capable of disrupting heterodimerization interactions and promoting apoptosis.

The abilities of BH3 peptides to disrupt heterodimerization interactions have been tested in a number of different systems. In *in vitro* heterodimerization assays, Diaz *et al.* (Diaz et al., 1997) showed that a 21-amino acid peptide representing the Bax BH3 domain was capable of disrupting Bax/Bcl-2 and Bax/Bcl-x_L interactions, as well as

homodimerization of Bcl-2 or Bcl-x_L. A 16-amino acid peptide derived from the Bak BH3 domain (a.a. 72-87) also inhibited each of these interactions (Diaz et al., 1997). Ottilie *et al.* (Ottilie et al., 1997) found that Bad BH3 peptide (21-mer; a.a. 103-123) was more effective than Bak BH3 (16-mer; a.a. 72-87) at disrupting Bax/Bcl-x_L heterodimerization interactions. Interestingly, Bad BH3 peptide (21-mer; a.a. 103-123) selectively inhibits Bax/Bcl-x_L heterodimerization without affecting Bax/Bcl-2 interactions, while Bax BH3 peptide (20-mer; a.a. 55-74) can inhibit both types of interactions (Shangary & Johnson, 2002).

Additional studies, using a variety of model systems, have demonstrated the abilities of BH3 peptides to activate apoptosis. In early studies, 16-mer BH3 peptides derived from Bax, Bak, and Bid were found to induce caspase activation in a cell-free system of *Xenopus* egg extracts (Cosulich et al., 1997). Subsequently, the release of cytochrome *c* from purified mitochondria has been used as a measure of apoptosis induction. Bax BH3 peptides have been found to induce cytochrome *c* release from mitochondria purified from sources such as rat liver (Narita et al., 1998), Jurkat cells (Shangary & Johnson, 2002), and neuronal cells (Polster et al., 2001), while Bid and Bim BH3 peptides induced cytochrome *c* release from mitochondria obtained from FL5.12 cells (Letai et al., 2002).

The use of BH3 peptides in whole cell studies, with an eye towards potential clinical application, has required the development of methods to facilitate cellular permeability. Various approaches have been used, including: (*i*) fusion of the BH3 peptide to short peptide transduction domains (PTDs) derived from proteins such as *Drosophila* Antennapedia protein Ant, (Holinger et al., 1999; Vieira et al., 2002) or fusion to polyarginine-containing peptides (Letai et al., 2002), (*ii*) conjugation of the BH3 peptide to fatty acids (Wang et al., 2000b), (*iii*) cationic lipid-mediated transduction (Shangary & Johnson, 2002), and (*iv*) electroporation (Finnegan et al., 2001). Holinger coworkers provided the first report of cell death-inducing activity of a cell-permeable BH3 peptide in experiments using Ant-Bak BH3 peptide (Holinger et al., 1999). Ant-Bak BH3 promoted caspase-dependent cell death in Bcl-x_L-overexpressing HeLa cells, and, when used at sublethal doses, enhanced sensitivity to Fas ligand (Holinger et al., 1999). Similarly, a 27-amino acid Bad BH3 peptide fused to decanoic acid induced cell death in Bcl-x_L-overexpressing HL60 cells, and reduced the growth of human myeloid leukemic cells in a SCID mouse model (Wang et al., 2000b). Strikingly, this peptide had insignificant effects on the viability of normal peripheral blood lymphocytes. Bax BH3 and Bak BH3 peptides have been found to induce cell death in the PC-3 hormone-refractory prostate cancer cell line, and in Jurkat cells that overexpress Bcl-2 or Bcl-x_L (Finnegan et al., 2001; Shangary & Johnson, 2002). Apoptosis induced by the Bax BH3 or Bak BH3 peptides was accompanied by disruption of intracellular Bax/Bcl-2 and Bak/Bcl-2 heterodimerization (Finnegan et al., 2001; Shangary, unpublished).

Although the precise mechanism of action of BH3 peptides remains to be determined, recent studies have provided some significant insights. Based on experimental evidence, Letai and colleagues have proposed that BH3 peptides derived from "BH3 domain-only molecules" can be divided into two distinct categories (Fig. 3) (Letai et al., 2002). The first category includes BH3 peptides (e.g. Bid BH3 and Bim BH3) that bind directly to Bax and Bak, inducing oligomerization and cytochrome *c* release. The second category includes BH3 peptides such as Bad BH3 that do not bind to Bax or Bak, but instead bind to Bcl-2 or Bcl-x_L and promote cellular sensitivity to Bid

and Bim. This second category of peptides may act by promoting the release of a bound pro-apoptotic factor from Bcl-2 or Bcl-x_L (Letai et al., 2002). Also, Moreau *et al.* (Moreau et al., 2003) have shown that Bax BH3 peptide does not bind to Bax, but likely acts by binding to Bcl-x_L, causing release of pro-apoptotic molecules. However, others have shown that Bax BH3 peptide binds to the adenine nucleotide translocator (ANT) present on the mitochondrial surface, and that binding to ANT can render ANT-containing liposomes permeable to small organic molecules (Vieira et al., 2002).

Lastly, although BH3 peptides derived from pro-apoptotic proteins can be potent inducers of cell death, their usefulness in the clinic is likely to be hindered by susceptibility to proteolytic degradation, poor permeability, and poor solubility. In particular, the peptide backbone consisting of L-amino acids is highly susceptible to cellular proteases. In this regard, it should be possible to enhance stability by employing D-amino acids or by cyclization of the peptide. Permeability can be significantly improved by fusion of the peptide to a PTD, but this also serves to increase the overall length of the peptide backbone. Moreover, different PTDs likely display cell-type specificity, limiting their use in certain cell types. Thus efforts to maximize delivery of BH3 peptides will benefit greatly from continued discovery and characterization of PTDs. In addition, continued structure/function studies of BH3 peptides and the BH3 binding pocket should help to define size-optimized BH3 peptides. Once size-optimized peptides have been determined, it may be possible to generate peptido-mimetic molecules with similar biologic activities. Such mimetics are likely to exhibit increased solubility, stability, and cell permeability relative to the parental peptide molecules.

5.3. Small-Molecule Inhibitors

The elucidation of the tertiary structure of Bcl-x_L through nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography, coupled with the screening of chemical libraries, has facilitated the identification and rational design of small-molecule inhibitors of Bcl-2 and Bcl-x_L (Muchmore et al., 1996) (Aritomi et al., 1997). Most of the currently available small-molecule inhibitors of Bcl-2 or Bcl-x_L have been identified using: a) cellular activity assays, b) fluorescence polarization (FP)-based or NMR-based binding assays, or c) computer-assisted molecular modeling. Additionally, certain small molecules such as polyphenols from green and black tea were identified as Bcl-2 or Bcl–x_L inhibitors on the basis of their known anticancer activities and their resemblance to previously identified polyphenols that bind to Bcl-2 or Bcl-x_L.

5.3.1. Inhibitors Identified By Cell-based Activity Assays

Tetrocarcin A (TC-A) was the first small-molecule inhibitor of Bcl-2 to be reported, and was identified by screening a library of natural products for compounds that induce apoptosis in Bcl-2-overexpressing cells (Nakashima et al., 2000). TC-A was originally characterized as an antibiotic (isolated from *Micromonospora chalcea*), but was also known to exhibit antitumor activity in mouse models of melanoma, hepatoma, and lung carcinoma (Tomita et al., 1980; Morimoto et al., 1982). Nakashima and coworkers have shown that TC-A sensitizes Bcl-2- and Bcl-x_L-overexpressing HeLa cells to chemotherapy- and death-ligand-induced cell death (Nakashima et al., 2000). Although,

TC-A possesses antimicrobial properties, TC-A derivatives that lack this property still retain Bcl-2 antagonistic activity (Kaneko et al., 2001). In T-ALL cell lines, TC-A-induced apoptosis is accompanied by upregulation of HSP-70 and activation of caspase-12, both of which are associated with ER stress signaling (Tinhofer et al., 2002). TC-A-induced apoptosis also involves Bid cleavage, Bax activation, and loss of mitochondrial membrane potential (Tinhofer et al., 2002). However, a direct interaction of TC-A with Bcl-2 or Bcl-x_L has not been demonstrated, and the precise mechanism of action of this compound remains unknown.

Antimycin A_3 was discovered by screening various inhibitors of mitochondrial respiration for molecules capable of triggering cell death in hepatocyte cell lines overexpressing Bcl-x_L (Tzung et al., 2001). Previously known as an inhibitor of the cytochrome bc1 complex ($CoQH_2$-cytochrome *c* reductase) and electron transfer, antimycin A_3 was found to induce a higher degree of cell death in Bcl-x_L-overexpressing murine hepatocytes than in cells expressing low levels of Bcl-x_L (Miyoshi et al., 1991; Tzung et al., 2001). By contrast, chemotherapy drugs were ineffective at killing the Bcl-x_L-overexpressing hepatocytes (Ziegler et al., 1997). Hypersensitivity to antimycin A has also been observed in Bcl-2-transfected L929 fibrosarcoid cells (Hennet et al., 1993). However, antimycin A failed to provoke potent killing in Bcl-x_L-overexpressing FL5.12 pro-B lymphoid cells (Vander Heiden et al., 1997). The death-inducing effects of antimycin A_3 in murine hepatocytes are accompanied by mitochondrial swelling and membrane depolarization, and in purified liposomes, antimycin A_3 inhibits pore-formation by Bcl-x_L (Tzung et al., 2001). In FP assays, antimycin A_3 was shown to compete with Bak BH3 peptide for binding to Bcl-2. Molecular modeling has verified that antimycin A_3 binds to the hydrophobic BH3 binding pocket of Bcl-2 and Bcl-x_L (Tzung et al., 2001; Kim et al., 2001).

5.3.2. Inhibitors Identified By Fluorescence Polarization-Binding Assays

A series of small molecules called BH3 inhibitors (BH3Is) were identified by screening a chemical library consisting of 16,320 compounds (Degterev et al., 2001). The library was screened using a high throughput FP-based assay to detect compounds capable of binding to recombinant Bcl-x_L. Seven distinct compounds were isolated, representing two different structural classes of molecules. NMR analyses revealed that, like BH3 peptides, BH3I compounds target the hydrophobic pocket of Bcl-x_L. Moreover, BH3Is were found capable of disrupting key intracellular heterodimerizations such as Bad/Bcl-x_L, Bax/Bcl-x_L, and Bad/Bcl-2 (Degterev et al., 2001). Apoptosis induced by these compounds is caspase-dependent, accompanied by cytochrome *c* release, and inhibited by overexpression of Bcl-x_L. The efficiency with which different BH3Is induce apoptosis has been shown to correlate with their affinity towards Bcl-x_L, supporting the contention that Bcl-x_L is the true intracellular target (Degterev et al., 2001).

Gossypol is a polyphenolic natural product derived from cotton seeds. Gossypol was previously known to possess antitumor activities *in vitro* and *in vivo*, and has shown limited activity in clinical trials of breast and adrenal cancers, glioblastoma and anaplastic astrocytoma (Flack et al., 1993; Van Poznak et al., 2001; Bushunow et al., 1999). However, the molecular target(s) of gossypol were unknown. Recently, gossypol and another plant product, purpurogallin (present in *Quercus nutgall*), were selected from

a library consisting of 50 natural products in FP-based Bcl-x_L binding assays (Kitada et al., 2003). Gossypol was found to exhibit 5-fold stronger binding to Bcl-x_L than purpurogallin. NMR spectroscopy and molecular modeling confirmed the binding of these compounds to the BH3 binding pocket of Bcl-x_L. Interestingly, the (-) stereomer of gossypol exhibits a higher affinity towards Bcl-x_L than the (+) stereomer, consistent with a reported tenfold higher cytotoxic activity of the (-) stereomer (Kitada et al., 2003; Qiu et al., 2002). Gossypol, but not purpurogallin, has been shown to induce cell death in breast cancer cell lines that coexpress high levels of Bcl-2 and Bcl-x_L. The poor cell permeability of purpurogallin has been suggested as a reason for a lack of activity, since purpurogallin derivatives predicted to have better permeability, acquire cell-killing activity (Kitada et al., 2003).

5.3.3. Inhibitors Identified by Structure-Based Virtual Screening

HA14-1 was the first small molecule Bcl-2 inhibitor discovered on the basis of the predicted 3-D structure of Bcl-2 (Wang et al., 2000a). HA14-1 was identified by computer-assisted virtual screening of a library of 193,833 compounds. The initial screen yielded 1000 compounds capable of binding to the BH3 binding pocket of Bcl-2, of which 28 compounds were chosen for subsequent testing in cellular assays. HA14-1 was ultimately selected based on its ability to potently induce cell death. Further studies have shown that apoptosis induced by HA14-1 is caspase-dependent, and is also dependent on expression of Bax and Bak (Wang et al., 2000a; Chen et al., 2002; An et al., 2004). Other studies have determined that HA14-1-induced apoptosis is characterized by generation of reactive oxygen species and redistribution of Ca^{2+} from the ER to the mitochondria, followed by release of cytochrome *c* into the cytosol (An et al., 2004).

Recent findings demonstrate that HA14-1 can synergize with inhibitors of the MEK/MAPK pathway and inhibitors of the peripheral benzodiazepine receptor (PBR) to promote apoptosis. Constitutive activation of MEK/MAPK and overexpression of PBR has been reported in a number of different cancers (Hardwick et al., 1999; Allen et al., 2003). PBR resides on the mitochondrial membrane and is part of the permeability transition pore complex (Vander Heiden & Thompson, 1999). Combination treatment of HA14-1 with the MEK inhibitor PD184352 or the PBR inhibitor PK11195 led to synergistic induction of apoptosis in HL60 cells (Milella et al., 2002; Chen et al., 2002). Additionally, sublethal doses of HA14-1 sensitized primary leukemic blasts to Ara-C (Lickliter et al., 2003). Co-treatment of multiple myeloma cell lines with the proteasome inhibitor (bortezomib) and HA14-1 also led to synergistic induction of cell death, accompanied by an upregulation of p53 and Bax, activation of caspases and JNK, and release of apoptogenic proteins from the mitochondria (Pei et al., 2003).

BH3I-1SCH3, an analogue of BH3I-1, was identified by computational methods as a putative Bcl-x_L-binding molecule (Lugovskoy et al., 2002). Lugovskoy and coworkers selected compounds from two online chemical libraries on the basis of structural similarity to BH3Is, and used a virtual screening approach based on shape-complementarity to identify BH3I-1SCH3 (Lugovskoy et al., 2002). However, information on the biological activity of this small molecule is not yet available.

5.3.4. Inhibitors Identified Using Combined Approaches

Enyedy and colleagues used a combination of structure-based computer modeling, FP assays, NMR chemical shift assays, and cell-killing assays to screen an NCI 3-D database containing 206 876 small organic molecules to identify inhibitors of Bcl-2 and/or Bcl-x_L (Enyedy et al., 2001). In an initial computer-assisted screen based on the 3-D structure of Bcl-2, 35 compounds were identified. The compounds were subsequently tested in *in vitro* FP-based Bcl-2 binding assays and 7 were found to disrupt Bak BH3 peptide/Bcl-2 interactions with IC_{50} values of less than 15μM. Screening of the 7 molecules for killing activity in cancer cell lines led to the identification of compound 6, a small molecule capable of killing breast cancer and leukemic cell lines at low doses. Compound 6 was also found to bind to Bcl-x_L in FP assays and binding to the Bcl-x_L hydrophobic pocket was revealed by NMR chemical shift studies (Enyedy et al., 2001).

5.3.5. Tea Polyphenols as Bcl-2/Bcl-xL Small-Molecule Inhibitors

Previous studies have reported the anticancer properties of green and black tea, but the compounds responsible for this activity, as well as the molecular targets, were not known (Jankun et al., 1997). Recently, NMR chemical shift- and FP-based assays, along with computer modeling studies have revealed the binding of certain tea polyphenols to Bcl-2 and Bcl-x_L (Leone et al., 2003). Of the 8 green tea polyphenols tested, 3 were found to bind to Bcl-2 and Bcl-x_L, and of the 4 black tea polyphenols tested, 3 bound to both proteins (Leone et al., 2003). Thus, certain tea polyphenols and other naturally-occurring polyphenols such as gossypol are now becoming known as binders and/or inhibitors of Bcl-2 and Bcl-x_L

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SURVIVIN, OTHER IAPS, SMAC/DIABLO, AND OMI/HTRA2 – MODULATION OF THE ADVANCING APOPTOTIC PROCESS

Chapter VI

Fengzhi Li*

1. INTRODUCTION

Many diseases including cancer, immune diseases (such as autoimmunity and immunodeficiency), cardiovascular diseases (such as restenosis, ischaemia and heart failure), and neurodegenerative disorders (such as Alzheimer's, Huntington's and Parkinson's Diseases) are attributed to a deficiency or an excessiveness of the organized cell suicide process or programmed cell death called "apoptosis". Mechanistically, the cause of these diseases is at least in part due to the deregulation of one or more members of the inhibitor of apoptosis (IAP) protein family. IAP family proteins are characterized by an evolutionarily conserved motif termed the baculovirus IAP repeat or BIR in short (~70 amino acids), originally found in baculoviral genomes in 1993 (Miller, 1999). Eight human IAP proteins have been described so far. They are c-IAP1, c-IAP2 (Rothe et al., 1995), XIAP (Duckett et al., 1996), NAIP (Liston et al., 1996; Roy et al., 1995), survivin (Ambrosini et al., 1997), apollon (Chen et al., 1999), ML-IAP/livin (Kasof & Gomes, 2001; Vucic et al., 2000) and ILP-2 (Richter et al., 2001). The corresponding orthologues of these human IAP proteins are found in mouse as well, with the exception of ILP-2 (Richter et al., 2001). It has been shown that mice with the deletion of *XIAP* (Harlin et al., 2001) or *NAIP* (Holcik et al., 2000) are developmentally normal. In contrast, mice with deletion of survivin are lethal during embryo development (Uren et al., 2000). The strikingly different roles of IAP proteins during development suggest that the role of survivin is functionally indispensable in development while some other IAP proteins may be dispensable during development or can be functionally compensated by a yet to be defined mechanism. Nevertheless, cell culture studies indicate that c-IAP1, c-IAP2,

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XIAP, NAIP and survivin all play critical roles in apoptosis inhibition (Deveraux & Reed, 1999; Li, 2003; Salvesen & Duckett, 2002). Moreover, the expression of IAP proteins can be regulated both transcriptionally and post-transcriptionally, and their protein function can be also modulated by the IAP regulatory proteins – Smac (Du et al., 2000) (DIABLO in mouse (Verhagen et al., 2000)) and Omi/HtrA2 (Suzuki et al., 2001). Therefore, the IAP proteins are considered as versatile apoptosis regulators. Manipulation of their expression or function is potentially able to control cell viability for treatment of apoptosis-associated human diseases. Thus, IAP proteins are currently considered important targets for the treatment of several human diseases, including cancer.

2. SURVIVIN AS A THERAPEUTIC TARGET

2.1. Survivin and its Significance in Cancer and Other Diseases

Survivin is a unique member in the IAP protein family since several features of survivin do not share with other members in this family. Survivin is the smallest member in this family containing 142 amino acids [140 amino acids in mouse (Li & Altieri, 1999)] with only one BIR domain (Ambrosini et al., 1997). Survivin has a long alpha-helical tail at the carboxyl terminal instead of a RING finger motif, and it homodimerizes in solution (Chantalat et al., 2000; Muchmore et al., 2000; Verdecia et al., 2000). Presumably, disruption of the homodimmerization of survivin may be a potential approach for abrogation of survivin function. Unlike other IAP proteins (Deveraux & Reed, 1999; Salvesen & Duckett, 2002), expression of survivin is undetectable in most normal adult tissues but is highly expressed in cancer. Many studies based on RT-PCR and immunohistochemical approaches revealed that survivin expression in cancer is associated with carcinogenesis, cancer progression, drug resistance and shorter patient survival [see reviews, (Altieri, 2003b; Li, 2003)], suggesting survivin is a novel cancer prognostic indicator. Moreover, a number of studies using DNA array approaches also obtained supportive data for this notion from human breast cancer (van't Veer et al., 2002), colorectal cancer (van de Wetering et al., 2002; Williams et al., 2003) and diffuse large B cell lymphoma (Kuttler et al., 2002). Expression of survivin is cell cycle-regulated with a robust increase in G2/M phase (Li & Altieri, 1999; Li et al., 1998). However, a number of studies indicate that survivin also plays a role outside of mitosis, and upregulation of survivin can be induced in interphase cells by various ligands (Fukuda & Pelus, 2002; Fukuda & Pelus, 2001; Ling et al., 2004; Suzuki et al., 2000), suggesting that survivin may play a role in all phases of the cell cycle. Survivin protein binds to the mitotic spindle (Li et al., 1998), centromere/kinetochore (Uren et al., 2000) or both (Fortugno et al., 2002) during mitosis. Consistent with its multiple foci of localization, survivin was shown to be involved in both inhibition of apoptosis and regulation of cell division (Li et al., 1999; Li et al., 1998), which have been validated by many studies (see reviews: Altieri, 2003b; Li, 2003), while the known functions of other IAP members are restricted to one of these two functions (Deveraux & Reed, 1999; Salvesen & Duckett, 2002). Together, these unique features make survivin an exciting target for cancer therapy. On the other hand, studies of survivin with endothelial and smooth muscle cells suggest that survivin also plays an important role in these cell

proliferation and/or migration up on the stimulation with various ligands. Therefore, manipulation of survivin expression or function may also benefit patients with cardiovascular diseases (see review Li, 2003).

2.2. Cancer Treatment Using Survivin as a Target

Plasmid-mediated expression of survivin in survivin-negative NIH3T3 cells suppresses taxol-induced cell death (Li et al., 1998). On the other hand, overexpression of survivin antisense RNA or a dominant-negative survivin mutant induces cell death and a cell division defect in survivin-positive HeLa cervical carcinoma cells (Li et al., 1999). These initial observations triggered enthusiasm to explore the possibility of cancer therapy using survivin as a target. Such studies could be classified into four different categories: 1) survivin immunotherapy, 2) inhibition of survivin expression, 3) suppression of survivin function and 4) using the survivin promoter for cancer gene-therapy.

2.2.1. Survivin Immunotherapy

Studies in this category have demonstrated the following concept [see recent review for detail, (Li, 2003)}. Monocyte-derived dendritic cells (DCs) pulsed with survivin protein can stimulate naïve T cells to mature into cytotoxic T lymphocytes (CTL), and these CTL cells can lyse survivin-positive but not survivin-negative cells *in vitro* (Schmitz et al., 2000). Tumors of different tissue origins are able to process and present the same set of survivin-derived peptide epitopes on the cancer cell surface via the class I MHC complex. CD8+ T cells and DCs can move into the primary solid tumor site *in vivo*, where the cancer cells in the primary tumor site may act as survivin-antigen presenting cells to mature CD8+ T cells into CTL cells (Andersen et al., 2001a). This notion is based on the observation that small clusters of CTL cells were detected in the primary tumor site. Metastatic tumor cells can also act as survivin antigen presenting cells to mature CD8+ T cells in the immune system (lymph node) into CTL, and CTL cells can be released into the circulation system and then travel to the primary tumor site (Andersen et al., 2001a; Andersen et al., 2001b). Recently, more survivin-derived CTL Epitopes have been identified (Reker et al., 2004), which would significantly increase the number of patients eligible for immunotherapy based on survivin derived peptides and reduce the risk of immune escape by HLA-allele loss. Interestingly, although survivin is expressed in CD34(+) hematopoietic progenitor cells (HPCs) (Carter et al., 2001; Fukuda & Pelus, 2001), survivin-specific CTLs with CD34(+) cells did not significantly decrease the colony-forming ability of HPCs (Pisarev et al., 2003). This observation further validates the feasibility for immunotherapy using survivin peptide epitopes as targets. Future studies focusing on identification and validation of the most effective endogenous proteasome-processed survivin peptides and/or modified ones will be also important for survivin antigen-specific cancer immunotherapy.

2.2.2. Inhibition of Survivin Expression

A number of approaches have been utilized to suppress survivin expression. These include the use of survivin antisense oligonucleotides (ASO) or survivin antisense expression vectors, RNA interference (RNAi), ribozymes, triplex DNA formation and anticancer agents.

Utilization of survivin antisense approaches for cancer treatment is being actively explored. Inhibition of survivin expression by survivin ASO (Ansell et al., 2004; Chen et al., 2000; Jiang et al., 2001; Li et al., 1999; Olie et al., 2000; Xia et al., 2002; Younis et al., 2004) or by survivin antisense expression vectors (Ambrosini et al., 1998; Grossman et al., 1999a; Grossman et al., 1999b; Kanwar et al., 2001; Tu et al., 2003; Yamamoto et al., 2002) in various cancer cells has been shown to induce tumor cell growth inhibition, cell death and/or to sensitize cells to death induced by chemotherapeutic drugs (Olie et al., 2000; Yamamoto et al., 2002) or immunotherapy (Kanwar et al., 2001). However, the mechanism of these effects has not yet been well defined. Additionally, recent studies indicated that inhibition of survivin expression in tumor cells suppresses tumor growth in a mouse xenograft model (Ansell et al., 2004; Tu et al., 2003). However, the results using a mouse lung metastatic model (Younis et al., 2004) suggest that the methods used for survivin ASO formulation and administration in animal models significantly affect the outcome. Much more work will be required before survivin ASO could be a feasible approach for cancer treatment.

RNAi-mediated silencing of mammalian gene expression is a recently recognized approach, which can be conducted either using in vitro synthesized 21-25 nucleotide (nt) double-stranded RNAs with 2-nt 3' overhangs (small interfering RNA, siRNA) or the vector-based expression of the 21-29 nt short hairpin double-stranded RNA (shRNA). The RNAi approach is believed to be more effective in gene silencing than the ASO approach. Recent studies showed that survivin expression could be silenced by both approaches (Ling & Li, 2004). RNAi-mediated survivin depletion induced cell growth inhibition, apoptosis (Ling & Li, 2004) and a cell division defect (Beltrami et al., 2004; Kappler et al., 2004). Although RNAi technology is a useful tool for the study of gene function and gene therapy, the much higher cost for siRNA synthesis than ASO may restrict its application in routine cancer treatment.

Similarly, targeting survivin by ribozymes (Choi et al., 2003; Pennati et al., 2003; Pennati et al., 2004; Pennati et al., 2002) and triplex DNA formation (Shen et al., 2003) induced cell growth inhibition and apoptosis, and also increased therapeutic drug sensitivity. These approaches are definitely alternative approaches for study of gene function but it is unlikely to apply these approaches to routine cancer treatment.

Another attractive approach to inhibit survivin expression is the application of survivin transcription inhibitors. Survivin transcription-specific inhibitors are currently not available. However, it was shown that doxorubicin indirectly inhibits survivin transcription by enhancing p53 protein (Hoffman et al., 2002; Mirza et al., 2002; Zhou et al., 2002). Our recent studies indicate that Hoechst33342, a drug that binds A/T rich regions within the DNA minor groove, upregulates survivin expression in HeLa and HCT116 cells while Hedamycin, a drug that binds G/C rich regions within the major groove, downregulates its expression (Wu et al., 2004). This finding suggested the concept that it may be possible to find certain chemical molecules that specifically inhibit

survivin transcription; this concept is worthy of a further exploration. In addition, it was recently shown that the ATPase domain of HSP-90 interacted with survivin BIR domain to form HSP-90-survivin complexes, and that disruption of this complex destabilized survivin protein (Fortugno et al., 2003). This finding may provide an alternative approach for modulating survivin expression via post-transcriptional control, such as dissociating HSP-90-survivin complexes.

2.2.3. Suppression of Survivin Function

This includes the approaches using survivin dominant-negative mutant (DNM) and pharmacological inhibitors. The first survivin DNM identified is survivin-C84A. Transfection-mediated expression of this mutant showed induction of apoptosis (Li et al., 1999; Tu et al., 2003) and mis-localization of survivin protein (Skoufias et al., 2000) with a cell division defect (Li et al., 1999). However, how the survivin-C84A mutant gains this ability is not clear. On the other hand, the functional mechanism of the survivin-T34A mutant has been well studied. The T34 position in survivin is a CDC2/cyclin B1 phosphorylation site. Phosphorylation of this site is required for survivin to interact with and to inhibit casapse-9 activity during mitosis (O'Connor et al., 2000). Abrogation of survivin phosphorylation on the T34 site either by mutation (O'Connor et al., 2000) or by pharmacological inhibitors of CDC2 (O'Connor et al., 2002; Wall et al., 2003) destabilizes survivin protein and abrogates the inhibition of caspase-9 by survivin, resulting in apoptotic cascade. A pharmacological inhibitor that can directly suppress survivin protein function is not yet available. However, based on the physical and functional relationship of survivin with caspase-9, it may be possible to develop pharmacological molecules that disrupt the interaction of survivin with caspase-9. Interestingly, it was recently shown that survivin forms complexes with a cellular protein (HBXIP) originally recognized for its association with the X protein of hepatitis B virus, and that survivin-HBXIP complexes, but not survivin itself, interact with pro-caspase-9, preventing activation of the mitochondrial/cytochrome *c* pathway (Marusawa et al., 2003). This finding provides an alternative therapeutic approach by disrupting the survivin-HBXIP complex. Additionally, a new survivin mutant (D53A) was recently identified that shows not only loss of antiapoptotic function, but also a gain of pro-apoptotic properties (Song et al., 2003a). The mechanistic function of this mutant is likely a result of the loss of its ability to interact with and to inhibit Smac (Song et al., 2003a) (see Smac/DIABLO section below).

2.2.4. Cancer Therapy Using the Survivin Gene Promoter

Consistent with the fact that survivin is highly expressed in cancer but not in normal adult tissues, reporter genes driven by the survivin promoter are highly expressed in cancer cells, but not in normal cells (Bao et al., 2002; Li & Altieri, 1999; Yang et al., 2004; Zhu et al., 2004). This suggests that the survivin promoter may be an ideal cancer-specific promoter for driving a cytotoxic gene expression for cancer therapy. A luciferase reporter driven by the survivin promoter in an adenovirus expression vector showed no or very low activity in liver and other normal tissues after transfection into mice (Zhu et al., 2004), suggesting utilization of the survivin promoter for cancer therapy will have a

minimal toxicity to normal tissues. Furthermore, expression of the toxic autocatalytic rev-caspase-3 gene driven by the survivin promoter specifically kills transfected cancer cells but not normal cells (Yang et al., 2004). Additionally, since the interior of solid tumors are hypoxic, the finding that hypoxia induces survivin expression (Yang et al., 2004) has significant consequences for utilization of the survivin promoter in gene therapy of cancer.

3. OTHER IAPS AS THERAPEUTIC TARGETS

c-IAP1 and c-IAP2 were initially identified as NF-κB-mediated survival components that interact with TRAF1 and/or TRAF2 in the TNF receptor 2 (TNF-R2) (Rothe et al., 1995) and TNF-R1 (Shu et al., 1996) signaling pathways (Chu et al., 1997). XIAP (Sanna et al., 1998), NAIP and ML-IAP (Sanna et al., 2002) were shown to activate JNK signaling as an alternative mechanism for antiapoptotic (Sanna et al., 2002; Sanna et al., 1998) or proapoptotic (Tang et al., 2001) properties. In addition, XIAP was shown to be involved in TGF-β signaling (Birkey Reffey et al., 2001; Yamaguchi et al., 1999) and the anti-apoptotic activity of XIAP is retained upon mutation of both the caspase 3- and caspase 9-interacting sites (Silke et al., 2001; Silke et al., 2002). However, strategies for using the above findings to treat cancer or other human diseases through targeting these IAPs have not yet been developed. In contrast, the antiapoptotic mechanism of c-IAP1, c-IAP2, XIAP (Deveraux & Reed, 1999; Salvesen & Duckett, 2002) and survivin (Li, 2003) as caspase inhibitors has been well defined, and several studies based on this information have attempted to treat human cancer and/or other human diseases.

XIAP, c-IAP1 and c-IAP2 have been shown directly to interact with and to inhibit caspases-3, -7 and -9 but not caspases-1, -6, -8 and -10 (Deveraux et al., 1998; Deveraux et al., 1997; Roy et al., 1997), indicating that the mechanism by which these IAPs block mitochondrial-mediated apoptosis differs from Bcl-2 family members (Duckett et al., 1998; Orth & Dixit, 1997) (refer to Chapters I and V for more information). Further studies indicated that the BIR2 domain of XIAP is involved in inhibition of caspases-3 and -7 (Deveraux et al., 1999; Sun et al., 1999; Takahashi et al., 1998), and the linker region between BIR1 and BIR2 is critical for inhibiting caspases-3 and -7, while the BIR2 domain may serve as a regulatory element (Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001; Sun et al., 1999). This structural basis for the interaction of XIAP with caspases-3 and -7 provides opportunities to design antagonists to disrupt the interaction for therapeutic purposes.

Identification of peptides targeting XIAP was recently reported (Tamm et al., 2003). Using recombinant full-length human XIAP or a fragment containing only the BIR2 domain, a consensus motif, C(D/E/P)(W/F/Y)-acid/basic-XC, was recovered from two independent screenings by using different phage peptide libraries. The authors demonstrated that CEFESC (K_D=1.8 nM) and CPFKQC peptide phages bound specifically to the BIR2 domain of XIAP but not to XIAP's BIR1, BIR3 or RING domain, nor to survivin, c-IAP1, c-IAP2 or NAIP. The binding of XIAP or BIR2 to CEFESC peptide phages could be displaced by adding free CEFESC peptides or capsase-7 protein in a concentration-dependent manner. However, this binding could not be displaced by adding an ARKGER control peptide or caspase-8 protein (Tamm et al.,

2003). This observation indicates that the CEFESC peptide targets a domain in BIR2 of XIAP relevant to the caspase-binding site. Consistent with this hypothesis, the authors pointed out that the core sequence EFES is homologous to a loop unique to caspase-3 and caspase-7 that are targeted by XIAP (Chai et al., 2001; Huang et al., 2001) (Riedl et al., 2001). Finally, the authors showed that a permeable version of the CPFKQC peptide could induce apoptosis in leukemia cells. However, since the K_D for CPFKQC was not presented in the report, it is not clear why the permeable version of the CEFESC peptide with a K_D of 1.8 nM has no significant effect on cell viability. Nevertheless, the phage-derived peptides may serve as prototypes for the design of small chemical compounds to inhibit IAP function.

Recently, using an assay based on disruption of the XIAP-caspase-3 interaction resulting in increased caspase-3 activity, high-throughput screening of a combinatorial chemical library led to the discovery of a novel small organic molecule (TWX024) that disrupts the interaction of XIAP/caspase-3 or XIAP/caspase-7 (Wu et al., 2003). The authors demonstrated that apoptosis induced by overexpression of CD95 in 293 cells could be inhibited by overexpression of XIAP in the absence of TWX024 but failed to do so in the presence of TWX024 (25 μM). However, treatment of 293 cells with TWX024 alone at even 40 μM did not induce apoptosis (Wu et al., 2003), suggesting that TWX024 directly inhibits the function of XIAP. Although the high IC_{50} (25 μM) of this compound may prevent its clinical application, TWX024 represents the first generation of small non-peptide molecules capable of releasing caspases 3/7 from XIAP and thus triggering apoptosis. This compound may serve as a prototype to facilitate the development of therapeutically useful agents that target XIAP function for the treatment of cancers since it is known that at least in some cancers, apoptosis resistance is due to overexpression of XIAP.

More recently, using a caspase-3 derepression assay similar to that above, screening of 11 mixture-based combinatorial chemical libraries containing about a million compounds identified eight compounds with significant XIAP-inhibitory activity from one library of 89,956 unique polyphenylureas (Schimmer et al., 2004). The authors showed that these compounds, but not inactive structural analogs, could trigger the activation of caspase-3 but not caspase-9. These authors characterized one of the eight compounds (1396-34) identified in depth and showed that it directly induced apoptosis in many types of tumor cell lines in culture with a LD_{50} (lethal dose-50%) range of 6-17 μM. 1396-34 significantly sensitized cancer cells to chemotherapeutic drugs (etoposide, doxorubicin, paclitaxel) and apoptotic inducer, TRAIL. Moreover, 1396-34 inhibited colony formation by >95% at 10 μM, and suppressed growth of established tumors in mouse xenograft models, while displaying little toxicity to normal tissues (Schimmer et al., 2004). This is the best study of IAP-targeting for cancer treatment reported so far. This study not only validated XIAP is a valid target for cancer drug discovery but the compounds identified also have a great potential to develop into chemotherapeutic drugs and/or chemosensitizers for molecular targeting of cancer therapy. The effective induction of apoptosis by 1396-34 compounds is consistent with the recent finding that in comparison with normal cells, tumor cell lines and cancer tissues simultaneously express high basal levels of both antiapoptotic factors (survivin & XIAP) and proapoptotic factors (caspase-3 and caspase-8 activities and active caspase-3

fragments) in the absence of apoptotic stimuli (Yang et al., 2003a). Therefore, disruption of the interaction of caspase-3 with XIAP for example presumably triggers apoptosis.

Studies of the interaction of XIAP with caspase-9 indicated that BIR3 domain of XIAP contributes to the inhibition of caspase-9 (Deveraux et al., 1999; Sun et al., 2000), that the 9 amino acid linker region prior to BIR3 appears important for caspase 9 inhibition (Sun et al., 2000), and that XIAP associates with the active caspase-9-Apaf-1 holoenzyme complex through binding to the amino terminus of the linker tetrapeptide on the small subunit of caspase-9, which becomes exposed after proteolytic processing of pro-caspase-9 at Asp315 (Srinivasula et al., 2001). The X-ray crystallographic structure reveals that the BIR3 domain forms a heterodimer with a caspase-9 monomer to block its homodimerization and that the monomeric caspase-9 is catalytically inactive (Shiozaki et al., 2003). This structural framework of BIR3-caspase-9 interaction implies that disruption of the interaction of the BIR3 domain of XIAP with the N-terminal linker tetrapeptide on the small subunit of caspase-9 may release caspase-9 from XIAP to trigger apoptosis. A recent study determined the affinity of the BIR3 domain of XIAP for a limited combination of the tetrapeptides derived from or based on its natural binding partners including human Smac, HtrA2/Omi and caspase-9 as well as Drosophila Reaper, Grim, Hid and Sickle (Kipp et al., 2002). The result indicated that most of the peptides tested have a K_D at or near the micromolar level. The best one (ARPF) derived from the tetrapeptide (ATPF) of human caspase-9 has a K_D at 20 nM (Kipp et al., 2002). Since this study used a limited tetrapeptide combination, it is not known whether ARPF is the best tetrapeptide among all possible tetrapeptides. Nevertheless, based on this study, we may expect that it is possible to use a tetrapeptide or a small chemical molecule mimicking the tetrapeptide to disrupt the interaction of BIR3 of XIAP with caspase-9.

In terms of cancer therapeutics using XIAP as a target, the critical question is whether disruption of the XIAP-BIR3/caspase-9 interaction will be a good approach for triggering apoptosis, even if an ideal tetrapeptide or a small chemical compound is available for the disruption. This question arises because the increased activity of caspases 3 and 7 triggered by the activated caspase-9 will be still inhibited by the linker-BIR2 domain of XIAP, which may result in the abrogation of apoptosis. In this regard, targeting downstream of caspases may be a better approach for triggering apoptosis. However, it was reported by Dr. Xiaodong Wang on the 95th AACR Meeting (Orlando, FL, March 27-31, 2004) that a small organic chemical molecule that disrupts the interaction of XIAP and Smac (the interaction is similar to caspase-9) effectively induces caspase 3 activation and apoptosis. This suggests that the mechanistic function of these inhibitory molecules in caspase activation and apoptosis requires further investigation and/or novel mechanisms for apoptosis induction remains to be defined.

Additionally, inhibition of XIAP expression may be an alternative approach to induce apoptosis. In this regard, it was reported that inhibition of XIAP expression by antisense approaches induced apoptosis (Li et al., 2001; Sasaki et al., 2000) and sensitized cancer cells to anticancer drug treatments in both cell culture (Bilim et al., 2003) and xenograft models (Hu et al., 2003).

NAIP was identified as a candidate gene potentially involved in spinal muscular atrophy (SMA), a neuromuscular disease with progressive motor-neuron cell death, since a high percentage of SMA patients show a defect in this gene (Hahnen et al., 1995; Roy et al., 1995). Exogenous expression of NAIP was shown to inhibit apoptosis (Liston et

al., 1996) and reduce ischemic damage (Xu et al., 1997), probably through its inhibition of caspases-3 and -7 (Maier et al., 2002). Moreover, it was demonstrated that NAIP and/or XIAP could prevent neuron cell death in several *in vivo* rat and/or mice models (Crocker et al., 2003; Crocker et al., 2001; Kugler et al., 2000; Perrelet et al., 2002; Perrelet et al., 2000; Xu et al., 1999). These studies suggest that IAPs may be utilized as a novel gene-therapy approach to reduce cell loss in various human diseases associated with excessive apoptosis.

4. SMAC/DIABLO AND OMI/HTRA2: WHAT IS THE DIFFERENCE IN THEIR FUNCTIONS?

Current studies indicate that, although Smac/DIABLO and Omi/HtrA2 seemingly have similar roles to enhance apoptosis by eliminating the inhibitory effect of IAPs on caspases during apoptosis induction, they have much different roles in normal physiology. For example, while Omi/HtrA2 likely has important physiological roles such as the neuromuscular disorder of mnd2 mutant mice is likely due to the loss of Omi/HtrA2 protease activity (Jones et al., 2003), Smac/DIABLO knockout mice do not show any abnormality in comparison with wild type mice (Okada et al., 2002). Therefore, their value in therapeutics of human disease is likely different.

4.1. Smac/DIABLO

Smac in human (Du et al., 2000) and DIABLO in mouse (Verhagen et al., 2000) were independently discovered as apoptosis inducers. Similar to cytochrome *c*, Smac/DIABLO is situated in the mitochondrial inter-membrane space and is released into the cytoplasm during apoptosis induction, and its release could be blocked by overexpression of Bcl-2. However, different from cytochrome *c*, release of Smac/DIABLO requires the presence of active caspases (Adrain et al., 2001), indicating that Smac/DIABLO release is a later event relative to cytochrome *c* release. The released/mature Smac/DIABLO removes the N-terminal 55 amino acids and exposes a new N-terminal, the first four amino acids of which are homologous to the N-terminal of matured Omi/HtrA2 (Suzuki et al., 2001), the small subunits of caspase-9 and several other Drosophila proteins involved in cell death controls (Srinivasula et al., 2001). It has been demonstrated that the mature Smac/DIABLO interacts with XIAP, c-IAP1, c-IAP2, survivin (Du et al., 2000; Song et al., 2003b) and ML-IAP/livin (Vucic et al., 2002). Binding of the N-terminal 4 amino acid peptide of mature Smac to the XIAP BIR3 domain is critical for the release of caspase-9 (Liu et al., 2000; Srinivasula et al., 2000; Wu et al., 2000). This competition is a result of a complete overlap in the binding site for caspase-9 binding of XIAP BIR3 (Srinivasula et al., 2001; Sun et al., 2000). Additionally, although less important, it was reported that mature Smac also activates caspases-3/-7 by interacting with the BIR2 domain of XIAP (Srinivasula et al., 2000), which likely requires a homodimer structure of Smac (Chai et al., 2000). Although Smac variants, Smacβ (Roberts et al., 2001) and Smac3 (Fu et al., 2003) may have alternative

mechanisms to potentiate apoptosis, the structural relationship among Smac, XIAP and caspase-9 provides an interesting opportunity for cancer therapeutics.

It was shown that forced expression of Smac/DIABLO or treatment with the mature Smac N-terminal peptide without linking to a cell-permeable sequence increased Epo B- and TRAIL-induced PARP-1 cleavage and caspase-3 activity in Jurkat cells (Guo et al., 2002), which was associated with increased apoptosis, although overexpression of mature Smac alone could not induce apoptosis (Guo et al., 2002). However, the permeability of the Smac peptide without conjugating a cell-permeable peptide was shown to be very poor in another report (Yang et al., 2003b). Expression of Smac or treatment with cell-permeable Smac peptide has been shown to sensitize resistant neuroblastoma or melanoma cells and patient-derived primary neuroblastoma cells to chemotherapeutic drugs- or TRAIL-induced apoptosis (Fulda et al., 2002). Moreover, local administration of cell-permeable Smac peptides strongly enhanced the antitumor activity of TRAIL in an intracranial malignant glioma xenograft mouse model. Complete eradication of established tumors and survival of mice was only achieved upon combined treatment with permeable Smac peptides and TRAIL, without detectable toxicity to normal brain tissue (Fulda et al., 2002). The cell-permeable Smac peptide was also shown to enhance the induction of apoptosis and long-term antiproliferative effects of various chemotherapeutic agents (paclitaxel, etoposide, SN-38 and doxorubicin) in breast cancer cells as well as in immortalized cholangiocytes (Arnt et al., 2002). It was also reported that the cell-permeable Smac peptide could restore the defect in apoptosome activity in H460 human lung cancer cells that overexpress XIAP, but it has no striking effect on the apoptosome activity of normal lung fibroblast cells, although these cells expressed modest amounts of IAP. The cell-permeable Smac peptide $SmacN_7$-$(R)_8$, but not the non-cell-permeable peptide $SmacN_7$, when used in combination with cisplatin, induced H460 cell death *in vitro* and suppressed tumor growth in a mouse xenograft model with little toxicity to the mice (Yang et al., 2003b). These studies indicate that Smac peptides are promising candidates for potentiation of cancer therapy.

Smac may also be involved in different mechanisms for enhancing apoptosis. It was recently shown that TNF-induced Smac release disrupts the TRAF2-cIAP1 complex (Deng et al., 2003), and Smac may differentially degrade IAPs (Yang & Du, 2004). Additionally, since Smac has been shown to interact with XIAP, c-IAP1, c-IAP2, survivin (Du et al., 2000; Song et al., 2003b) and ML-IAP/livin (Vucic et al., 2002), the effect of IAP-targeting Smac peptides on potentiating apoptosis is likely attributable to an overall antagonist effect on IAPs, rather than on a particular IAP, in most cases. Interestingly, a recent report showed that substitution of Proline3' in the Smac peptide (AV<u>P</u>IAQKSE) with (2S,3S)-3-methylpyrrolidine-2-carboxylic acid [(3S)-methyl-Proline] results in a peptide with 7-fold greater affinity for ML-IAP-BIR and about 100-fold specificity for ML-IAP-BIR relative to XIAP-BIR3 (Franklin et al., 2003). This new peptide may be an important reagent for studying the function of individual IAPs in cell survival. Regardless of mechanism, from a therapeutic perspective, utilization of IAP-targeting Smac peptides is an attractive approach to increase tumor cell death induced by chemotherapy. However, development of small molecule Smac peptide mimetics will be essential for routine clinical application of this approach in cancer treatment.

4.2. Omi/HtrA2

Human Omi/HtrA2 was initially described as a transformation-sensitive protein since it is expressed in human fibroblasts, but not in their matched SV40 transformed counterparts (Zumbrunn & Trueb, 1996). Omi/HtrA2 was found to be upregulated in cartilage from individuals with osteoarthritis (Hu et al., 1998). Structurally, Omi/HtrA2 is highly homology to the essential bacterial HtrA endoprotease that functions as a chaperone protein at normal temperatures and as a serine protease to degrade misfolded proteins at high temperatures (Spiess et al., 1999). Consistently, it was found that the protein expression and/or proteolytic activity of human Omi/HtrA2 were upregulated in response to stress induced by ischemia/reperfusion (Faccio et al., 2000a), heat shock or tunicamycin treatment (Gray et al., 2000). Interestingly, while Omi/HtrA2 is ubiquitously expressed in human tissues, an alternative splicing form of Omi called D-Omi, which lacks proteolytic activity, is predominantly expressed in the kidney, colon, and thyroid (Faccio et al., 2000b). These observations indicate potentially unique roles for Omi/HtrA2 in normal physiology.

Recently, however, several groups reported that Omi/HtrA2 from human (Hegde et al., 2002; Martins et al., 2002; Suzuki et al., 2001) or mouse (van Loo et al., 2002; Verhagen et al., 2002) is actually involved in promoting apoptosis in a manner very similar to Smac. Although it was shown that Omi/HtrA2 is localized in the endoplasmic reticulum (Faccio et al., 2000a) or nucleus (Gray et al., 2000), these authors consistently showed that Omi/HtrA2 is a mitochondrial protein, and mature Omi/HtrA2, after removal of the N-terminal 153 amino acids, was released from mitochondria during apoptosis induction. Mature Omi/HtrA2, which contains an N-terminal four amino acid motif similar to that of mature Smac, interacted with XIAP but not with survivin (Suzuki et al., 2001; Verhagen et al., 2002). This suggests that the antiapoptotic property of XIAP but not survivin may be affected by Omi/HtrA2. Mature Omi/HtrA2 interacts with both BIR2 and BIR3 but not BIR1 of XIAP (Suzuki et al., 2001; Verhagen et al., 2002) to activate both caspase-3 (Hegde et al., 2002; Suzuki et al., 2001; van Loo et al., 2002; Verhagen et al., 2002) and caspase-9 (Suzuki et al., 2001) *in vitro*; this is similar to the competitive interaction of mature Smac with BIR2 and BIR3 of XIAP to activate caspases-3, -7 and -9. It seems that Omi/HtrA2 is not able to induce apoptosis by itself, but it is able to sensitize cells to death induced by many apoptotic stimuli (Hegde et al., 2002; Suzuki et al., 2001; van Loo et al., 2002; Verhagen et al., 2002). Mature Omi/HtrA2 promoted cell death induced by UV (Martins et al., 2002; Verhagen et al., 2002) and other apoptotic stimuli (staurosponrine, Fas, TRAIL) (Hegde et al., 2002). Induction of apoptosis was determined by cell morphology (Hegde et al., 2002), Annexin V labeling (Martins et al., 2002; Verhagen et al., 2002) and DNA fragmentation cell death ELISA assay (Martins et al., 2002). It was shown that apoptosis contributed by Omi/HtrA2 is dependent on both the IAP inhibition and serine protease activities of Omi (Hegde et al., 2002; Verhagen et al., 2002). Overexpression of Omi/HtrA2 but not the protease-dead mutant of Omi in HEK293 cells was shown to induce a caspase-independent cell death (Cilenti et al., 2003; Suzuki et al., 2001), and a compound (ucf-101) identified in a high-throughput screening based on an assay to inhibit the serine protease activity of Omi/HtrA2 was found to inhibit Omi-induced cell death (Cilenti et al., 2003), indicating serine protease activity contributes to Omi-induced cell death. More recently, it was shown that binding of

Omi/HtrA2 to XIAP increases its serine protease activity (Martins et al., 2003) and results in degradation of IAPs *in vitro* and in cells (Srinivasula et al., 2003; Yang et al., 2003c), which may be an alternative mechanism contributing to apoptosis enhancement. Interestingly, the yeast homolog of mammalian Omi, Nma111p is situated in the nucleus, its pro-apoptotic activity also depends on its serine-protease activity, and yeast cells that lack Nma111p survive better at 50^0C than wild-type cells (Fahrenkrog et al., 2004).

Although recent studies have defined a major role of Omi/HtrA2 in promoting apoptosis, initial observations suggest Omi/HtrA2 likely plays a role in normal physiology. Consistently, neuromuscular degenerative disease in mnd2 (motor neuron degeneration 2) mutant mice was genetically mapped to the S276C missense mutation in the protease domain of Omi/HtrA2(Jones et al., 2003). The S276C mutant protein loses its protease activity (Jones et al., 2003). In contrast to the evidence above that the protease activity of Omi is involved in promoting apoptotic cell death (Hegde et al., 2002; Suzuki et al., 2001; Verhagen et al., 2002), these authors found that loss of the protease activity of Omi increases sensitivity to stress-induced cell death probably as a result of increased mitochondrial membrane permeability. This effect could be responsible for the massive loss of striatal neurons in mnd2 mutant mice (Jones et al., 2003). Although these results require further validation by comparison of the phenotype of Omi knockout mice with the phenotype of Omi S276C mutant mice, this study suggests that the protease activity of Omi is important in physiology for maintenance of normal mitochondrial permeability, which is essential for cell survival. However, during apoptotic induction, Omi will be released from mitochondria into the cytoplasmic compartment, and the protease activity of released Omi actually promotes apoptosis. In this regard, it is likely that the function of Omi as an apoptosis promoter is actually a result of a good guy present in the wrong place. Therefore, the cancer therapeutic value of Omi requires further investigation.

5. SUMMARY

Survivin appears to be a novel member in the IAP protein family since its structure, expression pattern, regulation, subcellular localization and function are unique. Although specific disruption of survivin function by small chemical molecules remains to be reported, a number of studies have shown significant consequences of interfering with survivin expression or function in apoptosis induction, which may provide extensive applications in cancer therapeutics (Altieri, 2003a; Li, 2003). However, in comparison with XIAP, less detail at the molecular level is known for survivin's mechanistic function in apoptosis as well as in cell division. Therefore, much more efforts will be required in the coming years to elucidate the mechanisms by which survivin is regulated in cancer versus normal cells, and it functions in cell survival and division. This will be essential for development of novel and highly effective approaches for cancer therapeutics using survivin as a target. On the other hand, the mechanistic function of XIAP at the molecular level is much clearer than that for other IAPs. As a result, several research groups have recently reported the feasibility using peptides or small organic molecules to specifically disrupt the inhibitory effect of XIAP on the activity and/or activation of caspases. Together, current studies have convincingly demonstrated that targeting IAPs is a feasible

and attractive novel approach for caner treatment. Additionally, overexpression of Smac or treatment with cell-permeable Smac peptides significantly potentiates apoptosis induced by antitumor drugs in various cancer cells, but not in normal cells, although this treatment alone is unable to induce apoptosis. This makes Smac an attractive molecule for cancer therapeutics and drug discovery. Although the IAP-targeting Smac peptide has disadvantages as a clinical drug for cancer treatment, it could act as a prototype drug to help the development of small organic molecule as Smac peptide mimetics. Overexpression of Omi/HtrA2 is able to induce cell death and potentiate apoptosis induced by chemotherapeutic drugs. However, due to the cellular localization of the endogenous Omi/HtrA2 is critical for its function in apoptosis induction or maintaining normal mitochondrial permeability, further studies will be required to evaluate its toxicity in normal cells versus cancer cells, and its application in human cancer therapy.

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THE LIFE CYCLE OF p53: A KEY TARGET IN DRUG DEVELOPMENT

Chapter VII

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1. SUMMARY

p53 is a key component of a stress-induced signalling pathway that is central to tumour suppression. Various gene products in this p53 pathway are mutated in human cancers leading to defects in the intrinsic apoptotic pathways that normally maintain tissue integrity and prevent cancer development. Being central to cancer suppression, components of the p53 pathway including upstream modifying kinases like ATM, p53 itself, or down-stream effectors proteins like cyclin-dependent kinase inhibitors, are targets of experimental strategies designed to modify the p53 pathway for therapeutic benefit. This review will highlight molecular properties of p53, regulatory stages in its activation and inhibition, putative anti-cancer drug targets in the p53 pathway, and the rationale for developing sets of drugs to proteins in the p53 pathway for treating human cancers.

2. INTRODUCTION

2.1. The Life Cycle of p53

p53 protein is a tumour suppressor whose gene is frequently inactivated by point mutation in human cancers (Olivier et al., 2002). It is generally held that p53 is not involved in normal cell proliferation or development, since p53-null animals generally develop to adulthood relatively normal. Nevertheless, what is striking is that p53-null animals develop cancers at an elevated rate indicating p53 plays a central role as a cancer suppressor (Donehower et al., 1992). However, there are other roles of p53 apart from tumour suppression including neural tube assembly and ischaemic injury-induced is apoptosis (Migliorini et al., 2002; Sakhi et al., 1994). p53 activity as a tumour suppressor

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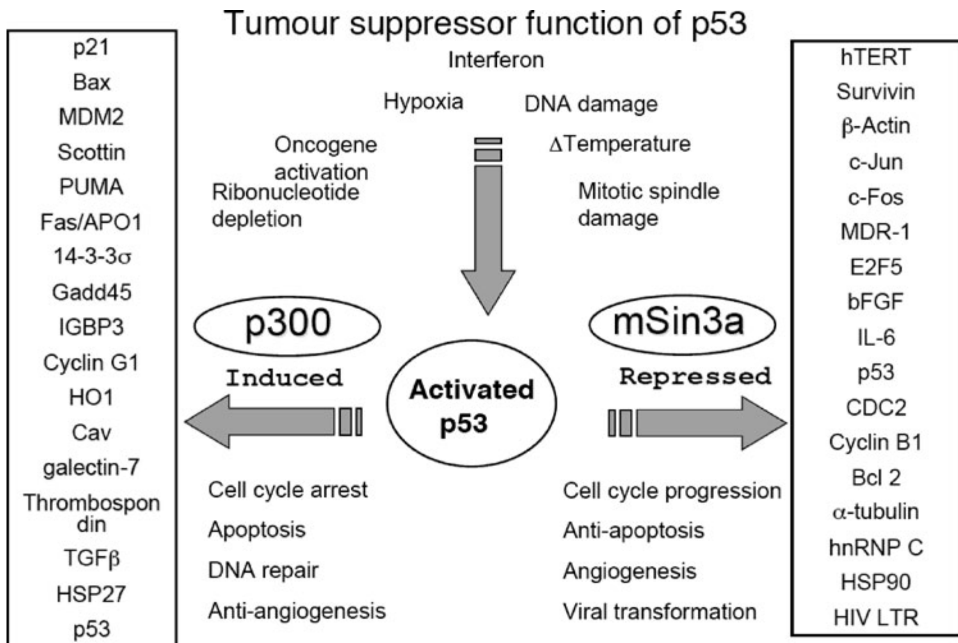

Figure 1. p53 is an inducible transcription regulator. p53 protein can be switched on by changes in the cellular microenvironment that can in turn change the expression of many genes implicated in tumour suppression (Kannan et al., 2001a; Kannan et al., 2001b; Zhao et al., 2000). Mutations in p53 prevent the activation of its transcription function and this prevents gene expression changes that would normally be associated with cancer suppression.

is linked to its activity as a transcription factor (Vogelstein et al., 2000). p53 activity is thought to be cryptic or latent, hence the viability of p53-null animals, yet activated by selected cellular stresses that can result in death of injured cells and maintenance of normal tissue integrity and/or cancer suppression. The p53 gene functions at a nodal point as an effector of the cellular response to changes in the microenvironment (Fig. 1). Such cellular stresses may be intrinsic stresses generated from evolving genome instability or interferon-mediated tumour suppression signals that can signal p53 activation. Extrinsic stresses that can signal to p53 include hypoxia, pro-oxidants, or DNA damaging agents (Fig. 1). Together, these cues recruit p53 to promoters to switch on pro-apoptotic genes in cooperation with the transcription coactivator p300. The number and types of genes co-ordinately induced and suppressed by p53 are vast and can be reviewed elsewhere (Kannan et al., 2001a; Kannan et al., 2001b; Zhao et al., 2000). It is the large number of cellular pathways that p53 can affect including protein degradation, RedOx status, apoptotic engagement, and mitotic checkpoints that explains why it functions at a nodal point and therefore why it is universally dysregulated as cancers develop.

The mechanism of p53 control is being dissected and there have been many stages identified in its life cycle (Fig. 2). This cycle includes: (*i*) its birth, where it is translated under control of MDM2 (Yin et al., 2002) and its assembly with chaperones into native

tetramers (Neckers, 2002); (*ii*) part of its life in the cytoplasm, where p53 can be stably stored in a cytoplasmic microtubule-associated pool in undamaged cells followed by damage induced and dynein-mediated nuclear entry (Giannakakou et al., 2002; Giannakakou et al., 2000); (*iii*) part of its life in the nucleus, where p53 is imbedded into promoters containing consensus p53-binding sites thus effecting stress-regulated gene

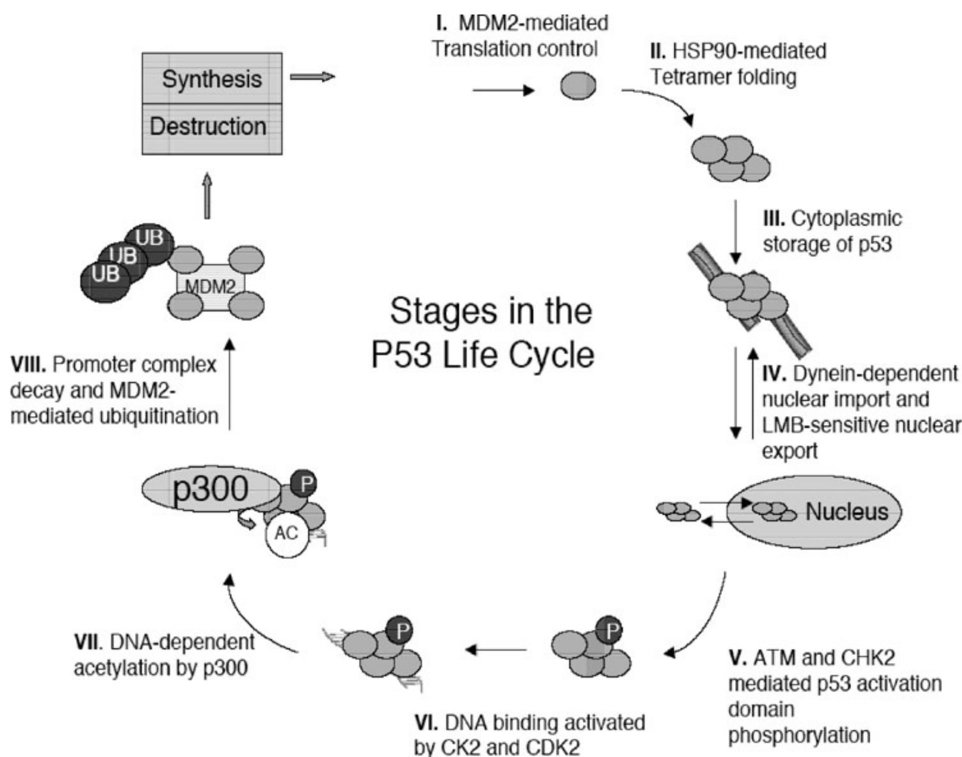

Figure 2. Stages in the assembly and disassembly of p53 protein. p53 protein is assembled by multiple protein-protein interactions including MDM2-mediated translation control (Yin et al., 2002); HSP-90-mediated control of native p53 tetramer assembly that involves dynein-dependent activity (Galigniana et al., 2004); cytoplasmic storage of p53 and related dynein-mediated nuclear import (Giannakakou et al., 2002; Giannakakou et al., 2000); ATM and CHK2-mediated p53 phosphorylation of the p53 transactivation domain (Bartek and Lukas, 2003); sequence-specific DNA binding controlled by C-terminal protein kinases (Blaydes et al., 2000; Blaydes et al., 2001); p300 docking and DNA-catalyzed acetylation of p53 at promoters (Dornan et al., 2003a; Dornan et al., 2003b); MDM2-mediated ubiquitination and degradation (Woods and Vousden, 2001). Anti-cancer drugs or drug lead can antagonize each of these reactions: (*i*) HSP-90-binding drugs like Geldanamycin can prevent p53 unfolding (Whitesell et al., 1997); (*ii*) microtubule disrupting drugs like taxol can compromise dynein-mediated nuclear import of p53 (Giannakakou et al., 2000) whilst export inhibiting drugs like leptomycin B can prevent p53 export and stabilize it in the nucleus (Hietanen et al., 2000); (*iii*) acetylation inhibitors can bind p300 and inhibit p53 activity, while de-acetylation inhibitors can bind HDAC's and stimulate p53 activity (Yu et al., 2002); and (*iv*) MDM2-binding drugs can stimulate p53 activity (Vassilev et al., 2004). Further drugs that bind p53 regulators like CDK's can either stimulate or attenuate p53 activity identifying distinct functions of CDK's in p53 control (Ferguson et al., 2004) and peptide-mimetics of p53-induicible gene products can alone induce apoptosis or growth arrest thus highlighting potential anti-cancer drug leads (Kontopidis et al., 2003).

expression; and (*iv*) its death, involving the MDM2 oncogene which enhances p53 turnover and degradation by the proteosome (Woods & Vousden, 2001). Upstream kinases including ATM and CHK2 can genetically cooperate with p53 and activate its transcription function (Bartek & Lukas, 2003), although the actual mechanism is only now being developed. The mechanistic data indicate that ATM and CHK2 can phosphorylate the transactivation domain of p53 and these phosphorylation events can stabilize the binding of the transcription coactivator p300 (Dornan & Hupp, 2001; Dornan et al., 2003b). This stabilization can result in a p300 docking-dependent and DNA dependent acetylation of p53 at promoters by p300 that in turn leads to chromatin remodelling and gene expression (Fig. 3). Thus, there appears to be a phosphorylation

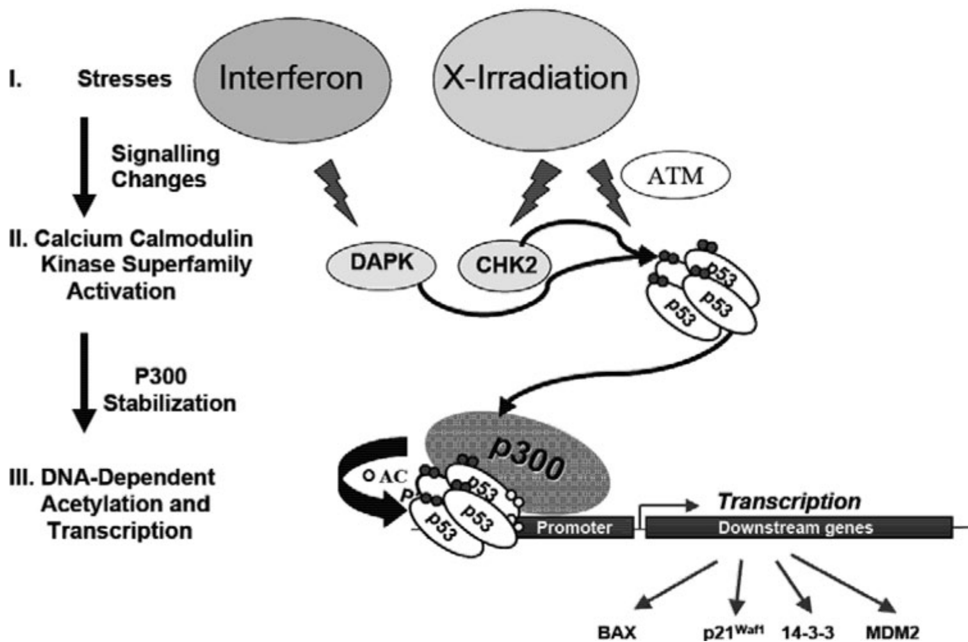

Figure 3. Stages in p53 Activation. Genetic disruption of p53 activators like ATM, CHK2 (Takai et al., 2002) or DAPK (Raveh et al., 2001) can attenuate p53 activity as a growth suppressor or apoptotic effector. Since these kinases can modify the transactivation domain of p53 (Shieh et al., 2000) (Craig et al., 2003) and this phosphorylation can stabilize p300-binding (Dornan and Hupp, 2001), then the mechanism of kinase activation is, in part, due to kinase stabilization of the p53-p300 complex. This in turn leads to p53 acetylation at promoters (Dornan et al., 2003a; Dornan et al., 2003b), that in turn can act to switch on an acetylation cascade that promotes chromatin remodelling and activation of gene expression (Barlev et al., 2001). The kinases that modify the p53 activation domain are part of the calcium calmodulin kinase superfamily and this group of enzymes can, in part, explain how different stresses can activate the p53 response. Disruption of these upstream kinases my mutation or by drugs would have the net effect of attenuating p53 and may be useful therapeutics to sensitize cells to damage (Bunz et al., 1999) under conditions in which p53 would normally be activated as a survival factor.

and acetylation cascade in activating p53, which can in turn imbed p53 within chromatin that confers resistance to its inhibitor MDM2. The dissociation of p53 from promoters presumably allows its access to its inhibitor MDM2 that catalyses the ubiquitination and degradation of p53. There are a number of unclear problems according to this model, such as how the cytoplasmic pool of p53 is stored in undamaged cells, how p53 can be protein signalled to enter the nucleus after DNA damage or other stresses, where ATM or CHK2 actually modify p53 in the cell, and how MDM2 protein can find p53 to promote its degradation. However the concept of its translation control, cytoplasmic anchoring, nuclear import, kinase modification, and ubiquitination produce a general framework that permits further dissection of the p53 life cycle.

This life cycle of p53 is disrupted at many stages in human cancers and this has the net effect of preventing p53 activation by upstream controlling kinases and associated promoter binding (Fig. 3). This can occur by inactivating mutation in the p53 gene itself in some cancers or retainment of the wild-type p53 gene along with either mutation in its upstream activators (like CHK2 or ATM) or epigenetic elevations in its proto-oncogenic inhibitors (Blandino et al., 1999). The p53 gene can be first activated by point mutation leading to a protein that is unfolded, inactive, hyperubiquitinated, and accumulated stably in the cancer cell. This mutated p53 protein can also function as a dominant negative gain-of-function oncogene that in turn gives enhanced survival to the cancer cell. Upstream components of the p53 pathway like ATM and CHK2 kinases can also be mutated in human cancers leading to reduction in wild-type p53 specific activity (Takai et al., 2002). Further, MDM2 protein can be amplified epigenetically in human cancers leading to enhanced wild-type p53 protein degradation and a reduction in wild-type specific activity (Momand et al., 1998). There are other recently identified p53 inhibitors like iASPP that binds to the core domain of p53 and inhibit its activity (Slee & Lu, 2003). Additionally, a gene amplified in breast cancers and co-expressed with the estrogen receptor, Anterior Gradient-2, can attenuate the ATM/DNA-PK mediated phosphorylation of p53 and inhibit p53 transactivation (Pohler et al., 2004). Since cancer develops over a relatively long time period, it is evident that even 50% changes in p53 activators or p53 inhibitors over time can tip the balance and effect cancer incidence in a human life span.

2.2. Therapeutic Strategies in the p53 Pathway

There are two thoughts in experimental cancer research that attempt to exploit the p53 pathway for therapeutic benefit. The first idea is that p53 is a pro-apoptotic factor whose activity needs to be stimulated or activated in order to kill cancer cells. This activation includes switching on wild-type p53 and associated pro-apoptotic pathways in cancers with defective intrinsic activating signals. Another activation strategy includes reactivation of unfolded mutant p53 leading to restoration of its pro-apoptotic activity. However, the p53 pathway is also a growth arrest, repair, and survival effector and it is also thought that inhibiting p53 activators like ATM or p53 itself can radio or chemo-sensitize cells in combination therapies. For example, in isogenic cell models, hypoxia kills p53-negative cells better than wild-type p53 containing cells (Achison & Hupp, 2003) and the DNA-damaging agent Adriamycin kills cells better which lack functional wild-type p53 (Bunz et al., 1999). Given these two precedents, it is imperative to develop

inhibitors of p53 activators (like ATM) as well as inhibitors of p53 inhibitors (like MDM2, iASPP, and AG-2), so that rationale choices can be made to either induce the pro-apoptotic function or sensitisation of cells for therapeutic benefit. The decision of a cancer to growth arrest and another to die when p53 is activated presumably is due to the variable expression of the growth arresting and apoptotic gene battery induced by p53.

3. DRUG TARGETS IN THE p53 PATHWAY

The life cycle of p53 involves the binding of many regulatory proteins that are the subject of intensive study (Fig. 2). Drugs that bind any of these key binding partners, regulators, activators, or inhibitors of p53, have the potential to stimulate p53 activity or inhibit its survival activity and sensitize cells to death in combination therapies. This review will highlight some of the drugs or drug mimetics that have been developed and which have the potential to modify the p53 pathway.

3.1. Reactivation of Mutant p53: a Protein Folding Problem

p53 protein is not often deleted or truncated in cancers like many tumour suppressor genes. Selective pressures during cancer progression actually retain a full-length but mutant p53 gene alongside the wild-type gene, indicating that a gain-of-function oncogenic property is required to promote clonal evolution of the cancer (Blandino et al., 1999). The loss-of heterozygosity by deletion of the wild-type allele is associated with cancers of enhanced metastatic potential and worse prognosis for the patient. The mutant p53 protein is often stabilized in the nucleus of the cancer cell (Gannon et al., 1990) and complexed to molecular chaperone complexes (Pinhasi-Kimhi et al., 1986; Whitesell et al., 1998). The mutant p53 protein is denatured or unfolded in cancers, providing the first molecular defect of the p53 pathway in a cancer cell and indicating that p53 protein is conformationally flexible *in vivo*. There is an ATP-dependent equilibrium between unfolded and folded p53 and co-oligomerization of mutant unfolded p53 drives the wild-type protein into the unfolded conformation (Milner & Medcalf, 1991). This allosteric nature of p53 protein provides realistic hope that its activity can be restored by small molecules that stabilize its oligomeric structure.

Human cancers arise due to genome instability and also accumulate misfolded proteins during the cellular aging process that may cause altered signalling required to drive cancer cell proliferation (Dyson & Wright, 2002). The cellular buffer that permits mutant or misfolded proteins to be stable and assemble into component pathways in vivo is the chaperone HSP-90 (Sangster et al., 2004; Wagner et al., 1999). Anti-cancer drugs have been developed that alter HSP-90 activity and that these drugs can refold mutant p53 into the wild-type conformation (Neckers, 2003). Manipulation of protein conformation in cells by small molecules provides a novel approach that is relatively distinct from classic anti-cancer drugs programmes which aim to damage DNA and kill cells, radiosensitize cells, or which competitively inhibit engines of the cell-cycle like mutant *ras* or cyclin-dependent protein kinases.

Recent ideas on protein conformation control have highlighted the idea that proteins can be intrinsically unstructured or partially unfolded and that native structure can be

acquired by an induced-fit mechanism (Wright & Dyson, 1999). This equilibrium model between unfolded and folded states is significant because it provides a mechanism to control p53 conformation post-translationally. For example, the de-stabilizing effects of the C-terminus or N-terminus may be neutralized by p53-binding proteins or by modifications like phosphorylation. The positive co-factor p300 may stimulate p53 activity in part by creating a structure in p53 by an induced-fit mechanism and stabilize the tetramer. P300 embraces multiple contact points on the p53 tetramer and the sequence-specific DNA-dependence in p300-catalyzed acetylation highlights a novel allosteric effect of DNA on p53 protein conformation and subsequent protein-protein interactions (Dornan & Hupp, 2001; Dornan et al., 2003a; Dornan et al., 2003b). In contrast to p300, negative regulatory co-factors like MDM2 protein could further de-stabilize the p53 tetramers, thus indicating that the equilibrium in p53 protein tetramer stability can be driven in two directions.

The discovery of a mechanism via antibody binding to a C-terminal negative regulatory domain of p53 to activate the one class of mutant p53 *in vivo* provides an exciting precedent for targeted activation of p53 (Abarzua et al., 1995; Abarzua et al., 1996; Hupp et al., 1995). This has been extended by using small peptides that stimulate sequence-specific DNA-binding by p53 and stimulate cell death (Kim et al., 1999; Selivanova et al., 1997), providing a precedent for small peptide regulation of p53. Most cancer-derived p53 mutants are thermodynamically unstable (Bullock et al., 1997) and one of the most oncogenic and unfolded protein is encoded by the HIS175 allele. This mutant cannot be activated by the same pathway that can be used for the allosteric class of p53 mutant due to the high degree of unfolding at physiological temperatures. Based on the models that the structural mutant p53 protein exists in a folded and unfolded state (both of which can be recognized by a distinct monoclonal antibody) a high throughout screen was used to identify a class of small molecules (e.g. CP-31398) that could stabilize the conformation of this class of mutant p53 (Foster et al., 1999). Although CP-31398 can stimulate the p53 pathway in cells, it has been questioned whether this drug actually stabilizes mutant p53 conformation *in vivo* (Rippin et al., 2002). This latter study claimed that CP-31398 does not bind to the core domain of p53. However, since the regulatory domains of p53 have effects on p53 tetramer stability (Bell et al., 2002), it is possible that CP-31398 does bind to p53 tetramers by interacting at sites outside the core domain thus stabilizing core domain conformation by allosteric effects. Further, a recent study has shown that p53 activation *in vivo* by CP-31398 does not damage DNA as defined by activation of the ATM-CHK2 pathway and that CP-31398 actually blocks p53 ubiquitination (Wang et al., 2003). Thus, these two studies are a very important precedent in p53 conformational control by small molecules since it is now established that small molecule screening can identify a compound that prevents unfolding of mutant p53 protein and stabilizes mutant p53 *in vivo*.

To complement the screening assay searching for molecules that stabilize the conformation of p53 would be to use a cell-based assay to screen for molecules that activate the transcription function of p53 *in vivo*. Such a screen has recently led to the discovery of a class of small molecule (PRIMA) from the NCI library that can refold the mutant conformation of p53 into the wild-type conformation *in vivo* and reactivate mutant p53-dependent transcription and apoptosis *in vivo* (Bykov et al., 2002; Bykov et al., 2003). Since p53 is so frequently mutated in human cancers, such molecules hold

high promise as a precedent for agents that can reactivate mutated p53 in cancers for therapeutic benefit. The disadvantage of that approach is that direct effects of the drug on p53 conformation cannot be established. As an example, such mutant p53 refolding molecules may act on the molecular chaperone pathways. The chaperones that bind mutant p53 function as a coordinated holoenzyme or protein-folding machine with discreet steps in the protein folding and unfolding process. The polypeptides of the chaperone complex can include HSP-90, HSP-70, HSP-40, $p60^{HOP}$, $p48^{HIP}$, immunophilins, and p23 with each having distinct functions involving substrate recognition and release, nucleotide-dependent binding and turnover, and conformational regulation of the protein complex organization (Hartl & Hayer-Hartl, 2002). Chaperones can function as anti-apoptotic effectors in cells exposed to otherwise toxic levels of damaging agent and the ability of anti-tumour antibiotics of the ansamycin class to bind to chaperones, which are overexpressed in tumour cell lines, to induce cell death further highlights their role in cell survival and the attraction for targeting chaperones for therapeutic effect. p53 conformation can be restored *in vivo* using the anti-tumour antibiotic Geldanamycin indicating that the potential exists to manipulate p53 conformation for therapeutic effect (Blagosklonny et al., 1996; Whitesell et al., 1997). Thus, two independent research approaches gave rise to Geldanamycin and PRIMA, both of which can permit refolding of mutant p53 protein *in vivo*.

The intrinsic degree of thermoinstability in p53 mutants suggests a common approach cold be used to reactivate the protein (Bullock et al., 1997). For example, the ability of chemical solvents to refold common p53 mutants *in vivo* (Brown et al., 1997) is consistent with studies that predicted a mild degree of intrinsic thermodynamic instability in p53 could be overcome to restore conformation. Modelling of the p53-stimulating factor BP2 into a fragment that binds the core domain of p53 has led to the development of small peptide that can stimulate the DNA-binding activity of wild-type p53 (Friedler et al., 2002). The unusual feature of this peptide is its binding site is adjacent to or within the active site for DNA-binding and its ability to stimulate DNA-binding can only be explained by an induced-fit mechanism. This results in the p53 core domain stabilization into an active conformation, followed by peptide dissociation, and high affinity DNA-binding. The demonstration that the core domain exists in at least two functional DNA-binding states (low affinity and high affinity) is consistent with the biochemical studies proposing an equilibrium exists between latent and partially-unfolded native tetramers (Bell et al., 2002). The ability of the p53 core-domain binding peptides to stimulate mutant p53 DNA-binding will undoubtedly lead to an expansion of the chaperone-rescue strategy for mutant p53 reactivation. It will important in future to further characterize other mutant p53 protein alleles in order to understand and group them into classes for specific targeted refolding strategies.

3.2. Stimulation of Wild-type p53 Function: Switching on Upstream Signalling Pathways

p53 protein is tetrameric, suggesting a relatively complex mechanism of assembly, function, and regulation. The tetrameric nature of p53 provides a tetravalent scaffold for the binding of regulatory proteins and expands upon possible regulatory permutations. By characterizing stages in the assembly of the p53 pathway, various rate-limiting steps have

been defined (Fig. 2). These include: (*i*) p53 monomer assembly into tetramers; (*ii*) equilibrium between the unfolded and folded tetramer that is controlled by factors such as HSP-90; (*iii*) C-terminal kinase phosphorylation that converts p53 protein from a low affinity to high affinity DNA-binding form; (*iv*) activation domain phosphorylation by CHK2 that promotes p300-catalyzed DNA-dependent acetylation, and (*v*) MDM2-mediated ubiquitination and associated nuclear export of p53. Each one of these reactions is regulated by enzymes or modifiers that are potential drug targets that can either sensitize p53 containing cells to damage-induced death or which can stimulate the p53 pathway (Fig. 2).

3.2.1. Ubiquitination Inhibitors

It appears on the surface easier to target "oncogenic" p53-inhibitors for novel anti-cancer drugs rather than inhibiting upstream p53-activators like ATM, or in fact reactivating mutant p53 itself. As such, much effort has been placed on identifying cellular proteins that inhibit wt-p53 function. MDM2 was one of the first cellular p53-binding proteins found (Momand et al., 1992), along with the heat shock protein complex (Pinhasi-Kimhi et al., 1986), and MDM2 has received the most attention as a model for oncogenic drug-development. MDM2 protein has a very short half-life and its binding to p53 tags the tumour suppressor for ubiquitination, degradation, and inactivation by the proteosome. MDM2 can bind to two regions on p53: an N-terminal activation domain binding site on p53 is important for transrepression of p53 activity (Kubbutat et al., 1997; Haupt et al., 1997) and an internal binding site in the DNA-binding domain of p53 plays a key role in p53 ubiquitination (Shimizu et al., 2002). Lead molecules have been designed that can bind MDM2, prevent MDM2 binding to the N-terminal transactivation domain of p53, and activate p53-depednent gene expression (Vassilev et al., 2004). Current reviews highlighting progress in this area extend the drug-development portfolio into ubiquitination-inhibitors, nuclear export inhibitors, and proteosome inhibitors (Chene, 2003). It is not known if these MDM2-binding drugs block p53 ubiquitination by MDM2 directly or effect other MDM2 protein interactions with factors such as E2F. Nevertheless, MDM2 has served as an example of how to disrupt a protein-protein interface that is normally thought to be generally beyond the possibility for small molecule drug design.

3.2.2. Histone de-Acetylase, Methylase, and Acetylase Inhibitors

p53 activation is associated in part by the binding of the co-activator p300 that is essential for p53 to induce genes that mediate its tumour suppressor activity. The p300/CBP family of proteins participate in many physiological processes, including proliferation and differentiation, and apoptosis (Janknecht, 2002). p300 functions as transcriptional co-activator, which is involved in multiple, signal dependent transcription events (Chan & La Thangue, 2001). Viral onco-proteins, such as the adenoviral E1A and SV40 T antigen possess high affinity for specific binding sites with p300, and results in loss of cell growth control, enhancement of DNA synthesis, and blocks in cellular differentiation (Roth et al., 2003; Shikama et al., 2003). p300 contains three broad functional domains including: (*i*) an acetyltransferase domain that mediates substrate

acetylation; (*ii*) a bromodomain, which is implicated in binding to acetylated amino acids; (*iii*) a variety of LxxLL, PxxP, and other transactivation peptide-binding domains (Grossman, 2001). The coordinated function of these domains is thought to mediate the role of p300 as a bridge, scaffold, and/or catalyst for binding to transcriptional components, like p53, and the activation of gene expression (Chan and La Thangue, 2001).

The tetrameric nature of p53, its allosteric properties, the enzymatic activity of p300 involved in its activation of p53, and the multiple transactivation-peptide binding domains of p300 make the p300:p53 complex an attractive model to understand the dynamic nature of a biological machine and for developing drugs that disrupt a protein-protein complex. The molecular mechanism by which p53 tetramers interact with p300 and how this modulates acetylation are beginning to be defined biochemically. Reconstitution of purified forms of the p300-p53 protein complex *in vitro* followed by validation *in vivo* have identified sequential stages in p53-p300 complex dynamics and include: (*i*) phosphorylation of the LxxLL motif at Thr18 or Ser20 by enzymes like CHK2 (Craig et al., 2003; Shieh et al., 2000); (*ii*) the stabilization of p300 to the phospho-LxxLL activation domain (Dornan & Hupp, 2001); (*iii*) docking of p300 docking to two contiguous phospho-LxxLL and PxxP activation domains in p53 (Dornan et al., 2003a); (*iv*) sequence-specific DNA-binding by p53 which induces a conformational change in the tetramer forcing acetylation to be DNA-dependent and PxxP-dependent (Dornan et al., 2003b); and (*iv*) acetylation of DNA-bound p53 that in turn stabilizes the p300:acetylated p53:DNA complex. How this multi-domain scaffold arranges itself onto an "octavalent" substrate like p53 is not known. However, the various phospho-LxxLL and PxxP-peptide binding domains of p300 map to regions distinct from the classic C/H1 and C/H3 p53-activation binding domains (Dornan et al., 2003a; Grossman, 2001). Some of these p300 miniproteins can function as dominant negative inhibitors of p300-coactivated stimulation of p53-dependent gene expression in cells and small peptides that bind to the p300 mini domain can inhibit p300-coactivated gene expression (Dornan & Hupp, 2001; Dornan et al., 2003b; Kung et al., 2000). These data provide leads and assays for drug screening for agents that can inhibit transcription factor activity by disruption of p300, similar in principle to ATM or CHK2 inhibitors, that could sensitize cells to death in combination therapies.

Antagonizing p300 coactivated gene expression and histone acetylation, are the so called histone de-acetylases like sin3A counteract the general and the specific functions of p300 and block the activation of the tumour suppressor transcriptome (Koumenis et al., 2001; Murphy et al., 1999; Zilfou et al., 2001). This effectively places the histone de-acetylase into a "proto-oncogenic" class of protein and histone de-acetylase inhibitors can stimulate tumour suppressor pathways and kill cells (Yu et al., 2002). Although such inhibitors may seem relatively non-specific inducers of gene expression, it should be noted that global suppression of tumour gene expression by promoter methylation is a widespread mechanism that promotes cancer progression in an epigenetic manner (Strichman-Almashanu et al., 2002; Suzuki et al., 2002). Human cancer progression is driven not only by mutations in tumour suppressor, but also by epigenetic silencing of tumour suppressor gene expression by G-C island methylation (Gaudet et al., 2003; Honorio et al., 2003; Waki et al., 2003). Methylation of genes like DAPK, p16, and E-cadherin are important steps in epigenetic cancer progression mechanisms. It is

encouraging that methylase inhibitors can restore tumour suppressor gene expression and a recent study identified a large panel of putative tumor suppressors as defined by genes which can be induced in tumour cells using methylase inhibitor drugs (Jones, 2003).

3.2.3. Cyclin-Dependent Kinase Inhibitors

p53 protein function as a growth suppressor can be antagonized by and itself can antagonize the main engines of the cell cyclin cyclin-dependent protein kinases (cdk's). These enzymes including cdk1, 2, 4, and 6 complex to a variety of cyclin subunit partners including cyclin A, B, D, and E and co-ordinately promote cell cycle progression (Malumbres et al., 2003). Both the cyclin subunit and the kinase subunit itself are the subject of independent drug-development programmes aimed at inhibiting these enzymes. The cyclin subunits function in part through a peptide-binding domain that can dock the kinase subunit onto its substrate protein (Kontopidis et al., 2003; Noble et al., 2004). This peptide binding activity of the cyclin can be exploited to inhibit cdk phosphorylation of substrates including p53 and pRB (Adams et al., 1999; Luciani et al., 2000), although small molecular weight drugs that mimic the cyclin-binding peptides and bind to the cyclin subunit have not yet been developed (Kontopidis et al., 2003). The kinase subunit has been the subject of more sophisticated drug design, since the natural plant product, Olomoucine, has been modelled and improved into a specific active site inhibitor (Meijer et al., 1997). Two such key drugs that bind to cdk, including Roscovitine (McClue et al., 2002) and NU2058 (Arris et al., 2000; Gibson et al., 2002) have different pharmacologies; both drugs can induce a growth arrest or apoptosis depending upon cell type, but one stimulates p53 activity and the other attenuates p53 activity (Ferguson et al., 2004). Such a difference could in theory be exploited in the clinic if it were known whether a particular cancer would respond better to p53 stimulation or to p53 inhibition.

3.3. Mimicking p53 Effectors

The p53-inducible gene product, the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf}$, has been the subject of anti-cancer drug design programmes, as is the most well characterized gene product induced by p53 (Dotto, 2000). Deletion of the $p21^{Cip1/Waf}$ gene does not alter cancer incidence like deletion of the p53 gene does. However, in a Wnt-1 mutant background, $p21^{Cip1/Waf}$ gene dosage has a significant impact on cancer development and $p21^{Cip1/Waf}$ is defined as a tumour modifier (Jones et al., 1999). The steady-state levels of $p21^{Cip1/Waf}$ protein are controlled in response to signalling changes after phosphorylation via PDK1 and a PKC signalling pathways (Scott et al., 2002). Further the ability of small inhibitory domains of $p21^{Cip1/Waf}$ to function as cell-cycle inhibitors has formed the basis for drug-discovery programmes aimed at replacing p53 function in cancer cells (Ball et al., 1997). Synthetic cyclin-dependent kinase inhibitors that mimic $p21^{Cip1/Waf}$ protein have been developed (McClue et al., 2002) and these can both activate the wtp53 response (Blaydes et al., 2000) as well have independent anti-proliferative effects (Zheleva et al., 2002), suggesting that cyclin-dependent kinase inhibitors will prove to be an important portfolio drug for cancer treatment. In effect, therefore, mimetics of $p21^{Cip1/Waf}$ can function as mediators of p53-driven tumour suppression. A similar peptide mimetic approach has led to the development of small

peptides derived form the p53-induicble gene product Bax (Cosulich et al., 1997) identifying a second effector of p53 that has been developed into small molecular weight mimetics.

4. CONCLUSION

The p53 pathway functions as a key coordinator of the cellular response to damage where it can be assembled or disassembled through a set of specific protein-protein interactions. Each one of these protein-protein complexes can in theory be the target of an agent that can stimulate p53 or inhibit p53, and thus provide therapeutic value depending upon tumour type. As one example, one natural molecule has been modelled into synthetic drugs like Roscovitine or NU2058 that inhibit cyclin-dependent kinases and halt cell-cycle progression, though with opposing effects on p53 activity. The p53-inhibitory pathways involved in ubiquitination or nuclear export have also been targeted for rationale drug-development that may form the basis for novel anti-cancer drug treatments in future.

Controlling p53 regulators like ATM or MDM2 seem obvious drug targets, but p53 is often mutated in human cancers and there is no necessarily obvious value in blocking MDM2 in a cancer with mutant and inactive p53. However, one of the most encouraging realizations in recent years is that mutant p53 protein is in an equilibrium between different conformational states, that the regulatory domains in p53 control its core DNA-binding domain conformation, and that cancer-prone mutations can alter the normal conformational equilibrium within the tetramer. The ability to exploit these properties provide a mechanism to reactivate mutant p53 protein has led to the identification of numerous small molecular weight agents that can activate mutant p53 pathway in cancer cells. Further, a second natural product Geldanamycin, which binds HSP-90 and alter mutant protein folding in cells, has been improved to also function as an effective anti-cancer agent. The continued accumulation of a battery of small molecules capable of stimulating both wild-type and mutant p53 *in vivo* raises expectations of developing anti-cancer drugs that can be used in combination with sophisticated diagnostics to treat cancer rationally.

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GROWTH FACTORS, RECEPTORS, AND KINASES: THEIR EXPLORATION TO TARGET CANCER

Chapter VIII

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1. INTRODUCTION

All cells have identical genetic compositions but perform different functions. Extracellular signals instruct cells on their functions from exposure to a wide range of stimuli from their external environment or from other cells. The balance between these often-competing signals determines the cell's fate. In an appropriate environment, cellular signals promote survival, proliferation, differentiation, adhesion, and migration. Growth factors induce signals in cells essential for these responses. When cells are damaged or are in an inappropriate environment such as withdrawal of growth factors from the extracellular environment, cells often undergo apoptosis. Alterations in this balance between cell survival and apoptosis promote the development of diseases such as cancer. Growth factor signaling in cancer cells is often altered, promoting cell survival through blocking apoptotic signaling pathways and contributing to cancer progression.

Growth factors illicit very specific cellular responses through binding to cell surface receptors. Cell surface receptor proteins bind growth factors (ligand) with high affinity and convert the extracellular event into one or more intracellular signals that alter the behavior of the target cell. These signaling pathways are regulated by the amount of ligand present, the level of cell surface receptors, and the present of both positive and negative signaling regulators. There are multiple growth factors and receptors. This indicates the complexity and specificity of signaling pathways and allows for a wide range of responses to signals. Some growth factors are highly specific in their function and the cell types they affect while others are broader in spectrum. Signals are transduced by activation of protein kinases. These kinases phosphorylate specific amino acids on target proteins eliciting a change in their function. This leads to further activation of other proteins resulting in a specific cellular function. In cancer cells, abnormal activation of growth factor induced signaling pathways involving protein kinase occur and are due to

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over expression of normal growth factor receptors (gene amplification or increased transcription), autocrine stimulation with overproduction of growth factors, increased activation of protein kinases, or constitutive activation of a mutant receptor (Barton et al. 2001). These abnormalities have been targeted by chemotherapeutic drugs to inhibit their functions and selectively induce apoptosis in cancer cells.

The regulation of apoptotic signaling pathways was extensively discussed in previous chapters. In this chapter, the understanding of the regulation of growth factor signaling in both normal and cancer cells will be explored, specifically growth factors contributing to cell survival (blockade of apoptosis) and to cancer progression. Finally, the development of a wide range of cancer therapeutic drugs designed to target growth factor signaling pathways will be described.

2. GROWTH FACTORS AS A SURVIVAL SIGNAL

Growth factors are signaling molecules that allow an external signal to be sent to the cell. This signal enables the cell to respond with an appropriate response. Different types of signals require a variety of different responses including differentiation, proliferation, migration, or survival. Growth factor signaling promotes cell survival since withdrawal of growth factors often causes cells to undergo apoptosis. Uncontrolled growth factor signaling also leads to a lack of apoptosis and unregulated cell growth contributing to the development of cancer. Described below are some growth factors that protect cells against apoptosis and contribute to cancer progression.

2.1. Epidermal Growth Factor

Epidermal growth factor (EGF) is a polypeptide growth factor that stimulates the growth and differentiation of epithelial and epidermal cells and is involved in cell survival. EGF receptor signaling has been found to be involved in different developmental processes such as cardiac and neural development, glial cell development and the later stages of mammary gland development during pregnancy when EGF-like ligands secreted by mesenchyme stimulate growth and branching of the mammary duct (Yarden 2001).

EGF sends extracellular signals to the cell by binding to EGF receptors, the ErbB receptor family. The ErbB family consists of four closely related tyrosine kinase transmembrane receptors:ErbB1/EGFR, ErbB2/HER2/neu, ErbB3/HER3, ErbB4/HER4. EGF-like ligands bind to ErbB1, heregulin-like ligands bind to ErbB3 and ErbB4, and EGF and neuregulin-like ligands bind to ErbB1 and ErbB4 (Vereb et al., 2002). No ligand has been found that directly binds ErbB2, indicating that it is a coreceptor. The ErbB receptors upon binding to their ligands form either homo- or hetero-dimers leading to activation of the receptors. ErbB receptors bound to ligand dimerize with ErbB2 increasing their signal-transducing and cell-growth stimulating abilities. Activated receptors tyrosine phosphorylate specific substrates that tranduce signals to the nucleus leading to cellular responses such as increased expression of anti-apoptotic genes.

Loss of function of EGF, as seen through loss of receptor binding, has been shown in a number of studies. Mice lacking EGF receptors have abnormal eyes and epidermal tissues and die due to defects in the development of epithelial organs. Loss of ErbB1 leads to embryonic or perinatal lethality with mice showing abnormalities in multiple

organs. ErbB2 null mice die due at midgestion due to malformation of the heart similar to ErbB3 knockout mice die due to defective valve formation in the heart as well as neural crest defect and lack of Schwann cell precursors (Olayioye et al., 2000). Increased levels of apoptosis could partially explain these defects.

Many human solid tumours (epithelial) express high levels of EGF receptors that often correlate to poor prognosis. The homology of EGF receptors and the v-erbB oncogene establishes the receptor as a cellular oncogene (Mendelsohn, 2001). Amplification or over expression of ErbB2 has been found in breast and ovarian cancer and correlates to poor clinical outcome including shortened survival and short time to relapse. ErbB2 activation causes drug resistance through blockade of both the extrinsic and intrinsic apoptotic signaling pathways and has been linked with increased chance of metastasis and micrometastatic bone marrow disease. Mutation in the tyrosine kinase receptor can also be oncogenic. A point mutation in the transmembrane region of the ErbB2 gene has been found to induce dimerization in the absence of a ligand. As a result, this dimer is continually autophosphorylated and active. This mutation is found in certain breast and ovarian cancers and is associated with poor prognosis.

2.2. Insulin-like Growth Factor

Insulin-like growth factor (IGF) is involved with cellular proliferation and survival. IGF1 stimulates the proliferation of fat cells and connective tissue cells and also is important in joint cartilage maintenance and repair. IGF1 is the major mediator of the effects of growth hormone, therefore having a strong influence on cell proliferation and differentiation and is a potent inhibitor of apoptosis.

The IGF family consists of two ligands, IGF1 and IGF2, two cell surface receptors, IGF1R and IGF2R, and several IGF-binding proteins. The availability of unbound IGF1 to react with its receptor is determined by an IGF-binding protein (IGFBP). Circulating IGFs are bound to IGFBPs. Cleavage of IGFBP by specific proteases modulates the levels of free IGFs and IGFBP, and therefore their actions. IGF1 is made and released from the liver in response to growth hormone and has amino acid sequences similar to insulin. This close relationship is further shown by the fact that IGF1 and IGF2 are able to bind to the insulin receptor and IGF1R forms heterodimers with the insulin receptor. Most IGF1-null mice die after birth. Those that survive exhibit a delay in ossification and muscle development and are infertile. IGF2 is expressed in embryonic and neonatal tissues and disruption causes a reduction in growth during embryogenesis (Furstenberger & Senn, 2002).

IGF1 and IGF2 expression have been found in normal and breast tumor tissues and may influence breast cancer cell responsiveness to estrogen. Both have been shown to stimulate estrogen receptor (ER) positive breast cancer cell proliferation. Estrogen also has been shown to induce the expression of IGF2, some IGF receptors and IGF receptor substrates (IRS-1). Once interaction has occurred with the receptor, IGF1 is the predominant signaling molecule activated in ER-positive human beast cancer cells. Higher serum IGF1 levels and higher IGF2 expression is reported to be associated with poor prognosis in breast cancer patients (Zhang & Yee, 2000). The tumor suppressor gene p53 has been found to activate IGF-binding protein 3 (IGFBP3) expression in response to DNA-damaging ionizing radiation. Transfection of cells with IGFBP3 cDNA causes an increase in apoptosis. This could at least in part explain why breast carcinoma cells lacking wild-type p53 are relatively resistant to radiation. (Furstenberger & Senn,

2002). High serum concentrations of IGF1 and IGFBP3 also have been found associated with increased risks of prostate, colorectal, and lung cancers. IGF1 accelerates the progression of precancerous changes to invasive lesions. High IGFs levels have been shown to prevent apoptosis in response to chemotherapeutics and radiation and over expression of IGF-1R has been linked to drug resistance in HER2 over expressing breast cancer cells (Nahta et al., 2003). Raised IGFBP-1 serum levels have been seen in patients with primary liver cancer and ovarian cancer. Treatment with tamoxifen results in reduction of IGF1 serum concentrations (Furstenberger & Senn, 2002). A decrease in serum IGF may help reduce the risks of breast, prostate, and colon cancer. Over expression of IGF2 in colon cancer is associated with an aggressive phenotype (Furstenberger & Senn, 2002). High levels of both IGF1 and IGFBP3 may be markers for colorectal cancer (Zumkeller, 2001). Thus the levels of IGF1 and IGFBP3 in cancer could contribute to cell survival and drug resistance.

2.3. Fibroblast Growth Factor

Fibroblast Growth Factor (FGF) is involved in inflammation, wound healing, hematopoesis, angiogenesis, and embryonic development. FGFs are a family of 21 similar proteins that play a prominent role in skeletal and nervous systems development as well as the development of the prostate. FGF's ability to regulate angiogenesis allows it to control the induction of new blood vessels during the early stages of tumor development. Formation of new capillaries involves endothelial cell proliferation and cell migration that work together to break down the extracellular matrix. FGF is an important regulator in these processes. FGF also contributes to cell survival in a variety of cell types and cancer cells.

FGF binds to heparan-sulfates and glycosaminoglycans of the extracellular matrix creating a reservoir of FGFs on the cell surface that protects them from denaturation and proteolytic degradation. FGFs are released from the cell surface by mechanical damage of the cell's plasma membrane, complexing with a carrier protein that is co-secreted. Signaling occurs by binding to fibroblast growth factor receptors (FGFR) (Beau-Faller et al., 2003). Some FGFs also have a nuclear location signal that is thought to be important for FGF signaling (Cronauer et al., 2003).

FGF has been shown to increase the invasiveness of a variety of tumour cell types including prostate, bladder, kidney, breast, and pancreas (Cronauer et al., 2003). FGF has also been shown to regulate the induction of metalloproteinases (MMP) that degrade extracellular matrix proteins facilitating tumor metastasis in prostate, bladder, and renal cancers. FGF-2 expressing lung carcinoma cells instigated a survival response and proliferated while control cells undergo apoptosis. This sustained apoptosis *in vivo* prevents growth of metastatic foci, while FGF-2 induction of cell survival is responsible for growth of the lung metastases. Serum deprivation in the presence of 24 kDa FGF-2 also improved carcinoma cell survival. This indicates that FGF is a potent survival factor (Thomas-Mudge et al., 2004).

2.4. Platelet Derived Growth Factor

Platelet-derived Growth Factor (PDGF) stimulates connective tissues and neuroglial cells. It is involved in normal physiological processes and pathological conditions such as atherosclerosis, rheumatoid arthritis, and abnormal wound repair. Platelet-derived growth

factor has important functions during the embryogenesis, in particular for the development of the kidneys, blood vessels, lungs, and CNS. In these organs, connective tissue-like cell types are dependent on PDGF, including mesangial cells, pericytes, alveolar fibroblasts, and glial cells. The role that PDGF plays in the formation of connective tissue is also important during wound healing (Heldin & Westermark, 1999).

PDGF exists as two polypeptides linked by disulfide bonds in homo- or heterodimers. PDGF is stored in platelet granules and released following platelet aggregation. It is a chemotactic agent to white blood cells and fibroblasts and stimulates wound healing. PDGF has a short half-life, produces local effects and is not released into general circulation making it very specific. In atherosclerosis, PDGF made by the vascular wall macrophages, endothelial cells, and platelets, may cause proliferation of smooth muscle cells and migration. There are two types of PDGF receptors (PDGFR), α and β PDGF receptors. Activation of the protein-tyrosine kinase activity of these receptors occurs by ligand-induced dimerization or heterodimeriztion of PDGF receptor types.

The gene encoding the receptor for PDGF is a proto-oncogene, and mutated versions have been found in cancer. Expression of the cognate ligand, PDGF A-chain, is dramatically increased from low or undetectable levels in low grade (I and II) to high levels in high grade (III and IV) tumors, suggesting a role in tumor progression. Bronzert et al. (1987) found PDGF expression in human breast cancer cells. They suggest that production of PDGF by breast cancer cell lines may be important in mediating paracrine stimulation of tumor growth and cell survival. PDGFR also is implicated in metastatic breast cancer for which a phase II study is being conducted (Nahta et al., 2003) and in prostate cancer (Mathew et al., 2004). High levels of α-receptor expression are particularly found in tumors without epidermal growth factor (EGF) receptor gene amplification. In virtually all cases of glioblastoma multiform (grade IV tumors), there is a high expression of either EGF receptors or PDGF α-receptors. Thus activation of either type of receptor may be of critical importance in cell survival and tumor progression.

Vascular Endothelial Growth Factor (VEGF) also is a member of the PDGF family. VEGF is angiogenic and increases the permeability of vascular endothelium. Its active form is comprised of homodimers linked by disulfide bonds. VEGF receptors share a cluster of seven extracellular Ig-like domains that are important for ligand binding. They are highly expressed in vascular endothelial cells and are critical for the physiological and pathological growth, development and maintenance of blood and lymphatic vessels. Wu and co-workers found that plasma VEGF should be considered as a tumor marker for breast cancer progression, and inhibitors of angiogenesis should be factored into the treatment protocol for patients who demonstrate increase in plasma VEGF levels at any stage of the disease (Wu et al., 2002). ErbB signaling has been shown to regulate angiogenesis through the transcriptional upregulation of VEGF (Yen et al., 2002; Yen et al., 2000). In particular, co-over expression of ErbB-2 or ErbB-2/ErbB-3 heterodimers with VEGF is more potent in inducing tumor vascularization as compared to other receptor combinations (Yen et al., 2002; Yen et al., 2000). This indicates that the angiogenic phenotype of a tumor may vary depending on whether the tumor over expresses single or multiple ErbB receptors.

Both VEGF and PDGF expression have also been implicated in blocking apoptosis, either through exogenous addition or in an autocrine manner. Leukemia and breast cancer cells are resistant to chemotherapeutic drug induced apoptosis in the presence of PDGF

or VEGF. Production of PDGF or VEGF during hypoxia has been demonstrated to block hypoxia induced apoptosis.

2.5. Hepatocyte Growth Factor

Hepatocycte Growth Factor (HGF) stimulates proliferation of hepatocytes, renal tubular epithelial cells, epidermal keratinocytes, and melanocytes making it essential for the development of the liver and other tissues in the mammalian embryo. A single chain precursor, pro-HGF, is cleaved to 70 kDa and 30 kDa chains that are linked by a disulfide bond. Because pro-HGF is similar to the heparin binding domain, secreted pro-HGF binds to the extracellular matrix. HGF activator (HGFA), a serine protease, is capable of binding to heparin molecules and converts proHGF to mature HGF (Ohnishi and Daikuhara, 2003) (Ohnishi & Daikuara, 2003). HGF is also known as scatter factor (SF) because it stimulates the motility of epithelial cells (Ohnishi & Daikuhara, 2003) and is involved in the control of cell dissociation and migration and invasion into extracellular matrices (Trusolino et al., 1998). Activation of HGF occurs when tissue is damaged resulting in blood vessel injury. This leads to thrombosis during which thrombin, a serine protease, cleaves the inactive pro-HGFA to form active HGFA. HGF is then activated by the active HGFA in the injured tissues (Ohnishi & Daikuhara, 2003). Co-localization of HGFA and HGF ensure that HGF is activated at the site of injury for tissue regeneration.

HGF acts through a transmembrane tyrosine-kinase receptor, the c-Met proto-oncogene. This is the only HGF receptor identified to date. Point mutations in c-Met have been identified in numerous cancers indicating its role in carcinogenesis (Ohnishi & Daikuhara, 2003). When stimulated in malignant cells, both are able to enhance cell motility, invasion, and eventually metastasis. HGF has been linked to lung cancer, especially adenocarcinoma of the lung (Beau-Faller et al., 2003). HGF has also been found to promote vascular endothelial growth factor (VEGF) expression and induce angiogenesis. Results by Horie et al. (1999) demonstrated that both HGF and c-Met mRNA were detected in renal cell carcinoma samples. They also found that HGF inhibited Fas-induced apoptosis, and that HGF enhanced invasiveness in vitro. This indicates that HGF/c-Met signaling pathway may promote tumor progression and cell survival of renal cell carcinoma cells.

2.6. Lysophosphatidic Acid

Lysophosphatidic acid (LPA) is a phospholipid growth and survival factor that plays a role in development in a wide range of tissues and cell types. LPA is responsible for cell responses such as survival, cell proliferation, attachment, migration, growth arrest, and smooth muscle contraction. LPA is generated and released during blood clotting and is involved in wound healing and tissue regeneration (Fang et al., 2002). Activated platelets, adiposcytes, fibroblasts, and endothelial cell can produce LPA. LPA is known to cause platelet aggregation to release more LPA as well as platelet-derived growth factor. This positive feedback system leads to the continuous growth of vascular smooth muscle cells (VSMCs). LPA also increases the intracellular concentration of free calcium in VSMCs and elevates blood pressure. LPA has been shown to protect the heart from ischemia and reperfusion-induced damage due to its anti-apoptotic effect. Two LPA receptors, LPA_1 and LPA_2, are expressed in the embryonic cerebral cortex, suggesting roles for LPA signaling in cortical formation.

LPA has been detected in the first stage of ovarian cancer, suggesting that it may represent a useful marker for the early detection of the disease. LPA is believed to stimulate DNA synthesis cell migration and the proliferation of ovarian cancer cells (Fang et al., 2002). LPA also is able to stimulate anchorage-dependent and anchorage-independent growth of ovarian cancer cell lines partially through inhibiting anoikis. Elevated levels of LPA have been found in multi-myeloma patients and contribute to cell survival in a number of leukemia cell lines. Thus, besides proteins, lipids such as LPA could act as survival factors in cancer.

2.7. Other Growth Factors

There are too many other growth factors to be covered in detail within this chapter but several other growth factors are worth mentioning. Interleukins are large family of growth factors that stimulate activation, differentiation, proliferation and survival of a variety of white blood cells and are secreted by activated white blood cells. Nerve Growth Factor (NGF) is a neurotrophic factor that promotes the differentiation and survival of neurons in the central and peripheral nervous systems. It is derived from and regulates the density of innervation of the target tissues. NGF attracts neurons to a tissue and protects neurons from death and enhances the growth of cholinergic neurons projecting to the forebrain. NGF also has been found to target non-neuronal tissues such as immune-related haematopoetic cells (Harmer et al., 2003). Withdrawal of NGF often induces apoptosis in neurons during development.

3. CELL SURFACE RECEPTORS

Growth factors transmit their effects on a cell by binding to three general types of cell surface receptors:tyrosine kinase receptors, non-tyrosine kinase receptors, or G-protein coupled receptors (Figure 1). Tyrosine kinase receptors activate signal transduction pathways by tyrosine phosphorylating their own receptor and specific substrates bound to the receptors. Non-tyrosine kinase receptors activate signal transduction by recruiting intracellular tyrosine kinases to the receptor that subsequently phosphorylates the receptor and proteins bound to the receptor. G-coupled receptors activate signal transduction by activation of G-proteins associated with the receptor. Inappropriately expressed growth factor receptors through point mutation, rearrangement, or over expression are often seen in tumors and lead to the development, growth, and reduced apoptosis of tumor cells and may predict poor prognosis for patients. These growth factor receptor alterations also contribute to drug resistance associated with blockade of apoptotic signaling pathways. Besides cancer, dysregulation of a number of receptor kinases has been demonstrated to be important for the genesis of a variety of diseases and developmental abnormalities. Thus, understanding the regulation of growth factor receptor function represents an important step in developing effective therapeutic interventions for these diseases especially cancer.

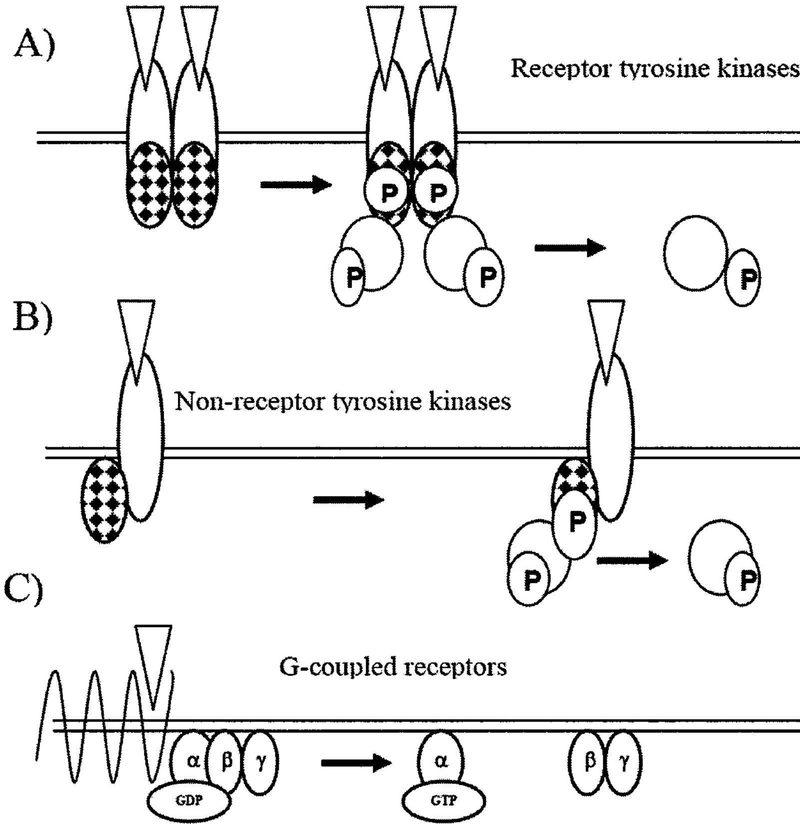

Figure 1 Three main types of cell surface receptors Cell surface receptors are generally classified into three large families, A) receptor tyrosine kinases, B) non-receptor tyrosine kinases, and C) G-coupled receptors. Receptor tyrosine kinases contain a tyrosine kinase domain (checkered region) on the intracellular portion of the receptor. Upon ligand binding (triangle), the receptors often dimerize causing tyrosine phosphorylate the receptor itself and/or other substrates bound to the receptor (denoted by a circular P). Non-receptor tyrosine kinases do not have an intracellular kinase domain but instead bind to intracellular tyrosine kinases (checkered circle) that become activated upon ligand binding (triangle). This leads to the tyrosine phosphorylation of many substrates (denoted by a circular P). G-coupled protein receptors are seven across the membrane receptors that bind to G proteins. Upon ligand binding, the G-proteins become activated through dissociation of the α subunit and the βγ dimer. This is induced by exchange of a GDP to a GTP in the α subunit. This dissociation leads to induction of downstream signaling pathways.

3.1. Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) represent a large family of evolutionarily conserved enzymes important in cell growth, differentiation, development and cell survival. They each have an extracellular growth factor binding domain, a tyrosine kinase domain that phosphorylates proteins on tyrosine residues, and a carboxyl terminal segment with multiple tyrosine residues for autophosphorylation. Binding of the ligand to the extracellular domain causes activation of its intrinsic kinase activity and rapid

internalization of the receptor-ligand complex into the cell. Receptors exist as monomers in the absence of a ligand. Ligand binding triggers receptor dimerization that leads to autophosphorylation of receptor kinases. The activated tyrosine kinase dimer phosphorylates the carboxy-terminal tyrosine residues on itself, then on other kinase proteins converting them into an active state. Many of these other proteins are also kinases and in this way a cascade of expanding phosphorylations occur within the cytosol. Some of these cytosolic kinases act directly on gene transcription by entering the nucleus and transferring their phosphate to transcription factors thus activating them. Others act indirectly through the production of second messengers. In this way an extracellular event gets converted into an intracellular signal that alters the behavior of the target cell.

Phosphorylation is an effective means of altering the activity of target proteins because it can occur in less than a second or it can take hours. The rate is regulated to match the physiological conditions. Phosphorylation can have amplified effects. A single activated kinase can phosphorylate many target proteins. This phosphorylation produces binding sites for proteins with Src Homology 2 (SH2) domains, setting off a cascade of biochemical reactions that ultimately leads to cell growth or survival. Although phosphotransfer reactions catalyzed by various RTKs are similar with regard to their basic mechanisms, their biological functions demonstrate a considerable degree of specificity.

Of the growth factors examined in this chapter, many interact with tyrosine kinase receptors including EGF, IGF, FGF, PDGF, and HGF. For example, ligand binding to EGF receptors results in dimerization of the receptors in different combinations. Homodimers of ErbB1 and ErbB2 have the weakest mitogenic activity. The ErbB2/ErbB3 heterodimer has the strongest mitogenic activity. Of all the receptors, ErbB2/HER2/neu forms the most stable complex by decreasing the rate of ligand dissociation from the receptor. This results in a stronger, prolonged activation of the EGF receptor signaling network (Karunagaran et al., 1996). Over expression or structural alterations of all ErbB family members have been found in many different types of cancer. Over expression of the ErbB3 receptor is associated with tumorigenesis. Amplification of ErbB2 leads to a constitutively activated receptor and transformation of cells. In breast cancer this is indicative of a more aggressive disease and drug resistance (Vereb et. al., 2002). The heterodimer of ErbB2 and ErbB3 is especially potent because they simultaneously stimulate the RAS/MAPK cascade and the PI3K/AKT pathway (see Signaling pathways section). The EGFR signal is inactivated through endocytosis, a process initiated by step-wise recruitment of two E3 ubiquitin ligases that culminates in the degradation of active receptors in the lysosome (Yarden, 2001). However, the ErbB2 and ErbB3 heterodimer is able to evade the restraining effect of receptor endocytosis.

3.2. Non-receptor Tyrosine Kinases

As mentioned earlier, non-receptor tyrosine kinase receptors signal through intracellular tyrosine kinases. In total, 32 genes encoding for non-receptor protein tyrosine kinases (PTKs) are present in the human genome including: Abl, Jak, Csk, Syk, Tec, Fak, Fes, Frk, and, Ack. Cellular Src (c-Src) was the first PTK identified and is homology to the Rous sarcoma virus oncogene protein pp60 (v-Src) which causes tumors in chickens (Brickell, 1992). Rous sarcoma virus is a tyrosine kinase that is constitutively active because it does not need to be phosphorylated by a receptor kinase. Oncogenic

forms of PTKs occur through altered regulation or expression of the endogenous protein and by virally encoded genes such as v-src. A lot of redundancy is built into this complex tyrosine kinase signaling system. In fact, when the action of Src protein is blocked in animals, there is no discernable effect. Other similar proteins appear to be able to fill in for the lost function, such as the Hck protein, and the Abl protein. c-Src has been shown to be elevated in epithelial cancer (colon and breast cancer, cell lines of ovarian, esophageal, lung, gastric) and associated with stage of disease or malignant potential (Summy & Gallick, 2003). Src is activated through mutation, phosphorylation, or other protein interactions. Tyrosine kinase receptors such as PDGFR can also activate non-receptor tyrosine kinases. Src SH2 domain binds to PDGFR when it is targeted to the cell surface, causing it to become active. Src also becomes active after treatment with PDGF or EGF and becomes associated with the actin cytoskeleton. PDGFR has been shown to phosphorylate tyrosine residues in the SH2 and SH3 domains of Src thereby altering its activity. Furthermore, studies using inhibitory mutant Src proteins or antibodies have shown that Src is important for mitogenic activity of PDFG and EGF specifically in cell cycle progression (Frame, 2002).

Intracelluar tyrosine kinases can be activated further downstream from cell surface receptors. Abl is another non-receptor tyrosine kinase that is activated upon DNA damage and growth factor activation but fails to associate direct with cell surface receptors. In chronic myelogenous leukemia (CML), a translocation of the abl gene from its normal location on chromosome 9 to a new location on chromosome 22 in the breakpoint cluster region (BCR) often occurs. This breakage and reattachment leads to an altered abl gene and the resulting *BCR-Abl* fusion gene encodes a chimeric protein with strong and constitutive tyrosine kinase activity (Pasternak et al., 1998). This so-called Philadelphia chromosomal translocation contributes to cancer progression.

3.3. G-coupled Receptors

The G protein coupled receptor family has over a thousand members and are activated by a wide variety of stimuli. Upon ligand binding, these receptors interact directly with guanosine triphosphate (GTP) binding proteins (G proteins). These trimeric G proteins consisting of α, β and γ subunits are bound to the receptor on the inner plasma membrane. The α subunit is bound to guanosine diphosphate (GDP), and less tightly bound to the β and γ subunits. The $\beta\gamma$ subunit forms a dimer. In the basal unstimulated state, the α subunit is bound to GDP. Upon stimulation the GDP is replaced with GTP molecule. This process is induced and accelerated by guanine nucleotide exchange factor. The GTP-α subunit dissociates with the $\beta\gamma$ dimer. Both the GTP-α and $\beta\gamma$ dimer are capable of stimulating substrates. The activated G proteins may 1) activate protein kinases such as intracellular tyrosine kinases, 2) interact with adenylyl cyclase to produce cyclic AMP (cAMP), 3) activate the phosphoinositide pathway, and 4) activate the MAP kinase pathway. Activation of these G protein coupled signaling pathways has been implicated in cell proliferation and cell survival.

4. SIGNALING PATHWAYS

We have discussed different types of growth factors and their corresponding receptors that initiates signaling pathway in the cell leading to survival. After binding of

the growth factors to their corresponding receptors, many tyrosine kinase receptors (RTKs) dimerize causing the phosphorylation of tyrosine residues on the receptor. Intracellular tyrosine kinases are also capable of tyrosine phosphorylating residues on both RTKs and non-receptor tyrosine kinases. These tyrosine phosphorylated sites bind to a region called the SH2 domain found in a number of intracellular signaling proteins. This SH2 domain directs the protein to the activated RTK. Phosphorylation produces binding sites for proteins with SH2 domains such as intracellular docking proteins or adaptor proteins such as Grb2, Nck and Shc. This leads to the activation of serine threonine kinases that activate other kinases and transcription factors. This kinase cascade results in amplification of the signal leading to cell survival and other cellular responses. Listed below are some of the signal transduction pathways that have been implicated in cancer progression and cell survival (Fig. 2).

4.1. MAP Kinase Pathways

MAPKs are a superfamily of protein serine-threonine kinases that are activated by diverse stimuli via protein kinase cascades. They are the final components of the cascades, activated by phosphorylation by mitogen-activated protein kinase kinases that in turn are activated by mitogen-activated protein kinase kinase kinases (MAP kinase kinase kinases). Families of these MAPKs include extracellular signal-regulated kinases (ERKs), stress-activated protein kinases (SAPKs) (also known as c-jun N-terminal kinases, JNKs), and p38-mitogen-activated protein kinases. MAP kinases can translocate into the nucleus and phosphorylate transcription factors (c-Fos and c-Jun) making them active and able to regulate gene expression (Minden et al., 1994).

Growth factors activate Erks through their ligation to cell surface receptors leading to cell survival. Tyrosine phosphorylation of cell surface receptors recruits adaptor proteins to the receptor such as GRB2. GRB2 is constitutively bound to son of sevenless (SOS) protein. SOS is a guanyl nucleotide-release protein (GNRP). SOS recruitment to the plasma membrane by the activated cell surface receptor causes the small G protein RAS to release GDP and exchange it for GTP. When RAS has GTP bound to it, it becomes active. Inactivating RAS requires a GTPase activating protein (GAP) that promotes the hydrolysis of GTP to GDP. Activation of RAS leads to the activation of a serine threonine kinase called RAF-1. RAF-1 kinase then phosphorylates and activates another cellular dual specific kinase called MEK. Activated MEK then phosphorylates and activates another serine threonine kinase called extracellular regulated kinase (ERK) a member of the mitogen-activated protein kinases (MAPK) family of kinases. This activation results in phosphorylation of a variety of substrates including 90 kDa ribosomal S6 protein kinase (Rsk), cytosolic phospholipase A2, and transcription factors c-Myc, NF-IL6, Tal-1, Ets-2, and Elk. This leads to the further phosphorylation of other proteins such as Rsk phosphorylation and inactivation of the pro-apoptotic protein Bad and increased expression of target genes such as anti-apoptotic Bcl-2 family members.

Unlike ERK activation, JNK and p38 MAPKs have been implicated in apoptotic signaling. In rat PC12 cells, withdrawal of NGF leads to sustained activation of JNK and p38 whereas Erk activation is inhibited. The effects of dominant-interfering or constitutively activated forms of various components of the JNK, p38 and Erk signaling pathways demonstrates that activation of JNK and p38 contributes to apoptosis and Erk activation contributes to cell survival. In contrast, many growth factors activate JNK and

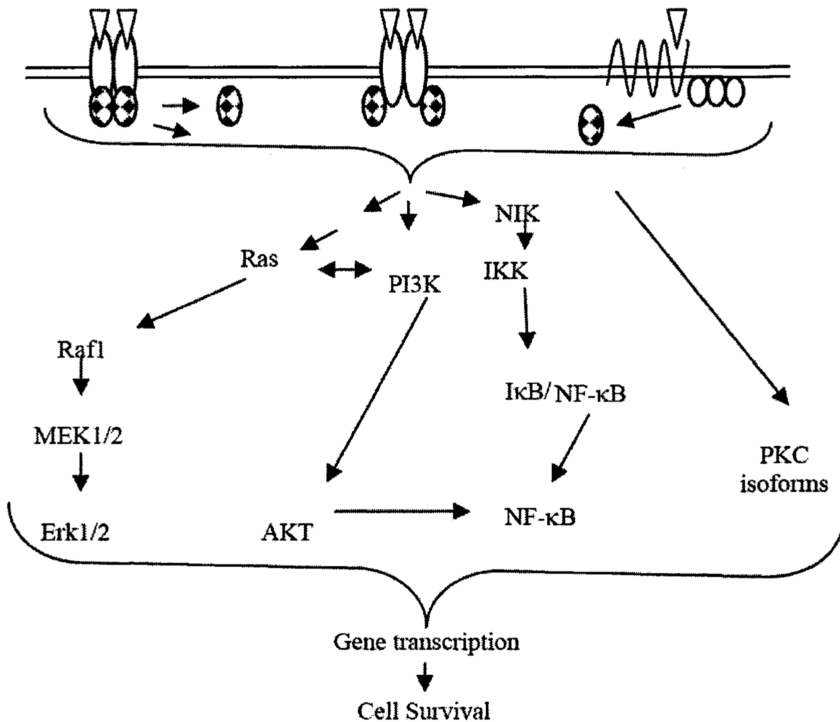

Figure 2 Signal transduction pathways leading to cell survival Ligand binding of all three types of cell surface receptors leads to the activation of intracellular kinases (checkered circles). This causes tyrosine phosphorylation of many substrates leading to the activation of small G-protein RAS that leads to activation of the serine threonine kinase RAF1. RAF1 phosphorylates and activates the kinases MEK1/2. MEK1/2 then phosphorylates the MAP kinases ERK1/2. PI3K is a lipid kinase that causes the activation of a serine threonine kinase AKT, this leads to gene transcription and inactivation of pro-apoptotic genes. Inhibitor of NF-κB kinase (IKK) becomes activated by several NF-κB inducible kinases (NIK). This leads to phosphorylation of the inhibitor of NF-κB (IκB) protein and degradation by proteasome complexes leading to NF-κB activation. Finally, ligation of cell surface receptors leads to the activation of PKC isoforms and cell survival.

p38 without inducing apoptosis. In certain circumstances, JNK and p38 could be protective against apoptosis through growth factor activation and seems to be dependent upon the transient activation of these MAPKs. Thus, the context of MAPK activation and cell type are critical for their function.

Deregulation of MAPK activation is often found in cancer. Activation of ERK has been observed in ErbB2 over expressing breast and ovarian cancer cells upon treatment with heregulin (Vereb et al., 2002). ERK activation is also elevated in leukemia, and epithelial cancers. HGF cell proliferation activity involves the ERK pathway mediated through the binding of Grb2 to the Tyr1356 of c-Met oncogene. Increased expression of IGF1 receptor and IRS-1 in cancer cells results in an enhanced response to IGF1 resulting in increased downstream signaling through ERK activation. Finally, elevated levels of LPA results in proliferation and survival dependent upon ERK activation (Fang et al., 2002). JNK signaling pathways may also contribute to cancer progression. c-Jun is highly expressed in many cancers and the JNK pathway seems to be important for c-Met

oncogene-induced transformation. The JNK kinase MKK4 is also activated in many cancers and is mutated in pancreas and lung carcinomas. The JNK pathway, in contrast, seems to sensitize cancer cells to chemotherapeutic drugs. Similar to JNK, p38 MAPK seems to have a complex role in cancer. For example, p38 promotes and suppresses proliferation in leukemia cells. Overall, MAPK pathways regulate cell survival in complex networks that depend upon the microenvironment of the cell and the cell type contributing to cancer progression.

4.2. PI3K/AKT Signaling Pathway

Growth factor signaling has been shown to prevent apoptosis (Gibson et al., 1999; Gibson, 2004). Many growth factor (EGF, PDGF, NGF, and IGF) receptors transmit survival signals through the Phosphatidylinositol-3 Kinase (PI3K) pathway. PI3K is able to transduce proliferation signals and is activated through receptor and non-receptor tyrosine kinases or G protein-coupled receptors. This is accomplished through recruitment of PI3K to the plasma membrane mediated by binding of its SH2 domain to tyrosine phosphorylated proteins. An example of this is Gab1 binding to PI3K and Gab1 binding to the growth factor receptor c-Met (Ohnishi & Daikuhara, 2003). PI3K activation catalyzes the transfer of a phosphate group from ATP to phosphatidylinositol generating a 3'-phosphatidylinositol phosphate (PIP). PIPs act as binding sites for proteins with pleckstrin homology (PH) domains. PI3K signaling is negatively regulated by PTEN that dephosphorylates PIPs. AKT is a serine/threonine kinase that contains a PH domain. AKT's PH domain binds to phosphatidylinositols generated by PI3K translocating AKT to the plasma membrane where it becomes phosphorylated and activated. PI3K can also activate RAS leading to activation of the ERK signaling pathways. This provides cross-talk between signaling pathways in cells.

AKT (c-AKT, also called protein kinase B or PKB) is serine threonine kinase that was first characterized as the human homologue of the viral oncogene *v-akt* from the transforming retrovirus AKT8 (West et al., 2002). AKT activation controls cellular function through phosphorylation of downstream targets. Many of these targets prevent apoptosis through different mechanisms. AKT phosphorylates the Bcl-2 family member Bad. Bad is a BH3 only Bcl-2 family member that upon over-expression induces apoptosis. Bad associates with anti-apoptotic Bcl-2 family members such as Bcl-2 blocking them from associating with and preventing apoptosis mediated by pro-apoptotic Bcl-2 family members such as Bax or Bak. AKT induced phosphorylation of Bad causes Bad to bind to 14-3-3 proteins, freeing anti-apoptotic Bcl-2 family members to bind to pro-apoptotic Bcl-2 family members and prevent apoptosis. AKT can also phosphorylate Inhibitor of NF-κB kinases (IKKα and β) leading to the activation of NF-κB transcriptional activity. NF-κB induces many anti-apoptotic proteins that promote cell survival (discussed in more detail below). Besides NF-κB, AKT also activates the transcription factor CREB that has been implicated in increased expression of anti-apoptotic genes such as Mcl-1. AKT also directly phosphorylates transcription factor Forkhead. This transcription factor regulates many pro-apoptotic proteins such as FasL and upon AKT phosphorylation its transcriptional activity is inhibited. Finally, AKT phosphorylates caspase-9 preventing its cleavage and protease activity. Thus, AKT activation blocks apoptotic signaling pathways in different ways.

Growth factor survival responses often involve the activation of AKT. EGF has been shown to block death receptor induced apoptosis. Kinase-inactive AKT expression blocked these EGF-protective responses. In contrast, inhibition of EGF stimulation of extracellular-regulated kinase (ERK) activity did not affect EGF protection (Gibson et al., 2002). Furthermore, EGF mediated activation of AKT induced expression of the anti-apoptotic protein Mcl-1 blocking death receptor induced apoptosis. Activation of IGF1 receptors has shown protection against cell death by inhibition of apoptosis through AKT activation (Zhang & Yee, 2000).

Activation of the PI3K/AKT pathway also contributes to the formation of cancer. PI3K is often mutated in cancers increasing its kinase activity. Similarly, PTEN is mutated in many cancers including 55% of prostate and endometrial cancers and 33% in breast cancer (Simpon & Parsons). These mutations block PTEN phosphatase activity thereby amplifying PI3K signaling. In mice lacking PTEN, the ability of cells to induce apoptosis is impaired through elevated AKT kinase activity. AKT is constitutively activated in prostate, breast, ovary, lung, and liver cancers (Hersey & Zhang, 2003) indicating its importance in maintaining cell survival and proliferative properties in cancer cells. Blocking the AKT pathway sensitizes ErbB2/Her-2/neu transformed cells with wild-type p53 to chemotherapeutic drugs (Zhou et al., 2001). AKT also participates in the anti-apoptotic signal in the oncogene c-Met. Inhibition of AKT can induce apoptosis and is a target for future drug development to treat cancer.

4.3. Transcription Factor NF-κB

NF-κB is a transcription factor that is the central mediator of the human immune, inflammatory and cell survival responses. NF-κB is activated by different signaling events that ultimately activate a serine / threonine kinase that phosphorylates inhibitor of NF-κB kinase (IKKα/β). Upon upstream activation, IKK phosphorylates IκB, which targets it for ubiquitintion and proteasomal degradation. This exposes the nuclear localization signals of NF-κB and allows it to translocate to the nucleus. Once in the nucleus it regulates the transcription of both pro- and anti-apoptotic genes. Some anti-apoptotic genes it activates transcription are: Bcl-2, Bcl-x_L, c-FLIP and the inhibitor of apoptosis (IAP) family (XIAP, c-IAP1, c-IAP2). Growth factors such as EGF activate NF-κB and protects against apoptosis. ErbB1 expression alone or in combination with ErbB2 in NIH3T3 cells up-regulated Mcl-1 following EGF treatment mediated by NF-κB preventing death receptor induced apoptosis (Henson et al., 2003). Inhibition of NF-κB sensitizes cancer cells to a wide variety of chemotherapeutic agents. NF-κB has been identified in many cancers and plays a role in drug resistance. Similar to MAPKs, NF-κB also contributes to apoptosis through up-regulation of pro-apoptotic genes including Fas, FasL, DR4, and DR5. This up-regulation seems to be dependent on stimuli used, type of cell, and subunit of NF-κB activated (Shetty et al., 2002). Overall, NF-κB activation seems to be important for cancer progression and many therapeutic drugs have been developed targeting this pathway to induce apoptosis in cancer cells.

4.4. Protein Kinase C

The Protein Kinase C (PKC) family of serine/threonine protein kinases is a ubiquitous group involved in complex intracellular signaling processes that are mediated

through phospholipid hydrolysis. PKC has been implicated in a multitude of physiological functions in the cell. It plays a fundamental role in signaling mechanisms leading to mitogenesis and proliferation of cells, apoptosis, platelet activation, remodeling of the actin cytoskeleton, modulation of ion channels, and antigen presentation to T-cells and cytokine secretion. At least 11 isozymes of PKC have been identified and they are products of multiple genes and alternative splicing. PKC isoforms consist of a single polypeptide chain containing four conserved regions and five variable regions. PKC has been implicated in cell proliferation and tumorigenesis with increased expression of PKC-α in human breast tumors. Growth factors activate PKC isoforms and this activation has been implicated in growth factor cellular responses such as proliferation and cell survival.

5. TARGETS FOR CANCER THERAPY

We have discussed the role of growth factors and their receptors and how they contribute to cancer development and progression. The understanding of growth factor activation has lead to the development of therapeutic drugs that modulate growth factor function for the treatment of cancer. These chemotherapeutic drugs interfere with the anti-apoptotic effect of growth factors causing induction of apoptosis preferentially in cancer cells. These growth factor modulating drugs can be grouped into five categories:monoclonal antibodies, kinase inhibitors, HDAC inhibitors, antisense oligonucleotides, and proteasome inhibitors (Fig. 3).

5.1. Monoclonal Antibodies

Monoclonal antibodies (mAb) are laboratory-produced antibodies that bind to specific proteins interfering with their function. These antibodies have been designed to target the extracellular domains in growth factor receptors or their corresponding ligands blocking receptor activation. This prevents cell survival signaling and promotes apoptosis.

As previously mentioned, ErbB2 expression is up-regulated in 25-30% of human breast cancers and over expression has been associated with chemotherapy resistance Because ErbB2 expression is associated poor prognosis and over expression is seen in the tumor cells but not normal cells, this receptor is an important target for monoclonal antibodies. Herceptin (trastuzumab) binds to the ErbB2/HER2/neu protein in breast cancer blocking EGF signaling leading to decreased AKT activity. Herceptin inhibits the proliferation of breast cancer cells mediated by the up-regulation of p27kip that inhibitors of cyclin -dependent kinases required for cell cycle progression. Herceptin also has been shown to accelerate the degradation of HER-2, reducing the level of activated kinase, and may induce immune-mediated effects such as cytotoxic T cell activation (Gibbs, 2000). Inhibition of EGF receptor activity allows activation of pro-apoptotic pathways such as caspase activation and deactivation of anti-apoptotic pathways such as decreased AKT activation. This will lead to anti-tumor activity when treated to patients. In addition, the anti-tumor activity of herceptin may be increased when used together with other chemotherapeutic drugs such as cisplatin, doxorubicin, and paclitaxel. Indeed, combinations of taxanes, or platinum salts, with Herceptin give synergistic apoptotic responses *in vitro* and initial data from phase II studies for Herceptin treatment in combi-

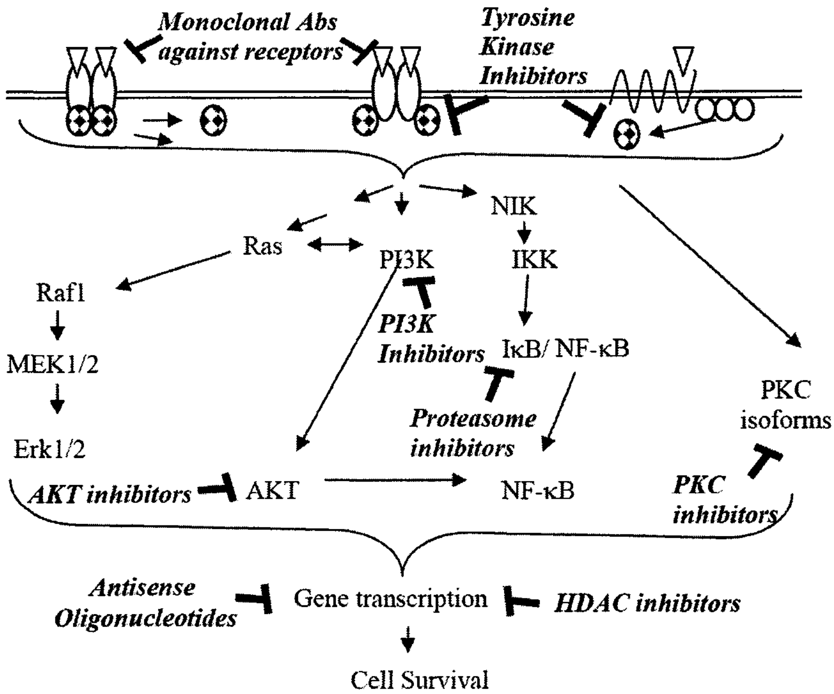

Figure 3 Chemotherapeutic drug targets in cell survival signaling pathways. Many chemotherapeutic reagents have been designed to block survival signaling pathways. The general reagent groups are denoted in bold italicized print. Monoclonal antibodies (Ab) against receptors block ligand binding and/or internalization of the receptor into cells. In either case, receptor activation is effectively blocked. Tyrosine kinase inhibitors block the activation of kinase domains in both receptor tyrosine kinases and non-receptor tyrosine kinases. Specific kinase inhibitors also block kinase activation in intracellular kinase such as PI3K, AKT, and PKC isoforms. Proteasome inhibitors prevent the degradation of proteins. One protein affected by this inhibitor is IκB. Blockage of its degradation prevents NF-κB activation. Targeting gene transcription by either antisense oligonucleotides designed to target specific genes or HDAC inhibitors that disrupt gene transcription, generally are effective at blocking regulation of both pro- and anti-apoptotic genes. All these reagents block cell survival signaling pathways and been effective at treating cancers in clinical trials.

nation with standard chemotherapy show a promising high response rate and increased time to progression in breast cancer patients (Nahta et al., 2003). In addition, Herceptin increases the amount of hypoxia induced cell death in breast cancer cells. Heceptin is a standard treatment for breast cancer patients with high levels of ErbB2 and aggressive disease.

Cross-talk between ErbB and prostaglandins suggests that a combination of cyclooxygenase (COX-2) inhibitors (Gupta & Dubois, 2001) and ErbB receptors may be another therapeutic approach. Mann et al. (2001) reported additive growth inhibitory effects on colorectal carcinoma cells when Herceptin was combined with a selective COX-2 inhibitor. This suggests that other combinations of anti-ErbB and inhibitors of pathways known to affect ErbB function should be examined.

IMC-C225 (cetuximab) is another mAb that blocks EGF receptor activity. It is directed against the extracellular epitope of the EGFR and inhibits the binding of EGF to

its receptor. When IMC-C225 is combined with chemotherapeutic drugs (cisplatin) or radiotherapy, significant response was seen in clinical trial patients (Mendelsohn, 2001). Synergistic anti-tumor effects was demonstrated when IMC-C225 was combined with doxorubicin. Phase I/II studies of IMC-C225 in EGFR overexpressing cancers achieved stable disease as a single agent and some partial remissions when combined with cisplatin or irinotecan in head and neck, colorectal, and non-small cell lung cancers. Phase II/III trials are currently in progress for various solid tumors (Nahta et al., 2003). Treatment with ABX-EGF of A431 tumor xenografts showed complete eradication. It suppressed growth of MDAMB468 xenografts, showing potential in treating breast cancer and is currently in phase I clinical trials of solid tumors (Nahta et al., 2003). Monoclonal antibodies against VEGF have been developed that specifically inhibit the activation of VEGF receptors (bevacizumab, rhumAb VEGF). In phase III clinical trials, metastatic colorectal cancer patients showed significantly better survival with modest toxicity when treated with bevacizumab and standard chemotherapy compared to patients only treated with standard chemotherapy. In metastatic breast cancer, another phase III study failed to show any significant differences in patients treated the bevacizumab in combination with chemotherapy compared to chemotherapy alone. Currently other solid tumors with elevated levels of VEGF receptors are being investigated.

Combining other toxic agents with monoclonal antibody treatment has been shown to increase their potency. Use of immunotoxins and oncotoxins with strong selectivity for HER2 binding has been used linked with herceptin. Radionuclides also have been attached to anti-HER2 antibodies. Immunotherapy also has been developed where two antibodies are linked together, one that binds HER2 and the other that binds an immune cell for more specific targeting of immune cells to HER2 over expressing cancer cells (Nahta et al., 2003). Another mode of delivering anti-ErbB2 antibodies to cancer cells is by immunoliposome therapy. Sterically stabilized liposomes have been developed and chemotherapeutic drugs such as doxorubicin can be loaded onto them. Doxorubicin-loaded anti-ErbB2 immunoliposomes work by accumulating in extracellular compartments of the tumor, the liposomes breakdown and release the drug, which diffuses into the tumor cells (Vereb et al., 2002).

5.2. Tyrosine Kinase Inhibitors

Deregulation of tyrosine kinase activity is common in cancer cells making it important to find ways to stop these rogue kinases. Original tyrosine kinase inhibitors competitively blocked ATP binding to the active site of kinases and prevented kinase signaling. These inhibitors had low specificity. New compounds have been developed to specific kinases that will not affect other kinases such as MAPKs thereby reducing toxic side effects (West et al., 2002). These therapies that specifically block tyrosine kinases in cancer cells offer the hope of sparing the normal, healthy cells of the body thereby having less severe side effects than traditional cytotoxic drugs.

The kinase inhibitor Gleevec® (Imatinib mesylate, STI571) is a notable success in treating chronic myelogenous leukemia (CML). The fusion protein BCR-ABL produced by the Philadephia chromosome constitutively activates the non-receptor tyrosine kinase

Abl. BCR-ABL would normally be activated only when the cell is stimulated by a growth factor such as PDGF. The Gleevec molecule fits into the active site of the Abl protein preventing ATP from binding. Without ATP as a phosphate donor, the Abl protein is unable to phosphorylate its substrate. A recently reported phase II study, found that almost 90% of the CML patients treated with Gleevec showed no further progression of their disease (Kantarjian et al., 2002). Gleevec may also be used for conditions where PDGFR is activated such as in metastatic breast cancer for which a phase II study is being conducted (Nahta et al., 2003) and phase I study in prostate cancer (Mathew et al., 2004). Angiogenosis is essential for tumor growth and disrupting angiogenosis is effective at eliminating tumors from animals. Li et al., (2002) reported that endostatin, a natural antiangiogenic protein that inhibits VEGF, together with other chemotherapeutic drugs was more effective in a squamous carcinoma model than each of these treatments given alone. This has lead to the development of drugs that specifically target VEGF. PTK787 (VEGF inhibitor) and SU11248, (VEGF and PDGF inhibitor) are at the stage of clinical trials. These drugs also have anti-angiogenic effects because VEGF and PDGF are important to many types of tumor to develop new blood supplies that are needed for tumor growth. Because ErbB signaling is also involved in regulation of VEGF and angiogenesis, ErbB inhibitors such as herceptin have also been found to have anti-angiogenic properties (Izumi et al., 2002; Petit et al., 1997).

Mutations in the EGFR ATP-binding site have been shown to eliminate receptor kinase activity and prevent cellular transformation. Therefore, tyrosine kinase inhibitors have been designed to competitively block ATP binding in EGF receptors. Quinazoline compounds competitively inhibit ATP-binding sites and are orally active, potent, and selective tyrosine kinase inhibitors. Because tyrosine kinase activity is required for EGFR mediated tumorigenicity and the poor prognosis of breast cancer patients with ErbB2 overexpression, tyrosine kinase inhibitors have been examined for treatment of breast cancer. Kinase inhibitors, ZD1839 and OSI-774 (EGFR specific) and CI-1033 (pan-Her inhibitor), that inhibit all four ErbB family receptors are the most studied. Blockage of EGFR by ZD1839 (Gefitinib, Iressa®) prevents transactivation of ErbB2, improving response rates to herceptin to treat herceptin resistant tumors. Phase I/II studies showed partial responses for renal and colon cancer and stable disease in prostate, cervical and head and neck cancers (Nahta et al., 2003). ZD1839 also has been shown to block the action of the EGF receptors on the cells of non-small cell lung cancer where it is in phase III studies. And when used in combination with radiotherapy on human colorectal cancer xenograft model, showed a significant increase in tumor growth inhibition (Williams et al., 2002). A number of other tyrosine kinase inhibitors have been examined for inhibition of ErbB kinase receptors. CI-1033 irreversibly inhibits a catalytic site conserved among all ErbB receptors. Both CI-1033 and PD168393 act against the ErbB1 and ErbB2 receptors. Current clinical trials with CI-1033 are being conducted in metastatic breast cancer patients who are resistance to herceptin therapy. PD153035, CP-358,774, and AG1478 also have been shown to inhibit receptor tyrosine kinase activity and proliferation of tumor cells with high levels of EGF receptors (Mendelsohn, 2001). SU-101 inhibits the kinase activity of the PDGF receptor and is in phase II for glioblastomas (Gibbs, 2000).

PI3K inhibitors have been investigated for cancer treatment. Wortmannin is a fungal metabolite and potent inhibitor of PI3K and has been shown to have in vitro and in vivo activity. In a study where wortmannin was administered to xenograft SCID mice with human or mouse mammary carcinoma cells or pancreatic carcinoma cells, it was shown to reduce tumour size and inhibit AKT phosphorylation by 50% (West et al., 2002). One disadvantage of wortmannin is its stability in aqueous environments, so new conjugates are being developed. Studies combining wortmannin with radiation or traditional chemotherapeutic drugs have showed enhanced apoptosis. LY294002 compound also inhibits PI3K by inhibiting lipid signaling of growth factor receptors by competitively binding at the ATP binding site of PI3K. When used in combination with farnesyltransferase inhibitors (blocks RAS signaling), it induced apoptosis in attached tumor cells (Gibbs, 2000). STI571 is a drug designed to inhibit AKT activity and causes decreased levels of the pro-survival proteins Bcl-2, and c-IAP, two genes whose transcription is at least partially controlled by AKT (West et al., 2002). AKT kinase inhibitors will be going into clinical trials in the near future.

UNC-01 and CGP 41251 are PKC kinase inhibitors. UNC-01 induces $p21^{Cip1/Waf}$ expression and leads to p53 independent apoptosis and mediates caspase-3 activation. UNC-01 has completed phase I clinical trials and is entering phase II trials (McLaughlin et al., 2003). Taken together, kinase inhibitors could provide a treatment regime that will be effective at controlling rogue kinases thereby inducing apoptosis selectively in cancer cells.

5.3. HDAC Inhibitors

Histone deacetylases (HDAC) are involved in regulating DNA transcription by deactylating histones in nucleosomes. This causes DNA to become transcriptionally inert, together with decreasing the activity of transcription factors (McLaughlin et al., 2003). HDAC inhibitors are a relatively new target for treating cancer and many are in preclinical or phase I and II clinical trials. Inhibition of HDACs allows the cell to express genes related to pro-apoptotic, anti-proliferative, or anti-angiogenic effects in cancer cells. These inhibitors have few toxicological side effects associated with their use at pharmacological concentrations (McLaughlin et al., 2003).

Suberoylanilide hydoxamic acid (SAHA) is a hydroxamate-containing small-molecule HDAC inhibitor. It interacts with the hydrophobic catalytic site of HDACs. SAHA has been shown to induce growth inhibition, cell cycle arrest, and apoptosis in breast cancer cells. This molecule has reached phase II clinical trials for the treatment of solid and hematological tumors (Aton Pharma, Inc.). SAHA also has shown synergy when combined with other types of cancer treatment. It produces an additive affect when combined with radiotherapy of human prostate spheroids. Other combinations of SAHA together with 5-FU, raltitrexed, and flavopiridol also demonstrated ability to target tumor cells. PXD101 and LAQ-824 are also hydroxamate-containing HDAC inhibitor molecules in phase I studies. PXD101 has been shown to slow tumor growth in xenograft models, particularly in leukemic mouse models. LAQ-824 has been shown to be effective in several xenograft models including breast (MDA-MB-435), colon (HCT116), and lung (A549) cell lines. Depsipeptide (FR-901228) is a cyclic peptide that is in phase II trials for cutaneous T cell lymphoma. Depsipeptide is a natural product purified from

Chromobacterium violceum that undergoes intracellular reduction to generate an active HDAC inhibitor (McLaughlin et al., 2003).

All these HDAC inhibitors deregulate gene expression of both pro- and anti-apoptotic genes and are effective at inducing apoptosis. The mechanism for how a general inhibitor increases histone acetylation leading to selective induction of apoptosis in cancer cells remains to be elucidated.

5.4. Antisense Oligonucleotide Targeting of Gene Expression

Antisense oligonucleotides have the ability to selectively target and inhibit gene expression. This is accomplished by antisense oligonucleotides binding to complementa-

Table 1. List of current chemotherapeutic drugs for the treatment of cancer that target growth factor mediated cell survival pathways.

Name of Drug	Type of Drug	Target	Cancer
Herceptin (trastuzumab)	Monoclonal Antibody	ErbB2 (HER2/neu)	Breast, Colon cancers
Bevacizumab	Monoclonal Antibody	VEGF	Colon Cancer
IMC-C225 (cetuximab)	Monoclonal Antibody	ErbB1 (EGFR)	Breast, Brain, Head and Neck, and Colon cancers
ABX-EGF	Tyrosine kinase inhibitor	ErbB1 (EGFR)	Breast cancer
Gleevac (Imatinib)	Tyrosine kinase inhibitor	Abl, Bcr-Abl, Kit or PDGFR	CML, breast, prostate cancers
PTK787 or SU11248	Tyrosine kinase inhibitor	VEGF or PDGFR	Gastrointestinal stromal tumors and renal cell carcinoma
ZD1839 (Iressa)	Tyrosine kinase inhibitor	ErbB1 (EGFR)	Breast, Renal, Colon, Prostate, Cervical, Lung and Head and Neck cancers
CP358 (Tarceva)	Tyrosine kinase inhibitor	ErbB1 (EGFR)	Squamous cell carcinoma, Head and Neck, Lung, Glioblastoma, and Breast cancer
CI-1033	Tyrosine kinase inhibitor	All ErbB receptors (pan-HER inhibitor)	Breast cancer
SU-101	Tyrosine kinase inhibitor	PDGFR	Glioblastomas
UNC-01	Kinase inhibitor	PKC	Myelogenous Leukemia
SAHA or Depsipeptide	HDAC inhibitor	HDAC	Lung, Colon, and Breast cancers and Leukemias
ISIS-321	Antisene oligonucleotide	PKC-α	Solid tumors
Velcade (Bortezomib, PS-341)	Proteasome inhibitor	Proteasome complex	Multiple Myeloma

ry mRNA and targeting it for degradation by endogenous nucleaseRNAaseH (Vereb et al., 2002). Antisense against IGFR indicates that IGF receptor inhibition may promote tumor apoptosis. (Gibbs, 2000). ErbB2 antisense showed down regulation of ErbB2 mRNA expression and inhibitory effects on cancer cell proliferation (Vereb et al., 2002). The antisense inhibitor to PKC-α, ISIS-321, is in phase II clinical trial (Gibbs, 2000). As well, the antisense against IGF-IR mRNA blocked proliferation of murine mammary carcinoma cells and has demonstrated antitumor effects in vivo (Nahta et al., 2003). Thus by specifically targeting growth factor signaling alterations in cancer, it is predicted that these cancer cells will have increased sensitivity towards chemotherapy.

5.5. Proteasome Inhibitors

Proteasome inhibitors block the intracellular protein-disposal system known as ubiquitin-mediated proteolysis (UMP). Ubiquitin ligase inhibitors are about five years in development behind kinase inhibitors. Velcade (bortezomib, PS-341) has been shown to reversibly inhibit the 26S proteasome's chymotryptic site. Intact 26S proteasome activity promotes the transcription of anti-apoptotic genes. Targeted inhibition (PS-341) of the 26S proteasome alone is also to block proliferation and induces apoptosis as pro-apoptotic kinases become activated. Velcade prevents proteasome degradation of IκB, causing NF-κB to remain in the cytosol inhibiting its transcriptional activity. This leads to the decreased expression of anti-apoptotic proteins such as Bcl-2 family members (Adams, 2002). Velcade's indiscriminate inhibition likely stalls the onset of drug resistance. When given to patients twice a week, treatment inhibited 70-80% of the cell's proteasomes. Velcade has been shown to shrink or stabilize tumors and is approved for refractory multiple myeloma treatment (Richardson et al., 2003). Other proteasome inhibitors are in development for treatment of many different tumors.

6. SUMMARY

Cell signaling is important for the survival of all cells. However, a cell must also be able to stop responding to a signal. For growth factor receptors, failure to be turned off can lead to uncontrolled growth and lack of apoptosis resulting in cancer. Understanding the interaction of growth factors, receptors and their cell survival signaling pathways has provided novel targets for cancer treatment. Indeed, many drugs that target these pathways are now routinely used to treat cancer and many more are under development. The targeted drugs currently being developed to stop uncontrolled survival signaling are only a starting point. As our knowledge of survival signaling pathways and how they interact with apoptotic signaling pathways expands more effective therapies will be developed that will make cancer cells undergo apoptosis without effecting healthy cells.

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TARGETS FOR APOPTOTIC INTERVENTION IN RHEUMATOID ARTHRITIS

Chapter IX

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1. INTRODUCTION

Rheumatoid arthritis (RA) is a disease primarily associated with chronic inflammation of the joints. Different from osteoarthritis (OA), which usually affects only a limited number of joints and is thought to arise largely from trauma or excessive use, RA is considered a systemic autoimmune condition that leads to erosive degeneration of the joint tissues. Increasing evidence suggests that the chronic persistence of the inflamed condition can be attributed to mechanisms that inhibit programmed cell death, or apoptosis, of activated cells within the joint tissues in RA (Pope, 2002). Because the cells that drive the pathology of RA are resistant to pathways that would otherwise enable their natural clearance, the inflamed condition is maintained indefinitely.

In normal joints, a fibrous capsule surrounds the articulating regions of the bones and encloses the joint space. A thin layer of synovium covers all the internal surfaces of the joint capsule, with the exception of the cartilage. The synovial tissue is comprised primarily of differentiated macrophages and synovial fibroblasts and is typically only a few cell layers deep. During the course of RA, the synovium becomes dramatically thickened and hyperplastic. The chronic presentation of inflammatory cytokines activates the cells within the expanded tissue, which develops a highly aggressive phenotype. The hypertrophied synovium attaches to the cartilage and subchondral bone and secretes a variety of proteinases that gradually degrade and erode these tissues. The pathogenesis of RA is slow, but progressive, and over time the prolonged erosion of articular tissues leads to disfigurement and loss of function (Arend, 2001).

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The underlying agent that initiates and drives the disease process is currently unknown. Although a variety of factors have been postulated, none has proven definitive. The prevailing opinion is that RA is an autoimmune disease triggered by an exogenous antigen that when presented to immune cells in the context of certain MHC allotypes, stimulates cross-reactivity or loss of tolerance to endogenous proteins found predominantly in articular tissues. Genetic analyses support this concept, as a predisposition to disease is associated with the third hypervariable region of the MHC class II genes, *DRB1*0401* and *DRB1*0404* (Angelini et al., 1992; Fries et al., 2002). However, the specific antigen(s) or causative agent has remained elusive. What is certain, however, is that there is a persistent production and release of inflammatory cytokines such as interleukin-1 (IL-1) or tumor necrosis factor α (TNF) into the joint tissues and circulation of RA patients (Arend, 2001). These cytokines occupy positions at the top of the inflammatory cascade, and their overproduction in transgenic animal models replicates the pathologies associated with RA in humans (Ghivizzani et al., 1997; Keffer et al., 1991). Currently, the most effective methods for treating RA are based on pharmacologics that inhibit directly or antagonize the effects of TNF and IL-1. Such treatments, though, are only effective in 40-70% of the afflicted population, and are not curative. They must be re-administered frequently, and cessation of treatment is met with the recurrence of disease.

Numerous histologic studies of joint tissues recovered from RA patients suggest that the occurrence of apoptotic events in the synovium of the rheumatoid joint is particularly rare (Firestein et al., 1995; Fraser et al., 2001; Matsumoto et al., 1996; Nakajima et al., 1995; Sugiyama et al., 1996), and that proteins which block apoptotic pathways are prevalent in cells and tissues of the afflicted joints. Thus, it is thought that therapeutic approaches that can effectively amplify apoptotic signals or, conversely, block apoptotic inhibitors in these cells hold promise as methods to down regulate the chronic inflammation and improve existing treatments (Baier et al., 2003; Pope, 2002). Experimental approaches to treatment involving apoptotic pathways fall under two general categories. The first is directed toward killing or elimination of cellularity at the site of disease, namely the inflamed synovium. The second focuses on apoptotic methods designed to block the development or maintenance of specific autoimmunity.

2. APOPTOTIC PATHWAYS IN RHEUMATOID ARTHRITIS

Apoptosis or programmed cell death is the process by which cells or cell populations are eliminated when they are no longer useful or pose a potential threat. This may occur by two general pathways: either through specific ligation of external death receptors on the target cell or via internal pathways originating from molecules released from the mitochondria (Fig. 1). Following induction, the process is driven by sequential activation of a cascade of cysteine proteases (caspases) which results in degradation of organelles and fragmentation of nuclear DNA.

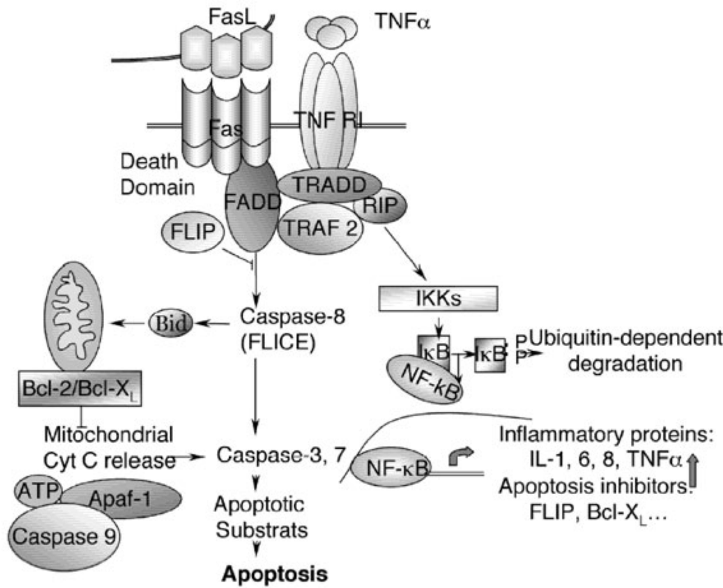

Figure 1. Apoptotic pathways in rheumatoid arthritis. Apoptotic pathways can be induced by a specific interaction ligand/receptor or by intracellular signaling. Following initial induction, the recruitment of several proteins, including FADD, induces the activation of a cascade of proteases called caspases. This results in the degradation of organelles and fragmentation of nuclear DNA. The association of TNFα with his receptor also activates the NF-κB pathway leading to the expression of inflammatory proteins and anti-apoptotic molecules. Apaf 1, apoptotic protease activator 1; Bid, BH3 interacting death-domain agonist; FADD, Fas associated death domain; FLICE, Fas ligand IL-1 converting enzyme, also termed caspase 8; FLIP, FLICE (caspase 8) inhibitory protein; IKKs: IκB kinases; NF-κB, nuclear factor κB; RIP, receptor interacting protein; TNFα : tumor necrosis factor α; TNFR, TNF receptor; TRADD, TNFR associated death domain; TRAF 2, TNFR associated factor 2.

2.1. Death Receptor-Mediated Apoptosis

Among the more studied of the death receptor signaling pathways is that of Fas:Fas ligand. Fas (CD95/Apo-1) is a type I membrane protein of a family of molecules known as death receptors (DR) (Nagata, 1997; Nagata & Golstein, 1995). The six identified DR proteins (DR1-6) are members of the TNF superfamily and are capable of inducing apoptotic pathways following binding to their specific ligand (Aggarwal, 2003). The Fas death receptor (DR2) is constitutively expressed on the surface of most cell types. Fas ligand (FasL), a trimeric, type II membrane protein, is expressed mainly by activated cells of the immune system, including T and B lymphocytes, macrophage and NK cells (Nagata, 1997). Fas, and FasL-induced apoptosis, are the primary means used for elimination of auto-reactive T and B cells (Abbas, 1996), and thus are critical to the normal development, proper function and regulation of the immune system. Similarly, aberrant function or specific inhibition of Fas/FasL apoptosis in the cells of the synovial tissue that drive the inflammatory process is thought to contribute to the chronic persistence of the inflamed state in the RA joint.

Ligation of the Fas receptor with FasL expressed on the surface of an apoptotic effector cell causes the Fas molecules to trimerize. The clustering of intracellular death domains (DD) on the Fas receptors that ensues, recruits the cytosolic adaptor protein, Fas associated death domain (FADD/MORT1). These molecules interact with Fas via their own death domains (Chinnaiyan et al., 1995; Zhang et al., 1998). FADD proteins also contain death effector domains (DED) which bind in tandem and activate caspase-8 (also termed FLICE, Fas ligand IL-1 converting enzyme), an "initiator" caspase of the apoptotic cascade (Boldin et al., 1996; Muzio et al., 1998). The complex of Fas/FADD and caspase 8 is termed the death-inducing signaling complex (DISC) (Kischkel et al., 1995). Following activation, caspase 8 directly or indirectly activates "effector" caspase 3, which then cleaves a variety of intracellular substrates resulting in cell death (Enari et al., 1996; Muzio et al., 1996; Villa et al., 1997).

Fas mediated apoptosis can be regulated at many steps along the pathway by a variety of anti-apoptotic proteins. Thus, following DR ligation the ultimate fate of the cell- death or survival- relies upon the balance between pro- and anti-apoptotic signals (Aggarwal, 2003). Fibroblasts and macrophage cells in the RA synovium both express Fas death receptor at significant levels, but remain resistant to apoptosis despite the presence of numerous adjacent cells that express FasL. Recent studies have shown that this resistance to cell death is due to the abundance of anti-apoptotic proteins in the RA joint. The roles of several of these will be discussed below in relation to their influence on specific cell types associated with pathogenesis in RA.

2.2. Mitochondrial-Mediated Apoptosis

Initiation of apoptotic signaling from mitochondria can occur in response to mediators activated by DR ligation, or independently from chemotoxicity or from genotoxic stress following exposure to x-rays and ultra-violet light. Members of the Bcl-2 family of proteins, including Bcl-2, and Bcl-x_L, contribute to cell survival through the regulation of mitochondrial-mediated apoptosis (Fig. 1). These proteins associate with apoptotic protease factor-1 (Apaf-1) on the mitochondrial membrane and prevent the release of apoptogenic molecules from the organelle. In response to toxic stress or cell damage, Apaf-1 is released from Bcl-2, permitting escape of cytochrome *c* from the mitochondrion (Li et al., 1997). Cytochrome *c* together with Apaf-1 serves to activate caspase 9, another of the "initiator" caspases, which then activates "effector" caspases-3 and 7, leading to apoptosis (Li et al., 1997). The importance of the Bcl-2 family proteins in cell maintenance and persistence of disease in RA is suggested by the increased Bcl-2 levels in RA synovium relative to that found in OA. Additionally, the knockdown of Bcl-2 expression in RA synovial fibroblasts in culture is associated with apoptotic cell death.

Mitochondrial apoptosis may also act cooperatively with death receptor signaling. Following activation as part of the DISC, caspase 8 may cleave BH3 interacting death-domain agonist (Bid) (Luo et al., 1998; Wang et al., 1996), another member of the Bcl-2 family, which leads to the loss of mitochondrial transmembrane potential ($\Delta\psi_\mu$) and the release of cytochrome *c*. Again, this leads to caspase-9 activation and induction of effector pathways (Green, 1998; Gross et al., 1999; Thornberry & Lazebnik, 1998). The overlap and cooperation between DR and mitochondrial apoptotic pathways provides numerous layers for redundant positive and negative regulation of apoptosis. This

complex network of proteins and signaling pathways also offers numerous potential targets for therapeutic intervention.

2.3. TNF Signaling and NF-κB: Apoptosis or Survival

Tumor necrosis factor is a primary mediator of the inflammatory cascade and a key contributor to the pathogenesis of RA. Its overexpression in transgenic mice leads to a highly destructive, chronic, polyarticular, inflammatory arthritis (Keffer et al., 1991). TNF is produced by macrophages, NK cells and activated fibroblasts and influences cellular biology via two receptor molecules, TNF-R1 and TNF-R2. Through these receptors TNF simultaneously activates apoptotic signals as well as pathways leading to apoptotic resistance, cellular activation and proliferation (Aggarwal, 2003). The proteins responsible for signaling TNF-mediated apoptosis are maintained constitutively and do not require novel protein synthesis for induction. The anti-apoptotic signals, however, require transcriptional events, mediated by the activity of the transcription factor, nuclear factor kappa B (NF-κB) (Karin & Lin, 2002). The balance between the pro- and anti-apoptotic signals eventually determines whether a cell is killed, or is activated and survives in response to TNF (Aggarwal, 2003; Karin & Lin, 2002).

TNF-R-mediated apoptosis is similar to that of Fas. For example, following ligation and trimerization of TNF-R1 (DR1), cytosolic TNF-R associated death domain (TRADD) is recruited by the receptors and activated. This complex subsequently activates FADD, which leads to caspase 8 activation and eventually apoptosis (Chen & Goeddel, 2002). Alternatively, TNF-R2 ligation may activate TNF-R associated factor 2 (TRAF2) which leads to receptor interacting protein (RIP) phosphorylation and downstream activation of NF-κB (Kelliher et al., 1998; Legler et al., 2003; Tada et al., 2001; Yeh et al., 1997). NF-κB (p50-p65) is a heterodimeric DNA binding protein well known for its involvement in inflammatory and innate immune responses. In the cytoplasm, NF-κB is held inactive, bound to its inhibitors, the IκB proteins. RIP, in response to TNF signaling, activates the IκB kinase (IKK) complex which then phosphorylates NF-κB-bound IκB. This leads to ubiquitin-dependent degradation of IκB and translocation of the liberated NF-κB to the nucleus.

NF-κB binding sites have been identified in the regulatory regions of numerous genes, and it participates in the expression of inflammatory cytokines such as IL-1, TNF, IL-6 and IL-8, as well as matrix-degrading enzymes and adhesion molecules (Baeuerle & Henkel, 1994). As such, NF-κB activation is important to the pathogenic activities specific to RA, including inflammation, synovial proliferation and tissue erosion. In addition to its contribution to inflammation, it also stimulates expression of proteins that inhibit apoptosis. These include FLICE (caspase-8) inhibitory protein (FLIP), Bcl-x_L, TNF receptor associated factor 1 (TRAF1), and, X-chromosome linked inhibitor of apoptosis protein (XIAP), among others (Stehlik et al., 1998; Tsukahara et al., 1999; Wang et al., 1998). Thus, NF-κB is also an important mediator of synovial hyperplasia (Miagkov et al., 1998).

NF-κB activation is a critical downstream link to inflammatory signaling by both TNF and IL-1. NF-κB is found at elevated levels in fibroblasts and macrophage cells in the synovial tissues of RA patients (Handel et al., 1995), and thus is a prime target for

therapeutic intervention. Evidence of the beneficial effects of NF-κB inhibition in synovial fibroblasts was initially provided from studies by Makarov et al. (Miagkov et al., 1998), They showed that intra-articular delivery of an adenoviral vector containing the cDNA for a super repressor form of IκBα profoundly enhanced apoptosis in the synovium of rats with streptococcal cell wall (SCW) induced arthritis (Miagkov et al., 1998). Similarly, administration of NF-κB decoy oligonucleotides was found to reduce the severity of recurrent SCW arthritis (Miagkov et al., 1998). In related work, Liu et al. showed that inhibition of constitutively activated NF-κB in macrophages led to enhanced apoptosis in these cells via a reduction in the level of Bcl-2 family member, A1 (Liu et al., 2004). This is associated with a loss in mitochondrial integrity and subsequent caspase-9 activation leading to apoptosis. Thus, local inactivation of NF-κB may offer a potent therapeutic strategy, providing reduced expression of inflammatory and erosive mediators while decreasing the numbers of inflammatory effector cells in the joint through apoptosis.

3. TARGET CELLS FOR INTERVENTION IN RHEUMATOID ARTHRITIS

The cell types that are considered to be the primary mediators of articular pathology in RA are synovial fibroblasts (SF), macrophage cells and T-lymphocytes. In each case, studies have found evidence that increasing the level of apoptosis could have therapeutic value. Neutrophils, on the other hand, are not generally considered to be high-priority targets in this disease. Although the acute stages of inflammation in RA are characterized by large numbers of infiltrating neutrophils (Liu & Pope, 2003), during the transition to the chronic inflammation these cells undergo apoptosis and are phagocytosed (Lawrence et al., 2001; Savill, 1997). Because neutrophils are not present in significant numbers in chronically inflamed tissues they are not believed to be critical to long-term maintenance of RA (Perlman et al., 2001).

3.1. Macrophage Cells

Macrophages together with synovial fibroblasts comprise the majority of cells in the rheumatoid synovium and are the primary producers of the principal inflammatory cytokines, TNF and IL-1. In contrast to synovial fibroblasts, macrophages are not thought to proliferate locally within the joint but to arise from monocytes in the peripheral blood that migrate to the joint tissues. Thus, for these cells their accumulation in the synovial tissues may not arise from aberrant local proliferation but rather from mechanisms that prevent their elimination by apoptosis (Pope, 2002).

In the periphery, monocytes express high levels of Fas and FasL and are sensitive to Fas receptor-mediated apoptosis (Perlman et al., 2001). However following TNF-induced activation in these cells, as would be expected to occur in the RA joint, the expression is induced of anti-apoptotic protein, FLICE inhibitory protein (FLIP) (Perlman et al., 1999). FLIP blocks apoptosis by binding to FADD and inhibiting caspase-8 activation following Fas ligation. It is highly expressed in macrophage cells isolated from the joints of RA patients (Perlman et al., 2001). The importance of FLIP to macrophage survival in vivo is further demonstrated in animal studies whereby, unstimulated macrophages isolated from

the peritoneal cavity of mice do not express FLIP and are sensitive to Fas mediated apoptosis. However, following activation in vivo FLIP expression is enhanced and these cells become resistant (Hohlbaum et al., 2001). Since macrophage cells express Fas and FasL and are in close proximity in rheumatoid synovium the suppression of FLIP within the joint may be a useful approach to induce apoptosis of macrophages (Pope, 2002).

3.2. Synovial Fibroblasts

Activated synovial fibroblasts are also key contributors to the pathology in RA and participate in the initiation and maintenance of the diseased state. In the rheumatoid joint much of the hypertrophied synovial tissue can be attributed to the proliferation and persistence of these cells. Different from fibroblasts from normal joints and from patients with osteoarthritis, RA-SF demonstrate a nearly transformed state, and aggressive, invasive behavior (Baier et al., 2003; Davis, 2003). These cells attach to, and directly erode articular cartilage. They also participate in the degradation of bone, both by the secretion of matrix degrading enzymes and via the activation of osteoclasts. A large body of evidence indicates that RA-SF are not just transiently activated by exposure to inflammatory cytokines, but rather adopt a stable activated state (Pap et al., 2000). This is supported by the severe combined immunodeficient (SCID) mouse model of RA. In this system RA-SF are implanted with pieces of normal human cartilage under the kidney capsule in SCID mice. Within this context, the implanted cells maintain their aggressive, erosive properties for over two months without the involvement of other inflammatory cells (Muller-Ladner et al., 1996; Pap et al., 2000).

RA-SF express high levels of the Fas death receptor, and up to 90% are sensitive to Fas mediated apoptosis in vitro (Kawakami et al., 1996; Nakajima et al., 1995). Indeed, no significant difference between Fas expression on synovial fibroblasts between RA and OA patients has been observed. Yet, despite the presence of FasL positive macrophage, histologic studies of tissue recovered from RA joints indicate apoptosis of RA synovial fibroblasts is particularly rare (Firestein et al., 1995; Fraser et al., 2001; Matsumoto et al., 1996; Nakajima et al., 1995; Sugiyama et al., 1996).

Recent work by Schedel et al. has shown that FLIP expression is elevated in RA-SF but not in normal synovium (Schedel et al., 2002). This was observed primarily in the lining and sublining and importantly at sites of cartilage and bone destruction. Although FLIP expression was also observed in synovial sections of some OA patients, its expression was mostly limited to the sublining layer (Schedel et al., 2002).

In addition to FLIP, another type of anti-apoptotic molecule has been found to be prevalent in RA-SF. This molecule, a small ubiquitin-like modifier (SUMO or sentrin-1) (Okura et al., 1996) interacts with certain proteins and inhibits their binding to specific substrates. As such, sentrin-1 has the capacity to regulate numerous protein/protein interactions and signaling pathways. These include p53 (Gostissa et al., 1999; Muller et al., 2000; Rodriguez et al., 1999) and IκB (Desterro et al., 1998), as well as the death domains of Fas and TNF-R1 (Okura et al., 1996). Due to its interaction with these death domains, cells are protected from DR induced apoptosis. Its participation in RA is suggested by immunohistochemistry where it has been observed mainly in cells in the synovial lining layer at sites of cartilage invasion (Baier et al., 2003).

The p53 tumor suppressor is a protein expressed in response to DNA damage that functions to arrest cell growth and direct cellular pathways toward DNA repair or apoptosis. A loss in p53 function is perhaps the single most frequent event that leads a cell toward tumor development; many of the common human cancers have been linked to mutations in the p53 tumor suppressor gene. Several lines of evidence suggest that aberrant p53 activity may also be linked to synovial cell proliferation and the persistence of the expanded synovium in rheumatoid joints (Aupperle et al., 1998). In normal cells p53 exists as a latent, inactive form and has a brief half-life; thus, it is rarely detected in non-malignant tissues. In contrast, immunohistochemical analyses of RA synovium show that p53 is present in large quantities (Firestein et al., 1996; Tak et al., 1999). This is likely the result of p53 accumulation induced by the genotoxic effects of reactive nitrogen and oxygen species, often produced at sites of chronic inflammation (Tak et al., 2000). In spite of the heightened presence of p53 in RA synovium and detection of numerous cells with DNA fragmentation, cellular turnover due to apoptosis occurs at low level (Firestein et al., 1995). In some RA patients specific mutations have been identified in as high as 40% of the p53 cDNAs generated from synovial mRNA (Yamanishi et al., 2002). Many of the point mutations were found to occur at positions similar to those in a variety of tumor types. This pattern of p53 expression however is not seen in the synovium of patients with osteoarthritis. These observations provide correlative evidence suggesting that inhibition of p53-mediated apoptosis may be a contributing factor in RA; however, the degree to which p53 function actually participates in the pathogenesis of this disease still remains unclear.

3.3. T Lymphocytes

T cell infiltration is another characteristic of RA synovium. However, according to their morphology, surface phenotype and cytokine profile, T cells in the RA joint do not appear activated, but rather to be resting. T cells from isolated RA patients appear to be anergic and exhibit down regulation of calmodulin, a protein essential to T cell activation (Ali et al., 2001). These resting T cells remain capable of activating synovial fibroblasts resulting in their increased production of inflammatory cytokines and matrix degrading enzymes (Yamamura et al., 2001).

T cells infiltrating the RA joint are resistant to Fas-induced apoptosis and display elevated levels of anti-apoptotic proteins from the Bcl-2 family (Firestein et al., 1995; Schirmer et al., 1998). Consistent with this, populations of CD4+ T cells in proteoglycan-induced arthritis in mice were found to be resistant to Fas-mediated apoptosis in spite of normal levels of Fas receptor. Further examination attributed this resistance to elevated production of FLIP.

Furthermore, although T cells contribute to the activation of RA-SF, the activated SF also release factors that inhibit T cell apoptosis (Zhang et al., 2001). It has been suggested that stromal cell-derived factor 1a (SDF-1a) produced by SF inhibits T cell apoptosis through PI3-kinase and mitogen-activated protein kinase pathways (Nanki et al., 2000). SDF-1a also is thought to elevate the migration of T cells to the RA joint (Baier et al., 2003; Nanki et al., 2000; Suzuki et al., 2001).

4. EXPERIMENTAL TREATMENTS FOR RA INVOLVING APOPTOSIS

A variety of experimental strategies involving induction of apoptotic pathways as a therapeutic tool have been tested in animal models of arthritis. For the most part these have been focused on two directions. The first involves methods intended to locally reduce the cellularity in the inflamed joint tissues. The second attempts to target apoptosis to specific cells at sites of immune activation, with the idea to specifically eliminate auto-reactive cell populations and thus remove the autoimmune response.

4.1. Synovial Ablation

Synovectomy, the physical removal or killing of synovial tissue, has demonstrated the ability to reduce the rate at which deformities occur and delay degradation of the rheumatoid joint. The various available methods, including surgery (Rosen & Weiland, 1998; Sculco, 1998), radiation (Deutsch et al., 1993; Taylor et al., 1997), and caustic chemicals (Hilliquin et al., 1996; Menkes, 1979) used as a means to ablate rheumatoid synovium are either invasive or have potential side effects. A treatment that could effectively reduce the cellularity of RA synovium without the disadvantages associated with current synovectomy techniques could be useful in the clinical management of RA.

4.1.1. Fas Ligand and p53

The effects of stimulating the Fas pathway to mediate apoptosis in the rheumatoid joint were originally studied in different murine model systems (Fujisawa et al., 1996; Okamoto et al., 1998; Sakai et al., 1998; Zhang et al., 1997). It was first shown that intra-articular injection of anti-mouse Fas monoclonal antibody into the paws of human T cell leukemia virus type 1 transgenic mice showed a reduction in paw swelling and disappearance of Fas-expressing cells from the synovium (Fujisawa et al., 1996). Later work by Zhang et al. showed that peri- and intra-articular injection of a recombinant adenovirus containing the cDNA for FasL into the paws of DBA/1 mice with collagen induced arthritis induced high levels of local FasL expression and ameliorated the effects of arthritis in this system (Zhang et al., 1997). This included a reduction in synovitis and synovial infiltration. Additional studies involving FasL gene transfer showed that in culture, RA-SF could be modified to express FasL and induce apoptosis in adjacent fibroblastic cells. Furthermore, direct intra-articular injection of an adenovirus containing FasL into the larger joints of rabbits with experimental arthritis showed evidence of apoptotic cell death in large regions of the expanded synovium (Yao et al., 2000). However, these effects were somewhat patchy, indicating that joints of human proportion would require methods that allow uniform distribution of apoptotic mediators across the breadth of the synovial tissues. Together, these studies showed that activated cells in the synovial lining express functional levels of Fas death receptor, and at least within the context of these model systems it is possible to override anti-apoptotic mechanisms to a functional degree.

In related work, it was found that gene delivery and overexpression of p53 in RA-SF in culture was an effective mechanism for induction of apoptosis in these cells. Delivery of an adenovirus encoding p53 to the joints of rabbits with either antigen-induced- or IL-

1β driven arthritis resulted in larger regions of apoptotic cell death than that observed with FasL (Yao et al., 2000). In this case, the cell killing appeared more evenly distributed throughout the joint capsule and was found to reduce leukocytic infiltration into the synovial fluid. Similar to the results with Fas-induced apoptosis above, these studies suggest that the mechanisms that inhibit cell death induced by these respective proteins are not insurmountable, and may be overcome by overstimulation of apoptotic signaling.

4.1.2. Induction of Apoptosis via TRAIL and Death Receptor 5

TNF related apoptosis inducing ligand (TRAIL) (Song et al., 2000) is a Type II transmembrane protein of the TNF superfamily that preferentially induces apoptosis in tumor cells. TRAIL binds to death receptors 4 and 5 (DR4 and DR5) (Chaudhary et al., 1997; Pan et al., 1997b; Walczak et al., 1997), and two decoy receptors, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) (Marsters et al., 1997; Pan et al., 1997a; Sheridan et al., 1997), which serve to titrate the death signal. It also binds osteoprotegerin (Emery et al., 1998). TRAIL and TRAIL receptors are constitutively expressed in various tissues, and are up regulated following cell activation (Strater et al., 2002; Zauli et al., 2003). Their actual participation in disease and health maintenance, however, is unknown. Relative to FasL, TRAIL is of lower toxicity to normal cells, which typically express low levels of the appropriate receptors (Baetu & Hiscott, 2002; LeBlanc & Ashkenazi, 2003). Most cancerous cells, though, express high levels of DR5 and are susceptible to DR5-mediated apoptosis. RA synovial fibroblasts share many features of malignantly transformed cells and likewise express high levels of DR5 (Ichikawa et al., 2003).

The potential for use of TRAIL, and DR5-mediated apoptosis in RA, is suggested in several animal models. First, TRAIL-deficient mice are hypersensitive to collagen-induced arthritis (Lamhamedi-Cherradi et al., 2003). Furthermore, constitutive blocking of TRAIL in mice, with soluble DR5, exacerbates autoimmune arthritis resulting in hyperproliferation of synovial cells and increased production of inflammatory cytokines (Song et al., 2000). Recent work has also shown that RA-SF are highly sensitive to anti-DR5 antibody-mediated apoptosis (Ichikawa et al., 2003). Gene transfer studies have found beneficial effects following delivery of the cDNA for TRAIL to the joints of animals with experimental arthritis. In mice with collagen induced arthritis, direct injection of an adenoviral vector containing TRAIL into the arthritic paws resulted in a dramatic reduction in disease severity that persisted for about 2 weeks (Song et al., 2000). Primary cultures of synovial fibroblasts from rabbits, and from a majority of RA patients were also found to be sensitive to TRAIL-mediated apoptosis following infection with adenovirus containing human TRAIL. Furthermore, in a rabbit model of arthritis, intra-articular gene transfer of TRAIL induced apoptosis in cells within the synovial lining, reduced leukocytic infiltration and stimulated new matrix synthesis by cartilage (Yao et al., 2003).

While each of the studies described above involving viral mediated gene transfer of apoptotic proteins to joint tissues demonstrate the feasibility of inducing apoptosis in the inflamed synovium, they were performed in acute models or were only followed for a few weeks downstream of application. Thus, the long-term benefits or effects of these experimental treatments are not known.

4.1.3. Protein Transduction Domains for Delivery of Apoptotic Agents to Synovium

As described briefly above, intra-articular gene transfer is a technique used experimentally to study the effects of pro-apoptotic proteins in hyperplastic synovial tissue in the joints of animals. In several studies, though, induction of apoptosis has been limited by the efficiency of gene delivery to the synovium. Furthermore, the viral systems useful for in vivo gene transfer typically generate adaptive immunity that prevents effective vector re-administration. Recent work, however, has identified a synovial-specific transduction peptide (HAP-1) that enables receptor-independent internalization of peptide conjugates in rabbit synovial fibroblasts and into rabbit synovium in vivo (Mi et al., 2003). Fusion peptides of HAP-1 with a pro-apoptotic peptide $(KLAK)_2$, that causes mitochondrial disruption, were found to effectively induce apoptosis of rabbit and human synovial fibroblasts in culture. Intra-articular injection of these peptides into the knees of rabbits with experimental arthritis was associated with a significant increase in synovial cell apoptosis, accompanied by a reduction in inflammation and synovitis (Mi et al., 2003). These results suggest an alternative method to gene transfer for delivery to synovium of pro-apoptotic agents that function intracellularly. Further, given the toxicity associated with the administration of non-specific apoptotic agents such anti-Fas antibody, the ability to target disease-specific cell types is an increased advantage to this system.

4.2. Autoimmune Modulation

Since RA is widely thought to arise from the loss of immune tolerance to specific "self" antigens in joint tissues, another potential strategy for therapeutic application of apoptosis is toward auto-reactive immune cells. One approach to achieve this involves the adaptation of dendritic cells as assassins that migrate to lymphoid tissues to eliminate antigen-specific T cells.

Dendritic cells (DCs) are professional antigen presenting cells that function to modulate immunologic activity. They interact directly with T cells in an antigen specific manner to induce activation, or conversely, to produce antigen-specific anergy or tolerance. During the transition from antigen acquisition to presentation, DCs are phenotypically altered from an immature to a mature state that controls T cell activation.

Genetically modified DCs have been used experimentally in several applications that have attempted to modulate immune activity in RA. In these cases the DCs were genetically modified to express cytokines such as IL-4, IL-10 and CTLA4-Ig which decrease T cell activation and immunity. In research directed toward treatment of autoimmune pathologies, the cDNA for FasL was delivered and expressed in DCs as a means to kill reactive T cell populations. Robbins et al. adopted this strategy in experiments directed toward treatment of collagen-induced arthritis in mice (Kim et al., 2002). It was found that a single administration of these DCs to mice with collagen induced arthritis was sufficient to reverse the pathologies of established disease, as measured by paw swelling, arthritic index and number of swollen paws. Analysis of the animals showed preferential depletion of collagen type II (CII) reactive T cells even though the DCs had not been pulsed with a specific antigen (Kim et al., 2002).

Recently, Liu et al. have adopted a similar approach involving the use of DCs genetically modified with adenovirus to express TRAIL under the control of a doxycycline inducible promoter (Liu et al., 2003). In these experiments Ad.TRAIL infected, mature DCs were pulsed with CII prior to intra-peritoneal injection into mice previously immunized with CII. In mice that received doxycycline in their drinking water, the incidence of arthritis was significantly reduced, and the time of arthritis development was delayed considerably. The treated animals also showed a reduction in CII specific antibody. Further examination of the effects of this procedure showed a specific elimination of antigen specific T cells in the spleen that prevented their migration to the joint tissues. The potent anti-arthritic effect of this treatment was attributed to antigen pulsing of the DCs that subsequently increased the specificity of the TRAIL-mediated cell killing (Liu et al., 2003).

5. CONCLUSION

As described above, considerable evidence supports the concept that the chronic stimulation by inflammatory cytokines in RA activates synovial fibroblasts, macrophages and T cells and enables their accumulation in the joint by increasing their resistance to apoptosis. The interconnectedness of inflammatory and anti-apoptotic pathways suggests that mechanisms that can effectively reduce inflammatory stimulation will heighten the sensitivity of these effector cells to apoptosis, enabling attenuation of disease. Conversely, treatments that can increase apoptosis in these cell types will likely reduce the amplitude of inflammatory signaling and the severity of disease.

Since therapeutic targeting of the etiological agent in RA is not yet possible, inhibition of the immune response that generates the inflammatory stimulus would the closest downstream objective. In this respect, the feasibility of adapting apoptotic pathways to eliminate auto-reactive immune cells has been demonstrated. This type of strategy has been achievable in defined animal models of arthritis whose pathogenesis is controlled by administration of specific exogenous materials such as CII. In RA, however, the "self" epitopes that drive the putative autoimmune response are as yet unknown, and indeed may be different among afflicted patients. As the results of further studies of genetics and predisposition to disease emerge, the viability of such a method toward the treatment of human disease should become more clear.

Targeted local delivery of pro-apoptotic agents to specific joints has also shown promise experimentally, but likewise is met with a number of challenges. The most apparent of which is limiting killing specifically to diseased cells against the background of normal tissues. The importance of this is demonstrated by the toxicity associated with delivery of anti-Fas antibody. To this end, systems analogous to that described for synovial-specific targeting peptides may be capable of averting non-specific toxicity. Gene delivery of apoptotic proteins has proven to be capable of eliciting a significant, therapeutic response in arthritic animals following intra-articular administration of appropriate recombinant viral vectors. Again, though, cell specificity and the containment of vectors is a concern. The use of disease restricted gene products such as TRAIL or vectors with limited tropism or with cell-type specific promoters or regulatory regions driven by inflammation may be capable of limiting collateral cytotoxicity.

Alternatively, the safest method may be to target NF-κB or its activation. This offers the potential to down-regulate expression of inflammatory and erosive proteins while increasing the vulnerability to apoptosis of cells involved in perpetuating disease pathology.

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THERAPEUTIC INHIBITION OF APOPTOSIS CAUSED BY STROKE

Chapter X

Seth Love*

1. INTRODUCTION

Stroke is the most common fatal neurological disorder and the leading cause of severe disability in the western world. The overall incidence is about 1.5-2 per 1000 people per year but the figure is much higher in the elderly: in people over 85 years it is about 18 per 1000 per year (Broderick et al., 1998; Rosamond et al., 1999; Thorvaldsen et al., 1995; Wolf & D'Agostino, 1993; Wolf et al., 1992). The prevalence of stroke rises almost exponentially from about the 5th decade. At all ages, the prevalence is greater in men than women. Of people surviving beyond 75 years of age, over 10% will have had a stroke. The term stroke encompasses any acute neurological deficit of vascular origin, including subarachnoid and parenchymal brain haemorrhage. However, about 80% of strokes are due to brain infarction, and it is with those that this chapter is concerned.

What I shall try to do in this chapter is (i) provide a simple overview of ischaemic neuronal death, to serve as a framework when considering the prospects for treatment by prevention of apoptosis, and (ii) summarise the current situation with regard to the acute treatment of ischaemic strokes and the prevention of neuronal apoptosis.

2. ISCHAEMIC INJURY AND CONSEQUENCES

It is conceptually helpful to subdivide the death of neurons after ischaemia into the processes responsible for cell injury, and those responsible for cell death (i.e. the consequences of fatal cell injury).

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2.1. Processes Responsible for Cell Injury

The precipitating event is usually thrombotic or embolic arterial occlusion. Less often strokes result from raised intracranial pressure, reduced cardiac output, or reduced content or availability of oxygen in the blood. Most strokes in man are due to prolonged, complete ischaemia in the greater part of the affected brain tissue. Whereas many experimental studies of stroke have involved brief or incomplete ischaemia, this accounts for only a small minority of strokes in clinical practice. Transient reduction in cardiac output (e.g. due to cardiac arrhythmia), and rapid thrombolysis after thrombotic arterial occlusion are the principal clinical contexts in which there is substantial reperfusion.

Because neurons have no capacity for anaerobic metabolism and contain negligible glycogen, they require a continuous haematogenous supply of oxygen and glucose to survive. Cessation of blood flow is rapidly followed by a fall in tissue pH, depletion of ATP, and slowing and then failure of the Na^+/K^+ pump (Erecinska & Silver, 1994; Silver, 1976; Silver & Erecinska, 1992). Na^+ moves into the cells and K^+ outwards, down their electrochemical gradients across the plasma membrane, and the membranes depolarise. Subsequent injury is mediated by a combination of energy depletion, glutamate-mediated excitotoxicity, oxidative stress, and inflammation (see below) (Dirnagl et al., 1999; Lo et al., 2003). The balance of these processes depends on the nature of the ischaemic insult and the proximity of the neuron to a supply of oxygenated blood.

In the permanently ischaemic core of a typical atherothrombotic infarct, the rapid depletion of oxygen and high-energy metabolites such as NADH soon leads to necrosis. Towards the periphery of the infarct, there is an unstable equilibrium between oxygen supply and demand, in which repeated repolarisation and depolarisation of neurons and release of glutamate lead to recurrent waves of spreading electrical depression, exacerbating consumption of oxygen and oxidative stress. Hypoxia also activates transcription factors and kinases, such as AP-1, HIF-1α, HIF-2, NF-κB, STAT3 and p38 MAPK, that in turn induce the expression of several pro-inflammatory molecules within the brain, including the cytokines TNF, and IL-1β, and the endothelial inflammatory cell adhesion molecules, P- and E-selectins and intercellular adhesion molecule-1 (ICAM-1) (Iadecola & Alexander, 2001). In an atherothrombotic infarct, this occurs especially towards the periphery of the infarct, where it causes intravascular accumulation of neutrophils and their subsequent infiltration of the parenchyma. If ischaemia is only transient, the release of pro-inflammatory cytokines and upregulation of inflammatory adhesion molecules after reperfusion has the potential to cause a more widespread inflammatory reaction, throughout the previously-ischaemic tissue.

2.1.1. Depletion of Oxygen and High-Energy Metabolites

The depletion of oxygen and high-energy metabolites from infarcted brain tissue results primarily from the lack of oxygenated blood but other factors also contribute. These include a tendency to hyperthermia after strokes, with an associated increase in the consumption of energy, and activation of DNA repair enzymes, especially poly(ADP-ribose) polymerase-1 (PARP-1), in response to oxidative damage to DNA (see below). Depletion of oxygen is also exacerbated by recurrent peri-infarct depolarisation and the effects of inflammation.

PARP-1 catalyses the cleavage of NAD into nicotinamide and ADP-ribose, and the addition of ADP-ribose groups to histones and several other nuclear proteins, including PARP-1 itself (de Murcia et al., 1997). This inhibits enzymes involved in transcribing and replicating DNA and may cause uncoiling of DNA at sites of damage, facilitating access of other repair proteins. By these means PARP-1 protects damaged cells from transcription and replication, and promotes DNA repair. The drawback is that the process consumes large amounts of energy (Eliasson et al., 1997; Endres et al., 1997). Each addition of ADP-ribose consumes the equivalent of 4 molecules of ATP, which are what are needed to regenerate NAD. This depletion of ATP may be critical in tissues of marginal energy supply and viability. In ischaemic brain the activity of other DNA repair proteins, such as DNA-dependent protein kinase, also consumes energy, although not to the same extent as PARP-1 does. In addition to its impact on energy consumption, PARP-1 activity increases expression of the pro-inflammatory endothelial adhesion molecule CD11a and influences the transcription of several other genes that play a critical part in the cellular response to ischaemia (Ha et al., 2002; Skaper, 2003). PARP-1 is cleaved and inactivated at an early stage of apoptosis by several caspases, including caspase-3. In man, PARP activity in neurons is particularly increased after transient brain ischaemia such as that resulting from a cardiac arrest, and in some forms of hypoxic-ischaemic neonatal brain damage (Love et al., 1998; Love et al., 1999; Love et al., 2000).

2.1.2. Excitotoxicity

This term refers to the cell damage that results from excessive activation of the receptors for glutamate, an excitatory neurotransmitter. The membrane depolarization and the change in the concentration gradients of Na^+ and K^+ across the plasma membrane cause reversal of the direction of action of the glutamate transporter proteins, as a result of which glutamate rapidly accumulates extracellularly until it reaches neurotoxic levels. Glutamate binds to several receptors, some (the ionotropic receptors) that are ligand-gated ion channels and others (the metabotropic, G-protein-linked receptors) that are coupled to phospholipase C or adenylyl cyclase (Schoepfer et al., 1994; Wisden & Seeburg, 1993). The ionotropic *N*-methyl-D-aspartic acid (NMDA) glutamate receptor, which incorporates a high-conductance Ca^{2+} channel, has a particularly important role in mediating acute excitotoxic damage in brain ischaemia. The rapid rise in intracellular Ca^{2+} that results from persistent activation of NMDA receptors activates a range of Ca^{2+}-dependent proteases (e.g. calpains), lipases (e.g. phospholipase A_2), kinases and nucleases, as well as nNOS: neuronal Ca^{2+}-dependent nitric oxide synthase. Several of these enzymes, particularly phospholipase A_2 and nNOS, are key mediators of another process that plays a major role in ischaemic brain damage, namely oxidative stress.

2.1.3. Oxidative Stress

Within the body there is normally a balance between the production of free radicals and other highly reactive, oxidizing chemical species – and the scavenging of free radicals and inactivation of oxidizing chemicals by enzymes and other antioxidants. Oxidative stress occurs when this balance is disrupted and the antioxidant defences are

overwhelmed. This occurs after brain ischaemia, particularly transient ischaemia such as might be expected after cardiac arrest or rapid thrombolysis, but also towards the periphery of atherothrombotic infarcts (Dirnagl et al., 1999; Lo et al., 2003; Love, 1999a).

Early contributors include Ca^{2+}-activated neuronal nitric oxide synthase (nNOS), phospholipase A_2 and xanthine oxidase; increased transcription and activity of cyclooxygenases (especially COX-2); and leakage of oxygen free radicals from the mitochondrial electron transport chain. On later activation of astrocytes and microglia, and infiltration of brain tissue by haematogenous inflammatory cells (see below), inducible nitric oxide synthase (iNOS) and NADPH oxidase also contribute.

NOS catalyses the conversion of L-arginine first to *N*-hydroxy-L-arginine, and then L-citrulline and nitric oxide (NO). These reactions are coupled to the donation of two electrons by NADPH. When NOS activity is uncoupled from electron donation by NADPH, as can occur under conditions of hypoxia, the synthesis of NO is accompanied by the production of superoxide and hydrogen peroxide. The reaction of superoxide with hydrogen peroxide leads to the formation of hydroxyl free radicals. When NO and superoxide are present together, they combine to form peroxynitrite. Although not a free radical, peroxynitrite is more highly reactive than is either of its precursors and causes oxidative damage to DNA, proteins, lipids and other macromolecules (Szabo, 1996).

Oxidation of lipids contributes to apoptosis through a range of mechanisms. The Ca^{2+}-mediated activation of phospholipases C and A_2 during ischaemia results in the hydrolysis of membrane phospholipids and release of arachidonic acid (AA) and other fatty acids (Bonventre et al., 1997; Rordorf et al., 1991; Umemura et al., 1992). Metabolism of AA leads to the production of prostaglandins, leukotrienes and platelet-activating factor, the activities of all of which contribute to oxidative stress and inflammation. Free radical-mediated peroxidation of the fatty acids generates 4-hydroxynonenal (Ji et al., 2001) and ischaemia, especially with reperfusion, causes activation of sphingomyelinase and hydrolysis of sphingomyelin to release ceramide (Herr et al., 1999). Both 4-hydroxynonenal and ceramide can cause mitochondrial release of cytochome *c*, activation of caspase-3 and apoptosis (Ji et al., 2001; Kruman et al., 1997; Richter & Ghafourifar, 1999).

2.1.4. Inflammation

As noted above, hypoxia induces the expression of multiple pro-inflammatory cytokines, including TNF and IL-1β, that cause activation of microglia, expression of adhesion molecules by vascular endothelium, accumulation, transendothelial migration and degranulation of neutrophils, and the later entry into the tissue of haematogenous macrophages (DeGraba, 1998; Iadecola & Alexander, 2001; Lindsberg et al., 1996; Love & Barber, 2001). The rolling of neutrophils across the endothelial surface of the blood vessels and their subsequent tethering to the endothelium are mediated by interaction between L-selectin on the surface of the leukocytes and P- and E-selectins on the endothelial cells (DeGraba, 1998). Adhesion is subsequently strengthened and transendothelial migration initiated by interaction between another inducible endothelial surface protein, ICAM-1, and a complex of leukocyte proteins (the CD18 complex). Multiple factors probably contribute to the deleterious effects of inflammation after brain

ischaemia. The adherent neutrophils reduce tissue perfusion by occluding small blood vessels and possibly also causing vasoconstriction. The neutrophils and other inflammatory cells produce IL-6, that promotes further inflammation, and cause direct tissue damage by the release of cytokines and proteolytic enzymes and the generation of free radicals.

2.2. Consequences: the Processes Responsible for Cell Death

The consequences of fatal ischaemic neuronal damage are usually presented as a dichotomy: apoptosis or necrosis. However, in considering approaches to therapy, it is worth noting that ischaemic neuronal death may involve a combination of apoptotic and necrotic processes even at the level of the individual neuron (Love, 2003).

2.2.1. Necrosis

This type of cell death is not energy-dependent and usually occurs *en masse* in a circumscribed volume of tissue with a hostile micro-environment. Necrosis predominates in prolonged, complete ischaemia, i.e. in most brain infarcts. Depletion of energy and failure of ion pumps cause swelling of the cytoplasm, of mitochondria and other organelles. This leads to rupture of plasma membranes and lysis of the cell, and induces an inflammatory reaction. Although death by necrosis does not need an exogenous source of energy, it is accelerated by the activity of several energy-dependent lytic enzymes, including Ca^{2+}-dependent proteases.

2.2.2. Apoptosis

This term describes the changes that occur when individual cells undergo programmed death, an energy-dependent process which is often a normal developmental or physiological response to local signals (Kerr et al., 1972; Wyllie et al., 1981). Apoptosis predominates after brief ischaemia with reperfusion (Charriaut-Marlangue et al., 1996; Fujimura et al., 1998; Li et al., 1997; Li et al., 1998; Vexler et al., 1997). In contrast, in a typical atherothrombotic infarct, apoptosis is largely restricted to small numbers of non-neuronal cells – glial, vascular and inflammatory (Love, 2003; Love et al., 2000). Both neuronal and glial apoptosis can occur in the peri-infarct region, but in man the extent of this is generally less than in experimental animals – even models of permanent focal ischaemia. The main clinical contexts in which neuronal and glial apoptosis tend to be relatively prominent are cardiac arrest followed by resuscitation, and neonatal hypoxic-ischaemic brain damage, both examples of ischaemia-reperfusion injury. Reperfusion is, of course, the objective of early thrombolysis, e.g. resulting from administration of recombinant tissue plasminogen activator (rTPA) in the first few hours of an ischaemic stroke, and it is quite likely that apoptosis may contribute to neuronal death in this clinical context also, although for obvious practical reasons this has not been confirmed in man.

Apoptosis in brain ischaemia is mediated through several intracellular pathways, involving the translocation of Bax, Bak and other pro-apoptotic members of the Bcl-2 family to the mitochondrial outer membrane; the actions of 4-hydroxynonenal and

ceramide; the inhibition of protein synthesis; possibly the activation of cyclin-dependent kinases and aberrant re-entry of neurons into the cell cycle; and the actions of caspases (cysteine-requiring aspartate-directed proteases) (Banasiak et al., 2000; Love, 2003; Schulz et al., 1999). Directly or indirectly, all of these cause release from the mitochondrial intermembrane space of cytochrome *c*, pro-caspase-9 and Apaf-1, which combine to form the apoptosome. This complex cleaves and activates pro-caspase-3 to form caspase-3, the key effector of caspase-mediated cell death. Caspase-3 and other effector caspases (caspase-6 and -7) cleave cytoskeletal proteins such as actin, α-fodrin and gelsolin, structural nuclear proteins such as the lamins, DNA repair proteins such as DNA-dependent protein kinase and PARP-1, anti-apoptotic members of the Bcl-2 family, and inhibitor of caspase-activated DNase (ICAD). This last action releases caspase-activated DNase and results in internucleosomal cleavage of nuclear DNA. Apoptosis can also be initiated by the binding of ligands to cell-surface death receptors – Fas/CD95/APO-1, tumour necrosis factor-α receptor (TNF-R), DR4 and DR5 - but research suggests that this latter pathway has only a very limited role in brain ischaemia.

The morphological consequences of the caspase activity are rounding and shrinkage of the cell, condensation of chromatin and the formation of cytoplasmic blebs at the surface of the cell, which fragments into multiple small membrane-bound bodies that contain intact organelles and dense clumps of condensed chromatin (Wyllie et al., 1981). Apoptotic cells are rapidly removed by phagocytosis, without eliciting an inflammatory reaction.

3. SCOPE FOR THERAPEUTIC INTERVENTION

Common sense indicates that the prospect of achieving a clinically useful outcome is greatest if we can intervene to prevent the processes responsible for cell injury, particularly the early processes such as vascular occlusion, and depletion of oxygen and high-energy metabolites. The likelihood of salvaging function is least if we try to prevent apoptosis or necrosis of cells that have mostly sustained irrecoverable damage. The interventions that act at an early stage are not specifically targeted at apoptosis or necrosis, but the effect of some, such as those that reduce oxidative stress, is predominantly anti-apoptotic. Targeted interference with apoptosis itself, usually by inhibition of effector caspases, has yielded moderate therapeutic benefits in animal studies, especially those involving relatively brief periods of ischaemia. However, inhibition of caspases may be ineffective if damaged neurons persist without recovery of function (Gillardon et al., 1999; Weishaupt et al., 2003) and counterproductive if the treatment has unpleasant side-effects or the damaged neurons provoke inflammation (Nicotera et al., 2000). Intriguingly, (Chopp et al., 1999) found that administration of the apoptosis-inducing drug staurosporine after transient focal cerebral ischemia in the rat not only increased the number of apoptotic cells but also reduced infarct volume and increased synaptogenesis. It is possible that, at least in some circumstances, diversion of irreparably damaged cells towards apoptosis rather than necrosis may be beneficial.

3.1. Prevention of Ischaemic Cell Injury

3.1.1. Thrombolysis – Reduction of Duration of Ischaemia

A recent Cochrane review concluded that thrombolytic therapy, rTPA in particular, reduces morbidity and mortality when given after ischaemic stroke (Wardlaw et al., 2003). The therapeutic time-window is still unclear (some studies suggest 3 hours and others 6 hours), as are the optimum dose and route of administration. Fibrinogen-depleting agents such as ancrod (produced from Malaysian pit viper venom) and defibrase may also be of benefit (Liu et al., 2003). The potential role of glycoprotein IIb/IIIa antagonists such as abciximab (the Fab fragment of a chimeric human-mouse antibody), eptifibatide (a cyclic heptapeptide), and tirofiban (a tyrosine-derived non-peptide molecule) is still under evaluation (Leclerc, 2002; Morris et al., 2003; Seitz et al., 2003; Sherman, 2004; Zhang et al., 2003). It is likely that most of the beneficial effects of thrombolysis are due to reduction of necrosis.

3.1.2. Prevention of Depletion of Oxygen and High-Energy Metabolites

Several experimental studies have shown that mild to moderate hypothermia reduces apoptotic as well as necrotic cell death after brain ischaemia, not only in the neonate but also in adults (Maier et al., 1998; Prakasa Babu et al., 2000; Yenari et al., 2003; Zhang et al., 2001). However, a Cochrane review in 2000 concluded that there was no evidence from randomised clinical trials to support the use of hypothermia in acute stroke (Correia et al., 2000). The reviewers also commented that experimental studies had shown a neuroprotective effect of hypothermia in experimental cerebral ischaemia in patients with severe closed head injury, and that trials of hypothermia for acute stroke were still needed. In a subsequent study comparing hemicraniectomy with moderate hypothermia for severe focal stroke, the latter was associated with increased mortality and complication rate (Georgiadis et al., 2002). Further trials are underway of body or head cooling for treatment of stroke (Wang et al., 2003; Weber et al., 2003). In contrast to the lack of evidence to date of clinical efficacy of hypothermia in focal atherothrombotic strokes, two prospective, randomized, controlled studies in cardiac arrest patients showed that induction of mild hypothermia (core temperature 32-34°C) for 12-24 hours after restoration of circulation reduced mortality and also improved neurological outcome (Bernard et al., 2002; Hypothermia after Cardiac Arrest Study, 2002). In a further study, the improvement in neurological outcome that resulted from mild hypothermic treatment of cardiac arrest patients was shown to be associated with a less sustained increase in serum levels of neuron-specific enolase, a marker of neuronal damage (Tiainen et al., 2003).

The potential of PARP inhibitors has still to be tested in stroke patients. Experimental evidence suggests that inhibition of PARP predominantly salvages neurons that would otherwise undergo necrosis rather than apoptosis (Ha & Snyder, 2000; Moroni et al., 2001) although this is probably an oversimplification and may, in any case, be an advantage in functional terms (see above). Observations from human autopsy studies suggest that cardiac arrest patients would benefit more than focal stroke patients from inhibition of PARP activity (Love et al., 1998; Love et al., 1999). Although several

inhibitors of PARP have been developed and used in animal studies, the most promising initial candidate for human trials is nicotinamide; although less selective than some other compounds, it has the advantages of a long track record of safe use in other human diseases such as pellagra, arthritis, insulin-dependent diabetes mellitus, and MELAS (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes), (Yang & Adams, 2004).

3.1.3. Prevention of Excitotoxicity

Recent Cochrane reviews of the use in stroke patients of calcium antagonists, or of agents that either reduce the release of glutamate or interfere with its binding to receptors, have concluded that no clinical benefit has yet been shown and that some of the drugs may increase mortality (Horn & Limburg, 2001; Muir & Lees, 2003). Trials of glycine, an NMDA receptor co-agonist, yielded promising but inconclusive results (Gusev et al., 2000). More recent trials of GV150526, a glycine antagonist which blocks the NMDA receptor at the glycine site, did not show benefit (Lees et al., 2001; Sacco et al., 2001). Clomethiazole (an anticonvulsant which probably acts on chloride channels to potentiate responses to GABA and glycine) seems not to improve outcome (Sareen, 2002); evaluation in stroke of another GABA agonist, diazepam, is still in progress (Lodder et al., 2000). Other drugs that interfere with the actions of glutamate and are undergoing clinical trials in stroke patients are magnesium, which interferes with glutamate release, voltage gated calcium channels and activation of NMDA receptors (Muir, 2002); YM872, a competitive antagonist of AMPA receptors (Akins & Atkinson, 2002); and ONO-2506, a homologue of valproic acid that modulates uptake capacity of glutamate transporters and expression of GABA receptors, and inhibits S100β synthesis and activation of astrocytes (de Paulis, 2003; Tateishi et al., 2002).

3.1.4. Prevention of Oxidative Stress

In many experimental animal studies, prevention of oxidative stress by administration of NOS inhibitors, phospholipase inhibitors, lipid-stabilizing compounds, *N*-acetyl cysteine (a precursor of glutathione) or free-radical scavenging agents has reduced ischaemic neuronal death, particularly apoptotic death after ischaemia-reperfusion injury. Beneficial effects in human stroke have been less consistent but have been demonstrated in several clinical studies. A modest, but statistically significant, benefit was seen when ingestion of ebselen, a seleno-organic antioxidant compound, was started within 24 hours of ischaemic stroke (Yamaguchi et al., 1998). A subsequent randomized, placebo-controlled, double-blind assessment of ebselen in patients with acute middle cerebral artery occlusion found a significant reduction in the volume of the cerebral infarct and an improvement in outcome of patients who started treatment within 6 hours of onset (Ogawa et al., 1999). Adminstration of the free radical-scavenging agent edaravone (MCI-186) to patients within 72 h of onset of ischaemic stroke was associated with a significant improvement in functional outcome, in a randomized, placebo-controlled, double-blind study (Edaravone Acute Infarction Study, 2003). Citicoline (cytidine-5'-diphosphocholine, an intermediate in the biosynthesis of phosphatidyl-

choline) is neuroprotective in several models of ischaemic, traumatic and other types of brain injury (Adibhatla et al., 2002). It seems to stabilize cell membranes and decrease lipid peroxidation, possibly by reducing phospholipase activity. A combined analysis of four prospective, randomized, placebo-controlled, double-blind clinical trials of oral citicoline for treatment of stroke found that administration of 2000 mg within 24 hours significantly increased the probability of complete recovery at 3 months (Davalos et al., 2002).

A promising addition to these agents is the nitrone spin-trap free radical scavenger, NXY-059. This has shown considerable neuroprotective efficacy in experimental studies of both transient and focal brain ischaemia (Ginsberg et al., 2003; Kuroda et al., 1999; Lapchak & Araujo, 2003; Marshall et al., 2003a; Marshall et al., 2001; Marshall et al., 2003b; Sydserff et al., 2002; Zhao et al., 2001). NXY-059 has been shown to maintain activation of AKT and to prevent mitochondrial cytochrome *c* release (Yoshimoto et al., 2002a; Yoshimoto et al., 2002b), and probably reduces both necrotic and apoptotic neuronal death. It is well tolerated in stroke patients, at and above concentrations shown to be neuroprotective in animals (Lees et al., 2003).

To date, NOS inhibitors have proven disappointing for treatment of stroke. A Cochrane review in 2002 concluded that there was insufficient evidence from randomised trials to recommend the use of these agents in acute stroke (Bath et al., 2002). More recently, several new NOS inhibitors have been shown to be neuroprotective in animals studies and it remains possible that this approach may prove useful clinically (Ding-Zhou et al., 2003; Marshall et al., 2003b).

3.1.5. Prevention of Inflammation

Effective anti-inflammatory strategies for ameliorating ischaemic brain damage in experimental models have included immunosuppression; removal of neutrophils or complement; interference with neutrophil function; inhibition of the proinflammatory caspase-1; blockade of inflammatory adhesion molecules or complement receptors; administration of IL-1β receptor antagonist or soluble TNF receptor; and inhibition of mitogen-activated protein kinase (MAPK), a downstream mediator of some of the effects of these cytokines (Arvin et al., 2002; Barone & Parsons, 2000; Basu et al., 2002; Chopp et al., 1996; Huang et al., 1999; Love, 1999b; Martin-Villalba et al., 2001; Mocco et al., 2002; Mulcahy et al., 2003; Rabuffetti et al., 2000; Takamatsu et al., 2001; Tikka & Koistinaho, 2001; Yrjanheikki et al., 1998). To date, no convincing evidence has been published that anti-inflammatory interventions benefit stroke patients. A randomized, controlled trial showed that enlimomab, a murine ICAM-1 antibody, worsened outcome when administered within 6 hours of onset of stroke (Enlimomab Acute Stroke Trial, 2001); however, there is some evidence that the adverse effects were due to immune activation induced by the heterologous protein, and trials of humanized blocking antibody may yield better results (Iadecola & Alexander, 2001). Trials of IL-1β receptor antagonist and of E-selectin nasal spray are still in progress.

3.1.6. Other Interventions Aimed at Reducing Cell Injury

Several neurotrophic proteins and peptides attenuate experimental ischaemic neuronal damage. These include insulin-like growth factor-1 (Tagami et al., 1997; Tamatani et al., 1998), basic fibroblast growth factor (bFGF) (Ay et al., 2001; Tamatani et al., 1998), glial cell line-derived neurotrophic factor (Miyazaki et al., 1999) and erythropoietin (Siren et al., 2001; Villa et al., 2003). None has yet been tested in human stroke. There have been three randomized trials of gangliosides in stroke, but these have not shown benefit (Candelise & Ciccone, 2001). Correction of hyperglycaemia probably helps to reduce cell injury after ischaemia. Hyperglycaemia has long been known to worsen neurological outcome and increase mortality after stroke (Kagansky et al., 2001). Infusion of glucose, potassium and insulin to treat mild to moderate hyperglycaemia after stroke may improve neurological outcome and reduce mortality (Scott et al., 2001; Scott et al., 1999).

3.2. Direct Interference with Apoptosis and Necrosis

Numerous experimental studies have shown that cerebroventricular administration of peptide inhibitors of caspases reduces delayed apoptotic neuronal death after brain ischaemia, especially if the ischaemia is only transient (Chen et al., 1998; Cheng et al., 1998; Endres et al., 1998; Fink et al., 1998). In some studies, caspase inhibitors have also provided some neuroprotection after permanent focal ischaemia, and newer peptide-based inhibitors are being developed that cross the blood-brain barrier and can be given intravenously (Deckwerth et al., 2001; OntAniente et al., 2003). These may be useful adjuncts to thrombolysis and antioxidants for the treatment of stroke in man. However, there remain some concerns as to the long-term benefit of therapeutic intervention at such a late stage in the sequence of intracellular events that lead to apoptosis (see first paragraph in section 3). Interventions apart from caspase inhibitors that have been used to interfere with apoptosis after experimental brain ischaemia include JNK inhibitors and antisense oligonucleotides (Borsello et al., 2003; Gu et al., 2001), pifithrin-α, a synthetic inhibitor of p53 (Culmsee et al., 2001), and viral vectors that over-express Bcl-2 (Zhao et al., 2003).

As noted above, there is evidence that apoptosis of some neurons may be preceded by their aberrant entry into the cell cycle. Furthermore, the use of cyclin-dependent kinase (CDK) inhibitors has been reported to reduce apoptotic neuronal death in models of ischaemia (Katchanov et al., 2001; Osuga et al., 2000; Park et al., 2000). However, these may prevent apoptosis by inhibiting CDK5, which is not involved in cell-cycle control (Weishaupt et al., 2003).

Lastly, non-caspase cysteine proteases, such as the calpains (Ca^{2+}-activated cysteine proteases) and cathepsins (lysosomal cysteine proteases), are involved in both apoptotic and necrotic cell death (Newcomb-Fernandez et al., 2001; Rami et al., 2000; Tontchev & Yamashima, 1999; Yamashima, 2000). In experimental studies, several calpain inhibitors have been shown to reduce ischaemic brain damage (Bartus et al., 1994; Markgraf et al., 1998; Wang et al., 1996).

4. CONCLUSION

The mainstays of acute management of stroke patients remain accurate, early diagnostic assessment, close clinical monitoring, skilled nursing care, maintenance of good cerebral perfusion, and the prevention of complications such hyperthermia and hyperglycaemia. However, the number of additional therapeutic interventions that are of proven efficacy is increasing. The constraints on their use are quite strict: in most cases the treatment needs to be commenced within the first 3-6 hours, and then only after exclusion of associated haemorrhage and various other contraindications. The most widely accepted of these interventions is rTPA-mediated thrombolysis, but several antioxidants seem also to improve clinical outcome, and cooling is of benefit in ameliorating ischaemic brain damage after cardiac arrest. PARP-1 inhibitors too seem likely to be of benefit after cardiac arrest, but their use in this context has not yet been evaluated clinically. Whilst early thrombolysis and reperfusion are essential if ischaemic tissue is to be salvaged, experimental studies suggest these are likely to produce excitotoxic, oxidative and inflammatory damage, in which case the addition of antioxidants and anti-inflammatory agents may be of benefit. However, combined intervention of this sort has not been assessed in clinical trials. The role of drugs that interfere specifically with apoptosis is still unclear. Promising results have been obtained in some experimental studies but other findings raise questions as to the utility of this approach. The most effective way of preventing apoptosis whilst also preserving function is to intervene at as early a point as possible in the metabolic pathways that lead from cell damage to cell death.

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MONOCLONAL AND BISPECIFIC ANTIBODIES AS NOVEL THERAPEUTICS

Chapter XI

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1. INTRODUCTION

Hybridoma technology followed later by gene technology has made it possible to produce monoclonal antibodies of almost any desired specificity in unlimited quantities and various formats. To talk of a monoclonal antibody revolution initiated by the ground breaking work of Köhler and Milstein is no exaggeration at least as far as basic biological sciences and medical diagnosis are concerned (Kohler & Milstein, 1975). But what of therapy, the prime interest of many of us and this book? What about the numerous strategies which have been put forward as possibilities to "translate" the unique antibody specificity into therapeutic specificity? A great challenge, indeed. Rather than contributing an exhaustive review, the following chapter is intended to provide the reader with a more personal view on this subject. According to the topic of the book the focus will be on a new approach developed in our laboratory for selectively engaging apoptotic pathways with bispecific antibodies. To make the foundations of this new strategy comprehensive we shall start with a brief overview of some of the principles governing the therapeutic use of monoclonal antibodies in general, and bispecific antibodies in particular.

2. "NAKED" ANTIBODIES

The physiological function of antibodies is to recognize and neutralize antigen. Occasionally neutralization can be achieved by mere recognition. In most cases, however,

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more than this is required and for this reason nature has attached the constant (Fc) effector part to the variable recognition part of the antibody molecule. The Fc region is capable of recruiting the complement system or Fc receptor carrying immune cells for the task of neutralization and destruction of antigen or antigen expressing cells.

Therapy with adoptively transferred monoclonal antibodies mimics this physiological situation: Sometimes the mere blocking by recognition approach may suffice, like in the case of TNF. This cytokine is involved in the pathogenesis of many inflammatory processes and in septic shock. Its specific inhibition by monoclonal antibodies or recombinant fusion proteins has provided an effective new treatment modality for various autoimmune conditions such as rheumatoid arthritis, inflammatory bowel disease and psoriasis (Keating & Perry, 2002; Weinberg, 2003; Nahar et al., 2003).

If the removal of cells, such as tumor cells, is required merely blocking the target antigen is not enough. The effector part of the antibody is certainly necessary, but does it also suffice? At the beginning of the era of monoclonal antibodies the answer seemed negative: In several clinical trials conducted in the early and mid-80s with mouse monoclonal antibodies directed to various tumor-associated antigens tumor regressions remained anecdotal (Verrill et al., 1986; Schroff et al., 1987; Eger et al., 1987; Dillman, 1989) with one notable exception. In a multicenter trial performed by Riethmüller and colleagues a significant prolongation of survival was observed if patients with colorectal carcinoma were treated in an adjuvant, rather than an advanced state of the disease (Riethmuller et al., 1994).

Nevertheless, improvement was necessary and it came from recombinant gene technology, which was used to exchange mouse constant regions with corresponding parts of the human IgG molecule. More recently, antibodies that are entirely human were generated using transgenic mice with completely exchanged Ig loci (Little et al., 2000; Clark, 2000; Hudson and Souriau, 2003). Compared to their murine counterparts, humanized antibodies promised not only to lower immunogenicity, but also to enhance efficiency of the 'now human' effector part. Did they live up to these expectations? In part certainly. Several of them now constitute approved drugs with established activity against certain types of lymphoma (anti-CD20, Rituxan), (Grillo-Lopez, 2003; Smith, 2003; Boye et al., 2003) and mammary carcinoma (anti-Her2neu, Herceptin) (Vogel et al., 2002; Ross et al., 2003; Albanell et al., 2003). Several more that are directed to tumor associated antigens such as CD30, the EGF-receptor or the EpCAM molecule, are currently being evaluated in phase I-II clinical trials (Glennie and Johnson, 2000; Brekke and Sandlie, 2003).

The success of antibody humanization confirms that the activity of monoclonal antibodies in cancer therapy is largely due to Fc mediated effects such as antibody dependent cellular cytotoxicity (ADCC) or complement mediated cytotoxicity (CDC). Nevertheless, it has been speculated that the reported capability of antibodies to CD20 and Her2neu to induce apoptosis in tumor cells in a multimerized form (Ghetie et al., 1997) may contribute to their therapeutic activity (Cragg & Glennie, 2003).

Regardless of the mechanisms responsible for their anti cancer activity, the most important strategic question is whether one can be satisfied with the current achievements using "naked" monoclonal antibodies? We think not, simply because the therapeutic activity of humanized antibodies as currently protocolled is transient or only marginal in many patients (Grillo-Lopez, 2003; Smith, 2003; Boye et al., 2003; Vogel et al., 2002;

Ross et al., 2003; Albanell et al., 2003), leaving ample room for improvement, in particular of the antibody effector function.

3. "ARMED" ANTIBODIES

The most obvious way of arming antibodies is to attach conventional cytotoxic reagents such as cytostatic drugs or radioactive isotypes to them. At first glance this approach seems feasible: Radioactively labeled monoclonal antibodies have been shown in numerous studies to be capable of targeting tumors not only in animal models (Halpern et al., 1983; Bernhard et al., 1983; Hwang et al., 1985), but also in humans (Matzku et al., 1989; Mach et al., 1983; Bischof-Delaloye et al., 1989). However, a closer look revealed this approach to be less encouraging. The amount of antibody specifically bound was usually found to be less than 1% of the total body dose (Matzku et al., 1989; Mach et al., 1983). These findings provide a lesson the importance of which cannot be emphasized enough: Antibody based targeting of effector molecules *in vivo* is insufficient if it relies merely on pharmacokinetics. Something more sophisticated is called for to ensure that the activity of effector molecules depends upon the binding of the targeting antibody. Obviously this is how the physiological effecor part of an antibody, its Fc portion, functions. It triggers complement activation and the Fc receptors on immune effector cells after- and not before binding to the respective target cell. There is a simple cell biological reason for this. Both the trigger molecule of the classical complement cascade, C1q, and Fc receptors, are activated by antigen-bound or multimeric and not by soluble monomeric antibody molecules. Thus, antibodies are not only specific but also selective as far as their effector function depends on binding to the antigen. Would it be possible to mimic this truly elegant mechanism by an artificial effector moiety attached to a monoclonal antibody thus achieving *in vivo*-selectivity by cell biology rather than by pharmacokinetics?

An interesting strategy was suggested in the pioneering work of E. Vitetta and I. Pastan and their groups already in the early 80s. Monoclonal antibodies were used to replace the binding (B) chain of highly effective bacterial toxins of the A/B type, resulting in antibody A chain immunotoxins (ITs) with an antibody defined specificity (Krolick et al., 1980; FitzGerald et al., 1983). Theoretically, such a reagent should act in a target-cell restricted way if the selected target antigen allows uptake of the IT into the cells. Recently, immunotoxins of this kind have been used successfully in the treatment of patients with hairy cell leukaemia (Kreitman et al., 2001). The toxicity of these reagents illustrates, however, that in practise the ideal of a complete dependency of the toxic principle upon antibody binding is difficult to achieve.

In an alternative strategy targeting antibodies were coupled with enzymes that catalyze the conversion of a prodrug. To obtain sufficient tumor to blood ratios of the immunoconjugate clearing antibodies had to be used. This again points to the insufficient antibody selectivity in vivo. Another problem of this type of IT is the relatively high toxicity of some of the activated prodrugs (Francis et al., 2002; Goodwin & Meares, 2001).

We and several other groups have favoured a third approach based on the development of bispecific antibodies. In general, this term describes a hybrid molecule

engineered by one of several available techniques to contain two different specificities. In the context discussed here the term is used more stringently to describe a reagent in which the Fc part of a targeting antibody is replaced by a second effector antibody with a peculiar "biomimetic" property.

4. BIOMIMETIC, BISPECIFIC ANTIBODIES AND THE BISPECIFIC PRINCIPLE

In several cases, monoclonal antibodies directed to certain cell surface receptors do not block but mimic the physiological function of the receptor ligand. These "biomimetic" antibodies can exert powerful biological effects. The following examples may illustrate this: (*i*) spontaneously arising antibodies to the thyroid stimulating hormone (TSH) receptor on thyroid cells induce an autoimmune disease of the thyroid gland (Graves disease) which is characterized by the uncontrolled production of thyroid hormones (Chen et al., 2003a). (*ii*) Immobilized monoclonal antibodies to the antigen-specific T-cell receptor (TCR)/CD3 complex are the most potent T-cell mitogens known (Tsoukas et al., 1985; Ledbetter et al., 1986; Meuer et al., 1984). Similarly, antibodies to other immune activating receptors such as the T cell associated molecules CD2 (Brottier et al., 1985; Ledbetter et al., 1988; Van Lier et al., 1988b) and CD28 (Van Lier et al., 1988a; June et al., 1990; Hara et al., 1985; Damle et al., 1988; Lenschow et al., 1996) and the various Fc receptors on monocytes, NK cells and granulocytes (Lanier et al., 1988; Fanger et al., 1989) induce profound agonistic effects in receptor-positive immune cells. (*iii*) The prototypic death receptor CD95 has been identified using the biomimetic function of specific monoclonal antibodies that are capable of inducing cell death in CD95 expressing, apoptosis-sensitive cell lines (Trauth et al., 1989; Yonehara et al., 1989).

In the context discussed above it is of paramount importance that in most of these cases, agonistic activity requires immobilization, that is multimerization, of the antibodies on cells or artificial surfaces. This is most likely a consequence of the fact that the ligands of the cell surface receptors recognized are themselves cell bound. Regardless of this, a biomimetic effector antibody whose activity depends on immobilization or multimerization on a target cell is exactly what is needed to arm a targeting antibody. In fact, in as much as the activity of the effector antibody depends upon its immobilization, the activity of the bispecific antibody containing it will depend upon its binding via the hybridized targeting antibody. Only if this "bispecific principle" is met will the biomimetic effector part of a bispecific antibody be as selective as the Fc part of a simple monospecific antibody. It is clear that a bispecific antibody of the kind described should be free of Fc parts in order to prevent activation of its effector function by binding to Fc receptor-positive rather than antigen positive cells (Fig. 1).

The biomimetic antibodies employed as effector components in bispecific antibodies consisted mainly of those listed in (*ii*); these were used to induce a target-cell restricted activation of the respective immune receptors. In several clinical studies with these reagents clearcut therapeutic effects were noted especially if the antibodies were applied

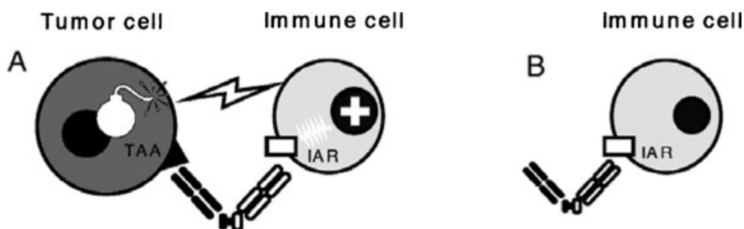

Figure 1. The bispecific principle: Bispecific antibodies with a targeting component directed to a tumor associated antigen (TAA) and a "biomimetic" effector component which triggers immune activating receptors (IARs) such as CD3, CD28, CD16 or CD64 induces immune cell activation and thus tumor cell killing (**A**). In as much as the agonistic activity of the effector antibody depends upon its multimerization, the activity of the bispecific antibody containing it depends upon its binding to the tumor target cells. Thus binding of the bispecific antibody to immune cells in the absence of the target cells will not lead to receptor activation (**B**). To ensure this principle of a strictly target cell-dependent activation, the antibodies need to be free of Fc parts (see text).

locally (Nitta et al., 1990; Canevari et al., 1995; Jung et al., 2001a). After systemic application, toxic side effects such as the cytokine release syndrome were observed which is induced in particular by bispecific antibodies containing an anti-CD3 specificity (Janssen et al., 1995; Tibben et al., 1996; Molema et al., 2000). This indicates that for this type of reagent at least, just as for the immunotoxins described above, the crucial prerequisite of strict target cell restriction is difficult to realize.

Let us now digress temporarily from the concept of recruiting immune cells with bispecific antibodies and turn to death receptors such as Apo-1/Fas/CD95.

5. TARGET CELL RESTRICTED ACTIVATION OF THE CD95 DEATH RECEPTOR WITH BISPECIFIC ANTIBODIES

As mentioned previously, "biomimetic" CD95 antibodies are capable of inducing apoptosis in CD95 positive and sensitive target cells. However, the application of such antibodies to mice rapidly killed the animals by inducing massive liver cell damage (Ogasawara et al., 1993). This illustrated dramatically that selectivity that is target cell restriction, is necessary if agonistic CD95 antibodies are to be used for tumor cell killing *in vivo*. Can the bispecific principle contribute selectivity? It is a well established fact that agonistic activity of CD95 antibodies requires antibody multimerization. As discussed above, this is an important prerequisite for the construction of target-cell-restricted bispecific antibodies (bsAb) in accordance with the bispecific principle. However, there is a difference between immune receptors and death receptors triggered by bsAbs. In the latter case, the target and effector antigen may be expressed on the same cell. How will bispecific antibodies behave in such a case? Will they allow a strictly target cell restricted stimulation of CD95? In our initial experiments the answer was clearcut. We constructed bispecific Fab_2 fragments directed to CD95 and different target antigens expressed on lymphoma cells of the B cell lineage (CD19, CD20 and CD40) and found that these antibodies induce pronounced apoptosis in SKW6.4 lymphoma cells carrying

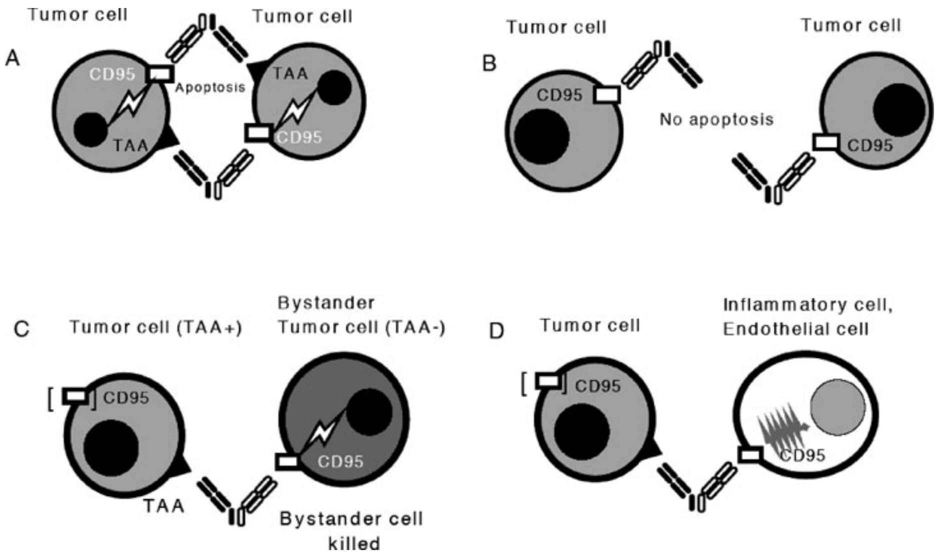

Figure 2. Examples of action evoked by bispecific antibodies with TAA X CD95-specificity. Bispecific antibodies with a biomimetic effector component directed against the death receptor CD95 can selectively induce apoptosis of cells expressing the TAA recognized by the targeting part. This is due to mutual crosslinking after "bicellular binding" as depicted in (**A**). If the target antigen is missing, this type of crosslinking cannot occur and apoptosis is not induced (**B**). However TAA-negative cells may be killed as bystanders in the neighbourhood of positive cells (**C**). Bi-cellular binding also allows stimulation of CD95 on immune cells or on vascular endothelial cells that can cause inflammation and anti-angiogenic effects (**D**). These pleiotropic effects do not depend on CD95 expression of the target cell, but they are target-cell restricted. This situation follows the bispecific principle as depicted in Fig. 1.

these target antigens but not in antigen negative, CD95 sensitive Jurkat cells. However, Jurkat cells were killed as bystander cells if SKW6.4 cells were present during the lytic assay (Jung et al., 2001b). This indicates that induction of apoptosis occurs after "bicellular" binding as depicted in Fig. 2A. Since hardly any tumor cell will express a given target antigen, the phenomenon of bystander killing is desirable as long as it is strictly dependent on the presence of antigen positive cells, which in our experiments is the case.

By using solid tumor cells with decreased expression of CD95, selective cell killing could still be observed. However, under these more stringent conditions we found that some target antigens, such as a melanoma-associated proteoglycan, are better than others (such as the EGF receptor) in presenting CD95 antibodies within a bispecific construct. We also found, that the effectiveness of such a construct depends on properties of the target antigen other than its expression level on the cell surface; its spatial arrangement in the membrane, for example. Thus the activity of a given bispecific antibody is difficult to predict. Moreover, and more importantly, we found that the killing of solid tumor cells was generally less impressive and depended largely on reagents that sensitize for apoptosis, such as cycloheximide (CHX). This problematic situation is not entirely unexpected: Tumor cells, in particular those freshly isolated from patients, are known to

acquire resistance to CD95-mediated cell death either because the molecule itself or parts of the intracellular signalling cascade associated with it are lost or damaged (Xerri et al., 1998; Bullani et al., 2002). In order for therapeutic CD95 stimulation to succeed as a therapeutic principle, it is thus not only the selectivity but also the sensitivity problem which needs to be addressed.

6. DEFINED SENSITIZERS MAY SUPPORT CD95 MEDIATED APOPTOSIS

Numerous compounds have been reported to increase the sensitivity of cells to apoptosis mediated by CD95 and other death receptors. These include such diverse substances as the standard sensitizing reagent cycloheximide (CHX), thalidomide (Mitsiades et al., 2002), several cytostatic drugs such as Topo isomerase inhibitors (Ciusani et al., 2002), adriamycin (Wu et al., 2000) and carboplatin (Mishima et al., 2003), demethylation agents (Fulda et al., 2001; Shaker et al., 2003), and newer reagents like the TLR7 agonist imiquimod (Schon et al., 2003), a farnesyltransferase inhibitor (Zhang et al., 2002), inhibitors of histon deacetylase (HDAC) (Peart et al., 2003; Imai et al., 2003) and cyclin dependent kinase (Kim et al., 2003) as well as Vitamin E derivatives (Yu et al., 2003). In most cases, only a few tumor cell lines have been tested for their sensitization and the extent to which the described findings can be generalized to a broad array of cells is not clear. Ideally, an optimal sensitizer would not induce apoptosis on its own. Particularly promising in this respect are recently developed reagents which interfere with CD95-mediated apoptosis induction in a defined and specific way.

6.1. Smac Peptides

Agonistic peptides derived from Smac/DIABLO, a protein which antagonizes the x-linked inhibitor of apoptosis (IAP) protein XIAP, were reported to sensitize cells for apoptosis mediated by the TNF-related apoptosis inducing ligand (TRAIL/Apo-2) and cytostatic drugs such as etoposide (Fulda et al., 2002; Arnt et al., 2002; Yang et al., 2003). For cell penetration the agonistic peptides had to be fused to special cargo sequences derived from the Drosophila antennapaedia and the Tat protein respectively. The development of peptide-derived therapeutics certainly represents an enormous challenge because not only effective penetration into cells, but also stability against proteolytic cleavage has to be ensured. Nevertheless the findings described above indicate that the interaction of Smac with XIAP, and possibly with other IAPs such as Survivin (Song et al., 2003), may provide an interesting target for the development of sensitizing reagents based either on peptides or on other modes of specific interference.

6.2. siRNAs

Gene silencing with small interfering RNAs is a rapidly evolving field, that has been used among others things for increasing apoptosis sensitivity of cells *in vitro* by silencing apoptosis related genes such as Survivin (Kappler et al., 2004) and Bcl-2 (Holle et al., 2004). Recently it was reported that siRNA derived from the caspase-8 sequence prevents acute apoptosis of liver cells in mice (Zender et al., 2003). Although this is definitely not

what is desired if one is aiming for therapeutic induction of apoptosis, this report demonstrates that siRNAs may be used to influence apoptotic pathways *in vivo.*

6.3. Oblimersen

Oblimersen is an 18mer phosphorothioate oligodeoxyribonucleotide which is targeted to the first six codons of the RNA for the human anti-apoptotic Bcl-2 protein. The drug has been shown to synergize with many cytotoxic reagents against tumor cells of different origin. It is currently evaluated in non-randomized clinical trials (Lai et al., 2003; Klasa et al., 2002).

Taken together, it is quite likely that in the near future reagents will become available which may synergize with selective antibody induced apoptosis in a defined and effective way.

7. POSSIBLE "PLEIOTROPIC EFFECTS" OF TUMOR X CD95-BISPECIFIC ANTIBODIES

In addition to the selective induction of CD95 mediated apoptosis, bispecific antibodies with Tumor X CD95 specificity may exert effects which do not depend on CD95 expression of the target cell. With that, we find ourselves in the middle of an ongoing controversy concerning the immunological consequences of apoptotic cell death in general and the role of CD95L expression on tumor cells in particular. Initially, the prevailing view was that apoptosis, in contrast to necrosis, is an immunologically innocent event and that stimulation of CD95 is immunosuppressive (Sauter et al., 2000; Gallucci et al., 1999). CD95L expressed on tumor cells was thought to kill activated T cells and provide a mechanism of tumor cell counterattack (Igney et al., 2000; Bennett et al., 1998). In the last few years numerous experiments with CD95L transfected cells have changed this view dramatically (Restifo, 2000). In most but not all (Chen et al., 2003b) of these experiments it was found that the transfected cells induced a vigorous influx of granulocytes and monocytes *in vivo* which eventually led to marked inhibition and not promotion of tumor cell growth (Owen-Schaub et al., 1998; Igney et al., 2003; Springer et al., 1998). In addition to the recruitment of innate immune effector mechanisms, the induction of specific immunity was also reported; this was probably due to the activation of dendritic cells via CD95 (Tada et al., 2002). Another report noted that dendritic cells transfected with CD95L induced a marked allospecific T-cell response *in vivo* (Buonocore et al., 2003). Even though these reports point to an enhancing rather than a suppressive effect of CD95 stimulation, controversial findings are still being reported (Chen et al., 2003b). We feel that careful consideration of the well established differential effects of membrane-bound vs. soluble CD95L (the former is stimulating, the latter suppressive, Hohlbaum et al., 2000) might help to resolve the controversy.

To complicate matters or make theme more interesting, there are more pleiotropic effects of CD95 stimulation than those exerted on immune cells: The CD95/CD95L-system is obviously also involved in angiogenesis. Inhibitors of neovascularization were reported to upregulate CD95L on endothelial cells, whereas inducers of angiogenesis enhance CD95 expression (Volpert et al., 2002). A recent paper by Janin et al. describes

the possible fate of endothelial cells after systemic CD95 stimulation. The authors reported the disseminated apoptosis of such cells in mice after application of either multimeric CD95L or the agonistic CD95 antibody Jo2 (Janin et al., 2002).

What can one expect from the *in vivo* application of bispecific antibodies with Tumor X CD95-specificity? The findings discussed above indicate that more may be achieved than selective killing of CD95-sensitive tumor cells. Indeed, our results demonstrate that bi-cellular binding can occur if CD95 and the target antigen are expressed on the same cell (Fig. 2A). This means that bispecific Tumor X CD95 antibodies can induce pleiotropic effects such as inflammation and possibly endothelial cell apoptosis, irrespective of CD95-expression on the target cell, but in a target cell restricted manner (Fig. 2C). This corresponds to the situation depicted in Fig. 1 in which bispecific antibodies selectively trigger immune receptors. In fact, the pleiotropic effects may be responsible for determining the therapeutic potential of anti-CD95-containing bispecific antibodies for better or for worse: The more potent the effects, the more important it is that the bispecific principle depicted in Fig.1 confers target cell restriction. The *in vitro* data available so far look promising in this respect. A thorough evaluation of pleitropic effects, however, demands the use of animal experiments. In our laboratory we are currently evaluating the activity of bispecific antibodies containing the CD95 antibody Jo2 in different syngeneic mouse tumor models.

8. CHANGING DEATH RECEPTORS AND THE BISPECIFIC FORMAT

The application of the above described principle to death receptors other than CD95, e.g. receptors for TNF or TRAIL, is tentative. Wajant et al. reported that a recombinant fusion protein composed of a targeting antibody (directed against the tumor associated FAP protein) and the TRAIL protein induced apoptosis predominantly in cells expressing a particular TRAIL receptor designated TRAIL-R2. TRAIL-R1-expressing cells could not be killed selectively since this receptor is triggered by soluble TRAIL protein (Wajant et al., 2001). Once again the problem of target cell restricted selective stimulation arises. More recently the same group generated a fusion protein which consisted of the anti-FAP antibody and CD95L. As bispecific antibodies with Tumor X CD95 specificity, these reagents induced selective CD95-mediated apoptosis in tumor cells *in vitro*. Moreover, they prevented the growth of human tumor cells in a nude mouse model (Samel et al., 2003). Notably, the fusion proteins did not induce toxicity in mice unless crosslinked with an anti-Flag antibody indicating that this protein may exert the type of selectivity required for successful *in vivo* application.

Several other groups demonstrated that fusion proteins consisting of antibodies and TNF stimulated TNF receptors with a certain degree of selectivity (Halin et al., 2003; Liu et al., 2004). Our view is that the attachment of an antibody to a soluble cytokine mainly generates the type of specificity which pharmacokinetics can provide and thus again sufficient target cell restriction may be difficult to achieve.

At present, these considerations may appear somewhat theoretical. Future investigations will have to select the bispecific reagent that is most suitable for selective stimulation of death receptors on tumor and possibly other cells *in vivo*.

9. BLOCKING CD95 MEDIATED APOTOSIS WITH ANTIBODIES

In some cases, it may be desirable to block rather than stimulate apoptosis. Since this topic is covered extensively in chapter X, we shall briefly focus here on an illustrative case involving antibodies.

Among the diseases associated with pathological CD95-mediated destruction of cells are brain damage after cerebral ischemia (Ferrer & Planas, 2003; Martin-Villalba et al., 2001) certain forms of acute hepatic failure (Galle et al., 1995), pulmonary silicosis (Borges et al., 2001) and toxic epidermal necrolysis (TEN) (Abe et al., 2003). The latter is a life threatening hypersensitivity reaction of the skin. In two studies, a marked improvement was reported after treatment of TEN patients with polyclonal immunoglobulin (Trent et al., 2003; Prins et al., 2003). These findings have been challenged, possibly because both the agonistic and antagonistic anti-CD95 activity in commercial immunoglobulin preparations is variable (Bachot & Roujeau, 2003; Altznauer et al., 2003). This suggests that the development of monoclonal antibody preparations which reliably block CD95 could be a highly worthwhile proposition. Given the refinements of present day antibody technology this task certainly appears surmountable.

10. CONCLUSIONS

In this chapter we have described a new strategy for the selective stimulation of the CD95 death receptor on tumor cells using bispecific antibodies with Tumor X CD95-specificity. Although this concept can be readily extended to other target cells and other death receptors, we believe that selective CD95 stimulation on tumor cells is particularly attractive. In addition to the selective induction of apoptosis it may allow the initiation of an inflammatory and anti-angiogenic response. If these effects could be induced *in vivo* in a tumor cell-restricted manner a promising new class of bispecific reagents would become available for experimental tumor therapy.

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TARGETING THE PROTEASOME IN CANCER THERAPY

Chapter XII

Robert Z. Orlowski*

1. INTRODUCTION

A variety of cellular constituents and metabolic processes have been exploited in the past as targets for cancer therapy. In that malignancies generally arise because of mutations of the genetic material, it is interesting to note that many of these therapies have themselves targeted DNA. Alkylating agents, one of the oldest classes of anti-tumor drugs, work in part by damaging DNA and preventing replication. Other drugs, such as taxanes and vinca alkaloids, interfere with microtubule function that is necessary to partition chromosomes after they have been faithfully copied. Still others, such as anthracyclines and camptothecins, interfere with topoisomerases, which are important in processes such as nucleic acid transcription and repair. Studies of metabolic pathways have provided many useful targets and therapeutic agents, such as folic acid metabolism, which is inhibited by methotrexate, and pyrimidine metabolism, which is inhibited by several drugs, including cytarabine. Agents that inhibit the unopposed action of certain hormones, such as the mixed anti-estrogen tamoxifen, have made important contributions to our chemotherapeutic armamentarium as well. More recent studies have focused in part on signal transduction pathways, with prominent successes such as the BCR-ABL tyrosine kinase inhibitor imatinib mesylate, which has revolutionized the care of patients with chronic myelogenous leukemia. Significant therapeutic benefits have also been achieved in targeting cell surface markers with monoclonal antibodies, most notably using rituximab, which recognizes CD20, a marker expressed commonly on B-lymphocyte-derived malignancies. One area of metabolism that has previously been relatively less explored as a possible target is that of intracellular protein turnover, which occurs predominantly through the ubiquitin-proteasome pathway. However, the

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proteasome has now been validated as a rational target for clinical cancer therapy in multiple myeloma, and is being studied further in a variety of other tumor types as well. This chapter will review the normal role of the ubiquitin-proteasome pathway in cellular homeostasis, and some of the molecular mechanisms by which proteasome inhibitors can exert anti-tumor efficacy. The current clinical use of one inhibitor, bortezomib, will be outlined, as will future promising directions for its development.

2. THE UBIQUTIN-PROTEASOME PATHWAY

2.1. Biologic Significance

Cellular protein synthesis is accomplished by macromolecular complexes called ribosomes, but to maintain normal homeostasis cells must also have a mechanism to remove proteins. This is necessary in some cases because of the synthesis of proteins that do not fold normally and are therefore inactive (Ciechanover et al., 2000; DeMartino & Slaughter, 1999; Tanahashi et al., 1999; Zwickl et al., 2000), while in others, proteins become damaged during normal metabolism, including by oxidation (Davies, 2001). Some proteins have short half-lives due to rapid proteolysis, without which their abnormal accumulation would have deleterious effects, such as the tumor suppressor p53, which can induce cell cycle arrest and apoptosis, and c-Myc, which can drive cells forward through the cell cycle (Ciechanover et al., 2000; DeMartino & Slaughter, 1999; Tanahashi et al., 1999; Zwickl et al., 2000). Proteolysis is required for the normal maturation of some proteins, such as the p50 subunit of the important transcription factor nuclear factor kappa B (NF-κB), (Palombella et al., 1994). At the level of the whole organism, protein degradation is also vital to normal immune function, since antigens must be processed for presentation in association with major histocompatibility (MHC) class I molecules (Kloetzel, 2001). For eukaryotes, all of these functions are performed in large part through the ubiquitin-proteasome pathway (UPP). In addition, UPP activity is essential to many other vital cellular processes, including mitosis through the orderly degradation of cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors (Hershko, 1997), angiogenesis (Oikawa et al., 1998), and programmed cell death (Orlowski, 1999). The fundamental importance of this pathway to normal homeostasis is underscored by the fact that it is highly conserved throughout evolution, with even some bacteria having recognizable, though less complex, counterparts. Moreover, inactivating mutations in the genes that encode most of the core components of the proteasome are almost invariably fatal and, by some estimates, the proteasome comprises up to 3% of the total cellular protein content (Ciechanover et al., 2000; DeMartino & Slaughter, 1999; Tanahashi et al., 1999; Zwickl et al., 2000).

2.2. Functional Components

2.2.1. The Ubiquitin Conjugation System

Though certain cellular proteins appear to be substrates for proteasome-mediated degradation without the need for prior ubiquitination, such as ornithine decarboxylase

(Murakami et al., 1992), polyubiquitination is a prerequisite for the turnover of most proteins through the UPP (Ciechanover et al., 2000; DeMartino & Slaughter, 1999; Tanahashi et al., 1999; Zwickl et al., 2000). Ubiquitin (Ub), itself a highly conserved 76-amino acid protein, is conjugated to many other proteins whose fate is to undergo degradation. This process begins with the ATP-dependent activation of Ub (Fig. 1) by an E1 ubiquitin-activating enzyme, which forms a high-energy thiolester bond between one of its own cysteine residues and Ub. In a reaction that maintains the high-energy thiolester bond, Ub is then transferred to a cysteine residue of an ubiquitin-conjugating enzyme (Ubc), or E2 protein. Some of these E2s can then directly transfer Ub to the target protein, forming an isopeptide bond between the ε-amino group of a lysine residue in the target and the first Ub molecule. More commonly, however, this transfer occurs in association with an E3 ubiquitin ligase, some of which act by first accepting Ub from the E2 and then transferring it to the target protein. In other cases the E3 acts as a scaffold protein, bringing together the target protein and the E2-Ub complex, and the E2 then itself transfers the Ub molecule. After this initial modification several additional cycles of ubiquitination occur, resulting in the formation of a chain of ubiquitin molecules. This polyubiquitin chain is the signal that is recognized by the proteasome as indicating a protein whose fate is to undergo proteolytic degradation.

Cells contain a single E1 Ub activating enzyme (Ciechanover et al., 2000; DeMartino & Slaughter, 1999; Tanahashi et al., 1999; Zwickl et al., 2000), and it is known that there are several E2 ubiquitin activating enzymes, but it appears that there are

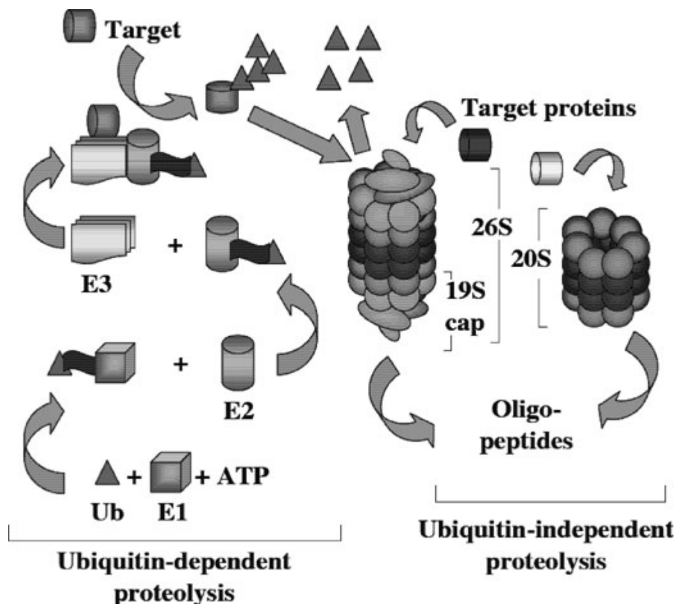

Figure 1. The ubiquitin-proteasome protein degradation pathway. Sequential function of the UPP results in the labeling of target proteins with chains of ubiquitin molecules, followed by proteasome-mediated degradation into oligopeptides, as detailed in the text. The 26S and 20S proteasomes may also be involved in ubiquitin-independent proteolysis of some protein substrates. Adapted from (Voorhees et al., 2003), with permission.

hundreds of E3 ligases. This step is one of the rate limiting points in protein turnover, and also provides specificity to this process since each E3 is responsible for a small group of client proteins. Three classes of E3s have been described to date, one of which is the HECT-domain containing E3s that have sequences which are homologous to the E6AP carboxy-terminus. They include the prototype papillomavirus E6-associated protein (E6-AP) that is involved in p53 degradation (Rolfe et al., 1995; Scheffner et al., 1993), and usually bring together the target and Ub-E2 using domains from a single E3 protein. A second class of E3s are the SCF family of ligases that contain S-phase kinase-associated protein 1 (Skp1), Cullin-1 (Cul1), regulator of cullins 1 (Roc1), and an F-box protein such as Skp2, with the latter being involved in turnover of $p27^{Kip1}$ (Tsvetkov et al., 1999). These ligases use a series of components to bridge the gap between the target protein and the Ub-E2 complex. Finally, RING-finger domain-containing proteins are the third class, with examples such as breast cancer growth suppressor protein BRCA1 (Hashizume et al., 2001) and murine double minute 2 (Mdm2) (Shmueli & Oren, 2004), which also use different domains of the same polypeptide to juxtapose the target with an Ub-E2 complex. In that p53 can be targeted for ubiquitination and ultimate degradation by both E6-AP and Mdm2, it is clear that some proteins may be substrates for several types of E3s.

It is also noteworthy that the Ub conjugation system is involved in a related process called monoubiquitination but, unlike polyubiquitination, the covalent attachment of a single Ub moiety does not seem to be involved in signaling for proteolysis. Though the functional consequences of monoubiquitination are only beginning to be explored, it has become clear that one of the associated processes is receptor endocytosis and trafficking, and subcellular protein localization (Haglund et al., 2003). The p53 protein, for example, appears to be a substrate for monoubiquitination by Mdm2 when the latter protein is expressed at low levels, and this results in nuclear exclusion of p53 (Shmueli & Oren, 2004). Another process in which monoubiquitination may be involved is control of DNA damage repair through the Fanconi anemia pathway (Gregory et al., 2003).

2.2.2. The multicatalytic proteinase complex

Once a target protein is polyubiquitinated, this polypeptide chain is recognized by constituents of the 19S cap structure present at each end of the mature 26S complex (Fig. 1) (Ciechanover et al., 2000; DeMartino & Slaughter, 1999; Tanahashi et al., 1999; Zwickl et al., 2000). Deubiquitinating enzymes in the cap cleave off this tag, and the ubiquitin molecules are recycled for later reuse. Proteins are then unwound in an ATP-dependent process, and enter a central channel in the proteasome, where they come into contact with the catalytic proteases encoded by some of the β subunits of the inner two proteasome rings. Up to five different activities have been described (Orlowski & Wilk, 2000), including a chymotrypsin-like activity which cleaves after hydrophobic amino acids such as phenylalanine and leucine, a trypsin-like activity that cleaves after basic amino acids including lysine and arginine, a peptidyl-glutamyl-peptide hydrolyzing activity that cleaves after acidic residues such as aspartate and glutamate, as well as a branched chain amino acid preferring activity and a small neutral amino acid preferring activity. Together, these activities are capable of hydrolyzing proteins into oligopeptides, though the chymotrypsin-like activity appears to be the most important, rate-limiting step

in this process. Once generated, oligopeptides then exit the proteasome, and are subject to further hydrolysis into their constituent amino acids by endopeptidases and aminopeptidases.

2.3. UPP Function in Cell Biology

Up to 80% or more of cellular proteins are degraded through the UPP (Rock et al., 1994) and, as alluded to above in the discussion of the E3 ubiquitin ligases, there are many different proteolytic paths taken by target proteins on their way to the proteasome where they are reduced into their constituent amino acids. To provide the reader with one example that is relevant to cancer, the role of the UPP in $p27^{Kip1}$ degradation will be reviewed.

2.3.1. $p27^{Kip1}$

Progression of cells through the cell cycle is governed by the function and interplay of cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (Cdkis) (Murray, 2004). For cells to exit from the resting G_0 or G_1 phase and progress into mitosis they must traverse the so-called G_1/S checkpoint. This process begins in early G_1 when mitogens induce D-type cyclins that bind and activate CDK4 and CDK6. Subsequently, cyclin E-CDK2 and then cyclin A-CDK2 complexes are activated, leading to entry of the cell into the S-phase, where chromosomal material is duplicated. Two families of Cdkis contribute to the regulation of cyclin and CDK activity, including the inhibitor of CDK4 family, and the kinase inhibitor protein (KIP) family. Among the members of the latter group are the proteins $p21^{Cip1/Waf}$, $p27^{Kip1}$, and $p57^{Kip2}$, which bind and inhibit cyclin E-CDK2 and cyclin A-CDK2 complexes.

One of the most important control mechanisms used by both normal and malignant cells to regulate the levels of $p27^{Kip1}$ at G_1/S is by its regulated proteolysis through the UPP (Bloom & Pagano, 2003). Upon mitogenic stimulation of quiescent cells, $p27^{Kip1}$ becomes phosphorylated at Thr-187 by cyclin E-CDK2, and perhaps by cyclin A-CDK2 as well. It is also notable that phosphorylation at Ser-10 in G_0 and G_1 that is mitogen-independent may be important in determining the fate of p27. This event leads to export of nuclear $p27^{Kip1}$ to the cytoplasm, possibly freeing enough cyclin E-CDK2 complexes to phosphorylate p27 at Thr-187. Once phosphorylated, $p27^{Kip1}$ is recognized by SCF^{Skp2}, the E3 ligase responsible for its ubiquitination. While Skp-2 binds p27, the E2 Ubc3 is brought into proximity through a bridge made up of Skp1, Cul1, and Roc1. Ubc3 then catalyzes the addition of multiple ubiquitin moieties forming polyubiquitin chains, possibly at lysine residues 134, 153, and 165 (Shirane et al., 1999). Polyubiquitinated $p27^{Kip1}$ is then recognized by proteins in the cap structure of the proteasome, and subjected to degradation as described above.

Dysregulation of cell cycle progression is a common hallmark of the transformation process (Laiho & Latonen, 2003), and one of the more frequently affected proteins is $p27^{Kip1}$ (Nho & Sheaff, 2003). Multivariate analysis has shown that loss of p27 protein is an independent prognostic marker for a poor outcome in several malignancies, including both solid tumors such as breast cancer (Fredersdorf et al., 1997; Porter et al., 1997; Tan et al., 1997), and hematologic malignancies such as mantle cell non-Hodgkin's

lymphoma (Chiarle et al., 2000) and multiple myeloma (Filipits et al., 2003). While $p27^{Kip1}$ deregulation can occur through a variety of mechanisms, including gene deletion, cytoplasmic mislocalization, and sequestration by other proteins, most cases of decreased p27 concentration appear to be due to accelerated proteolysis (Nho & Sheaff, 2003). Indeed, decreased concentrations of $p27^{Kip1}$ have been noted in up to 60% of human cancers, and Skp2 has itself been identified as an oncogene (Signoretti et al., 2002), underlining the importance of the regulation of proteolysis to the biology of both normal and transformed cells.

3. ANTI-TUMOR ACTIVITY OF PROTEASOME INHIBITORS

Given the central role of the ubiquitin-proteasome pathway in maintaining homeostasis it is probably not surprising that inhibition of the proteasome is deleterious to cells. Perhaps more surprising is the finding that there is a differential sensitivity with enhanced effects on transformed cells in comparison with normal cells. This has been reported in a number of human tumor-derived model systems, including non-Hodgkin's lymphoma (Orlowski et al., 1998), chronic lymphocytic leukemia (Masdehors et al., 1999), multiple myeloma (Hideshima et al., 2001b), and acute myeloid leukemia (Guzman et al., 2002). In such studies proteasome inhibitors have been noted to impact upon a variety of processes that may contribute to their anti-tumor efficacy. These include adhesion, angiogenesis, apoptosis and survival, and invasion and tumor metastasis. An overview of the evidence that proteasome inhibitors affect these pathways, as well as the suspected mechanisms by which they act, is presented below.

3.1. Adhesion

Adhesion of both non-malignant as well as transformed cells to basement membrane elements, or to other cells such as stroma, is an important part of normal cellular homeostasis and, in some cases, of factors that contribute to tumorigenesis. An early role for the proteasome in this process was described by Cobb and colleagues, who noted that proteasome inhibition blocked the interleukin (IL)-1-induced activation of reporter constructs containing the promoter regions of intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 (Cobb et al., 1996). This was accompanied by decreased expression of these molecules in human umbilical vein endothelial cells (HUVECs). Expression of VCAM-1, ICAM-1, and E-selectin in HUVECs induced by other cytokines, including either tumor necrosis factor α (TNF) or lipopolysaccharide, was also reported to be decreased by proteasome inhibition (Kalogeris et al., 1999). These effects may contribute to the anti-angiogenic properties of proteasome inhibitors, which are described in the next section.

Other targets for proteasome inhibitors that may impact upon cellular adhesion include P-selectin (Xia et al., 1998), lymphocyte function-associated antigen-1 (Katagiri et al., 1999), and endothelial cell adhesion molecule (Dagia & Goetz, 2003). However, it should be noted that the expression of some proteins in this category may be enhanced by proteasome inhibition. Beta catenin, for example, a central component of the cadherin cell adhesion complex, is a target for ubiquitin-proteasome mediated degradation (Aberle

et al., 1997), and increases in abundance with proteasome blockade (Marschitz et al., 2000). Since high-molecular-weight beta-catenin species appear, which are presumably polyubiquitinated, these may not functional due to their post-translational modification, but this possibility has yet to be tested experimentally.

Studies demonstrating the important contribution of proteasome inhibitor-mediated changes in cellular adhesion molecule expression to their anti-tumor efficacy have been most rigorously performed in multiple myeloma. Adhesion of myeloma cells to bone marrow stroma stimulated the NF-κB-dependent induction of the important myeloma growth and survival factor IL-6 (Chauhan et al., 1996). This led Hideshima and colleagues to investigate the benefits of blockade of NF-κB activation through proteasome inhibition in this model system. They showed that the inhibitor bortezomib decreased adherence of myeloma cells to bone marrow stroma, and decreased stromal production of IL-6 (Hideshima et al., 2001b). This agent also blocked TNF-induced activation of NF-κB, and expression of ICAM-1 and VCAM-1 (Hideshima et al., 2001a). In that such adherence in part mediates resistance to chemotherapies, this activity also contributed to chemosensitization to a variety of drugs used in myeloma therapy, including anthracyclines and alkylating agents (Mitsiades et al., 2003).

The central role of NF-κB in expression of many of these molecules would certainly suggest that inhibition of its translocation should be an important part of the mechanism by which proteasome inhibitors impact upon adhesion molecule expression in non-malignant cell model systems as well. Consistent with this possibility, Jobin and colleagues noted that inhibition of TNF-induced degradation of IκBα led to an increase in ICAM-1 expression in an intestinal epithelial cell line (Jobin et al., 1998). In other models, however, it is notable that investigators found no associated inhibition of NF-κB nuclear translocation (Cobb et al., 1996), suggesting the possibility that there may be some cell-type or even proteasome inhibitor-dependent specificity.

In addition to contributing to the anti-tumor efficacy of proteasome inhibitors, the ability to impact upon cell adhesion may be important in other potential clinical applications of these agents. One notable such area is inflammation, which contributes to the pathogenesis of several processes, including both coronary artery and central nervous system occlusive and ischemic events. Inhibition of the proteasome in a model of coronary artery occlusion inhibited P-selectin expression on vascular endothelial cells, thereby decreasing leukocyte-endothelial cell interactions (Campbell et al., 1999). This may have contributed to the improvement in coronary flow and indices of cardiac contractile function which were seen in comparison with vehicle-treated controls following reperfusion in this model system. Similarly, in a model of cerebral artery occlusion followed by reperfusion, proteasome inhibition decreased E-selectin and ICAM-1 expression (Berti et al., 2003). This was associated with a neuroprotective effect against ischemic brain injury in rats, possibly due in part to a decreased inflammatory response. Proteasome inhibitors have also been noted to impact upon inflammation and cell adhesion molecule expression in other model systems, such as *Streptococcal* cell wall-induced polyarthritis (Palombella et al., 1998). The reader interested in a more complete description of anti-inflammatory applications of this class of compounds is referred to several recent excellent reviews (Di Napoli & Papa, 2003; Elliott et al., 2003).

3.2. Angiogenesis

A role for the proteasome in angiogenesis was first described by Oikawa and colleagues (Oikawa et al., 1998) using a non-malignant model system. They noted that lactacystin was able to almost completely prevent *in vivo* neovascularization in a model of the developing chick embryo chorioallantoic membrane. Vascular endothelial tube formation on Matrigel was inhibited as well, as was production of plasminogen activator, an important protease for angiogenesis. Lactacystin, as well as a tri-peptidyl aldehyde proteasome inhibitor, were subsequently noted to block basic fibroblast growth factor- and fetal bovine serum-induced induction of S-phase in HUVECs (Kumeda et al., 1999). This was associated with an increase in cellular levels of p53 and the cyclin dependent kinase inhibitor $p21^{Cip1/Waf}$, leading to CDK2 inactivation. Moreover, this growth inhibition occurred preferentially in HUVECs, with lower concentrations needed in these cells than those required to effect similar changes in normal fibroblasts and carcinoma cells. It is also notable that proteasome inhibitors decreased the expression of the vascular endothelial cell growth factor (VEGF) receptor Flt-1 on explant cultures of the developing chicken pecten oculi (Mezquita et al., 2003), and also on human microvascular endothelial cells.

An impact of proteasome inhibition on tumor angiogenesis was reported by Sunwoo and colleagues using a model of human squamous cell carcinoma (Sunwoo et al., 2001). They found a decrease in microvessel density in tumors treated with the dipeptide boronic acid derivative PS-341 (bortezomib, also known as VELCADE®) (Adams et al., 1999). This was associated with a marked tumor growth inhibition of both human and murine squamous cell carcinoma cell lines *in vivo*. Other model systems of human malignancies in which proteasome inhibitors have been noted to inhibit angiogenesis as part of their anti-tumor activity include breast carcinoma (Shibata et al., 2002), multiple myeloma (LeBlanc et al., 2002), pancreatic carcinoma (Nawrocki et al., 2002), prostate carcinoma (Williams et al., 2003), and colon carcinoma (Stoklosa et al., 2004). Combination therapy using a proteasome inhibitor and docetaxel has also been noted to decrease tumor microvessel density and increase apoptosis of tumor-associated endothelial cells (Nawrocki et al., 2004). The latter study provides a strong rationale for chemosensitization to standard agents by addition of a proteasome inhibitor (see subsequent section).

While proteasome inhibitors have direct effects on vascular endothelial cells as noted above, it is likely that at least part of their anti-angiogenic properties are due to effects on the tumor cells themselves. Treatment of a squamous cell carcinoma model with PS-341 was associated with inhibition of nuclear translocation of NF-κB (Sunwoo et al., 2001), and expression of the pro-angiogenic cytokines growth-related oncogene-alpha and VEGF. The ability of proteasome inhibitors to suppress angiogenesis by blocking VEGF induction, presumably through blockade of NF-κB, has also been documented in the MDA-MB-231 breast carcinoma model (Shibata et al., 2002), the L3.6pl and Mia-PaCa-2 pancreatic carcinoma models (Nawrocki et al., 2002), and the PC-3 (Bosland, 2003; Levine et al., 2003) and LNCaP (Williams et al., 2003) prostate carcinoma cell model. Another pathway whose proteasome inhibitor-mediated blockade could result in inhibition of angiogenesis is the p44/42 mitogen-activated protein kinase (MAPK), which occurs in part through induction of MAPK phosphatases (MKPs) (Orlowski et al.,

2002a). Down-regulation of p44/42 MAPK results in inhibition of VEGF secretion and angiogenesis in a number of model systems, including multiple myeloma (Giuliani et al., 2004).

Proteasome inhibition has not consistently been associated with anti-angiogenic effects, however, in the setting of anti-tumor efficacy (Golab et al., 2000). Also, in one instance, proteasome inhibitors were reported to activate VEGF secretion (Stoklosa et al., 2004) in the setting of decreased angiogenesis, prompting the authors to hypothesize that VEGF might lead to activation of endothelial cells, which would be more susceptible to the pro-apoptotic effects of proteasome inhibition. Nonetheless, it seems very likely that the therapeutic benefits of proteasome inhibitors occur, at least in part, by inhibition of angiogenesis due to effects on both tumor cells as well as their local microenvironment. The latter is particularly interesting in that the differential sensitivity of HUVECs to proteasome inhibitors suggests that chronic, low-dose therapy could be effective in a maintenance setting to slow tumor relapse and progression from other therapies.

3.3. Apoptosis and Survival

Since the first demonstrations that inhibition of the proteasome can result in the induction of apoptosis in both cell line (Imajoh-Ohmi et al., 1995) and *in vivo* (Orlowski et al., 1998) models of human malignancies, many investigators have documented that activation of programmed cell death is an important component of the anti-tumor efficacy of proteasome inhibitors. Given the central role of the ubiquitin-proteasome pathway in maintaining cellular homeostasis, it is not surprising that proteasome inhibitors have been reported to impact upon many pro- and anti-apoptotic pathways. A full review of these effects is beyond the scope of this chapter, but an overview is provided in Table 1, and the interested reader is referred to several recent excellent works (Adams, 2002; Voorhees et al., 2003). In addition, the sections below will discuss the roles of the NF-κB, p44/42 MAPK and JNK, and p53 pathways in apoptosis, since these may be some of the more important contributors to this process.

Table 1. Some of the pro- and anti-apoptotic targets of proteasome inhibitors.

Target	Impact of proteasome inhibition	Role in apoptosis	References
Bax	Accumulates due to inhibition of degradation, and also due to increased p53-mediated transcription	Interaction with Bcl-2 and Bcl-x_L promotes release of mitochondrial cytochrome *c*	(Li & Dou, 2000)
Bcl-2	Induces phosphorylation and caspase-dependent cleavage	Abrogates Bcl-2 mediated survival and resistance to chemotherapy and radiation	(Ling et al., 2002; Pei et al., 2003; You et al., 1999; Zhang et al., 1999)
Bim-E_L	Stabilized by proteasome inhibition	May contribute to cell death through the pro-apoptotic functions of Bim- E_L	(Luciano et al., 2003)

Table 1. (continued).

c-FLIP	c-FLIP levels are reduced by proteasome inhibition in some model systems	Augments caspase-8 activation and apoptosis	(Sayers et al., 2003)
HSP-72	Activates HSP-72	Inhibits apoptosis induced by proteasome inhibitors, possibly through interaction with JNK	(Meriin et al., 1998; Robertson et al., 1999)
NF-κB	Blocks processing of p105 into p50; stabilizes IκB, inhibiting nuclear translocation of NF-κB	Decreases NF-κB-dependent transcription of genes involved in proliferation, survival, invasion, metastasis, and angiogenesis	(Orlowski & Baldwin, 2002)
$p21^{Cip1/Waf}$ and $p27^{Kip}$	Both accumulate due to inhibition of degradation; $p21^{Cip1/Waf}$ is also a target for p53 transactivation	Induce arrest at G_1/S, leading to apoptosis	(Adams et al., 1999; Li and Dou, 2000)
p53	Enhanced p53 expression due to inhibition of degradation	Induces cell cycle arrest in part through $p21^{Cip1/Waf}$, and apoptosis in part through Bax	(Shinohara et al., 1996; Williams and McConkey, 2003)
p44/42 MAPK	Activates transcription of MKP-1, inhibiting p44/42 activity; may also lead to caspase-mediated cleavage of upstream receptors	Inhibits p44/42-mediated cell proliferation and survival signaling; may contribute to anti-angiogenic effects	(Hideshima et al., 2003; Orlowski et al., 2002a)
JNK	Activates JNK through effects on upstream kinases	Impacts upon mitochondria-dependent apoptotic processes, including release of cytochrome *c* and Smac	(Meriin et al., 1998; Yu et al., 2004)
Smac/ DIABLO	Accumulates as a result of proteasome inhibition	Binds and inhibits members of the IAP family of proteins, potentiating activation of pro-caspase-9	(Chauhan et al., 2003b; MacFarlane et al., 2002)
tBid	Accumulates as a result of proteasome inhibition	Induces a conformational change in Bak, promoting mitochondrial release of cytochrome *c*	(Breitschopf et al., 2000)
TRAIL death receptor 4 and 5	Up-regulated by proteasome inhibition	Contributes to activation of caspase-8	(He et al., 2004; Johnson et al., 2003)

3.3.1. NF-κB

NF-κB is a ubiquitous transcription factor which plays important roles in a variety of processes important to both normal and transformed cells. Among these are angiogenesis through targets such as VEGF, cellular proliferation through transactivation of cyclin D1, metastasis by influencing expression of several matrix metalloproteinases (MMP), and suppression of apoptosis by activation of the Bcl-2 homologs A1/Bfl-1, and Bcl-x_L (Lin & Karin, 2003; Orlowski & Baldwin, 2002). Typically, NF-κB is present in the cytoplasm as a heterodimer of p50 and p65 (RelA) subunits, and is prevented from translocating to the nucleus because it is bound to the inhibitory protein IκBα, which masks a nuclear localization signal on p65. In a variety of tumors NF-κB is constitutively

activated, including hematologic malignancies such as Hodgkin's Disease, certain leukemias, and multiple myeloma, as well as solid tumors such as breast, prostate, colorectal and ovarian cancers. This activation commonly occurs by stimulation of the IκB kinase complex, resulting in phosphorylation of IκBα at two serine residues, Ser-32 and –36. Dually phosphorylated IκBa is a substrate for the E3(SCF^{Fbw1}) complex, which attaches polyubiquitin chains to lysine residues at positions-21 and -22. Polyubiquitinated IκBα is then degraded through the proteasome, thereby exposing the nuclear localization signal of p65, and allowing nuclear translocation of NF-κB. Proteasome inhibition therefore results in inhibition of NF-κB through the stabilization of NF-κB–bound phospho-IκBa, and there may also be a contribution from inhibition of processing of the p105 precursor protein into the mature p50 subunit (Palombella et al., 1994).

In addition to the many anti-tumor effects that would therefore be expected of agents that inhibit NF-κB based on this transcription factor's roles in the processes outlined above, proteasome inhibitors are able to directly impact upon both capsase-8 and –9-dependent apoptosis pathways, probably at least in part through NF-κB. Such dual activation has been demonstrated in several model systems, most notably by Mitsiades and colleagues in multiple myeloma (Mitsiades et al., 2002a; Mitsiades et al., 2002b; Zhang et al., 2004). With respect to caspase-8, NF-κB activates the cellular FLICE-inhibitory protein c-FLIP, which inhibits processing of pro-caspase-8 (Lin & Karin, 2003; Orlowski & Baldwin, 2002). Proteasome inhibitors would therefore be expected to reduce c-FLIP levels and promote activation of caspase-8 (Sayers et al., 2003). This would be potentiated by relief of NF-κB-mediated induction of members of the cellular inhibitor of apoptosis (c-IAP) family proteins, which interact directly with caspase-8 and effector caspases to block programmed cell death (Lin & Karin, 2003; Orlowski & Baldwin, 2002). Inhibitors such as lactacystin may also be able to directly activate caspase-8 in some model systems (Yamada et al., 2000), resulting in cleavage of Bid into its pro-apoptotic, truncated form tBid. Generation of tBid would lead to activation of mitochondrial-dependent apoptosis (Li et al., 1998), since tBID induces mitochondrial release of cytochrome *c*, thereby linking the extrinsic and intrinsic apoptosis pathways. Moreover, because degradation of tBid normally occurs through the proteasome, tBid would be stabilized (Breitschopf et al., 2000) resulting in further amplification of the apoptotic process.

Focusing on caspase-9-dependent pathways of programmed cell death, relief of NF-κB-mediated induction of Bcl-x_L and A1/Bfl-1 through proteasome inhibition would promote mitochondrial release of cytochrome *c* (Lin & Karin, 2003; Orlowski & Baldwin, 2002). This would lead to activation of effector caspases, which would be potentiated by a loss of inhibition by X-linked IAPs, which are also transcribed in an NF-κB-dependent manner. Down-regulation of X-IAPs and gadd45β would also relieve inhibition of the c-Jun-N-terminal kinase (JNK), which is known to play a role in proteasome inhibitor-mediated apoptosis (Meriin et al., 1998). JNK induces mitochondrial release of cytochrome *c* (Tournier et al., 2000) in part by promoting Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins (Tsuruta et al., 2004). Additional contributions may come from JNK-dependent phosphorylation of pro-apoptotic Bim proteins (Putcha et al., 2003) that bind Bcl-2. It is also notable that

proteasome inhibition stabilizes members of the Bim family such as Bim-E_L (Luciano et al., 2003), which as with tBid may amplify the apoptotic process further.

3.3.2. p44/42 MAPK

The p44/42 MAPK pathway is one of the most ubiquitous cellular signal transduction cascades, and is involved in a variety of crucial functions including cell growth and survival in response to mitogenic stimuli (Chang et al., 2003; Johnson & Lapadat, 2002). These pathways are also involved in tumorigenesis, and in addition to contributing to transformation in a variety of malignancies have also been noted to play roles in angiogenesis, cell migration, and tumor invasion (Reddy et al., 2003). Studies of the impact of proteasome inhibition on p44/42, also called the extracellular signal-regulated kinases (ERK), revealed that signaling through this pathway was suppressed (Hideshima et al., 2001b; Orlowski et al., 2002a). An impact of p44/42 activity has been particularly well studied in breast cancer, where up-regulation of signaling through this pathway is frequently seen and may have clinical prognostic significance (Santen et al., 2002). Mechanistic studies have shown that in models of breast carcinoma decreased ERK activity appears to be due not to effects on upstream kinases, but to transcriptional induction of MKP-1 and –2 (Orlowski et al., 2002a), which decrease levels of the dually phosphorylated, active p44 and p42. This dephosphorylation plays a role in inhibitor-mediated apoptosis, since expression of phosphatase-resistant mutant MKPs was able to protect cells from programmed cell death. In lymphoma cell lines another mechanism may contribute, since proteasome inhibition has been reported to induce caspase-dependent cleavage of gp130, which dimerizes and is phosphorylated after IL-6 binds to its receptor and activates p44/42 phosphorylation (Hideshima et al., 2003).

3.3.3. p53

While several proteolytic pathways contribute to the degradation of the p53 tumor suppressor, including the calcium-activated calpains (Gonen et al., 1997; Kubbutat & Vousden, 1997; Pariat et al., 1997; Zhang et al., 1997), the UPP is the major determinant of p53 turnover (Salvat et al., 1999). Proteasome inhibition results in the accumulation of p53, and in some model systems induction of apoptosis is dependent on this protein (Lopes et al., 1997), which induces programmed cell death through a variety of mechanisms (Slee et al., 2004). These include effects that are likely due to transcriptional activation of targets such as the cyclin dependent kinase inhibitor $p21^{Cip1/Waf}$ and Reprimo (Ohki et al., 2000), resulting in cell cycle arrest, Bax and Noxa (Oda et al., 2000), pro-apoptotic proteins that interact with Bcl-2, and p53 upregulated modulator of apoptosis (Nakano & Vousden, 2001). Other effects may occur through transcriptional repression of targets such as Bcl-2 itself, and also through direct binding of Bcl-2 and Bcl-x_L. Accumulation of p53 would normally result in activation of a negative feedback loop through Mdm2, which as the E3 ligase for p53 (Honda et al., 1997) would induce ubiquitination and proteasome-mediated degradation of p53 (Shmueli & Oren, 2004). However, in the presence of a proteasome inhibitor, this mechanism is abrogated, preventing this pro-apoptotic cascade from being down-regulated.

It is interesting to note, however, that studies in many model systems have documented that proteasome inhibitors induce apoptosis in a manner that is independent of cellular p53 status, such as in p53-null HL60 promyelocytic leukemia cells (Drexler, 1997). This may in part be explained by the fact that some of the down-stream effectors of p53 such as Bax (Chang et al., 1998) and $p21^{Cip1/Waf}$ (Blagosklonny et al., 1996) are themselves substrates for UPP-mediated degradation. Moreover, since binding of $p21^{Cip1/Waf}$ to cyclins normally accelerates its proteasome-mediated proteolysis (Cayrol & Ducommun, 1998), inhibition of the proteasome deregulates other mechanisms of $p21^{Cip1/Waf}$ clearance. Thus, though their accumulation might be enhanced by p53 due to increased mRNA transcription and then translation, their levels would nonetheless rise due to inhibition of degradation even in p53-mutant or -null cells. The role of $p21^{Cip1/Waf}$ has been studied, and it too is dispensable for proteasome inhibitor-mediated apoptosis (Wagenknecht et al., 1999). One possible explanation is the ability of $p27^{Kip1}$ accumulation to result in cell cycle arrest and apoptosis (Kudo et al., 2000). Here again, however, p27 may be dispensable for programmed cell death induction in some model systems (Drexler, 2003; Drexler & Pebler, 2003). These results probably do not indicate a lack of a contribution of these pathways to apoptosis, but rather are likely a testament to the multiple pro-apoptotic targets of proteasome inhibitors. This degeneracy precludes the ability of the absence of contributions from one or perhaps even several targets, such as p53, from abrogating programmed cell death due to this class of agents.

3.4. Invasion and Tumor Metastasis

Preclinical studies have shown in several model systems that proteasome inhibition, either alone or in combination with other agents, is able to decrease tumor metastasis. The first such demonstration was by Teicher and colleagues in their studies of PS-341 using the Lewis lung carcinoma model (Teicher et al., 1999). Proteasome inhibition resulted in a decrease in the number of lung metastases to as low as 22-35% of vehicle-treated controls. A trend towards a lower percentage of large vascularized metastases was noted, and combinations of PS-341 with 5-fluorouracil, cisplatin, and doxorubicin seemed to act with enhanced efficacy as well. This impact was likely in part due to many of the effects discussed herein, including angiogenesis and apoptosis. Given the role of NF-κB in the induction of proteins such as MMP-9, it seems likely that proteasome inhibitors impact on other processes associated with metastasis such as invasion, but this area has not yet been the subject of extensive investigation. Supporting an effect of proteasome inhibition in this regard are recent studies showing that a peptidyl-aldehyde inhibitor enhanced IL-10-induced expression of tissue inhibitor of metalloproteinase-1 (Stearns et al., 2003). As a result, in a modified Boyden chamber assay using prostate carcinoma cell lines, invasion by tumor cells was blocked. However, in studies of an immortalized mouse mammary epithelial cell line, lactacystin has been reported to markedly enhance the expression of MMP-2 (Park et al., 2001). Further studies are needed to determine the net effect of proteasome inhibition on these and other proteases that contribute to the development of tumor metastases.

4. COMBINING PROTEASOME INHIBITION WITH OTHER THERAPIES

The multiple mechanisms by which proteasome inhibitors can impact upon transformed cells and tumors has engendered a great deal of interest in such agents as cancer chemotherapeutics. Equally exciting, however, if not more so, is emerging information that modulation of proteasome function is a rational means to enhance the anti-tumor activity of a variety of other cancer therapies, including chemotherapy and radiation therapy, as well as vaccine therapy.

4.1. The Proteasome and Chemotherapy and Radiation Resistance

One of the major obstacles to the effectiveness of cancer therapies is the presence of *de novo* resistance, or the development of either acquired or inducible drug and radiation resistance. Several mechanisms used by transformed cells and tumors to evade the therapeutic effects of these treatment modalities have been noted to be abrogated by proteasome inhibitors, thereby contributing to sensitization. Examples include stabilization of topoisomerase II, whose enhanced degradation by some tumors decreases their susceptibility to agents such as anthracyclines and epipodophyllotoxins (Ogiso et al., 2000), and stabilization of camptothecin-toposiomerase I cleavable complexes, which are normally repaired in a proteasome-dependent fashion (Desai et al., 2001; Desai et al., 1997), and in the absence of which agents such as irinotecan may have enhanced cytotoxicity. It is also notable that, unlike other drugs which can be less effective at killing cancer cells grown as spheroids, when studied the proteasome inhibitor PS-341 showed no such limitation (Frankel et al., 2000). This suggests that agents in this class can circumvent multicellular drug resistance, and show activity against solid tumors with low growth fractions *in vivo* whose centers may be relatively hypoxic. More detailed discussions are presented below of some of the mechanisms by which proteasome inhibitors may abrogate resistance mediated by Bcl-2, NF-κB, p44/42 MAPK, and P-glycoprotein, and the interested reader is also referred to several recent excellent reviews (Voorhees et al., 2003; Yang et al., 2003).

4.1.1. Bcl-2

Bcl-2 and others of its related anti-apoptotic family members such as Bcl-x_L represent one of the most important mechanisms of resistance to the effects of cancer therapy, and their activation often occurs as part of the transformation process and contributes to *de novo* drug and radiation resistance (Pommier et al., 2004). These proteins are overexpressed in a wide range of tumors, including hematologic malignancies such as chronic lymphocytic leukemia, multiple myeloma, and non-Hodgkin's lymphoma (Kirkin et al., 2004), as well as solid tumors such as colorectal, lung, and prostate carcinomas. Through the formation of homo- or heterodimers in the inner mitochondrial membrane they inhibit the release of pro-apoptotic proteins such as cytochrome *c*, which would normally induce oligomerization of apoptosis protease activating factor-1, or Apaf-1, resulting in the recruitment of pro-caspase-9 molecules and auto-activation of caspases-9. Release of second mitochondria-derived activator of caspases (Smac) and the serine protease HtrA2/Omi is also suppressed by Bcl-2 proteins.

During programmed cell death Smac/DIABLO binds to members of the IAP family, and potentiates apoptosis by relieving IAP-mediated inhibition of caspases-3, -7, and -9. Thus, blockade of Smac release has the net effect of inhibiting apoptosis (Pommier et al., 2004).

In several model systems inhibition of the proteasome has been reported to induce programmed cell death in a fashion that is independent of the Bcl-2 expression status (for example, (An et al., 1998; Herrmann et al., 1998)). Studies of a human non-small cell lung carcinoma cell line showed that targeting the proteasome with one of several structurally distinct inhibitors induced phosphorylation of Bcl-2 at an early stage in the apoptotic process (Ling et al., 2002). This was followed by cleavage of Bcl-2 into two different fragments, one of which was generated through a caspase-dependent mechanism, while the other cleavage was caspase-independent. Induction of Bcl-2 cleavage by proteasome inhibition was recently confirmed in a model of multiple myeloma in combination with a direct Bcl-2 inhibitor (Pei et al., 2003). Cis-trans peptidyl-prolyl-isomerase (Pin-1) may contribute as well, since proteasome-mediated degradation of Pin-1 is linked to conversion of phospho-Bcl-2 to its native form (Basu et al., 2002). Inhibition of this process may therefore promote accumulation of phospho-Bcl-2, possibly promoting its cleavage, though this hypothesis has not been tested experimentally. Proteasome inhibitor-induced cleavage of Bcl-2 into smaller fragments that presumably cannot block mitochondrial release of cytochrome *c* or Smac may in part explain their ability to induce apoptosis independent of Bcl-2 status. In addition, the pro-apoptotic Bcl-2 family protein Bax is normally degraded in a proteasome-dependent fashion, and after proteasome inhibition promotes its accumulation it interacts with Bcl-2 (Li & Dou, 2000) and probably contributes to the abrogation of Bcl-2-mediated cytoprotection. Finally, in regards to Smac, during apoptosis IAP proteins serve as E3 ubiquitin ligases for Smac thereby inducing its proteasome-mediated degradation (MacFarlane et al., 2002). Inhibition of the proteasome, however, would stabilize Smac, further potentiating programmed cell death.

4.1.2. NF-κB

NF-κB interferes with programmed cell death by enhancing transcription of targets such as the Bcl-2 homologs A1/Bfl-1, Bcl-x_L, and X-linked IAPs, and is important in the transformation process of tumors that have an increased incidence of activation of NF-κB, as noted earlier (Lin & Karin, 2003; Orlowski & Baldwin, 2002). However, further activation of nuclear NF-κB translocation is induced by several therapies commonly used in treatment of a variety of malignancies, including both radiation therapy as well as cytotoxic agents, particularly those that damage DNA. This leads to a phenomenon called inducible chemoresistance, through which some therapeutic interventions are limited in their own ability to activate programmed cell death by their stimulation of this NF-κB-mediated survival program. Thus, inhibitors of NF-κB activation have potential both as therapeutic agents in and of themselves, and also as radiation- and chemo-sensitizers.

In strong support of a role for NF-κB as part of inducible chemoresistance, treatment of fibrosarcoma cells with ionizing radiation or the anthracycline daunorubicin led to nuclear translocation of NF-κB, while inhibition of this process with a non-degradable

form of IkB potentiated apoptosis dramatically (Wang et al., 1996). A cytotoxic chemotherapeutic targeting topoisomerase 1, CPT-11, also activated NF-κB, and while in an *in vivo* model xenografts were not responsive to treatment with either CPT-11 or the super-repressor IκB, the combination led to significant tumor growth inhibition (Wang et al., 1996). Comparable results have been obtained in a number of human tumor model systems, including with colon cancer (Cusack et al., 2001) and multiple myeloma (Ma et al., 2003; Mitsiades et al., 2003).

4.1.3. p44/42 MAPK

Activity of the p44/42 pathway is another important determinant of cellular resistance to chemotherapy and radiation therapy that, like NF-κB, involves mechanisms that impact in part upon the mitochondrial contribution to programmed cell death. One of the better-characterized links between ERK activity and apoptosis is through the downstream targets p90 ribosomal S6 kinase (RSK) and Bad (Bonni et al., 1999; Jan et al., 1999; Scheid et al., 1999; Zha et al., 1997). p44/42 activation results in p90RSK phosphorylation, and p90RSK then phosphorylates the protein Bad. In its phosphorylated form the latter is sequestered in the cytoplasm by 14-3-3 proteins, without which it would translocate to mitochondria and bind Bcl-x_L, promoting release of mitochondrial cytochrome *c*. Several groups of chemotherapeutics, most notably taxanes (MacKeigan et al., 2000) and anthracyclines (Small et al., 2003; Zhu et al., 1999), activate signaling through the p44/42 MAPK pathway resulting in an anti-apoptotic effect, the latter in part by repression of MKP-1 (Small et al., 2003). The ability of proteasome inhibitors to induce MKPs might indicate that these agents would be useful in enhancing taxane- or anthracycline-mediated apoptosis by down-regulating p44/42 MAPK activation. In support of this possibility, early evidence from our laboratory shows that the proteasome inhibitor bortezomib blocks doxorubicin-mediated activation of p44/42 MAPK in a murine xenograft model of breast cancer. Furthermore, this correlates with the ability of the combination therapy to enhance apoptosis and anti-tumor efficacy.

4.1.4. P-glycoprotein

One of the more common acquired mechanisms of resistance is overexpression of the P-glycoprotein (P-gp) (Lin & Yamazaki, 2003), which along with related molecules such as lung resistance-related protein, is involved in multidrug resistance. P-gp is a transporter expressed at the cell membrane whose function is to act as an efflux pump to promote the removal of intracellular toxins. Unfortunately from the standpoint of cancer chemotherapy, members of several classes of drugs are recognized as substrates by the P-gp, including anthracyclines, vinca alkaloids, and epipodophyllotoxins. As a result they are extruded from cells, thereby lowering their intracellular concentrations and possibly compromising their anti-tumor efficacy. In many model systems, overexpression of P-gp has been associated with an enhanced resistance to chemotherapy, including in multiple myeloma (Bellamy et al., 1995). Clinically such overexpression is commonly seen in tumors that are primarily resistant to chemotherapy (Leonard et al., 2003). In addition, tumors with low basal levels at the time of their initial diagnosis that relapse after therapy often have increased levels of P-gp expression, presumably due to the elimination of drug

sensitive clones. This has prompted great interest in agents that modulate P-glycoprotein function in combination with standard chemotherapeutics that would normally be P-gp substrates.

Interestingly, studies of misprocessed mutants of P-gp revealed that they accumulated as core-glycosylated intermediates in the endoplasmic reticulum, where they were rapidly degraded (Loo & Clarke, 1998). Perhaps not surprisingly, inhibition of the proteasome stabilized these normally short-lived intermediates, but more notably such inhibition also prevented the maturation of normal P-gp molecules due to accumulation of immature forms. These immature forms have been shown to lack the ability to extrude chemotherapeutics (Loo & Clarke, 1999). This suggests that proteasome inhibitors may be able to sensitize tumors that overexpress P-gp to standard chemotherapeutics by increasing the intracellular concentration of these drugs. It is also possible that the use of proteasome inhibitor-based regimens as initial therapy may abrogate the emergence of drug resistant clones if they can overcome the survival benefit of those cells that express multidrug resistance.

4.2. The Proteasome and Vaccine Therapy

Processing of protein antigens for presentation in conjunction with MHC class I molecules occurs predominantly through the proteasome, with some contributions from other proteases (Rock et al., 2002). One might suspect therefore that proteasome inhibition, like many other therapies used in the treatment of human malignancies, would be immune suppressive. While this is an area that still needs investigation both pre-clinically and clinically, it is interesting to note that proteasome inhibition may be of benefit in strategies using vaccine therapy. In studies of a murine metastasis model, Wong and colleagues showed that increasing the density of specific peptide MHC class I complexes improved antigen presenting cell (APC) function of APCs loaded with unfractionated peptides derived from poorly immunogenic, highly metastatic tumor cells (Wong et al., 1998). One of these strategies was to pre-treat dendritic cells with proteasome inhibitors, which suppressed their endogenous peptide generation, allowed loading of specific epitopes, and resulted in a dramatic enhancement of primary cytotoxic T-lymphocyte (CTL) induction. A similar strategy was utilized subsequently in a murine model of lung metastases, where proteasome-inhibited and modified peptide-loaded fibroblasts induced a peptide-specific CTL response (El-Shami et al., 2000). This significantly delayed primary tumor progression, and protected mice which were pre-immunized against the development of lung metastasis following surgical removal of the primary tumor. Thus, while prolonged inhibition of the proteasome may well prove to be deleterious to the host immune response, transient inhibition may have an application for enhancing the ability to generate CTL responses to specific peptide vaccines.

5. ANTI-APOPTOTIC EFFECTS OF PROTEASOME INHIBITORS

The ability of proteasome inhibitors to down-regulate inducible chemotherapy resistance through the NF-κB pathway has been one of the driving hypotheses behind the development of proteasome inhibitor-based regimens. It is therefore interesting to note that recent studies have revealed that proteasome inhibitors themselves have pleiotropic

effects, and in addition to activating pro-apoptotic pathways such as JNK, also activate anti-apoptotic survival signals. These latter are interesting both mechanistically and also clinically, since they may provide novel avenues through which the anti-tumor efficacy of proteasome inhibitors may be enhanced. One of the first such described involved members of the heat shock protein (HSP)-70 family, whose induction by a brief incubation with a peptidyl-aldehyde inhibitor was reported by Meriin and colleagues to interfere with certain subsequent pro-apoptotic stimuli (Meriin et al., 1998). These included JNK-dependent processes such as further proteasome inhibition, ethanol, and heat shock, but not JNK-independent stimuli such as TNF. Thus, HSP-70 appeared to be inhibiting the intrinsic, caspase-9-dependent, but not the extrinsic, caspase-8-dependent pathway of programmed cell death. Subsequent studies indeed showed that induction of HSP-70 inhibited JNK activation and cytochrome *c*-mediated processing of pro-caspase-9 in a manner dependent on its chaperone function (Mosser et al., 2000). Supporting the possibility that HSP-70 might be a target for sensitization, cells treated with specific anti-sense oligonucleotides enhanced proteasome inhibitor-mediated apoptosis in both a control and a Bcl-x_L overexpressing IL-3 dependent cell line (Robertson et al., 1999).

Another set of heat shock proteins that influence programmed cell death are the small proteins of the HSP-27 family. These have been noted to inhibit apoptosis due both to activation of death receptor pathways such as Fas (Mehlen et al., 1996), as well as caspase-9-dependent pathways, the latter by inhibiting the cytochrome *c*-dependent activation of pro-caspase-9 (Garrido et al., 1999). Recently, HSP-27 was reported to block release of Smac, and contributed to dexamethasone resistance in multiple myeloma cells (Chauhan et al., 2003a). This pathway may also oppose proteasome inhibitor-mediated apoptosis, since anti-sense down-regulation of HSP-27 could overcome resistance to PS-341 in a model of non-Hodgkin's lymphoma (Chauhan et al., 2003c). Since HSP-27 is overexpressed in some isolates of primary, patient-derived multiple myeloma cells (Chauhan et al., 2003a), it would appear to be another potential target to enhance the anti-tumor efficacy of proteasome inhibitors.

Our recent research has focused on a third protein which forms part of the stress response pathway, MKP-1, which is transcriptionally induced by proteasome inhibition (Orlowski et al., 2002a). While it contributes to apoptosis by inactivating p44/42 MAPK, MKP-1 also can oppose programmed cell death induced by a variety of stimuli, in part through inhibition of activation of JNK (Guo et al., 1998a; Guo et al., 1998b; Ishibashi et al., 2001). Stable expression as well as Adenoviral-mediated overexpression of MKP-1 decreased the ability of proteasome inhibitors to induce apoptosis in comparison with controls (Fig. 2). Also, cell lines stably expressing a small interfering RNA specific for MKP-1 (Small et al., 2003), underwent enhanced levels of apoptosis when exposed to a proteasome inhibitor, thereby validating MKP-1 as another target for sensitization of cells to agents that inhibit proteasome function. One class of drugs that impact on MKP-1 are anthracyclines (Small et al., 2003), which down-regulate MKP-1 protein by repressing the activity of the promoter for this phosphatase. This provides further support for the testing of anthracycline/proteasome inhibitor regimens, since proteasome blockade would abrogate anthracycline-mediated anti-apoptotic activation of NF-κB, while anthracyclines would suppress proteasome inhibitor-mediated induction of anti-apoptotic MKP-1. In addition, the finding that these three heat shock- and stress-response proteins oppose programmed cell death mediated by proteasome inhibitors supports the investigation of

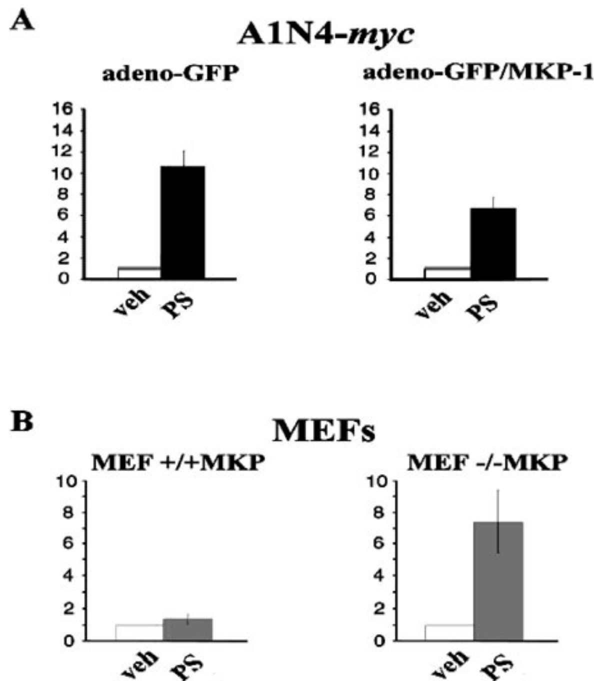

Figure 2. MKP-1 and proteasome inhibitor-mediated apoptosis. (A) A1N4-*myc* human mammary epithelial cells were infected with Adenovirus inducing the expression of either green fluorescent protein (GFP) as a control, or MKP-1 and GFP as indicated. They were then treated with either vehicle (veh) or the proteasome inhibitor PS-341 (PS), and apoptosis was determined by a DNA fragmentation assay. Vehicle-treated cells were arbitrarily set at 1.0, and the fold-increase in apoptosis due to PS-341 is shown. Infection with the MKP-1 Adenovirus inhibited programmed cell death, in association with decreased JNK activation (not shown). (**B**) MKP-1 knockout (-/-) mouse embryo fibroblasts (MEF) and wild-type (+/+) MEF were treated with vehicle or PS-341, and evaluated as above. The MKP-1 knockout MEFs were more sensitive to proteasome inhibitor-mediated apoptosis.

the roles for other members of these pathways, especially those that can be targeted with currently available agents. For example, geldanamycin and its analogues are able to inhibit the activity of HSP-90 (Maloney & Workman, 2002; Neckers, 2002), and if HSP-90 activation by proteasome inhibitors were to induce survival pathways the clinical testing of these two classes of drugs in combination would be warranted. Pre-clinical studies have already provided an excellent rationale in this regard, with findings in a model of multiple myeloma that proteasome inhibition sensitized transformed cells to the effects of agents targeting HSP-90 (Mitsiades et al., 2002a).

6. PROTEASOME INHIBITION IN THE CLINICAL ARENA

The first inhibitors of the proteasome were synthesized predominantly as laboratory tools to probe the various functions of this protease, and were peptidyl-aldehydes that

mimicked the sequence of natural substrates (Vinitsky et al., 1992). These inhibitors targeted the active site of catalytic proteasome subunits, forming a reversible hemiacetal with the N-terminal threonine amino acid (Kisselev & Goldberg, 2001). As a result this threonine, which would normally act as a nucleophile in breaking the peptide bond of a target protein, was no longer available for this function. In addition, the bound inhibitory peptide blocked access of potential substrates to the proteolytic active site. While these inhibitors were the first agents targeted to the proteasome which were shown to have anti-tumor activity *in vivo* (Orlowski et al., 1998), their poor potency and specificity detracted from their potential for clinical application. Several other peptide derivatives that inhibit the proteasome and overcome these obstacles, however, have since been synthesized, including peptidyl-boronic acids, peptidyl-epoxyketones, and peptidyl-vinylsulfones (Kisselev & Goldberg, 2001). One of the peptidyl-boronic acids, originally known as PS-341 (Adams et al., 1999) and since renamed bortezomib (VELCADE®), has already entered the clinical arena, and is being studied in a variety of trials. Based on encouraging results in multiple myeloma this agent has received approval by the Food and Drug Administration for the therapy of patients with this plasma cell dyscrasia if they have had at least two prior treatment regimens, and progressed on the second of these. As outlined in the sections below, promising results are also being obtained with bortezomib as a single agent in other hematologic disorders such as non-Hodgkin's lymphoma, and with bortezomib in combination with other therapies in patients with hematologic malignancies as well as solid tumors.

6.1. Phase I Trials with Single Agent Bortezomib

Novel agents that are felt to have potential as cancer chemotherapeutics are first evaluated in the context of phase I trials, whose primary objectives are to identify the toxicity profile of the drug, and also its maximum tolerated dose. Secondary objectives in these studies, which usually enroll a heterogeneous group of patients with different disease entities, generally also include studies of the pharmacokinetics and pharmacodynamics of the drug being tested. The results of two phase I studies with bortezomib have been published, the first of which evaluated a population of heavily pre-treated patients with solid tumors (Aghajanian et al., 2002). A total of 43 patients were treated with bortezomib given as a rapid intravenous bolus over three to five seconds administered twice weekly for two weeks, generally on days one, four, eight, and eleven, followed by a ten day recovery period. For the first patient cohort a dose of 0.13 mg/m^2 was used, with escalations pursued in subsequent groups ultimately ending at a dose of 1.56 mg/m^2, which was identified as the maximum tolerated dose. Side effects that precluded a further increase in the bortezomib dose, or dose-limiting toxicities, included grade 3 diarrhea in two out of twelve patients, and a painful sensory neuropathy, also seen in two patients. Of the latter two patients, both had a pre-existing neuropathy, likely due to prior exposure to other neurotoxic agents. Other toxicities noted included mild to moderate fatigue, fever, decreased appetite, nausea, vomiting, rash, itching, and headache. In addition, mild to moderate decreases were seen in the peripheral blood counts of some of these patients, including episodes of neutropenia, anemia, and thrombocytopenia. To evaluate the impact of bortezomib on proteasome function a pharmacodynamic assay was developed (Lightcap et al., 2000), and applied to lysates of

whole blood obtained from patients at various time points. This revealed both a time- and dose-dependent inhibition of the proteasome (Aghajanian et al., 2002), which reached 68% at the 1.56 mg/m^2/dose level. While efficacy is usually a secondary endpoint in phase I trials, evidence of anti-tumor activity was seen in one patient with bronchoalveolar lung carcinoma, who had a 50% reduction in measurable disease burden and an improvement in symptoms related to his disease. Three other patients also showed evidence of a benefit, including one with melanoma, one with nasopharyngeal carcinoma, and one with renal cell carcinoma, all of whom had previously progressive disease that was stabilized with bortezomib therapy.

A second phase I study was performed focusing on patients with advanced hematologic malignancies and used a different schedule, with bortezomib delivered twice weekly for four consecutive weeks, followed by a seventeen day recovery period (Orlowski et al., 2002b). This more intensive schedule was evaluated starting at a dose level of 0.40 mg/m^2 and was escalated through 1.38 mg/m^2, with 1.04 mg/m^2/dose identified as the maximum tolerated. Side effects that precluded use of the higher doses included moderate, grade 3 hyponatremia, hypokalemia, malaise, and fatigue, and thrombocytopenia was seen frequently as well. Other serious adverse events that were seen in patients receiving more than one cycle of therapy included postural hypotension, a systemic hypersensitivity reaction, and transaminitis, all of which were reversible upon discontinuation of bortezomib. Less severe adverse events that were commonly seen included thrombocytopenia, fatigue, nausea, anemia, leukopenia, headache, diarrhea, neutropenia, constipation, vomiting, arthralgias, dyspnea, and fever. Many of these occurred during the third or fourth weeks of therapy and resolved in the subsequent recovery period, suggesting a schedule of two weeks of treatment followed by a ten-day recovery, such as that used in the previously described study, would allow patients to tolerate a higher dose, while maintaining the same dose intensity over a six-week stretch. Out of the twenty seven patients treated there were nine with refractory plasma cell malignancies who were evaluable for a response, including eight with multiple myeloma and one with Waldenstrom's macroglobulinemia. All nine of these patients showed evidence of anti-tumor efficacy as judged by a decrease in either their serum monoclonal protein, their marrow plasmacytosis (Fig. 3), or both. One of these nine patients had a durable complete remission, which is a rare finding in patients with advanced, heavily pre-treated multiple myeloma. Among patients with different diseases one with a follicular non-Hodgkin's lymphoma, and another with a mantle cell non-Hodgkin's lymphoma had partial responses, with at least a 50% reduction in their disease burden.

6.2. Phase II Trials with Single Agent Bortezomib

Phase II clinical trials are studies in which the dose and schedule of a drug that have been determined to be safe in prior phase I studies are tested in a more homogeneous, sometimes larger population, often consisting of patients with a type of tumor against which some activity was previously seen. Renal cell carcinoma in its advanced, metastatic form is an incurable disease, and the finding of one patient in the solid tumor phase I trial whose disease was stable on bortezomib therapy led to follow-up phase II trials. The results of the first of these has recently been reported (Davis et al., 2004), in which twenty three patients with stage IV disease were treated using bortezomib at an

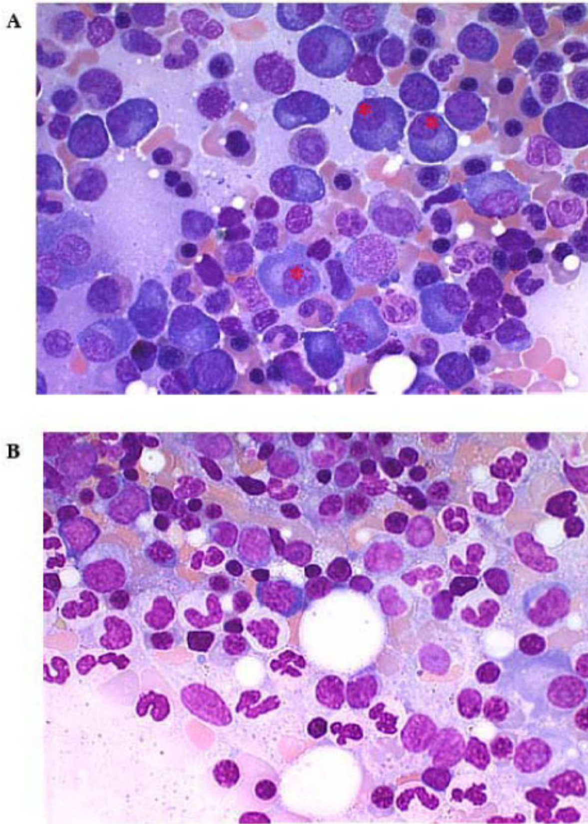

Figure 3. Response of a patient with multiple myeloma to bortezomib. (**A**) A bone marrow aspirate is shown from a patient with multiple myeloma prior to starting therapy with bortezomib. Nuclei of some of the plasma cells, which constituted 28% of the total marrow cellularity, are indicated with a white asterisk. (**B**) After two cycles of therapy with bortezomib, a repeat bone marrow aspirate was performed and showed less than 5% plasma cells.

initial dose of 1.5 mg/m^2 administered twice weekly for two weeks every twenty one days. Severe, grade 4 toxicities that were seen included arthralgias, diarrhea, and vomiting, while moderate, grade 3 toxicities included thrombocytopenia with one hemorrhage, anemia, febrile neutropenia, gastrointestinal toxicity, pain, fatigue, sensory neuropathy in one patient and a mixed sensorimotor neuropathy in another, and electrolyte disturbances. Three patients experienced progression of their disease after two cycles, leaving eighteen who were able to complete at least three cycles of therapy, which was required for an evaluation of their response. Among these an objective response was seen in only one patient, leading the authors to recommend against further evaluation in this disease, though the results from other studies in this patient population have not yet been reported in their final form.

The very encouraging results from phase I in patients with multiple myeloma, as well as the pioneering preclinical studies performed in models of myeloma reviewed

earlier, certainly supported further evaluation of bortezomib in this patient population. A multi-center phase II study was therefore performed, and enrolled 202 patients with relapsed, refractory disease who had received at least two prior therapies (Richardson et al., 2003). These patients were treated with bortezomib at 1.3 mg/m^2/dose on days one, four, eight, and eleven, followed by a ten-day recovery period every twenty one days, which constituted one cycle of therapy. Toxicities seen in at least one-quarter of patients that were felt to be drug-related included nausea, diarrhea, fatigue, thrombocytopenia, peripheral neuropathy, vomiting, and anorexia, but most of these were mild to moderate. The only side effects that were grade 3, or moderately severe, and seen in at least one-tenth of patients, included thrombocytopenia, fatigue, peripheral neuropathy, and neutropenia, and these and other toxicities led to drug discontinuation in 18% of the study patients. Bortezomib's activity was evaluated by an independent review committee using the stringent response criteria of the European Group for Blood and Marrow Transplantation (Bladé et al., 1998). Among the 193 patients who were evaluable for a response 10% overall achieved a complete response, with 4% of patients having a negative serum and/or urine protein electrophoresis and immunofixation, while another 6% of patients had a trace protein visible on immunofixation. An additional 18% of patients had a partial response, leading to an overall response rate, including those with complete and partial responses, of 27%. Other measures of response that were studied included the median time to disease progression, which for all 202 patients was 6.6 months, and was more than double the median time to progression of 3 months on whatever had been each patient's prior treatment regimen. Those patients who achieved a complete or partial response had the greatest benefit in time to progression, which was thirteen months in this subgroup. Finally, the median duration of response for patients having at least a minor response was 12 months, while the median survival for the entire population was 16 months. Responding patients also benefited in other ways, including improvements in their thrombocytopenia and anemia, elevations in normal immunoglobulins, enhanced performance status, and a decrease in symptoms such as pain and fatigue, which were disease-related. Based on these encouraging results, bortezomib was approved by the Food and Drug Administration for patients with multiple myeloma who have received at least two prior treatment regimens, and progressed on the second of these.

Several other phase II trials with bortezomib used as a single agent have been performed, the final results of which have not yet been reported. Encouraging preliminary data have been presented in some subtypes of non-Hodgkin's lymphoma, documenting durable partial and some complete responses in patients with either refractory indolent or mantle cell lymphoma variants (Goy et al., 2003; O'Connor et al., 2003). The latter results have led to the initiation of a nationwide phase II study of bortezomib in patients with relapsed and refractory mantle cell lymphoma that is currently ongoing. There is also preliminary data from a phase II trial of bortezomib in previously untreated patients with multiple myeloma (Jagannath et al., 2003) showing a 75% response rate in the first twelve evaluable patients. These findings suggest the possibility that bortezomib may have a role in patients with relapsed or refractory non-Hodgkin's lymphomas, as well as in the up-front therapy of patients with multiple myeloma.

6.3. Phase III Trials with Bortezomib

In order to identify the appropriate use of novel agents in our chemotherapeutic armamentarium, drugs that show activity in phase II trials are often then studied in a phase III setting. These studies generally randomize patients to receive either the drug of interest or a currently accepted standard regimen, with the primary goal of directly comparing their anti-tumor efficacy. One such trial involving bortezomib has been completed, and randomized patients with refractory multiple myeloma who had received no more than three prior therapies to receive either high dose dexamethasone or bortezomib. Though the final results have not yet been presented, the study was terminated earlier than expected after an independent review committee performed an interim analysis and found that patients receiving bortezomib had a significantly longer time to disease progression, and allowed patients on the dexamethasone arm to crossover to the bortezomib treatment regimen.

6.4. Clinical Trials with Bortezomib-Based Combinations

The ability of proteasome inhibitors to impact upon chemotherapy resistance pathways such as NF-κB, thereby enhancing chemosensitivity (Voorhees et al., 2003; Yang et al., 2003), has engendered great interest in the incorporation of bortezomib into regimens using currently available agents. Phase I trials of several such bortezomib-based combinations with a variety of chemotherapeutics in patients with both hematologic malignancies and solid tumors, and also in combination with radiation therapy, are either underway, or have already been completed. None of these have yet been reported in their final form, and since their main goals were to determine the toxicities and maximum tolerated dose of these regimens, none were designed to formally test the hypothesis that bortezomib was acting as a chemo- or radio-sensitizer. Preliminary results from two of these trials, however, do suggest that this may indeed be the case. In one of these the combination of bortezomib and thalidomide was studied in patients with multiple myeloma who had relapsed after autologous peripheral blood stem cell transplantation (Barlogie et al., 2004; Zangari et al., 2003). Of the 56 patients who were treated 81% had been previously exposed to thalidomide, and were determined to be resistant to this agent. After administering bortezomib alone for the first cycle of therapy, thalidomide was then added in the second and subsequent cycles. Major responses were seen in 57% of these patients, including either complete or partial responders, with complete responses documented in 22%. This response rate compared favorably with the expected 10% complete response rate of bortezomib alone (Richardson et al., 2003), and given that almost all of these patients were previously refractory to thalidomide it is unlikely that this agent by itself would have induced even a single complete response in this population.

A second study whose preliminary data support the possibility that bortezomib may be enhancing chemosensitivity *in vivo* combined bortezomib and pegylated, liposomal doxorubicin (Orlowski et al., 2003). In this trial, a total of forty two patients with a variety of advanced hematologic malignancies were treated, and included twenty four patients with multiple myeloma. For this subpopulation a 36% complete response rate was reported, with an overall response rate including both partial and complete

responders of 73%. Thirteen of these patients had previously received an anthracycline-containing regimen through which their disease had either progressed or at best been stable, and five of these patients, or 38%, had a complete response to the anthracycline-proteasome inhibitor combination. These rates are again significantly higher than what would be expected with either bortezomib alone, or with an anthracycline-containing regimen, given that the patients were previously anthracycline non-responsive. The results of these studies certainly need to be confirmed in larger, multi-center trials, but they do support the hypothesis that bortezomib-based combination therapies may demonstrate enhanced anti-tumor efficacy.

Bortezomib's potential as a chemosensitizer is also being studied in several phase I trials directed at patients with solid tumor malignancies. A preliminary report of data from a study of this proteasome inhibitor in combination with pegylated, liposomal doxorubicin (Dees et al., 2003) indicated that responses were seen in patients with breast cancer, including both some who had previously received anthracyclines, and others who were anthracycline-naïve. In patients with recurrent ovarian cancer a regimen of bortezomib and carboplatin also induced responses (Aghajanian et al., 2003), again including some patients who were platinum-naïve, as well as others who were platinum-refractory. Further testing of these and other combination regimens in solid tumor patients is ongoing.

7. CONCLUSIONS

The intricacies of the ubiquitin-proteasome pathway are only beginning to be explored, but it is already clear that this system has the potential for yielding many novel targets for cancer therapy. Consistent with this hypothesis, the proteasome itself has been validated as a target for therapy, providing an excellent example of the successful translation of laboratory-based findings into the clinical arena in a systematic, rational fashion. Recently, this process has culminated in the approval, both in the United States and European Union, of the proteasome inhibitor bortezomib for patients with multiple myeloma who have received two prior therapies, and have progressed on the second of these. This drug has the potential of radically changing the current treatment paradigm for multiple myeloma (Orlowski, 2004), which previously was an universally fatal, incurable disease. Moreover, there are encouraging indications of activity in other hematologic diseases, such as non-Hodgkin's lymphoma, as well as solid tumor patients, where studies are ongoing in a variety of populations. With the greater understanding of the molecular mechanism of action of this agent that is emerging, it may be possible to design additional rational combinations to enhance its efficacy. In addition, pharmacogenomic studies may be able to identify genetic characteristics of tumors that would be predictive of response. Such findings would allow us to tailor therapy to each patient as an individual, rather than following the same algorithm for everyone with a certain disease. It is this type of individualized care that is likely to be able to improve the outcomes for patients with a variety of malignancies, and hopefully lead to an enhanced likelihood of curing a greater proportion than is currently possible.

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D-RNAi-BASED THERAPEUTICS
Chapter XIII

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1. INTRODUCTION

The role of messenger RNA (mRNA), an intermediate between genomic DNA and its coding protein, is thought to be merely a biological bridge for connecting a genetic code to its cellular function. Nevertheless, numerous recent studies of small mRNA fragments reveal a novel RNA-mediated gene regulation system, which involves several distinctive RNA structures including short interfering RNAs (siRNA), small hairpin RNAs (shRNA), microRNAs (miRNA) and shape-shifting RNAs (ssRNA). These small RNA molecules usually function as a gene silencer, interfering with intracellular expression of genes either complementary or homologous to the small RNAs. In principle, siRNAs are double-stranded RNAs capable of degrading target gene transcripts with almost perfect complementarity (Parrish et al, 2000; Holen et al., 2002). Unlike the stringent complementarity of siRNAs to their RNA targets, shRNAs and miRNAs are single-stranded and pair with target RNAs that have partial complementarity to the small RNAs (Hutvagner et al., 2002; Zeng et al., 2003). Both shRNAs and miRNAs can trigger either translation repression or RNA degradation depending on the degree of complementarity with their targets. Many native miRNA structures are identical to shRNAs and have been demonstrated in diverse eukaryotes from yeast (*Schizosaccharomyces pombe*), plant (*Arabidopsis spp.*), nematode (*Caenorhabditis elegans*), fly (*Drosophila melanogaster*), mouse to human, mostly involved in defense against viral infections and regulation of certain gene expression during development (Volpe et al., 2002; Hall et al., 2002; Llave et al., 2002; Rhoades et al., 2002; Lee et al., 1993; Reinhart et al., 2000; Lau et al., 2001; Brennecke et al., 2003; Xu et al., 2003; Lagos-Quintana et al., 2002 & 2003; Mourelatos et al., 2002; Zeng et al., 2002). In contrast, native siRNAs and ssRNAs have mainly been discovered in plants and low-

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level animals (worms and flies), and their existence in mammalians remains to be investigated (Zeng et al., 2003). Some ssRNAs may act like miRNAs while others functionally resemble ribozymes, antibodies or DNA-binding proteins (Winkler et al., 2002; Knight J., 2003). Because of the widespread detection of shRNAs and miRNAs in eukaryotes, these small RNAs have recently been used to design novel therapeutics against cancers and viral infections (Lin et. al., 2001a and 2001b). In fact, gene silencing mechanisms involving these small RNAs have been proposed to be an intracellular defense system for eliminating undesired transgenes and foreign RNAs, such as viral infections and retrotransposon activities (Lin et. al., 2001b; Carthew R.W., 2001).

A novel therapeutic and preventive approach which has recently been demonstrated to effectively inhibit cancer progression is the use of specific DNA-RNA hybrid agents. The D-RNA (hybrids of messenger RNA and complementary DNA) interference effect, a newly found posttranscriptional gene silencing phenomenon by treating a cell with the D-RNAi (cDNA-mRNA hybrid interference) agent, was originally observed in silencing the anti-apoptotic function of *bcl-2* oncogene on phorbol ester-induced programmed cell death (apoptosis) in human prostate cancer LNCaP cells (Lin et al., 2001a). The D-RNAi phenomenon has also been demonstrated in chicken embryos and human $CD4^+$ T cells, H9 and peripheral blood mononuclear cells (PBMC) from AIDS patients (Lin et al., 2001b and 2004). The *in-vivo* transduction of anti-β-catenin D-RNAi molecules was shown to knock down more than 90% endogenous β-catenin gene expression in chicken embryonic livers and skins, while the *in-cell* and *ex-vivo* transfection of anti-HIV *gag*-p24 D-RNAi agents completely eliminated HIV-1 type B (HIV-1b) replication in acute phase (< 2-week) infection. D-RNAi was found to offer highly specific gene silencing effects, potentially resulting from a posttranscriptional mechanism that involves homologous exchange between target RNAs and the RNA components of the D-RNA construct. Such homologous exchange can trigger the production of miRNA-like molecules through type-II RNA polymerase (Pol-II) activity and other RNA excision mechanisms in eukaryotes (Lin et al., 2001b and 2003b). These findings suggest an intracellular defense network potentially useful for treatment against cancers and viral infections; however, the profound interaction among RNA transcription, excision and miRNA generation remains to be determined.

2. DEVELOPMENT OF DNA-RNA INTERFERENCE

Gene silencing, including specific molecule degradation or expression inhibition of mRNA transcripts, tRNAs, hnRNAs, viral RNA genomes and other pathogenic RNAs, holds great therapeutic promise to cure cancers and viral infections. Approaches to suppressing or quelling specific gene activities are by means of posttranscriptional gene silencing (PTGS) and RNA interference (RNAi) phenomena, which have been applied to a variety of *in vivo* systems, such as plants, *Drosophila melanogaster, Caenorhabditis elegans,* zebrafish and mammals. (Grant, S.R., 1999; Kennerdell et.al., 1998; Misquitta et.al., 1999; Pal-Bhadra et.al., 1999; Tabara et.al., 1999; Ketting et.al., 1999; Grishok et.al., 2000; Wargelius et.al., 1999; Wianny et.al., 2000; Xia et al., 2002; McCaffrey et al., 2003). Both PTGS and RNAi actions are suspected to involve an intracellular defense system that is useful against viral infection and retrotransposon activities. In general, the PTGS phenomena involve the

transfection of a plasmid-like DNA structure or double-stranded DNA (transgene) into cells, while the RNAi phenomena involve the transfection of double-stranded RNA (dsRNA) into cells. These phenomena appear to evoke an intracellular sequence-specific RNA degradation process, affecting all highly homologous transcripts, called co-suppression. It has been proposed that such co-suppression results from the generation of small interfering RNA products sized about 19-25 base-pairs (bp) by an RNA-directed RNA polymerase (RdRp) and/or an RNase III-familial endoribonuclease (Dicer) activity on an aberrant or double-stranded RNA template, derived from the transfection of synthetic nucleic acids or viral infections. (Grant, 1999; Ketting et al., 1999; Bosher et al., 2000; Zamore et.al., 2000; Elbashir et al., 2001). The small interfering RNAs are further targeted by an RNA-induced silencing complex (RISC) for the fast degradation of its complementary gene transcripts (Knight et al., 2001). Since the use of long dsRNAs (>25 bp) causes interferon-induced non-specific RNA degradation in mammalian cells (Stark et.al., 1998; Elbashir et al., 2001), researchers in the field prefer to use synthetic or Dicer-digested siRNAs for better gene-specific silencing results. However, it would be difficult to deliver such small sized dsRNAs *in vivo* due to the low siRNA stability and high dsRNase activities in mammals (Hutvagner et al., 2001; Lieberman et al, 2003).

The recent development of type-III RNA polymerase (Pol-III)-directed RNAi systems has been shown to overcome the instability problem of siRNAs by constitutive expression from U6 or H1 RNA promoter-driven plasmids and thus provide a more sufficient and consistent RNAi effect for silencing disease-associated gene expression in human cells (Jacque et al., 2002; Miyagishi et al., 2002; Lee et al., 2002; Paul et al., 2002; Xia et al., 2002; Benerjea et al., 2003). For gene therapy, a functional gene is preferably delivered into an animal or human being by expression-competent vector vehicles, including retroviral vector, lentiviral vector, adenoviral vector, adeno-associated viral (AAV) vector and so on. The main purpose of such a vector-based approach is to maintain long-term consistent gene modulation *in vivo*. Although several *in vivo* studies (Xia et al., 2002; McCaffrey et al., 2003) attempting to use the Pol-III-directed siRNA-expressing system have succeeded in maintaining constant RNAi efficacy, their delivery strategies fail to provide global effectiveness for the targeted cell population. Moreover, due to the demanding requirement of Pol-III-based transcription activity, there are still several unsolved problems. Firstly, because the read-through effect of a Pol-III transcription machinery may occur on a short template if a proper termination codon is missing, cellular Pol-III could occasionally synthesize RNA products longer than a desired siRNA (< 25 bp) and then cause unexpected interferon cytotoxicity (Grunnery et al., 1995; Geiduschek et al., 2001; Schramm et al., 2002). Such a problem can also result from the competition between the Pol-III promoter and another viral promoter of the plasmid or viral vector (i.e. LTR and CMV promoters). Furthermore, despite the widespread existence of Pol-III RNA promoters, the activity of Pol-III transcription machinery is not always consistent in numerous mammalian cell types, causing great difficulty in regulating adequate expression amounts. It has been noted in our PBMC study that high dosage of siRNAs (>250 nM) can also trigger the interferon-induced cytotoxicity similar to that of long dsRNAs. These disadvantages actually hinder the use of vector-based RNAi gene silencing for therapeutic purposes.

Consequently, there remains a need for an effective strategy with novel compositions for reducing or inhibiting an undesired gene function of interest. We thus developed a novel vector-based D-RNAi strategy and D-RNAi-derived miRNA agents for silencing selected

oncogene functions or viral genome activities without interferon-related side-effects of previous RNAi therapies. The utilization of D-RNAi agents as an anti-cancer drug or vaccine may provide potential major breakthroughs for cancer therapy and metastasis prevention. The D-RNAi gene silencing phenomenon is based on the transfection of a sequence-specific D-RNAi (mRNA-cDNA hybrid >300 bp) agent instead of a transgene (PTGS) or aberrant RNA fragment (i.e. long dsRNA and short siRNA) for inhibiting intracellular gene expression or genome function of interest. The D-RNAi gene silencing mechanism likely shares some similarities to the long-term PTGS process in plants and low–level animals. As described by Grant (1999), there are three major phenomenal effects for PTGS, including initiation, spreading and maintenance, all of which are also observed in numerous inheritable

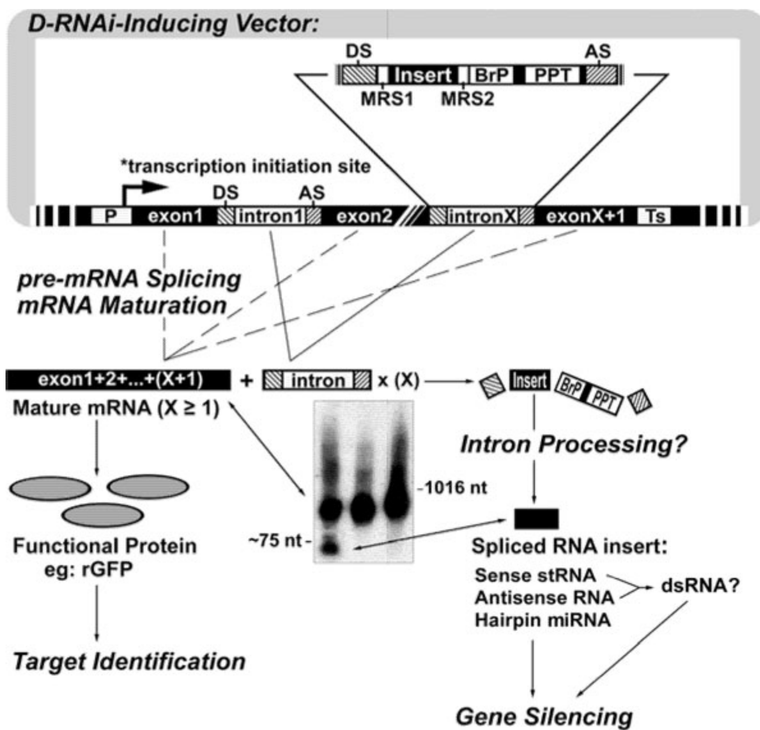


Figure 1. Experimental evidences for possible D-RNAi-induced miRNA-like molecule generation. Artificial introns, SpRNAi, were excised out of a vector-based co-expressed gene, rGFP, and processed into miRNA-like small RNAs for silencing the genes either homologous or complementary to the small RNAs. The low-stringent northern blot analysis (middle bottom) showed the generation of mature rGFP mRNA (~900 nt) and the miRNA-like molecules (~15-45 nt). The release of 15-45 nt RNA fragments was observed only from the spliced gene transcripts (left), but not from an intronless rGFP mRNA (middle) or a splicing-defective SpRNAi-rGFP pre-mRNA (right), indicating the requirement of RNA splicing and processing machineries for miRNA-like molecule generation. There is an unknown mechanism for processing of a minimal 90-nt spliced intron to small miRNA-like fragments.

RNAi phenomena. Briefly, initiation states that the onset of PTGS takes a relatively longer period of time (1~3 days), possibly to generate sufficient amounts of small RNAs for silencing target genes. In human cells, the onset of D-RNAi takes 42-48 hours, which is even longer than that of siRNA-induced RNAi (12-24 hours) under the same tested conditions. Additionally, the effect of PTGS/RNAi may spread from a transfected cell to neighboring cells (spreading) and can be maintained for a much longer time (weeks to lifetime) in a mother cell as well as its daughter cells (maintenance). The maintenance and spreading effects of PTGS/RNAi phenomena can facilitate cells to generate long-lasting/inheritable suppression of oncogenes and their polymorphisms. Although the detailed mechanism of D-RNAi is still unclear, a vector-based RdRp-dependent model has been successfully tested and warrants further investigation. Based on our current understanding, D-RNAi is most likely to be involved in not only a novel intracellular defense system but also a genomic evolution system for transgene adaptation (Lin et. al., 2001b and 2003b).

Since our previous study (Lin et al., 2001b), which demonstrated the involvement of type-II RNA polymerases (Pol-II) in the D-RNAi mechanism, a coupled interaction of nascent Pol-II pre-mRNA transcription and intron excision was observed within a certain region proximal to genomic DNA (i.e. perichromatin fibrils), providing potential evidence for D-RNAi-associated miRNA generation. In an effort to examine such a process, we constructed an artificial intron which mimics the natural structure of a pre-mRNA intron (Lin et al., 2003b). The artificial intron additionally contains a 5'-proximal insert sequence that is either homologous or complementary, or both, to a targeted exon (Fig. 1). This portion of the intron would normally represent a region unrecognized by snRNPs during RNA splicing and processing. By co-expression of the artificial intron with a recombinant red fluorescent protein (rGFP) gene in several mammalian cells, we observed that the pre-mRNA maturation of such intron-inserted rGFP transcripts induces strong suppression of genes homologous to the 5'-proximal insert sequence of the artificial intron. The splicing and processing of the introns containing inserts in either sense or antisense conformation produced equivalent gene silencing effects, while that of a palindromic hairpin insert containing both sense and antisense strands resulted in synergistic effects. We further detected that the splicing-processed introns possess an average length of 15-45 base nucleotides (nt) comparable to the general sizes of Dicer-processed miRNA intermediates, which are usually involved in developmental regulatory events. According to the variety and complexity of natural miRNA structures, there was no artificial means to produce intracellular miRNA-like molecules before the finding of this intron splicing-mediated gene silencing mechanism. These findings indicate a new function of mammalian genomic introns in intracellular miRNA generation and gene silencing, which can be used as a tool for analysis of gene function and development of gene-specific therapeutics.

3. *IN VITRO* THERAPEUTIC MODEL FOR PROSTATE CANCER

Prostate cancer accounts for 20% of all male malignancies and 11% of cancer deaths in men in the United States and it becomes more prevalent as the population ages. The pathogenesis remains mostly obscure. At present, limited knowledge of the basic factors responsible for its initiation, transformation and progression is known. The mainstay

treatment of advanced cancer progression is to remove androgenic stimulation surgically (orchiectomy) or medically (estrogens, gonadotropin releasing hormone [GnRH] agonists, anti-androgens). However, the response to hormone therapy is temporary and progression is characterized by development of hormone independent disease which is commonly unresponsive to chemotherapy. Such an increase of multiple-drug resistance in prostate cancer possess a great challenge and financial burden for current cancer therapy programs. Although many therapies involving ribozyme, antisense oligonucleotide and adjuvant immunoglobin treatments have been proposed to treat prostate cancer, none of them have so far produced convincing results for preventing or eliminating drug resistance and cancer metastasis. A recent approach using siRNA-induced RNAi showed great suppression of oncogenes *in vitro*; however, highly stringent complementarity between target RNA and siRNA must be maintained for full efficacy and thus impossible to overcome cancer polymorphisms *in vivo*. On the other hand, the RNAi-associated endonuclease Dicer in eukaryotes produces two classes of functionally distinct small interfering RNAs, siRNA and miRNA. Unlike the stringent complementarity of siRNA to its RNA targets, miRNAs are single-stranded and pair with target RNAs that have partial complementarity to the miRNA. Depending on the degree of complementarity, the miRNA is able to trigger either translation repression or RNA degradation of the target gene(s). We therefore tested an improved RNAi approach to overcome the polymorphisms of prostate cancer using the gene silencing effect of miRNA. These studies should facilitate the development of anti-cancer drugs for other cancer therapy.

As known in the literature, normal human prostatic secretory epithelial cells express limited bcl-2 protein, whereas neoplastic prostate tissues from androgen-ablation patients show an elevated level of this apoptosis-suppressing oncoprotein (Raffo et al., 1995). It was noted that over-expression of bcl-2 protects prostate cancer cells from apoptosis *in vitro*, and confers resistance to androgen depletion *in vivo* (Raffo et al., 1995; Colombel et al, 1993). The tumorigenic and metastatic potentials of LNCaP cells are also significantly increased after bcl-2 stimulation by either androgen priming or transgenic bcl-2 treatment (Raffo et.al., 1995; Berchem et al., 1995; McConkey et al., 1996). Such inhibition of apoptosis can be abolished by transfection of antisense *bcl-2* oligonucleotides, but not treatment with anticancer etoposide drugs or apoptotic stimuli such as phorbol ester (Raffo et al., 1995; Berchem et al., 1995). Thus, we tested the utilization of D-RNAi agents against *bcl-2* over-expression in androgen-primed LNCaP cells, with the expectation to increase cancer cell susceptibility to apoptotic stimuli and reduce tumorigenic cell outgrowth (Fig. 2A). The LNCaP cells were pre-treated short-term (2 days) with dihydrotestersterone (100 nM 5α-anrostan-17β-ol-3-one) to block the apoptotic effect of phorbol ester (10 nM phorbol-12-myristate-13-acetate). When further treated with various anti-*bcl-2* nucleic acid hybrid constructs (>300 bp), the cells responded with apoptosis only in the mRNA-cDNA hybrid-treated conditions (Fig. 2B) (Lin et al., 2001a). Although the morphology of dsRNA-transfected cells was also shown to be significantly changed, bcl-2 expression was not specifically inhibited, probably due to interferon-induced non-specific RNA degradation which has been found to interfere with the specificity of gene silencing (Stark et al., 1999; Elbashir et al., 2002).

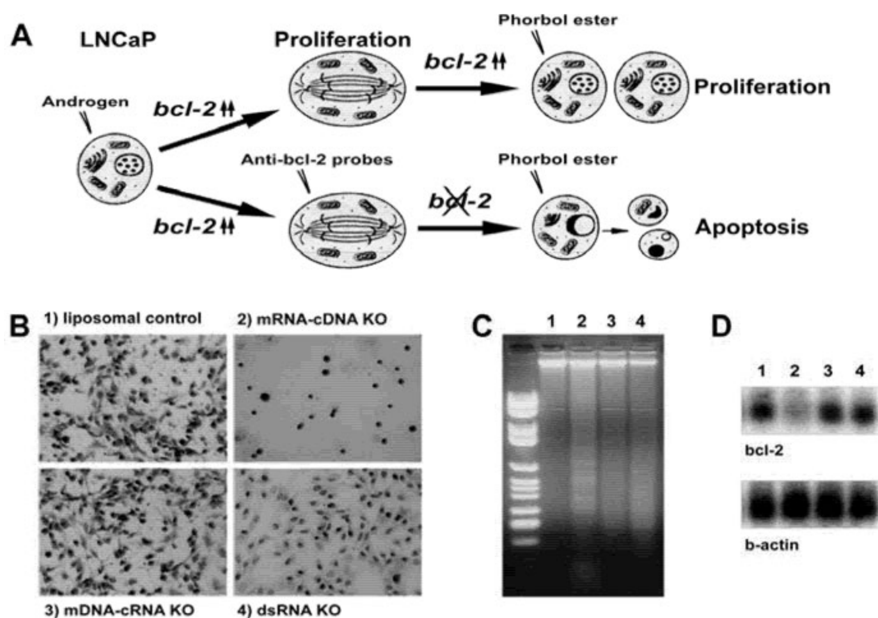


Figure 2. Discovery of D-RNAi phenomena in androgen-primed human prostate cancer LNCaP cells. (**A**), the strategy for silencing *bcl-2* oncogene in order to re-sensitize drug-resistant cancer cells to the cytotoxic effect of an anti-cancer drug, phorbol ester, was tested (n = 4). The chromosomal DNAs were stained with propidium iodide to show apoptotic condensation. (**B**), evidence of the gene silencing effect was observed only in the mRNA-cDNA transfection, which abolishes the anti-apoptotic effect of *bcl-2* and causes DNA laddering fragmentation after the treatment of phorbol ester (lane 2 of **C**). (**D**), northern blot analysis confirmed the silencing of *bcl-2* gene expression in B. Blank control without any nucleotide treatment was shown in the lanes 1 of **B**, **C** and **D**. Although the dsRNA transfection (lane 4 of **B**) also comparatively changes cell morphology and growth, a non-specific effect of interferon-induced cytotoxicity has been reported to inhibit the silencing specificity of RNAi, showing a necrotic smearing pattern of genomic DNAs in the lane 4 of **C**.

We are the first research group that demonstrated the feasibility of using a PTGS/RNAi-based approach in treating cancers (Lin et al., 2001a). The finding of D-RNAi gene silencing phenomena in human cancer cells is even a breakthrough development in the field of RNA interference studies. When D-RNAi hybrid molecules of larger than 150 bp were used, a significant long-term (>6 days) PTGS/RNAi-like gene silencing effect was observed 36-h post-transfection. As demonstrated in figure 2A, drastic changes of cell proliferation rate and morphology of the LNCaP cells were observed in the (2) D-RNAi (mRNA-cDNA hybrid) experiment versus other treatments in (1) blank control; (3) sense DNA-antisense RNA (mDNA-cRNA hybrid); and (4) dsRNA. Figure 2B shows genomic laddering patterns of the transfected cells, demonstrating significant and specific apoptosis induction by the anti-*bcl-2* D-RNAi transfection, while figure 2C presents northern blot analysis related to figure 2B, showing a strong gene silencing effect of the mRNA-cDNA hybrid transfection on *bcl-2* expression. The transfection of anti-*bcl-2* D-RNAi agents at a low dose of 5 nM into

LNCaP cells was sufficient to silence >80% *bcl-2* expression and cause apoptosis (chromosomal condensation and genomic DNA laddering fragmentation), which were not observed in the dsRNA or mDNA-cRNA transfections. Due to the limited transfection efficiency in commercially available liposomal transfection reagents that offer only 30~40% transfection rate, one transfection treatment is usually not sufficient to reach the entire cell population. A more complete inhibition of cell growth by the anti-*bcl-2* D-RNAi agent is usually achieved after double transfections, indicating no spreading effect of the D-RNAi gene silencing phenomena.

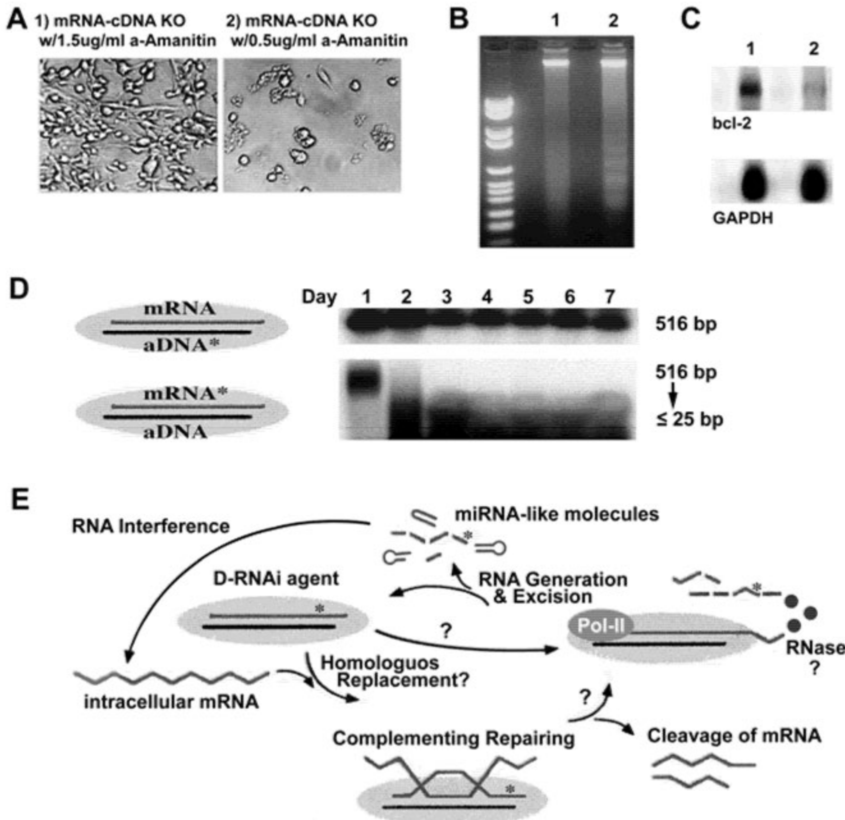
Mammalian Pol-II has been reported to possess RdRp activity (Filipovska et al., 2000; Modahl et al., 2000), capable of binding to an A/T-rich domain of hepatitis D viral RNA genome to produce viral RNAs. In conjunction with the reverse transcription activity of DNA polymerases (Herbert et al., 1999), the synthesis of DNA-RNA hybrids can be intracellularly processed to trigger D-RNAi phenomena. In experiments, the addition of α-amanitin (1.5 μg/ml), a Pol-II-specific inhibitor derived from a mushroom *Amanita phalloides* toxin, was found to abrogate the apoptosis induction of anti-*bcl-2* D-RNAi agents (Fig. 3A). Given the changes of cell proliferation rate and morphology by α-amanitin post-D-RNAi transfection (n = 3), a significant reduction of D-RNAi-induced apoptosis was detected in the 1.5 μg/ml α-amanitin addition, but not in the non-effective dosage of 0.5 μg/ml α-amanitin addition, indicating a dose-dependent release of D-RNAi inhibition on phorbol ester-treated, *bcl-2* over-expressing cancer cell growth. Figure 3B shows genomic laddering patterns of the transfected cells, demonstrating the blocking of apoptotic induction of the anti-*bcl-2* D-RNAi transfection by the α-amanitin treatment, while figure 3C shows northern blot analysis relative to figure 3B, confirming the abolishment of D-RNAi-induced anti-*bcl-2* silencing by α-amanitin. Previous studies have shown that the use of concentration up to 3.5 μg/ml can cause partial transcriptional inhibition without significant α-amanitin-induced apoptosis in the dihydrotestersterone-treated LNCaP cells. These findings suggest that the Pol-II or an α-amanitin-sensitive RdRp is responsible for the activation of D-RNAi in human LNCaP cells.

4. POTENTIAL GENE SILENCING MECHANISMS

We postulated that homologous exchange between intracellular mRNAs and the RNA components of a D-RNAi agent construct probably accounted for one of its specific gene silencing effects (Lin et.al., 2001b). We observed that the [P^{32}]-labeled DNA

Figure 3. The involvement of α-amanitin-sensitive RdRp in D-RNAi-induced gene silencing. The dose-dependent treatment of Pol-II inhibitor α-amanitin abrogated the silencing effect of anti-*bcl2* D-RNAi transfection and thus prevented the apoptotic DNA laddering effect of phorbol ester treatment (lanes 1 of **A** and **B**). Northern blot analysis confirmed the abrogation of D-RNAi-induced *bcl-2* silencing by α-amanitin addition (lane 1 of **C**). Pol-II is likely capable of generating miRNA-like molecules based on the long template of certain RNA-DNA hybrid constructs. (**D**), the replacement of RNA component in an RNA-DNA hybrid was found to correlate with the generation of miRNA-like molecules, while the DNA component remains intact and attached to the RNA component. (**E**), a proposed model of the D-RNAi mechanism contains a systemic interaction among Pol-II transcription, RNA excision and probably complementing-repairing machineries. The resulting miRNA-like molecules are able to silence intracellular RNA homologues and thus deliver the D-RNAi effect.

component of a D-RNAi agent construct was intact in a hybrid duplex during the effective period of a D-RNAi phenomenon, while the labeled RNA part was replaced by cold homologues and degraded into small RNAs within a 3-day incubation period (Fig. 3D). It is possibly that the D-RNAi agent construct can facilitate the degradation of non-recombined parts of its mRNA targets as shown in figure 3E. Alternatively, the newly recombined mRNA component of the D-RNAi agent may be further processed by intracellular Pol-II and an unknown RNA excision machinery to generate miRNA-like molecules for long-term gene silencing. This suggestion is supported by the fact that both D-RNAi-derived small RNAs and Pol-II RNA splicing-processed intron fragments possess an average length of 15-45 nucleotides (nt) comparable to the general sizes of Dicer-processed miRNA intermediates. Additionally, both kinds of small RNAs isolated by guanidinium-chloride ultracentrifugation can elicit strong, but short-term gene silencing effects to genes homologous to the small RNAs in transfected cells, indicating the possible miRNA-related interfering property of these small RNAs. Since the small miRNA-like RNAs are constitutively derived from the large templates of mRNA-cDNA or precursor mRNA-genomic DNA hybrids, the long-term effect of D-RNAi phenomena



may be maintained by accumulation of sufficient small miRNA-like RNAs rather than the stability of these resulting small RNAs. This suggestion also explains the delayed initiation observed in both D-RNAi-induced gene silencing and intron splicing-mediated PTGS phenomena (Lin et al., 2001a and 2003b).

Previous studies (Zhang et al., 1994; Spector, 1996; Parfenov et al., 2000; Ghosh et al., 2000) have demonstrated that a coupled interaction of nascent Pol-II pre-mRNA transcription and intron excision occurs within nuclear perichromatin fibril regions of genomic DNA, indicating a potential location for D-RNAi-associated miRNA generation in cells. The spliced RNA introns are not completely digested into monoribonucleotides for transcriptional recycling since approximately 10~30% of the introns can be found in the cytoplasm with a moderate half-life (Nott et al., 2003). In order to understand the function of these spliced introns before nuclear export and also to test our D-RNAi hypothesis, we constructed an artificial intron mimicking the natural structure of a pre-mRNA intron for evaluating splicing-directed small RNA generation (Lin et al., 2003b). As shown in figure 4A, the splicing-competent artificial intron, SpRNAi, is flanked with a splice donor (DS) and acceptor (AS) site, and contains a branch-point domain (BrP), a poly-pyrimidine tract (PPT) and at least one intronic insert located in the 5'-proximal domain of the artificial intron. To facilitate the accuracy of pre-mRNA splicing, the SpRNAi also contains a translation stop codon in its 3'-proximal region, which if present in a cytoplasmic mRNA, would signal the diversion of the defective pre-mRNA to a non-sense mRNA degradation (NMD) pathway. As a result of low stringency northern blotting (middle bottom of figure 1), the intracellular processing of a spliced intron into miRNA-like small fragments was detected to be highly efficient. The release of small 15-45 nt RNA fragments was observed only from the intron-containing gene transcripts (left), but not from an intronless mRNA (middle) or a splice-defective pre-mRNA (right; a positive example of NMD). These small miRNA-like RNAs are able to trigger translation repression or sometimes RNA degradation depending on the degree of complementarity and homology with their gene targets. The process of such miRNA-like small interfering RNA generation is thus different from that for the dsRNA-induced RNAi; however, we cannot rule out the possible involvement of an RNAi mechanism in that some small RNAs might form siRNAs by complementary hybridization within a localized compartment in cells.

5. VECTOR-BASED D-RNAI EFFECTS *IN VITRO*

Our rationale of developing a therapeutic D-RNAi-inducing vector is based on the observation that D-RNAi-associated gene silencing has improved upon several properties of siRNA-induced RNAi (Lin et al., 2001b). In mammals, it has been noted that interferon-induced non-specific RNA degradation represses the specificity of dsRNA-mediated RNAi effects when the dsRNA size is larger than 30 bp or its concentration is too high (Stark et al., 1998; Elbashir et al., 2001). Such a cellular response usually triggers global, non-specific mRNA degradation and may cause strong cytotoxic effects in the cells with high levels of interferon. The dsRNA-induced interferon production is used as one of the most effective adjuvant therapies to kill cancer cells. Moreover, the Pol-III-based vector delivery of siRNA expression for gene therapy *in vivo* is almost impractical due to the incompatibility between

the Pol-III promoter and another RNA promoter in the vector or from endogenous viruses. The safety issue will be a great challenge for using multiple RNA promoter-driven expression systems, which are likely to disturb the global balance of intracellular gene regulation. In contrast, the use of Pol-II-based D-RNAi-inducing vectors offers the advantages of low dosage, high potency, long-term efficacy, and lack of overt toxicity for both *in vitro* and *in vivo* applications as discussed previously. Since the use of artificial introns for inducing D-RNAi phenomena by cellular Pol-II transcription cannot affect the normal exon functions of genes unrelated to the artificial intron, the specificity of cellular gene regulation can therefore be well maintained. For extreme safety concerns, the artificial intron can be inserted into the non-regulatory region (mostly the 5'-proximal non-snRNP-binding area) of a gene intron for co-expression and co-regulation along with the gene of desire. The intron insertion can be performed through homologous recombination or transposon integration, but not necessarily through the utilization of viral vectors. Thus, this novel RNA interference approach may provide an alternative gene therapy in combating cancers and viral infections, and is currently applied to the studies of cancer research, developmental biology and anti-viral drug development.

To identify the intracellular production of Pol-II RNA splicing-processed, miRNA-like intron fragments, the artificial intron (SpRNAi) shown in figure 1 was further incorporated into a mutated HcRed1 red fluorescent membrane protein (rGFP) gene to form a recombined SpRNAi-rGFP gene, in which the functional fluorescent structure was disrupted by the intron insertion. Thus, we were able to determine the occurrence of intron splicing and rGFP-mRNA maturation through the appearance of red fluorescent emission on the membranes of transfected cells. There is no homology or complementarity between the SpRNAi-rGFP gene and its expression vectors. Upon transfection of SpRNAi-rGFP genes containing synthetic inserts homologous to a target exon, we found that the insert homologues in either sense or antisense conformation of the exon produced equivalent silencing effects, while a hairpin insert comprising both sense and antisense strands resulted in synergistic effects of gene silencing with potentially maximal efficacy. As shown in figure 4B, the transfection of various SpRNAi-containing rGFP (SpRNAi-rGFP) genes targeting the nt 279-303 open-reading-frame region of *Aequorea victoria* green fluorescent protein (eGFP) was found to be highly significant ($n = 4$, $p < 0.01$) in silencing eGFP protein expression. The use of eGFP-positive HCN-A94-2 rat neuronal stem cells offered an excellent visual aid to observe the decreased green fluorescent emission of eGFP in the red fluorescent rGFP-expressing cells. Silencing of eGFP was detected at 42-48 hours after transfection, indicating a potential requirement for precise timing of the production of sufficient small interfering intron-inserts from the SpRNAi-rGFP gene. Quantitative knockdown levels of eGFP protein (27 kDa) were also shown to be significantly altered (Fig. 4C), with reduction rates of 56 ± 6% for the transfection of inserts homologous to the sense strand of the eGFP target (sense-eGFP), 50 ± 4% for that of the antisense strand of the eGFP target (antisense-eGFP) and 81 ± 2% for that of hairpin inserts containing both strands of the eGFP target (hairpin-eGFP). No knockdown specificity to eGFP was detected by the transfection of intron-free rGFP gene (rGFP(−)), or SpRNAi-rGFP gene containing hairpin inserts homologous to either integrin β1 exon 1 (hairpin-ITGb1 mock) or to HIV-1 *gag*-p24 gene (hairpin-HIV p24). The western results shown in figure 4C confirmed the gene knockdown specificity observed in figure 4B and demonstrate that such a gene silencing effect is determined by the homology and complementarity of an insert to the targeted gene transcript, regardless of insert orientation.

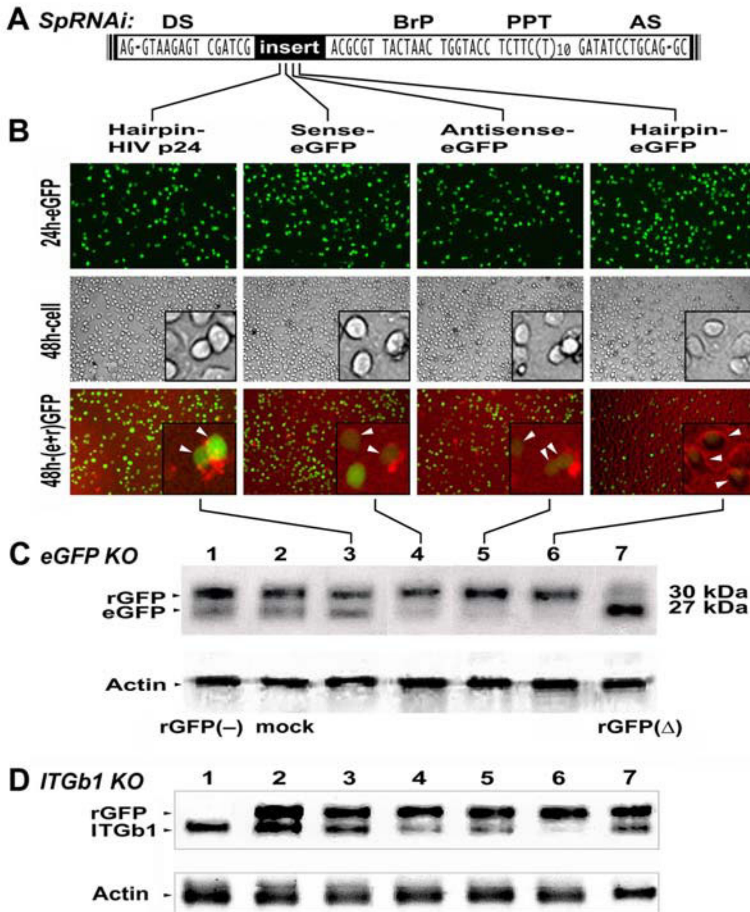


Figure 4. Strategy for analysis of endogenous D-RNAi-like mechanisms using artificial introns. A constitutive gene expression system was generated by recombination of an artificial intron, SpRNAi into rGFP gene. (**A**), the SpRNAi is shown to be flanked with a splice donor (DS) and acceptor (AS) site, and contains a branch-point domain (BrP), a poly-pyrimidine tract (PPT) and at least one intronic insert located in the 5'-proximal domain of the artificial intron. By posttranscriptional splicing and processing of the intronic RNAs against eGFP as shown in (**B**), different D-RNAi effects were detected corresponding to the degrees of knockdown potency as: hairpin-insert >> sense-strand   antisense-strand >> mock control. There is no visual difference 24-h post-transfection, whereas the ratio of eGFP to rGFP light emission was significantly changed 48-h post-transfection, reflecting a lag period of time for the induction of eGFP silencing in the rGFP-transfected cells. A close-up view of rGFP-transfected cells (white arrows) is provided in the lower small windows. (**C**), Western analysis of the D-RNAi silencing effects on eGFP in rat HCN-A94-2 neuronal stem cells confirmed the above results of (**B**). (**D**), The same approach for silencing integrin   1 (ITGb1) in human LNCaP cancer cells was also found to be consistent with the findings of (**B**) and (**C**), indicating that D-RNAi is most likely to be a global phenomenon for gene regulation in mammals.

In low-level eukaryotes, miRNA represses mRNA translation usually without causing significant mRNA destruction, whereas the siRNA mainly triggers mRNA degradation. Unlike the stringent complementarity of siRNA to its mRNA targets, miRNAs are originated to be single-stranded and pair with target mRNAs that have partial complementarity to the miRNA, i.e. small RNAs in palindromic hairpin constructs. Since the miRNA-induced gene silencing occurs most efficiently at the level of protein synthesis and the physiological response of cells is mainly dependent on protein function, the above demonstration of D-RNAi-derived gene silencing function at the protein level provides strong support for the functional and physiological significance of miRNA. Additionally, similar observations were made in human prostatic cancer LNCaP cells using SpRNAi inserts that target nts 244-265 of integrin β1 gene (ITGb1; 29 kDa), as shown in figure 4D. The expression levels of ITGb1 protein were significantly reduced by the transfection of SpRNAi-rGFP genes containing sense-ITGb1 (52 ± 2%), antisense-ITGb1 (51 ± 3%) and hairpin-ITGb1 (86 ± 1%) inserts ($n = 3$, $p < 0.01$). No knockdown specificity was detected by the transfection of either intronless rGFP gene (blank controls) or SpRNAi-rGFP gene containing hairpin-HIV p24 insert (negative mock controls). The consistent findings in two different mammalian cell lines with two different target genes suggest that this gene silencing mechanism may be a universal cellular response to intronic sequences with homology or complementarity to existing exons in cells. Because of the diversity of miRNA structures and the complexity of genomic introns, further investigation into the mechanism by which cells process a minimal 90 nt intron sequence into small miRNA-like molecules may shed light on not only a novel intracellular defense system but also one of the stringent gene regulation systems in eukaryotic cells.

6. *IN VIVO* D-RNAi PHENOMENA

The foregoing establishes the fact that the D-RNAi agents can be used as an effective strategy to silence specific target genes *in vivo*. As an example, the β-catenin gene was selected because its protein products play a critical role in the biological development and oncogenesis (Butz et al., 1995). β-catenin is known to be involved in the growth control of skin and liver tissues in chicken embryos. As shown in figure 5, experimental results demonstrated that D-RNAi (mRNA-cDNA) agents were capable of inhibiting β-catenin gene expression in the liver and skin of developing chicken embryos. The anti-β-catenin D-RNAi molecule was generated against the central region of the β-catenin coding sequence (aa 306–644) according to an RNA-polymerase cycling reaction (RNA-PCR) procedure (Lin et al., 1999a). Using embryonic day 3 chicken embryos, a dose of 25 nM of the D-RNAi agent or reverse control hybrids of sense DNA-antisense RNA (mDNA-cRNA) was injected into the ventral body cavity, which is close to where the liver primordia would form (Fig. 5A). After injection, eggs containing the chicken embryos were sealed and cultured in a

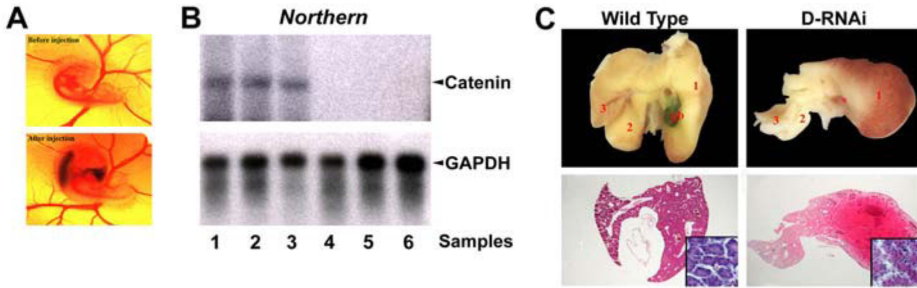


Figure 5. ***In vivo*** **gene silencing effects of an anti- -catenin D-RNAi agent in embryonic chicken.** (**A**), micro-injection of the anti- -catenin D-RNAi agent into the ventral body cavity proximal to liver primordial area was shown by Fast green staining. (**B**), northern blot analysis using total RNAs from dissected livers showed that -catenin in the control livers remains expressed (lanes 1-3), whereas the level of -catenin mRNA is decreased dramatically (lanes 4-6) after treatment with the D-RNAi agent directed against -catenin. Controls used include liposome alone (lane 1) and similar concentrations of the control mDNA-cRNA hybrids (lanes 2,3). (**C**), the morphological alteration of D-RNAi-transfected embryonic livers was correlated to the result of -catenin knockout.

humidified incubator at 39-40°C until day 12 when the embryos were removed, examined and photographed under a dissection microscope. While there were malformations, the embryos survived and there was no visible overt toxicity or overall perturbation of embryo development. The liver was the closest organ to the injection site and was most dramatically affected in its phenotype, as shown in figure 5B. Other regions, particularly the skin, were also affected by the diffused D-RNAi agent molecules. By localizing the gene silencing effect within a specific organ, we can now prevent the lethal outcome of transgenic -catenin knockout animals and provide a feasible means for evaluation of the relative gene function *in vivo*.

Ten days post-injection with the anti- -catenin D-RNAi agent, the embryonic chicken livers showed an enlarged and engorged first lobe, but the sizes of the second and third lobes of the livers were dramatically decreased (Fig. 5C). Histological sections of normal livers showed hepatic cords and sinusoidal space with few blood cells. In the anti- -catenin D-RNAi-treated embryos, the general architecture of the hepatic cells in lobes 2 and 3 remained unchanged; however, there were islands of abnormal regions in lobe 1. The endothelium development appeared to be defective and blood leaked outside of the blood vessels. Abnormal types of hematopoietic cells were also observed between the space of hepatocytes, particularly dominated by a population of small cells with round nuclei and scanty cytoplasm. In severely affected regions, hepatocytes were disrupted (Fig. 5C, small windows). The results discussed above showed that the anti- -catenin D-RNAi agent was very effective in knocking out the targeted gene expression *in vivo* at a very low dose of 25 nM and remained in effect over a long period of time (>10 days). Furthermore, such a gene silencing effect appeared to be highly specific as non-targeted organs appear to be normal, indicating that the D-RNAi hybrid compositions herein possessed no overt toxicity *in vivo*. The gene silencing *in vivo* by the D-RNAi agent

We have recently observed that patched albino (white) skins of melanin-knockout mice (W-9 strain) can be created by a succession of intra-cutaneous (i.c.) transduction of about 50 nM anti-tyrosinase (tyr) mRNA-cDNA hybrids for 4 days (total 200 nM). Tyr, a type-I membrane protein and copper-containing enzyme, catalyzes the critical and rate-limiting step of tyrosine hydroxylation in the biosynthesis of melanin (black pigment) in skin and hair. After 14-day incubation, the expression of melanin was blocked due to loss of its intermediates resulting from the tyr silencing effect, whereas the blank control and dsRNA-transfected mice presented normal skin color (black), indicating that the anti-tyr D-RNAi agent was stable enough to inhibit melanin production *in vivo*. Moreover, northern blot analysis using RNA-PCR-derived mRNAs from hair follicles showed a 76.1±5.3% reduction of tyr expression 2-day in the area posterior to the D-RNAi transfection, consistent with the immunohistochemistry results from the same skin area, whereas mild, non-specific degradation of common gene transcripts was detected in the dsRNA-transfected skins, as detected by smearing patterns of both house-keeping control GAPDH and targeted tyr mRNAs in northern blots. Thus, the results show that utilization of D-RNAi agents provides a powerful new strategy for *in vivo* gene therapy, potentially to melanoma. At the same dosage (200 nM in total), the D-RNAi transfections did not cause detectable cytotoxic effects, while the dsRNA transfections induced notable non-specific mRNA degradation, as in previous reports (Stark et al., 1998; Elbashir et al., 2001). This underscores the fact that mRNA-cDNA hybrids are effective even under *in vivo* systems without the side-effects of dsRNA. The results also indicate that this gene silencing effect is stable and efficient in knocking out the target gene expression over a relatively long period of time since the hair regrowth required at least a ten-day recovery period. Further, it was observed that non-targeted skin hairs appear to be normal, which implies that the compositions herein possess high specificity and no overt toxicity. Thus, D-RNAi-based gene manipulation offers the advantages of low *in vivo* dosage, stability, long-term effectiveness, and lack of overt toxicity.

7. *EX VIVO* THERAPY AGAINST HIV-1 INFECTION

The D-RNAi hybrid agents have been tested to show a great potential as antiviral drugs or vaccines for the prevention and treatment of HIV infection in conjunction with IL-2 adjuvant therapy (Fig. 6A). In this anti-viral approach, specific D-RNAi-inducing vectors were generated against the *gag-pol* region nts +2110 to +2450 of HIV-1 type B (HIV-1b) viral genome. The viral genes located in this target region include 3'-proximal $Pr55^{gag}$ polyprotein (i.e. matix p17 + capsid p24 + nucleocapsid p7) and 5'-proximal $p66/p51^{pol}$ polyprotein (i.e. protease p10 + reverse transcriptase), all of which have critical roles in viral replication and infectivity. In the early infection phase, the viral reverse transcriptase transcribes the HIV RNA genome into a double-stranded cDNA sequence and then together with matrix, integrase and viral protein R (Vpr) forms a preintegration complex for nuclear import and integration into the host chromosome, thus establishing the HIV provirus. Although HIV virus contains few reverse transcriptase and matrix proteins during its first entry, the co-suppression of $Pr55^{gag}$ and $p66/p51^{pol}$ expressions is expected to prevent the production of infectious viral particles at the late infection phase. The silencing of $Pr55^{gag}$ also further inhibits the assembly of intact viral

particles due to the lack of matrix and capsid proteins. Moreover, we anticipate that the silencing of protease in p66/p51^{pol} can prevent the maturation of some major viral proteins. As the HIV expresses about nine viral gene transcripts encoding at least 15 various proteins, the resolution of a polyprotein into individual functional proteins requires the viral protease activity. Therefore, by targeting against the HIV *gag-pol* nts 2113–2453 region, we are able to knock down multiple viral gene expressions at one time for a better therapeutical effect. The following experiments have proven the feasibility of this therapeutic strategy in a patient-extracted T lymphocyte culture model

Figure 6. Strategy and experimentation of silencing HIV-1 replication by an anti-HIV D-RNAi agent in conjunction with IL-2 adjuvant therapy. **(A)**, anti-HIV D-RNAi transfection causes degradation of HIV-1 RNA genome and suppresses early activation of *gag-pol* genes needed for retroviral replication. A sequential IL-2 treatment further stimulates normal T cell immunity and also quickens the turnover consumption of HIV-infected T cells. A net result of normal T cell outgrowth takes place to reach total clearance of HIV-1 infection. **(B)**, inhibitory effects on HIV-1b replication were shown by northern blot analysis 96-h post-transfection using various D-RNAi agents, including liposome only (blank control; lanes 2, 3, 1' and 2'), anti-HIV vDNA-aRNA (lanes 4 and 4'), anti-HIV vRNA-aDNA (lanes 6, 3', 5' and 6'), anti-HTLV vRNA-aDNA (mock control; lane 5), and anti-HIV antisense DNA (lanes 7 and 7') constructs, respectively. The D-RNAi agent targeting against HTLV-1 contains no homologous region to the HIV-1 genome in order to indicate the specificity of the above D-RNAi effects. All samples were normalized to the expression amount of β-actin.

ex vivo. As the D-RNAi agents were designed with several hundreds of base pairs (≥300 bp) in length, it would be very advantageous to overcome the daunting challenge of high frequent HIV genome mutations leading to drug resistance to small molecule drugs.

The northern blot analysis of figure 6B demonstrate the *ex-vivo* gene silencing results of anti-HIV D-RNAi transfection (n = 3 for each set) in $CD4^+$ T lymphocytes extracted from acute and chronic phase AIDS patients. In the acute infection phase (≤ two weeks), the treatment of 5 nM anti-HIV viral RNA-antisense DNA hybrid (vRNA-aDNA) agent transfection (lane 6) knocked down most of viral replication, while those of 5 nM reverse hybrid vDNA-aRNA (lane 4) and traditional antisense DNA (lane 7) transfections had very minor effects. The transfection of an anti-HTLV-1 vRNA-aDNA agent (lane 5) did not affect the expression of the HIV-1 genome, indicating that the anti-HIV D-RNAi agent transfection was very specific in the silencing of undesired viral infection. However, a less potent silencing effect was found in the chronic phase with about a two-year infection, probably due to the long half-life of latently infected T cells (Chun et al., 2003). The treatment of 5 nM anti-HIV vRNA-aDNA agent (lane 3') transfection knocked down 55.8% viral gene expression, while that of 250 nM traditional antisense cDNA (lane 7') had no visible silencing effects. When the anti-HIV D-RNAi agent concentration was increased to 25 nM and 250 nM (lanes 5' and 6'), the transfections knocked out almost all viral gene expression without detectable induction of cytotoxicity. The full size of pure HIV-1b RNA genome was displayed on the lane 1 of the acute phase northern blot. Normal total RNAs extracted from non-infected T cells were used as negative controls shown in lanes 2 and 1', whereas infected total RNAs extracted from HIV-seropositive patients as positive controls were shown in lanes 3 and 2', respectively. The expression of cellular house-keeping gene, β-actin, was at its normal level and showed no interferon-induced non-specific RNA degradation. These results suggest that the designed anti-HIV D-RNAi agent is highly specific and efficient in inhibiting the HIV-1b genome replication. Thus, it is possible that the specific anti-HIV D-RNAi treatments can be used in conjunction with the intermittent IL-2 adjuvant therapy (Marchetti et.al., 2002) to promote the outgrowth of non-infected T lymphocytes over HIV-infected ones *in vivo*. These findings suggest that the anti-HIV D-RNAi agent may be useful in the therapeutic treatments for both acute and chronic HIV infections in AIDS.

8. MULTIPLE GENE APPROACH FOR CANCER THERAPY

Unlike the single targeting approach in anti-viral therapy, cancer therapy usually takes multiple gene targets in order to deal with cancer polymorphisms generated during different stages of cancer progression. Cancer formation is a long-term process of multiple gene mutations, resulting in initiation, promotion and differentiation of cancer cell growth. Cancerous tissues often display massive heterogenecity that causes great difficulty in development of a common treatment for eliminating all of the cancer cells. At present, there is no RNAi methodology for targeting against multiple diseased genes. Based on our current experiences in gene silencing using vector-based D-RNAi strategy (Lin et al., 2003b), we started to investigate the possibility of using a D-RNAi-inducing vector to express multiple different kinds of SpRNAi introns for silencing various stage-

specific oncogenes, thus, inhibiting the progression of cancer formation at all stages. We have identified and confirmed numerous differential gene targets from LNCaP *in vitro* and micro-dissected prostate cancer cells *in vivo* (Lin et al., 1999a and 1999b). D-RNAi therapies based on these gene targets may provide a synergistic silencing effect on the genes facilitating cancer progression. Since a multiple targeting D-RNAi therapy against AIDS-facilitating cellular genes has been proven to be feasible for HIV therapy (Lin et al., 2004), the same approach in prostate cancer therapy is most likely to be promising as well. However, the results of this new approach remain to be determined.

9. CONCLUSION

The link between D-RNAi-induced posttranscriptional gene silencing and pre-SpRNAi-directed miRNA generation reveals a very complicated intracellular network for defending against undesired transgenes in probably all eukaryotic cells. This systemic network involves interactions among pre-mRNA transcription, RNA splicing and processing, and homologous complementing-repairing machineries. Our current findings may shed light on a corner of the whole interactive mechanism. Firstly, we discovered that a long mRNA-cDNA hybrid template can trigger the posttranscriptional silencing of *bcl-2* oncogene expression to re-sensitize drug-resistant prostate cancer cells to the apoptotic effect of phorbol ester. The involvement of Pol-II was identified to play a potential role of RdRp in this D-RNAi mechanism. Secondly, the same approach was used in embryonic chickens to successfully knock down one of critical organ morphogens, β-catenin, and thus create a localized transgenic silencing effect *in vivo*. Then, the treatment of anti-HIV D-RNAi agents was proven to be effective and safe in T cells *ex vivo*, providing the first evidence for RNAi-like AIDS therapy. Recently, we developed a vector-based D-RNAi strategy and found that both mRNA-cDNA and pre-mRNA-genomic DNA templates can induce miRNA-like molecule generation in mammalian cells. These findings suggest that the D-RNAi mechanism is most likely associated with miRNA activities. Since there are more than 135 kinds of miRNA structures in mammals and many of them are functionally uncharacterized, the investigation of length requirement, structural determinant and mismatch tolerance of these miRNA-like molecules in the D-RNAi mechanism may reveal one of the largest gene regulation systems in eukaryotic genomes.

We are continuously looking for the missing details of this proposed D-RNAi mechanism. For example, the requirement of multiple intracellular systems remains to be further investigated. How do eukaryotic cells process a single-stranded D-RNAi-derived RNA to its active form of various hairpin constructs, either by complementing-excision proteins or RNase III-familial endonucleases? Where is the intracellular compartment for this processing? How do the D-RNAi-processed RNAs execute miRNA-like functions to silence specific gene expression? And how many seqμences in the human genome can serve as a D-RNAi template for miRNA generation? There are too many questions to be solved in such a short time of study until now. Since pre-mRNA transcription, RNA splicing-processing, and complementing-repairing machineries were found to be involved, a more thorough understanding of the D-RNAi mechanism must rely on a broad scope of research in the interactions among these systems, whose connections may

be far more beyond the current findings. According to the recent discovery of intron-mediated gene silencing effects, a comprehensive encyclopedia of intron-derived miRNA functions is essential to utilize the D-RNAi mechanism fully in order to establish a better understanding of human biological processes, to predict potential disease risks, and to stimulate the development of new therapies and interventions to prevent and treat diseases. Treatments based on such a cellular gene modulation system can advance current therapeutic design and provide a safer means for gene therapy since intron production is tightly regulated by intracellular transcription and splicing machineries. In summary, we have shown, for the first time, a new strategy for miRNA-mediated gene therapy in mammalian cells. The targeted gene domain subject to the therapy can be inserted into the non-snRNP-binding region of a gene intron, serving as a convenient tool for analysis of gene function and development of gene-specific therapeutics. This strategy may be useful for investigating the potential role of endogenous gene-homologous introns in regulating gene function and also prevention of undesired gene activities, steering cells away from malignancies due to oncogenes and viral genes.

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NUCLEAR TRANSCRIPTION FACTOR-κB: A DRUG TARGET FOR ALL SEASONS

Chapter XIV

Amit K. Garg[1] and Bharat B. Aggarwal[1*]

1. INTRODUCTION

Since its discovery in 1986, nuclear transcription factor kappa B (NF-κB) has been recognized as a key mediator of the immune and inflammatory response to cellular stress (Mercurio & Manning, 1999). While its role has been established in normal physiologic processes, its role has also been established in the pathogenesis of a wide variety of diseases, most notably cancer (Fig. 1). NF-κB regulates a number of genes involved in cancer initiation, progression, and metastasis, and has shown to be a major regulator of apoptosis signaling. In fact, the first NF-κB-specific inhibitor used for refractory multiple myeloma has been approved by the Food and Drug Administration (FDA). Because of its involvement in multiple steps in tumorigenesis, NF-κB has become a marked target in the development of therapeutics for many other solid and hematopoietic cancers. We will focus on the role of NF-κB in a diverse tumorigenic processes and different methods used for its inhibition in pursuit of targeted therapeutics.

2. WHAT IS NUCLEAR FACTOR-κB?

NF-κB was first identified in the nuclei of mature B lymphocytes as a transcription factor that binds an 11-bp DNA sequence in the κ-light chain enhancer GGGACTTTCC (Sen & Baltimore, 1986). Mammalian cells have five distinct NF-κB subunits based on a highly conserved 300 amino acid dimerization domain called the rel homology domain,

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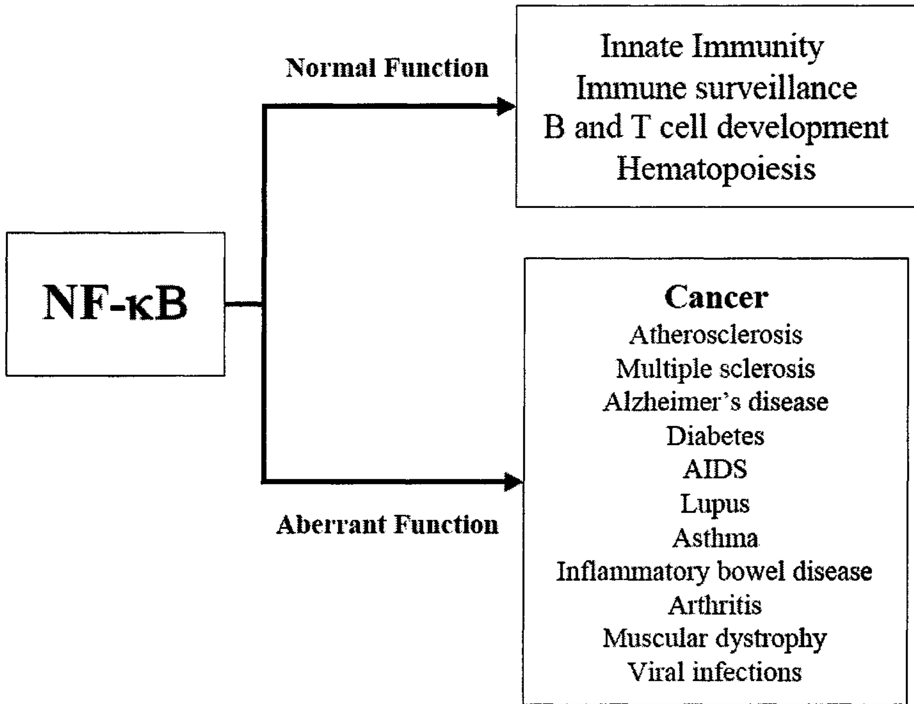

Figure 1. Major physiologic and pathologic roles of NF-κB.

which is required for binding DNA and mediating the transcription of over 200 target genes. These subunits may be classified into two functional groups, one containing the NF-κB1 (p105/p50) and NF-κB2 (p100/p52) subunits and the other containing the RelA (p65), Rel B, and c-Rel subunits. Members of the second group share a carboxy-terminal transactivation domain usually required within the Rel/NF-κB structure to promote transcription. Members of the first group exist as precursor subunits p105 and p100 (105 and 100 kDa), which contain a series of 5 to 7 ankyrin repeat domains that mask the nuclear localization signals (NLS) within the rel homology domain. They must be cleaved to the active p50 and p52 subunits (from p105 and p100, respectively) before allowing the translocation of the NF-κB complex from the cytoplasm to the nucleus, where transcription takes place.

Other inhibitory subunits that utilize a similar ankyrin repeat domain (but do not contain the rel homology domain of the five subunits above) include IκBα (most common), IκBβ, IκBγ (derived from the C-terminal of p100), IκBε, Bcl-3, pp40 (chicken homologue), and avian swine fever virus protein p28.2. More recently, another IκB-like subunit called IκBζ, with a six ankyrin repeat domain, was discovered and was found to retain the NF-κB proteins in the nucleus instead of the cytoplasm.

Several different structural combinations of subunits in the cytoplasm are referred to as NF-κB with the most common heterodimer consisting of a Rel A subunit (p65), a NF-

κB1 subunit (p105/p50), and the IκBα inhibitory subunit (Verma et al., 1995). On activation, degradation of IκBα exposes nuclear localization signals (NLS) on the p50-p65 heterodimer, leading to nuclear translocation and binding to a specific sequence in the DNA, which in turn results in gene transcription (see below). This pathway is well conserved, both in structure and function, from Drosophila to humans (Chen & Ghosh, 1999).

3. MECHANISM OF NF-κB ACTIVATION

The cellular pathways leading to NF-κB activation have become increasingly more complex in the past 10 years. A wide variety of diverse stimuli leading to NF-κB activation reveal that NF-κB is a common pathway for cellular adaptation to stress (Pahl, 1999). The stimuli include inflammatory cytokines, immune-related stress such as bacterial infection of S. *aureus* and their products such as lipopolysaccharide (LPS), viruses such as HIV-1 and their products such as hemagluttinin of the flu virus, physiologic stress such as ischemia, physical stress such as UV irradiation, environmental hazards such as cigarette smoke, many therapeutic drugs such as taxol or haloperidol, therapeutic radiation, apoptotic mediators such as anti-Fas, growth factors such as insulin,

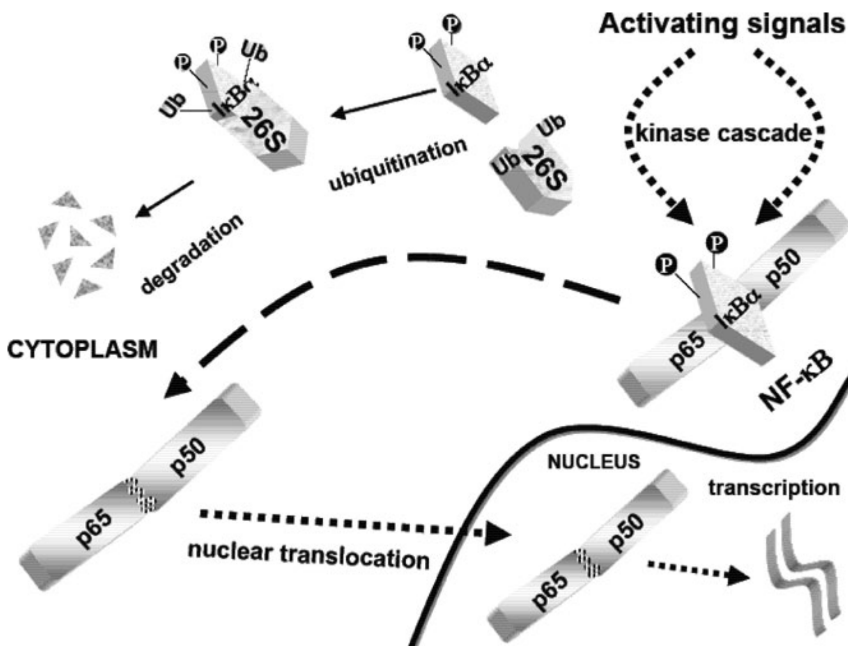

Figure 2. Classic mechanism leading to NF-κB activation. NF-κB is activated through a pathway that involves phosphorylation and subsequent ubiquitination and degradation of the inhibitory protein IκBα by the 26S proteosome, the site at which proteosome inhibitors work.

physiologic mediators such as angiotensin II or PAF, oxidative stress such as exposure to hydrogen peroxide, and many more.

Depending on the stimulus, the mechanism of activation involves overlapping and nonoverlapping steps. When not activated, NF-κB exists in the cytoplasm of cells as a homo- or heterodimer of five members of the Rel/NF-κB family that include p65/RelA, RelB, c-Rel, NF-κB1/p50, and NF-κB2/p52 (Bharti & Aggarwal, 2002b; Karin, 1999). Most commonly, NF-κB exists as a p65/p50 heterodimer that is retained in its inactive state by its association with the inhibitory protein IκBα. Activating stimuli activate an intracellular kinase cascade that phosphorylates IκBα (Fig. 2). The phosphorylation results in dissociation of IκBα from the p65/p50 heterodimer. Most of the NF-κB activation pathways converge on the phosphorylation of IκBα by the IκBα kinase complex (IKK, see below) (Karin, 1999). The IKK complex phosphorylates IκBα at serines 32 and 36, which leads to ubiquitination at lysines 21 and 22, and this leads to the degradation of IκBα by the 26S proteosome, resulting in the translocation of NF-κB to the nucleus, where it binds to its consensus sequence (5'-GGGACTTTC-3') and activates gene expression.

The central role of the IKK complex in different NF-κB activation pathways has placed it at the focus of intense study. An IKK complex consists of three subunits including IKKα, IKKβ, and IKKγ (also called NEMO). IKKβ is an inducible catalytic

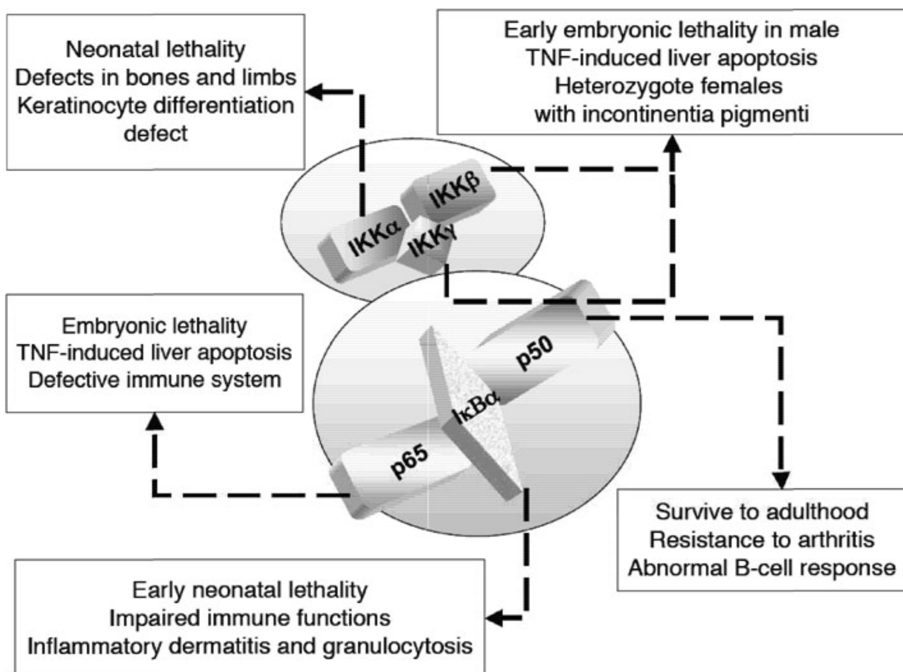

Figure 3. Effects of gene deletion of specific NF-κB proteins in mice. These results, when taken together, indicate distinct roles of the NF-κB proteins in regulation of innate and adaptive immune responses, lymphocyte functions, and cell survival.

subunit that actually phosphorylates IκBα at serine 32 and 36 and causes the subsequent degradation of IκBα, leading to the activation of NF-κB. Physiologic roles of IKKβ via gene deletion studies have shown IKKβ to be integral in liver development and protection of T-cells from tumor necrosis factor α (TNF) induced apoptosis (Fig. 3) (Senftleben et al., 2001b). IKKα has recently been shown to be involved in the activation of NF-κB via an IκBα independent pathway that involves the direct phosphorylation of NF-κB2 (p100 precursor) in response to upstream kinases (Senftleben et al., 2001a). Gene deletion studies of IKKα have shown that it plays an unexpected role in skin and skeletal development (Takeda et al., 1999). IKKγ (a.k.a. NEMO or IKKAP1) is a regulatory subunit without intrinsic kinase activity and was found to play an integral role in the activation of NF-κB as well via modulation of IκBα degradation pathways (Rothwarf et al., 1998). Gene deletion studies (X-linked) reveal that IKKγ is imperative for male survival in mice and important in lymphocyte development and persistence (Schmidt-Supprian et al., 2000). The novel IKK related kinase called IKKε/ IKKι is an LPS and PMA inducible kinase whose role in NF-κB activation is less well defined likely involves the unique preferential phosphorylation of only serine 36 (and not serine 32) on IκBα. Its mechanism may involve interaction with the TRAF interacting protein/TRAF family member-associated NF-κB activation pathway as well as interactions with unidentified upstream and downstream kinases.

Perhaps the most well-known pathway is the mechanism by which TNF activates

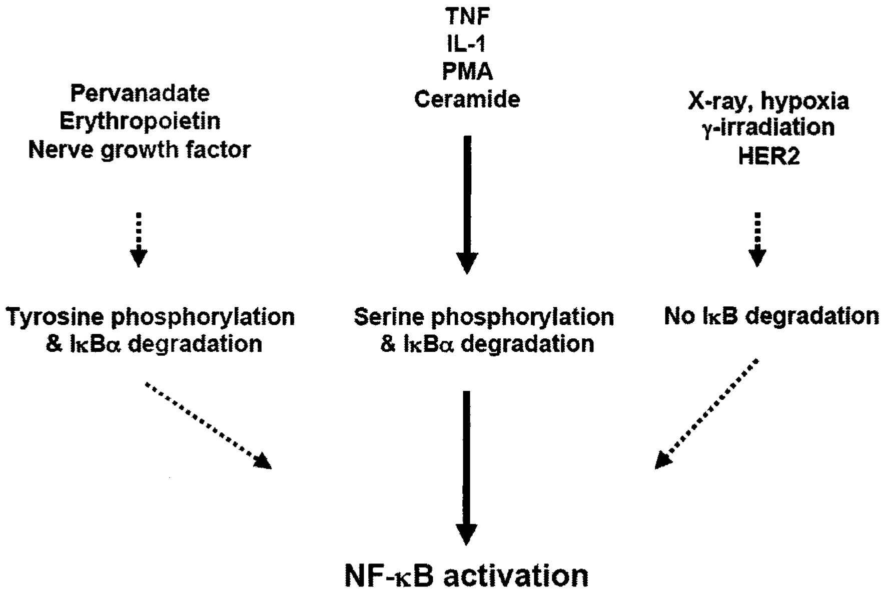

Figure 4. Various mechanisms of NF-κB activation. Other than the classic mechanism of NF-κB activation (center pathway) via serine phosphorylation of IκBα (inhibitory protein), NF-κB may be activated via tyrosine phosphorylation or an IκBα-independent pathway depending on the stimuli.

NF-κB. This pathway involves the interaction of the ligand with its receptor at the cell surface (TNFR), which then recruits a protein called TNF receptor-associated death domain (TRADD). This protein binds to TNF receptor-associated factor (TRAF)-2, which activates receptor-interacting protein (RIP). RIP interacts with mitogen-activated protein kinase kinase kinase 3 (MEKK3) to phosphorylate and activate the IκBα kinase complex (IKK) which then follows as above leading to the translocation of NF-κB to the nucleus. Other mechanisms of NF-κB activation that do not require IκBα degradation have been identified (Fig. 4), including those induced in response to such stimuli as oxidative stress and X-rays (Raju et al., 1998). Additional mechanisms, although not precisely understood, likely involve tyrosine phosphorylation of IκBα instead of the traditional serine phosphorylation that causes its subsequent degradation (Singh et al., 1996a). Interestingly, it was found that erythropoietin (EPO) activates NF-κB through phosphorylation of tyrosine and serine residues of IκBα and this is mediated through Janus kinase-2 (JAK2), the only protein tyrosine kinase thus far implicated in the activation of NF-κB (Neubauer et al., 1998; Parganas et al., 1998).

4. NF-κB MEDIATES TUMORIGENESIS

NF-κB is a worthy target for anticancer drug development for several reasons. Cancer is a hyperproliferative disorder that involves transformation, initiation, promotion, angiogenesis, invasion, and metastasis. The diversity of its clinical presentation, aggressiveness, and current treatment strategies imply an equally diverse number of potential targets in the molecular pathways leading to its formation. NF-κB activation

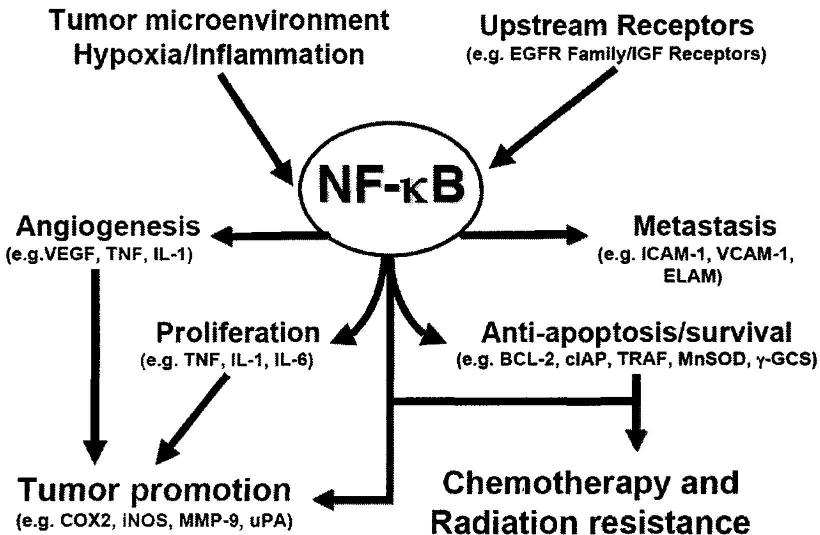

Figure 5. Pathways leading to tumor progression and resistance to therapies. Through multiple signaling pathways, NF-κB is directly and indirectly involved in processes leading to tumor promotion and cellular resistance to treatments.

participates at multiple steps in these pathways shown below and its suppression may lead to the suppression of cancer development. First, NF-κB mediates the expression of genes that are involved in tumor promotion, angiogenesis, and metastasis (Fig. 5). Second, it has been shown that NF-κB is activated by hypoxia and acidic pH, both indigenous to the tumor microenvironment. Third, the activation of NF-κB blocks apoptosis and promotes cell proliferation. Fourth, many tumor types show a constitutive (persistent) nuclear activation of NF-κB. Fifth, NF-κB activation has been shown to induce resistance to radiation and various chemotherapeutic agents. Sixth, NF-κB gene products can be oncogenic when aberrantly expressed and are implicated in a number of tumor types.

4.1. NF-κB Regulates Cancer Promoting Genes

The development of cancer is generally categorized into three stages: tumor initiation, tumor promotion, and tumor metastasis. Besides regulating a number of genes involved in prolonged cell survival, NF-κB regulates many genes involved in the promotion of cancer (i.e. clonal expansion, growth, diversification, angiogenesis, adhesion, extravasation, degradation of extracellular matrix, etc). For example, NF-κB may regulate the production of prostaglandins via the pro-inflammatory gene cyclooxygenase-2 (COX-2), which has been shown to be overexpressed in a variety of cancers including colorectal cancer and mesothelioma (Kalgutkar & Zhao, 2001; Marrogi et al., 2000). Similar studies have been found for many other pro-inflammatory genes regulated by NF-κB including TNF, interleukin-1 (IL-1), inducible NO-synthase (iNOS), matrix metalloproteinase (MMP-9), urokinase-type plasminogen activator (uPA), and many other chemokines (Strieter, 2001).

4.2. NF-κB Regulates Angiogenesis and Metastasis

Tumor cells, just like normal cells, need oxygen to survive and thus can be a limiting factor to progression of tumors. Vascularization of tumors requires the release of angiogenic growth factors (e.g. VEGF, MCP-1) from tumor cells and/or inflammatory cells such as macrophages and neutrophils or in response to pro-inflammatory cytokines (e.g. TNF). NF-κB regulates the expression of such growth factors and cytokines (VEGF, TNF, MCP-1) necessary for angiogenesis providing another pathway for which inhibition of NF-κB may be justified in anti-cancer therapy (Ueda et al., 1994). The metastasis of cancer requires the migration of cancerous cells both into and out of the vessel walls that transport them to other parts of the body. The ability to cross vessel walls is mediated by specific molecules that are expressed in response to a number of signals from inflammatory cells, tumor cells, etc. Among those special molecules are ICAM-1, ELAM-1, and VCAM-1, all of which have been shown to be expressed in response to NF-κB activation (Iademarco et al., 1992; van de Stolpe et al., 1994; Whelan et al., 1991).

4.3. Induction of NF-κB by the Tumor Microenvironment

The stress of fluctuation in blood flow in the microenvironment of solid tumors and the resultant intermittent hypoxia has been shown to activate NF-κB (Koong et al., 1994). Since oxygen is needed for a tumor to grow, the tumor must secrete chemotactic signals such as growth factors and cytokines in order to induce neovascularization (Folkman, 1993). Many of these growth factors and necessary signals for tumor progression are target genes of NF-κB and utilize its activation for their transcription. The cellular signals and precise mechanism of activation have not been elucidated, but the pathway of NF-κB activation by hypoxia is somewhat unique. The more "traditional" pathway of activation in response to stimuli (e.g. TNF IL-1) via IκB inhibitor phosphorylation, ubiquitination and degradation requires the phosphorylation of serines on IκB (see above) but Koong et al have shown that hypoxia stimulates the phosphorylation of tyrosine groups instead. The addition of this pathway leading to NF-κB activation adds to the belief that NF-κB may involve additional undiscovered pathways.

4.4. NF-κB Inhibits Apoptosis and Promotes Tumor Cell Proliferation

In 1996, three separate reports appeared implicating NF-κB in the inhibition of apoptosis (Beg & Baltimore, 1996; Wang et al., 1996). Since then, a flurry of reports, mostly supporting the original conclusions, have confirmed NF-κB's role as a mediator of inhibition of apoptosis in many cell types. Tumor initiation begins with the prolonged survival of a cell, and so, given its role in apoptosis, NF-κB has obvious implications for cancer.

An anti-apoptotic role of NF-κB has been linked to T-cell lymphoma, osteoclasts (Jimi et al., 1998), melanoma (Bakker et al., 1999), pancreatic cancer (McDade et al., 1999), bladder cancer (Sumitomo et al., 1999b), and breast cancer (Sovak et al., 1997). Cell types (not necessarily oncogenes) that display an anti-apoptotic role for NF-κB include B-cells (Arsura et al., 1996; Lee et al., 1995), T-cells (Boothby et al., 1997; Esslinger et al., 1998), granulocytes, macrophages (von Knethen et al., 1999), neuronal cells (Bales et al., 1998; Maggirwar et al., 1998), and smooth muscle cells (Erl et al., 1999).

Although rare, there are systems in which NF-κB has been shown to play a pro-apoptotic role in addition to its more common anti-apoptotic role. Examples of its pro-apoptotic effects in cells include those found in B-cells (Abbadie et al., 1993), T-cells (Dumont et al., 1999), neuronal cells (Schneider et al., 1999), and endothelial cells (DeMeester et al., 1998).

The opposing effects of NF-κB are thought to be cell type specific and/or dependent on the inducing signal (e.g. IL-1, TNF, and UV radiation). Different activation pathways of NF-κB may cause the expression of proteins that promote apoptosis (e.g. Fas, c-myc, p53, IκBα) or inhibit apoptosis (e.g. TRAF2, IAP proteins, Bcl-2-like proteins) (Chan et al., 1999; Qin et al., 1999). In addition NF-κB activation variably controls the regulation of cell-cycle proteins (e.g. cyclin D1 and CDK2 kinase) (Bash et al., 1997; Guttridge et al., 1999) and the interaction with various cellular components (e.g. p300 and p53) that promote or induce apoptosis (Ravi et al., 1998; Yang et al., 1999b).

4.5. NF-κB is Constitutively Active in Cancer

Another potential mechanism through which NF-κB could play a role in tumorigenesis involves its constitutive activation. As explained above, the activation of NF-κB occurs as it is transported from the cytoplasm to the nucleus upon degradation of the inhibitory subunit. In the nucleus it binds to specific κB sites on the DNA and mediates the expression of a number of genes involved in the cellular response to various stresses. Thus when NF-κB is found persistently in the nucleus, it is referred to as constitutive activation. Constitutive activation is shown in a wide variety of tumor types including breast cancer (Nakshatri et al., 1997; Sovak et al., 1997), prostate cancer (Palayoor et al., 1999; Sumitomo et al., 1999a), lung cancer (Batra et al., 1999; Mukhopadhyay et al., 1995), colon cancer (Cadoret et al., 1997), pancreatic cancer (Wang et al., 1999c), skin cancer (Shattuck-Brandt & Richmond, 1997), liver cancer (FitzGerald et al., 1995; Iimuro et al., 1998), head and neck cancers (Ondrey et al., 1999), bladder cancer (Sumitomo et al., 1999b), ovarian cancer (Bours et al., 1994), thyroid cancer (Visconti et al., 1997), Hodgkin's disease (Bargou et al., 1997; Bargou et al., 1996), multiple myeloma (Feinman et al., 1999), and several types of leukemias and lymphomas (Kordes et al., 2000; Mori et al., 1999; O'Connell et al., 1995). A higher level of NF-κB binding activity was found in 86% of nuclear extracts from mammary tumors that were induced in rats versus normal rat mammary glands and higher levels of NF-κB were found in estrogen receptor-negative breast cancer cell lines in rats and humans which correlated with tumorigenesis. Also, increased expression of NF-κB was found in papillary, anaplastic, and follicular thyroid cancer cell lines versus normal cells. Furthermore, the inhibition of p65 in these cancer cells led to a decrease in c-myc expression and a decrease in growth. Finally, constitutive activation was found in 83% of human pancreatic cancer cell lines. Taken together, these results suggest a strong correlation between NF-κB expression and tumor formation making the inhibition of NF-κB a valid therapeutic frontier. It should be noted, however, that constitutive activation of NF-κB is not limited exclusively to tumors as evidenced by the existence of normal cells which show constitutive activation.

The precise role of constitutive activation in tumors is not known but has been linked to resistance to apoptosis in human cutaneous T-cell lymphoma cells (Giri & Aggarwal, 1998). It is tempting to believe that a similar mechanism accounts for the progression of all tumors that constitutively express NF-κB, but such a link has yet to be clearly identified. Normally, activation of NF-κB has been shown to be stimulus dependent, such that some stimuli such as TNF cause its activation in certain cells whereas other stimuli such as IL-1 or hypoxia cause its activation in other cells. While many NF-κB stimuli have been identified, the stimulus responsible for constitutive activation of NF-κB in most cell types is not understood. Cells that express constitutively activated NF-κB are resistant to various chemotherapeutic agents.

4.6 Alterations of NF-κB Proteins in Cancer

While the expression of a large number of genes involved in the development of cancer are regulated by NF-κB, the genes that code for individual NF-κB proteins

themselves have also been implicated in the development of several types of cancers, both hematopoietic and solid tumors. These genes are expressed aberrantly i.e. amplification of gene on chromosome, rearrangement, overexpression, substitution, mutation, truncation, etc (Rayet & Gelinas, 1999). Alterations in NF-κB proteins have been found in a wide variety of tumors cell lines including lung cancer (Bours et al., 1994; Mukhopadhyay et al., 1995), prostate cancer (Motokura & Arnold, 1993), breast cancer (Nakshatri et al., 1997; Wang et al., 1999b), brain cancers (Motokura & Arnold, 1993), bone cancers (Motokura & Arnold, 1993), head and neck cancers (Mathew et al., 1993), thyroid cancer (Visconti et al., 1997), stomach cancer (Wang et al., 1999b; Wang et al., 1996), multiple myeloma (Trecca et al., 1997), and several types of leukemias and lymphomas (Barth et al., 1998; Cabannes et al., 1999; Houldsworth et al., 1996; Joos et al., 1996; Liptay et al., 1992; Lu et al., 1991; Rao et al., 1998). For instance, amplification of rel A (p65) has been linked to squamous head and neck carcinoma and adenocarcinomas of the breast and stomach. Other alterations of the Rel A protein (e.g. overexpression, mutation, substitution) have been linked to thyroid carcinoma cell lines, multiple myeloma and non-small cell lung carcinoma. Aberrations in c-Rel are present in non-Hodgkin's lymphomas, thymic B-cell lymphoma, and follicular large cell lymphoma, follicular lymphoma and human diffuse lymphoma cell lines, and non-small cell lung carcinoma. NF-κB1 (p105/p50) alterations are present in acute lymphoblastic leukemias, T-cell leukemias, and non-small cell lung carcinoma, and in cell lines of colon, prostate, breast, brain, and bone cancers. Aberrant NF-κB2 (p100/p52) expression is linked to B-cell non-Hodgkin's lymphoma, chronic lymphocytic leukemia, multiple myeloma, and cutaneous T-cell lymphoma, as well as breast cancer, and colon cancer. Finally, aberrant IκBα expression has been identified in Hodgkin's disease. Thus, the altered expression of the NF-κB gene products may play a critical role in tumorigenesis.

5. LESSONS LEARNED FROM NF-κB GENE DELETION

Mouse models with a deletion of one or more of the genes that code for specific Rel/NF-κB proteins (termed "knockout mice") have provided valuable insight into the function and relevance of various NF-κB gene products (Fig. 3). Overall, individual knockouts have caused either mild to severe immune-related deficiencies (e.g. p105/p50, p100/p52, Rel A, Rel C, IκBα), liver apoptosis (Rel A), or various other developmental abnormalities (e.g. IκBα, IKK). When p105/p50 is knocked out, there are functional defects in the immune system despite an otherwise normal development and phenotype. More specifically, p105/p50 is essential for the survival of non-activated B cells but not essential for all B-cell activated pathways (Grumont et al., 1998; Snapper et al., 1996). For example, p50-deficient mice are susceptible to *L. monocytogenes* and *S. pneumoniae* infections and do not proliferate in response to LPS but do respond to *Haemophilus influenzae* and *Escherichia Coli* (Jefferies et al., 2001). Knocking out the Rel A subunit causes embryonic lethality as a result of fetal liver cell apoptosis and granulopoiesis (Beg & Baltimore, 1996). This implicates Rel A in cell survival, specifically in response to the cytotoxic effects of TNF via induction of IκBα. Also, Rel A has been shown to be

important in induced lymphocyte proliferation and isotype switching but not basal transcription (Doi et al., 1997).

C-rel knockout mice show normal development but B and T cell deficiencies (Kontgen et al., 1995). Specifically, c-rel-deficient B cells cannot proliferate in response to immunogens due to a cell-cycle block at G1 and more prevalent activation-induced apoptosis due to a failure to upregulate A1 (homologue of Bcl-2), a prosurvival protein (Grumont et al., 1999). C-rel has also been shown to cause a tissue-specific deficiency of various cytokines and growth factors in T-cells and macrophages affecting both innate and humoral immune responses in the host (Gerondakis et al., 1999; Grigoriadis et al., 1996; Grumont et al., 1999). Mice deficient in the NF-κB2 gene (p100/p52) have mainly defects in lymph node and splenic architecture although development is normal (Caamano et al., 1998; Franzoso et al., 1998). This leads to antigen presentation impairment from accessory cells such as dendritic cells and macrophages but does not affect B or T cells directly (Gerondakis et al., 1999). Knocking out the major inhibitory subunit IκBα produces severe runting (1/3rd of normal weight) despite normal development, death by day 8 of life due to widespread dermatitis and granulocytosis, scaly appearing skin with significant sloughing, extensive post-natal granulopoiesis, small spleen size caused by depletion of cells of erythroid and lymphoid lineages (not myeloid though), and elevated levels of NF-κB in hematopoietic tissues and some NF-κB dependent target genes (implying that additional transcriptional factors are involved) (Beg et al., 1995a; Klement et al., 1996).

Recently, IKKα and IKKβ knockouts have demonstrated that IKKβ is the major subunit involved in NF-κB activation in response to a majority of stimuli (i.e. pro-inflammatory cytokines) (Li et al., 1999; Tanaka et al., 1999). Gene deletion studies have also revealed that IKKα plays little role in NF-κB activation but has proven to play an unexpected role in skin and skeletal development (Hu et al., 1999; Karin, 1999).

6. NF-κB AND CANCER TREATMENT

6.1. NF-κB Activation Induces Chemoresistance

There are several lines of evidence which suggest that NF-κB activation mediates chemoresistance. As previously indicated, NF-κB expression inhibits apoptosis through either direct or indirect regulation of a number of important genes in the apoptotic pathway (Fig. 5) (Beg & Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996). *In vitro* and *in vivo* studies have shown that constitutive activation of NF-κB inhibits chemotherapy-induced apoptosis of a number of tumor types (Bharti & Aggarwal, 2002a; Bharti & Aggarwal, 2002b; Wang et al., 1996). For example, up-regulation of NF-κB-inducible genes was found to protect MDA-231 breast cancer cells from paclitaxel-induced and radiation-induced apoptosis (Newton et al., 1999; Wang et al., 1996).

In addition to constitutive activation of NF-κB, chemotherapy and radiation may themselves increase NF-κB activation, causing treatment resistance (Barkett & Gilmore, 1999; Beg & Baltimore, 1996; Pahl, 1999; Wang et al., 1996). For example, a recent study of four tumor cell lines each treated with four different chemotherapy regimens,

found that cell survival correlated with the level of NF-κB activity induced by the drugs (Chuang et al., 2002). Specific mechanisms of NF-κB activation that account for differences in activation patterns and kinetics among the four drugs were unclear. However, most cytotoxic agents exert their effects through direct DNA damage and such damage has shown to activate NF-κB through DNA-dependent protein kinase and ataxia telangiectasia mutated (ATM) protein kinase (Basu et al., 1998; Kuo et al., 1998; Zandstra et al., 1999). Furthermore, its enhanced activity has been shown to be linked to decreased apoptosis and higher resistance via expression of the anti-apoptotic gene A1/Bfl-1 and enhanced expression of the multiple-drug resistance gene product or MDR gene (prevents the intracellular accumulation of toxic drugs such as those used in chemotherapy), both likely factors in the progression of tumors (Wang et al., 1999b).

One strategy to overcome chemotherapy and radiation induction of NF-κB expression is to increase expression of its inhibitory protein, IκBα. For example, overexpressing IκBα in MDA-231 breast cancer cells (thus permanently sequestering NF-κB in the cytoplasm) increased paclitaxel-induced G2/M arrest and apoptosis (Patel et al., 2000). In addition, these cells had lower expression of the NF-κB-regulated antiapoptotic genes TRAF-1, c-IAP2, DAD-1, and Mn-SOD. Other groups have increased IκBα in cells using adenovirus-mediated gene transfection and have also demonstrated the potentiation of chemotherapeutic efficacy in both *in vitro* and *in vivo* models of gastrointestinal malignancies (Cusack et al., 2000; Wang et al., 1999a), human glioma cells (Weaver et al., 2003), and pancreatic cancer cells (Arlt et al., 2003). These results are consistent with the hypothesis that treatment resistance can result from therapy-induced expression of NF-κB, which leads to transcription of downstream inhibitors of the apoptotic pathway (Biswas et al., 2003).

6.2. Suppression of NF-κB Induces Chemosensitization

One strategy to overcome the effects of NF-κB inhibition in cells is adenovirus-mediated transfection of an IκBα super-repressor gene. However, adenovirus-mediated transfection is not an effective method for providing a systemic treatment. A second strategy for increasing IκBα levels is to prevent its degradation. The 26S proteosome, responsible for the degradation of the inhibitory IκBα protein and subsequent activation of NF-κB (Fig. 1), has been the subject of intense study as a therapeutic target in cancer (Lenz, 2003; Mitchell, 2003). In fact, the Food and Drug Administration recently approved PS-341 (bortezomib), a potent 26S proteasome inhibitor, for use in refractory multiple myeloma (Mitchell, 2003). In a variety of cancer cells, PS-341 has been shown to enhance chemotherapy- and radiation-induced apoptosis through NF-κB inhibition (Oyaizu et al., 2001; Russo et al., 2001; Teicher et al., 1999; Wang et al., 1999a). Furthermore, proteosome inhibition was shown to increase the sensitivity of various tumors to gemcitabine, cisplatin, paclitaxel, irinotecan, cyclophosphamide, and radiation (Adams, 2002; Teicher et al., 1999).

There are also over 150 additional exogenous inhibitors of NF-κB , derived from natural and synthetic compounds (Fig. 6). These compounds may have potential therapeutic benefits and can be more easily combined with chemotherapy and radiation. Examples of such inhibitors include curcumin (Aggarwal et al., 2003; Bharti et al., 2003;

Chuang et al., 2002), parthenolide (Patel et al., 2000), peroxisome proliferator activator receptor-γ (PPARγ)-agonists (Badawi & Badr, 2002; Mehta et al., 2000), peptide inhibitors (May et al., 2000), and others (Bharti & Aggarwal, 2002a). Curcumin, a common ingredient in Asian food, has been successful as a chemopreventive agent in preclinical models and is currently being evaluated in clinical trials. Parthenolide, a natural plant extract has been shown to increase paclitaxel-induced breast cancer cell kill (Weldon et al., 2001).

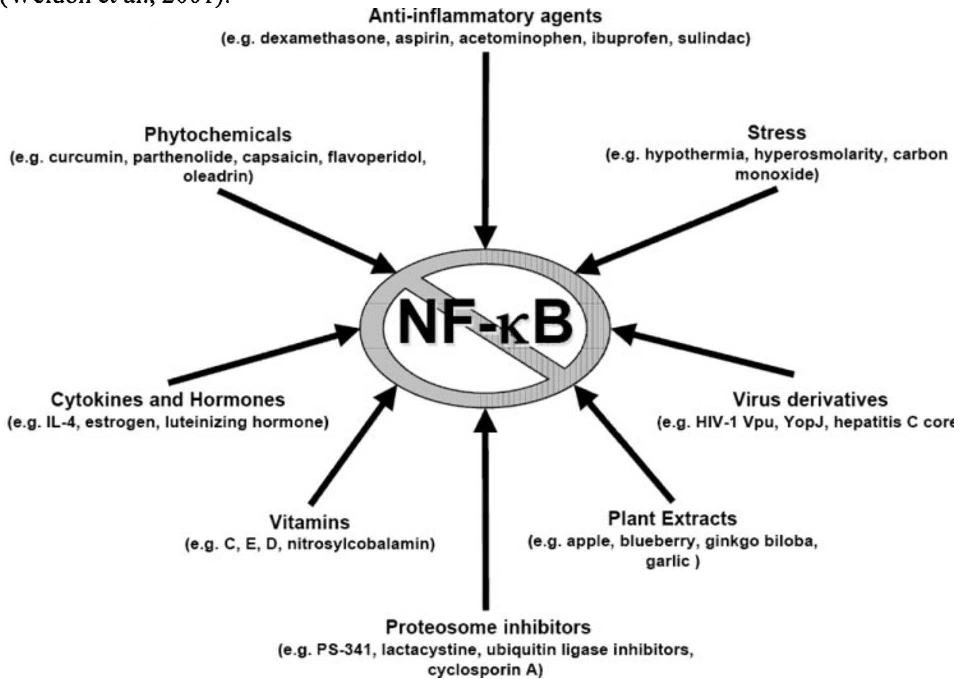

Figure 6. Various exogenous inhibitors of NF-κB. These inhibitors, and many others, may be studied as potential chemopreventive and anti-tumorigenic agents.

6.3. NF-κB Activation Induces Radioresistance

As is the case for chemotherapy, radiation-induced NF-κB activation has been reported in a variety of cancer cell types leading to decreased apoptosis and radioresistance (Jung & Dritschilo, 2001; Nakshatri et al., 1997; Sommer et al., 2003). Both ionizing and non-ionizing radiation have been shown to activate NF-κB. Similar to chemotherapy-mediated activation, radiation-induced NF-κB activation differs from the activation caused by pro-inflammatory cytokines. For example, NF-κB activation in response to chemotherapy or radiation occurs over hours, rather than the immediate response seen after TNF exposure (Brach et al., 1991; Russell et al., 2002).

Inhibition of NF-κB may increase cellular radiosensitivity for some cancers. For example, fibrosarcoma cells expressing genetically manipulated IκBα ("super-repressor," not able to be phosphorylated) are more sensitive to radiation induced apoptosis than in controls (Beg & Baltimore, 1996). Also, glioblastoma cell lines (A172, M054) that express the super repressor are more susceptible to radiation treatment (Yamagishi et al., 1997). While the precise mechanism leading to induction of radiation resistance is not clear, these examples make the development of inhibitors to NF--κB a clinically important endeavor. In a preclinical study, use of protease inhibitors increased radiation-induced apoptosis of lymphoma cells (Kurland & Meyn, 2001). Furthermore, specific mechanisms involved in radiation-induced apoptosis have become clearer with the use of specific inhibitors in the NF-κB pathway (Kurland et al., 2003). Interestingly, COX-2 inhibitors, whose therapeutic activity has been shown to utilize an NF-κB dependent pathway, are being studied for their radiosensitizing effects at our institution with promising results (Milas et al., 2003a; Milas et al., 2003b; Milas et al., 2003c).

6.4. Targeted NF-κB Inhibition

From the discussion above, it is clear that targeted inhibition of the NF-κB pathway has significant therapeutic potential, most likely due to its role in anti-apoptosis signaling. Molecular strategies include gene transfer, decoy oligonucleotides, and transgenic animal models. Also, a wide variety of pharmacologic compounds (both natural and synthetic) have been screened for their ability to suppress NF-κB. These compounds block NF-κB activation through multiple mechanisms by intercepting various steps leading to NF-κB activation. How specific some of these inhibitors are and whether they block other signaling pathways (Epinat & Gilmore, 1999) remain to be elucidated. The following gives examples for each of the strategies shown to block NF-κB activation in response to one or more stimuli.

6.4.1. Blockers of IκB Phosphorylation as Therapeutics

Because phosphorylation of IκBα is critical for NF-κB activation, compounds that block this phosphorylation prevent IκBα's ubiquitination and further degradation. Examples include aspirin (sodium salicylate) (Frantz & O'Neill, 1995; Kopp & Ghosh, 1994), ibuprofen (Palayoor et al., 1998), nitric oxide (Katsuyama et al., 1998; Matthews et al., 1996), prostoglandin A1 (Rossi et al., 1997), sanguinarine (Chaturvedi et al., 1997), and YopJ (encoded by *Yersinia pseudotuberculosis*) (Schesser et al., 1998). Recently, 4-hydroxy-2-nonenal, a lipid peroxidation product, has been shown to block phosphorylation by direct inhibition of IKK (Ji et al., 2001). Also, a novel peptide that selectively blocks the association of IKK-γ (NEMO) with the rest of the IKK complex has been shown to inhibit NF-κB activation in response to pro-inflammatory cytokines in mice while preserving basal NF-κB activity (May et al., 2000). Various pharmaceutical companies are employing IKK as a target to design structure-based inhibitors.

6.4.2. Up-regulate inhibitory subunit

Up-regulating the amount of IκBα is another strategy employed by various compounds to inhibit NF-κB activation. Examples include β-amyloid (found in Alzheimer's) (Bales et al., 1998), glucocorticoids such as dexamethasone or prednisone (Auphan et al., 1995; Brostjan et al., 1996), IL-10 (Ehrlich et al., 1998; Lentsch et al., 1997), and IL-13 (Manna & Aggarwal, 1998a).

6.4.3. Block Binding of NF-κB to the DNA

The most direct strategy for blocking activation of NF-κB is to block its binding to specific κB sites on the DNA. This is assessed by assaying the amount of NF-κB protein that is able to bind to DNA after administration of inhibitor. One mechanism is the use of a transcription factor decoy (TFD) peptide, called double-stranded oligodeoxynucleotides (ODN), that binds the same complementary region of specific DNA sites, competitively inhibiting NF-κB binding (D'Acquisto et al., 2000). Other examples of inhibitors that block DNA binding of NF-κB either directly or indirectly include atrial natriuretic peptide (ANP) (Gerbes et al., 1998), IL-4 (Manna & Aggarwal, 1998b), metals such as chromium, cadmium, gold, mercury, and zinc (Shumilla et al., 1998), ribavirin (Fiedler et al., 1996), vascular endothelial growth factor (VEGF) (Gabrilovich et al., 1998; Oyama et al., 1998), caffeine acid phenylether ester (CAPE) (Natarajan et al., 1996), and vasoactive intestinal peptide (VIP) (Delgado et al., 1998).

6.4.4. Block Proteasome from Degrading IκB Inhibitory Subunit

A more upstream strategy for blocking the activation of NF-κB is by affecting the inhibitory pathways in NF-κB activation. Proteasome inhibitors block the 26S proteasome necessary to degrade the IκB inhibitory subunit after its phosphorylation and ubiquitination in the cytoplasm and thus its release from the NF-κB complex (Palombella et al., 1994; Russo et al., 2001). Examples of these include peptide aldehydes such as ALLnL, LLM, Z-LLnV, and Z-LLL (Grisham et al., 1999), lactacystine (Fenteany & Schreiber, 1998), PS-341 (Cusack et al., 2001), ubiquitin ligase inhibitors (Yaron et al., 1997), and cyclosporin A (Frantz et al., 1994). Other IκB degradation inhibitors, whose mechanisms are unknown, include capsaicin (Singh et al., 1996b), core protein hepatitis C virus (Shrivastava et al., 1998), fungal gliotoxin (Pahl et al., 1996), IL-13 (Manna & Aggarwal, 1998a), and pervanadate (Singh & Aggarwal, 1995b).

6.4.5. Block Nuclear Translocation

Another approach to inhibiting NF-κB activation is to use cell-permeable peptides that block the nuclear localization of the NF-κB complex. The mechanism works by mimicking the sequence of p50 responsible for transporting the NF-κB complex from the cytoplasm to the nucleus to block the normal import machinery (Lin et al., 1995). Examples of these include SN-50 (Pieper & Riaz 1997) and o,o'–bismyristoyl thiamine disulfide (Shoji et al., 1998).

6.4.6. Suppression of NF-κB by Gene Transfer

Another strategy to block the activation of NF-κB is through the transfer of genes that code for proteins shown to suppress NF-κB activation. The most direct target is the IκBα gene. This entails the modification of IκBα at the specific phosphorylation sites (ser 32 and 36 switched with ala) and ubiquitination sites (lys 21 and 22 switched with arg) to prevent its degradation. This "super-repressor" keeps the NF-κB complex in the cytoplasm indefinitely (Bentires-Alj et al., 1999; Van Antwerp & Verma, 1996). Recently, a nonphosphorylatable form of IκBα was shown to inhibit osteoclastogenesis and block bone resorption when injected into bone marrow macrophages (Abu-Amer et al., 2001).

Another potential target for gene transfer has been recently shown to be HDAC3, a histone deacetylase that acts directly upon nuclear Rel A (part of the combined active p50/Rel A complex) enabling its association with IκBα and its subsequent export from the nucleus. Expression of HDAC3 in TNF stimulated HeLa cells repressed both NF-κB DNA binding and levels of Rel A with a corresponding increase in inactive cytoplasimic IκBα/NF-κB complexes (Chen et al., 2001). This mechanism has shown to control the duration of NF-κB activation and thus may be a potential weapon against constitutive NF-κB activation.

Finally, the presence of pro-apoptotic cellular proteins have been shown to inhibit the anti-apoptotic function of NF-κB as described earlier, serving as potential targets for gene transfer. Erg-1, a transcription factor that is activated by similar stimuli as NF-κB, has recently been shown to block NF-κB activation both in vitro and in vivo. Erg-l dimerizes with the p65 (Rel A) subunit of NF-κB via a specific zinc finger domain and prevents NF-κB from binding to its promotor regions on DNA (Chapman & Perkins, 2000; Cogswell et al., 1997). The RAI (Rel A associated inhibitor) gene also encodes a protein that associates with p65 (Rel A) and inhibits the anti-apoptotic activity of NF-κB. RAI shares a homologous region with 53BP2, a protein involved in apoptosis regulation (Yang et al., 1999a). Par-4 (prostate apoptosis response-4) is another recently identified inhibitor of NF-κB at the level of IKK and activator of the Fas apoptotic pathway (Chakraborty et al., 2001; Diaz-Meco et al., 1999). In addition, Par-4 inhibits Bcl-2, a well known anti-apoptotic oncogene (Camandola & Mattson, 2000). E2F-1 is another transcription factor whose expression has been correlated to increased apoptotic activity via mechanisms including the inhibition of NF-κB. E2F-1 promotes cell cycle progression and its aberrant expression is present in most tumors (Phillips et al., 1999). Finally, p53 (the classic gatekeeper of cell cycle progression) and Cyclin E-CDK2 may inhibit the anti-apoptotic action of NF-κB via the transcriptional co-activator protein CRB/p300 (Chen & Li, 1998; Mayo et al., 1997; Webster & Perkins, 1999). Before NF-κB binds to its promotor regions on DNA, it is acetylated by CRB/p300 which maintains its presence in the nucleus. p53 and Cyclin E-CDK2 compete for the finite CRB/p300 complexes and prevent its interaction with NF-κB. It should be noted however that the classic antagonism between the pro-apoptotic p53 and the anti-apoptotic NF-κB has been the subject of debate after p53 was found to activate NF-κB and correlate with the ability of p53 to induce apoptosis (Ryan et al., 2000).

6.4.7. Further Genetic Manipulation of Proteins Involved in the Activation Cascade

It has been possible to block the activation of NF-κB by manipulating the genes that encode proteins (e.g. TRAF2, TRAF6, I-TRAF, NIK, MEKK1, and IKK) found directly in the known activation pathways (Epinat & Gilmore, 1999). TRAF2 (TNF receptor associated factor) and TRAF6 interact with TNF receptors and serve as adapters for the activation of NF-κB. Dominant negative mutants of TRAF2 and TRAF6 have been shown to repress NF-κB activity in response to TNF and IL-1, respectively (Cao et al., 1999; Hsu et al., 1996). I-TRAF interacts with TRAF2, and its overexpression inhibits TRAF2 activation of NF-κB (Rothe et al., 1996). NIK (NF-κB inducing factor) is induced by several proteins in the activation pathway, including TRAF2 and TRAF6, and activates IKKα (as does MEKK1). Therefore, it has been shown that a dominant negative mutant of NIK (and MEKK1) represses NF-κB activation (Jang et al., 1998; Song et al., 1997). The IKKs (IκB kinases, α and β) phosphorylate IκBα to subsequently cause its ubiquitination and ultimate degradation. Thus, alterations in the ATP-binding site of the IKK complex (IKKβ more so than IKKα) or its activation loop have been shown to block the activation of NF-κB as well (Ling et al., 1998; Regnier et al., 1997).

6.4.8. Antioxidants

Various antioxidants have also been shown to inhibit NF-κB activation in response to a variety of stimulants (i.e. TNF, IL-1, phorbol ester, LPS, UV) through diverse and largely unknown mechanisms (Garg & Aggarwal, 2002; Li & Karin, 1999; Pahl, 1999). Examples include disulfiram (Schreck et al., 1992), curcumin (Singh & Aggarwal, 1995a), glutathione (Natarajan et al., 1996), and vitamin C (Staal et al., 1993).

7. PHYSIOLOGIC CONSEQUENCE OF NF-κB INHIBITION AND FUTURE DIRECTION

The inhibition of NF-κB with the methods illustrated above represents a theoretical approach to the more complicated issue of creating drug therapies that are effective in preventing or attenuating tumorigenesis. So far several agents have been shown to utilize the modulation and/or inhibition of NF-κB to carry out some part of their therapeutic purpose such as glucocorticoids, nonsteroidal anti-inflammatory agents (NSAID), vitamin E, curcumin, thiols, cyclosporin, rifampicin, dithiocarbamates, methotrexate, thalidomide, leflunomide and various fungal and bacterial metabolites (Fig. 6) (Baeuerle & Baichwal, 1997). It should be noted however that many of these drugs influence the NF-κB pathway among others, and the interactions between these different pathways in vivo likely play a role in their characteristic effects.

While we show that NF-κB is potentially capable of causing much distress, the elements in its pathways that we have targeted for inhibition above (e.g. ubiquitin ligase inhibitors, proteasome inhibitors, IκBα super-repressors, etc.) may be valuable parts for other pathways that are required for the normal functioning of our body. For example, the turnover of the outer layers of our skin requires the function of a specific ubiquitin ligase

complex, SCFβ-TrCP, which is also part of the traditional activation cascade for NF-κB (Maniatis, 1999). Ironically, the consequences of its inhibition include oncogenesis. Furthermore, the complete inhibition of NF-κB via genetic knockout may produce severe toxicity as evidenced from mice studies using dominant negative Rel A among others (Fig. 3) (Beg et al., 1995b). These Rel A deficient mice die during embryogenesis due to pathologic apoptosis of the liver. Other gene knockouts that involve the NF-κB complex and its inhibitors have shown us that its complete deficiency likely results in severe immunodeficiencies and accelerated apoptosis.

It is clear that for effective drug development, targeting NF-κB must focus on its partial and specific inhibition with respect to toxicity. This level of specificity is achievable given our current progress, as in the example of glucocorticoids (Auphan et al., 1995), which targets the inhibition of NF-κB in lymphoid cells making it the most effective, although not ideal anti-asthmatic therapy.

8. CONCLUSION

In recent years there have been major developments in our understanding of how NF-κB is activated and also how it may contribute to the development of cancer. Activation of NF-κB is emerging as one of the major mechanisms of tumor cell resistance to cytokines and chemotherapeutic agents. Until now most of our knowledge about NF-κB and its role in cellular physiology is based on in vitro experimentation. Future in vivo studies may demonstrate the true importance of NF-κB and allow that knowledge to be used in clinical medicine. The development of specific inhibitors that can block NF-κB activation may have great potential in improving cancer therapy.

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MONITORING OF PROGRAMMED CELL DEATH *IN VIVO* AND *IN VITRO*, –NEW AND OLD METHODS OF CANCER THERAPY ASSESSMENT

Chapter XV

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1. INTRODUCTION

Cells can die either by apoptosis, necrosis or by a form of cell death that displays morphologic characteristics of both forms (Los et al., 2002; Proskuryakov et al., 2002; Sreedhar & Csermely, 2004). Furthermore, both necrosis (where the cell membrane loses its integrity and the cell contents are released causing an inflammatory response) and apoptosis (where the cell content remains "well-packed" in the apoptotic bodies and inflammation does not occur): (*i*) can be caused by related (patho)-physiological stimuli; (*ii*) can be partially prevented by anti-apoptotic mechanisms; and (*iii*) can shift from one form to the other (Los et al., 2002). Thus, depending on which form of cell death prevails, an appropriate detection method should be chosen that accurately detects and quantifies the process. Furthermore, some methods will detect only one form of cell death, whereas other methods are not able to distinguish between different forms. Anticancer drugs may not necessarily induce classical apoptosis in cancer cells. In addition, some components of the apoptotic machinery may participate in processes

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unrelated to apoptosis, such as immune response or cell activation (Boissonnas et al., 2002; Kreuter et al., 2004; Los et al., 2001). In this chapter, we provide a short introduction concerning programmed forms of cell death, followed by description of methods that detect and quantify processes that are an integral part or result of the dying process.

Apoptosis is an energy-dependent, ubiquitously observed physiological process genetically controlled by the expression of evolutionarily conserved genes. These genes either mediate or suppress the process. Apoptosis is well characterized by distinct morphological and physiological changes. The morphological changes include nuclear condensation, cell shrinkage, and membrane blebbing. The physiological changes are fragmentation of nuclear DNA due to activation of specific endonucleases, cleaving nuclear DNA into 180–200 oligonucleosomal fragments and activation of caspases, resulting in partially digested proteolytic protein products (Lawen, 2003; Shivapurkar et al., 2003). Apoptosis is a highly regulated process that mainly responds to the initial stimulus followed by a cascade of events. Hence, it can be divided into 3 phases: (a) the initiation phase (or signaling phase), which involves the activation of surface death receptors (mainly the tumor necrosis factor family members, so called extrinsic pathway), or the initiation of apoptosis by the mitochondrial (intrinsic) pathway or other signaling cascades by a selected stimuli (e.g. those affecting the endoplasmic reticulum); (b) the signal transduction phase (or preparation phase), where activation of initiator caspases and certain kinases/phosphatases takes place; followed by (c) the execution phase (or death phase), which involves the activation of effector caspases (Los et al., 1999). Apoptosis detection and quantifying approaches usually detect events within the signal transduction phase or the execution phase of cell death. Since death receptor dependent death pathways typically activate, at least to some extent, the mitochondrial death pathway via processing of Bid molecule by caspase-8 (Sadowski-Debbing et al., 2002), mitochondria may actively participate in some forms of necrosis (Los et al., 2002), so we will provide some basic information about the physiologic changes that occur in the mitochondria of dying cells.

1.1. Mitochondrial Death Pathway

Although initial studies from the early 1990s suggested that organelles like mitochondria, Endoplasmic reticulum (ER), and lysosomes do not play a major role during apoptosis, it was found later that mitochondria are the central coordinators of apoptotic events (Fleischer et al., 2003; Sadowski-Debbing et al., 2002). Currently, the majority of anticancer strategies used to induce changes in cell physiology lead to the activation of the intrinsic, mitochondrial death pathway (Los et al., 2003). Furthermore, in addition to anticancer drugs, many other stimuli converge on the mitochondria to induce mitochondrial membrane permeabilization, cytochrome *c* release and activation of apoptotic-proteolytic cascades (Fig. 1). There are several competing models to explain the release of cytochrome *c* from the mitochondrial intermembrane space. Some models predict the permeabilization of the outer mitochondrial membrane as a result of the opening of the mega-channel, called the permeability transition pore complex (PTP). The adenine nucleotide translocator and the voltage-dependent anion channel in the outer membrane are the major components of the PTP and are responsible for the loss of in

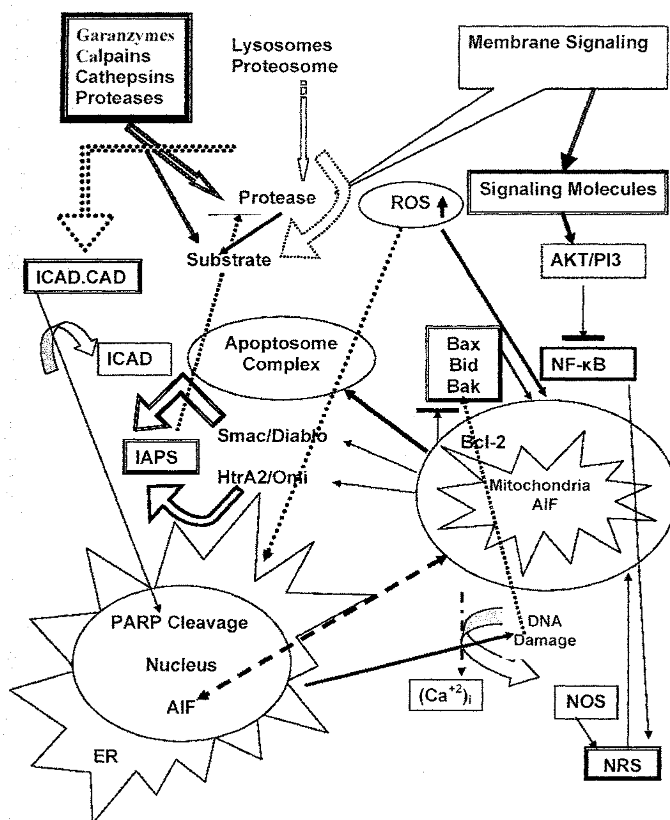

Figure 1. The "apoptotic soup" - major pathways of mitochondrial apoptosis. Many proapoptotic and signal transduction pathways converge on mitochondria to induce mitochondrial membrane permeabilization. The PTP induces the mitochondrial translocation and multimerization of the proapoptotic protein, Bax. Bax, in turn, helps the permeabilization of the inner mitochondrial membrane resulting in the leakage of cytochrome *c* and other mitochondrial inter-membrane proteins that give a "go" signal for the execution phase of apoptosis. The second mitochondria-derived activator of caspases (Smac/DIABLO) is a mitochondrial protein that inhibits the IAP (such as XIAP, c-IAP1, and c-IAP2) after its release to the cytosol. IAP are known to block the processing of the effector caspases, caspase-3 and -9. Apart from Smac/DIABLO, there is another mitochondrial protein, HtrA2/Omi, that also inhibits IAP. The release of cytochrome *c* from mitochondria drives the assembly of the high molecular weight caspase-activating complex called apoptosome. The apoptosome contains oligomerized Apaf-1, which in presence of (d)ATP and caspase-9, recruits and helps the autoactivating cleavage of caspase-3, an executioner of apoptosis. On the figure, several elements of ROS-induced apoptosis are also included, such as the formation of the highly toxic nitrogen reactive species (NRS). Finally, in the executioner phase of apoptosis, endonucleases, such as CAD, are activated (see: Sadowski-Debbing et al., 2002; Sreedhar & Csermely, 2004, for more details).

mitochondrial membrane potential (Renz et al., 2001; Sreedhar & Csermely, 2004). According to a different model, the PTP-opening induces the mitochondrial translocation and multimerization of the proapoptotic protein Bax (Bcl-2-associated X protein). Bax, at least in cell-free systems, can form channels, thus it may facilitate the leakage of cytochrome *c* and other mitochondrial intermembrane proteins that regulate apoptotic

processes (Fleischer et al., 2003; Vande Velde et al., 2000). Models still exist that propose simple osmotic rupture of the outer mitochondrial membrane caused by intense influx of water. None of the models fully explain all the events observed in the course of cell death with mitochondrial proteins. The release of cytochrome *c* from the mitochondria drives the assembly of the high molecular weight caspase activating complex called apoptosome. The apoptosome contains oligomerized Apaf-1, which in the presence of (d)ATP and caspase-9, recruits and helps the autoactivating cleavage of caspase-3, an executioner of apoptosis (Acehan et al., 2002), (see chapters V and VI for more details). Recently, it has been shown that cytochrome *c* may leave the apoptotic cell (Renz et al., 2001) and may be implicated in the bystander killing of other cells, at least in the brain (Ahlemeyer et al., 2002). The extracellular release of cytochrome *c* has led to the development of a new method that monitors the burden of apoptosis *in vivo* (see below).

Although most research that focuses on the mitochondrial/intrinsic death pathways investigates the release of cytochrome *c*, mitochondria are the source of several other molecules capable of modulating the death process. The second mitochondria-derived activator of caspases (Smac/DIABLO) is a mitochondrial protein that inhibits the "inhibitors of apoptosis proteins" (IAPs, such as XIAP, c-IAP1, and c-IAP2) after its release to the cytosol. IAPs are known to block the processing of the effector caspases, caspase-3 and -9 (Cassens et al., 2003). Apart from Smac/DIABLO, there is another mitochondrial protein, HtrA2/Omi, that also inhibits IAP (Festjens et al., 2004; Los et al., 2003). At least in some experimental models, mitochondria have been implicated in the release of ROS that lead to DNA damage and subsequent necrosis (Los et al., 2002).

1.2. Apoptosis in Tumor Cells

When Bcl-2 was discovered as an oncogene involved in the promotion of cell survival, it was thought that anti-apoptotic genetic lesions are necessary for tumors to arise (Philchenkov et al., 2004; Sreedhar & Csermely, 2004). This hypothesis has been supported by murine transgenic models and *in vitro*-cell culture experiments. Thus, acquired resistance to apoptosis is a hallmark of most, if not all, types of cancers. Although tumor cells are resistant to apoptosis, they are not completely devoid of death. Local environmental conditions that differ significantly from those in healthy tissues, play an important role in shaping the mode and biochemistry of cell death in the tumor. Cell death in tumor cells is frequently associated with cellular senescence and may involve caspase-independent routes of apoptosis or necrosis. A tumor cell may escape from caspase-mediated apoptosis either by overexpressing antiapoptotic proteins or by debilitating mutations in proapoptotic factors. For example, the antiapoptotic Bcl-2 is overexpressed in many tumors (Philchenkov et al., 2004; Sreedhar & Csermely, 2004). In the case of Hodgkin's Disease, mutations of the Fas receptor were found (Philchenkov et al., 2004; Sreedhar & Csermely, 2004). Caspase-8 is also frequently mutated in neuroblastoma, a childhood tumor of the peripheral nervous system (Philchenkov et al., 2004; Sreedhar & Csermely, 2004). Besides changes in caspase-dependent apoptotic pathways, tumor cells also undergo changes to prevent caspase-independent apoptosis. For example, the IAP family member, survivin, is also highly expressed in many tumors. Some authors associate survivin expression with poor prognosis (Yamamoto et al., 2002).

Since the central parts of tumors are frequently hypoxic, some tumors mutate a BNIP3 molecule. BNIP3 is a proapoptotic Bcl-2 family member that is induced under hypoxic conditions (S.B. Gibson, pers. communication). This mechanism may allow cell to evade apoptotic mechanisms under hypoxic conditions.

2. SELECTED METHODS OF APOPTOTIC CELL DEATH DETECTION

While the word "apoptosis" was coined in the early 1970s, most methods for precise detection of apoptosis were developed in late 1980's or 1990's. We will describe selected methods that we frequently use in our lab. Some of them will specifically detect apoptosis, whereas others will just detect cell viability or cell death. The readership is invited to modify/adjust these protocols to their specific experimental conditions.

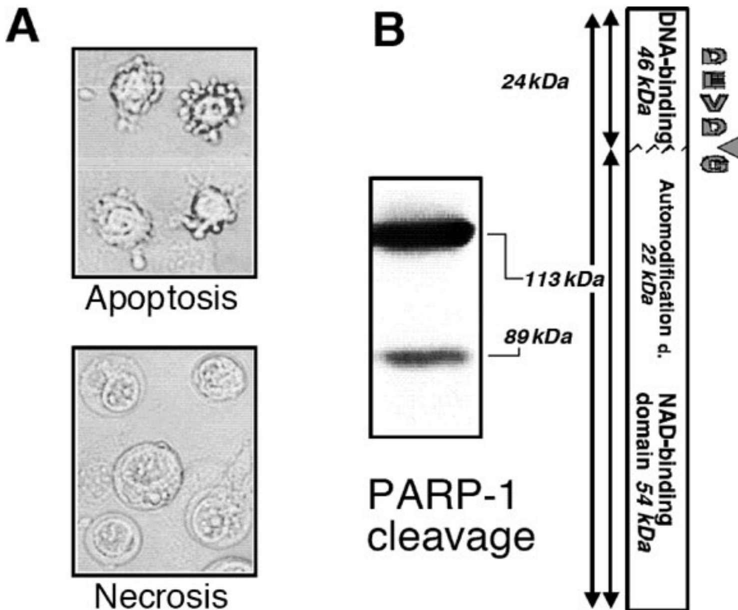

Figure 2. Morphologic and biochemical features of apoptosis and necrosis. (A) Morphologic features of apoptosis (top) and necrosis (bottom). Please note that apoptotic cells are smaller and more condensed, cell membrane blebbing with single apoptotic bodies separating is clearly visible by some cells. Necrotic cells in contrast are round, and bigger, and sometimes present ring-like forms with the nucleus pushed to the periphery. Such morphologic shapes of cells can be achieved by treatment with a mild detergent. Here L292 murine fibrosarcoma adherent cells were treated with anti-CD95 (Apo-1/Fas) antibodies to induce apoptosis, and with the combination of TNF and caspase inhibitor z-VAD-fmk to induce necrosis. (**B**) Western blot detection of PARP-1 cleavage (left) and schematic representation of the cleavage point within the PARP-1 molecule (right). The cleaved peptide sequence DEVD^E is also indicated. PARP-1 is a substrate of caspase-3, -7 and -9, and thus its cleavage is often used as an indirect indicator of activity of these caspases during cell death.

2.1. Morphologic Assessment of Apoptotic Cells, (Apoptotic Index)

A wide variety of techniques have now been utilized to assess apoptotic cells in tissue sections (Stadelmann & Lassmann, 2000). Electron microscopy is the most precise technique to identify apoptotic cells, but this is not practical in pathological specimens. Thus, the majority of studies have instead counted apoptotic cells using light microscopy. This technique is not straightforward and there is some subjectivity in the recognition of apoptotic cells. They are usually detected as having a condensed, shrunken cytoplasm and prominent nuclear changes including homogeneously condensed chromatin around the nuclear membrane or as solid masses (Fig. 2A). Usually they are documented as a small number of individual cells surrounded by surviving tumor cells, but detached from them in a vacuolar structure. Problems arise in areas close to necrosis where debris can be mistakenly identified as an apoptotic body. Apoptotic cells in the stroma are also problematical as they may have a lymphocyte origin. **The apoptotic index (AI) is typically reported as a ratio of apoptotic cells/100 non-apoptotic (tumor) cells, per unit area, per grid, per field or as a grade.** The apoptotic index is often a small number and thus a significant number of cells need to be counted to minimize errors and take into account heterogeneity. Most reports count ten high magnification microscopy fields. Therefore, the majority of methods target various components of the apoptotic pathway to judge the effectiveness of anticancer therapy. In the following sections other methods for the detection of caspase activation or activity have been described.

2.2. Detection of Caspase Activation by Western Blot

Western blotting is preferred when detecting the expression level and the degree of proteolytic activation of pro-caspases. It works well only if a sufficient amount of biologic material is available (at least 10^6 cells per sample). Either the increase of a catalytic subunit and/or the decrease of the pro-enzyme are monitored. Many companies offer poly- and monoclonal isoenzyme-specific antibodies that recognize either the pro-enzyme form, a catalytic subunit, or both. Special attention to be paid to the appearance and detection of intermediate processed caspase forms that may remain inactive, but may have comparable size to the activated forms of caspases (e.g. an inactive, 19-20 kDa cleavage fragment of caspase-3 generated by other proteases, may be mistaken for the active, p17 subunit of the caspases). In addition, caspase activity can be monitored by the detection of cleavage products of various caspase substrates. By far the most popular indicator of caspase activity (specific for caspase-3, -7 and -9) is the cleavage of poly(ADP-ribose) polymerase-1 (PARP-1), (Fig. 2B), (Los et al., 2002), but principle cleavage products of any protein known to be a caspase substrate can be used to monitor the activity of certain caspase family members. Anti-caspase antibodies can be obtained from the following companies: Alexis, Biocat, Calbiochem, Pharmingen, Promega, Roche, Transduction Laboratory, and Upstate Biotechnology. Anti-PARP-1 antibodies, which specifically recognize the active p89 fragment, are available from Calbiochem and Promega. To quickly identify suppliers of necessary antibodies, or other reagents, www.google.com, or another internet search engine is recommended. Usually specific information about Western blot conditions is included with the shipment, and the standard protocol given below should be modified accordingly. For quick identification

of caspases involved in a pathway, they can be found in pathway databases, like e.g. www.ncgr.org/pathdb/ or www.bind.ca/cgi-bin/bind/dataman.

2.2.1. Materials and Equipment

- Isotonic lysis buffer: 50mM HEPES (pH 7.6), 150mM NaCl, 1mM EDTA, 1% NP40 (Roche, #1754599), 20μl/ml protease inhibitor coctail (Roche, #:1836153). The protease inhibitor cocktail from Roche can be replaced by the mixture of: 3mg/ml aprotinin, 3mg/ml leupeptin, 3mg/ml pepstatin, and 2mM phenylmethylsulfonyl fluoride (PMSF).
- Alternative hypertonice lysis buffer *(works well with less stable proteins, but sometimes gives less sharp bands):* 20mM HEPES (pH 7.9), 0.5mM EDTA, 0.1mM EGTA, 350mM NaCl, , 0.5mM DTT, 20 % glycerol, 20μl/ml protease inhibitor cocktail (Roche, #:1836153).
- Bradford Reagent, (BioRad, #:500-0006).
- Nitrocellulose membranes (e.g. Amersham-Pharmacia Biot.) or polyvinylidenedi-fluoride (PVDF) membranes (e.g. Amerscham-Pharmacia Biotech, #:RPN303F)
- 10—20% polyacrylamide gradient gels (e.g. Sigma).
- SDS-PAGE electrophoresis apparatus and power supply (e.g. Biorad or Höfer).
- 5x Tris-glycine running buffer: 25mM Tris, 250mM glycine, 0.1 % SDS, pH 8.3.
- Semi dry blotter (Biorad)
- Blotting buffer: 39mM glycine, 48mM Tris base, 0.037% SDS, 20% methanol
- 2x SDS loading dye: 100mM Tris-HCl (pH 6.8), 4% SDS, 0.2% bromophenolblue, 20% glycerol, 200mM β-mercaptoethanol.
- Horseradish peroxidase (HRP)-conjugated anti-Ig antibody.
- 10 x TBS: 250Tris-HCl (pH 7.4), 1.5M NaCl.
- Blocking buffer: 1x TBS, 5% (w/v) skim milk (from Merck, *non-fat dry milk from a local grocery store may sometimes produce equal or better results for a fraction of the cost, this option should therefore also be tested*).
- 1x TBST: 1x TBS, 0.05% Tween 20.
- Enhanced chemiluminescence Western blotdetection system (Amersham-Pharmacia Biotech, #: RPN 2209)
- Autoradiography films (Amersham-Pharmacia Biotech) and cassette or appropriate phosphorimage cassestte and a Phosphorimage reader; our group uses "Storm 860" from Molecular Dynamics.

2.2.2. Methodology

There are many more or less similar Western blot methods described that may work for the detection of caspses (for other examples of protocols please see (Burek et al., 2003; Stroh et al., 2002). We give an example of the protocol that we use. Special attention should be given to the antibody suppliers recommendations.

- 10^7 cells (10^6 cells/ml) are washed two times with 1x phosphate-buffered saline (PBS) and then resuspended in 40-100μl lysis buffer.

- The protein concentration is determined by the Bradford-Protein Assay kit according to the protocol of the manufacturer.
- Mix protein samples (50μg per lane) with 2x SDS loading dye (or 20-25μg per lane if "mini-gel" system is being used).
- Heat the samples at 95°C for 2min to denature by SDS.
- For Western analysis 50μg of protein are separated on 10-20% polyacrylamide gels depending on the size of the protein to be analyzed. Gradient gels are recommended if protein fragments of significantly different length are expected (e.g. for PARP-1, the signal is detected at ~113 kDa, proteolysis fragments: ~89kDa and sometimes also ~25kDa).
- Proteins are then blotted onto nitrocellulose or PVDF membranes according to standard protocols (100V for 1h or 30V overnight).
- Membranes are then incubated in 10-20ml blocking buffer (membrane must be covered with solution). Gently agitate for 1h at RT (or at 4°C overnight) using a rocker platform.
- Wash the membrane two times in 20ml TBST for 5min with gentle agitation.
- Incubate the membrane in 10-20ml blocking buffer containing the first antibody. The monoclonal or polyclonal anti-caspase antibodies are diluted according to the manufacturer's instructions. Incubate with gentle agitation for 1h to overnight. If the incubation is done overnight, we recommend to be performed at 4°C.
- Wash the membrane two times in 20ml TBST for 5min with gentle agitation.
- Incubate the membrane in 10-20ml blocking buffer containing the secondary antibody. Secondary antibodies are usually anti-mouse or anti-rabbit IgG_1 coupled to horseradish peroxidase at a dilution of 1:5000. Incubate with gentle agitation for 1h.

IMPORTANT: NaN_3 will inhibit Horseradish peroxidase. It is frequently used for conservation of antibodies, so it cannot be added to horseradish peroxidase-coupled antibodies. However, it can be used to conserve the primary antibodies, since it will later be washed away.

- Wash the membrane two times in 20ml TBST and one time in 20ml TBS for 5min, respectively, with gentle agitation.
- Antibody reactive bands are detected by using the enhanced chemiluminescence Western detection system (Amersham-Pharmacia Biotech). Mix the detection solution 1 with solution 2 at a ratio of 1:1 (v/v). Incubate the membrane for 1min at RT and detect the produced chemiluminescent light by using appropriate films or phosphorescence equipment.
- Blotted membranes can be stripped from antibodies used for the detection of specific proteins for 5min in 0.2M NaOH at room temperature and they can be re-probed with antibodies specific for another protein. The stripping process may however slightly reduce the quality (signal) of future Western blots.

2.3. Detection of Hypodiploid Nuclei as a Hallmark of Apoptosis – "Nicoletti Method".

This method takes advantage of DNase activation and internucleosomal DNA-degradation that becomes clearly visible in advanced apoptotic processes. It is a variation of a method that was first described by Nicoletti and colleagues in the early 90s (Nicoletti et al., 1991). Although some DNA-degradation also occurs during necrosis, the method is not sensitive enough to sufficiently detect that process, and thus, it is apoptosis-specific. The cells are lysed and samples may be treated with RNase to remove residual RNA. However, there is usually enough residual RNase in the cell to remove the majority of RNA. After staining samples (to this point cells have been converted into DNA clumps) with 40-50μg/ml of Propidium Iodide (PI), the fluorescence signal is measured using a flow cytometer equipped with a laser that supplies light with λ=488nm (we use FACS Calibur, Becton-Dickinson). Cells with normal DNA-content show typical G1-M-G2 cell-cycle-specific DNA-content histograms. Cells with decreased (hypodiploid) DNA-content are considered apoptotic (Fig. 3). We normally use 1-5x 10^5 cells per sample, but experiments can be done successfully with samples of 4x 10^4 or even fewer cells. It is recommended to perform and measure the experiment in duplicate samples. This method will not work well in cells (e.g. MCF7) and tumor samples (Philchenkov et al., 2004) with mutations of caspase-3 or CAD-DNase, since both components are necessary for apoptotic DNA degradation.

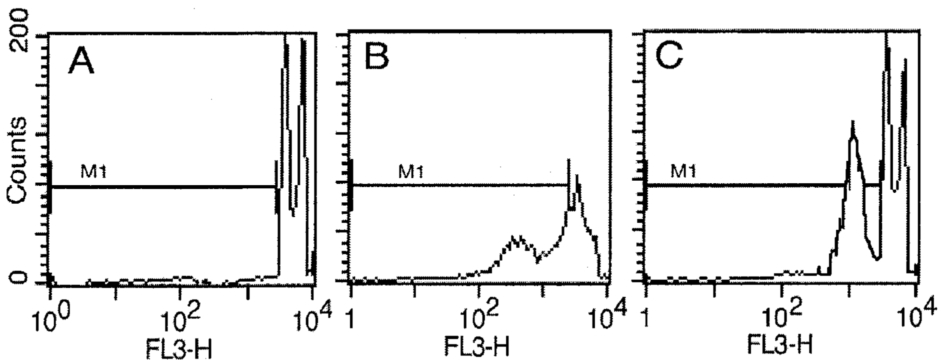

Figure 3. "Nicoletti" method - DNA histograms. (**A**) Typical control DNA-histogram. M1 (statistical marker) has been placed to mark sub-diploid DNA that is almost absent on this histogram. The diploid (G1), and tetraploid (G2) DNA is clearly visible in the form of 2 peaks in the far-right part of the histogram. (**B**, and **C**) show examples of typical apoptotic DNA-histograms. In (**B**) most cells underwent apoptosis, therefore the G1 and G2 peaks are fused together and shifted to the left and a wide sub-diploid peak of cells with advanced apoptosis is clearly visible in the middle-right part of the histogram. (**C**) shows a typical experiment with apoptosis affecting only a fraction of cells. G1 and G2 peaks are still preserved and sub-diploid peak corresponding to apoptotic cells is also clearly visible to the left from both peaks that represent normal cells, please compare to (**A**).

Staining Method A: This standard method can be used for most samples.

Reagents and solutions: Hypotonic PI-lysis buffer: 0.1% (w/v) Natruim Citrate, 0.1% (v/v) Triton-X100, 40μg/ml PI.

The procedure:

- Lyse and stain cell pellets with 0.1-0.5ml of hypotonic PI lysis buffer for ~1min.
- Transfer cells in the staining solution into flow-cytometer compatible tubes.
- Vortex shortly and incubate for 10min at room temperature.
- Vortex again, transfer on ice and measure by flow cytometry the PI emission peak wavelength λ=635nm. The signal can also be measured in a broad range between ~500-670nm, that corresponds FL2 and FL-3 channels by FACS flow-cytometers produced by Becton-Dickinson).

Staining Method B: This method is slightly more expensive and laborious. It is recommended for samples with higher intrinsic DNase activity, or if "Method A" results in high levels (over 20-25%) of apoptotic cells in control samples.

Reagents and solutions: Lysin/Fixing buffer set: Cold Acetone-Metanol 1:1 (stored at –20°C), RNase 10mg/ml in PBS, PI 2mg/ml, PBS (cold, ~4C°).

The procedure:

- Fix cells with 1ml of cold Acetone:Methanol solution in 6ml tubes compatible with the flow cytometer, for 10min on ice or overnight at –20°C.
- Wash 2-3 times with cold PBS to remove organic solvents.
- Resuspend pellets in 400μl of RNase solution and incubate at 4°C for 1h.
- Add PI to final concentration of 40μg/ml, vortex briefly and incubate on ice for 10-20min. Samples can then be measured by flow cytometry, (PI emission peak is λ=635nM, but the signal can be measured in a broad range between ~500-670nm, that corresponds to FL2 and FL-3 channels by FACS flow-cytometers produced by Becton-Dickinson).

2.4. Measurement of Cell viability by Flow Cytometry

Staining cells with 0.5-1μg/ml of PI can quickly assess cell viability. The method takes advantage of the fact that an intact cell membrane is PI-impermeable. Thus, only necrotic and late-apoptotic cells will be stained by this method. We usually use 1μg/ml of PI to stain suspension cells, whereas 0.5μg/ml of PI will stain adherent cells that were previously detached by scraping, or treatment with trypsin. PI is added directly to a cell suspension and cells are measured by flow cytometry. Living cells are PI-negative, whereas dead cells are PI-positive (stronger fluorescence signal).

If dual-laser flow cytometry equipment is available, apoptosis can be induced more precisely and measured using a modified version of the method combining PI with Hoechst33342 that is semi-permeable for apoptotic cells, but impermeable for healthy cells (Los et al., 1998). Briefly, cells are loaded with 1μg/ml of Hoechst33342 (Molecular Probes, Eugene, OR) and 0.5μg/ml of PI for 5 min at room temperature. Then cells are transferred from ice while protected from light, and measured using a dual-laser flow cytometer (e.g. FACS Vantage, Becton-Dickinson). Hoechst33342 has to be excited at 360nm, and emission is detected at 450nm. PI has to be excited at 488nm, and emission

is detected at ~610nm. The cell population that is positive for Hoechst33342 staining but still negative for PI is considered "early-apoptotic". Later, cells become positive for both PI and Hoechst dye as apoptosis advances. Fixed cells cannot be assessed by this method since their membranes are no longer intact and impermeable to these dyes.

2.5. Fluorescent Measurement of Caspase Activity in Intact Cells

The methodology is based on the fluorescence increase of $(Aspartyl)_2$Rhodamine-110 (D_2Rhodamine), (supplied by Alexis, Calbiochem, Biocat) upon removal of aspartyl groups from the substrate (Hug et al., 1999). Liberation of each NH_4- group (previously occupied by aspartate) increases the fluorescence intensity of Rhodamine-110 one hundred fold. Theoretically, the signal from cells carrying active caspases should shift four decades on the logarithmic scale. In reality, not all the substrates that entered the cell are processed and the signal is partially absorbed by cytoplasmic contents. Therefore, the increase of signal intensity is usually lower than expected (1-1.5 on a log scale). The hypo-osmotic condition used in the protocol facilitates substrate loading into cells. β-mercapto ethanol or dithiothreitol (DTT) protects the cysteine residue in the active center of the caspases from oxidation. Please note that Rhodamine-110 has a wide fluorescence spectrum, and the signal collected in the FL-2 channel is only about 20% weaker than in the FL-1 channel (which is usually used to detect the dye). Therefore, in three-channel flow cytometers only two-color fluorescence should be performed with the second signal being collected in the FL-3 channel (e.g. cy5 surface staining or 7-AAD).

One has to be aware that D_2Rhodamine cannot distinguish between different caspases. If necessary, DABCYL-xxxxxxx-EDANS -based substrates can be used where the caspase-specific sequence is placed between DABCYL and EDANS (i.e. DABCYL-DEVDAPK-EDANS) for caspase-3 or DABCYL-YVADAPK-EDANS for caspase-1. For the design of new substrates, the authors recommend previous publications (Lamkanfi et al., 2002; Los et al., 1995; Pennington & Thornberry, 1994). 7-amino-4-trifluoromethyl-coumarin (AFC) -based substrates have also been used successfully for detection of caspase activity in cell extracts (Burek et al., 2003). The appropriate kit can be obtained for example from Clontech, (Palo Alto, CA). For the best results, start the staining procedure about 1h before typical apoptotic morphology is observed.

2.5.1. Materials and Equipment

- D_2Rhodamine, (50mM); stock solution is stable for at least one month at -20°C or for at least 8 years at -70°C. A stock solution is prepared by diluting 63mg of powdered D_2Rhodamine in 1ml DMSO before 1ml of absolute ethanol is added; the stock solution is then 50mM. The dye dissolves very well in DMSO and the addition of ethanol keeps the stock liquid even at low temperatures, saving time upon stock usage. Since the dye is not very stable in H_2O, be careful not to contaminate the stock solution with it. Higher concentrated stock solutions (i.e. 100-200mM) are easily made as well. D_2Rhodamine is usually used at the final concentration of 50-60μM, with approx. 5x 10^5 cells in 1ml.

- ß-mercapto ethanol (100mM); Dissolve 347µl of absolute ß-mercapto ethanol in 50ml of PBS. The solution is stable at 4°C for at least three months, protect from light.
- PBS; Dissolve in 900ml H_2O:
  8g of NaCl
  0.2g of KCl
  1.44g, Na_2HPO_4
  0.24g of KH_2PO_4
- Adjust pH to 7.3, and the volume to 1 L, store at 4°C (you will need it cold).
- dH_2O; deionised H_2O, store at RT.
- FACS® Brand Lysing Solution; (Becton Dickinson, San Jose, CA)
- Flow cytometer (e.g. FACS-Calibur, Becton Dickinson) equipped with blue-light laser (488nm) and compatible plastic-ware, (e.g. FALCON #2058).
- Centrifuge compatible with plastic-ware for flow cytometer.
- Pipetting equipment.

2.5.2. Methodology

2.5.2.1 Detection of caspase activity in isolated PBMC and other cells and cell lines

- Dilute an aliquot of D_2Rhodamine to 1mM with H_2O.
- Prepare 10^5-10^6 cells in 420µl of culture medium. To discriminate between effector and target cells: (*i*) FSC/SSC criteria can be used (which is quite difficult and not very precise); (*ii*) if cells differ significantly in their DNA content, PI or 7-amino-actinomycin-D (7-AAD) staining can be used; (*iii*) pre-staining with antibodies against a specific surface marker for one of two cell populations can be used if it is sufficiently stable (please note that dead cells may stain non-specifically, so target cells should be stained). It is recommended to stain duplicate samples and to calculate the mean of two probes for the same sample. When staining adherent cells, it is better to pre-stain them for caspase activity in a twelve-well-plate or a Petri dish, then scrape them off and transfer to FACS-compatible tubes. Staining of adherent cells is often less successful since detaching may cause spontaneous activation of caspases due to accompanying damage.
- Add ß-mercapto-ethanol to a final concentration of 10mM (50µl of 100mM stock sol.).
- Add D_2Rhodamine to a final concentration of 60µM (30µl of 1mM stock sol.).
- Add 750µL of dH_2O (osmotic loading), (alternatively 1-2ml of FACS® Brand Lysing Solution can be used).
- Incubate 8-10min, 37°C.
- Stop the reaction by pipetting 3ml of cold PBS (≤4°C). Keep cells on ice and proceed with additional staining steps e.g. for detection of specific cell subsets if desired.
- Measure samples by flow cytometry (e.g. use FL-1 channel when FACS-calibur is employed).

IMPORTANT: The samples have to be measured within three hours.

2.5.2.2 Detection of caspase activity in PBMC from whole blood

- Prepare a 1mM solution of D_2Rhodamine in H_2O from the stock solution.
- Take 200µl of whole blood (heparinized). The FSC/SSC signal will change slightly due to osmotic loading. For identification of specific blood cell sub-populations, it is advisable to pre-stain a given cell population with antibodies against a specific surface marker. Usually, we used 200µl of blood per sample, but staining is also possible with 50µl of blood. One can then either reduce the amount of used reagents or dilute the whole sample with PBS (room temp.) and follow the above protocol.
- Add ß-mercapto-ethanol for a final concentration of 10mM (20µl of 100mM stock sol.).
- Add D2Rhodamine for a final concentration of 150µM (30µl of 1mM stock sol.).
- Add an appropriate amount of mAb if desired, and then 2ml of FACS® Brand Lysing Solution, (the staining works also with 1ml of the lysing solution, but a significant amount of erytrocytes will not be lysed).
- Incubate 8-10min 37°C.
- Stop the reaction by pipetting 2ml of cold PBS (≤4°C), (smaller amount of cold PBS, i.e. 0.5ml or 1ml can be added if this simplifies the following staining procedures). From now on cells have to be stored on ice.
- Perform the additional individual staining procedures (if desired).
- At the end of staining, resuspend blood cells in about 50µl of cold PBS and measure samples by flow cytometry (i.e. FL-1 channel when FACS Calibur is employed).

IMPORTANT: The samples have to be measured within three hours.
Inconsistency in your results can often be caused by carry-over of the dye on the external wall of the pipette tip.

2.5.2.3 Detection of caspase activity with D2R in adherent cell lines

- Approximately 90% of confluent adherent cells in six well plates are washed once with 1ml 1x PBS and detached with 30µl Trypsin-EDTA (Biochrome).
- Cells are resuspended in 1ml of RPMI which is supplemented with 10% FCS and centrifuged.
- The cell pellet is then resuspended in 1 ml of caspase-dye-solution (1x PBS, 10mM β-mercapto-ethanol, 25µM D_2Rhodamine).
- Cells are incubated for 10min at 37°C.
- After adding 5ml of 1x PBS, the cells are centrifuged. The supernatant is removed, but 200µl are left for the pellet to be resuspended in for flow cytometric analysis.
- The cellular Rhodamine-110 green fluorescence (λ=515-545nm) is measured upon excitation by a 488nm argon laser on a FACScan flow cytometer in channel 1 (Becton Dickinson), (Hug et al., 1999). The fluorescence intensities of Rhodamine-110 measured in channel 1 and channel 2 are similar. A minimum of 10000 events per sample are acquired, stored in listmode files and

subsequently analyzed with Cellquest® software (Becton Dickinson). Fluorescence can also be detected by confocal laser scanning microscopy.

2.6. Detection of Caspase Activity in Cell Extracts

A number of caspase substrates have been successfully used for detecting caspase activity in cellular extracts. Contrary to the detection of caspase activity in intact cells, fluorescent substrates used for measurement of caspase activity in cellular extracts do not need to be cell-permeable. This allows application of bigger-, peptide based-, caspase-sub-family-specific substrates like YVAD-AMC [N-acetyl-Tyr-Val-Ala-Asp-aminomethyl-coumarin] (Caspase-1), VDVAD-AMC (Caspase-2), DEVD-AMC (Caspase-3), VEID-AMC (Caspase-6), IETD-AMC (Caspase-8), etc., as well as substrates based on other dyes, e.g. "-pNA" based caspase substrates (Ghavami et al., 2004). Unlike Western blot based detection of caspases, the data obtained by utilizing fluorometric- or colorymetric assays should be interpreted cautiously. Most, if not all of these substrates are not completely specific for a single caspase, but at best they preferentially detect active members of a given caspase sub-family.

2.6.1. Materials and equipment

- CASPASE FLUORESCENT SUBSTRATES:
  DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-aminomethyl-coumarin) and other AMC-based substrates were obtained from Bachem, a 66mM stock solution was prepared in DMSO.
  D_2Rhodamine, 50mM of stock solution was prepared in DMSO, prior to measurement 1mM aliquots of D_2Rhodamine were prepared by diluting the stock solution with H_2O.
- LYSIS BUFFER:
  50mM HEPES pH 7.3
  1% Triton X-100
  10mM DTT
  The buffer without DTT can be stored at RT, DTT should be added directly before use.
- CASPASE BUFFER:
  50mM HEPES
  100mM NaCl
  0.1% CHAPS (3-[cyclohexylamino]-1-propanesulfonic acid),
  10mM DTT
  10% Sucrose
  Prepare freshly before use.
- Bench-top centrifuge.
- Spectrofluorometer, (the authors used spectrofluorometer GEMINI-SX (Molecular Devices).
- Water bath.

Cytosolic cell extracts were prepared by lysing cells in a buffer containing 0.5% NP40, 20mM HEPES pH 7.4, 84mM KCl, 10mM $MgCl_2$, 0.2mM EDTA, 0.2mM EGTA, 1mM DTT, 5μg/ml aprotinin, 1μg/ml leupeptin, 1μg/ml pepstatin and 1mM PMSF. Caspase activity was determined by incubation of cell lysates with 50μM of the fluorogenic substrate DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin, Bachem, Heidelberg, Germany) in 200μl of buffer containing 50mM HEPES with pH 7.3, 100mM NaCl, 10% sucrose, 0.1% CHAPS and 10mM DTT. The release of aminomethyl-coumarin was measured by fluorometry using an excitation wavelength of 360nm and an emission wavelength of 475nm.

2.6.2. Methodology

- Prepare ~10^6 cells per sample. Perform desired experimental procedures.
- At the end of an experiment pellet cells in eppendorf tubes (ETs) by centrifuging at 1000 rpm, 4°C, and for 10min in a bench-top centrifuge.
- Discard supernatant, dry ETs briefly by inverting on paper towel (if necessary remove the rest of liquid with a pipette or a piece of Whatman paper).
- Lyse cells in 100μl of lysis buffer for 10min on ice, vortex samples vigorously about every two minutes.
- Centrifuge extracts at 12000*g* for 20min at 4°C and transfer supernatant into fresh ETs. Measure protein concentration (e.g. by BioRad protein assay according to the manufacturer's instruction), and adjust samples to the same concentration with the lysis buffer. Transfer 30μl of extract into fresh ET and dilute it 1:10 with caspase assay buffer.
- Add the dye (D_2Rhodamine to a final concentration of 10μM-3μl of 1mM solution; or the DEVD-AMC to final concentration of 50μM) and incubate for 10-20min at 30°C.
- Meanwhile switch on the spectrofluorometer (the lamp usually needs 5-10min to warm-up).
- Terminate the reaction by placing the tubes on ice.
- Transfer probes into 500μl quartz cuvettes (glass cuvettes can be used if excitation wavelength is 488nm or longer), and measure in spectrofluorometer (for D_2Rhodamine excitation: 488nm; emission: 550nm, for DEVD-AMC excitation: 360nm, emission: 475nm). If 1ml cuvettes need to be used, samples can be diluted with 300μl of cold H_2O or PBS (600μl of total volume is usually sufficient to fill 1ml cuvette).

Blank cuvettes should be filled with 30μl of lysis buffer, 270μl of caspase buffer, and 3μl of 1mM D_2Rhodamine, or 50μM of DEVD-AMC respectively (do not forget to dilute the blank in case the experimental samples are diluted to a total volume of 600μl). Some cell lines express large quantities of caspases, which can get activated partially during lysis. If the signal from experimental samples is very high compared to the blank cuvette, but there is no difference between control and samples induced with dye, it is recommended to, (*i*) shorten the incubation step at 30°C to 10 min, (*ii*) repeat the entire experiment by using ~2x 10^5 cells, (*iii*) harvest cells earlier.

2.7. Detection of Cytochrome *c* in Supernatants of Apoptotic Cell

Cytochrome *c* is released not only from the mitochondria of apoptotic cells, but can also leave the cell and serve as an apoptosis-specific marker in extracellular medium or blood plasma (Ahlemeyer et al., 2002; Renz et al., 2001). We have been detecting cytochrome *c* either by immunoprecipitation or by cytochrome *c* specific ELISA.

2.7.1. Detection of Cytochrome c by Immunoprecipitation

Standard Western blot equipment and software capable of a densitometric evaluation of signal intensity are required to perform this assay. In order to determine the cellular cytochrome *c* content, cells were collected by centrifugation, washed in cold PBS, and extracted in cold lysis buffer (50mM Tris, pH 7.4, 150mM NaCl, 1% Triton X-100 containing 3mg/ml aprotinin, 3mg/ml leupeptin, 3mg/ml pepstatin, and 2mM phenylmethylsulfonyl fluoride). Extracellular fractions and cell lysates were pre-cleared of particulated elements by centrifugation at 10000*g* and 4°C for 15min prior to immunoprecipitation. Supernatants were kept at -70°C until use. Immunoprecipitations were used in a volume of 4ml in a rotator at 4°C for 4h using anti-cytochrome *c* mAb 6H2.B4 (Pharmingen) at a final concentration of 0.5mg/ml. The cytochrome *c*–mAb complexes were precipitated for 1h with 40µl of a 50% slurry of protein G-Sepharose in PBS. Precipitates were harvested by short centrifugation (2000 rpm, 10s, 4°C) and washed 4 times with cold washing buffer (20mM HEPES, pH 7.4, 150mM NaCl, 10% glycerol, 0.1% Triton X-100, 1mg/ml aprotinin, 1mg/ml leupeptin). Proteins were eluted by boiling the precipitates in SDS-loading buffer containing β-mercaptoethanol, by separating under reducing conditions on a 12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferring to a polyvinylidene difluoride (PVDF) membrane (Amersham). The detection of cytochrome *c* was performed using anti-cytochrome *c* mouse mAb 7H8.2C12 (Pharmingen). See above Western blot procedure for details. The quantity of immunoprecipitated cytochrome *c* was determined by densitometric analysis of cytochrome *c*-specific signals. Cytochrome *c* standards (10, 50 and 100ng) were loaded on each Western blot gel in order to provide references for densitometry.

2.7.2. Cytochrome c ELISA

ELISA reader and multi-channel pipettes are required to perform this method. The above described immunoprecipitation method is simple, sensitive, but quite laborious. Therefore, recently we adapted the marked cytochrome *c* ELISA ("module set", Bender MedSystems) so that it can be used to detect the protein in various specimens (e.g. in serum and cell culture supernatants). To increase sensitivity and reliability we exchanged the primary coating antibody. Plates were coated with anti-cytochrome *c* antibody (Pharmingen, San Diego, CA) and diluted in PBS (final concentration: 2µg/ml). This modification increased the sensitivity up to 40pg/ml of serum. Manufacturer instructions were followed to perform the ELISA procedure.

This sensitive method can be widely used to monitor apoptosis in *in vitro* experiments (cell culture supernatants), in clinical samples, e.g. to monitor cell death in

patients under chemotherapy (plasma, serum, urine samples) and can also be applied to monitor cell death in experimental animals (e.g. blood samples of mice under experimental tumor therapy).

2.8. MTT-Assay

The MTT assay is a "cell survival" or better, a "mitochondrial oxidation" assay, rather than a death assay (Sladowski et al., 1993). Nevertheless, due to its popularity it should be considered in some experimental settings. This method is recommended to monitor cell death in cells (e.g. MCF7) and tumor samples (Philchenkov et al., 2004) with mutations of caspase-3, other caspases, or CAD Dnase since some of the methods described previously may not work well under such experimental conditions. The method utilizes oxidation of yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasolium bromide (MTT), to the dark blue formazan product in mitochondria. The formazan salt is insoluble in water and it can be observed under a microscope in its crystalline form. Thus, it requires solubilization prior to spectrophotometric readings. MTT assays have been used in the clinic for a long time to evaluate the sensitivity of neoplastic cells towards planned chemotherapy.

- The method requires a 96-well-plate-compatible spectrophometer equipped with filters λ=570nm (measurement) and λ=630nm (reference), and a multi-channel pipette.
- The experiment is performed in a 96-well plate (flat bottom preferred).
- Prepare MTT solution, 5mg/ml in PBS and filter or centrifuge to remove particulate, insoluble elements.
- Towards the end of the experiment add the MTT solution to cells with a ratio of 1:10 (e.g. 10μl of the MTT solution to 100μl of cells in medium).
- Incubate for 3-5h at 37°C, and you may evaluate the progress of staining under the microscope.
- At the end of the incubation time, spin down the cells to the bottom of the 96-well-plate at 90*g* for 10 min and discharge the cell culture medium by rapid inverting of the plate.
- Add 150μl of ethanol:DMSO mix (1:1) per well and mix the plate on a shaker for 20min to solubilize the formazan crystals.
- Measure using a spectrophometer equipped with filters λ=570nm (measurement) and λ=630nm (reference).

The method itself only provides information about cell viability. However, if combined with another method capable of qualitatively confirming an apoptotic processes, it is fast and powerful assay for investigating e.g. chemotherapy sensitivity or other cell viability related issues.

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CURRENT STRATEGIES IN TUMOR-TARGETING

Chapter XVI

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1. INTRODUCTION

The recent advances in assay and instrument technologies have provided the means for the high throughput screening of large numbers of compounds (Lam, 1997, Cox et al., 1997). These techniques have accelerated the discovery of potent drugs. However, the standard screening methods identify compounds depending on factors such as potency of an enzyme inhibitor or the binding affinity of a receptor ligand. Other factors such as solubility and hydrophobicity, and in particular pharmacokinetics are not sufficiently considered when choosing novel pharmaceutical leads. Regardless of their *in vitro* performance, the success of novel therapeutics is doomed if pharmacokinetics are not considered in the early stages of drug design. To bridge the gap between *in vitro* an *in vivo* drug development, it is suggested to consider tissue specific mechanisms of either drug delivery or drug release. In order to advance the design of novel drugs that target apoptotic pathways, this chapter will introduce the basic principles of drug targeting as they have evolved to date.

The term "chemotherapy" was coined by Paul Ehrlich at the end of the 19th century for the treatment of infectious diseases. From the second half of the twentieth century the linguistic usage of „chemotherapy" was extended to comprise of anti-neoplastic therapies. The first observations of anti-neoplastic effects came from animal experiments and subsequently from the chemical warfare agent mustard gas. In world-war II bone marrow aplasia was observed in American soldiers who were exposed to mustard gas. This led to therapy attempts with derivatives of this agent in patients with malignant hematological diseases (deVita, 1978). In the 40's the antineoplastic effect of folic acid

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antagonists was discovered. In 1948 Farber reported the first remission of an acute lymphatic leukemia after administration of the folic acid antagonist aminopterin (Farber et al., 1949). In the 60's it became clear that some forms of leukemia, particularly those of infancy can be healed by chemotherapy (Laszlo, 1995). Unfortunately, the hope of healing all cancers by chemotherapy was followed by disillusionment due to the exceedingly slow progress in drug development. Despite the immense progress of cancer research in the areas of tumor development and diagnostics, only few tumors (e.g. acute lymphatic leukemia) can presently be cured, and the vast majority of cancers remain incurable.

The chemotherapeutic agents used in systemic cancer therapy exert their effect mainly on proliferating cells so that not only tumors, but also normal tissue that is physiologically proliferating, such as bone marrow, intestinal or dermal epithelia is affected. Consequently, the systemic side effects of chemotherapeutic agents in healthy tissues represent one of the foremost problems in cancer chemotherapy. These side effects of chemotherapy limit dosing, i.e. drug plasma levels, and compliance of patient's during therapy. Drug targeting which pinpoints tumors specifically, holds the potential of reducing the systemic toxicity of chemotherapy. Unfortunately, most of the currently used chemotherapeutics are not selectively delivered into their target tissues.

In 1906 Paul Ehrlich introduced the expression "magic bullet" (Ehrlich, 1906) for the search of optimized treatment strategies. Since then, research in the sense of drug targeting has focused on the development of carrier systems that increase the therapeutic concentration of a drug in target tissue such as tumors or pathogens thus lowering the side effects of the organism (Gregoriadis, 1977). Ideally, drug targeting[1] should fulfill the following criteria:

- exclusive transfer of the drug to the required site of action
- a minimum of toxic effects for the remaining organism
- use of a pharmacologically inactive vector

In order to carry a drug to a tumor, different strategies are pursued. These are for example, using prodrugs, from which the pharmacologically active part is released in the target tissue by tissue-specific enzymes. A further possibility is to couple effective, non-tissue-specific drugs to tissue-specific, but pharmacologically inert carrier systems like receptor affine peptides or colloidal particles. In the following section the most important drug targeting concepts are presented.

2. LOCAL RELEASE FROM PRODRUGS

The assumption that tumors possess metabolic pathways differing from those found in normal tissues is the basis for the so-called prodrug monotherapy. If for example a

[1] Loco-regional therapy is an alternative to systemic drug targeting. In loco-regional therapy the drug is brought as near as possible to the tumor in order to achieve a high regional concentration and to thus decrease the systemic toxicity. Examples are the intravascular application, (e.g. isolated perfusion, by which the organ concerned is isolated from the remainder of the circulation and perfused with blood and cytostatics) and the intracavitary application, where the drug is given e.g. intraperitoneal, intrapericardial or intravesicular so that little active substance can escape into circulation. The direct application of cytostatic drugs into the vessels supplying the tumor represents the simplest case of loco-regional therapy.

tumor secretes an enzyme that does not occur in healthy tissue, this enzyme may activate the prodrug and thus cause the tumor-specific release of the active cytotoxic substance. Apart from enzymatic release, the prodrugs can also be activated due to altered physiological characteristics such as pH or RedOx potential in which tumors differ from healthy tissue. Examples of four different prodrugs are found in Figure 1.

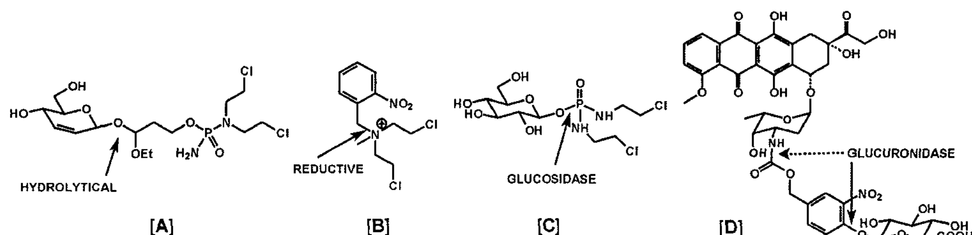

Figure 1. Structures of prodrugs with different release mechanisms. The arrows show the unstable link between the drug and the carrier moiety. Under acidic conditions the acetal-glucoside [**A**] (Tietze et al., 1989) is cleaved. In [**B**] (Kriste et al., 2002). reducing conditions trigger the release of mechlorethamine (nitrogen-lost). Glucosidases are believed to be involved in the release ifosfamid mustard from [**C**] (Becker et al., 2002; Haberkorn et al., 1998, Niculescu-Duvaz et al., 2002). The release of doxorubicin from [**D**] (Bosslet et al., 1998 Murdter et al., 1997) is initiated by enzymatic cleavage of the phenolic glucuronide, leading to a destabilization of the carbamate linkage.

2.1. Glucuronides

In 1947 Fishman and Anlyan showed that many tumors produce increased amounts of β-glucuronidase (Fishman & Anlan, 1947). In healthy tissues the β-glucuronidase is located intracellularly in lysosomes where it is involved in the hydrolytic breakdown of glycoproteins. In tumor necrosis, cells express large quantities of β-glucuronidase before they disintegrate. In the final stages of necrosis this enzyme is released into the surrounding tumor tissue. The locally elevated β-glucuronidase may cleave and thus activate prodrugs containing a glucuronidated cytostatic (Houba et al,. 2001). An example of a prodrug that can be activated by glucuronidases is shown in Figure 1D.

2.2. Glycosides

A metabolic switch accompanies malignant transformation from aerobic to anaerobic carbohydrate metabolism. Since anaerobic glycolysis yields less ATP than oxidative glycolysis, less energy per molecule of glucose is produced in tumors than in healthy tissue. Together with the high rate of proliferation, this leads to an intensified need of glucose in tumors. In cancer cells the glucose utilization is often more than ten times greater than in normal tissue.

The altered glucose utilization is reflected by the enzymatic status of malignant cells. Malignant tumors usually express higher levels of the key enzymes of glycolysis, but reduced levels of gluconeogenetic key enzymes. In addition to the glycolytic enzymes, the number of saccharide transporters in the cell membrane is increased in tumor cells (Flier et al., 1987).

Consequently, the potential of glycosides and saccharide conjugates as 6-[bis(2-chloroethyl)-amino]-6-deoxy-D-glucose (Lazarus et al., 1986) or 6-[bis(2-chloroethyl) amino]-6-deoxy-D-galactose (Schein et al., 1987) was thoroughly explored for therapeutic purposes. Wießler and coworkers successfully targeted tumors with a conjugate consisting of ifosfamid mustard, the alkylating metabolite of ifosfamid, linked via a glycosidic bond to β-D-glucose (Pohl et al., 1995; Engel et al., 2000). The cellular uptake of this conjugate named glufosfamide is mediated by SAAT-1, a transmembrane transport protein for glucose (Veyhl et al., 1998). It is believed that uptake by SAAT-1 contributes to the increased glucose utilization of tumor cells, and to the selectivity of glufosfamid. Presently glufosfamid is being examined in phase II-studies in patients with pancreatic cancer and non-small cell lung cancer.

With 2-[^{18}F]-2-deoxy-D-glucose (FDG) (Fig. 2) it has become possible to visualize and quantify the regional glucose utilization of tumors *in vivo* by positron emission tomography (PET). Imaging the tumor-specific accumulation of FDG allows the diagnosis, staging and therapy monitoring of cancers (Delbeke 1999; Delbeke et al., 1999). Consequently, FDG has become an established PET-imaging agent that has its main application in clinical oncology. Due to the increased metabolism of glucose, FDG is avidly consumed by most cancer cells that exhibit the upregulated glucose transporter 1 (GLUT-1) (Yamamoto et al., 1990). The tumor retention of the tracer is due to a metabolic trapping mechanism that is attributed with the formation of FDG-6-phosphate catalyzed by type II hexokinase (Goel et al., 2003; Haberkorn et al., 1994) (Fig. 2). In addition, hydrolysis of FDG-6-phosphate mediated by glucose-6-phosphatase is negligible within the physical lifetime of the label because this enzyme is downregulated in malignant tumors.

Besides its function as a biological marker for glucose transport and hexokinase activity, FDG uptake is considered to correlate to some degree with the number of viable tumor cells (Spaepen et al., 2003). Consequently, FDG uptake has been proven to be a sensitive tool for the evaluation of glucose metabolism during or soon after treatment of malignant tumors.

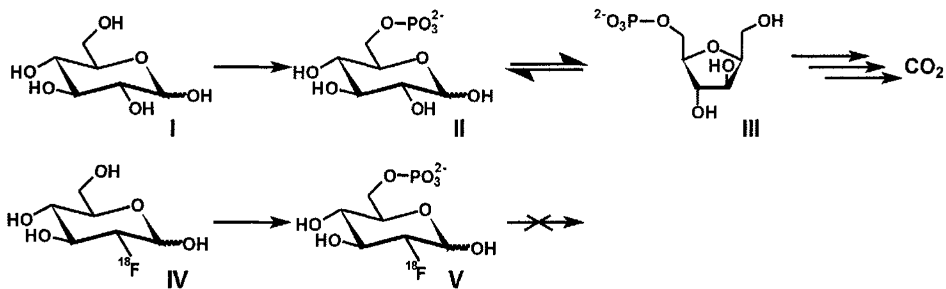

Figure 2. Intracellular metabolism of glucose (I) and FDG (IV). Absence of a 2-hydroxy group prevents FDG-6-phosphate (V) to be transformed to the fructose derivative by glucose-6-phosphate isomerase. The FDG-6-phosphate (V) is too polar to penetrate cellular membranes and is therefore trapped inside the cell.

2.3. Agents Activated by Hypoxia

Hypoxic areas with diameters >2 cm are frequently observed in advanced solid tumors. The hypoxic areas are a consequence of anomalies of the blood vessels grown within the process of tumor vascularization. During tumor growth these anomalies lead to a disturbed blood supply and an increasingly longer distance between blood vessels and tumor cells. Since the oxygen transport capacity of blood is limited, many solid tumors show tumor hypoxia (Rockwell, 1992). The hypoxic areas are heterogeneously distributed over the tumor mass. Tumor cells proliferate slower in an hypoxic environment.

Since radio- and chemotherapy affect proliferating cells, reduced therapeutic effectiveness must be expected in hypoxic, less proliferating tumor regions. However, prodrugs that are activated in an environment rendered reductive by hypoxia, may be activated in tumor areas of low oxygen partial pressure. The cell cycle phase dependency of radiotherapy and cytostatics in non-proliferating or slowly proliferating tumor tissue is not observed for agents activated by hypoxia.

Activation of prodrugs occurs, for example, by reduction of linkages in which the transformation of a nitro group to an amino group leads to a destabilization of a cytotoxic conjugate. The postulated mechanism of the enzyme-dependent transformation of a nitro compound into the activated amine is shown in Figure 3.

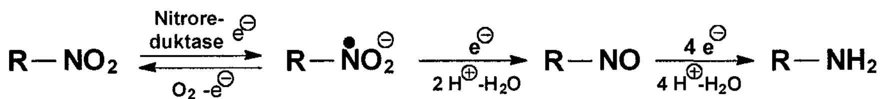

Figure 3. Reduction of nitro compounds. The transfer of the first electron can be catalyzed by nitroreductase which is inhibited by high concentrations of oxygen (Mason et al., 1975). In addition, high oxygen concentrations cause the reoxidation of the amine to the initial nitro compound.

2.4. pH-Selective Conjugates

About 70 years ago Warburg found that in tumors lactate is formed in higher concentrations than in healthy tissue (Warburg, 1927). The switch from aerobic metabolism to anaerobic glycolysis leads to an increased concentration of glycolytic enzymes and thus to an increased level of lactate in tumors. A key enzyme of glycolysis, the pyruvate kinase, is quantitatively increased as a tetrameric M2-pyruvate kinase-isoenzyme in tumor tissues. The lactate acidosis is increased in tumor cells as they are unable to activate the glucose-6-phosphatase. It is amazing that tumor cells, despite their energetic disadvantage[1], outgrow normal cells (Brand & Hermfisse, 1997). Glycolysis can be stimulated by intravenous administration of large quantities of glucose. The resulting hyperglycemia increases the rate of lactate formation in tumors (Volk et al., 1993). For a long time it was accepted that lactate formation would lead to lowering pH

[1] It could be shown that pyruvate, which is formed during anaerobic glycolysis in a far higher concentration than during oxidative glucose utilization, does act as a radical scavenger. Thus anaerobic glycolysis protects the cell from oxidative stress. This might confer a selection advantage in the process of malignant transformation.

in tumor cells. This hypothesis was the basis for the synthesis of prodrugs whose active part is set free by hydrolysis under acidic conditions (Tietze, 1989).

However, it was not possible to realize this concept in the anticipated manner. Although interstitial space in tumors is acidic, tumor cells maintain a physiological intracellular pH by proton pumps (Griffith, 1991). A physiological intracellular pH was demonstrated in tumor cells using selective probes, for example by nuclear magnetic resonance (NMR) techniques (Stubbs et al., 1994). An activation of conjugates sensitive to low pH values will thus occur in the interstitial space. pH-selective conjugates can only be applied successfully if the activated form penetrates into the surrounding cells.

2.5. Antibody-Directed Enzyme Prodrug Therapy (ADEPT)

The increased expression and release of enzymes such as β-glucuronidase in tumors is the basis of the prodrug monotherapy. Tumors failing to overexpress a specific enzyme cannot be treated by this approach. In order to target such tumors specifically, the enzyme needed for the activation of the prodrugs must be artificially transferred into these tumors prior to therapy. Antibody directed enzyme prodrug therapy (ADEPT) is a strategy used to transfer the required enzyme (Syrigos et al., 1999).

Tumors that express a specific cell-surface antigen, are amenable to ADEPT. The systemically applied antibody enzyme conjugate accumulates over several days in the tumor due to the affinity for the tumor-specific antigen. The excess antibody remaining in circulation is removed after several days, either by endogenous mechanisms or by means of a second antibody directed against the primary conjugate (Bagshawe, 1994). The prodrug is then applied and activated locally by the enzyme previously transferred.

3. GENE TRANSFER INTO TUMORS

Currently, different gene therapy approaches are being evaluated in experimental and clinical studies. These approaches are primarily based on the transduction of modulating cytotoxic genes into tumor cells:

- Improvement of the tumor-specific immune response by genetically modifying tumor cells and/or tumor-infiltrating immune competent cells. The transduction of genes coding for immunogenic determinants on the tumor cell surface may elicit an immune response leading to the elimination of genetically modified as well as non-modified tumor cells. If tumor-infiltrating lymphocytes or tumor cells are transduced by genes coding for cytokines, an enhanced recruitment and activation of immune competent cells will be expected due to elevated intratumoral cytokine concentrations.
- By introducing tumor suppressor genes or by suppressing oncogene expression either event results in reversion of the malignant phenotype. The inactivation of oncoproteins may be achieved by use of antisense oligonucleotides and ribozymes, or by transduction of genes coding for oncogene specific intracellular antibodies (intrabodies).

- Direct killing of tumor cells by introducing cytotoxic or prodrug-activating genes (suicide genes).

A broad range of suicide systems have been developed (Altmann & Haberkorn, 2003). The respective genes usually code for non-mammalian enzymes that systemically convert applied nontoxic prodrugs into highly toxic metabolites at the tumor site. Most studies employed cytosine deaminase (CD) which is expressed only in yeasts and bacteria, or Herpes Simplex Virus thymidine kinase (HSV-tk) as suicide genes.

Cytosine deaminase converts the antifungal agent 5-fluorocytosine (5-FC) to the highly toxic chemotherapeutic drug 5-fluorouracil (5-FU). 5-FU interferes with the transcription, translation or replication of uracil by 5-FU in RNA, and inhibition of thymidilate synthetase by 5-fluorodeoxy-uridine monophosphate which results in impaired DNA biosynthesis. The pharmacological effects of 5-FC are limited and high therapeutic doses are permitted. While 5-FC is uniformly distributed lacking tissue-specific accumulation or appreciable affinity to plasma proteins, 5-FU is not produced due to the lack of CD activity in mammalian cells. However, after conversion of 5-FC to 5-Fluoruracil (5-FU) by the intestinal microflora, side effects may occur.

The transduction of the herpes simplex virus thymidine kinase (HSVtk) has been carried out in a variety of tumor models *in vitro* and *in vivo* (Altmann & Haberkorn, 2003). In contrast to the human counterpart, HSVtk efficiently phosphorylates nucleoside analogs, such as acyclovir and ganciclovir to their monophosphate metabolites, which cannot leave the cell and are subsequently phosphorylated by cellular kinases to the di- and triphosphates. Due to the incorporation of the triphosphate metabolites into DNA during replication, chain termination occurs followed by cell death.

However, these approaches require vectors leading to efficient infection as well as to tissue specific expression of the genes.

4. COUPLING TO CARRIER MOLECULES

4.1. Antibody Conjugates

Tumor cells express specific antigens on their surface and consequently possess well-defined immunological properties. Antibodies against tumor specific antigens can be coupled to cytotoxic drugs (Sezaki & Hashida, 1984). Since their affinities are usually high, antibodies bind avidly to tumor cells and should be ideal shuttles for cytostatic drugs. Unfortunately, when used clinically, antibodies show unfavorable pharmacokinetic characteristics caused by their high molecular weight. Due to the high antibody selectivity only the antigen-expressing tumor cells are attacked which eventually lead to a selection of antigen-negative, resistant tumor cells.

4.2. Targeted Transport Using Carrier Peptides

Theoretically a wide range of peptides should be suitable carriers of radionuclides or cytostatics. The application of peptide carriers requires an overexpressed receptor and a sufficient *in vivo* stability of the peptide. In addition, the drug moiety should not interfere

with the binding sequence of the peptide. Besides peptides which by now have been examined in detail, such as somatostatin, gastrin, luteinizing hormone releasing hormone (LHRH), epidermal growth factor (EGF), substance P, corticotropin releasing factor (CRF) and insulin. Novel peptides with high specificity can be found by the screening of phage display libraries (Zhang et al., 2001, Koivunen et al., 1999).

4.3. Receptor Binding Peptides, Suited for the Targeting of Tumors

Depending on the ontogenic origin, tumors overexpress a variety of peptide receptors. This differential receptor expression distinguishes tumors from nontransformed tissue (Vogelstein & Kinzler, 1993). Examples are receptors of the vasoactive intestinal polypeptide (VIP) which are overexpressed in neuroendocrine and several other tumors (adenocarcinoma, lymphoma, astrocytoma, glioblastoma and meningioma) (Hessenius et al., 2000). By autoradiography cholecystokinin b (CCKB)/gastrin receptors were found in the majority of medullary thyroid carcinomas and small cell lung cancers, tumors of the ovaries, astrocytomas and several adenocarcinomas (Reubi et al., 1997).

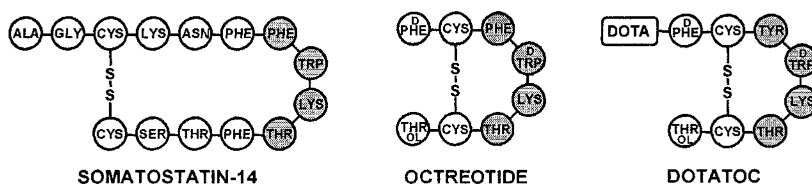

Figure 4. Schematical illustration of native somatostatin, octreotide and DOTATOC ($DOTA^0$-D-Phe^1-Tyr^3-octreotide). The amino acids essential for receptor binding are shaded in grey.

Receptors of gastrin releasing poly-peptide (GRP) are expressed by prostate carcinomas (Schally et al., 2000). Neurotensin (NT) receptors are expressed in Ewing's Sarcomas, Meningiomas, Astrocytomas and exocrine pancreatic tumors (in particular ductal pancreatic adenocarcinomas) (Reubi et al., 1999). The majority of chest tumors express calcitonin receptors (Gillespie et al., 1997). Somatostatin receptors are found in the majority of neuroendocrine tumors such as neuroblastomas and pheochromocytomas and, some medullary thyroid carcinomas, prostate tumors, and small cell lung cancers.

As the clinical use of somatostatin is severely limited due to its *in vivo* half-life of 2-3 min (Wangberg et al., 1991) smaller cyclic peptides have been developed which incorporate D-amino acids to prolong the *in vivo* half-life by inhibiting amino- and carboxypeptidases. One of these discoveries is octreotide (Sandostatin®), a cyclic eight-membered peptide with a disulfide bridge, which has an *in vivo* half-life in humans of 60-90 min (del Pozo et al., 1986) and is 2000 times more effective than somatostatin at inhibiting growth hormone secretions (Bauer et al., 1982). A number of derivatives of octreotide have been introduced to diagnose and treat somatostatin receptor-positive tumors. The most important derivatives are [^{111}In-DTPA-D-Phe^1]-octreotide (Krenning et al., 1993) (Octreoscan®) and $DOTA^0$-D-Phe^1-Tyr^3-octreotide (DOTATOC) (Henze et al., 2001).

Table 1. A selection of peptides suitable for tumor targeting.

Ligand	tumor type & selectivity
Somatostatin	neuroendocrine tumors, non-Hodgkin lymphoma, melanoma, breast cancer
α-melanocyte-stimulating hormone	Melanoma
luteinizing hormone releasing hormone	prostate cancer, breast cancer
vasoactive intestinal polypeptide /pituitary adenylate cyclase activating polypeptide	small cell lung cancer, colon cancer, pancreatic cancer
endomorphines	small cell lung cancer, neuroblastoma, breast cancer
cholecystokinin-B/gastrin	medullary thyroid carcinoma, small cell lung cancer, pancreatic cancer, astrocytoma, ovarian stromal tumor
Neurotensin	small cell lung cancer, colon cancer, exocrine pancreatic carcinoma
bombesin/gastrin releasing peptide	small cell lung cancer, colon cancer, glioblastoma, prostate cancer
substance P	glioblastoma, astrocytoma, medullary thyroid carcinoma, breast cancer

4.4. Membrane Permeable Peptides

The conjugation of drugs to small, highly basic transport peptides results in their rapid translocation[1] into cells. Importantly, these conjugates have been effective in a number of animal models suggesting their general utility for drug transport *in vitro* and *in vivo*. Several cell-penetrating peptides such as TAT (Vives et al., 1997), antennapedia peptide (Derossi et al., 1994) and transportan (Pooga et al., 1998) have been used to noninvasively transport cargoes like oligonucleotides and peptides. The ability of these peptides to convey cargoes into living cells has been studied in detail. Until now the only common feature of these peptides is their ability to gain cell entry in an energy-independent manner. Some believe peptide internalization is not mediated by

Table 2. Amino acid sequence of selected membrane permeable peptides.

Peptide	amino acid sequence
tat-peptide (48→60)	GRKKRRQRRRPPQ
antennapedia peptide (43→58)	RQIKIWFQNRRMKWKK
Transportan	GWTLNSAGYLLKINLKALAALAKKIL
amphipatic model peptide	KLALKLALKALKAALKLA

[1] Recently the ability to translocate has been challenged. In fluorescence microscopy studies an endosomal distribution of fluorescently labeled peptides was observed in unfixed cells (Richard *et al.*, 2003).

endocytosis, chiral receptors, or proteins in general, and is not dependent on temperature. Interestingly, synthetic model peptides that mimic the properties of the transport peptides can be designed (Oehlke et al., 1998).

5. COLLOIDAL PARTICLES AS CARRIERS

Colloidal systems such as liposomes have the ability to integrate cytostatics into a closed carrier. Thus, a drug is protected against degradation *in vivo.* To increase the biological half life and enhance the tissue specificity of liposomes, the membrane properties need to be considered in the design of liposomes. *In vivo* unmodified liposomes accumulate rapidly in liver and spleen through uptake by the reticuloendothelial system. When using liposomes with defined diameters and distinct membrane phospholipids, their scavenging by the reticuloendothelial system is substantially affected. The degradation of liposomes can be slowed down through the integration of polyethyleneglycols (PEG) into the liposomal membrane and thus the biologic half-life in plasma is greatly extended (Harrington et al., 2000). (Pegintron®, a PEGylated interferon alfa-2b (Tilg et al., 2003), which is used for the monotherapy of chronic hepatitis C). The "PEGylation" of liposomes is another option to stabilize compounds, in particular proteins in circulation.

By integrating tumor-specific antibodies into the liposomal membrane, it is possible to vary the tissue specificity of the particles (Bendas 2001, Huwyler et al., 2002) depending on which tumor is being treated. The biodistribution of the drug is thus completely different from that of the free drug. As a result of better targeting, the toxic side effects are reduced. For example Doxil® and Caelyx®, and liposomal formulations of doxorubicin clearly decrease the particularly acute and chronic cardiac toxicity of doxorubicin (Muggia, 2001).

Alternatively, the organ specificity of the particles can be determined by coupling them to ligands for known receptors or transport proteins. By integration of specificoligosaccharide structures or folic acid derivatives, it is possible to target selectins (Hutchinson & Jones, 1988) or the folate receptors (Gabizon et al., 1999, Gabizon et al., 2003) that are both overexpressed in numerous tumors (Fig. 5).

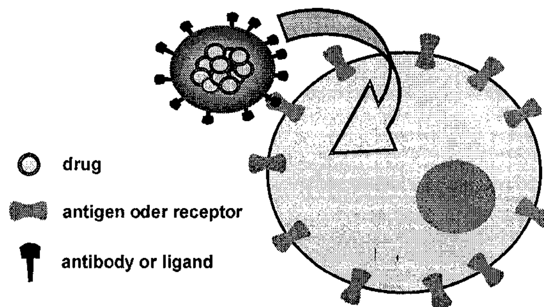

Figure 5. Schematic representation of tissue selective targeting of a liposomally packed drug by receptor mediated uptake.

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STRATEGIES OF PROTECTION OF NORMAL CELLS DURING CHEMO- AND RADIO-THERAPY BASED ON MODULATION OF CELL CYCLE AND APOPTOTIC PATHWAYS

Chapter XVII

Mikhail V. Blagosklonny[1*] and Zbigniew Darzynkiewicz[1]

1. INTRODUCTION

The ultimate goal of cancer therapy is to selectively kill cancer cells, while sparing normal cells. Therefore, major research efforts have been aimed at the discovery of molecular targets that are specific for cancer cells. One such target is BCR-ABL, a product of a chromosomal translocation (Philadelphia chromosome), which is both specific and vital for chronic myelogenous leukemia (CML). Therefore, BCR-ABL is the most validated target in oncology (Druker, 2002; Druker and Lydon, 2000; Daley, 2003). Gleevec (Imatinib, STI571), an inhibitor of BCR-ABL and c-Kit, have demonstrated excellent clinical effectiveness, causing remission in more than 90% patients with chronic phase of CML (Kantarjian et al., 2003). Without doubt, such a successful treatment of BCR-ABL -expressing leukemia with the BCR-ABL kinase inhibitor Gleevec is the most spectacular achievement in modern oncology. Unfortunately, this is the only example. In fact, BCR-ABL is an unique target. Other targets are either non-specific for cancer cells or dispensable for cancer growth and survival. Most drugs either affect normal cells (e.g. inhibitors of histone deacetylases and farnesyltransferase) or do not affect cancer cells (some inhibitors of growth factor receptors and cyclin dependent kinase 2). This is not co-incidental. First, almost all anticancer drug targets are present in normal cells. Second, cancer cells have multiple genetic alterations. Therefore, a single growth factor receptor may be dispensable for cancer cell proliferation (Fig. 1). As emphasized by Richard

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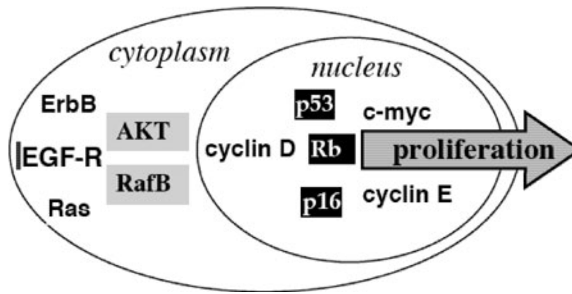

Figure 1. Multiple genetic and epigenetic traits that are common to most cancer types. The common feature of cancer is the presence of activated oncogenes and/or loss of tumor suppressors (the most frequently altered nuclear and cytoplasmic oncogens/tumor suppressors are marked). This results in autonomous cell proliferation independent of the presence of mitogens/growth factors and of mitogenic kinases (e.g. the EGF receptor kinase, ErbB2, PI3K). This also renders cells resistant to apoptosis and thereby to chemotherapy.

Klausner, a former NCI director, we continue to have very few effective and specific ways to treat the vast majority of common tumors once local therapy is not an option (Klausner, 2002). Although numerous inhibitors of growth factor receptors, tyrosine kinases, RAS, PI3K, RAF-1, MEK, mTOR, and cyclin-dependent kinases have been entered in clinical trials, with a few exceptions, they have not shown clinical activity as a single agent. With an exception of trastuzumab (Herceptin) in HER-2-overexpressing cancer, most agents were not effective in drug combinations (Tan and Swain, 2003; Wilkinson, 2002; Twombly, 2002; Baselga, 2002b). The number of all possible drug combinations, their doses and sequences of administration far exceed the ability of trial-and-error methods. For example, the combination of Trastuzumab (an antibody against ErbB2) and Vinorelbine (antitubulin agent) was found to be effective in therapy of Erb2-positive metastatic breast cancer. As the clinical investigator commented: "It was a lucky pick, because subsequently it was shown in the laboratory that they are synergistic" (Warnock, 2002). Among the most successful agents, were several anti-EGFR agents that are in clinical development. Three drugs are currently in Phase II and III development as single agents, or in combination with other anticancer modalities: IMC-225 (Cetuximab/Erbitux; ImClone), a monoclonal antibody which blocks activation of the EGFR; OSI-774 (Erlotinib/Tarceva; Genentech/OSI/Roch) and ZD1839 (Gefitinib/Iressa; AstraZeneca), two small molecules that are EGFR-selective inhibitors (Rowinsky, 2003). Cetuximab (Imclone), an antibody against the EGF receptor, has been recently approved by the FDA to treat irinotecan refractory metastatic colorectal cancer. Iressa is the first EGFR-targeting agent to be registered as an anticancer drug, but it surprisingly failed phase III. However, there is nothing wrong with Iressa *per se*. It inhibits the EGF receptor in patients (Baselga, 2002b). There are at least twenty EGFR inhibitors (and hundreds of kinase inhibitors) "in pipeline" of the drug development (Dancey and Sausville, 2003). But, if a selective agent such as Irresa is not sufficiently effective in the clinic, then it is reasonably expected that similar agents will fail too. On the other hand, these mechanism-based agents when combined with cell cycle-dependent chemotherapy can be used to (*i*) increase sensitivity of cancer cells and (*ii*) to protect normal cells.

2. CYCLOTHERAPY: COMBINING SELECTIVE CYTOSTATIC AGENTS WITH CHEMOTHERAPY THAT TARGETS PROLIFERATING CELLS

Currently, standard chemotherapy is still a cornerstone of medical oncology. As we will discuss, these agents target either DNA and DNA metabolism (alkylating agents, antimetabolites, intercalating agents, topoisomerase I and II inhibitors) or mictrotubules, in particular, mitotic spindles (vinblastine, vincristine, paclitaxel, docetaxel). In addition, most new mechanism-based drugs also target non-specific targets for instance: histone deacetylases and proteasomes. Yet, such a non-specific therapy can be curative. The most effective antitumor drugs target cells progressing through the cell cycle (Halicka et al., 1997; Huang et al., 2003). However, the frequency of cell proliferation and the kinetics of the cell cycle progression in some normal tissues (e.g. epithelial cells of most of the digestive tract, bone marrow) is higher than in most tumors (Gartler, 1977; Greider et al., 1983). Cell cycle-dependent drugs, such as DNA topoisomerase inhibitors, DNA antimetabolites or microtubule poisons are expected to preferentially eradicate the rapidly cycling normal cells and spare tumor cells. How can therapeutic effects be explained?

One of the mechanisms for increased sensitivity of tumor cells to the cell cycle-targeting drugs is the inherent difference in the regulation of the cell cycle between normal and tumor cells. Namely, the vast majority of human cancers have abnormalities in the retinoblastoma (Rb) pathway (Chen et al., 1999; Nevins, 2001; Sherr, 1999, 2002). The abnormalities make proliferation of these tumors autonomous, independent of mitogenic factors from the cell environment, as well as the cell cycle checkpoints (Hartwell and Kastan, 1994; Bartek et al., 1999; Dixon and Norbury, 2002). Thus, exposure of normal cells to the drug effectively stops at the checkpoint allowing for the damage to be repaired. The arrest of the cells with the defective checkpoints is incomplete (Stewart et al., 1999). Their progression in the presence of the drug leads to a cumulative increase in the extent of damage. For example, progression though the S phase in the presence of DNA topoisomerase inhibitor results in a collision between the DNA replication forks and the "cleavable" complexes of DNA-topoisomerse stabilized by the drug. Such collisions result in dsDNA breaks, which are lethal cell lesions (Hsiang et al., 1989; Holm et al., 1989; Li et al., 1994; Deptala et al., 1999; Wang et al., 2002). Apoptosis is then triggered at a certain threshold of damage severity.

One would expect that this protection mechanism for normal cells is most effective at low doses of antitumor drugs when cells with defective checkpoints progress through the cell cycle, but normal cells arrest growth (Traganos et al., 1992; Bruno et al., 1992; Darzynkiewicz, 1995). At higher concentrations, the drugs may arrest tumor cells (e.g. through their direct effect on DNA replication machinery) and may also kill normal cells. Perhaps optimal implementation of this strategy may involve low doses of cytostatic agents (as a hypothetical example, pretreatment of the patient with a low dose of X-irradiation to the critical parts of the body hosting the most rapidly proliferating normal cells) followed by infusion of the drugs targeting cycling cells.

The success of chemotherapy also requires proclivity of the tumor cells to undergo apoptosis (Woynarowska and Woynarowski, 2002). Hence, the most curable tumors are the apoptosis-prone tumors such as some leukemias. In testicular cancer, childhood leukemias and chorioepithelioma, chemotherapy with DNA damaging and microtubule-active agents can cure disease (Frei, 1985; Cohn and Herzog, 2000). These tumor cells

readily undergo apoptosis (Martin and Green, 1994; Sellers and Fisher, 1999). Yet, in most common malignancies, cancer cells avoid apoptosis. Therefore, chemotherapy more likely kills normal hematopoietic and epithelial cells than cancer cells. In most cases, the toxicity to normal cells limits therapy of cancer.

So, how can we preferentially kill cancer cells, thus minimizing side effects of chemo- or radio-therapy? One solution is to combine cytostatic agents, which inhibit proliferation, with chemotherapeutic drugs that target proliferating cells. The proliferation of normal cells absolutely depends on growth factors (mitogens). In contrast, most cancer cells have an impaired restriction point in the cell's cycle (Pardee, 1974; Sherr, 1999) and can proliferate in the absence of growth factors (autonomous growth). Since loss of cell cycle check- and restriction-points is the most universal alteration in human cancer, "common strategies might be developed against a wide variety of cancers, as a more promising approach than unspecific attempts to block cancer cell cycle" (Nurse, 2000). Autonomous cancer cells do not depend on growth factors and likely will not respond to inhibitors of mitogenic kinases as well. In contrast, normal cells will get arrested. Then cytotoxic drugs that target proliferating cells will kill only cancer cells. While protecting normal cells, inhibitors of mitogenic kinases can render apoptosis-resistant cancer cells sensitive to therapy (Fig. 2).

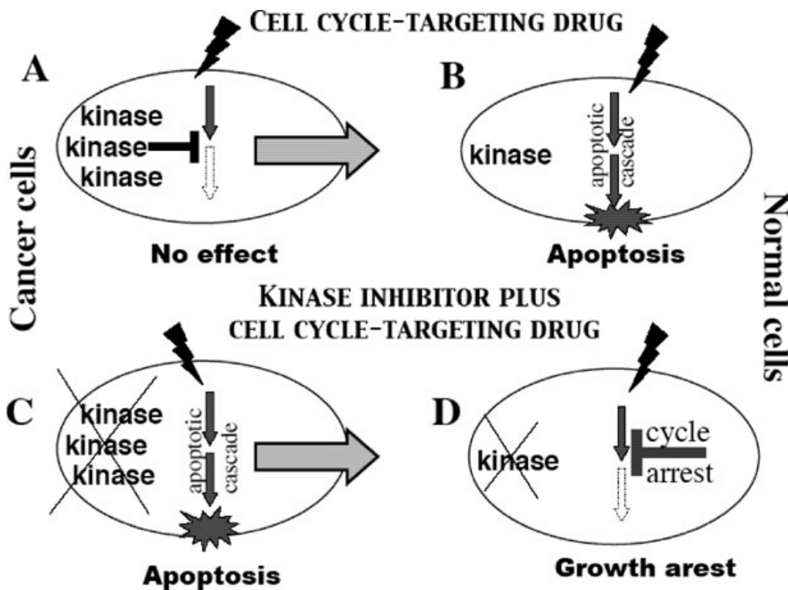

Figure 2. The strategy of protection of normal cells concurrent with exposure of cancer cells to chemotherapy. (**A, B**) due to overexpression and/or overactivation of mitogenic and anti-apoptotic kinases, cancer cells (A) are resistant to apoptosis and growth arrest caused by the cell cycle-targeting chemotherapy (e.g. topoisomerase inhibitors, mitotic poisons). Normal cells may undergo apoptosis and this causes side effects (toxicity). (**C, D**) inhibitor of mitogenic kinases (e.g. LY294002) sensitizes cancer cells to apoptosis but does not arrest their proliferation making them susceptible to the cell cycle-targeting chemotherapy. Normal cells are arrested in the cycle and thereby protected by the inhibitor.

3. AUTONOMOUS GROWTH OF CANCER CELLS

In normal cells, growth factors are absolutely necessary for proliferation. By activating multistep kinase signaling pathways, growth factors induce cyclin D1, which is a sensor of GF (Sherr, 1999; Sherr, 2000; Sherr, 2002). Following induction of cyclin D1, activation of CDK4/6 by cyclin D, phosphorylation of Rb (which blocks its activity), release of E2F, induction of cyclin E which activates CDK2 then completing phosphorylation of Rb (Blagosklonny and Pardee, 2002). This complex network represents the Rb pathway. Following phosphorylation (inactivation) of Rb, a cell passes the restriction point of the cell cycle and does not require GF to complete the cell cycle (Bartek et al., 1999). This restriction point is lost in cancer. Different alterations can be responsible for the loss of the restriction point. Thus, cyclin D 1 is often overexpressed in cancer (Sherr, 1999). In fact, the unscheduled expression of cyclin D is the most characteristic feature of tumor cell lines compared with normal cells (Juan et al., 1996). The deregulation in the expression and activity of cyclin E has been associated with a number of cancers (Keyomarsi and Herliczek, 1997; Keyomarsi and Pardee, 1993). Low molecular isoforms of cyclin E are especially active in cancer cells (Wingate et al., 2003). Also, cancer cells show loss of CDKs inhibitors such as p16 (an inhibitor of CDK4/6). Mutations in p16 lead to elevation of cyclin D/CDK4 activity and Rb phosphorylation. High frequencies of p16 gene alterations are observed in many primary tumors. Defective p16 is a major oncogenic change in human cancer, rivaled in frequency only by the p53 tumor-suppressor gene (Liggett and Sidransky, 1998). Loss of p53 seen in 50% of cancers prevents "activation of the restriction point" in response to DNA damage. Loss of Rb function contributes to a wide array of human cancers. The abrogation of the restriction point is the primary function of each of the DNA tumor virus oncoproteins, including SV40 T antigen, E1A and E7 (Nevins, 2001). Cancers acquire multiple genetic alterations with activation of downstream signaling pathways that by itself promote proliferation, making the cells independent of the upstream mitogenic signals (Fig. 1). One can predict, therefore, that inhibition of mitogenic kinases will not stop proliferation of such cancer cells. This in turn could be exploited for selective killing by chemotherapy.

4. HISTORICAL PERSPECTIVE ON PROTECTION OF NORMAL CELLS

Given that some chemotherapeutic agents preferentially kill proliferating cells, growth arrest can protect cells against such cyclo-dependent therapy. Since cytostatic agents cannot inhibit cycling of cancer cells as easily as cycling of normal cells, one can arrest normal cells without affecting cancer cells (Pardee and James, 1975; Darzynkiewicz, 1995). When initial experiments were performed, growth arrest in normal cells was achieved with non-specific inhibitors of metabolism (Pardee and James, 1975). These protectors were too toxic to be exploited clinically and also were not selective for normal cells. At that time, this approach was limited by the lack of molecular therapeutics that targets the cell cycle. In initial work, normal baby hamster kidney (BHK) cells but not tumorigenic polyoma virus-transformed BHK cells (PyBHK) were arrested by either serum deprivation or by metabolic inhibitors (Pardee and James,

1975). Such pretreatments prevent normal cells from entering S and M phases, and thus protect them against S-phase and M-phase specific agents. Growth arrest and protection of cells was also achieved by using non-selective cell cycle inhibitors such as cycloheximide, an inhibitor of translation (Pardee and James, 1975). Slapak *et al* (1985) investigated the effect of cycloheximide on granulocyte/macrophage progenitors (CFU-GM) after in vitro Ara-C exposure using normal human bone marrow, malignant progenitors from patients with chronic myelogenous leukemia (CML), and human leukemia cell lines HL-60 and KG-1 (Slapak et al., 1985). Cells were incubated for one hour with cycloheximide, followed by addition of Ara-C. Normal CFU-GM survival was significantly increased. Cycloheximide pretreatment of CML progenitors and HL-60 and KG-1 leukemia cells failed to protect them from Ara-C-induced cytotoxicity (Slapak et al., 1985). Warrington *et al* (Warrington et al., 1984) have shown that L-histidinol protected a variety of phenotypically normal cell lines from certain proliferation-dependent anticancer drugs without decreasing the toxicity to cancer cells. L-histidinol is a structural analogue of the essential amino acid L-histidine and a reversible inhibitor of protein biosynthesis. L-histidinol modulated the toxicity of 1-β-D-arabinofuranosyl-cytosine (ara-C) and 5-fluorouracil (5-FU) in tissue culture systems by its ability to arrest growth of normal cells. Histidinol conferred substantial protection upon bone marrow cells of mice treated with ara-C and 5-FU. Histidinol-mediated protection to bone marrow cells persists in L1210 leukemia-bearing mice treated with Ara-C or 5-FU without diminishing the toxicities of these agents for leukemia cells (Warrington et al., 1984). Furthermore, L-histidinol not only protected the marrow cell population from both ara-C and 5-FU, but also increased the toxicities of these agents for the intrafemoral tumor cells. Thus, L-histidinol mediates a substantial increase in the specificities of ara-C and 5-FU in mice bearing an established bone marrow leukemic condition (Warrington and Fang, 1985). In mice bearing a murine lymphocytic leukaemia line P388, combined treatments with L-histidinol and either BCNU or cisDDP (alkylating DNA damaging drugs), doses of the alkylating agents were ineffective when used alone, but when used together, they were curative. Dose-response studies showed that L-histidinol conferred dose-dependent, synergistic improvements on the capacities of both BCNU and cisDDP to increase the life-span of mice bearing P388 leukemia (Warrington and Fang, 1989). L-histidinol, N,N-Diethyl-2-[4-(phenylmethyl) phenoxy]ethanamine HCl (DPPE) (4 mg/kg) protected murine bone marrow progenitors from doxorubicin or fluorouracil, while doses of 4-50 mg/kg significantly enhanced the antitumor activity of doxorubicin and daunorubicin in murine models of early cancer (Brandes et al., 1991). In another study, the administration of L-histidinol protected the CD8F1 mouse from 5-FU-associated leukopenia, body weight loss, and ultimately, from mortality (Stolfi et al., 1987). However, in contrast to results reported in the L1210 leukemic system, L-histidinol also reduced the cytotoxic activity of FUra against CD8F1 breast tumors. Although, the dose of 5-FU that could be administered safely was higher in mice receiving L-histidinol, the therapeutic results of the combination of 5-FU and L-histidinol were not superior to those obtained with 5-FU alone at a lower dose (Stolfi et al.,' 1987).

However, these cytostatic (protective) agents are not very selective to normal cells and may non-specifically "freeze up" cycling of cancer cells, precluding cytotoxic effects of therapy (antagonism). In fact, non-specific inhibitors of metabolism may arrest growth of cancer cells. For example, pretreatment of human breast cancer cells with fluorouracil

antagonized effects of paclitaxel in vitro (Grem et al., 1999). This explains "cell cycle-mediated drug resistance" (Shah and Schwartz, 2001). For example, paclitaxel (PTX) arrests cells in mitosis leading to cell death, while cisplatin (cis-Pt) can arrest cells in G2. Then, sequence (cisplatin - PTX) is antagonistic secondary to a G2 arrest caused by cisplatin (cisP) (Shah and Schwartz, 2001). As another example, camptothecins (CPT), inhibitors of topoisomerase I, induce their primary cytotoxicity during DNA synthesis (Wang et al., 2002). Cells in S-phase are 100-1000 times more sensitive to CPT than cells in Gl or G2 (Shah and Schwartz, 2001). Flavopiridol can inhibit the entry into mitosis, protecting cells from paclitaxel (Bible and Kaufmann, 1997). Therefore, the challenge is to develop mechanism-based cytostatitc agents that arrest normal cells, but not cancer cells.

5. TRANSFORMING GROWTH FACTOR BETA (TGF-β) FOR PROTECTION OF NORMAL CELL

Normal bone marrow and epithelial cells are particularly vulnerable to chemotherapy. Damage to the normal replacing tissues (specifically, the gastro-intestinal tract and bone marrow) causes severe side-effects. Transforming growth factor beta (TGF-β) inhibits proliferation of hematopoietic progenitor cells and normal epithelial cells (Massague et al., 2000; Siegel and Massague, 2003). TGF-β protects these cells from the toxicity caused by vinblastine, vincristine, etoposide, paclitaxel, Ara-C, methotrexate, or 5-FU (McCormack et al., 1997). The protected cells could re-enter the cell cycle. TGF-β1 and 2 can reversibly inhibit the proliferation of hematopoietic progenitor cells *in vivo*. Such quiescent progenitors might be more resistant to high doses of cell cycle active chemotherapeutic drugs, thereby allowing dose intensification of anticancer agents. The administration of TGF-β2 protected recovering progenitor cells from high concentrations of 5-FU *in vitro*. This protection coincided with the finding that significantly more progenitors for colony-forming unit-culture (CFU-c) and CFU-granulocyte, erythroid, megakaryocyte, macrophage (GEMM) were removed from S-phase by TGF-β in mice. TGF-β protected up to 90% of these mice undergoing hematologic recovery from a re-challenge in vivo with a high dose of 5-FU. Pretreatment of mice with TGF-β1 or TGF-β2 also protected 70-80% of mice from lethal doses of doxorubicin hydrochloride (Grzegorzewski et al., 1994), and reduced the severity and duration of oral mucositis induced by 5-FU *in vivo*. TGF-β protected small intestinal clonogenic stem cells from radiation damage, reduced diarrhea and animal mortality (Booth et al., 2000). Mucositis is a common, dose-limiting complication in patients receiving cancer chemotherapy. It appears to be a consequence of the rate of epithelial proliferation. TGF-β3 administration reduced proliferation of oral epithelium in vitro and in vivo. Topical application of TGF-β3 to the oral mucosa of hamster prior to chemotherapy significantly reduced the incidence, severity, and duration of oral mucositis, reduced chemotherapy-associated weight loss, and increased survival (Sonis et al., 1994). TGF-β protects rodent small intestinal crypt stem cells and animal survival during methotrexate treatment and after irradiation (Booth et al., 2000; Van't Land et al., 2002) by possibly reducing stem-cell cycling. The gastrointestinal tract, with its rapid cell

replacement, is sensitive to cytotoxic damage and can be a site of dose-limiting toxicity in cancer therapy. In one study (Potten et al., 1997), TGF-β3, an inhibitor of cell cycle progression through G1, was used to alter intestinal crypt stem cell sensitivity before 12-16 Gy of gamma irradiation. It was shown that the administration of TGF-β3 over a 24-h period before irradiation increased the number of surviving crypts by 4- to 6-fold. After 14.5 Gy of radiation, only 35% of the animals survived within a period of about 12 days, while prior treatment with TGF-β3 provided significant protection against this early gastrointestinal animal death. 95% of the treated animals survived for more than 30 days (Potten et al., 1997). For prevention of chemotherapy-induced mucositis, TGF-β is entering clinical trials (Wymenga et al., 1999).

The sensitivity to TGF-β is lost during transformation of epithelial cells (Markowitz et al., 1995; Baldwin et al., 2003). Furthermore, cancer cells may respond to TGF-β by proliferation, instead of growth arrest (Cui et al., 1996; Tang et al., 2003). Thus, TGF-β seems to be an excellent agent to protect normal cells from cycle-dependent chemotherapy and to expose cancer cells to such a therapy. Problems with its distribution may decrease TGF-β usefulness. TGF-β is a protein not a small molecular therapeutic. This limits its ability to reach target normal cells. Small molecular agents that similarly inhibit proliferation of hematopoietic and epithelial cells will be especially valuable.

6. INHIBITORS OF MITOGENIC KINASES AND AUTONOMOUS GROWTH

6.1. General Considerations

With exception of a subset of CLLs, most human cancer cells have multiple genetic alterations (Steinberg, 2002). Many mitogenic kinases lie upstream from major oncogenic alteration in cancer (Fig. 1). Therefore inhibitors of certain mitogenic kinases will not, in theory, interrupt proliferation of cancer cells because such cancer cells are autonomous. Are cancer cells actually resistant to clinically relevant doses of inhibitors? Thus, concentrations of inhibitors that exceed those normally required to inhibit mitogenic pathways can inhibit growth by non-specific mechanisms. For example, while 0.1-1 μM AG1478 inhibited the EGF receptor, 50 times higher concentrations were required to arrest growth of nasopharyngeal carcinoma cells (Zhu et al., 2001). At relevant concentrations, EGF kinase inhibitors do not inhibit growth of most cancer cells (Bishop et al., 2002). Therefore, although at supra-pharmacological doses inhibitors inhibit all cells, these effects are neither clinically relevant nor attributed to their intended targets.

Second, inhibition of mitogenic pathways may cause apoptosis (instead of growth arrest) in certain cells that are resistant to growth arrest. For example, lovastatin induces apoptosis in cells that cannot undergo growth arrest (Kim et al., 2000). In contrast, low concentrations of lovastatin cause growth arrest without cell death in lovastatin-sensitive cells (Jakobisiak et al., 1991). Similarly, rapamycin-induced apoptosis in tumor cells is a consequence of continued G1 progression despite mTOR inhibition. The G1 phase arrest protects against apoptosis (Huang et al., 2001).

6.2. Inhibitors of Mitogenic Kinases for Selective Arrest of Normal Cells

6.2.1. Inhibitors of EGF/ErbB

Non-malignant MCF-10A breast epithelial cells depend on EGF for their proliferation. These cells undergo G_0/G_1 arrest following EGF withdrawal. In contrast, MCF-7 cancer cells do not need EGF and proliferate autonomously. Therefore, low concentrations of the inhibitor of EGF receptor kinase AG1478 arrest MCF-10A cells but not MCF7 cells. As a result, AG1478 can protect MCF-10A cells, but not MCF7 cells from paclitaxel which kills cells in mitosis (Blagosklonny et al., 2000a). It was initially expected that inhibitors of the EGF receptor would arrest cancer cells with high levels of the EGF receptor. Although, these inhibitors actually inhibited the EGF receptor kinase and mitogen-activated kinases, they did not cause growth arrest in most cancer cell lines (Bishop et al., 2002).

6.2.2. Inhibitors of MEK

PD098509, a synthetic inhibitor of MEK, inhibited proliferation in normal fibroblasts and in cells transformed by oncogenes whose activity strictly depends on MEK (Dudley et al., 1995). In contrast, PD098509 inhibited MEK without arresting growth of some cancer cells (Busse et al., 2000). Thus, whereas this agent inhibits MEK at low micromolar concentrations, it does not inhibit proliferation of SKBr3 cells (Blagosklonny, 1998) and HL60 cells (Blagosklonny et al., 1999; Bishop et al., 2002) unless 60-100 μM concentrations of PD098509 were used. The MEK inhibitor PD184352 completely inhibited the MEK/ERK pathway with an IC_{50} below 1 μM (Squires et al., 2002). Though, in most cancer cell lines, higher doses were needed to cause growth arrest.

6.2.3. Rapamycin

mTOR is a target of rapamycin. mTOR is downstream from PI3K and is activated by mitogens. As expected, normal cells which depend on mitogens are sensitive to rapamycin. For example, normal chromaffin cells depend on NGF, whereas PC12 pheochromocytoma cells (malignat conterpart of chromaffin cells) proliferate in the absence of mitogens. Rapamycin suppressed normal chromaffin cell proliferation, whereas PC12 cells were refractory to the antiproliferative effect of rapamycin although rapamycin inhibited mTOR. This suggests, as expected, that a proliferative signal normally requiring mTOR either is unnecessary in PC12 cells or is provided by a downstream pathway (Powers et al., 1999). Rapamycin (at doses as low as 0.1-1 nM) inhibited growth of the nonmalignant hematopoietic cell line BAF-1 (Metcalfe et al., 1997). Since rapamycin is already clinically used as an immunosuppressant, its antiangiogenic potential and prevention of re-stenosis were intensively studied in endothelial cells, lymphocytes and endothelial cells. Indeed, rapamycin inhibits proliferation of normal lymphocytes and prevents their response to mitogens, thus causing immunosuppression (Hidalgo and Rowinsky, 2000). At low concentrations (10 ng/ml), rapamycin also inhibited endothelial cell proliferation and their response to GFs

including VEGF (Yu and Sato, 1999; Guba et al., 2002). It prevented induction of cyclin D1, which is necessary to start the transition to S phase. At these doses, rapamycin did not inhibit tumor cell proliferation (Guba et al., 2002). In most transformed cells, mitogenic signaling bypasses rapamycin-sensitive steps. The target of rapamycin mTOR kinase is downstream of the PI3 kinase/AKT-signaling pathway, which is up regulated in multiple cancers because of loss of the PTEN tumor suppressor gene. Therefore, only PTEN-deficient cancer cells and cancer cells transformed by PI3K and AKT were sensitive to the rapamycin derivative CCI-779 in vitro and *in vivo* (Neshat et al., 2001; Podsypanina et al., 2001). Rapamycin effectively blocked oncogenic transformation induced by either PI3K or AKT but transformation by 11 diverse oncoproteins was refractory to inhibition by rapamycin (Aoki et al., 2001). Moreover, transformation by RAS and Myc was enhanced (Aoki et al., 2001). It has been shown that normalization of Ki-ras-induced transformed phenotypes by U0126 (a kinase inhibitor) is a consequence of concurrent inhibition of MAPK and mTOR pathways. Simultaneous inhibition of the MAPK pathway and the mTOR pathway by PD98059 in conjunction with the mTOR inhibitor rapamycin restored the normal phenotype. U0126 or the combination of PD98059 and rapamycin flattened morphology of v-src-transformed cells, but did not reverse anchorage independence. The results suggest that normalization of Ki-ras-induced transformed phenotypes by U0126 is a consequence of concurrent inhibition of the MAPK and mTOR pathways. Simultaneous blockade of more than one signal transduction pathway by combining selective inhibitors might be effective in suppressing uncontrolled tumorigenic growth (Fukazawa and Uehara, 2000).

6.2.4. Staurosporine and UCN-01

Low concentrations of staurosporine (kinase inhibitor) arrested growth of normal, but not malignant cells (Bruno et al., 1992; Chen et al., 1999). Tumor cells displayed little to no inhibition of growth at low UCN-01 concentrations (i.e. <80 nM). In cancer cells, UCN-01 caused growth arrest at concentrations between 200-1000 nM (IC_{50} >1000 nM in the prostate cancer cell line LNCaP). In comparison, in normal prostate epithelial cells and endothelial cells, IC_{50} was found to be around 25-35 nM (Blagosklonny et al., 2001; Kruger et al., 1998-99). Similarly, normal mammary epithelial cells were very sensitive to UCN-01 with IC_{50} (10 nM). Rb was found to be essential for UCN-01-mediated G1 arrest in normal cells (Chen et al., 1999). Rb status was also a determinant of response to high concentrations of UCN-01 in non-small cell lung carcinoma (Mack et al., 1999). *In vivo,* 5 mg/kg UCN-01 induced inhibition of BrdU incorporation in intestinal epithelial cells and G1 arrest of bone marrow cells. This may predict protection from topotecan-induced bone marrow toxicity (Redkar et al., 2001).

6.2.5. Inhibitors of PI3 Kinase

At 5 μM the wortmannin analog LY294002, a PI3K inhibitor, arrested normal lymphocytes but not leukemia cell lines (Du et al., 2001). Such cancer cell lines as DLD-1 and HCT15 were resistant to 20 μM LY294002 (Semba et al., 2002).

7. MECHANISM-BASED CYTOSTATIC AGENTS PROTECT FROM CELL CYCLE-DEPENDENT CHEMOTHERAPY

7.1. G1 Arrest Protects from S-Phase Active Agents

Staurosporine causes selective and reversible G1 arrest, which can selectively protect normal proliferating cells against chemotherapy with camptothecin (Chen et al., 2000), (Fig. 3). In contrast, cancer cells are killed by campthothecin in the presence of staurosporine (Chen et al., 2000). Also, 5 μM of LY294002, a PI3K inhibitor, arrests lymphocytes, but not leukemia cells that selectively protect normal cells against chemotherapy with camptothecin and Ara-C (Du et al., 2001). Similarly, topical 1,25-dihydroxyvitamin D3 protected mice against cyclophosphamide-induced alopecia (hair loss) (Chen et al., 1998). Disruption of mitogenic signaling either upstream, at, or downstream of RAS resulted in a temporal interruption of the cell cycle in normal cells, while those cells transformed by oncogenes, or tumor cells themselves often were not affected (Weyman and Stacey, 1996). During these cell cycle blocking treatments the cells were exposed to the DNA topoisomerase II inhibitor, m-AMSA. This anticancer drug is selectively toxic to cycling cells. In each case, the tumor cells were selectively killed (Weyman and Stacey, 1996). The $p21^{Cip1/Waf}$ -induced cycle arrest in G1 protected cells from apoptosis induced by UV-irradiation or RNA polymerase II blockage (Bissonnette and Hunting, 1998).

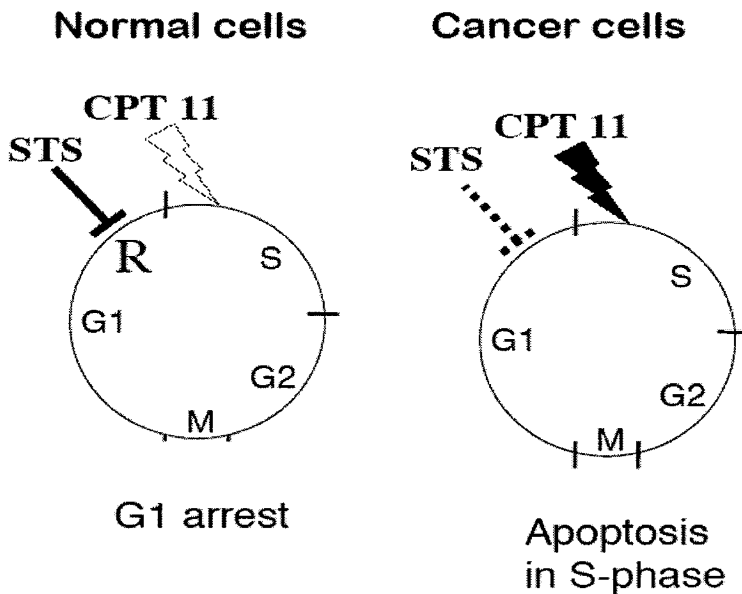

Figure 3. Exploiting loss of G1-restriction point for therapy with the S-phase targeting drugs. Low concentrations of staurosporine (STS) arrest normal cells in G1, protecting them from the cytotoxicity of topoisomerase I inhibitor (CPT-11), while cancer cells progressing through S remain sensitive to CPT-11.

7.2. G2 Arrest Protects from Paclitaxel

It has been shown that $p21^{Cip1/Waf}$ protected cells from paclitaxel (Barboule et al., 1997; Stewart et al., 1999; Yu et al., 1998; Blagosklonny et al., 2002a). Low concentrations of doxorubicin induce p53 and $p21^{Cip1/Waf}$ caused G2 arrest in HCT116 cells (Bunz et al., 1998; Bunz et al., 1999; Blagosklonny et al., 2002b), thus protecting HCT116 cells from paclitaxel (Blagosklonny et al., 2000b), (Fig. 4). HCT116 cells lacking either p53 or $p21^{Cip1/Waf}$ (a defective G2 checkpoint) still entered mitosis and were killed by paclitaxel (Blagosklonny et al., 2000b). Low concentrations of flavopiridol (40-50 nM) induced $p21^{Cip1/Waf}$ and caused growth arrest in $p21^{Cip1/Waf}$ -hypersensitive cells (LNCaP, HL60, and SKBr3), while protecting these cells from paclitaxel (Blagosklonny et al., 2002a). Unlike LNCaP cells, the highly autonomous PC3M cells were not protected from paclitaxel (Blagosklonny et al., 2002a).

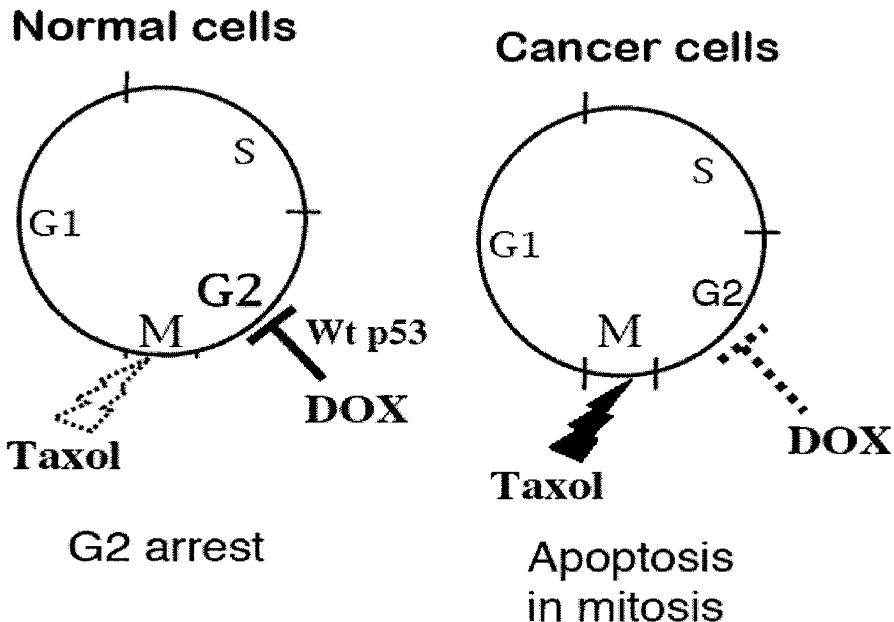

Figure 4. Exploiting loss of G2-checkpoint for therapy with M-phase targeting drugs. Low concentrations of doxorubicin (DOX) arrest normal cells having wtp53, in G2, protecting them from the cytotoxicity of the microtubule-targeting drugs (Taxol). Cancer cells that lack p53 enter mitosis where they become entrapped and ultimately undergo apoptosis by Taxol.

8. TWO BIRDS WITH ONE STONE: SELECTIVE SENSITIZATION TO APOPTOSIS

There are many mechanisms used to avoid apoptosis including, inactivation of caspases and activation of antiapoptotic kinases that inhibit caspases. These include activated receptor tyrosine kinases (RTKs). In turn, RTKs activate numerous mitogen-

activated pathways such as the RAF-1/MEK signaling pathway and the PI3K/AKT/mTOR pathway. These pathways transduce mitogenic and survival signaling. Constitutive activation of mitogen-activated kinases is often associated with cancer.

When induced by downstream oncogenes (e.g., c-myc, E2F, cyclins), apoptosis can be prevented by upstream signaling (e.g., growth factors, RAS, RAF, PI3K and AKT) (Kauffmann-Zeh et al., 1997; Bonni et al., 1999; Ballif and Blenis, 2001). Mitogens block apoptotic cascade on several levels: phosphorylation of BAD, release of cytochrome *c* and other pro-apoptotic molecules from mitochondria and inhibition of apoptosis at a postmitochondrial level (downstream of cytochrome *c*) including inhibition of caspases (Harada et al., 2001; Cardone et al., 1998; Erhardt et al., 1999). In addition, AKT suppresses c-myc-induced apoptosis downstream of Fas (and FADD), but upstream of caspase-8 (Rohn et al., 1998). Thus, autonomous growth and apoptosis avoidance is typically achieved by activation of cooperative oncogenes (Evan and Littlewood, 1998). C-myc, E2F, cyclin A or loss of Rb promote proliferation, but also may render cells vulnerable to apoptosis. Forced over expression of "downstream" oncogenes such as c-myc induces apoptosis in certain cells in the absence of mitogenic signaling. In cancer cells, "downstream" oncogenes such as c-myc or E2F drive proliferation, whereas mitogenic kinases (ErbB, MEK, RAF-1, PI3K, AKT, mTOR) inhibit apoptotic cascades (Evan and Littlewood, 1998).

9. THIRD BIRD WITH THE SAME STONE: INHIBITION OF ANGIOGENESIS

Angiogenesis is absolutely required for tumor growth. Therefore, inhibition of angiogenesis (antiangiogenic therapy) can prevent tumor growth (Folkman, 1971; Hanahan and Folkman, 1996; Harris, 2002; Blagosklonny, 2004). Tumor-derived endothelial cells can be targeted selectively (Browder et al., 1999). Endothelial cells are highly sensitive to inhibitors of proliferation. Moreover, proliferating tumor-nourishing endothelial cells and remodeling vessels are intrinsically more sensitive to antiangiogenic agents than resting endothelial cells. Thus, antiangiogenic therapy may have few side-effects and low toxicity.

UCN-01, a kinase inhibitor, and rapamycin (RAPA), an inhibitor of TOR, both directly target endothelial cells and inhibit production of VEGF (Kruger et al., 1998-99; Zhong et al., 2000; Guba et al., 2002; Adjei and Rowinsky, 2003; Potter and Harris, 2004; Melillo, 2004). Most importantly, RAPA is already used in the clinic for different applications. It is namely used to inhibit proliferation of normal lymphocytes to cause immunosuppression. RAPA was shown to inhibit metastatic tumor growth and angiogenesis in *in vivo* mouse models. In addition, immunosuppressive (low) doses of RAPA effectively controlled the growth of established tumors (Guba et al., 2002). RAPA showed antiangiogenic activities linked to a decrease in production of VEGF and to a markedly inhibited response of endothelial cells to stimulation by VEGF (Guba et al., 2002). Thus, at low concentrations (10 ng/ml), RAPA is able to directly inhibit basal, as well as VEGF-induced proliferation of endothelial cells and to decrease levels of VEGF.

In any case, RAPA did not directly inhibit tumor cell proliferation (Guba et al., 2002). This indicates that RAPA may have indirect antitumor effects in animals. Furthermore, current chemotherapeutic agents inhibit growth of endothelial cells (Kerbel

and Folkman, 2002). Therefore, at low doses and with long term chemotherapy (antiangiogenic schedule), conventional chemotherapeutics can be used as antiangiogenic agents. Even if tumor cells are resistant to anticancer drugs, a tumor may respond to therapy due to the inhibition of vessel formation (Kerbel, 1997).

10. INHIBITION OF MITOGEN-ACTIVATED SIGNALING SENSITIZES CANCER CELLS TO CHEMOTHERAPY

Normal cells undergo growth arrest following withdrawal of growth factors, whereas oncogene-transformed cells may undergo cell death (Baserga, 1994). For human cancer cells, inhibition of mitogenic kinases may render these cells sensitive to chemotherapy. In other words, the inhibition of mitogenic signaling "releases" apoptotic pathways (Fig. 2). For example, human pancreatic adenocarcinoma cell lines PK1 and PK8 are resistant to gemcitabine, a cytidine analog. Like primary pancreatic cancers, these cell lines carry constitutively active Ki-RAS and overexpress multiple tyrosine kinases. Both genetic abnormalities may potentially up-regulate the activity of the PI3K/AKT cell survival pathway. After exposure to 20 μM of gemcitabine for 48 h and in continuous presence of the drug, treatment with the PI3K inhibitors wortmannin (50-200 nM) and LY294002 (15-120 μM) for 4 h substantially enhanced apoptosis (Ng et al., 2000). As another example, Trastuzumab (antibody against ErbB2) can effectively sensitize ErbB2-overexpressing breast cancer cells to paclitaxel by reversing the antiapoptotic function of ErbB2 (Yu and Hung, 2000). Inhibition of MEK and PI3K/AKT pathways increased radiosensitivity (Tenzer et al., 2001) and also sensitized non-small cell lung cancer cells to chemotherapy and radiation (Brognard et al., 2001; MacKeigan et al., 2000; MacKeigan et al., 2002). Protein kinase C (PKC) can activate both MEK and PI3K/AKT pathways. Therefore, agents that inhibit PKC circumvent resistance to 1-β-D-arabinofurano-sylcytosine-induced apoptosis in human leukemia cells (Wang et al., 1997). The MAPK pathway is a potential route to radio- and chemo-sensitization of tumor cells resulting in the induction of apoptosis and loss of clonogenicity (Dent et al., 1998). LY294002 and wortmannin (both inhibitors of PI3K) can decrease apoptosis-resistance of cells when receptor RTK is over activated (Bacus et al., 2002).

ZD1839 is a quinazolone that inhibits the EGF receptor with an IC_{50} of 0.02 μM. This leads to a blockade of EGFR downstream signal transduction pathways, including the MAPK and the PI3K/AKT pathways, cell cycle arrest, inhibition of angiogenesis, and augmentation of the antitumor effects of chemotherapy and radiation therapy (Baselga, 2002a).

In phase I study, ZD1839 (an inhibitor of the EGF receptor) was administered for 4 consecutive days (Ranson et al., 2002). Common side effects were a skin rash and diarrhea. Similarly, OSI-774 (another EGFR inhibitor) induced skin rashes and diarrhea. The dose-limiting toxicity occurred at 700 mg/day which is a dose well above the dose range required to achieve complete inhibition of the EGF receptor and downstream receptor signaling in skin biopsies of patients participating in ZD1839 clinical trials. There were partial responses in 4 of 16 patients with non-small cell lung cancer at doses ranging from 300 to 700 mg/d. ZD1839 inhibits EGFR activation *in vivo* at doses well below the one producing unacceptable toxicity. This finding strongly supports

pharmacodynamic assessments to select optimal doses instead of a maximum-tolerated dose for definitive efficacy and safety trials (Albanell et al., 2002). As summarized by Baselga, "the observed response rates with ZD1839, although exciting, are modest. It is likely that only a subset of tumors will be sensitive to EGFR inhibition. Other unresolved issues include the best administration schedule (intermittent versus continuous) and the optimal combinations of chemotherapy and EGFR tyrosine kinase inhibitors that will result in a greater synergistic interaction" (Baselga, 2002a).

How would we suggest combining ZD1839 with chemotherapy to increase therapeutic index? The side effects indicate that the inhibition of normal cell cycling had been achieved. Lack of therapeutic responses indicates that cancer cells were not affected. Since ZD1839 alone caused side-effects, the dose of ZD1839 (700 mg/day) was too high or duration of therapy (14 days) was too long. The answer is to use a pretreatment dose below 700 mg with co-administration of a chemotherapeutic that targets cycling cells (e.g, paclitaxel). Then, pulse-treatment with ZD1839 will cause selective and protective growth arrest of normal cells. Simultaneously, this may sensitize cancer cells to pro-apoptotic stimuli. Then, chemotherapy can be performed with a wider therapeutic index and a higher therapeutic index may be translated in a curative outcome.

11. CONCLUSIONS

- Certain chemotherapeutic drugs at certain concentrations preferentially kill proliferating (both normal and cancer) cells (e.g. vinblastine kills cells in mitosis and camptothecin in S-phase).
- Loss of restriction and checkpoints of the cell cycle (e.g. due to loss of Rb or overexpression of Myc) is a hallmark of cancer.

Therefore, using a mechanism-based cytostatic agent, the goal is: (*i*) To reversibly arrest normal cells. (*ii*) To reactivate apoptotic pathways in cancer cells. Then, a chemotherapeutic agent that induces apoptosis in proliferating cells will kill cancer cells selectively. Furthermore, arresting normal cells is expected to have anti-angiogenic effect, additionally suppressing growth of cancer.

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Appendix 1.

Summary Table: New therapeutic agents designed by exploring the recent progress on our understanding of cell death pathways.

Pathway /Enzyme	Ch.[1]	Chemical Compound, Mode of Action	Code- or Brand Name	Phase of Development	Affiliation of the Research Project
Caspases	I, IV, XV	irreversible pan-caspase inhibitor z–VAD-fmk		-in a rat-model, a broad spectrum caspase inhibitor, z-VAD-fmk (dose: 3 mg/kg, i.v.) when co-injected with endotoxin, completely prevented endotoxin-induced myocardial dysfunction evaluated at 4 h and 14 h following endotoxin challenge	INSERM, France, (non-profit, gov.-sponsored org.)
				-early phase, prove of principle experiments, EFFECTIVE in experimental models of heart infarct (Yaoita et al., 1998; Dumont et al., 2001; Chapman et al., 2002)[2], traumatic brain injury (Felderhoff-Mueser et al., 2002; Bittigau et al., 2003; Fink et al., 1999), amyotrophic lateral sclerosis (Li et al., 2000), Huntington's Disease (Chen et al., 2000), systemic lupus erythematosus (Seery et al., 2001), autoimmune disease (Saegusa et al. 2002; Iwata et al. 2003), autoimmune encephalomyelitis (Furlan et al., 1999), peritonitis (Catalan et al., 2003), sepsis (Catalan et al., 2003), pneumococcal meningitis (Braun et al., 1999), pulmonary fibrosis (Kuwano et al., 2001), endotoxin-induced liver failure (Valentino et al., 2003), Apo-1/Fas-mediated induced liver failure (Rodriguez et al., 1996; Kunstle et al., 1997), endotoxin-induced myocardial dysfunction (Fauvel et al., 2001; Neviere et al., 2001). The effectiveness in neuronal transplantation and stroke is currently being debated -ADVERSE EFFECTS: exacerbated TNF-mediated liver failure, counteracted HIV clearance from peripheral blood	Supplied by Enzymes Systems Products, Dublin, CA, USA
More information on peptide-based caspase inhibitors can be found in Table 3, Ch. IV.					
Caspase-1 & -4	IV, IX	selective inhibitor originated from specific substrate peptide motif	Pralnacasan, VX–740, HMR–3480	-in a Type II collagen-induced rat rheumatoid arthritis model, pralnacasan is effective at 50 mg/kg, for over 60 days; well tolerated in animal models, (Randle et al., 2001) -encouraging results in phase I clinical studies, currently in phase II trials for rheumatoid arthritis treatment	Vertex Pharmaceuticals Inc. / Aventis Pharma AG

Summary Table: (continued)

Caspases-3 & -7	IV	(S)-(+)-5-[1[(2-methoxymethylpyrrolidinyl) sulfoyl]isatin, reversible inhibitor of Caspases-3 and -7	MMPSI	-in experimental models, reduced ischemic injury in isolated rabbit hearts or cardiomyocytes, at conc. 0.1-10 μM evoked a concentration-dependent reduction in infarct size (up to 56% vs. control; IC(50)=0.2 μM), inhibited rec. human caspase-3 with an IC(50)=1.7 μM, reduced apoptosis after 16 h simulated ischemia and 2 h simulated reperfusion in isolated adult rabbit cardiomyocytes (IC-50=1.5 μM), (Chapman et al., 2002)	Pfizer Inc., Groton, CT, USA
Caspase-3 & -7, to lesser degree Caspase-4, -4, -6, 8 & -10	IV	(3*S*)-3-({2*S*)-2-[5-*tert*-butyl-3-{[4-methyl-1,2,5-oxadiazol-3-yl)methyl]amino}-2-oxopyrazin-1(2H)-yl]butanoyl}amino)-5-[methyl(pentyl)-amino]-4-oxopentanoic acid, reversible inhibitor	M867	-EFFECTIVE in experimental models of sepsis and peritonitis (Methot et al., 2004; Hotchkiss et al., 2000)	Merck Frosst Centre for Therapeutic Research, Montreal, Qc, Canada
Caspase-3, to lesser degree -1, -7, -8, -4, -6, and -5	IV	Active center caspase inhibitor	M920 / L–826,920	-strongly (~80%) reduces mortality in a murine and rat sepsis model by preventing from sepsis-related apoptosis of B- and T-cells, (Hotchkiss et al., 2000) -EFFECTIVE in experimental models of peritonitis and sepsis (Methot et al., 2004)	Merck Frosst Canada & Co.
Caspase-3	IV	highly selective caspase-3 inhibitor	M791 / L-826,791	-strongly (~80%) reduces mortality in a murine and rat sepsis and peritonitis models by preventing from sepsis-related apoptosis of B- and T-cells, (Hotchkiss et al., 2000; Methot et al., 2004)	Merck Frosst Canada & Co.
Caspase-1	IV	Caspase-1 active site mutant C285G, (dominant negative)		-prevents the activation of endogenous caspase-1 -early phase, prove of principle experiments, EFFECTIVE in experimental Huntington's Disease (Ona et al., 1999; Fink et al. 1999), stroke (Friedlander et al., 1997), traumatic brain injury (Fink et al. 1999), Parkinson's Disease (Klevenyi et al., 1999)	Harvard Med. Sch., Boston, Massachusetts, USA
Caspase-3 & -7	IV	NAIP fragment containing BIR-domains		-early phase, prove of principle experiments, EFFECTIVE in experimental models of stroke, reduces ischemic damage in the rat hippocampus, counteracts the loss of function (Xu et al., 1997)	Dept. of Cell. & Mol. Med., Univ. Ottawa, Ontario, Canada

Summary Table: (continued)

Caspase-3, -7 & -9	IV	XIAP, whole molecule		-early phase, prove of principle experiments, EFFECTIVE in experimental models of stroke, attenuates ischemia-induced cellular and behavioral deficits (Xu et al., 1999; Du et al., 2001), ocular hypertension-mediated neuronal damage (McKinnon et al. 2002), see also chapter IV, table 1.	Depart. Cell. & Mol. Med., Univ. Ottawa, Ontario, Canada; Dept. Pharm. & Tox., Indiana Univ. Sch. Med., Indianapolis; Dept. Ophthalm., Univ. Texas, San Antonio, Texas, USA
Caspase-3, -7 & -9	IV	XIAP fused to the protein transduction domain of TAT (cell membrane permeable)	PTD-XIAP	-early phase, prove of principle experiments, EFFECTIVE in experimental models of stroke (Onteniente et al. 2003)	INSERM U421/IM3, Universite Paris-Val-de-Marne, Cedex, France
Caspase-9, to lesser degree –2, –8, -1, -6 & -3	IV	*N*-[(indole-2-carbonyl)-alaninyl]-3-amino-4-oxo-5-fluoropentanoic acid, Irreversible inhibitor of caspase-9	IDN-1529	-EFFECTIVE in experimental models of heart infarct & septic shock (Dumont et al., 2001; Grobmyer et al., 1999)	Idun Pharmaceuticals Inc.; Cardiovasc. Res. Ins., Maastricht, the Netherlands
Caspase-1	IV	benzyloxycarbonyl-Val-Ala-Asp-(O-Et)-CH_2O-dichlorobenzoate, *in vivo* it is rapidly hydrolysed to VE–16,084, an irreversible pept. caspase-1 inhibitor	VE-13,045	-early phase, prove of principle experiments, EFFECTIVE in experimental models of acute inflammation, collagen-induced arthritis (Ku et al. 1996), and severe hemorrhagic pancreatitis (Norman et al. 1997)	Vertex Pharmaceuticals, Inc., Cambridge, MA, USA
Caspases		caspase inhibitor	IDN-5370	-protective towards apoptosis induction in cortical- and synaptic neurons -reduces infarct size in a rodent cardiac ischemia/reperfusion model, by more than 50%	Idun Pharmaceuticals Inc.

Summary Table: (continued)

Caspases, most potent against –9, -6 & 8	IV	caspase inhibitor N-[(1,3-dimethylindole-2-carbonyl)valinyl]-3-amino-4-oxo-5-fluoropentanoic acid	IDN-1965	-ED_{50} by i.p. administration is 0.14 mg/kg, by i.v. administration is 0.04 mg/kg and by oral administration is 1.2 mg/kg -beneficial in experimental models of liver transplantation (Natori et al., 1999) -protects from anti-CD95-induced death and liver damage in murine system, (Hoglen et al., 2001; Mazur et al., 1998) -increased survival in a Gaq-40 transgenic mouse model of heart failure (left ventricular hypertrophy, left ventricular dysfunction) -all treated animals showed improved fractional shortening and reduced left ventricular end-diastolic diameter compared with control, placebo-treated animals (Dumont et al., (2001) -ineffective in experimental models of septic shock (Grobmyer et al., 1999)	Idun Pharmaceuticals Inc. / Mayo Foundation
Caspases		caspase inhibitor	VX-799	-a potent small molecule caspase inhibitor -VX-799 was very effective in several animal models of bacterial sepsis -clinical trials in preparation	Vertex Pharmaceuticals Inc.
Caspases	IV	peptidomimetisc	IDN-6556	-bioavailable in all tissues, highest concentration in liver (Hoglen et al., 2003) -EFFECTIVE: Fas-mediated liver injury, mild hepatic impairment (clinical trial, see Saegusa et al., 2002) -ADVERSE EFFECTS: Phlebitis after intravenous administration to clinical trial patients (Saegusa et al., 2002)	Idun Pharmaceuticals Inc.
Caspase-3		selective activation of caspase-3		-caspase-3 zymogen is maintained in an inactive conformation by a regulatory triple–Asp-motif, so called "safety-catch" localized within a flexible loop near the large-subunit/small-subunit junction (Roy et al., 2001, Proc Natl Acad Sci USA, 98: 6132-7) -the inhibitory mechanism depends on electrostatic interaction -screen for "small molecules" capable of disrupting the interaction is in progress	Merck Frosst Canada & Co
Caspase-3 (preferen-tially)	IV	irreversible inhibitor of casppase-3 & to lesser degree caspase-1, Quinoline-Val-Asp(OMe)-CH_2-OPH	OPH-001	-early phase, prove of principle experiments, EFFECTIVE in experimental models of acute tubular necrosis (Melnikov et al. 2002)	Dept. Med., Univ. Colorado Sch. Med., Denver, Colorad, USA

Summary Table: (continued)

Caspase-3		recombinant caspase-3 linked to an antibody		-recombinant caspase-3 linked to the antibody Herceptin (Genentech Inc) tested in animal tumor model	Immunex
Caspase-1, -3, -6, -7, –8, -9	IV	z-VD-fmk benzyloxycarbonyl-Val-Asp-fluoromethylketone), less potent than tripeptide or tetrapeptide inhibitors	MX1013 / CV1013	-early phase, prove of principle experiments, EFFECTIVE in experimental models of CD95-mediated liver failure, stroke, heart attack (Yang et al., 2003b), endotoxin-induced liver failure, (Jaeschke et al., 2000)	Cytovia, Inc. & Maxim Pharmaceuticals, Inc., San Diego, CA, USA.
Caspases		caspase activator	MX-2060	-"small molecule" caspase activator, a potential anticancer agent, disrupts electrostatic interactions within caspase-3 secondary structure - tested in human cancer xenograft animal models	Maxim Pharmaceuticals Inc.
Caspases	IV, X	tetracycline derivative, caspase inhibitor	Minocycline	-lipophilic, penetrates the blood-brain barrier, currently used for treating rheumatoid arthritis, EFFECTIVE in experimental models of spinal cord injury (Lee et al., 2003), amyotrophic lateral sclerosis (Zhu et al., 2002; Kriz et al., 2002; Van Den Bosch et al., 2002; Zhang et al., 2003), and in some models of Parkinson's Disease (Du et al., 2001). The effectiveness in Huntington's Disease is hotly debated. -ADVERSE EFFECTS: Exacerbated MPTP-induced damage to dopaminergic neurons (Yang et al. 2003)	Korea Inst. Sci. & Techn., Seoul, Korea; Dept. Pharmacol. & Tox., Indiana Univ. Sch. Med., Indianapolis, IN; Weill Med. Col., Cornell Univ., NY, USA; Lab. Neurobiol., Campus Gasthuisberg, Leuven, Belgium
Aspartases	IV	z-D-fmk (Boc-Asp-fluoromethylketone)		-early phase, prove of principle experiments, EFFECTIVE in experimental models of stroke (Cheng et al. 1998) and heart infarct (Huang et al. 2000)	Dept. Neur., Washington Univ. Sch. Med., St. Louis, USA; Merck Frosst, Dorval, Canada
Calpain	X	z-Leu-Abu-CONH-CH_2CH_3, inhibits calpain	AK275	-75% reduction in infarct volume when direct AK275 was perfused directly onto the infarcted tissue up to 3 h after middle cerebral artery occlusion in the rat (Bartus et al., 1994)	

Summary Table: (continued)

Calpain	X	carbobenzylzoxy-Val-Phe-H, inhibits calpain	MDL 28,170	-reduced infarct volume when administered up to 6 h intravenously after temporary middle cerebral artery occlusion in the rat (Markgraf et al., 1998)	
Calpain	X	3-(4-iodophenyl)-2-mercapto-(z)-2-propenoic acid, inhibits calpain	PD150606	-attenuated hypoxic/hypoglycemic injury to cerebrocortical neurons in culture and excitotoxic injury to Purkinje cells in cerebellar slices (Wang et al., 1996)	
XIAP	VI	organic molecule (caspase activator)	TWX024	-Enhance CD95-induced apoptosis in XIAP overexpressed cells (Wu et al., 2003)	The Scripps Research Institute, La Jolla, CA, USA
XIAP	VI	polyphenylureas (caspase activator)	1396-34	-induced apoptosis in many cancer cell lines *in vitro* -sensitizes cancer cells to chemotherapeutic drugs -inhibits tumor growth in mouse xenograft model *in vivo* -little toxicity to normal tissues (Schimmer et al., 2004)	The Burnham Institute, La Jolla, CA, USA
XIAP	VI	peptides (caspase activator)		-finish screening from two different phage-peptide libraries. -permeable version of the CPFKQC peptide induces apoptosis in leukemia cells (Tamm et al., 2003)	The Burnham Institute, La Jolla, CA, USA
XIAP		cell permeable small molecule of herbal origin, XIAP–inhibitor		-discovered by screening of 3D–databases, obtained from the Jap. Ardisia herb -binds to the XIAP BIR3 domain with an affinity similar to that of the natural Smac peptide -inhibits cell growth, induces apoptosis and activates caspase-9 in prostate cancer cells (Nikolovska-Coleska et al., J Med Chem. 2004, 47:2430-40.	Univ. Michigan Comprehensive Cancer Center, Ann Arbor, Michigan, USA.
TRIAL pathway	III	mAbs against DR5 and DR5	HGS-ERTR1, HGS-ERTR2	-phase I clinical trials in UK and USA. The studies in UK are on solid tumors whereas the studies in the USA are on leukemias. -preclinical studies on cell lines have been published as an abstract at 2004-ASH conference.	Human Genome Sciences

Summary Table: (continued)

Bcl-2	II, V, XI	antisense 18-mer-oligonucleotide targeting codons 1-6 (phosphorothioate)	G3139, Genasense	-phase I/II studies genasense have demonstrated an excellent safety profile with toxicity observed in 20% of patients, fatigue in 10% and rash in 5%, the symptoms reverse upon withdrawal of treatment -in phase I trials for NHL (Webb et al., 1997; Waters et al., 2000) and prostate cancer (Morris et al., 2002) showed safety as a single agent -in phase I trial of chemoresistant AML/ALL (Marcucci et al., 2003), in phase I-II study of malignant melanoma (Jansen et al., 2000), phase I trial of hormone-refractory prostate cancer (Chi et al., 2001; Tolcher et al., 2001), G3139 was well-tolerated and effective in combination with standard chemotherapy. -in phase III trials for malignant melanoma, (Banerjee et al., 2001) -also successful in animal studies in breast cancer (Lopes de Menezes et al., 2000; Lopes de Menezes et al., 2003) colon cancer (Tortora et al., 2001), gastric cancer (Wacheck et al., 2001)	Genta Inc.
Bcl-x_L	V	antisense 20-mer-oligonucleotide	ODN 4259	-induced apoptosis in NSCLC cell line (Leech et al., 2000)	University Hospital, Zurich, Switzerland/ Novartis Pharma
Bcl-2/ Bcl–x_L	V	bispecific 20-mer antisense	ODN 4625	-slowed tumor growth in animal studies of breast and colon cancer (Zangemeister-Wittke et al., 2000; Gautschi et al., 2001) -induced apoptosis in melanoma cell lines and cultured patient samples (Olie et al., 2002; Starsberg Rieber et al., 2001)	University Hospital, Zurich, Switzerland/ Novartis Pharma
Bcl–x_L/ Bcl-x_S	V	antisense 20-mer oligonucleotide shifts splicing in favor of Bcl–x_S	ISIS 22783	-shifts splicing, and allows a decreased production of Bcl-x_L and increased production of pro-apoptoic Bcl-x_S. Sensitized lung cancer cells to chemotherapy (Taylor et al., 1999)	Isis Pharmaceuticals, CA, USA
Bcl-x_L/ Bcl-x_S	V	antisense 18-mer oligonucleotide shifts splicing in favor of Bcl–x_S	5'Bcl-x AS	-shifts splicing, induces cell death, and promotes chemosensitivity of breast and prostate cancer cell lines (Mercante et al., 2001; Mercante et al., 2002)	Lineberger Compreh. Cancer Center, Dept. Pharmacol., Univ. North Carolina/ NIH
Bcl-2 and or Bcl-x_L	V	Bad BH3 Peptide 27-mer	cpm-1285	-induced cell death of leukemic cell lines *in vitro* and reduced the growth of leukemic cells in a mouse model of AML (Wang et al., 2000)	Kimmel Cancer Center, Philadelphia, USA

Summary Table: (continued)

Bcl-2, Bcl–x_L	V	small mol. identified by cell-based screening of the inhibitors of mitoch. respiration	Antimycin A_3	-binds to the surface pocket in Bcl-2 and Bcl-x_L -selectively kills Bcl-2- and Bcl-x_L-overexpressing hepatocyte cell lines (Tzung SP et al., 2001)	Fred Hutchinson Cancer Research Center, USA/ University of Seattle, USA/ NIH
Bcl-2, Bcl–x_L	V	small molecule inhibitor of Bcl-x_L, identified in Bcl-x_L binding assays	BH3Is	-bind to Bcl-x_L, disrupt heterodimerization between pro-and anti-apoptotic Bcl-2 family proteins, and induce apoptosis in cell lines (Degterev et al., 2001)	Harvard Medical School, USA/ MIT, USA
Bcl-2, Bcl–x_L	V	small molecule identified in Bcl-x_L binding assays	Gossypol	-binds to Bcl-x_L, and exhibits antitumor activity in cell lines, and in animal models of cancer (Kitada et al., 2003)	The Burnham Institute, CA, USA
Bcl-2	V, XII	small molecule identified based on 3-D structure of Bcl-2	HA14-1	-binds to Bcl-2 (Wang et al., 2000) and sensitizes primary leukemic cells to chemotherapy (Lickliter et al., 2003). -in leukemic and myeloma cell lines, HA14-1 synergizes with small-molecule inhibitors of MEK (Milella et al., 2002) or PBR (Chen et al., 2002) or the proteasome (Pei et al., 2003)	Kimmel Cancer Center, Philadelphia, USA
Bcl-2	X	over-expression of Bcl–2	viral vectors that over-express Bcl-2	-direct injection of viral vectors over-expressing Bcl-2, into the infarct margin improved neuronal survival in transient or permanent focal ischemia in the rat (Zhao et al., 2003)	
Retinoid_ receptor-driven transcr., synergy with TRAIL		retinoid acid derivative: 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid	CD-437 AHPN	-facilitates mitochondria and caspase-3 dependent apoptosis -increases expression of Bad and down-regulates Bcl-2 expression -synergy effect between recombinant TRAIL and CD-437 observed in a number of cancer cell lines and in human tumor xenografts	Anderson Cancer Center, USA / CIRD Galderma
Survivin		antisense oligodeoxynucleotides		-following transfection of antisense oligonucleotides to mouse survivin mRNA, a time- and dose-dependent increase in polyploidy of approx. 2- to 3-fold and induction of apoptosis were observed in most of the tumor cell lines (Chen et al., 2000, Neoplasia, 2: 235-41)	Isis Pharmaceuticals / Abbott Laboratories

Summary Table: (continued)

Smac/ DIABLO		exclusive rights patented		-exclusive rights to develop Smac-based therapy have been patented, -Smac inhibitor screening program have been started	Idun Pharmaceuticals
p53	VII	stabilizes p53 by inhibition of ubiquitination without altering phosphorylation at Ser 15 or 20 or MDM2 binding	CP-31398	-rescues destabilized mutant p53 expression and to promote the activity of wild-type p53	Pfizer
p53	VII	restores mutant p53 to its active conformation and induces apoptosis in mutant p53 carrying cells	PRIMA	-a low-molecular-weight compound, capable of inducing apoptosis in human tumor cells through restoration of the transcriptional transactivation function to mutant p53 -restored sequence-specific DNA binding and the active conformation to mutant p53 proteins in vitro and in living cells -*in vivo* studies in mice revealed an anti-tumor effect of PRIMA-1 with no apparent toxicity (Bykov et al., 2002)	
p53	VII	HSP-90 inhibitor, prevents proper folding of (mutated) p53 and other molecules requiring assistance of chaperons	Geldanamycin	-geldanamycin analogue in phase I clinical trials, enrolled are patients with advanced cancer	
p53	X	inhibits p53	Pifithrin-α	-mice given pifithrin-α exhibited increased resistance of cortical and striatal neurons to focal ischemic injury (Culmsee et al., 2001)	

Summary Table: (continued)

ErbB2 (HER2 /neu)	VIII, XI	monoclonal antibody	Herceptin/ Trastuzumab	-approved in September 1998 for treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have received one or more chemotherapy regimens for their metastatic disease. Trastuzumab in combination with paclitaxel is indicated for treatment of patients with metastatic breast cancer whose tumors overexpress HER2 protein and who have not received chemotherapy for their metastatic disease -in clinical trials for other types of cancer, including osteosarcoma and cancers of the lung, pancreas, salivary gland, colon, prostate, endometrium, and bladder.	Genentech, Inc, South San Francisco, CA, USA
VEGF	VIII	monoclonal antibody	Bevacizumab/ Avastin	-used to treat metastatic cancer of the colon or rectum. Avastin is given along with the chemotherapy combination known as IFL. IFL consists of irinotecan, 5-fluorouracil (5-FU), and leucovorin	Genentech, Inc, South San Francisco, CA, USA
ErbB1 (EGFR)	VIII	monoclonal antibody	IMC-C225 (Cetuximab), Erbitux	-used to treat cancer of the colon or rectum that has spread to other parts of the body. Erbitux is given with irinotecan, another anti-cancer medicine, or alone in patients who cannot tolerate irinotecan. -tested among others in: breast-, brain- and head & neck cancers.	ImClone Systems Inc., Branchburg, NJ, USA
ErbB1 (EGFR)	VIII	tyrosine kinase inhibitor	ABX-EGF/ Panitumumab	-tested for breast-, renal-, and colorectal cancer treatment -phase II clinical trials (in combination with paclitaxel and carboplatin) for the treatment of carcinoma, non-small-cell lung cancer, metastatic lung cancer	(Amgen), Immunex Corp., Seattle, WA, USA., Abgenix, Inc., Fremont, CA, USA.
Abl, BCR-ABL, Kit or PDGFR	VIII, XII, XVII	tyrosine kinase inhibitor	ST1571 Gleevac (Imatinib)	-approved for the treatment of CML, breast, prostate cancer and gastrointestinal stromal tumors	Novartis Pharmaceuticals Corp.
VEGF or PDGFR	VIII	1-[4-chloroanilino]-4-[4-pyridylmethyl] phthalazine succinate, tyrosine kinase inhibitor	PTK787 or SU11248	-tested for the treatment of gastrointestinal stromal tumors and renal cell carcinoma -under consideration for the treatment of rheumatoid arthritis -phase II trials for the treatment of unresectable malignant mesothelioma -phase I/II trial (in combination with either temozolomide or lomustine) for the treatment of recurrent glioblastoma multiforme	Novartis Pharmaceuticals Corp.

Summary Table: (continued)

ErbB1 (EGFR)	VIII, XVII	tyrosine kinase inhibitor, 4–Quinazolinamine, N-(3-chloro-4fluoro-phenyl)-7-methoxy-6-[3-4-morpholin propoxy]	ZD1839, Gefitinib, Iressa	-used for the tratment of osteosarcoma, liver cancer, lymphoma, melanoma, ovarian cancer, rhabdomyosarcoma, sarcoma, stomach cancer, teratoma, testicular cancer, thyroid cancer, uterine cancer, breast, renal, colon, prostate, cervical, lung and head & neck cancers	AstraZeneca
ErbB1 (EGFR)	VIII, XVII	tyrosine kinase inhibitor	CP358, Erlotinib, (Tarceva)	-experimental therapy of squamous cell carcinoma, head & neck, lung, glioblastoma, and breast cancer -phase I/II trial for the treatment of advanced non-small cell lung cancer (NSCLC) in a combined regimen of bevacizumab (AvastinTM) and erlotinib (TarcevaTM) -phase I B trial of gemcitabine and erlotinib HCL in patients with advanced pancreatic adenocarcinoma and other potentially responsive malignancies	Genentech, OSI, Roche
all ErbB receptors (pan-HER inhibitor)	VIII	irreversible inhibitor of pan-erbB family of tyrosine kinases, 4–anilinoquinazoline, (covalently binds to the ATP binding site in the TK domain of ErbB family)	CI-1033	-phase I clinical and pharmacokinetic (PK)/food effect study of oral CI-1033, a pan-erb B tyrosine kinase inhibitor, in patients with advanced solid tumors (colon cancer, non-small cell lung cancer, breast cancer, ovarian cancer, rectal cancer and 12 patients with other cancers)	Pfizer Global Research and Development, Ann Arbor, MI, USA
PDGFR	VIII, XIV	tyrosine kinase inhibitor	SU-101 (Leflunomide)	-phase I and pharmacokinetic (PK) study of the tyrosine kinase inhibitor SU-101 in patients with advanced solid tumors -phase III randomized study of leflunomide (SU-101) versus procarbazine for glioblastoma multiforme	Sugen
PKC	VIII, XVII	kinase inhibitor	UNC-01	-increases tumor sensitivity when combined with cisplatin -tested for experimental therapy of myelogenous leukemia	Dana Farber Cancer Institute, Harvard Med. School, Boston, Massachusetts

Summary Table: (continued)

HDAC	VII, VIII	HDAC inhibitor	FR901228, NSC 630176, SAHA, Depsipeptide	-phase I trial for the treatment of advanced thyroid, lung, colon, breast cancer, leukemia and other advanced cancers	Fujisawa Company
PKC-α	VIII	antisene oligonucleotide inhibiting the expression of PKC-α	LY900003, (ISIS-321)	-phase I/II trials for the treatment (in combination with paclitaxel and carboplatin) of advanced non-small cell lung cancer (NSCLC) -planned phase III trial of LY900003 plus gemcitabine and cisplatin versus gemcitabine and cisplatin in patients with advanced, previously untreated NSCLC -phase II trial of the efficacy and safety of ISIS 3521/LY900003 in patients with low-grade, non-Hodgkin's lymphoma -phase I trials for other solid tumors under consideration	Eli Lilly & Co.
c-Jun N-terminal kinase (JNK)	X	blocks access of JNK to targets within cell	JNK peptide inhibitor	-strong neuroprotection with intraventricular or systemic administration in two models of middle cerebral artery occlusion: transient occlusion in adult mice and permanent occlusion in 14-day-old rat pups (Borsello et al., 2003)	
c-Jun N-terminal kinase (JNK)	X	inhibits transcription of JNK mRNA	antisense oligonucleo-tides	-cerebroventricular infusion of JNKI / 2 antisense oligonucleotides significantly decreased CAI pyramidal cell death after transient brain ischaemia in the rat (Gu et al., 2001)	
Cyclin-dependent kinases (CDKs)	VII, X	inhibition of CDKs	Olomoucine	-subcutaneous infusion reduced neuronal death after transient focal brain ischaemia in the mouse (Katchanov et al., 2001)	Alexis, Grünberg, Germany
Cyclin-dependent kinases (CDKs)	X	inhibition of CDKs	Flavopiridole	-intraventricular infusion reduced neuronal death after transient focal brain ischaemia in the rat	National Cancer Center, Japan
Proteasome complex	VIII, XII, XIV	proteasome inhibitor	Velcade (Bortezomib, PS-341)	-approved for the treatment of relapsed multiple myeloma	Millennium Pharm.

Summary Table: (continued)

NF-κB	XI, XII, XIV	prevention of IκBα phosphorylation	Thalidomide	FDA-approved, currently being studied in multiple myeloma, colon cancer	Serrono Pharmaceuticals Inc.
NF-κB	XI, XIV	Prevention of IκBα phosphorylation	Rituxan Celebrex	-studied as potentially synergistic therapies when used with chemotherapy and radiation for a variety of hematopoietic and solid tumors	Genentech Pharmacia
AMPA receptors	X	competitive antagonist of AMPA receptors	YM872	-i.v. infusion significantly reduces infarct volume after permanent middle cerebral artery occlusion in the rat (Takahashi et al. 1998) -phase II clinical trial in progress	Yamanouchi Pharma America
Glutamate transporters, GABA receptors, astrocytes	X	modulates uptake capacity of glutamate transporters and exp. of GABA recs., inhibits S100β synthesis and activation of astrocytes	ONO-2506	-intravenous administration of ONO-2506 (10 mg/kg) abolished delayed infarct expansion between 24 and 168 h after permanent middle cerebral artery occlusion in the rat (Tateishi et al., 2002) -phase II and III clinical trials in progress	Ono Pharma
Free radicals	X	seleno-organic compound with antiox. activity, glutathione peroxidase-like action	Ebselen	-early treatment with oral ebselen improved the outcome of acute ischemic stroke Yamaguchi et al., 1998 Ogawa et al., 1999) -phase III clinical trials in progress	Daiichi Pharmaceutical Co. (Japan); Rhône-Poulenc Rorer (Avantis)
Free radicals	X	free radical-scavenging agent	Edaravone	-adminstration to patients within 72 h of onset of ischaemic stroke was associated with a significant improvement in functional outcome, in a randomized, placebo-controlled, double-blind study (Edaravone Acute Infarction Study, 2003, Cerebrovasc Dis, 222-229)	Mitsubishi-Tokyo Pharmaceuticals
Membrane phospholi-pids	X	stabilizes cell membranes and decreases lipid peroxidation	Citicoline	-combined analysis of four prospective, randomized, placebo-controlled, double-blind clinical trials of oral citicoline for treatment of stroke found that administration of 2 g within 24 h significantly increased the probability of complete recovery at 3 months of observation (Davalos et al., 2002)	Interneuron Pharmaceuticals, Lexington, MA

Summary Table: (continued)

Free radicals	X	nitrone spin-trap free radical scavenger	NXY-059	-neuroprotective efficacy in several experimental studies of both transient and focal brain ischaemia (Ginsberg et al., 2003; Kuroda et al., 1999; Marshall et al., 2003; Sydserff et al., 2002; Zhao et al., 2001) -phase II and III clinical trials are in progress	AstraZeneca
(mainly) IL–1β	X	competitively inhibits pro-inflammatory cytokine	IL-1 receptor antagonist, IL-1RA, Kineret, (Anakinra)	-delayed administration of interleukin-1 receptor antagonist protects against transient cerebral ischaemia in the rat (Mulcahy., et al., 2003) -clinical trial in progress -approved to treat rheumatoid arthritis -phase I study of anakinra mediated tumor regression and angiogenesis inhibition in patients with cancers producing interleukin-1	Amgen Inc.

[1]chapter number where the compound is described

[2]see chapter (indicated in the second column) for full literature listing

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