Oxidative Stress in Applied Basic Research and Clinical Practice

Douglas R. Spitz
Kenneth J. Dornfeld
Koyamangalath Krishnan
David Gius *Editors*

Oxidative Stress in Cancer Biology and Therapy

Humana Press

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Editor-in-Chief
Donald Armstrong

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All books in this series illustrate point-of-care testing and critically evaluate the potential of antioxidant supplementation in various medical disorders associated with oxidative stress. Future volumes will be updated as warranted by emerging new technology, or from studies reporting clinical trials.

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Editors

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Editors
Douglas R. Spitz, PhD
Free Radical and Radiation
Biology Program, Department of Radiation
Oncology, Holden Comprehensive
Cancer Center, The University of Iowa
Iowa City, IA, USA
douglas-spitz@uiowa.edu

Kenneth J. Dornfeld, MD, PhD
Duluth Clinic Radiation Oncology
and Department of Biochemistry
and Molecular Biology
University of Minnesota
Duluth, MN, USA
kdornfeld@smdc.org

Koyamangalath Krishnan, MD
Department of Internal Medicine
East Tennessee State University
Johnson City, TN, USA
krishnak@etsu.edu

David Gius, MD, PhD
Departments of Cancer Biology
and Pediatrics and Radiation Oncology
Vanderbilt Medical School
Nashville, TN, USA
david.gius@vanderbilt.edu

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Preface

Oxidative Stress and Cancer Biology: A Historical Perspective

Driven by Warburg's observation of increased glucose metabolism in cancer cells [1] as well as decades of research in the first three quarters of the twentieth century by Weber and many other investigators (reviewed in [2, 3]), cancer was thought to have at its origins fundamental defects in glycolytic and respiratory metabolism. This theoretical construct was based on the proposal that cancer cells had fundamental defects in their respiratory processes (O_2 metabolism) that were believed to be compensated for by increases in glycolytic metabolism. This dependence on glycolysis was thought to keep cancer cells from being able to properly regulate the switching between glycolysis and respiration which was thought to inhibit the cancer cell's ability to engage in normal higher order differentiated cellular functions.

With the discovery of oncogenes [4, 5] and tumor suppressor genes [6] in the last quarter of the twentieth century the critical importance of the accumulation of genetic alterations in the process of carcinogenesis and maintenance of the malignant phenotype became clearly evident. In this theoretical construct, cancer is believed to be a multistep genetic disease in which mutations resulting in the aberrant expression of cellular homologues of oncogenes (i.e., Ras, c-Fos, c-Jun, and c-Myc, etc.) associated with growth and development as well as tumor suppressor genes (i.e., p53) gradually accumulated over time, eventually resulting in immortalization, the loss of control of cell proliferation, and progression to the malignant phenotype.

During the same era that the genetic theory of cancer was blossoming, Oberley et al. [7–10], formally proposed the Free Radical Theory of Cancer which incorporated critical aspects of both metabolic and genetic theories of cancer. In this theoretical construct, cancer cells were proposed to have aberrant mitochondrial respiration leading to increased steady-state levels of superoxide and hydrogen peroxide that caused damage (both genetic and epigenetic) leading to the activation of oncogenes that governed signaling pathways controlling the malignant phenotype. This proposal was then followed by the recognition that free radicals and reactive

oxygen species produced by O_2 metabolism could act as both initiators and promoters of carcinogenesis as well as contribute to the process of cancer progression [11–13]. Also it was confirmed that cancer cells appeared to exist in a chronic condition of metabolic oxidative stress characterized by increased steady-state levels of mitochondrial respiratory chain-dependent superoxide and hydrogen production [14–16] that stimulated signaling pathways affecting the malignant phenotype that were compensated for by increased levels of glucose and hydroperoxide metabolism [16–19]. At the end of the twentieth and the beginning of the twenty-first centuries, these and similar findings from other investigators have led to the realization that free radical biology and cancer biology are two integrally related fields of investigation that can greatly benefit from cross-fertilization of theoretical constructs.

The current volume of scientific reports was assembled under the heading of "Oxidative Stress in Clinical Practice: Cancer" in order to stimulate discussion of how the well-established role of oxidative stress in cancer biology as well as in mechanisms by which radiation therapy and chemotherapy kills cancer cells can be utilized to design interventions to enhance therapeutic responses while causing fewer treatment limiting complications. The data gathered in the last 30 years which is summarized in the chapters contained in this volume, supports the hypothesis that selective enhancement of oxidative stress in cancerous tissues based on fundamental differences in oxidative metabolism between cancer vs. normal cells can be used as a target for enhancing therapeutic outcomes as well as sparing damage to normal tissues. In addition, since oxidative stress is believed to be causally involved with initiation, promotion, and progression of carcinogenesis, interventions designed to limit oxidative stress may also hold promise for limiting the numbers of cancers that are induced as well as delaying the progression of cancers once they are formed.

Finally, we would also like to dedicate this volume of work to the memory of Dr. Larry W. Oberley (1946–2008) who was the originator of the Free Radical Theory of Cancer [7–10]. Dr. Oberley diligently championed this theoretical construct during the difficult early phases of development when the field of study was being established. He also involved his students, colleagues, and junior faculty collaborators at every step of the way in his academic journey, which resulted in Dr. Oberley being integrally involved with training and mentoring of a generation of Free Radical Cancer Biologists. Dr. Oberley was an exceptional theoretician, scientist, and educator whose many contributions to the study of oxidative stress in cancer biology are clearly evident in the excellent chapters contained in this volume.

Iowa City, IA, USA — Douglas R. Spitz
Iowa City, IA, USA — Michael L. McCormick
Madison, WI, USA — Terry D. Oberley

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Contents

Contributors

Maher Y. Abdalla, PhD Department of Biology and Biotechnology, The Hashemite University, Zarqa, Jordan

Munjal Acharya, PhD Department of Radiation Oncology, University of California, Irvine, CA, USA

Iman M. Ahmad, PhD Department of Medical Imaging, The Hashemite University, Zarqa, Jordan

Mark E. Anderson, MD, PhD Cardiovascular Medicine, Department of Internal Medicine, The University of Iowa, Iowa City, IA, USA

Maaike Berbée, MD Department of Radiation Oncology (Maastro), Maastricht University Medical Center, Maastricht, The Netherlands

Theresa M. Busch, PhD Department of Radiation Oncology, Hospital of the University of Pennsylvania, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

Vincent Caiozzo, PhD Department of Orthopaedic Surgery, University of California, Irvine, CA, USA

Valerian E. Cagan, PhD Center for Free Radical and Antioxidant Health, Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA, USA

Sharon E. Campbell, PhD Department of Biochemistry and Molecular Biology, East Tennessee State University, Johnson City, TN, USA

Daret K. St. Clair, PhD Graduate Center for Toxicology and Markey Cancer Center, University of Kentucky, Lexington, KY, USA

Joseph J. Cullen, MD Department of Surgery, University of Iowa College of Medicine, Iowa City, IA, USA

Frederick E. Domann, PhD Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, Carver College of Medicine, The University of Iowa, Iowa City, IA, USA

Kenneth J. Dornfeld, MD, PhD Department of Biochemistry and Molecular Biology, University of Minnesota, Duluth, MN, USA

Duluth Clinic Radiation Oncology, Duluth, MN, USA

Neife Ap. Guinaim dos Santos, PhD Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, USP, Ribeirão Preto, SP, Brazil

Antonio Cardozo dos Santos, PhD Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, USP, Ribeirão Preto, SP, Brazil

Kevin L. Du, MD, PhD Department of Radiation Oncology, Hospital of the University of Pennsylvania, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

Bilikere S. Dwarakanath, PhD Division of Biocybernetics, Institute of Nuclear Medicine and Allied Sciences, Delhi, India

Michael W. Epperly, PhD Department of Radiation Oncology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Jeffrey Erickson, PhD Cardiovascular Medicine, Department of Internal Medicine, The University of Iowa, Iowa City, IA, USA

Melissa A. Fath, PhD Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, Iowa City, IA, USA

Rentian Feng, PhD Department of Pharmaceutical Sciences and Drug Discovery Institute, University of Pittsburgh School of Pharmacy, Pittsburgh, PA, USA

Division of Hematology/Oncology, University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA

John R. Fike, PhD Departments of Neurological Surgery and Radiation Oncology, Brain and Spinal Injury Center, University of California, San Francisco, CA, USA

Jarod C. Finlay, PhD Department of Radiation Oncology, Hospital of the University of Pennsylvania, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

Qiang Fu, MD, PhD Division of Radiation Health, University of Arkansas for Medical Sciences, Little Rock, AR, USA

David Gius, MD, PhD Departments of Cancer Biology and Pediatrics and Radiation Oncology, Vanderbilt Medical School, Nashville, TN, USA

Ruth K. Globus, PhD Space Biosciences Division, NASA Ames Research Center, Moffett Field, CA, USA

David J. Grdina, PhD, MBA Department of Radiation and Cellular Oncology, The University of Chicago, Chicago, IL, USA

Joel S. Greenberger, M.D Department of Radiation Oncology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Heather C. Hatcher, MD Department of Cancer Biology, Wake Forest School of Medicine, Winston-Salem, NC, USA

Martin Hauer-Jensen, MD, PhD Division of Radiation Health, University of Arkansas for Medical Sciences, Little Rock, AR, USA

Geraldine Jacobson, MD, MPh, MBA Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, Iowa City, IA, USA

Paiboon Jungsuwadee, PhD Graduate Center for Toxicology and Markey Caner Center, University of Kentucky, Lexington, KY, USA

Yasushi Kataoka, PhD Department of Radiation and Cellular Oncology, The University of Chicago, Chicago, IL, USA

Divya Khaitan, PhD Division of Biocybernetics, Institute of Nuclear Medicine and Allied Sciences, Delhi, India

Koyamangalath Krishnan, MD Department of Internal Medicine, East Tennessee State University, Johnson City, TN, USA

K. Sree Kumar, PhD Armed Forces Radiobiology Research Institute, Uniformed Services University of the Health Sciences, Bethesda, MD, USA

Ninh M. La-Beck, Pharm D Division of Pharmacotherapy and Experimental Therapeutics, School of Pharmacy, University of North Carolina, Chapel Hill, NC, USA

Suzanne Lentzsch, MD, PhD Division of Hematology/Oncology, University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA

Charles Limoli, PhD Department of Radiation Oncology, University of California, Irvine, CA, USA

Antonio Macciò, MD Department of Medical Oncology, University of Cagliari, Cagliari, Italy

Clelia Madeddu, MD Department of Medical Oncology, University of Cagliari, Cagliari, Italy

Joshua M. Madsen, PhD Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, Iowa City, IA, USA

Giovanni Mantovani, MD Department of Medical Oncology, University of Cagliari, Cagliari, Italy

Michael L. McCormick, PhD Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, Iowa City, IA, USA

Yusuf Menda, MD Department of Radiology, Carver College of Medicine, University of Iowa, Iowa City, IA, USA

Jeffrey S. Murley, PhD Department of Radiation and Cellular Oncology, The University of Chicago, Chicago, IL, USA

Terry D. Oberley, MD, PhD Molecular and Environmental Toxicology Center, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA

Department of Pathology and Laboratory Medicine, University of Wisconsin School of Medicine and Public Health , Madison, WI USA

Pathology and Laboratory Medicine Service, William S. Middleton Memorial Veterans Hospital, Madison, WI, USA

Kevin P. Orcutt, MD Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, Iowa City, IA, USA

James Peterson, PhD Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA, USA

Peter M. Scarbrough, PhD Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, Iowa City, IA, USA

Weihua Shan, PhD Molecular and Environmental Toxicology Center, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA

Andrean L. Simons, PhD Department of Pathology, Holden Comprehensive Cancer Cente, The University of Iowa, Iowa City, IA, USA

Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, Iowa City, IA, USA

Douglas R. Spitz, PhD Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, Iowa City, IA, USA

William L. Stone, PhD Department of Pediatrics, East Tennessee State University, Johnson City, TN, USA

Jamie M. Swanlund, BS Pathology and Laboratory Medicine Service, William S. Middleton Memorial Veterans Hospital, Madison, WI, USA

Melissa L. Teoh-Fitzgerald, PhD Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, Carver College of Medicine, The University of Iowa, Iowa City, IA, USA

Frank M. Torti, MD, MPH Department of Cancer Biology, Wake Forest School of Medicine, Winston-Salem, NC, USA

Comprehensive Cancer Center, Wake Forest School of Medicine, Winston-Salem, NC, USA

Suzy V. Torti, PhD Comprehensive Cancer Center, Wake Forest School of Medicine, Winston-Salem, NC, USA

Department of Biochemistry, Wake Forest School of Medicine, Winston-Salem, NC, USA

Mary Vore, PhD Graduate Center for Toxicology and Markey Cancer Center, University of Kentucky, Lexington, KY, USA

William C. Zamboni, Pharm D, PhD Division of Pharmacotherapy and Experimental Therapeutics, School of Pharmacy, University of North Carolina, Chapel Hill, NC, USA

Weixiong Zhong, MD, PhD Department of Pathology and Laboratory Medicine, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA

Pathology and Laboratory Medicine Service, William S. Middleton Memorial Veterans Hospital, Madison, WI, USA

Timothy C. Zhu, PhD Department of Radiation Oncology, Hospital of the University of Pennsylvania, University of Pennsylvania School of Medicine, Philadelphia, PA, USAMolecular and Environmental Toxicology Center, University of Wisconsin School of Medicine and Public Health, Madison, WI USA

Part I
Oxidative Stress and Tumor Response (Preclinical)

Chapter 1
Mitochondria-Mediated Oxidative Stress and Cancer Therapy

Iman M. Ahmad and Maher Y. Abdalla

Abstract Most cancer cells demonstrate increased rates of glucose metabolism when compared to normal cells. Glucose metabolism leads to the formation of pyruvate and NADPH both of which function in the cellular detoxification of hydroperoxides. Therefore, tumor cells may increase their metabolism of glucose as a compensatory mechanism to protect against hydroperoxides generated as byproducts of mitochondrial metabolism. Recent studies have shown that glucose deprivation preferentially induces cytotoxicity and oxidative stress in human cancer cells, relative to normal cells. Mitochondria have been hypothesized to be the site of prooxidant production during glucose deprivation. The preferential cytotoxicity and oxidative stress seen during glucose deprivation in cancer cells, relative to normal cells, has been hypothesized to have implications in designing more effective combined modality cancer therapies involving inhibitors of glycolytic metabolism and agents that enhance ROS production. Many drugs currently used to treat cancer cells (i.e., ionizing radiation, Cisplatin, Doxorubison, and azidothymidine, etc.) have been proposed to increase superoxide and hydrogen peroxide production and could also be combined with inhibitors of glucose metabolism and peroxide detoxification. The application of these findings to developing new combined modality cancer therapy protocols will be discussed as well as the clinical implications of using glucose metabolism and FDG-PET imaging to predict tumor responses to therapy.

I.M. Ahmad, PhD (✉)
Department of Medical Imaging, The Hashemite University, Zarqa, Jordan
e-mail: iman_maher@yahoo.com

M.Y. Abdalla, PhD
Department of Biology and Biotechnology, The Hashemite University, Zarqa, Jordan

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1.1 ROS Production by Cancer Cell Mitochondria

1.1.1 ROS and Mitochondrial Electron Transport Chain Proteins

The mitochondrial respiratory chain consists of a series of redox catalysts (pyridine nucleotide, flavoproteins, iron sulfur proteins, ubiquinone, and cytochromes) that are assembled at the mitochondrial inner membrane. The components of mitochondrial electron transport chain (ETC) can be viewed as if they are arranged in thermodynamic chain according to their reduction potential. As electrons are passed from one complex to another, energy can be extracted to drive the central processes of life by producing a proton gradient across the inner mitochondrial membrane. Mitochondria have long been known to be one of the major cellular sources of $O_2^{\bullet-}$ [1–6]. It has been estimated during normal respiration that 1–2% of the electrons traversing the respiratory chain contribute to the formation of $O_2^{\bullet-}$ ions [6]. Much of the original research into ROS production from mitochondria suggested that the majority of $O_2^{\bullet-}$ originates from Complex III [7, 8]. However, recent work clearly demonstrates that Complex I also play a major role in the production of $O_2^{\bullet-}$ in mitochondria [9]. In addition, Ishii et al. [10] have suggested, based on their work with nematodes, that $O_2^{\bullet-}$ may also arise from Complex II. Clearly, there are a number of potential sources of ROS within the mitochondrial respiratory enzyme chain. Normal mitochondria produce $O_2^{\bullet-}$ when exposed to different ETC blockers, such as antimycin A (AntA), which is known to inhibit the transfer of electrons from cytochrome *b* to coenzyme Q (CoQ), myxothiazole (Myx), which prevents the transfer of electrons from the reduced CoQ to FeS (III) protein and then to cytochrome *c* [11], and rotenone (Rot) which is a specific blocker of NADH-dehydrogenase activity at Complex I of the mitochondrial ETC [12].

Few studies have been done on the production of $O_2^{\bullet-}$ in tumor cells. Tumor cells also produce $O_2^{\bullet-}$ when exposed to ETC inhibitors or uncouplers [13, 14]. However, Docampo et al. [15] have studied $O_2^{\bullet-}$ production in intact mitochondria from mouse sarcoma 180 cells in the presence of NADH without respiratory chain inhibitors, and they found that the tumor mitochondria produced $O_2^{\bullet-}$. Dinescu-Romalo and Mihai [16] performed a study of $O_2^{\bullet-}$ production in several tissues from normal and Guerin T_8 ascites tumor-bearing rats without inhibitors as well. They found that the tissues from tumor-bearing animals produced $O_2^{\bullet-}$ at a much higher rate than the same tissues from nontumor-bearing animals. These results suggest that tumor cell mitochondria produce more $O_2^{\bullet-}$ than normal cells. Moreover, the mitochondria of malignant human tumor cells have been shown to exhibit histological pleomorphism when compared to the mitochondria from normal human cells [17]. This pleomorphism is manifested in the abnormal arrangements of the mitochondrial cristae, mitochondrial hypertrophy, and the fragmentation of mitochondria.

1.1.2 Mitochondrial ETC Proteins Mutations and Cancer

Growing evidence exists that cancer cells are under increased intrinsic metabolic oxidative stress due in part to mitochondrial malfunction [18, 19]. Since the mitochondrial ETC complexes are major sources of ROS in cells, it is logical that ROS-mediated damage to mtDNA may be a mechanism for increased oxidative stress in cancer cells. MtDNA is a highly vulnerable target due to its proximity to the ETC complexes. SDH (Complex II) is made of four subunits A, B, C, and D and is involved in the Krebs cycle. Recently, Ishii et al. suggested mutations in SDH might increase $O_2^{\bullet-}$ production substantially, contributing to increased sensitivity to oxidative stress as well as accelerated aging in nematodes [10]. In addition, mutations in three of the four subunits (B, C, and D) have been linked to familial forms of two human cancers (paraganglioma and pheochromocytoma) [20–23]. Furthermore, many tumors, including epithelial cancers (i.e., colon, breast, as well as head and neck), have been shown to have high rates of mtDNA mutations (relative to normal human tissues) and this has been suggested to contribute to increased $O_2^{\bullet-}$ and H_2O_2 production [24, 25]. As more mutations in genes coding for ETC proteins accumulate, complexes in the ETCs are thought to become less efficient at passing electrons due to stoichiometric mismatches between the proteins in each complex and changes in accessibility of electrons to form $O_2^{\bullet-}$ [26]. These disruptions in electron flow would be expected to increase residence times of electrons on sites capable of mediating one-electron reductions of O_2 to yield $O_2^{\bullet-}$ and H_2O_2 [26]. The resulting increased fluxes of ROS could then contribute to genomic instability and the progressive accumulation of mutations that are the hallmark of malignancy [27, 28].

1.2 Glucose Metabolism and Cancer

It has been found that cancer cells demonstrate altered metabolism when compared to normal cells [29–32]. The metabolic disruptions appear to involve metabolism of glucose and the loss of regulation between glycolytic metabolism and respiration [29–32]. Glucose metabolism has been shown to be involved in cellular sensitivity to oxidative stress mediated by hydroperoxides, presumably via the formation of pyruvate and NADPH [33]. The main pathways for glucose metabolism are glycolysis and pentose phosphate cycle (PPC) after the formation of glucose-6-phosphate [32]. Glycolysis results in the formation of pyruvate, which has been shown to scavenge H_2O_2 and other hydroperoxides [5] One of the major products from the PPC is NADPH, which has been shown to participate in the glutathione peroxidase (GPx)-mediated metabolic decomposition of H_2O_2 and organic hydroperoxides [34]. Therefore, besides its well-known role in energy production, glucose metabolism is also related to the metabolic detoxification of intracellular hydroperoxides formed as byproducts of oxidative metabolism.

Many cancer cells have altered metabolism, relying heavily upon glycolysis for ATP production [29]. Glucose deprivation would be expected to cause metabolism to shift to oxidative phosphorylation in order to meet the metabolic demand for ATP. This shift to mitochondrial respiration would be expected to increase one-electron reduction of oxygen from ETCs leading to increased $O_2^{\bullet -}$ [35]. Superoxide anions can dismutate to H_2O_2 spontaneously. Hydrogen peroxide can pass through mitochondrial membranes readily to reach the cytosol [36]. Hydrogen peroxide causes cell damage through hydroxyl radical generation via the Fenton reaction [37, 38].

$$Fe^{2+} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}.$$

In the absence of adequate levels of MnSOD, as in most cancer cells, the catalyst (Fe^{3+}) is recycled to (Fe^{2+}) through $O_2^{\bullet -}$ [39]:

$$O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+},$$

leading to an increase in $^{\bullet}OH$ generation that could lead to cytotoxicity and oxidative stress.

1.3 Glucose Deprivation Studies

1.3.1 Glucose Deprivation and Signal Transduction Pathways

Recently, it was discovered that simply removing glucose from the cell culture medium (i.e., glucose deprivation) induced cytotoxicity in the MCF-7/ADR human multidrug-resistant breast carcinoma cell line [1, 40]. Cytotoxicity induced by glucose deprivation in this model system was found to be preceded by activation of several signal transduction pathways such as: mitogen-activated protein kinase (MAPK, ERK1/ERK2) [1], Ras, Lyn kinase, and c-Jun N-terminal kinase (JNK1) [41]. Furthermore, glucose deprivation in this model system caused an increase in the DNA binding activity of the AP-1 transcription factor as well as an increase in the expression of the angiogenic factor, basic fibroblast growth factor (bFGF), and c-Myc gene expression [2]. These observations demonstrated that removal of glucose from these human tumor cells results in cytotoxicity as well as activation of signaling gene expression pathways, thought to be involved in neoplastic transformation.

1.3.2 Glucose Deprivation and Metabolic Oxidative Stress

The role of oxidative stress as a primary initiator has been shown in glucose deprivation-induced cytotoxicity and activation of signal transduction (Lyn, JNK, and MAP kinases ERK1/ERK2); in transcription factor activation (AP-1); in the

increased gene expression of bFGF; and in the induction of a cellular homolog of an oncogene (c-Myc) [1–3]. These results indicated that glucose deprivation causes oxidative stress as evidenced by increased production of prooxidants as well as increases in total glutathione, glutathione disulfide (GSSG), γ-glutamylcysteine, and cysteine [2]. The logical explanation for the increases in GSSG content in MCF-7/ADR cells is that the intracellular mitochondrial production of hydroperoxide exceeds the metabolic capabilities of the glutathione peroxidase/glutathione reductase/NADPH system to maintain glutathione in the reduced form, and therefore GSSG accumulates. Furthermore, addition of *N*-acetylcysteine (NAC) inhibited oxidative stress by suppressing prooxidant production and the oxidation of glutathione as well as the activation of signal transduction pathways and gene expression [1, 2]. Also, it has been shown that NAC can protect MCF-7/ADR cells from cell killing induced by glucose deprivation [1, 2]. The ability of the thiol antioxidant NAC to block both the cytotoxicity and alterations in signal transduction induced by glucose deprivation supports the hypothesis that glucose deprivation-induced oxidative stress and disruption of thiol metabolism are causally related to the biological effects that were observed. There are several ways in which NAC could block oxidative stress during glucose deprivation. NAC can function directly as an antioxidant to scavenge radicals generated by hydroperoxides undergoing Fenton chemistry. NAC could also increase intracellular cysteine pools, which could directly scavenge radicals as well as provide a vital substrate for increased synthesis of glutathione. Finally, by increasing intracellular reduced thiol pools, NAC could block oxidative stress-induced changes in signal transduction by maintaining redox-sensitive proteins in their reduced form [42]. Another major finding of these studies is that glutamate was capable of reversing the glucose deprivation-induced oxidative stress and alteration in signal transduction in the MCF-7/ADR cells [1]. Glutamate could therefore rescue the cells from glucose deprivation by directly entering the TCA cycle via glutamate dehyrogenase regenerating NADPH as well as increasing the formation of α-ketoacids, such as α-ketoglutarate and pyruvate, which have been shown to be excellent scavengers of hydroperoxides [5]. Glutamate is also required for the first committed step in the synthesis of glutathione catalyzed by γ-glutamylcysteine synthetase. Finally, the hypothesis that metabolism of O_2 to hydroperoxides could represent the source of increased prooxidants during glucose deprivation was supported by the fact that a scavenger of hydroperoxides, pyruvate, suppressed prooxidant production and cytotoxicity during glucose deprivation [1, 2].

1.3.3 Glucose Deprivation in Normal vs. Cancer Cells

Spitz et al. showed that glucose deprivation-induced cytotoxicity and oxidative stress occurs in several human transformed cell lines, in addition to MCF-7/ADR, such as SV40 transformed fibroblasts (IMR90, GM00637G) and colon cancer cells (HT-29) [3]. These data allow for the speculation that susceptibility to glucose deprivation-induced cytotoxicity and oxidative stress could represent a general phenomenon and

therefore not limited to a single type of transformed human cell type. Furthermore, transformed cells appeared to be more susceptible to glucose deprivation-induced cytotoxicity and oxidative stress as compared to their normal matched pair cells [3]. These results allow for the speculation that normal cells may be less susceptible to glucose deprivation-induced cytotoxicity and oxidative stress than cancer cells. This supports the speculation that cancer cells exhibit a defect in oxidative metabolism, which could be used to gain a therapeutic advantage when trying to kill cancer cells and spare the normal tissues.

1.4 Mitochondrial Involvement in Glucose Deprivation-Induced Cytotoxicity

To determine the role of mitochondria in glucose deprivation-induced cytotoxicity and oxidative stress, three different approaches were utilized [43]. First, the effect of mitochondrial ETC blockers (known to increase $O_2^{\bullet-}$ and H_2O_2 production in isolated mitochondria) was determined in intact cells during glucose deprivation. Second, rho(0) human cancer cells, deficient in functional mitochondrial ETCs, were utilized. Finally, adenovirus-mediated transduction of mitochondrially targeted catalase (MitCat) or MnSOD was utilized to over express enzymatic scavengers of $O_2^{\bullet-}$ and H_2O_2 in human tumor cells prior to glucose deprivation.

1.4.1 Mitochondrial ETC Blockers Enhance Glucose Deprivation-Induced Cytotoxicity and Oxidative Stress

The effects of mitochondrial ETC blockers along with glucose deprivation in GM00637G SV40 transformed human fibroblasts compared to the parental GM00037F normal human fibroblasts were investigated [43]. These cells were chosen in order to make direct comparisons between responses in normal vs. transformed cells derived from the same parental cell line. ETC blockers had previously been shown to increase $O_2^{\bullet-}$ and H_2O_2 production in isolated mitochondria [6, 7], leading to the expectation that these blockers would enhance glucose deprivation-induced cytotoxicity to a greater extent in transformed vs. normal cells if mitochondrial ROS production were mechanistically involved with these biological phenomena. The mitochondrial ETC blockers utilized were antimycin A (AntA), myxothiazole (Myx), and rotenone (Rot). The proton ionophore 2,4-dinitrophenol (DNP) that uncouples ETC activity from ATP synthesis, without blocking electron flow, was included as a control for inhibition of ATP synthesis [43]. It was shown that by 2 h of glucose deprivation, 10 μM AntA, 10 μM Myx, and 50 μM Rot were significantly toxic in the transformed cells (relative to DMSO controls), but these ETC blockers were not toxic for 2 h in the presence of glucose [43]. No toxicity was

seen in cells treated with DNP in the presence or absence of glucose, indicating that no correlation existed between ATP levels and glucose deprivation –induced cytotoxicity [43]. By contrast, normal cell toxicity was unaffected by 2 h exposure to ETC blockers in the presence or absence of glucose [43]. Furthermore, an increase in the mean fluorescence intensity (MFI) seen in the presence of ETC blockers when the transformed cells were labeled with an oxidation sensitive probe ($CDCFH_2$) in the absence of glucose for 2 h as compared to same cells labeled with oxidation insensitive probe (CDCF) where no changes in MFI were seen, are indicative of changes in dye oxidation, and not caused by changes in uptake, ester cleavage, or efflux. These results can be interpreted as an indication of increased prooxidant production in cells exposed to ETC blockers in the absence of glucose. When comparing the results of this study to literature results obtained using ETC blockers in isolated mitochondria from normal tissues [6, 7], some interesting points emerged. In glucose-deprived transformed fibroblasts, a 5.5-fold increase in prooxidant production was measured after treating the cells with 10 μM Ant A (as determined by MFI using sensitive probe) and in the presence of Myx, a 6.5-fold increase in prooxidant production was noted. This result was not consistent with the previous findings using isolated mitochondria from normal cells [6–8, 44] which suggested that treatment with Myx would inhibit electrons reaching ubisemiquinone ($Q^{\bullet}$) resulting in less prooxidant production in the presence of Myx, than in the presence of AntA. Based on this phenomena, one-electron reductions of O_2 to produce $O_2^{\bullet -}$ in the presence of Myx would then only be expected from Complexes I and II. When SV40 transformed cells were exposed to Rot during glucose deprivation, a threefold increase in prooxidant production was noted. The results with Rot combined with the surprising results using AntA and Myx, allow for the speculation that Complex II might be responsible for up to 50% of the prooxidant production seen in the presence of Myx and the other 50% of the prooxidant production may come from Complex I since Rot only backs up electron flow into Complex I and allows electrons to flow through Complexes II, III, and IV. However, the results obtained with normal human fibroblasts in terms of prooxidant production were different as compared to results obtained with transformed fibroblasts, which raise several interesting points. First, the absolute amount of prooxidant production was greater by a factor of 2 in the transformed cells, relative to the normal both in presence and absence of glucose. These results are, however, consistent with a previous report of increased prooxidant production by human cancer cells, relative to normal human cells [45]. Second, 50% of the prooxidant production seen in glucose-deprived normal cells exposed to AntA comes from $Q^{\bullet}$ in Complex III and 50% comes from Complex I, with no detectable signal originating from Complex II, based on the results obtained with Myx and Rot showing the same absolute value of prooxidant production. These results are consistent with previous results using isolated normal mitochondria [6–8, 44]. Glucose deprivation in the presence of 10 μM AntA for 6 h significantly enhanced cytotoxicity and parameters indicative of oxidative stress in other human cancer cell lines such as PC-3, DU145, MDA-MB231, and HT-29 [43]. These results show the generality of the results obtained with mitochondrial ETC blockers in SV40 transformed human fibroblasts.

1.4.2 Rho(0) Cells as a Tool for Studying the Role of Mitochondrial Metabolism in Glucose Deprivation-Induced Cytotoxicity and Oxidative Stress

Electron leakage from mitochondrial ETCs is believed to be the primary source of ROS generation in nonphagocytic cells. To provide evidence that glucose deprivation-induced cytotoxicity and oxidative stress were dependent on functional mitochondrial ETCs, glucose deprivation-induced cytotoxicity and oxidative stress was measured in human osteosarcoma cells rho(0) deficient in fully functional mitochondrial ETC [43]. The cells that are depleted of mitochondrial DNA lack the ability to generate ATP via oxidative phosphorylation and demonstrate greatly reduced O_2 consumption [46, 47]. No extensive characterization of the ability of rho(0) human cancer cells to produce $O_2^{\bullet-}$ and H_2O_2 has been reported. However, a few reports suggest that rho(0) cells may produce less $O_2^{\bullet-}$ and H_2O_2 than their wild-type counterparts [48–50]. Mitochondrial deficient rho(0) and the parental rho(+) human osteosarcoma cells were deprived of glucose for 6 and 8 h in the presence of 10 μM AntA. The results show that rho(+) cells demonstrated 40–70% cell killing during 6–8 h of glucose deprivation in the presence of AntA. By contrast, rho(0) cells exhibited only 20–30% cell killing during 6–8 h of glucose deprivation in the presence of AntA. Furthermore, following 6 or 8 h of glucose deprivation in the presence of AntA, GSSG and %GSSG was greater in rho(+) cells than in rho(0) cells [43]. These results are consistent with the hypothesis that fully functional mitochondrial ETCs significantly contribute to glucose deprivation-induced cytotoxicity and oxidative stress in human cancer cells.

1.4.3 The Effect of Manipulating Cellular Antioxidants on Glucose Deprivation-Induced Cytotoxicity and Oxidative Stress

To determine if specific ROS (i.e., $O_2^{\bullet-}$ and H_2O_2) are causally involved with glucose deprivation-induced cytotoxicity and oxidative stress, human prostate cancer cells (PC-3) were transduced with 10 and 50 MOI of AdMnSOD and/or AdMitCat, respectively, and then treated in the presence and absence of glucose. Each group infected with virus containing antioxidant(s) was compared to the appropriate control using the same MOI of the empty vector (AdBglII) [43]. The results obtained demonstrate a ~tenfold change in either or both enzymatic activities of infected cells. The results showed also that over expression of MnSOD and/or MitCat activity slightly protected PC-3 cells from clonogenic inactivation induced by 48 h of glucose deprivation, but when both enzymes were over expressed, significantly more protection was seen than with either enzyme alone [43]. Furthermore, the results showed that over expression of either antioxidant enzyme alone slightly suppressed GSSG accumulation by ~20% during glucose deprivation, relative to the appropriate vector control. By contrast, those cultures over expressing both MnSOD and MitCat

demonstrated a ~50% suppression in the accumulation of GSSG during glucose deprivation, relative to the appropriate vector control [43]. These results provide strong evidence that both mitochondrial $O_2^{\bullet-}$ and H_2O_2 contribute to the cytotoxicity and oxidative stress associated with glucose deprivation in this model system. It is interesting to note that despite a ~tenfold increase in PC-3 catalase and MnSOD activity, only partial protection against cytotoxicity and accumulation of GSSG was noted during glucose deprivation. Since the genes were delivered on separate vectors, the most likely explanation for this is that not all cells that were infected in the co-transduction experiments expressed both genes.

1.4.4 The Ability of 2DG to Mediate Biological Effects Similar to Glucose Deprivation in Normal vs. Transformed Cells

To determine the potential clinical utility of the mechanistic information gathered during the study of glucose deprivation-induced cytotoxicity and oxidative stress, the glucose analog 2-deoxy-D-glucose (2DG) was used. 2DG has been known for many years to be cytotoxic to cancer cells as well as enhancing the cytotoxicity of ionizing radiation toward cancer cells in culture, tumors in animals, and in clinical trials [51–53]. The mechanisms responsible for 2DG-induced cytotoxicity and radiosensitization were thought to involve an inhibition of DNA repair pathways after exposure to radiation [53]. However, the obvious mechanism proposed to explain this inhibition of DNA repair (impairment of ATP production and energy metabolism) has not been supported by the data gathered to date [53]. Furthermore, a recent report has also shown that 2DG-induced apoptosis in leukemia cells is selectively inhibited by overexpression of the mitochondrial phospholipid hydroperoxide glutathione peroxidase enzyme [54]. This previous report provided the first direct evidence that glutathione peroxidase-sensitive decomposition of hydroperoxides originating from mitochondrial metabolism might be an important contributor to 2DG-induced apoptosis.

New therapeutic strategies using 2DG preferentially kill cancer cells via metabolic oxidative stress combined with inhibitors of hydroperoxide metabolism to induce radio- and chemosensitization was developed. Many current studies showed that the competitive inhibitor of glucose metabolism, 2DG, is both cytotoxic and capable of inducing radiosensitization in human cancer cells in vitro [52, 53]. In addition, Gius et al. showed for the first time that simultaneous treatment with the thiol antioxidant, NAC, suppressed both the cytotoxicity and radiosensitization induced by 2DG in a human cancer cell line [4]. In addition, 2DG exposure was shown to induce disruptions in thiol metabolism that were partially reversed by exposure to NAC. Finally, the cytotoxicity and radiosensitization induced by exposure to 2DG appeared to be more pronounced in an oncogene-transformed cell line, when compared with the parental cell line, and this radiosensitization was also inhibited by treatment with a thiol antioxidant [4]. The results of these studies are consistent with the previous observations obtained with glucose deprivation [1–3]

and support the hypothesis that 2DG-induced cytotoxicity and radiosensitization are mediated by disruptions in thiol metabolism resulting from metabolic oxidative stress. In this regard, cancer cells were hypothesized to have a fundamental defect in their ETCs, leading to increased leakage of electrons to form $O_2^{\bullet -}$ and H_2O_2, relative to normal cells [3]. Aykin-Burns et al. showed the differential effects of 2DG on cytotoxicity in normal vs. cancer intestinal epithelial cells [55]. During 72 h exposure, 2DG was not overtly toxic to normal cells but 30% of the cancer cells were metabolically inactivated during the same frame as determined by trypan blue exclusion. These results suggest that 2DG is more cytotoxic to human cancer cells relative to the normal cell type from the same tissue origin and is consistent with the data using rat cells in previous studies [4].

1.5 Clinical Significance of Glucose Deprivation-Induced Oxidative Stress in Cancer Therapy and Imaging

1.5.1 Glucose Deprivation and Cancer Therapy

Cancer therapy is based upon the concept that certain cytotoxins kill cancer cells preferentially, relative to normal cells, because of cell physiological characteristics peculiar to the cancer cells and/or the localized delivery of cytotoxic agents to cancer cells. To date, one of the most common abnormal biochemical characteristics associated with cancer cells is the increased utilization of glucose. Glucose deprivation-induced oxidative stress was more pronounced in transformed vs. normal human cells [3]. Glucose deprivation-induced oxidative stress was hypothesized to involve impairment of hydroperoxide metabolism, because the products of glycolysis (pyruvate) and the pentose cycle (NADPH) have been shown to be integrally related to peroxide detoxification [3]. Pyruvate can directly react with peroxides to detoxify them [5], and NADPH serves as the source of reducing equivalents for the glutathione-dependent decomposition of hydroperoxides via the glutathione peroxidase/glutathione reductase system, as well as the thioredoxin-dependent decomposition of hydroperoxides via the thioredoxin peroxidase/thioredoxin reductase system [34, 56].

Using this theoretical construct, cancer cells were hypothesized to increase glucose metabolism (relative to normal cells) to enhance the metabolic decomposition of ROS formed as byproducts of oxidative energy metabolism [3]. Therefore, inhibition of glucose metabolism would be expected to sensitize cancer cells (relative to normal cells) to agents that increase levels of hydroperoxides (i.e., ionizing radiation and chemotherapy agents known to produce ROS) [57]. Although it is not possible to deprive cells of glucose in vivo, it is possible to treat tumor bearing animals and humans with 2DG, a relatively nontoxic analog of glucose that competes with glucose for uptake via the glucose transporters as well as being phosphorylated by hexokinase at the entry point to glycolysis. Although there are reports that the phosphorylated form of 2DG (2DG-6-P) can proceed through the first step in the pentose cycle

(glucose-6-phosphate dehydrogenase) leading to the regeneration of one molecule of NADPH [58], it appears to be incapable of further metabolism in the pentose cycle as well as incapable of metabolism to pyruvate. Administration of 2DG to mice has been shown to be an effective way to inhibit glucose metabolism without causing toxicity until very high levels are achieved (LD50≥2 g/kg body weight) [59]. Therefore, using 2DG as an inhibitor of glucose metabolism in vivo may provide a relatively nontoxic and effective addition to multimodality cancer therapies designed to limit hydroperoxide metabolism for the purpose of enhancing radio- and chemosensitivity in human cancers. It has been shown that the cytotoxic effects of ionizing radiation and some chemotherapeutic agents (i.e., such as Adriamycin, cisplatin, and AZT) involve the formation of ROS capable of causing oxidative DNA damage and cell death [57, 60–65]. Furthermore, it has been known for more than 20 years that 2DG treatment sensitizes tumor cells to the cytotoxic effects of ionizing radiation both in vitro and in vivo [4, 52, 53, 66] and this has led to phase I/II clinical trials in humans testing 2DG as an adjuvant to radiation therapy [66]. 2DG treatment of cancer cells with high rates of glucose metabolism would be expected to cause greater sensitization to agents that cause oxidative stress because of the added oxidative burden that would be expected when hydroperoxide metabolism (via glucose dependent processes) was compromised. Cisplatin is one of the most commonly used drugs in the management of locally advanced or recurrent head and neck cancer [67]. The combination of 20 mM 2DG and 0.5 μM cisplatin showed what appeared to be greater than additive cell killing in FaDu human head and neck cancer cells compared to 2DG or cisplatin alone [68]. Furthermore, the drug combination caused perturbations in parameters indicative of oxidative stress, including decreased intracellular total glutathione and increased % of GSSG, suggesting that oxidative stress was involved [68]. Moreover, the effects of 2DG + cisplatin-induced cytotoxicity and %GSSG were reversed with 15 mM NAC treatment [68].

L-Buthionine-[S,R]-sulfoximine (BSO) is a drug being tested in clinical trials for cancer therapy that inhibits GSH synthesis and resulting in GSH depletion and chemosensitization [69]. It was found that 1 mM BSO enhanced the toxicity of 2DG and cisplatin, when used alone or in combination [68]. Furthermore, thiol analysis shows that BSO depleted total glutathione in all groups and increased the %GSSG significantly in both the 2DG and 2DG + cisplatin groups, in which 15 mM NAC treatment reverse these effects [68].

Gene therapy vector (i.e., p53) was also combined with 2DG as a new cancer therapy modality. It was chosen because p53 has been suggested to kill colon cancer cells by induction of oxidative stress via enzymes capable of increasing steady-state levels of H_2O_2 [70, 71]. Ahmad et al. showed that 2DG significantly enhanced the cytotoxic effects of treatment with Adp53 in prostate cancer cell lines (p53 minus androgen independent) and the % of cells killed by 2DG in the presence of Adp53 appeared to be greater in DU145 cells, relative to PC-3 cells treated identically [72]. In addition, DU145 cells consumed glucose from the tissue culture media at twice the rate of PC-3 cells as determined with a glucose analyzer [72]. These results support the hypothesis that the combination of 2DG and Adp53 is more cytotoxic to human prostate cancer cells that consume glucose at a higher rate

in vitro, relative to those that consume glucose at a lower rate. Furthermore, treatment with 2DG resulted in increases in total GSH and GSSG in both cell lines suggesting that (1) glutathione synthesis was enhanced in response to 2DG-induced oxidative stress and (2) the cells' ability to maintain newly synthesized glutathione in the reduced state may have been compromised. Treatment with Adp53 alone as well as the combination of 2DG and Adp53 demonstrated significantly greater increases (approximately threefold) in the accumulation of GSSG in DU145 cells compared to PC-3 cells [72]. These results support the hypothesis that 2DG and Adp53 causes greater oxidative stress in human prostate cancer cells that consume more glucose. DU-145 cells were also pretreated with PEG-SOD, AdGPx, or AdMitCat, before and during labeling with DHE or CDCFH2, respectively [72]. The significant increases in PEG-SOD-inhibitable DHE oxidation as well as AdGPx or AdMitCat-inhibitable CDCFH2 oxidation that were observed strongly support the hypothesis that increases in steady-state levels of $O_2^{\bullet-}$ and hydroperoxides did occur in prostate cancer cells treated with 2DG and Adp53. These results suggested that both $O_2^{\bullet-}$ and hydroperoxides were capable of contributing to the oxidative stress and cytotoxicity seen during 2DG and Adp53 treatment. These results are also consistent with the previous observations that p53-dependent apoptosis was preceded by the induction of the enzyme proline oxidase and increased respiration, presumably leading to the increased generation of $O_2^{\bullet-}$ and hydroperoxides [73–76].

1.5.2 Glucose Deprivation and Cancer Imaging

Fundamental differences in glucose metabolism between transformed and normal cells are used clinically to image cancerous tissues by using tracer amounts of FDG with PET imaging [77, 78]. In addition, many cancers have shown robust signals using FDG-PET imaging that vary between patients [78–80]. The significance of the variability in FDG-PET images obtained from cancers of the same type in different patients is currently not well understood but has been suggested to relate to therapeutic response [53, 66, 77–81]. Studies have shown that there may be strong correlations between glucose uptake, glycolysis, and treatment resistance in tumors [82]. More specifically, tumors with lower FDG uptake tended to respond better to standard treatments than those with greater FDG uptake [83, 84]. These results suggest that new adjuvants to chemoradiotherapy are needed to treat patients with tumors with high FDG uptake. If cancer cells (relative to normal cells) increase glucose metabolism to form pyruvate and NADPH as a compensatory mechanism in response to ROS formed as byproducts of oxidative energy metabolism then: (1) the uptake of glucose (or FDG) would be expected to be proportional to intracellular levels of hydroperoxides and disruptions in mitochondrial metabolism leading to metabolic oxidative stress. Using this logic, 2DG should sensitize tumors with greater FDG uptake to a greater extent to agents that further increase hydroperoxide production and metabolic oxidative stress relative to low-FDG-uptake tumors. Simons et al., showed that the head and neck carcinoma, Cal-27 xenograft tumors showed

greater pretreatment FDG uptake and increased disease-free survival when treated with 2DG + CIS relative to head and neck FaDu xenograft tumors [85]. These results show the potential for FDG uptake to predict tumor sensitivity to 2DG + CIS. Meanwhile, a vast majority of cancer cell lines have increased mitochondrial membrane potentials [86]. Although the precise mechanism responsible for the increased membrane potential is not completely known, cancer imaging using 99mTc-Sestamibi is based on this difference between normal and malignant cells. 99mTc-Sestamibi-SPECT uptake and retention is based in part on mitochondrial membrane potential and agents known to damage mitochondria and disrupt the ETC alter 99mTc-Sestamibi-SPECT uptake. The high rate of mitochondrial DNA mutations seen in cancer cells also suggests a difference between normal and transformed cells. If mtDNA damage and mutation are responsible for the elevated production of $O_2^{\bullet -}$ and H_2O_2 seen in cancer cells, additional mtDNA damage (using chemotherapy agents such as AZT, Cis) may augment metabolic differences leading to ROS production between normal and cancer cells affording the opportunity for more effective therapy. Just as altered glucose metabolism can be manipulated with 2DG and monitored with FDG-PET, mitochondrial function and mtDNA integrity can be manipulated with AZT/cisplatin and imaged with 99mTc-Sestamibi-SPECT. This strategy has proven useful in following responses to cisplatin-based chemotherapy [87]. Taken together, these observations suggest mitochondrial function as a difference between normal cells and cancer cells that can be imaged, manipulated, and augmented to improve cancer therapy.

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Chapter 2
The Role of Akt Pathway Signaling in Glucose Metabolism and Metabolic Oxidative Stress

Andrean L. Simons, Kevin P. Orcutt, Joshua M. Madsen, Peter M. Scarbrough, and Douglas R. Spitz

Abstract Glucose metabolism plays an important role in hydroperoxide detoxification and the inhibition of glucose metabolism has been shown to increase prooxidant production and cytotoxicity in cancer cells. Increased Akt pathway signaling has been shown to be directly correlated with increased rates of glucose metabolism observed in cancer cells versus normal cells. These observations have led to the proposal that inhibition of Akt signaling would inhibit glycolysis and increase hydroperoxide production which would preferentially kill tumor cells versus normal cells via oxidative stress. The current study shows that inhibition of the Akt pathway inhibits glucose consumption and induces parameters indicative of oxidative stress such as glutathione disulfide (%GSSG) and thioredoxin reductase (TR) activity in human head and neck cancer (HNSCC) cells. A theoretical model to explain the results is presented and implications for the use of Akt pathway inhibitors in combination with glycolytic inhibitors and/or manipulations that increase prooxidant production are discussed.

A.L. Simons, PhD (✉)
Department of Pathology, Holden Comprehensive Cancer Center, The University of Iowa, 200 Hawkins Drive, Iowa City, IA 52242, USA

Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, B180 Medical Laboratories, Iowa City, IA 52242, USA
e-mail: andrean-simons@uiowa.edu

K.P. Orcutt, MD • J.M. Madsen, PhD • P.M. Scarbrough, PhD • D.R. Spitz, PhD
Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, B180 Medical Laboratories, Iowa City, IA 52242, USA

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2.1 Introduction

Glucose metabolism plays an important role in hydroperoxide detoxification and the inhibition of glucose metabolism has been shown to increase prooxidant production and cytotoxicity in cancer cells. Increased Akt pathway signaling has been shown to be directly correlated with increased rates of glucose metabolism observed in cancer cells versus normal cells. These observations have led to the proposal that inhibition of Akt signaling would inhibit glycolysis and increase hydroperoxide production which would preferentially kill tumor cells versus normal cells via oxidative stress. The current preliminary studies explore how the inhibition of the Akt pathway would disrupt glucose consumption and induce parameters indicative of oxidative stress such as glutathione disulfide (%GSSG) and thioredoxin reductase (TR) activity in human head and neck cancer (HNSCC) cells. A theoretical model to explain the results is presented and implications for the use of Akt pathway inhibitors in combination with glycolytic inhibitors and/or manipulations that increase prooxidant production are discussed.

2.2 Upregulation of Glucose Metabolism in Cancer Cells

Cancer cells exhibit increased glucose metabolism and pentose phosphate cycle activity compared to normal untransformed cells [1–3]. The most consistent of these observations is that cancer cells metabolize glucose into pyruvate producing excess lactate even though the supply of oxygen is adequate to support mitochondrial respiration [1–3]. Warburg referred to this phenomenon as aerobic glycolysis [1]. He hypothesized that these metabolic abnormalities were due to "damage" to the tumor cell respiratory mechanism (now known to be mitochondria) and cancer cells compensated for this defect by increasing glycolysis [1]. Warburg originally proposed that cancer cells upregulated glycolysis to compensate for a deficit in ATP production due to compromised oxidative phosphorylation and hypoxic microenvironments [1]. However, conflicting evidence regarding Warburg's hypothesis involving ATP deficits in transformed cells has limited the utility of these concepts in enhancing cancer therapy.

2.3 Glucose Metabolism and Detoxification of Hydroperoxides

Several studies have focused on alternative mechanisms contributing to the Warburg effect [4–7]. Of particular interest is glycolysis' role in the detoxification of hydroperoxides. Glycolysis plays an important role as a source of electrons for energy metabolism in cells under normal steady-state conditions. However, glucose metabolism also plays a major role in the detoxification of hydroperoxides produced as byproducts of O_2 metabolism [8–12]. The major pathways of glucose

metabolism following the formation of glucose 6-phosphate (via the action of hexokinase) include glycolysis and the pentose phosphate cycle [13]. Glycolysis results in the formation of pyruvate (PYR) and the pentose phosphate pathway results in the regeneration of NADPH from NADP+ [13]. PYR, in addition to being a substrate for energy metabolism via the tricarboxylic acid (TCA) cycle and mitochondrial oxidative phosphorylation, has been shown to scavenge H_2O_2 and other hydroperoxides directly [8, 11, 12]. NADPH, by virtue of being the source of reducing equivalents for the glutathione and thioredoxin antioxidant system, has also been shown to participate in the detoxification of H_2O_2 and organic hydroperoxides [14, 15]. Therefore, glucose metabolism appears to be integrally related to the detoxification of intracellular hydroperoxides. In fact, increasing glucose concentrations up to 10–20 mM in tissue culture media has been shown to render cells resistant to H_2O_2-induced cytotoxicity [10].

Given that glucose metabolism appears to be involved with the detoxification of intracellular hydroperoxides and other investigators have suggested that cancer cells demonstrate increased intracellular hydroperoxide production [16], we proposed tumor cells may increase the metabolism of glucose to compensate for increased intracellular hydroperoxides caused by a defect in mitochondrial respiration [17]. Furthermore, we proposed that therapeutic interventions designed to inhibit glucose metabolism and hydroperoxide detoxification combined with manipulations that increase prooxidant production would preferentially kill tumor cells versus normal cells via oxidative stress [17, 18].

2.4 PI3K/Akt Signaling Pathway

The Akt pathway has garnered significant interest in HNSCC research given that increased Akt activity accounts for up to 60% of all HNSCC [19, 20]. Akt is a member of a serine-threonine specific kinase family in mammalian cells [21–24] and Akt is able to activate and deactivate many downstream targets involved in key cellular processes such as cell growth, survival, cell cycle progression, and metabolism [25, 26].

Signaling through the Akt pathway can start at the cell membrane from the binding of ligands with their receptor, which include human epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2/neu, or ErbB-2) [27]. The receptors are activated which then activate phosphoinositide 3-kinase (PI3K), which converts phosphatidylinositol-4,5-bisphosphate (PIP_2) into phosphatidylinositol-3,4,5-trisphosphate (PIP_3) [28, 29]. Akt and phosphoinositide-dependent kinase 1 (PDK1) are recruited to the plasma membrane by binding to PIP_3 via their pleckstrin homology (PH) domains [30]. After localizing to the membrane, Akt is phosphorylated at Thr 308 and Ser 473 by PDK1 and PDK2 also known as mammalian target of rapamycin complex 2 (mTORC2), respectively [30]. Activated Akt can then go on to activate or deactivate many downstream proteins which control cell proliferation and survival. Akt activity is negatively regulated by

the tumor suppressor gene PTEN (phosphatase and tensin homolog deleted on chromosome 10), which acts as a phosphatase to dephosphorylate PIP_3 back to PIP_2 [31]. PTEN inhibits the ability of Akt to be localized to the cell membrane [32–34]. Without this localization, Akt activation decreases, along with all the other downstream pathways that depend on Akt for activation [32–34].

2.5 Role of Akt Signaling in Glycolysis

Akt hyperactivation is believed to be associated with increased rates of glucose metabolism observed in tumor cells [35–37]. Akt signaling exerts a direct influence on glycolysis in cancer cells by several mechanisms. Akt has been shown to regulate the localization of the glucose transporter GLUT1 to the plasma membrane [38–42] and regulate hexokinase expression, activity, and mitochondrial interaction [43–45]. In addition, Akt may indirectly activate the glycolysis rate-controlling enzyme phosphofructokinase-1 (PFK1) by directly phosphorylating phosphofructokinase-2 (PFK2) [46], which produces the product, fructose-2.6-bisphosphate (Fru-1,6-P2), which is the most potent allosteric activator of PFK1. In support of these findings, the activity of Akt was found to be correlated to the degree of glycolysis in glioblastoma cells, i.e., the higher the Akt activity, the higher the rate of glycolysis [36]. In addition, activation of Akt signaling in glioblastoma cells previously without constitutive Akt activity stimulated a high level of aerobic glycolysis without increasing oxygen consumption [36].

From these observations, it is clear that Akt activation causes an increase in glycolysis in cancer cells, and this may be the key step in the metabolic transformation of cells to increase glycolysis (originally observed by Warburg) in the development of cancer. This was supported by work showing that lymphoma cells with mitochondrial respiration defects lead to activation of the Akt pathway but not in the parental cells lacking mitochondrial respiration defects [47]. Therefore, the increased glycolytic rates observed by Warburg in cancer cells exhibiting mitochondrial respiration malfunction compared to normal cells may involve activation of the Akt pathway.

2.6 Role of EGFR Signaling in Glycolysis

There is also a strong relationship between EGFR and glucose metabolism. High concentrations of epidermal growth factor (EGF) have been shown to increase glucose consumption and cause selective cytotoxicity in MDA-468 human breast cancer cells, a cancer cell line over expressing EGFR, compared to MCF-7 breast cancer cells, which have a very low expression of EGFR [48]. EGFR may additionally influence with glucose metabolism as it has been found that the physical presence of EGFR within the membrane stabilizes the sodium glucose cotransporter (SGLT1) that may cause increased glucose consumption by EGFR over expression independent of EGFR signaling initiation [49].

2.7 Role of Glucose Metabolism and Oxidative Stress in Akt Pathway Signaling

Since glucose metabolism appears to play an important role in hydroperoxide detoxification then therapeutic interventions designed to inhibit glucose metabolism would be expected to increase prooxidant production and cytotoxicity in cancer cells. Furthermore, if increased Akt pathway signaling is correlated with increased rates of glucose metabolism observed in cancer cells versus normal cells, then the inhibition of Akt pathway signaling would be expected to inhibit glycolysis and increase hydroperoxide production which would preferentially kill tumor cells versus normal cells via oxidative stress. Based on these assumptions, the combination of Akt pathway inhibitors with glycolytic inhibitors and/or manipulations that increase prooxidant production should further and preferentially cause cytotoxicity in cancer cells, with minimal to no toxicity to normal cells.

The preliminary studies presented here were designed to determine:

1. If inhibition of EGFR, PI3K, and Akt signaling inhibited glucose consumption.
2. If inhibition of EGFR, PI3K, and Akt signaling induced oxidative stress.
3. If the glycolytic inhibitor, 2-deoxyglucose (2DG), would enhance the cytotoxicity induced by Akt pathway inhibitors via metabolic oxidative stress.

To accomplish these goals, we performed select experiments in FaDu and Cal-27 human head and neck squamous carcinoma cells.

2.7.1 Materials and Methods

2.7.1.1 Cells and Culture Conditions

FaDu and Cal-27 human head and neck squamous cell carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and 4.5 g/L glucose with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Cultures were maintained in 5% CO_2 and humidified in a 37°C incubator.

2.7.1.2 Drug Treatment

2-Deoxy-D-glucose (2DG), *N*-acetyl cysteine (NAC), LY294002 (LY), and L-buthionine-[S,R]-sulfoximine (BSO) were obtained from Sigma Chemical Co. (St. Louis, MO). Perifosine (PER) was obtained from Cayman Chemical (Ann Arbor, MI). Erlotinib (ERL) was obtained from OSI Pharmaceuticals (Long Island, NY). All drugs were used without further purification. Drugs were added to cells at a final concentration of 20 mM 2DG, 20 mM NAC, 5 μM LY, 1.0 mM BSO, 5 μM PER,

and 10 μM ERL. 2DG and BSO were dissolved in PBS. NAC was dissolved in 1 M sodium bicarbonate (pH 7.0). LY, ERL, and PER were each dissolved in DMSO then diluted with 0.9% sodium chloride (Hospira, Lake Forest, IL). The required volume was added directly to complete cell culture media on cells to achieve the desired final concentrations. All cells were placed in a 37°C incubator and harvested at the time points indicated.

2.7.1.3 Glucose Consumption

FaDu cells (100,000) were plated and grown for 24 h prior to treatment with 20 mM 2DG, 5 μM LY (LY5), 5 μM PER, or 10 μM EDRL for 24 h. Glucose levels were measured on 20 μL samples with an ACCU-CHEK® Aviva glucometer (Roche; Mannheim, Germany) before and after 24 h of drug treatment.

2.7.1.4 Glutathione Assay

Following treatment, medium was collected and centrifuged to harvest floating cells, and attached cells were scrape harvested in ice-cold PBS and centrifuged at 4°C, the supernatant was discarded, the cell pellets were transferred to 1.5 mL tubes, and frozen at 20°C until biochemical analysis was performed. Cell pellets were thawed and homogenized in 50 mM PO_4 buffer (pH 7.8) containing 1.34 mmol/L diethylenetriaminepentaacetic acid (DETAPAC) buffer. Total glutathione content was determined by the method of Anderson [50]. GSH and GSSG were distinguished by the addition of 2 μL of a 1:1 mixture of 2-vinylpyridine and ethanol per 30 μL of sample followed by incubation for 1 h and assayed as described previously [51]. All glutathione determinations were normalized to the protein content of whole homogenates using the method of Lowry et al. [52].

2.7.1.5 Clonogenic Cell Survival Experiments

Floating cells in medium from the experimental dishes were collected and combined with the attached cells from the same dish that were trypsinized with 1 mL trypsin–EDTA (CellGro, Herndon, VA) and inactivated with DMEM containing 10% FBS (Hyclone). The cells were diluted and counted using a Coulter counter. Cells were plated at low density (300–1,000 per plate), and clones were allowed to grow in a humidified 5% CO_2, 37°C environment for 14 days in complete medium, and in the presence of 0.1% gentamicin. Cells were fixed with 70% ethanol and stained with Coomassie blue for analysis of clonogenic cell survival as previously described [53]. Individual assays were performed with multiple dilutions with at least four cloning dishes per data point.

2.7.1.6 Thioredoxin Reductase Activity Assay

Thioredoxin reductase (TR) activity was determined spectrophotometrically using the method of Holmgren and Bjornstedt [54]. Enzymatic activity was determined by subtracting the time-dependent increase in absorbance at 412 nm in the presence of TR activity inhibitor aurothioglucose from total activity. One unit of activity was defined as 1 μM TNB formed/(min mg protein). Protein concentrations were determined by the Lowry assay [52].

2.7.1.7 NADPH Measurements

Treated cells were washed with PBS and scrape harvested in PBS at 4°C. After centrifugation at $200 \times g$ for 5 min, cell pellets were resuspended in extraction buffer containing 0.1 M Tris–HCl (pH 8.0), 0.01 M EDTA, and 0.05% (v/v) Triton X-100. The cell suspension was sonicated at a duty cycle of 34% (Sonics Vibracell, VC750) in ice water. The solution was centrifuged at $2{,}300 \times g$ for 5 min. The supernatants were collected and analyzed immediately for NADPH as described previously [55]. Results were obtained by comparison with a standard curve using genuine NADPH and normalized per milligram of cellular protein.

2.7.1.8 Statistical Analysis

Statistical analysis was done using GraphPad Prism version 4 for Windows (GraphPad Software San Diego, CA). To determine differences between three or more means, one-way ANOVA with Bonferroni posttests were performed. Two-way ANOVA was used to determine differences over different time points and treatment groups. Error bars represent ±1 standard deviation. All statistical analysis was performed at the $p < 0.05$ level of significance.

2.7.2 Results

2.7.2.1 Effect of Akt Pathway Inhibitors on Glucose Metabolism

In order to determine if Akt pathway inhibitors would affect glucose metabolism in cancer cells, we used the PI3K inhibitor LY294002 (LY), the Akt inhibitor perifosine (PER), and the EGFR inhibitor Erlotinib (ERL) and compared their effects on glucose consumption to that of the glycolytic inhibitor 2-deoxyglucose (2DG) in FaDu human head and neck cancer cells (Fig. 2.1). We found that 5 μM LY (LY5), 5 μM PER, and 10 μM ERL were all able to inhibit glucose consumption in FaDu cells compared to untreated control cells over a 24 h period (Fig. 2.1). We have also duplicated these results in other head and neck cancer cell lines such as Cal-27,

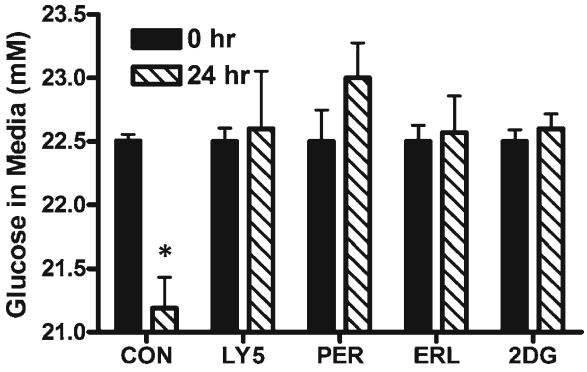

Fig. 2.1 Glucose consumption in FaDu cells. 100,000 FaDu cells were plated and grown for 24 h prior to treatment with 5 μM LY294002 (LY5), 5 μM perifosine (PER), 10 μM Erlotinib (ERL), or 20 mM 2-deoxyglucose (2DG) for 24 h. Glucose levels were measured with a glucometer before and after 24 h of drug treatment. *Error bars* represent ±1 SD of at least $N = 3$ experiments. *$p < 0.05$ versus time 0 h

SCC-25, and SQ20B cells (data not shown). These results suggest that EGFR/PI3K/Akt pathway inhibitors do in fact inhibit glucose consumption and they do so just as well as 2DG, suggesting a possible role of glucose metabolism in all 3 of these pathways (EGFR, PI3K and Akt).

2.7.2.2 Effect of Akt Pathway Inhibitors on Oxidative Stress Parameters

We examined if oxidative stress could be contributing to the effects of EGFR/PI3K/Akt pathway inhibitors by measuring glutathione (GSH/GSSG) levels in the cells. The GSH/GSSG redox couple represents a major small molecular weight thiol-disulfide redox buffer in cells [56]. The amount of total GSH that was oxidized (GSSG) was used to calculate the percentage of GSSG (%GSSG). Consequently, an increase in %GSSG is believed to signify a shift toward a more highly oxidizing intracellular environment indicative of oxidative stress [56]. We analyzed %GSSG levels in FaDu cells after treatment with LY5, PER, ERL, and 2DG for 24 h. 2DG has previously been shown to disrupt glutathione metabolism and induce an increase in %GSSG in head and neck cancer cells and we were able to repeat those findings here (Fig. 2.2a, 18). We additionally observed that all agents tested induced a significant increase in %GSSG, with ERL inducing the greatest increase compared to untreated control cells (Fig. 2.2a). These results suggest that thiol oxidation was induced in these cells in response to EGFR/PI3K/Akt pathway inhibitors (Fig. 2.2a). To further investigate this phenomenon, we pretreated the cells for 1 h with 20 mM of the thiol antioxidant NAC. NAC has been previously shown to increase GSH production in FaDu cells and may function by enhancing GSH-mediated hydroperoxide metabolism [18]. NAC in combination with each agent tested, suppressed the

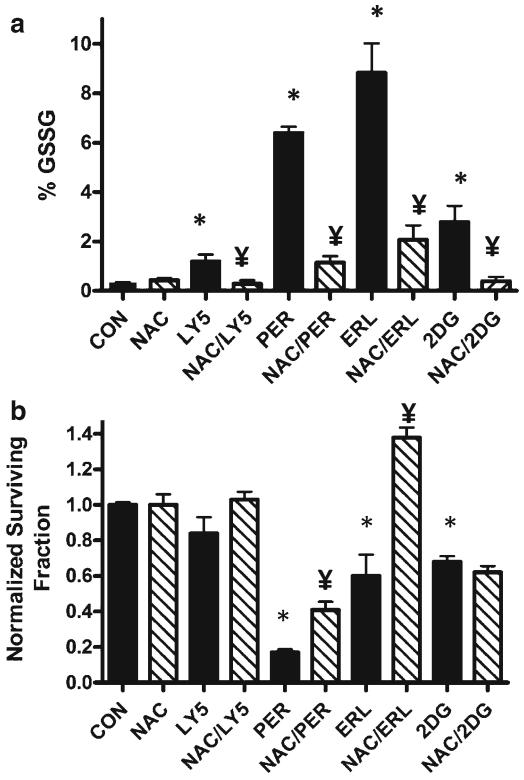

Fig. 2.2 Effect of LY294002 (LY5) on percentage glutathione disulfide (**a**) and cell survival (**b**) in FaDu head and neck cancer cells. FaDu cells were treated with 5 μM LY294002 (LY5), 5 μM perifosine (PER), 10 μM Erlotinib (ERL), or 20 mM 2-deoxyglucose (2DG) for 24 h then harvested for glutathione analysis using the spectrophotometric recycling assay (**a**) or plated for clonogenic survival (**b**). Clonogenic cell survival data were normalized to control (CON). *Error bars* represent ±1 SD of $N=4$–6 experiments performed on different days with at least two cloning dishes taken from one treatment dish. *$p<0.05$ versus control (CON), ¥$p<0.05$ versus respective treatment without NAC

increase in %GSSG induced by the EGFR/PI3K/Akt pathway inhibitors and 2DG (Fig. 2.2a) in FaDu cells further supporting the role of thiol oxidation in the mechanism of Akt inhibitors.

2.7.2.3 Effect of Akt Inhibitors on Survival

To investigate the effect of EGFR/PI3K/Akt inhibitors on FaDu cell growth, cells were analyzed for clonogenic survival after 24 h of treatment with LY5, PER, ERL, and 2DG. We observed that LY5 caused a slight decrease in survival but PER, ERL, and 2DG significantly decreased survival in FaDu cells compared to control ($p<0.01$, Fig. 2.2b). These results show that EGFR/PI3K/Akt inhibitors are able to induce varying degrees of cytotoxicity in FaDu head and neck cancer cells. When we analyzed the effect of NAC on the cytotoxicity induced by these agents, NAC partially but significantly rescued the cytotoxicity induced by PER, and completely rescued the cytotoxicity induced by LY5 and ERL (Fig. 2.2b). NAC was not able to rescue 2DG-induced cytotoxicity in FaDu cells, but we have shown in other studies that antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT)

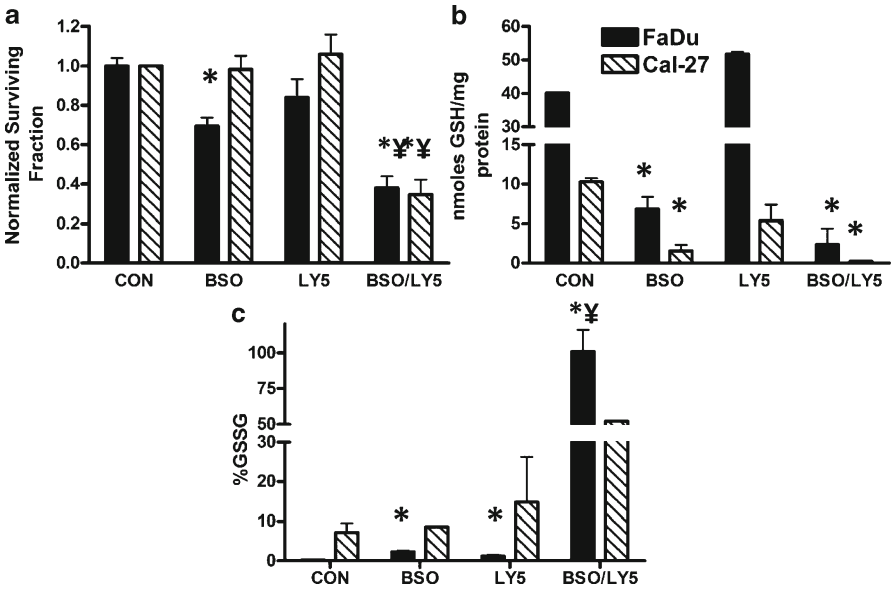

Fig. 2.3 Effect of buthionine sulfoximine (BSO) on LY294002 (LY5)-induced toxicity (**a**), total glutathione (**b**), and percentage of glutathione disulfide (**c**) in FaDu head and neck cancer cells. (**a**) FaDu cells were treated with 5 μM LY5 for 24 h with or without treatment with 1 mM BSO for 1 h before and during LY5 exposure. Clonogenic cell survival data were normalized to control (CON). *Error bars* represent ±1 SD of $N=3$ experiments performed on different days with at least four cloning dishes taken from one treatment dish. (**b**, **c**) Cells were treated as stated above and harvested for total glutathione (GSH) levels (**b**) and percentage glutathione disulfide (%GSSG) levels (**c**) using the spectrophotometric recycling assay. *Error bars* represent ± 1 SD of $N=3$ experiments. *$p<0.001$ versus control; ¥$p<0.05$ versus respective treatment without BSO

were able to completely rescue 2DG-induced cytotoxicity [18]. It is also worth mentioning that pretreating FaDu cells with exogenous glucose prior to drug treatment was also able to rescue the cytotoxicity induced by LY5, PER, ERL, and 2DG (data not shown). Taken together, Fig. 2.2 supports the hypothesis that the EGFR/PI3K/Akt inhibitors induce disruptions in thiol metabolism consistent with oxidative stress, which was reversed by NAC, and EGFR/PI3K/Akt inhibitor-induced cytotoxicity in FaDu cells may be due in part to increases in oxidative stress and the inhibition of glucose metabolism.

2.7.2.4 PER-Induced Cytotoxicity Is Enhanced by Buthionine Sulfoximine

To further probe the involvement of thiol metabolism in the mechanism of Akt pathway inhibition, an inhibitor of GSH synthesis, BSO, was used in combination with LY294002 in FaDu and Cal-27 cells. Pretreatment of cells with 1 mM BSO sensitized both cell lines to LY294002 (Fig. 2.3a), which was associated with

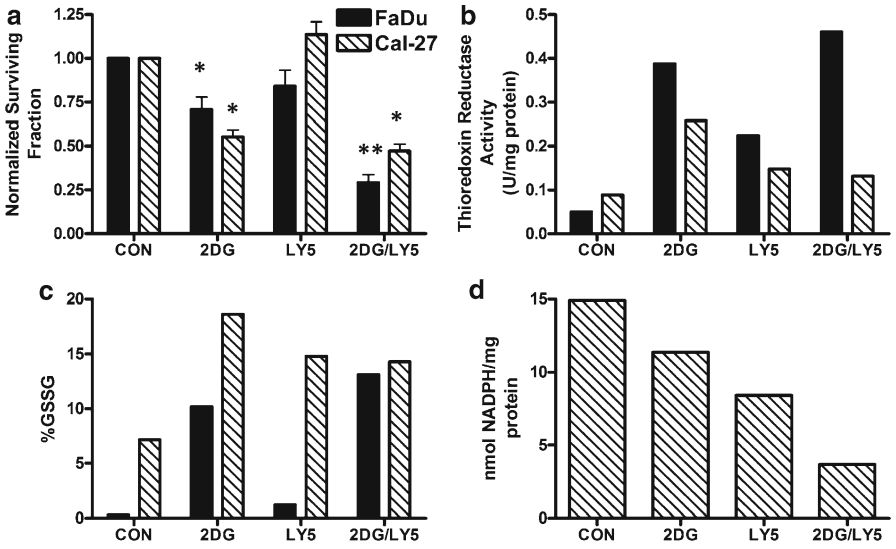

Fig. 2.4 Effect of 2-deoxyglucose (2DG) in combination with LY294002 (LY5) on cytotoxicity (**a**), thioredoxin reductase activity (**b**), percentage glutathione disulfide levels (%GSSG) levels (**c**), and NADPH levels (**d**) in FaDu and Cal-27 head and neck cancer cells. Cells were treated with 5 μM LY5 and/or 20 mM 2DG for 24 h with or without treatment with 20 mM NAC for 1 h before and during LY5 exposure. Clonogenic survival data were normalized to control (CON). *Error bars* represent ±1 SD of $N=3$ experiments performed on different days with at least two cloning dishes taken from one treatment dish. *$p<0.05$ versus control; **$p<0.001$ versus 2DG or LY5

depletion of GSH (Fig. 2.3b) and significant increases in %GSSG (Fig. 2.3c). These results suggest that BSO is enhancing LY294002-induced oxidative stress by limiting hydroperoxide metabolism through the GSH antioxidant system. Overall, the results in Figs. 2.2 and 2.3 strongly suggest that LY294002 is inducing oxidative stress via disruptions in thiol metabolism in our head and neck cancer cell model.

2.7.2.5 2DG-Induced Sensitization to LY294002

Since we observed that both 2DG and LY294002 (LY5) inhibited glucose consumption and increased thiol oxidation, we next determined the effects of 2DG in combination with LY5 in FaDu and Cal-27 cells. The cells were treated with 20 mM 2DG in combination with LY5 for 24 h and then analyzed for clonogenicity (Fig. 2.4a), %GSSG (Fig. 2.4c), thioredoxin reductase (TR, Fig. 2.4b), and NADPH levels (Fig. 2.4d). LY5 caused no significant decrease in clonogenicity as a single agent in either cell line but significant sensitization was observed with 2DG in combination with LY5 compared to either agent alone in FaDu cells (Fig. 2.4a). In addition, %GSSG increased profoundly when treated with 2DG in combination with LY5 (Fig. 2.4c), which suggests that 2DG sensitized FaDu cells to LY5 by increasing

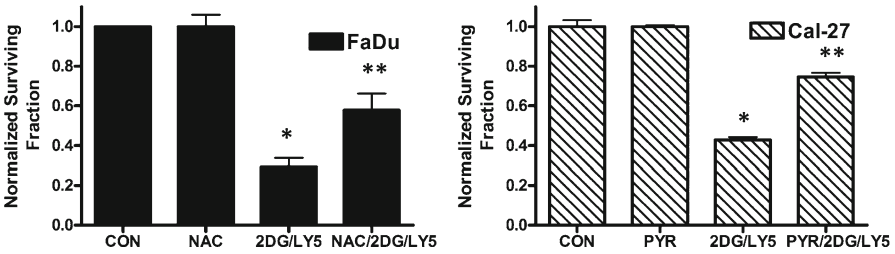

Fig. 2.5 Effect of *N*-acetylcysteine (NAC) and pyruvate (PYR) on 2-deoxyglucose (2DG) and LY294002 (LY5)-induced cytotoxicity in FaDu (**a**) and Cal-27 (**b**) human head and neck cancer cells. Cells were treated with 5 μM LY5 and/or 20 mM 2DG for 24 h with or without treatment with 20 mM NAC (**a**) or 1 mM PYR (**b**) for 1 h before and during LY5 exposure. Clonogenic survival data were normalized to control (CON). *Error bars* represent ± 1 SD of $N = 3$ experiments performed on different days with at least two cloning dishes taken from one treatment dish. *$p < 0.05$ versus control; **$p < 0.001$ versus 2DG + LY5

thiol oxidation. Since the thioredoxin (TRX) antioxidant system is another major antioxidant system in the cell [54], we analyzed TR activity to determine if the TRX system was a target in 2DG + LY5-induced cytotoxicity. TR catalyzes the reduction of TRX using NADPH as a reducing agent [54]. We observed a dramatic increase in TR activity in response to 2DG and 2DG + LY5 (Fig. 2.4b) suggesting that TR activity was being increased to counteract the increase in oxidative stress induced by 2DG + LY5 in FaDu cells.

Although 2DG only slightly enhanced sensitivity of Cal-27 cells to LY5, 2DG as a single agent was very effective at causing cytotoxicity, while inducing %GSSG and TR activity in Cal-27 cells compared to FaDu cells (Fig. 2.4a). Furthermore, NADPH levels in Cal-27 cells were significantly decreased in response to 2DG and/or LY5 suggesting that in addition to glucose metabolism being inhibited, pentose phosphate cycle activity was also being inhibited by 2DG and/or LY5 (Fig. 2.4d).

To follow up on these observations, we determined if the cell killing seen with 2DG in combination with LY5 could be mediated by oxidative stress in both FaDu and Cal-27 cells. The cells were treated with 20 mM 2DG and/or 5 μM LY5 for 24 h with or without 20 mM NAC, for 1 h before and during 2DG and LY5 exposure. We show in Fig. 2.5a that NAC partially but significantly protected FaDu cells from the cytotoxicity associated with 2DG + LY5. Treatment of Cal-27 cells with NAC was cytotoxic to these cells and we therefore pretreated the cells with the antioxidant pyruvate (PYR) which is also a byproduct of glycolysis. PYR was able to significantly protect Cal-27 cells from 2DG + LY5-induced cytotoxicity (Fig. 2.5b).

The overall data support the hypothesis that inhibition of glycolysis with 2DG or Akt pathway inhibitors induced metabolic oxidative stress in head and neck cancer cells. Furthermore, since the thiol antioxidant NAC and PYR inhibited the cytotoxicity associated with 2DG + LY5, these results suggest that a causal relationship exists between Akt pathway inhibition, inhibition of glucose metabolism, metabolic oxidative stress, and enhanced cancer cell killing.

2.8 EGFR/PI3K/Akt Pathway in Tumorigenesis

Alterations in the Akt pathway can lead to abnormal cell signaling, leading to cell proliferation, differentiation, survival, and/or migration. The result of such uncontrolled cell signaling promotes the acquisition of a cancerous phenotype. Although Akt gene mutations are rare in human cancer, several studies have shown Akt amplifications in human ovarian, pancreas, breast, and gastric malignant tumors [57–60]. It is possible that Akt gene amplification may lead to increased response to ambient levels of growth factors. However, gene amplification of PI3K has been reported in glioblastoma, human ovarian, cervical, and gastric cancers [27, 57, 61–65] and also represents about 40% of early genomic aberrations observed in HNSCC [20].

PTEN, the negative regulator of Akt activation, has been shown to be frequently deleted or mutated in a wide variety of human tumors notably in glioblastoma, prostate, endometrial cancers, and HNSCC [27, 65–74]. PTEN acts as a tumor suppressor gene, and in addition to p53, it is one of the most important tumor suppressor proteins [75, 76]. Over expression of PTEN has been shown to inhibit cell growth and enhance apoptosis in different cancer types and is also thought to be a prognostic indicator of clinical outcome [77].

Alterations upstream of the Akt pathway are also able to lead to Akt activation. For example, receptor tyrosine kinases (RTKs), the initiating signaling elements of the Akt pathway, are highly activated and dysregulated due to over expression, truncation, or mutation in certain cancer types [78–80]. Of particular interest are the members of the EGFR family (also known as Erb B tyrosine kinase receptors), which include EGFR (ErbB-1), HER2/neu (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4), which have been linked to the development and growth of up to 90% of HNSCC tumors [81]. EGFR, which exists on the cell surface, may be activated by binding of specific ligands such as EGF and transforming growth factor (TGFα). Upon activation, EGFR transitions from an inactive monomeric form to an active homodimer. EGFR may also dimerize with other EGFR family members to form an activated homodimer [78]. ErbB-2 containing heterodimers are potent activators of the Akt pathway, and tumors overexpressing ErbB-2 show constitutive Akt activity [78].

EGFR gene amplification is one of the predominant mechanisms leading to EGFR overexpression in tumors [82]. Other mechanisms of EGFR overexpression include increased activation of EGFR with its ligands, most notably TGFα, which is frequently observed in HNSCC, and the expression of mutant forms of EGFR. As a result, EGFR overexpression leads to an increase in its kinase activity by spontaneous dimerization and the activation of downstream signaling pathways which include the Akt pathway [82–86]. In addition, EGFR overexpression has been shown to be correlated with decreased sensitivity to chemotherapy and radiation, and increased risk of recurrence [87].

Resistance to many therapeutic agents may be associated with Akt hyperactivity, as seen, for example, with EGFR signaling inhibitors and in the case of head and neck cancer, cisplatin [88, 89]. Thus, inhibition of the Akt pathway is an attractive

therapeutic strategy and has potential in monotherapy and in combination with existing therapies. The preliminary studies presented here demonstrate that the mechanism of action of various agents that result in the inhibition of Akt pathway signaling (i.e., PI3K, Akt and EGFR inhibitors) involves inhibition of glucose metabolism and the induction of metabolic oxidative stress in head and neck cancer cells. In our studies, we used the PI3K inhibitor LY5 (LY), the Akt inhibitor perifosine (PER), and the EGFR inhibitor Erlotinib (ERL) and compared their effects on glucose consumption to that of the well-known glycolytic inhibitor 2DG in FaDu human head and neck cancer cells.

2.9 Effect of EGFR/PI3K/Akt Pathway Inhibitors on Glucose Metabolism

LY is a derivative of the flavonoid quercetin and is a commonly used pharmacological inhibitor of PI3K in vitro [31, 90]. LY acts by targeting the ATP binding site in the p110 catalytic subunit of PI3K which in most cases results in the inhibition of Akt activity [31, 90]. We found that 5 μM LY (LY5) was able to inhibit glucose consumption in FaDu cells compared to untreated control cells over a 24 h period (Fig. 2.1). In support of these findings, LY was found to decrease hexokinase activity in murine blastocysts [91] and inhibit GLUT1 mRNA and protein expression in human pancreatic cancer cells [92], both of which may be the mechanisms of action responsible for our observed results.

PER is a synthetic alkylphospholipid which inhibits translocation of Akt to the cell membrane preventing Akt's activation [93–96]. PER is the most developed and best characterized Akt inhibitor to date and has shown in phase I trials to be tolerable with the dose-limiting toxicity being gastrointestinal toxicity [97–102]. PER as a single agent has shown favorable responses in patients with advanced soft tissue sarcomas [99] and Waldenstrom macroglobulinemia [103]. However, responses to PER in patients with common solid tumors have been disappointing and have not justified the further investigation of PER as a single agent. Inhibition of Akt with 5 μM PER was also able to inhibit glucose consumption in FaDu cells compared to control cells (Fig. 2.1). As mentioned before, Akt is able to induce the expression of hexokinase, which catalyzes the first step of glycolysis and is able to activate the most important rate-controlling enzyme in glycolysis – phosphofructokinase-1 [43–46]. Therefore, Akt seems to have multiple mechanisms for controlling glucose metabolism in cells.

Erlotinib is a small molecule tyrosine kinase inhibitor of EGFR [104, 105]. Erlotinib binds the intercellular ATP binding domain and prevents the autophosphorylation of EGFR and subsequent signal progression [104, 105]. Since Akt could be activated by EGFR signaling, we proposed that inhibition of EGFR with Erlotinib would inhibit signaling down the Akt pathway, thus inhibiting glucose metabolism. Figure 2.1 shows 10 μM ERL was indeed capable of inhibiting glucose consumption in FaDu cells during 24 h of exposure. Previous results have shown that EGFR

is a stabilizer of an active glucose transporter, SGLT1, and activation of EGFR in the epithelium of intestine led to active transport of glucose [49]. Although ERL does inhibit Akt expression in FaDu cells (data not shown), it remains to be determined if ERL inhibited glucose consumption in our Fadu cells by the destabilization of SGLT1 or by the inhibition of Akt. We have duplicated these results in other head and neck cancer cell lines such as Cal-27, SCC-25, and SQ20B cells (data not shown) all of which over express Akt and EGFR.

These results all suggest that EGFR, PI3K, and Akt inhibitors do in fact inhibit glucose metabolism comparably to 2DG, which is a commonly used glycolytic inhibitor. More importantly, based on these results, increased Akt pathway signaling may have a significant role in the Warburg effect and this phenomenon may be exploited to selectively target cancer cells for the purpose of enhancing radio- and chemo-sensitivity in cancer therapy.

2.10 Redox Regulation of the Akt Pathway

Intracellular redox status plays a vital role in the reversible activation and inactivation of the Akt pathway [47, 106–109]. For example, moderate levels of ROS activate Akt pathway signaling and promote cell survival, but high or chronic oxidative stress inhibits this pathway resulting in apoptosis [47, 106–109]. Activation of the Akt pathway occurs mainly through the oxidative inactivation of Cys-dependent phosphatases (CDPs) or the direct oxidation of pathway kinases [106, 107]. For example, the phosphatase PTEN, the main phosphatase involved in the negative regulation of the Akt pathway, has been shown to be inactivated by oxidation by both H_2O_2 and nitrosylation, posttranslational modifications which would hyperactivate the Akt signaling pathway [107, 110]. Akt is also directly activated by oxidative stimuli. H_2O_2 and peroxynitrite treatments have both been shown to promote Akt activity via posttranslational modification of Akt [107, 111].

Since cancer cells are under increased metabolic oxidative stress compared to normal cells and the Akt pathway may be activated for survival under these oxidizing conditions, we proposed that inhibition of the Akt pathway would increase oxidative stress to such an extent that would render cancer cells sensitive to further increases in oxidative stress.

We investigated the effects of LY, PER, ERL, and 2DG on oxidative stress by analyzing glutathione (GSH/GSSG) levels. LY, PER, and ERL induced significant increases in %GSSG in FaDu cells compared to control cells (Fig. 2.2a) which indicated an increase in oxidative stress and suggests that inhibition of Akt may be involved in increasing oxidative stress. These results also implicate the role of glycolytic inhibition as a mechanism of action in Akt-induced oxidative stress, since 2DG was also able to induce %GSSG (Fig. 2.2a). To further support this idea, the thiol antioxidant NAC was able to completely suppress the increase in %GSSG induced by all three agents (Fig. 2.2a). In addition, NAC was able to significantly reverse the cytotoxicity induced by LY, PER, and ERL in FaDu cells suggesting that

increased oxidative stress was responsible for the cytotoxicity induced by these agents (Fig. 2.2a). NAC was not able to rescue 2DG-induced cytotoxicity in FaDu cells (Fig. 2.2b), but we have shown in other studies that antioxidant enzymes such as SOD and catalase (CAT) were able to rescue 2DG-induced cytotoxicity [18]. It is also worth mentioning that pretreating FaDu cells with exogenous glucose or pyruvate prior to drug treatment was also able to rescue the cytotoxicity induced by LY5, PER, ERL, and 2DG (data not shown), suggesting that inhibition of glucose metabolism and thiol oxidation are involved in the mechanism of action of Akt inhibitors.

To further probe the role of thiol metabolism in the effects of Akt inhibitors, we used BSO, an inhibitor of glutamate cysteine ligase, which is believed to be the rate-limiting enzyme in the synthesis of GSH [112, 113]. Previous studies in our laboratory have shown that BSO significantly depleted GSH pools in breast and head and neck cancer cells while sensitizing cancer cells to chemotherapy agents [18, 114]. BSO has also been used in clinical trials for cancer therapy to enhance the cytotoxicity of chemotherapeutic agents [115]. In the present studies, BSO was found to significantly increase the cytotoxicity induced by LY in FaDu and Cal-27 cells (Fig. 2.3a). As expected, BSO significantly decreased total GSH levels (Fig. 2.3b) and increased %GSSG as a single agent and in combination with PER (Fig. 2.3c), which suggests that inhibition of GSH synthesis further enhanced the oxidative stress induced by LY and further sensitized these cells to the toxicity of LY in FaDu and Cal-27 cells.

2.11 Role of Akt Signaling and FOXO Proteins on Glycolysis

The results in Figs. 2.1–2.3 all suggest that inhibition of glucose metabolism and increased oxidative stress are involved in the inhibition of the Akt pathway. Although Akt inhibition has been shown to have direct effects on glucose metabolism by disrupting glucose transport and inhibiting enzymes involved in glycolysis, it is possible that downstream substrates of Akt may also exert an effect on glucose metabolism. For example, Akt pathway activation results in the cytoplasmic accumulation of the forkhead box O (FoxO) family of transcription factors, FOXO1 and FOXO3a, occluding these factors from the nuclear genes that they activate [116]. FOXO1 has been shown to stimulate expression of gluconeogenic genes (i.e., phosphoenolpyruvate carboxykinase [PEPCK] and glucose-6-phosphatase) and suppress expression of genes involved in glycolysis (i.e., glucokinase) in liver cells [117]. In addition, some of the genes that FOXO3a regulates include antioxidant enzyme genes such as MnSOD, CAT, Peroxiredoxin, Sesn3, and iNOS [111]. Since FOXO1 is involved in the suppression of glycolysis and Akt inhibits FOXO1 activity, then inhibition of Akt may stimulate FOXO1 activity, which would then suppress the expression of enzymes involved in glycolysis. In addition, inhibition of Akt and activation of FOXO3a would increase expression of target antioxidant enzymes which may be in response to the oxidative stress induced by the inhibition of Akt.

Taken together, it is clear that in addition to increased metabolic oxidative stress, disruptions in glucose metabolism are directly and indirectly involved in the inhibition of Akt pathway signaling.

2.12 Effect of LY5 in Combination with 2-Deoxyglucose

The glucose analogue 2DG is a well known and clinically relevant inhibitor of glucose metabolism [118, 119]. We hypothesized that 2DG in combination with LY may act to inhibit critical aspects of thiol-mediated hydroperoxide metabolism leading to increased steady-state levels of ROS and enhanced tumor cell killing via metabolic oxidative stress. 2DG is a clinically relevant analogue of glucose that competes with glucose for uptake and entry into glucose metabolic pathways [119–122]. 2DG can therefore create a drug-induced state of glucose deprivation, although it does not completely inhibit the regeneration of NADPH from NADP+ because it is a substrate for glucose-6-phosphate dehydrogenase [122]. Inhibition of glucose metabolism has been observed in animals administered 2DG without toxicity until high levels (>2 g/kg body weight) were achieved [120], and 2DG has been shown to be tolerable in humans when administered up to 200 mg/kg [123].

We predicted that the combination of 2DG and LY would have an additive and possibly synergistic effect on clonogenic cell killing in FaDu and Cal-27 human head and neck squamous carcinoma cells by enhancing metabolic oxidative stress. Accordingly, treatment with 2DG has been shown to induce cytotoxicity, significant increases in prooxidant production, and profound disruptions in thiol metabolism in head and neck cancer, colon, breast, cervical, and prostate cancer cells, suggesting that oxidative stress was involved with the mechanism of action [5, 17, 55, 114, 124, 125].

In the current study, we found that the combination of 2DG and LY showed at least additive (and possibly more than additive) cell killing in FaDu cells compared with 2DG or LY alone (Fig. 2.4a). However, Cal-27 cells were more resistant to LY, and 2DG + LY-induced cell killing was only slightly increased compared to 2DG alone (Fig. 2.4a). These observations in Cal-27 cells suggest that 2DG is more cytotoxic in this cell line compared to FaDu and that the cytotoxicity we see with 2DG + LY in Cal-27 is mostly due to 2DG (Fig. 2.4a).

The increase in %GSSG induced by 2DG + LY (Fig. 2.4c) compared to control in both cell lines suggests that oxidative stress is involved. 2DG alone seemed to cause the greatest increase in %GSSG in Cal-27 cells which again indicates that 2DG is more effective in this cell line (Fig. 2.4b). Nevertheless, we believe that 2DG and the combination of 2DG + LY cause an increase in steady-state levels of hydroperoxides and this increase exceeds the metabolic capabilities of the glutathione system to maintain glutathione in the reduced form.

To further determine the role of 2DG and LY on hydroperoxide metabolism, we investigated the thioredoxin (TRX) antioxidant system. The TRX system is a highly conserved, ubiquitous system composed of thioredoxin reductase (TR), thioredoxin

(TRX), thioredoxin peroxidases (a.k.a. peroxiredoxins) and NADPH [126]. The TRX system plays an important role in the redox regulation of multiple intracellular processes and resistance to cytotoxic agents that induce oxidative stress [127, 128]. TrxRed is a selenocysteine-containing protein that catalyzes the reduction of Trx using NADPH as a reducing agent [126]. TrxRed has been shown to initiate signaling pathways in response to oxidative stress that play a role in protecting the cell from oxidative stress and is therefore believed to be a potential target for cytotoxic agents that induce oxidative stress [127, 129, 130].

We observed that 2DG and LY alone and in combination increased TR activity in both cell lines, with the greatest effects observed in FaDu cells (Fig. 2.4b). Again 2DG alone was more effective in Cal-27 cells by showing the greatest increase in TR activity compared to the other treatment groups (Fig. 2.4b). These results suggest that TR activity may be increased in response to the oxidative stress induced by 2DG and/or Akt pathway inhibitors. In addition, inhibition of glucose metabolism with 2DG and/or Akt pathway inhibitors may induce oxidative stress by disrupting glutathione and thioredoxin metabolism.

We next measured NADPH levels in Cal-27 cells since NADPH is the source of reducing equivalents for the glutathione and thioredoxin antioxidant systems [9, 12, 15]. We observed that 2DG and/or LY greatly decreased NADPH levels with 2DG + LY causing the greatest decrease in NADPH compared to the other treatment groups (Fig. 2.4d). The results in Fig. 2.4 suggest that inhibition of glucose metabolism with 2DG and LY inhibited the pentose phosphate cycle resulting in decreased NADPH, increased %GSSG, and increased TR activity.

Finally, to further demonstrate that oxidative stress was involved in 2DG + LY-induced cytotoxicity, we show that NAC significantly protected FaDu cells from 2DG + LY-induced cytotoxicity (Fig. 2.5a). Since NAC was very toxic in Cal-27 cells in the presence of 2DG, we treated these cells with pyruvate (PYR). Pyruvate, in addition to being a substrate for energy metabolism via the TCA cycle and mitochondrial oxidative phosphorylation, has been shown to scavenge H_2O_2 and other hydroperoxides directly [8, 11, 12]. We observed that PYR did protect Cal-27 cells from 2DG + LY-induced cytotoxicity (Fig. 2.5b). This protection with PYR has also been observed in other head and neck cancer cells (unpublished data).

2.13 Rationale for Glucose Consumption and Oxidative Stress in Akt Signaling

Overall, the data provided here suggest that inhibition of glucose metabolism and increased intracellular oxidative stress contributes to the toxicity of Akt pathway inhibitors, which is similar to the mechanism of actions observed for 2DG. The results also suggest that manipulating intracellular redox levels (i.e., glutathione) may affect cellular responses to LY and other Akt inhibitors. The scheme shown in Fig. 2.6 illustrates some of the hypothetical relationships between glucose metabolism,

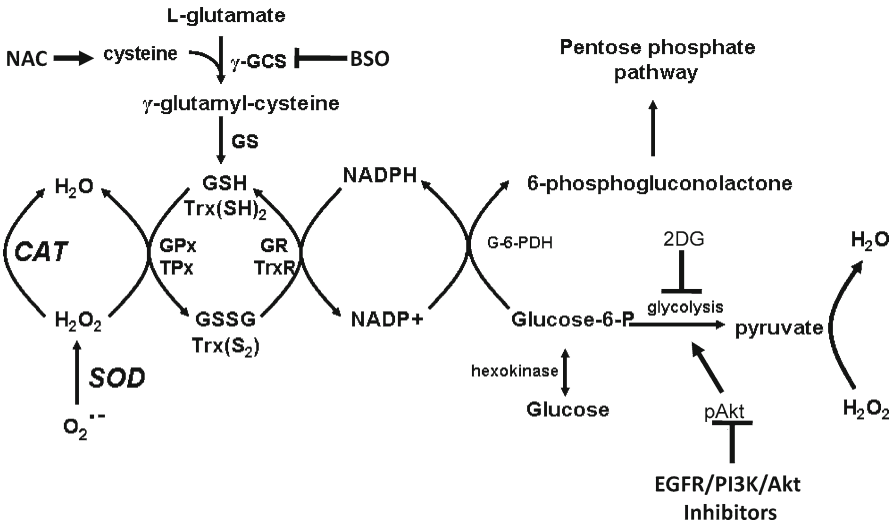

Fig. 2.6 Hypothetical biochemical rationale to explain the role that Akt plays in glucose metabolism and hydrogen peroxide metabolism. The activated form of Akt (pAkt) activates glycolysis by direct and indirect mechanisms thereby increasing glucose transport into the cell and hexokinase activity allowing for the phosphorylation of glucose to glucose-6-phosphate (Glucose-6-P). Glucose-6-phosphate continues into glycolysis to form pyruvate, a known scavenger of hydrogen peroxide (H_2O_2). Glucose-6-P proceeds through the first step in the pentose phosphate cycle via glucose-6-phosphate dehydrogenase (G-6-PDH) to 6-phosphogluconolactone leading to the generation of NADPH from NADP+. NADPH is a source of reducing equivalents for the glutathione system consisting of GSH, GSSG, glutathione peroxidase (GPx), and glutathione reductase (GR) and the thioredoxin system consisting of reduced thioredoxin ($Trx(SH)_2$), thioredoxin disulfide ($Trx(S_2)$), thioredoxin peroxidase (TPx), and thioredoxin reductase (TrxR). The glutathione and thioredoxin systems participate in the detoxification of H_2O_2 and organic hydroperoxides. 2-Deoxy-D-glucose (2DG) competes with glucose for uptake into the cell and phosphorylation by hexokinase into 2-deoxy-D-glucose-6-phosphate (2DG-6-P). 2DG-6-P is unable to continue down the glycolytic pathway but is able to proceed through the first step in the pentose phosphate cycle via G-6-PDH to 6-phospho-2-deoxygluconolactone. 6-Phospho-2-deoxygluconolactone cannot go further in the pentose phosphate cycle. Agents that inhibit Akt signaling (i.e., EGFR, PI3K, or Akt inhibitors) are also able to inhibit glycolysis. Superoxide dismutase (*SOD*) converts superoxide ($O_2^{\bullet-}$) to H_2O_2 which is converted to H_2O by catalase (*CAT*) or GPx. *N*-Acetyl-cysteine (*NAC*) provides cysteine which reacts with L-glutamate catalyzed by glutamate cysteine ligase (GCL, inhibited by L-buthionine-[S,R]-sulfoximine [*BSO*]) to form ã-glutamyl-cysteine. Glutathione synthetase (GS) converts ã-glutamyl-cysteine into GSH

ROS metabolism, Akt pathway inhibitors, and antioxidants suggested by the results of the current study. Inhibiting glucose metabolism with Akt pathway inhibitors and/or 2DG in cancer cells is hypothesized to limit the production of pyruvate and the regeneration of NADPH leading to increased steady-state levels of H_2O_2 and hydroperoxides from metabolic sources resulting in cytotoxicity. BSO is thought to further enhance the toxicity of Akt pathway inhibitors by inhibiting the synthesis of GSH that is required for GSH peroxidases and GSH transferases, both of which are believed to protect against oxidative stress. Finally, the antioxidants NAC and PYR

are able to protect against LY and 2DG+LY by acting to augment small molecular weight intracellular thiols that are capable of scavenging toxic species and by directly scavenging H_2O_2 and other hydroperoxides directly, respectively.

Overall, the results of this study support the hypothesis that Akt pathway inhibitors disrupt glucose metabolism and induce metabolic oxidative stress in head and neck cancer cells. These data also strongly support the potential therapeutic use of Akt pathway inhibitors in combination with chemotherapeutic agents that increase prooxidant production, as well as the new biochemical rationale shown in Fig. 2.6 for combining Akt pathway inhibitors and inhibitors of glucose and hydroperoxide metabolism for enhancing the cytotoxicity of anticancer agents thought to cause injury via oxidative stress.

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Chapter 3
Enhancement of Cancer Therapy Using Ketogenic Diet

Melissa A. Fath, Andrean L. Simons, Jeffrey Erickson, Mark E. Anderson, and Douglas R. Spitz

Abstract Ketogenic diets, which are low in protein and carbohydrates and high in fats, result in elevated ketones (β-hydroxybuturate and acetoacetate; precursors to Acetyl-CoA) forcing cells to rely more heavily on mitochondrial metabolism for energy production. It has been hypothesized that cancer cells, relative to normal cells, exist in a condition of chronic metabolic oxidative stress mediated by $O_2^{\bullet-}$ and H_2O_2, with a major site of pro-oxidant production being mitochondrial electron transport chain complexes. If cancer cells (relative to normal cells) have defective mitochondrial O_2 metabolism that results in chronic metabolic oxidative stress and ketogenic diets forcing cancer cells to rely more heavily on mitochondrial O_2 metabolism, then ketogenic diets would be expected to selectively cause oxidative stress in cancer cells. The increased steady-state level of oxidative stress in turn would be expected to selectively sensitize cancer cells to conventional cancer therapeutic agents that cause cell killing via oxidative stress. The current study demonstrates that a ketogenic diet increases parameters indicative of oxidative stress, as demonstrated by an increase in oxidized proteins in the plasma of mice fed a ketogenic diet, and also sensitizes FaDu head and neck cancer xenograft tumors to the antitumor effects of cisplatin. These results support the hypothesis that ketogenic diets may be useful adjuvants for improving outcomes with cisplatin in head and neck cancer therapy.

M.A. Fath, PhD (✉) • A.L. Simons, PhD • D.R. Spitz, PhD
Free Radical and Radiation Biology Program, Department of Radiation Oncology,
Holden Comprehensive Cancer Center, The University of Iowa,
B180 Medical Laboratories, Iowa City, IA 52242, USA
e-mail: melissa-fath@uiowa.edu

J. Erickson, PhD • M.E. Anderson, MD, PhD
Cardiovascular Medicine, Department of Internal Medicine,
The University of Iowa, Iowa City, IA 52242, USA

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3.1 Introduction

3.1.1 Metabolic Oxidative Stress and Cancer

An increased rate of glucose uptake in cancer cells is the most general metabolic difference between cancer and normal cells. Originally, it was theorized that the increased glucose utilization in cancer cells was due to increased demand for ATP due to a defect in respiration [52]. However, no clear defects in tumor cells' ability to generate ATP by respiration have been demonstrated [22, 32a]. In addition to its role in ATP production, glucose metabolism is known to play a major role in detoxification of peroxides via the formation of pyruvate (which scavenges peroxides directly) and through regeneration of NADPH (from glucose-6-phosphate dehydrogenase activity in the pentose cycle) which acts as the cofactor in glutathione (GSH)- and thioredoxin (Trx)-mediated peroxide detoxification reactions. The glutathione peroxidase and peroxiredoxin pathways utilize the reducing equivalents in GSH and Trx, respectively, to scavenge hydroperoxides capable of causing oxidative stress ([4]; 32b [7, 31, 41, 44, 51]).

During oxidative phosphorylation within the inner mitochondrial membrane, electrons are shuttled down electron transport chain complexes I–IV resulting in the production of a transmembrane proton potential gradient which is coupled to the production of ATP through the ATP synthase in complex V. In normal cells, as much as 1% of the electrons flowing through ETCs are thought to undergo one-electron reductions of O_2 to form $O_2^{\bullet-}$ which can undergo further chemical and enzymatic dismutation reactions to become H_2O_2 and other reactive oxygen species (ROS) [8, 35, 50]. Cellular antioxidant detoxification pathways scavenge ROS and prevent them from causing intracellular damage, including lipid peroxidation, DNA damage, and protein oxidation [3, 7, 13, 44]. In cancer cells, the ability of mitochondria to consume O_2 and produce ATP does not appear to be compromised; however, mitochondrial structure and mitochondrial DNA integrity have been reported to be abnormal [22]. The mitochondria of malignant human tumor cells have been shown to exhibit significant histological abnormalities characterized by unusual arrangements of mitochondrial cristae, mitochondrial hypertrophy, and fragmentation when compared to normal human cells [48]. Furthermore, many tumors have been shown to have high rates of mitochondrial DNA mutations (relative to normal human tissues) [17, 36] and this has been suggested to lead to increased $O_2^{\bullet-}$ and H_2O_2 production [25, 39]. In support of this idea, mitochondria from human colorectal cells have been shown to have higher levels of ROS as well as having increased oxidative damage, as measured by TBARS and protein carbonyl content compared with mitochondria from normal adjacent colon tissue [43].

Alterations in mitochondrial ETC structure and function could, therefore, result in increased steady-state levels of ROS implying that cancer cells may exist in a chronic condition of metabolic oxidative stress that is compensated by increasing hydroperoxide metabolism using reductants derived from glucose [2, 24, 38, 47]. In support of this hypothesis, breast and colon cancer cells have been found to

demonstrate significantly increased steady-state levels of ROS (i.e., $O_2^{\bullet-}$ and H_2O_2), relative to normal colon and breast cells, that were more pronounced in the presence of mitochondrial electron transport chain blockers [5]. In addition, colon cancer cells were more susceptible to oxidative damage caused by 2-deoxyglucose, a competitive inhibitor of glucose metabolism, than were normal colon epithelia cells in vitro [5]. Furthermore, the combination of electron transport chain blocker with 2-deoxyglucose decreased tumor growth rate in an in vivo model of colon cancer [15]. Therefore, it is possible that forcing tumors cells to increase dependence on mitochondrial electron transport for energy production (by lowering glucose and utilizing fats through feeding a ketogenic diet) while treating them with conventional chemotherapy agents that are known to induce oxidative stress may selectively enhance cancer cell killing without damaging normal tissue.

3.1.2 Ketogenic Diet

Ketogenic diets are low in protein and carbohydrates and high in fats. Ketogenic diets have been used safely and effectively for 80 years for the treatment of epilepsy [20]. Side effects associated with ketogenic diets are usually minor and include mild acidosis, constipation, modest reduced blood glucose, as well as elevated cholesterol and increased incidence of kidney stones with chronic (≥6 years) use [21, 29, 42]. Ketogenic diets derive their name from the elevation in blood ketones, mainly β-hydroxybutyrate (βHB) and acetoacetate (AA), which are precursors for acetyl Co A, the first step in the Krebs cycle and which occur due to increased fatty acid oxidation in the liver. Ketones are transported through the blood to the tissues, where they provide energy to the cell. Because of the low carbohydrate nature of ketogenic diets, there is often a modest reduction of blood glucose levels and overall greater glycemic control [53]. The combination of the reduction in blood glucose and the rise in ketones forces cells to rely more heavily on mitochondrial respiration than on glycolysis for energy production. It has been demonstrated that the rates of glucose uptake as well as rates of flux into the glycolytic pathway are reduced in rats fed a ketogenic diet [9, 23].

3.1.3 Ketosis and Oxidative Stress

Elevated levels of ketones have been implicated in generation of cellular metabolic oxidative stress. Hyperketotic diabetic humans have a higher level of lipid peroxidation in red blood cells and a significant decrease in cellular glutathione relative to normal ketonic diabetic controls [27]. Lim et al. also found elevated indices of lipid peroxidation in cultured human endothelial cells treated with AA [26]. AA was also found to deplete cellular glutathione, increase intracellular ROS, and activate cellular signaling pathways associated with oxidative stress in primary rat hepatocytes [1]. Finally, chronic exposure to βHB was shown to increase ROS production in cardiomyocytes [37].

3.1.4 Ketogenic Diets' Use in Cancer

Recently, ketogenic diets have been used in the treatment of cancer in both mouse models and humans. Beck et al. saw a decrease in tumor weight and an improvement in cachexia in mice bearing MAC16 colon cancer cells fed a ketogenic diet that supplied 80% of the calories as medium-chain triglycerides [6]. In a study using both an orthotopic and xenograft model of astrocytoma and malignant glioma, mice were fed a standard rodent diet, a ketogenic diet, or a caloric-restricted ketogenic diet. In the restricted ketogenic diet, tumors were significantly reduced in size and there was an increase in animal survival. The tumors from the mice fed caloric-restricted ketogenic diet had a decrease in tumor vascularity indicating that this diet was antiangiogenic [54]. More recently, Freedland et al. saw an improvement in tumor size and survival in mice fed a ketogenic diet on equal caloric intake when compared to a high-fat, high-carbohydrate "western" diet in a mouse model of prostate cancer [19]. In humans, a case report in the literature indicated that two female pediatric patients with advanced-stage malignant astrocytoma tumors had clinical improvement and an average decrease in tumor FDG uptake by PET scans of 21.8% when fed a ketogenic diet composed primarily of medium-chain triglycerides [34]. These observations have stimulated the opening of two phase I clinical trials, one in Tuebingen Germany testing the effects of ketogenic diet on recurrent glioblastoma and another testing a very low carbohydrate "Atkins style" diet for advanced cancers at Albert Einstein College in New York [16, 40]. Despite interest in ketogenic diets as an adjuvant in cancer therapy, the mechanism(s) responsible for enhanced therapeutic response are not well-understood and the role that oxidative stress may play in this process has not been investigated.

If cancer cells (relative to normal cells) have defective mitochondrial O_2 metabolism that results in chronic metabolic oxidative stress and ketogenic diets force cancer cells to rely more heavily on mitochondrial O_2 metabolism to generate energy, then ketogenic diets would be expected to selectively cause oxidative stress in cancer cells which in turn would be expected to selectively sensitize cancer cells to cancer therapeutic agents like IR and cisplatin that are known to cause cell killing via oxidative stress. Furthermore, if cancer cells increase their utilization of glucose to compensate for increased metabolic production of hydroperoxides, then an increase in glycemic control would be expected to further enhance the anticancer effects of ketogenic diets combined with radio/chemotherapy.

3.2 Experimentation and Results

3.2.1 The KetoCal® Diet Induces Ketosis in Nude Mice and Can Be Used Safely

In order to establish a mouse model for studying the effects of ketogenic diets, three athymic, 6–8-week-old, nude female mice were fed *ad libitum*, a standard rodent diet (NIH-31 modified 25% protein, 21% fat, 54% carbohydrate), and three mice

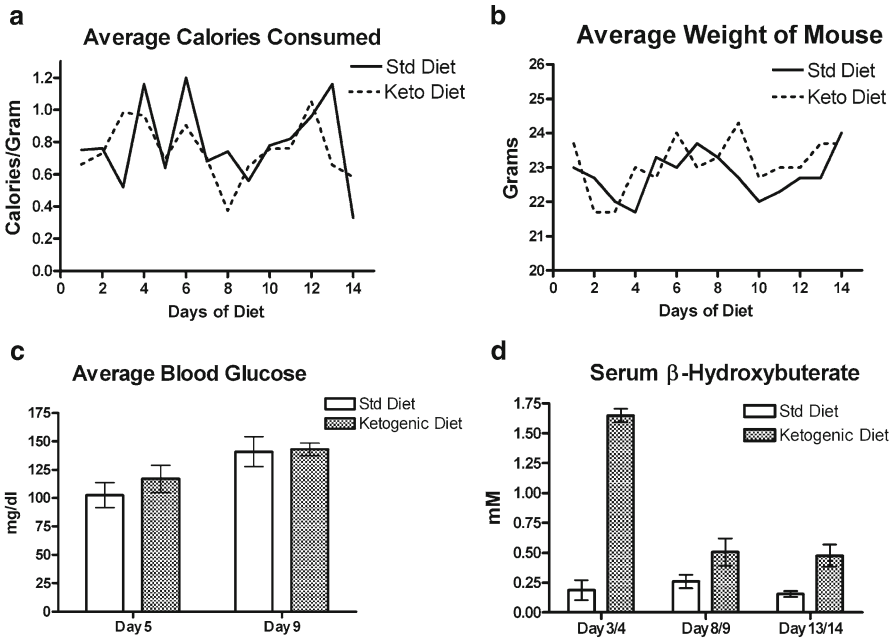

Fig. 3.1 Ketogenic diet is well-tolerated by nude mice and results in elevated serum ketones. Mice on ketogenic diet consumed equal calories per gram of mouse (**a**) and maintained equivalent weight (**b**) as mice on standard rodent chow. Blood glucose levels also did not differ significantly between the diet groups (**c**). Serum βHB levels were significantly higher in mice fed ketogenic diet compared to standard chow (**d**). $n = 3–6$ mice on each diet ± SEM

were fed ketogenic diet consisting of KetoCal® (Nutricia North America, a formula designed for children with epilepsy). This diet has a ketogenic ratio (fats: proteins + carbohydrates) of 4:1 (energy distribution: fat 90%, carbohydrate 1.60%, and protein 8.40%) and the fat is derived from soybean oil (100% long-chain fatty acids). KetoCal® was prepared as a paste on a culture dish lid by adding water (water:KetoCal; 1:2), then placed upside down in the food hopper, and attached to assure the animals access. The food remaining in the hopper the next day was replaced with fresh food, dried and weighed to determine caloric intake. Mice fed ketogenic diet consumed equivalent calories per gram of mouse as mice fed standard diet (Fig. 3.1a); there was no significant change in animal weight in either group (Fig. 3.1b) and all mice appeared healthy. Interestingly, there was no significant differences in blood glucose levels measured via glucometer on days 5 and 9 (Fig. 3.1c). Serum βHB levels (as an indicator of ketosis) were elevated by day 3 or 4 of the diet, and then dropped somewhat before rising again, but remained significantly elevated compared to levels in mice on standard diet throughout the experiment (Fig. 3.1d). This study demonstrates that nude mice easily tolerate a diet consisting of KetoCal® paste, as determined by measurements of body weight, and the ketotic state can easily be confirmed in mice using blood βHB levels on days 3–14 of the experiment.

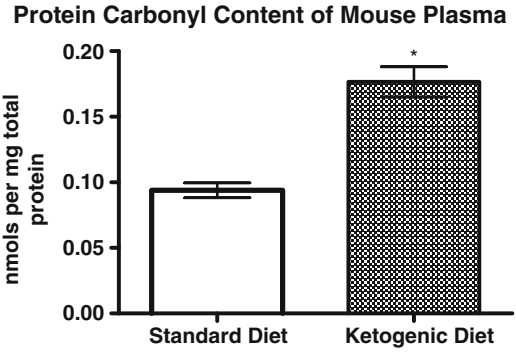

Fig. 3.2 Ketogenic diet results in increased protein carbonyls compared to mice fed standard rodent chow. Nude female mice were fed a ketogenic diet or a standard rodent diet for 5 days. Mouse plasma was derivatized with dinitrophenylhydrazine and analyzed spectrophotometrically at 370 nm. $n = 3$ mice on each diet, *error bars* represent ± SEM, *asterisk* $p = <0.01$

3.2.2 Evidence of Systemic Oxidative Stress Caused by Ketogenic Diet

Three athymic, adult, nude female mice were fed *ad libitum*, a standard rodent diet, and three mice were fed ketogenic diet consisting of KetoCal® as before. On day 5 of the diet, the mice were subjected to terminal cardiac bleeds and the resulting plasma was assayed for protein carbonyls using the assay kit from Cayman Chemical Company (Ann Arbor, MI). Protein carbonyl content is a commonly used marker of general oxidative stress in animals [49]. Redox-cycling cations, such as Fe^{2+} or Cu^{2+}, can bind to proteins and with the aid of further attack by H_2O_2 or O_2 can transform side-chain amine groups on several amino acids (i.e., lysine, arginine, proline, or threonine) into carbonyls. The resulting side-chain carbonyls can be detected by reaction with 2,4-dinitrophenylhydrazine, forming a Schiff base and corresponding hydrazone, which can be analyzed and quantified spectrophotometrically [30]. Increased protein carbonyl content has been associated with aging [11] and multiple diseases, including diabetes [28], cancer [12], and cardiovascular disease [46]. Conversely, dietary restriction, which has been shown to increase animal longevity, is associated with a decrease in protein carbonyls [33]. In the current study, plasma from mice fed a ketogenic diet had a significant increase in protein carbonyl content when compared to mice fed a standard diet (Fig. 3.2). These data support the hypothesis that increasing production of energy through oxidative phosphorylation and decreasing glycolysis can result in general systemic oxidative stress.

3.2.3 Mouse Tumor Model of Human Head and Neck Cancer

Squamous cell carcinoma of the head and neck (HNSCC) comprises roughly 3–5% of all cancers that occur in the USA and is newly diagnosed in over 40,000 patients

annually with a 5-year survival rate of 56% [10]. The management of locally advanced or recurrent HNSCC usually involves treatment with cisplatin (CIS) alone or in combination with other chemotherapeutic agents or radiotherapy (IR) [18]. In order to investigate the tolerability and effects of a ketogenic diet combined with IR or CIS in the human head and neck xenograft model, 4×10^6 FADU squamous cell head and neck carcinoma cells were injected into the right flank of female nude mice 6–8 weeks old [45]. When the tumors reached approximately 0.06 cm^3, mice were assigned (six mice per group) to control (100-μl sterile saline i.p.), CIS (2 mg/kg i.p. every other day for 2 weeks), or IR (2 Gy on days 3, 5, 10, and 12) and fed either standard rodent chow or KetoCal® *ad libitum* for 2 weeks during therapy. The flank xenograph tumor model not only allowed for easy tumor monitoring using Vernier calipers, but also allowed for shielding of the body with lead allowing only the tumor to be irradiated. Mean tumor volumes were calculated and plotted versus time to determine response to therapy. Mice were removed from the study due to tumor growth when the tumors reached 15 mm in diameter; the day was recorded and used to plot Kaplan–Meier survival.

3.2.4 *Evidence of Systemic Oxidative Stress Caused by Cancer and/or Treatment with CIS, IR, and Ketogenic Diet*

Recently, a polyclonal antibody was developed which recognizes the oxidized methionine residues on Ca^{2+}/Calmodulin-dependent kinase II (ox-CaMKII; M281/282) [14]. The fidelity of the antiserum has been validated using purified wild-type and M218/V282 mutant CaMKII protein with and without treatment with H_2O_2. This polyclonal antibody also shows significant reactivity to the oxidized methionine epitope of other proteins, including contrapsin, a serine protease abundant in mouse serum (unpublished observation), and therefore can be used as a general systemic marker for oxidative stress in vivo. Western blot analysis of plasma harvested from the animals at the time of removal from the therapy study (10–30 days after the end of therapy), was accomplished using this polyclonal antibody serum. Animals bearing FaDu tumors demonstrated an increased level of immuno-reactive protein recognized by the ox-CaMKII antibody, relative to the total protein recognized by a commercially available antibody against unoxidized protein, compared to animals that were cancer free (Fig. 3.3). The ratio of oxidized to total protein detected with this antibody was further increased in tumor-bearing animals treated with ketogenic diet or IR supporting the hypothesis that these treatments induce increases in systemic oxidative stress.

3.2.5 *Feeding a Ketogenic Diet Sensitizes FaDu Human Head and Neck Cancer Xenografts to Cisplatin*

All mice treated with IR or IR + ketogenic diet appeared healthy and maintained weight throughout the experiment (Fig. 3.4). Surprisingly, mice in the CIS + ketogenic

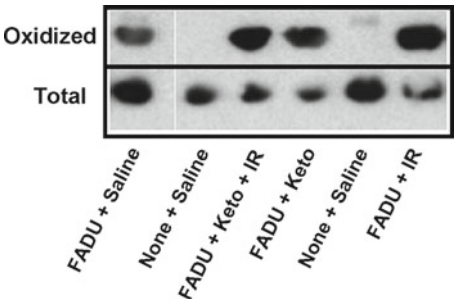

Fig. 3.3 Cancer-bearing mice with and without treatment demonstrate increases in immunoreactive, methionine-oxidized protein in serum. Serum samples from control (no tumor) or FaDu tumor-bearing mice on standard or ketogenic diet treated with IR were harvested 1–4 weeks following treatment. Samples were analyzed using Western blotting and probed with antibodies against total protein or methionine-oxidized protein

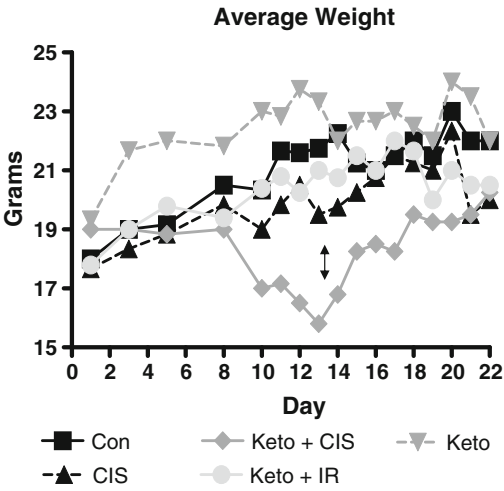

Fig. 3.4 The average weight of mice on ketogenic diet, IR + keto diet, or CIS + standard diet was not significantly different from that of control group. Mice treated with CIS + keto diet lost weight during therapy; however, they quickly regained weight when fed standard chow (*arrow*) at the end of the treatment period

diet group did exhibit significant weight loss at 8–13 days of therapy (Fig. 3.4) requiring the last two scheduled doses of CIS to be reduced by 50%. However, the mice quickly regained weight and appeared completely healthy (Fig. 3.4) after therapy was completed and they were returned to standard rodent chow at the end of 2 weeks (arrow in Fig. 3.4; day 13).

Mice treated with the modest total dose of IR (4×2 Gy fractions) showed no inhibition of growth compared to control tumors or mice fed ketogenic diet (Fig. 3.5a).

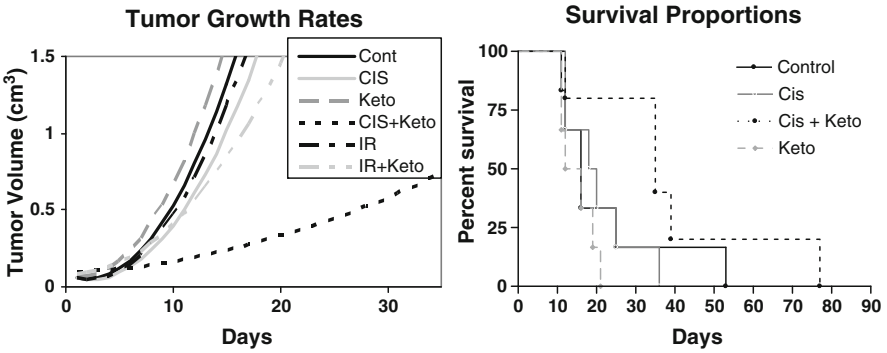

Fig. 3.5 Ketogenic diet + CIS is effective in decreasing tumor growth of FADU human head and neck cancer cells in vivo. FADU cells were injected into hind-leg region of nude mice. Mice were fed *ad libitum* standard rodent chow or ketogenic diet and treated with injections of saline (Cont), CIS, or exposure to IR (total 8 Gy). *Curves* represent tumor growth estimates from the linear mixed effects regression analysis (**a**). Kaplan–Meier survival plots for control, ketogenic, CIS, and CIS + ketogenic treatment groups are presented in (**b**)

In addition, this IR dose also did not significantly alter tumor growth when combined with ketogenic diet ($p = 0.189$; Fig. 3.5a); however, one mouse in this group had complete regression of tumor.

The mice given CIS + ketogenic diet demonstrated significantly longer tumor growth delay ($p < 0.01$; Fig. 3.5a) as well as significantly longer ($p < 0.01$) median survival (36 days) compared to mice on CIS + standard diet (19 days) or ketogenic diet groups (14 days) (Fig. 3.5b). Overall, these results clearly demonstrate that ketogenic diet sensitizes FaDu head and neck tumors to the antitumor effects of CIS therapy in vivo.

3.3 Summary and Conclusions

3.3.1 Clinical Significance of Ketogenic Diet and Metabolic Oxidative Stress in Cancer Therapy

The current results demonstrate the potential for utilizing ketogenic diets to enhance cancer cell responses to conventional chemotherapeutic interventions in vivo and suggest that the mechanism responsible for this chemosensitization may involve metabolic oxidative stress. Traditional cancer therapy is based on the concept that certain cytotoxins kill cancer cells preferentially, relative to normal cells, because of physiological characteristics peculiar to the cancer cell. By combining a ketogenic diet (and potentially other high-protein/high-fat diets) that forces cancer cells to utilize mitochondrial oxidative metabolism, we can potentially take advantage of

fundamental biochemical differences in mitochondrial production of ROS to selectively sensitize cancer cells to traditional chemoradiation therapies that cause oxidative stress. Given the safety of ketogenic diets in humans, the strategy of utilizing these diets to selectively induce chemo- and/or radiosensitization during cancer therapy may allow for both improved cancer control as well as the de-escalation of doses of cytotoxic agents, thereby limiting normal tissue injury.

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Chapter 4
Superoxide Dismutase and Cancer Therapy

Melissa L. Teoh-Fitzgerald and Frederick E. Domann

Abstract The primary superoxide scavenger, SOD, has become an important potential therapeutic modality for cancer treatment in view of the link between reactive oxygen species (ROS) and carcinogenesis. Ironically, these same ROS also play a critical role in anticancer therapies that depend on ROS generation for their action rendering the use of SOD, in these settings, as a potential interfering factor that could decrease therapeutic efficacy. This complicating situation arises since ROS are well recognized for playing a dual role as both beneficial signaling molecules as well as deleterious damaging species in biological systems. A growing body of evidence has shown that ROS act as secondary messengers in intracellular signaling cascades, which stimulate and support the oncogenic phenotype of cancer cells. On the other hand, ROS also function as antitumorigenic species since they can induce cellular senescence and death. Therefore, the appropriate application of these antioxidant enzymes is a critical consideration in designing proper strategies for both prevention and treatment of malignant disorders. This chapter reviews evidence that suggests SOD as an emerging therapeutic agent as well as a target for cancer treatment. A discussion is also devoted to the practical challenges that limit the use of SOD manipulation as an anticancer approach.

4.1 Introduction

Since the discovery of the antioxidant enzyme superoxide dismutase (SOD) by McCord and Fridovich [1] four decades ago and the subsequent evidence demonstrating the importance of free radicals in living systems, there has been an accelerated

M.L. Teoh-Fitzgerald, PhD (✉) • F.E. Domann, PhD
Free Radical and Radiation Biology Program, Department of Radiation Oncology,
Holden Comprehensive Cancer Center, Carver College of Medicine, The University of Iowa,
Iowa City, IA 52242, USA
e-mail: Laitee-Teoh@uiowa.edu

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interest in the role of SOD in experimental and clinical medicine. Free radicals are chemical species capable of independent existence that contain one or more unpaired electrons in their molecular orbital. In the mammalian system, free radicals are mainly produced by incomplete reduction of oxygen during metabolic respiration, by chemical or enzymatic reactions, by exposure to radiation, or by release from activated immune cells in response to pathological invasion or cancer cells. These molecules are mainly represented by a family of reactive oxygen species (ROS) which include superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) as well as reactive nitrogen species such as nitric oxide (NO) and peroxynitrite ($ONOO^-$). Because of the presence of unpaired electrons, free radicals are chemically reactive and can cause detrimental damage to biological structures, particularly polyunsaturated membrane lipids, DNA, and amino acids. A disturbance of redox balance between these free radical pro-oxidants and cellular antioxidant systems can result in oxidative stress, which has been implicated in many disease processes, including cardiovascular diseases, aging, and cancer. Accumulation of free radical-mediated damage to bio-molecules is a slow but progressive pathological process in vivo. Therefore, application of antioxidants to prevent or treat free radical-induced diseases presents a logical and attractive clinical approach.

4.2 ROS and Cancer

4.2.1 ROS in Biological System

As damaging as superoxide radical and its reactive derivatives are at high levels, there is a different profile for lower levels of this species. Superoxide radical is not simply a noxious cytotoxic byproduct, but has long been recognized to function as a physiological mediator and signaling molecule in regulating inflammation, stimulating cell proliferation, and regulating apoptosis [2–4]. Since ROS are by nature both oxidants and reductants, they influence the cellular redox status and may cause either a positive response (stimulation of cell proliferation) or a negative response (induction of growth arrest and cell death) according to their concentrations. The dose-dependent effects of ROS on cellular homeostasis and proliferation are postulated to be bimodal [5]. Small changes in the redox environment, say toward a mild oxidative state, can stimulate a variety of factors that support greater cell proliferation. In contrast, when the oxidative stress is pushed beyond the cellular "threshold" level capable of detoxifying the damaging effects of ROS, cellular proliferation decreases followed by induction of cell death by apoptosis, necrosis, or mitotic catastrophe. Damage beyond that level necessary to trigger apoptosis leads to necrotic death. This concept of a progression of behavior from "proliferation to apoptosis to necrosis," linked to an increased rate of oxidation has recently gained prominent recognition in the redox biology field, as it provides a good explanation for the seemingly contradictory literature on the effects of ROS on cellular

biology. Therefore, a better understanding of the pathological changes induced or inhibited by ROS and their modulation by antioxidants is of great importance for the prevention and treatment of malignant disorders.

4.2.2 ROS as Carcinogens

ROS have been widely implicated in neoplastic transformation due to marked changes in metabolism, with some of the most profound changes being related to disruption of glucose metabolism [6–8]. The increased glycolysis seen in cancer cells together with a reduced rate of respiration has been shown to be associated with heightened mitochondrial superoxide production leading to enhanced oxidative stress [9–13]. The resulting change to a more oxidizing redox status in the transformed cells is thought to increase cell proliferation, stimulate development of the malignant phenotype, and facilitate immortalization. Free radicals are usually involved in the initiation and promotion steps, with genetic alterations in DNA regarded as a critical process in the etiology of cancer. Elevated levels of oxidative DNA lesions have been noted in various tumors with more than 100 oxidized DNA products identified to date. This ROS-induced DNA damage includes single- or double-stranded DNA breaks, modifications of purine, pyrimidine, or deoxyribose, and DNA cross-links. This DNA damage can result in loss of transcriptional regulation, replication errors, genomic instability, and induction/impairment of signal transduction pathways, all of which are associated with carcinogenesis [8]. ROS have also been known to act as oncogene activators as well as secondary messengers for triggering redox-sensitive signaling cascades [14–16]. ROS primarily oxidize phosphoserine/threonine phosphatases, phosphotyrosine phosphatases, and phospholipid phosphatases, likely by interacting with sulfhydryl groups on their cysteine residues, forming either intra- or intermolecular disulfide bonds [17]. These modifications lead to deregulation of critical signaling cascades such as MAPK, PI3, and growth factor kinase-dependent signaling pathways. These signaling cascades subsequently affect the downstream redox-sensitive transcription factors such as AP-1, NF-κB, p53, and HIF-1 [15, 17–22].

In support of the role of ROS in the etiology of cancer, a body of investigations have demonstrated elevated oxidative status in many types of cancer cells [23, 24], and that the introduction of chemical [25, 26] and enzymological [24] antioxidants can inhibit tumor cell proliferation, pointing to a critical role of free radicals in mediating loss of growth control in tumor cells. Since the generation of superoxide in mitochondria is believed to be a major source of free radicals in aerobic cells, MnSOD is particularly important in the prevention of free radical accumulation and subsequent damage to cells since it is localized to the mitochondria. The findings that most cancer cells have lower levels of MnSOD compared to their normal counterparts has lead to a hypothesis that normalization of MnSOD in these cancer cells will reverse the tumor phenotypes [23]. This hypothesis has been repeatedly tested by Oberley's group as well as his collaborators with regards to over-expression of MnSOD inhibiting cancer cell phenotypes in various cancer cell lines [24, 27–36] as discussed in the following section.

4.2.3 ROS as Anticancer Agents

Since ROS possess both proproliferative as well as prodeath activities, manipulations of ROS levels have been exploited to selectively kill cancer cells. The antitumor effects of pro-oxidants have been demonstrated both in vitro and in vivo and have been implicated in the beneficial effects of radiation therapy [37–39], chemotherapy [40, 41], immunotherapy [37, 40], hormone therapy [42], photodynamic therapy [43], and hyperthermia [44]. Based on these observations that most nonsurgical anticancer therapies kill tumor cells via the production of cytotoxic prooxidants, a "threshold concept of cancer therapy" has been proposed by Kong et al. in which the appropriate use of inhibitors of antioxidants or free radical generating compounds could be used as an alternative combined modality approach to cancer treatment [45]. The authors rationalized that if the ROS level reaches the "threshold level" that overwhelms the antioxidant and the repairing capacities, irreversible damage will occur and cell death via apoptosis/necrosis will be initiated in cancer cells. A similar concept of inducing cytotoxic ROS in solid tumors has also been suggested by Iyer's group in Japan [46]. An interesting discussion on the potential use of polyethylene glycol (PEG) conjugated $O_2^{\cdot-}$ or H_2O_2 generating enzymes developed by this group as a promising anticancer therapy is presented in their review article [46].

Although this threshold/pro-oxidative concept sounds appealing and promising, there are challenges regarding the practical application of this therapy. First and foremost, determination of an optimum treatment dose is of critical importance. Since high levels of ROS are also toxic to normal cells, their usefulness could be limited in cancer therapy. In addition, technical difficulty in effectively delivering this pro-oxidant therapy to the tumor is an important consideration in terms of tumor targeting.

4.3 SOD and Cancers

4.3.1 Biological Importance of SODs

SODs (EC1.15.1.1) constitute the only known mammalian antioxidant enzymes that convert superoxide to hydrogen peroxide at rate constants higher than the spontaneous dismutation rate. The importance of the SOD enzymes is seen in the different forms that exist, as well as in their different distribution in the cellular compartments, protecting cells against all possible sources of superoxide generation. There are three forms of SOD in mammalian cells: a copper- and zinc-containing CuZnSOD, a manganese-containing MnSOD, and an extracellular copper- and zinc-containing EcSOD. These SODs have impressive rate constants in the order of $10^9\ M^{-1}S^{-1}$ in catalyzing the dismutation of $2O_2^{\cdot-} + 2H^+$ into $H_2O_2 + O_2$. Although these SODs catalyze similar chemical reactions, they are thought to play different biological roles due to their differential distribution in cells and tissues. CuZnSOD comprises approximately 90% of total SOD activities in eukaryotic cells [47].

Besides its primary distribution in the cytosol, a fraction of this enzyme has been found in cellular organelles such as lysosomes, peroxisomes, the intermembrane space of mitochondria, and the nucleus [48–50]. In contrast to the "broad distribution" of CuZnSOD, MnSOD is limited to the matrix of mitochondria [51]. MnSOD, although existing in lower concentration in most cell types, is considered the most critical SOD necessary for life in O_2, since animals deficient in MnSOD cannot live breathing O_2 more than a few weeks.

Extracellular SOD (EcSOD) utilizes copper and zinc in a similar fashion as CuZnSOD but the expression of EcSOD is highly restricted to specific cell types and tissues such as lung, heart, kidney, plasma, lymph, ascites, and cerebrospinal fluids [52]. EcSOD differs from CuZnSOD in that EcSOD is a glycosylated high molecular weight homotetramer (135 kDa) and CuZnSOD is an unglycosylated homodimer (32 kDa). As the name implies, EcSOD is the only antioxidant enzyme that scavenges $O_2^{\cdot-}$ in the extracellular space. The concept of "compartmentalization of ROS signaling" has arisen based on the distribution of these antioxidant enzymes [53–55]. The fact that pathophysiological conditions caused by a deficiency in one of these SODs cannot be corrected with the other SOD isoforms highlights the uniqueness of the individual SOD enzymes where the loss of one enzyme cannot completely compensate by the other. For instance, over-expression of CuZnSOD did not prevent neonatal lethality in mice lacking MnSOD [53].

In an attempt to investigate the interplay between these SOD enzymes on compartmentalization of ROS signaling, Missirlis et al. [54] have shown that the mitochondria and the cytosolic SODs provide independent protection to compartment/organelle-specific protein iron–sulfur clusters against oxidative damage generated by superoxide within different compartments. Based on the observation that oxygen can accept unpaired electrons to generate $O_2^{\cdot-}$, this radical is thought to act as a "sink" for intracellular radicals and that SODs provide a critical primary defense [56]. Compared to other radicals, $O_2^{\cdot-}$ is a relatively weak oxidant. However, a potent oxidizing agent, OH, can be generated from $O_2^{\cdot-}$ and H_2O_2 through reactions driven by transition metals such as Fe and Cu via the Harber–Weiss reaction. Therefore, disruption in the redox balance regulated by these SODs could lead to important biological consequences.

Knockout studies in mice have provided vital tools toward the understanding of the importance of SODs in mammalian systems. Mice deficient in MnSOD exhibit dramatically reduced growth rates, increased susceptibility to mitochondrial injury in metabolically active tissues, and die prematurely once they breath O_2 [57–61]. This demonstrates that MnSOD is essential for protecting against mitochondrial generated $O_2^{\cdot-}$ and is ultimately critical for the survival of aerobic organisms. Although CuZnSOD constitutes the major proportion of total cellular SOD activity, deleting this gene from mice is not lethal and the animals develop normally to adulthood [62]. However, these mice are extremely vulnerable to motor neuron loss after axonal injury, indicating that this cytosolic SOD plays a critical role under oxidative stress conditions. Furthermore, fetal fibroblasts derived from the CuZnSOD homozygote knock out fetuses show substantially diminished cell growth in culture and display hypersensitivity to $O_2^{\cdot-}$ generating agents [57]. Mice lacking EcSOD

have shorter survival than control animals and developed enhanced lung damage after exposure to high oxygen tension [63]. These differential effects seen from the knock out studies further emphasize the significance of compartmentalization of ROS signaling and damage.

4.3.2 SOD Levels in Cancer

The role played by SODs in cancer biology is not well understood, except that all tumor cells examined to date clearly demonstrate an altered redox balance, with most studies suggesting a more oxidative state [5], characterized by elevated intracellular ROS, in particular, superoxide [64]. The reported studies that investigated the relationship of SODs to malignant cells and tumors show significant inconsistencies in results between cell lines suggesting that many different pathways may contribute to these effects. Many studies have found low SOD activity in primary human tumors while others have found high levels of SODs in metastatic tumors that have been associated with aggressive characteristics. The in vivo and in vitro findings on SODs in malignancy have been well reviewed [5, 65, 66].

4.3.2.1 MnSOD

The first report demonstrating that the diminished activity of MnSOD in transformed cells when compared to their normal counterpart was reported in 1974 [67]. Since then, numerous investigations have demonstrated altered levels of antioxidant enzymes in cancer cells. These studies have been reviewed previously [23, 65, 68]. In general, primary cancer cells are nearly always low in MnSOD [69–71] and this phenomenon has recently been shown to be associated with mutations in the MnSOD promoter [72], aberrant methylation of the MnSOD promoter [73, 74], loss of heterozygosity [75, 76], as well as mutations in the MnSOD coding sequence [77, 78]. However, reduced expression and/or activity of MnSOD is by no means a universal feature of cancer cells. There are reports with controversial findings on MnSOD levels compared with their normal counterparts. For instance, in an investigation that involved 31 human diploids and neoplastic cell lines, Marklund et al. [79] found that the MnSOD level was variable and did not appear to be significantly lower in malignant cell lines compared to normal tissues. In fact, mesothelioma was found to have a substantially higher MnSOD level than normal cells in the same study. A list of cancer cells that showed elevated MnSOD levels compared to their normal counterparts was described in a review by Hileman et al. [66]. It was suggested that the up-regulation of MnSOD expression observed in some cancer cells is associated with intrinsic oxidative stress in cancer cells as a mechanism to tolerate the increased ROS stress [80].

In addition to altered expression levels, a polymorphic variant of MnSOD has been associated with enhanced risk of cancer development. In this polymorphism,

valine is replaced by alanine at position 16 in the mitochondrial targeting sequence (A16V) [81]. This residue is nine amino acids upstream of the cleavage site and has often been designated as A-9V polymorphism [82]. This polymorphism has also been designated as V16A [83]. MnSOD with an Ala residue at this position is more efficiently targeted to the mitochondria, whereas the Val form is partially arrested in the inner mitochondrial membrane leading to a reduction in the formation of active MnSOD in the mitochondrial matrix [84]. In view of these findings, expression of the Val form is expected to result in higher ROS stress and thus individuals with this polymorphism may be predisposed to a greater risk for cancer development. However, some reports find associations between the Val form and higher cancer risk while the majority of studies have shown a higher risk for different types of cancer with the Ala form [85]. No overall association of this polymorphism with cancer risk has been reported in a meta-analysis from 13 published case–control studies [86]. As described earlier, dismutation of O_2^- by SOD leads to production of H_2O_2. If not properly converted to water molecules by the peroxide removal systems such as glutathione peroxidases (GPx) and catalases, accumulation of H_2O_2 can result in molecular changes that promote carcinogenesis [87]. Therefore, Bag et al. suggested that polymorphisms of MnSOD as well as GPx, catalase, and peroxiredoxin should be considered in combination for the analysis for cancer risk association studies. Other MnSOD polymorphisms have been reviewed recently [50]. Although varying effects were seen in different cancers, the intriguing changes in cancer expression related to MnSOD polymorphism indicate that MnSOD and ROS stress may play a significant role in predisposition to carcinogenesis.

4.3.2.2 CuZnSOD

Similar to the findings on MnSOD, lowered CuZnSOD levels appear to be a common, but not universal characteristic of cancers. H6 hepatoma was found to have a lower CuZnSOD level than in the normal liver [69]. The activity of cytosolic SOD in hepatocellular carcinoma is significantly lower than the normal hepatic tissue [88]. In a breast cancer study, a concomitant increase of plasma lipid peroxidation and decreased CuZnSOD activity in blood cells was reported [89]. Lower expression of CuZnSOD as well as MnSOD was also reported in prostatic intraepithelial neoplasia and prostate carcinoma than in benign epithelium, suggesting that oxidative stress is an early event in carcinogenesis [90]. More examples also demonstrated a decreased expression of this cytosolic antioxidant enzyme in cancer cells compared to their normal counterparts including Marklund's study of the 31 different human diploid and neoplastic cell lines [79], gynecological disorder and endometrium carcinoma studies [91], and a report on malignant stomach and squamous cell carcinomas [92]. The exceptions to the lowered CuZnSOD found in cancers include leukemia [93] and glioblastomas [94] in which this enzyme has been shown to be elevated compared to their normal tissues. Over 90 mutations in CuZnSOD have been characterized to date but they are mostly associated with central nervous

system manifestations in the case of familial amyotrophic lateral sclerosis [95]. None of these polymorphisms have been studied or linked in terms of association with cancer risk.

4.3.2.3 EcSOD

EcSOD, despite being discovered more than two decades ago [52], has not been as widely studied as the other SODs, particularly in cancer research. This is likely due to its restricted expression in certain cell types and tissues [96]. A study examining EcSOD levels in cancer tissues compared to its normal counterpart was carried out by Svensk et al. [97] in lung cancer, in which the researchers have found that EcSOD immunoreactivity was 70% lower in those cancer tissues than in nonmalignant lung tissues, suggesting that decreasing EcSOD may contribute to tumor progression. Future studies investigating the level of this antioxidant enzyme in cancerous cells will aid in our understanding of the role played by EcSOD in the etiology of cancer.

An arginine 213 to glycine (R213G) polymorphism has been described for human EcSOD [50]. Interestingly, this variation occurs in the heparin binding domain of the protein leading to decreased anchoring of this protein to the negatively charged heparan sulfate proteoglycan on the cell surface and extracellular matrix, hence resulting in an increase in plasma EcSOD levels [98]. No immediate physical or clinical abnormalities have been observed in subjects carrying the R213G mutation. Although the consequences of this mutation are unclear, it has been shown to be associated with the poor outcome in diabetic patients who require hemodialysis and an increased risk factor associated with cardiovascular disease [99, 100]. This R213G variant has also been shown to confer resistance to the development of chronic obstructive pulmonary disease (COPD) in some smokers, which was suggested to be linked to a greater availability of the circulating EcSOD-R213G to provide an antioxidant or anti-inflammatory response [101]. To date, no association between this polymorphism and cancer risk has been reported.

4.4 SOD and Cancer Therapy

The metabolic and oxidative state differences between cancer and normal cells provide a biochemical basis for therapeutic strategies by exploiting these characteristics. Currently, the “free radical biology” approach to cancer therapy has been primarily focused on two main separate paths. One path aims at increasing the removal of oncogenic superoxide radical by up-regulating SODs while the other approach takes the opposite strategy by therapeutically manipulating ROS levels to a lethal level in cancer cells. Since SOD is the only class of enzymes known to scavenge superoxide, this function makes SOD an attractive target for pharmacological intervention.

4.4.1 SOD as a Clinical Drug for Cancer Treatment

The findings that most cancer cells have lower levels of MnSOD compared to their normal counterparts have lead to a hypothesis that normalization of MnSOD in those cancer cells will reverse the malignant phenotype. Oberley and Buettner were the pioneers in suggesting SOD as a therapeutic drug for cancer treatment [23]. This hypothesis has long been tested with regards to over-expression of MnSOD in suppressing tumor phenotypes in various cancer cell lines [24, 27–29].

The first study examining the role played by MnSOD was performed by introducing chromosome 6 into human melanoma cells [102]. Suppression of tumor growth in culture as well as in animals was observed. Although not specifically tested, this study supports the idea that one of the genes located on chromosome 6, with MnSOD being a likely candidate, influenced the malignant phenotype of human melanoma. A direct introduction of MnSOD cDNA into mouse embryonic fibroblasts was later tested by St Clair et al. [103] and found to suppress radiation-induced neoplastic transformation, supporting the role of mitochondrial ROS in inducing carcinogenesis. In a subsequent study, over-expression of MnSOD in melanoma cell lines resulted in the loss of their ability to form colonies in soft agar and produced a more differentiated phenotype [104]. The ability of MnSOD to reduce the in vitro plating efficiency and inhibit the in vivo tumor growth of breast cancer cells formed the basis for the proposal by Oberley that MnSOD was a tumor suppressor [105]. Since then, numerous studies have been conducted that provided further evidence to support this concept. Examples of these studies include stable clones of a human fibroblast cell line in which over-expression of MnSOD showed slower growth rate, reduced plating efficiency, and a more differentiated morphology [106]; human glioma cells transfected with MnSOD became less malignant and showed retarded growth in nude mice, relative to control transfected cells [27]; constitutive over-expression of MnSOD in oral squamous carcinoma cells caused reduced growth both in vitro and in vivo [28]. Similar results have been demonstrated in human prostate carcinoma cells [36], fibrosarcoma cells [107], papilloma [34], pancreatic adenocarcinoma cells [32], and breast cancer cells [29]. Thus the evidence appears substantial that over-expression of MnSOD can suppress the malignant phenotype in a great variety of tumors, supporting the potential use of this antioxidant enzyme for cancer treatment. However, the general statement of MnSOD as a tumor-suppressor has not been unambiguously established.

Studies that showed elevated levels of MnSOD in some aggressive cancer cells compared to their normal counterparts as discussed earlier make the statement that MnSOD is a tumor suppressor questionable. Although some of these cancer cells such as glioma and ovarian cancer actually possess higher levels of MnSOD than their normal tissues, over-expression of MnSOD has still been shown to inhibit tumor growth [27, 108, 109]. However, contradictory findings showing that up-regulation of MnSOD actually increased tumorigenicity have been reported. In HeLa cervical carcinoma cells, over-expression of MnSOD did not suppress the

growth of the cells but it promoted growth and protected the cells from death induced by serum starvation [110]. In addition to growth stimulation, over-expression of MnSOD was also found to induce the invasion potential of some cancer cell lines. For instance, invasiveness of hamster cheek pouch carcinoma cells was increased when MnSOD was overexpressed [35]. Over-expression of MnSOD in fibrosarcoma cells and bladder tumor cells significantly enhanced both the in vitro and in vivo invasive potential of the cells [111]. Further over-expression of catalase inhibited the MnSOD-induced invasion, indicating that this invasive phenotype is H_2O_2 dependent. In addition to over-expression of MnSOD, some studies have also used an opposite approach that disputes the concept of MnSOD as a tumor suppressor. Down-regulation of MnSOD expression with antisense transfection in squamous cell carcinomas resulted in a slower growth and longer survival in mice compared to controls [112]. Inhibition of either CuZnSOD or MnSOD in our hands with RNA interference technique also caused an induction of growth and plating efficiency in a pancreatic cancer study [113]. Taken together, these seemingly contradictory results reveal the complexity of ROS signaling in cancer biology and emphasize the importance of the balance within the multiple mechanisms regulating oxidative stress during carcinogenesis.

Recently, the role played by CuZnSOD in cancer biology has also been examined but similar to the findings on MnSOD, the results are inconsistent. In recent reports, over-expression of CuZnSOD, either via adenovirus constructs or by cDNA transfection, resulted in decreased platting efficiency, prolonged cell doubling time, reduced clonogenic fraction in soft agar, and most significantly, inhibition of tumor formation in nude mice of human malignant glioma cells [114], breast carcinoma cell lines [29], as well as pancreatic cancer cell lines [113]. These observations have led the investigators to suggest that this cytosolic SOD, similar to the mitochondrial SOD, has tumor suppressive properties. Growth suppression by CuZnSOD over-expression has also been demonstrated by others in a hepatocellular cancer cells study [115]. Targeting CuZnSOD with antisense resulted in a higher motility of human tongue carcinoma cells [116]. Down-regulation of CuZnSOD expression with siRNA transfection has also been shown to induce growth and clonogenic survival in our pancreatic cancer study [113]. These findings further suggest that CuZnSOD may play an important role in growth control regulation and disruption in its expression may contribute to tumorigenesis. In addition, CuZnSOD has been shown to significantly increase antimetastatic effects of chemotherapeutic agents such as adriamycin in a mouse sarcoma model, leading to a suggestion that this SOD has an antimetastatic therapeutic potential [117]. In a mouse model recombinant CuZnSOD reduced pulmonary metastasis of fibrosarcoma cells [118]. In contrast, increased colon carcinoma metastasis to the liver was observed in another mouse model when recombinant CuZnSOD was administered [119]. Transgenic mice overexpressing CuZnSOD in a GPx background also demonstrated higher tumor incidence and lower tumor regression in a DMBA/TPA two-stage skin carcinogenesis model [120]. Despite its more “general” cellular distribution positioning CuZnSOD as the primary enzyme protecting cells against

cytosolic-generated superoxide, the role played by this antioxidant enzyme in tumorigenesis is not clear. While some studies have shown decreased levels of CuZnSOD in tumors and cancer cell lines compared to their normal counterparts, other studies found no significant change in its expression whereas a subset of studies reported elevated levels of this enzyme as discussed earlier. Furthermore, although over-expression of CuZnSOD has been found to suppress growth and metastasis of some human cancer cells, other studies have shown promising results in suppressing tumor progression by targeting this enzyme as is presented in a later section of this chapter. These inconsistent findings in these differing systems emphasize the need for further investigation to support the suggestion that CuZnSOD is a tumor suppressor.

Due to its extracellular distribution and important function in maintaining vasodilation, EcSOD has been widely implicated in various cardiovascular diseases [121]. It is not as well studied as the other two SODs in terms of its role in tumorigenesis. Little is known regarding the expression level of this antioxidant enzyme in cancer cells compared to their normal counterparts and there is a limited report investigating the effect of EcSOD over-expression in human tumor or cancer cell lines. However, the potential use of this enzyme as an antioxidant cancer therapy need not be understated. Based on the unique properties possessed by this enzyme, EcSOD could provide superior therapeutic benefits than the other antioxidant cancer therapies. First, EcSOD over-expression has been shown by our group and others to suppress the growth of pancreas cancer cells [113], breast cancer cells [122], and melanomas cells [123]. In the pancreatic cancer cell xenograft, EcSOD actually provides the best tumor suppression and survival rate among all the SODs [113]. Besides inhibiting tumor growth, EcSOD has also been shown to have both antiangiogenic and antimetastatic effects. Over-expression of EcSOD via adenovirus infection resulted in a reduction in in vivo vascular endothelial growth factor (VEGF) expression that blunted tumor growth in the melanoma study [123]. Subcutaneous inoculation of EcSOD secreting fibroblasts suppressed metastatic lung nodules of methylcolanthrene-induced fibrosarcoma and Lewis lung carcinomas in mice [124]. Unlike the other SODs which are ubiquitously expressed by all mammalian cell types, EcSOD expression is cell and tissue specific as mentioned earlier. EcSOD is also synthesized and secreted by a variety of fibroblast cell lines, glial cell lines, and endothelial cell lines. Therefore, cells that do not express EcSOD can still acquire this antioxidant enzyme through their cell surface heparin sulfate proteoglycan as has been shown in a variety of cells both in vivo and in culture systems [125]. This implies that due to its extracellular localization and its ability to be acquired by cells through its heparin-binding domain, EcSOD gene therapy does not require direct tumor targeting but could be applied systemically. A longer plasma half life of this antioxidant enzyme compared to MnSOD and CuZnSOD also makes EcSOD better suited as a therapeutic candidate [126]. However, further investigations are needed in examining the expression level of this enzyme in human tumors and cancer cell lines as well as elucidating the effects of EcSOD over-expression in human carcinomas.

4.4.2 SOD as a Chemo- and Radio-protector

In addition to suppressing tumorigenesis, SODs have also been shown to have potential use in providing differential protection for normal cells or tissues against the side effects induced by anticancer therapies. Radiation is a common modality used for the treatment of malignancies, but injury to surrounding normal tissues is a major limitation that dictates the ultimate dose, volume, and technique of radiation. Radiation-induced fibrosis (RIF), a late effect of therapeutic irradiation, is well known in clinical practices. It is characterized by local inflammation and swelling, which in time will undergo step-wise aggravation and becomes established fibrosis. The fibrosis is often associated with atrophy and gradual destruction of cutaneous and subcutaneous normal tissues. Since oxidative damage is a central pathogenic process in RIF [127], application of SODs have been tested in an attempt to reverse or mitigate this process. Many reports have shown radio-protective effects of SODs in various experimental models. Intraperitoneal injection of CuZnSOD in mice decreased the severity of acute radiation skin damage in mice [128]. Intramuscular introduction of liposomal CuZnSOD and MnSOD resulted in regression of established radiation skin fibrosis in pigs [129]. The same group also found similar regression of radiation-induced skin fibrosis in humans with the use of liposomal CuZnSOD [130]. Irradiation protection by MnSOD in mouse lung, esophagus, oral cavity, and intestine has also been well documented by Greenberger and colleagues using adenovirus vector and a unique plasmid/liposome vector developed by the group [131–135]. These investigators showed that mitochondrial localization of MnSOD is important in the protection suggesting that oxidative damage to mitochondria is a critical step during RIF. Besides the intracellular SODs, the extracellular EcSOD has also been shown to be a potential therapeutic agent for radioprotection during cancer treatment. Over-expression of EcSOD was reported to confer protection against radiation-induced lung injury in transgenic mice [136]. In a separate study, Rabbani et al. showed that over-expression of EcSOD in mice ameliorates acute radiation-induced injury by attenuating macrophage-induced inflammatory response and inhibiting Transforming Growth Factor beta 1 (TGFβ1) activation with a subsequent down-regulation of the profibrotic TGF pathway [137]. The role of EcSOD has also been examined in a chemotherapeutic drug-induced pulmonary fibrosis using both the EcSOD-transgenic mice and -null mice models. Targeted EcSOD over-expression in the lungs of mice significantly protects these mice against bleomycin-induced lung injury [138] while enhanced bleomycin-induced pulmonary damaged was observed in EcSOD-deficient mice [139]. Lung is one of the tissues that expresses a high level of EcSOD [140]. This enzyme is found in the matrix of conducting airways and blood vessels as well as in the matrix and surface of alveolar septa [141]. The alveolar parenchyma is the primary location of fibrosis in human and mouse models of pulmonary fibrosis. The localization of EcSOD in the matrix and cell surface of alveolar suggests that its effects on pulmonary fibrosis are likely modulated in these locations. Since many matrix components are sensitive to oxidative modification/degradation and increased turnover of

the extracellular matrix is a hallmark of pulmonary fibrosis, it is suggested that the primary mechanisms in which EcSOD prevents pulmonary fibrosis is by preserving the integrity of the matrix [142]. Physical association of EcSOD through its heparin binding domain to cell surface and extracellular matrix components such as heparan sulfate proteoglycan [143], collagen [144], and hyaluronan [145] have been shown to be protective against oxidative degradation of these polyanionic molecules.

Despite the promising evidence discussed above, application of SOD does present some practical technical challenges as a clinical drug for cancer treatment. Proteins, in general, make poor drugs due to their rapid renal clearance and poor extravasation. SODs are no exception which is the primary reason why SOD-based antioxidant therapy has not made a greater and more rapid impact on clinical medicine. SOD gene therapies or protein drugs also suffer from pharmacokinetic difficulties such as delivery, inaccessibility of intracellular targets, chemical stability, and immunogenicity.

Furthermore, the varying dosage response of SODs that affects the overall outcome of the targeted cells is a more fundamental problem for clinical application.

4.4.3 SOD Mimetics as Clinical Drugs

Although the SOD enzymes have shown promising anticancer properties for cancer treatment, there are drawbacks and issues associated with their use as therapeutic agents and pharmacological tools. Considering the superoxide scavenging effect of SOD that modulates tumorigenesis, various classes of small-molecule SOD mimetics have been developed. These antioxidant catalysts that display SOD activity include metal-based manganese(III) salens, manganese(III) meso-porphyrins, and copper(II) diisopropylsalicylate (CuDIPS) and nonmetal-based nitroxide free radicals [146, 147]. Derivatives with dual SOD- and catalase-mimetic activity such as salen EUK-134 (Proteome Systems Ltd) have also been designed [148]. Two nonpeptidyl mimics of SOD, CuDIPS and manganese(III) tetrakis-(5,10,15,20)-benzoic acid porphyrin (MnTBAP) have been shown to increase the cytotoxic activity of anticancer drugs such as oxaliplatin, paclitaxel, and 5-fluorouracil, by increasing H_2O_2 levels [26]. The same investigators later demonstrated that another SOD mimetic, mangafodipir, with catalase- and glutathione reductase-like properties also improved the therapeutic index of these anticancer agents [149]. Mangafodipir is approved as a clinical contrast agent for hepatic MRI application. One general limitation of these SOD mimetics is that they react not only with superoxide, but also with a wide range of other ROS.

A new class of highly active and selective SOD mimetics has been developed with M40403 being the prototypical complex. This compound is a manganese(II) containing pentaazamacrocyclic ligand-based SOD mimetic that has the function and catalytic rate of the native SOD enzymes, but with the advantage of being a much smaller molecule (M_r = 483 Da) [150]. M40403 removes superoxide selectively at a high rate without reacting with other oxidizing species [151]. It has been

tested for the prevention of side effects associated with IL-2, an immune-stimulating cytokine drug that is approved for use in metastatic melanoma and renal carcinoma [152]. The use of IL-2 is limited by its side effects, primarily hypotension, which is related to overproduction of superoxide. A double-blind, placebo-controlled phase I clinical trial of intravenous M40403 showed no dose-limiting effects, demonstrating the promising use of this SOD mimetic in therapeutic use for superoxide-related diseases. However, a phase I/II trial assessing the effectiveness of M40403 as a co-therapy with IL-2 in patients with advanced skin and end-stage renal carcinomas was suspended due to severe systemic side effects caused by IL-2 [153]. The efficacy of M40403 has been shown in a wide variety of animal models of inflammation [150], ischemia–reperfusion injury [154], and pain [155, 156]. Recently, this compound has also been reported to attenuate radiation-induced oral mucositis in hamster, indicating the potential of M40403 for decreasing the severity and duration of this condition in patients undergoing radiotherapy for cancer treatment [157].

Low molecular weight nitroxides are SOD mimetics that are widely used as electron paramagnetic resonance (EPR) spin trapping in biological research. Through one-electron redox cycles, nitroxides can act catalytically as SOD and catalase mimetics [158]. Selective cytotoxicity of the nitroxide TEMPO against various cancer cell lines has been well documented. The chemotherapeutic efficacy of TEMPO has been demonstrated in xenograft models of human hormone-dependent and -independent prostate carcinomas [159]. FC-TEMPO (4-ferrocenecarboxyl-2,2,6,6-tetramethyl piperidine-1-oxyl), a nitroxide derivative, has also been shown to selectively induce cytotoxicity in highly metastatic lung tumor cells [160]. A dramatic delay in the onset of thymic lymphoma in ataxia telangiectaxia mutated (ATM –/–) mice by a nitroxide antioxidant, 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl (CTMIO), was observed [161].

4.4.4 Targeting SOD

Recently, there is an increased interest in targeting MnSOD based on its effect as a negative modulator of cellular apoptosis and as a survival factor for cancer cells [162]. ROS have long been known as inducers of programmed cell death. Upon triggering of apoptosis, release of cytochrome *c* from mitochondria activates the effector caspase 3, in a process that switches from the normal 4-electron to 1-electron reduction of oxygen, leading to production of superoxide anions [163]. Superoxide in turn amplifies the apoptotic cascade by damaging mitochondria and inducing the oxidative opening of the permeability transition pores and the further release of cytochrome *c* and apoptosis inducing factors (AIFs). This mode of action implies that the mitochondrial MnSOD plays an important role in inhibiting or at least delaying cell death, by preventing mitochondrial oxidative catastrophe. In support of this idea, MnSOD has been shown to promote cancer cell survival in experimental models of apoptosis. It has been demonstrated that MnSOD gene induction by Tumor Necrosis Factor Alpha (TNFα) confers resistance of cells to TNF-induced

apoptosis [164, 165]. This induction of MnSOD by TNF was shown to be mediated mainly by NF-κB, which is a transcription factor crucial for survival signaling in tumor cells [166]. In keeping with this view, over-expression of MnSOD protects cells from ionizing radiations [108] and chemotherapeutic drugs [167], and from cell death induced by serum deprivation [110]. This suggests that up-regulation of MnSOD may confer a selective advantage to tumor cells against cytotoxic effects imposed by therapy or nutrient restriction. Prevention of p53-dependent cell death has also been shown to be one of the important mechanisms involved in MnSOD-mediated survival signaling [168]. The fact that p53 negatively regulates the transcription of MnSOD, and MnSOD levels are elevated in p53-deficient cells as well as in tumors expressing inactive p53 [168, 169] further suggests that MnSOD may have a role in promoting malignancy and that inhibition of this enzyme may prove useful in the attempt to trigger spontaneous tumor cell apoptosis, and to increase tumor susceptibility to current anticancer treatments. Recent studies in this direction have indicated promising therapeutic potential with this strategy. Inhibition of MnSOD expression by genetic means has proven advantageous in inducing apoptotic death in squamous cell carcinoma cells by anticancer drugs and radiation [112].

In addition to targeting the expression of SOD, chemical agents that inhibit the enzymatic activity of SOD have also been tested for anticancer effect. Compounds that chelate metal ions or directly target the catalytic site of SOD such as cyanide, H_2O_2, azide, and diethyldithiocarbamate (DETCA) have been shown to inhibit SOD activity [170–172] but their use as anticancer agents are limited. Safety concerns related to the use of azide and cyanide makes them undesirable for therapeutic use. Since CuZnSOD and MnSOD contain different metal ions in their catalytic sites, they show variable sensitivity to these inhibitors. Furthermore, these agents suffer specificity issues due to their inhibiting effect on other metal-containing enzymes. Another copper chelator, a choline salt of tetrathiomolybdate (ATN-224), has been shown to be a promising clinical drug as a SOD inhibitor. ATN-224 specifically targets and inactivates CuZnSOD in tumors, leading to elevation of intracellular superoxide levels and induction of in vitro and in vivo apoptotic death [173]. The fact that the SOD mimetic, MnTBAP, was able to reverse the effect of ATN-224 shows the selectivity of its action. ATN-224 is currently being tested in two phase I studies for solid and advanced hematological malignancies and two multicenter phase II studies for advanced melanoma and prostate cancer [146].

As discussed above, certain types of cancer cells express a higher level of SOD than do the normal cell types from which they originate. Coupled with the high oxidative stress background, this situation may render cancer cells more vulnerable if oxidative stress is further heightened by impairing the ROS scavenging system. Huang et al. [80, 174] have demonstrated that established leukemia cell lines as well as primary leukemia cells isolated from patients are much more sensitive than normal cells to the inhibition of SOD by 2-methoxyoestradiol (2-ME). This inhibition resulted in an accumulation of superoxide in leukemia cells and subsequently damaged the mitochondrial membrane, leading to release of cytochrome *c* and

activation of apoptotic cascades. In contrast, a lower basal level of superoxide stress in the normal lymphocytes renders the normal cells more tolerant to the inhibition of SOD by 2-ME. This differential susceptibility of cancer cells supports the concept of selective killing of cancer cells by targeting SOD. The antitumor activity of 2-ME was discussed in a review by Hileman et al. [66]. However, the issue regarding the specificity of 2-ME's action on SOD was raised and the proapoptotic activity of this agent was subsequently attributed to other mechanisms that increase ROS stress [175]. A nanocrystal colloidal dispersion derivative of 2-ME has recently been evaluated in phase II clinical trials for hormone-refractory prostate cancer, recurrent glioblastoma multiforme, and metastatic renal cell [146]. Another pro-oxidant agent, 1,6-bis[4-(4-amino-3-hydroxyphenoxy)phenyl] diamantane (DPD) has also been shown to induce apoptotic death in leukemia cells both in cell culture and in mouse xenograft by specifically inhibiting CuZnSOD expression [176]. However, the mechanism underlying the SOD suppressive effect of these agents remains to be elucidated. It is noteworthy that even though high SOD levels were detected in some malignant cancer cells, relatively high ROS stress was still seen in these cells. This suggests that the malignant cells may heavily depend on SOD for elimination of superoxide to keep the ROS levels within a tolerable range, which makes the concept of SOD inhibition for cancer therapy an appealing approach.

As promising as a therapeutic target for cancer treatment, inhibition of SOD should not be considered as a general approach to cancer therapy. This approach is not likely to be effective in cancer cells that express a lower level of SOD activity due to insufficient target molecules. In addition, caution should be exercised in developing more potent SOD inhibitors as a cancer therapy, as complete inhibition of cellular SOD may result in a toxic effect not only to cancer cells but could also be harmful to normal cells. Inhibition of SOD may increase intracellular superoxide level that leads to nonlethal damage to DNA, proteins, and lipids. Mutation may hence occur in damaged cells that eventually evolve into cancer cells. ROS-mediated DNA damage may also contribute to genetic instability and accelerate disease progression. The major challenge in this approach is in defining the appropriate doses and the duration for drug exposure to kill the target cells while sparing the normal cells. Since different cancer types may possess various redox states, it is important to identify which types of cancers will properly response to SOD inhibition therapies. The potency and stability of SOD inhibitors at an effective concentration are also two important considerations for future development of new SOD inhibitors as anticancer drugs.

4.5 Future Perspective

The balance of intracellular ROS and antioxidants plays a critical role in maintaining homeostasis in both normal and malignant cells. ROS not only promote carcinogenesis but paradoxically are key players in nonsurgical anticancer treatments by

inducing tumor cell death. Similarly, antioxidant enzymes including SODs, not only protect normal cells against superoxide-mediated tumorigenesis, but also favor tumor survival. What can be concluded from the above evidence is that changing the levels of ROS can have complex and pleiotropic effects on cell signaling. A mild prooxidant state seems to promote cellular proliferation/carcinogenesis while also suppressing apoptosis. However, as the ROS levels further increase, the concentration of hydrogen peroxide will reach a level sufficient to activate apoptosis signals via ROS-mediated oxidative stress. This can induce programmed cell death. The exposure to even more severe oxidative stress, caused by even higher levels of ROS (especially hydroxyl radicals), will ultimately lead to necrotic death as a result of oxidative damage to biological molecules. This issue raises the question of which SOD manipulation approach, be it the antioxidant or pro-oxidant pathway should be utilized as the anticancer strategy. The answer is far from simple or universal but a more complex consideration is needed based on the intricate interplay between the pro-oxidants and antioxidants in the particular cancer type.

SODs are important enzymes that provide the first line of defense against ROS. Compelling evidence suggests that these antioxidant enzymes, particularly the MnSOD, have tumor suppressive property. However, contradictory findings showed that targeting SODs enhanced chemotherapy- and radiation therapy-mediated tumor cell killing. Despite this paradox, a universal notion has emerged in which targeting the intrinsic oxidative stress in cancer cells appears to be a central focus. Antioxidant or pro-oxidant anticancer approach with SOD manipulation can be successful if and only if the knowledge of the redox signaling signature of the particular cancer cells is available.

Therefore, therapeutic intervention that targets the emerging redox vulnerabilities of cancer cells is promising in selectively delivering therapeutic benefits against tumors that display significant deviations from normal redox homeostasis. An ideal redox-modulating therapeutic agent should provide selective and specific cancer cell killing without causing harm to noncancerous cells. To achieve that, development of validated tumor redox biomarkers that will provide guidance for drug selection and predict therapeutic benefits of a certain redox chemotherapeutic agent is of crucial need. This targeting of the redox Achilles heel of the individual tumor by redox profiling, i.e., redox phenotyping and genotyping, or determining the "redoxome," to guide the selection of novel redox therapy as has been suggested by Cabello et al. [146] may greatly improve outcomes for cancer patients. Although facing many challenges ahead, this "patient-specific redox therapy" based on SOD manipulation holds great promise for future investigations in cancer therapy.

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Chapter 5
Radiosensitization and Chemosensitization of Multicellular Tumor Spheroids by 2-Deoxy-D-Glucose is Stimulated by a Combination of TNFα and Glucose Deprivation-Induced Oxidative Stress

Divya Khaitan and Bilikere S. Dwarakanath

Abstract Selective sensitization of tumor cells to radiation and chemotherapeutic drugs by the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) arises from differential modifications of multiple damage response pathways in tumor and normal cells. Heterogeneity in responses to the combined treatment (2-DG + radiation/chemotherapeutic drugs) among mice bearing the same tumor has prompted studies on the roles of tumor physiology and host–tumor interactions. Using multicellular tumor spheroids (MTS), which mimic the microenvironment of tumors, we have addressed the issue of tumor physiology and found that the radio- and chemosensitization by 2-DG in MTS generated from a human glioma cell line (BMG-1) was nearly 2.5-fold higher than in monolayer cultures (MLCs), that correlated well with enhanced glycolysis in MTS. The enhanced sensitivity of MTS was accompanied by a profound induction of apoptotic and necrotic death as compared to cytogenetic damage-linked mitotic death as the major death pathway in MLC. Radio- and chemosensitization by 2-DG in MTS arose from endogenous TNFα-mediated oxidative stress and glucose deprivation-induced oxidative stress (due to the depletion of lactate) synergizing with oxidative stress induced by radiation and the chemotherapeutic drugs (like etoposide). Stimulation of MLC with TNFα elicited responses similar to MTS and supported its involvement in enhanced radio- and chemosensitization by 2-DG in MTS. Taken together, the available evidence suggests that alterations in cell signaling linked to tumor physiology, particularly related to endogenous and induced oxidative stress, contribute significantly to the radio- and chemosensitization of tumors by 2-DG.

D. Khaitan, PhD • B.S. Dwarakanath, PhD (✉)
Division of Biocybernetics, Institute of Nuclear Medicine and Allied Sciences, Delhi 110 054, India
e-mail: bsd@inmas.drdo.in

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5.1 Introduction

It is increasingly becoming clear that resistance of tumors to various therapeutic agents as well as systemic complexity arising out of normal tissue toxicity limits the efficacy of contemporary cancer therapies [1]. The scenario is accentuated further in the treatment of solid tumors, where spatial heterogeneity of clonal population as well as constraints in drug delivery add another dimension to the problem. Therefore, approaches which have a higher potential for differentially enhancing therapeutic responses in tumor cells/tissues plays an important role in improving cancer therapy [2]. However, an equally important role is played by the deployment of appropriate relevant model systems that facilitate the evaluation and understanding of the mechanisms underlying tumor response, leading to the evolution of newer and more effective therapies [3–7]. Therefore, newer interventional approaches and the models employed for experimental studies need to be critically evaluated from the view point of their potential for enhancing therapeutic gain as well as the appropriateness of the model being employed. We briefly review here the use of multicellular tumor spheroids (MTS) in experimental oncology, focusing on the modifications of radiation and chemotherapeutic drug responses by the glucose analogue and glycolytic inhibitor 2-deoxy-D-glucose (2-DG). We particularly emphasize the synergetic effects of endogenous oxidative stress related to TNFα and induced oxidative stress generated by primary therapeutic agents (radiation and chemotherapeutic drugs) as well as 2-DG in radio- and chemosensitization of MTCs that may have important implications on the responses of tumors in vivo to the combined treatment of 2-DG and radiation or chemotherapy.

5.2 Radio- and Chemosensitization of Tumors

The fact that tumors develop resistance to cancer treatments has stimulated the pursuit of agents that can sensitize tumor cells to the treatment, e.g., drugs which target resistance mechanisms. The goal of treatment sensitization is to increase the efficacy of the therapy, in addition to decreasing the drug doses needed and thereby reducing toxic side effects of the treatment. Many approaches using normal tissue protection with radioprotectors containing sulfhydryl groups [8, 9] or aminothiols [10] and radio/chemosensitizer like imidazoles, thymidine analogue, etc. had limited success due to acute toxicity of these compounds at therapeutically effective doses in the clinics.

Based on the differences in the bioenergetics of normal and tumor cells, particularly with reference to glucose metabolism [11–13], a novel approach has been proposed for improving cancer therapy that uses glycolytic inhibitors as modifiers of therapeutic response [14]. The cellular processes of repair and recovery from damage caused by radiation [14], certain anticancer drugs, as well as drug efflux are energy dependent [15, 16]. Since malignant cells derive most part of their energy

from glycolysis, it has been suggested that inhibitors of glycolysis like 2-DG can differentially inhibit repair processes and drug efflux, thereby enhancing tumor cell death by radiation and chemotherapeutic agents [14, 16, 17]. Recent studies showing a strong correlation between elevated glucose metabolism and unfavorable clinical outcome, including resistance to therapeutic agents [18, 19], have supported the potential of this approach to enhance the efficacy of radio- and chemotherapy.

5.2.1 Strategies Targeting Glucose Metabolism in Cancer

Glycolysis occurring in the cytosol is one of the two main sources of cellular ATP production. Targeting glycolysis directly is, therefore, a straightforward approach to disrupting the energy metabolism of tumor cells for antitumor activity. There are several approaches that have been taken, including inhibiting glycolytic enzymes and glucose transporters and induction of release of HK II from the mitochondrial receptor [VPAC]. The compound 2-DG is an antimetabolite and a glycolysis inhibitor that is also preferentially taken up by tumors, similar to the uptake of glucose. 2-DG has been shown to cause cancer cell apoptosis in vitro and to also further upregulate Glut-1 [20] mimicking glucose deprivation. Although 2-DG alone seems to have very little antitumor activity, therapeutic strategies using combinations of 2-DG with other chemotherapeutic agents, for example, adriamycin and taxol, have demonstrated antitumor activity in animal models [21, 22]. One of the advantages of 2-DG over many other conventional treatments is that it is considered a nontoxic agent and may potentially offer better quality of life than other cytotoxic agents. Clinical studies have demonstrated that it was well-tolerated up to single doses of 300 mg per kg body weight [23] and seems to have clinical benefits when combined with radiation [23, 24]. A chemotherapy drug conjugated to glucose is also being tested [25] to take advantage of the increased glucose uptake by tumor for specific targeting of chemotherapy. There are other agents, besides 2-DG that target glucose metabolism, which have been either investigated in preclinical studies or suggested for evaluation. HK inhibitors, for example, 3-bromopyruvate, have been shown to have impressive activity against liver cancers [26]. Phloretin, a Glut-1 inhibitor, has been shown to suppress glucose uptake by 60% and sensitize cancer cells to daunorubicin.

5.3 Multicellular Tumor Spheroids as In Vitro Models of Cancer

To translate the findings from basic cellular research into clinical applications, cell-based models need to recapitulate both the heterogeneity and the microenvironment of the tumor. Various in vitro models have been used to study the effects of oxidative damage on cancer cells, and provide information about the biochemistry

of cancer cells. However, tumor responses to many therapeutic agents and their combinations often differ from the responses of established tumor cell lines as monolayer cultures (MLCs) cannot mimic realistically the complex environmental conditions generated by an inadequate and inhomogeneous vascular supply leading to a heterogeneous cell population and processes that influence therapeutic efficacy [27, 28]. It has long been recognized that the cell-based models used in basic cellular research need to recapitulate both the three-dimensional organization and multicellular complexity of the tumor and also the organs to translate findings from these studies into clinical applications. Three-dimensional cultures have been utilized in biomedical research since the first half of the twentieth century to gain deeper insight into the mechanisms of organogenesis and expression of malignancy [27, 28]. The MTS is one of the best-described three-dimensional, in vitro tumor model system, which depicts many of the physiological and tissue characteristics of tumors and allows reproducible experiments. This system was adapted in cancer research during the early 1970s by Sutherland and colleagues and is widely used in many laboratories throughout the world today. MTS show strong similarities in morphology and mimic functional characteristics of in vivo solid tumors [27, 28].

Spheroids are sphere-shaped cell colonies that permit growth and functional studies of diverse, normal, and malignant tissues. MTS growth mimics the growth of naturally occurring human tumors as they contain an extensive extracellular matrix and network of cell-to-cell and cell-to-matrix interactions that differs in the relative amount and assembly from the corresponding MLCs [7]. They are characterized by high cell density, close packing, and 3-D tumor-like structure, which lead to severe diffusion limitations for molecules as small as glucose and oxygen leading to a gradient of oxygen and glucose in different zones of spheroids as in solid tumors [27]. In addition, solid spheroids also endogenously generate ROS that are used in signaling cascades involved in the regulation of tumor cell growth. Spheroids, therefore, serve as a good model bridging the condition between monolayers and in vivo tumors to gain further insight into the mechanisms involved in the response to various therapies, particularly the ones that are mediated by oxidative stress [29].

5.3.1 Role of Oxidative Stress in Radiosensitization by 2-DG in Spheroids

Oxidative stress has been found to induce apoptosis in a number of cell systems and plays an important role as a mediator of apoptosis in diverse models [30–32]. The functional importance of ROS generation for the activation of death mechanisms has also been supported with studies using antioxidants and inhibitors of specific enzymes in MLCs [33]. Another mechanism of the induction of apoptosis by ROS involves activation of FAS receptors belonging to the tumor necrosis factor family (TNFα) resulting in upregulation of extrinsic apoptotic pathway [34]. TNFα is a strong inducer of oxidative stress and also NfκB-mediated prosurvival responses [35]. While this signaling plays an important role in the response of solid tumors in vivo, it cannot be mimicked in MLC models due to negligible levels of TNFα.

On the other hand, MTS present with varying levels of TNFα depending on the extent of necrotic cells and is influenced by the age and size of spheroids. Indeed, a significant difference in the levels of TNFα and corresponding endogenous oxidative stress has been noted in the spheroids of a human glioma cell line (BMG-1). Observations suggest the proposition that both the extent of cellular responses as well as the mechanisms involved could significantly differ between the MLCs and MTS and strongly suggest the need for an appropriate in vitro model to evaluate the tumor responses besides elucidating the mechanisms of actions.

Results from our earlier studies have shown that the ROS levels in spheroids generated from a human glioma cell line (BMG-1) were not significantly different from MLCs, although a 29% decrease in the GSH levels was evident [3]. Further, TNFα levels were also higher in spheroids suggesting a possible increase in the ROS generation. However, a two- to threefold increase in the glucose consumption and lactate production observed in the BMG-1 spheroids [3] suggested that the enhanced lactate from the enhanced glycolytic flux adequately compensated for the decrease in GSH in the nonenzymatic antioxidant defense [36]. Since the clonogenic survival of the MTS cells was not significantly compromised as compared to the MLCs, it appears that under unperturbed conditions there is homeostasis between generation of oxidative stress and apoptotic defense and/or prosurvival factors.

Extrinsic signals, such as death-inducing ligands, as well as intrinsic signals, like macromolecular damage (for example, DNA damage), generation of ROS, and metabolic catastrophe, cause cell death through multiple mechanisms. However, exposure of cells to the same perturbing agent can elicit diametrically opposite responses under different conditions. Such responses have important implications for the design of therapy using such agents. Our results clearly shows that exposure of 2-DG enhances the oxidative stress in spheroids due to leakiness of ROS from the damage to mitochondria. In vivo [37] as well as in vitro [37, 38] studies have revealed that sensitivity of tumor cells to TNFα is increased under conditions of reduced glucose metabolism, thereby enhancing cell death. Earlier studies have clearly shown that the 7-day-old BMG-1 spheroids manifest elevated glycolysis and express higher levels of c-Myc and TNFα [3], thereby making spheroid cells more susceptible to glucose deprivation-induced apoptosis.

The radiation-induced ROS in BMG-1 spheroids was significantly lower than the MLCs, along with an increase in the glycolysis that perhaps contributed to the radioresistance of the spheroids reported earlier [3]. However, the radiosensitization by the glycolytic inhibitor 2-DG was significantly higher in spheroids, which was mainly due to the enhanced apoptotic cell death, while the moderate sensitization in monolayers was primarily due to enhanced cytogenetic damage [39]. The enhanced apoptosis in spheroids correlated well with the sustained elevation of ROS observed under these conditions, suggesting that stimulation of ROS-induced cell death and compromised glycolysis-linked antiapoptotic response contributed to enhanced radiosensitization. The differential induction of apoptosis in spheroids and cytogenetic damage (micronuclei) in monolayers following exposure to gamma radiation and 2-DG are consistent with the idea that these processes are regulated by different proteins and depend on different signaling pathways which may not be mutually exclusive.

Table 5.1 Spiking monolayer cultures with TNF enhance their sensitivity to radiation and 2-DG similar to the responses in spheroids

	Surviving fraction			Relative MFI values of ROS		
	MLS			MLS		
Treatment	Without TNF	TNF	MTS	Without TNF	TNF	MTS
None	1 ± 0.05	0.80 ± 0.1	1 ± 0.07	1.00 ± 0.8	1.00 ± 0.7	1.00 ± 0.1
2-DG	0.98 ± 0.3	0.74 ± 0.08	0.71 ± 0.08	1.27 ± 0.3	1.00 ± 0.4	1.04 ± 0.08
5 Gy	0.21 ± 0.04	0.11 ± 0.03	0.46 ± 0.03	2.18 ± 0.9	1.76 ± 0.6	1.09 ± 0.07
2-DG + 5 Gy	0.13 ± 0.02	0.08 ± 0.03	0.15 ± 0.02	1.10 ± 0.4	3.18 ± 1.02	20.90 ± 2.9

Existence of hypoxic and necrotic regions in the spheroids, which stabilize HIF1Alpha and secrete TNFα, could be one of the reasons for increased apoptosis in spheroids as stabilization/expression of these factors have also been shown to increase apoptosis in the glucose deprivation conditions [40]. Glucose metabolism results in the formation of not only ATP, but also regulates the redox potential through NADPH and helps the cell to detoxify intracellular hydroperoxides formed as by-products of oxidative metabolism in mitochondria [41]. Glucose deprivation leads to accumulation of pro-oxidants, like superoxide and hydrogen peroxide, due to the metabolic shift to oxidative phosphorylation, leading to glucose deprivation-induced oxidative stress which activates signal transduction pathways causing apoptosis often driving cells to apoptosis [42]. It appears that a significantly higher degree of radiosensitization induced by 2-DG in spheroids (two- to threefold) as compared to monolayers [1.4-fold] arises from a synergy between endogenous TNFα-mediated oxidative stress and glucodeprivation-induced oxidative stress due to a reduction in the lactate level. Support to this proposition came partly from the observations in MLCs, where stimulation by exogenously added TNFα elicited responses similar to spheroids (Table 5.1). Therefore, a synergy between endogenous oxidative stress (linked to TNFα and induced oxidative stress stimulated by 2-DG (linked to glucodeprivation) as well as radiation contributes to the radiosensitization, particularly in tumor tissues, whose behavior is mimicked by spheroids. Since tumor cells are generally associated with a disruption of intracellular oxidation/reduction reactions as well as enhanced glucose metabolism [12, 13, 37], results obtained using spheroids culture can be used for obtaining a near-realistic estimate of the oxidative stress-related responses of tumors to the combined treatment of therapeutic agents, particularly metabolic modulators.

5.3.2 Chemosensitization of Spheroids by 2-DG

Tumor response to chemotherapeutic drugs is determined by a multitude of factors that include drug penetration to different parts of the tumor, status of cell signaling determined by intercellular interaction and tumor cell–extracellular matrix interaction,

hypoxia, etc. Major emphasis on experimental oncology has been given to the mechanisms of cellular resistance to drugs using MLCs which are partly responsible for the limited success of many chemotherapeutic drugs in clinics that have otherwise been found effective against the tumor cells. Due to their three-dimensional architecture, MTS have been found to be very useful in drug sensitivity testing [43, 44], which addresses many of the issues related to drug resistance. Since spheroids also mimic the heterogeneous nature of individual tumors to a very great extent [4–7], it can also measure the drug sensitivities of specific cell types of cultured tumors.

MTS, coupled with the analysis of metabolic status, have been extensively used for investigating the treatment response of tumors to chemotherapeutic drugs. Most of the studies have shown that spheroidal cells are more resistant to etoposide (a topoisomerase II poison) than the monolayer cells [45]. Contrary to these findings, BMG-1 spheroids were found to be more sensitive to etoposide [IC50 = 1 μM] than their monolayer counterparts (IC50 = 10 μM). Immunofluorescence studies of topoisomerase II enzyme clearly showed a decrease in the topo II levels in spheroids as compared to monolayers. Furthermore, most of the topo II was localized in the cytoplasm of the spheroidal cells while it localized predominantly in the nucleus of the monolayer cells. The enhanced sensitivity was accompanied by a significant increase in the DNA damage and apoptosis in spheroids [46]. Oxidative stress has been found to induce apoptosis in a number of cell systems and play an important role as a mediator of apoptosis in diverse models [30–32]. The functional importance of ROS generation for the activation of death mechanism has also been supported with studies using antioxidants and inhibitors of specific enzymes [33]. Another mechanism of the induction of apoptosis by ROS involves activation of FAS receptors belonging to the TNFα resulting in upregulation of extrinsic apoptotic pathway [34]. Therefore, triggering of apoptosis by enhanced ROS levels induced by etoposide, perhaps synergizing with TNFα-related oxidative stress, appears to be partly responsible for higher sensitivity of BMG-1 spheroids to this chemotherapeutic drug. Exposure of spheroids to 2-DG following etoposide treatment shifted the apoptotic death pathway to necrotic cell death. Moreover, exposure of spheroids to 2-DG resulted in a persistent oxidative stress which was effectively quenched by pyruvate (Table 5.2), but not *N*-acetyl cysteine (NAC; data not shown). This is consistent with the understanding that death by apoptotic mode requires energy for its execution and a higher level of oxidative stress shifts the mode of cell death from apoptosis to necrosis [47]. Under conditions of glucose deprivation and persistent oxidative stress induced by etoposide and 2-DG, spheroidal cells were induced into necrotic death as opposed to apoptosis induced by either of the agents alone. Existence of hypoxic and necrotic regions in the spheroids stabilizes HIF1α and secretes TNFα, which could be one of the reasons for increased apoptosis in spheroids as stabilization/expression of these factors have also been shown to increase apoptosis in the glucose deprivation conditions [40]. Moreover, TNFα increased the sensitivity of monolayers to etoposide and induced the oxidative stress. These studies suggest that oxidative stress induced by etoposide alone or in combination with 2-DG synergizes with endogenous TNFα-mediated oxidative stress activating multiple death pathways that ride over the prosurvival responses, resulting in a profound cell death in spheroids.

Table 5.2 Pyruvate rescue spheroids against cell death due to oxidative stress induced by a combination of radiation and 2-DG

	Surviving fraction		Relative MFI values of ROS	
Treatment	Without pyruvate	Pyruvate	Without pyruvate	Pyruvate
None	1.00 ± 0.06	1.08 ± 0.05	1.00 ± 0.9	1.00 (0.32[a])
2-DG	0.71 ± 0.05	0.87 ± 0.04	1.62 ± 0.9	1.00 ± 0.04
Etopo	0.50 ± 0.03	0.61 ± 0.07	3.03 ± 1.1	1.00 ± 0.07
Etopo + 2-DG	0.13 ± 0.01	0.51 ± 0.08	4.63 ± 1.9	1.00 ± 0.06

[a] Value relative to untreated controls

5.4 Summary

Taken together, the available evidences suggest that alterations in cell signaling linked to tumor physiology, particularly related to endogenous and induced oxidative stress, contribute significantly to the radio- and chemosensitization of tumors by 2-DG. Further, in vitro MTS model could be very useful in predicting responses of in vivo tumors to bioenergetics-based therapeutic modalities (like, for example, 2-DG), besides providing insight into the possible mechanisms involved. Since tumor cells are generally associated with a disruption of intracellular oxidation/reduction reactions as well as glycolytic metabolism [11], the results obtained from MTS models linking glucose metabolism to cytotoxicity via oxidation/reduction reactions may have far-reaching implications in understanding the contribution of metabolism to the phenotypic changes associated with cancer as well as suggesting metabolic targets for the design of novel therapeutic interventions.

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Part II
Oxidative Stress in Normal Tissue Response (Preclinical)

Chapter 6
Chemotherapy-Induced Oxidative Stress in Nontargeted Normal Tissues

Paiboon Jungsuwadee, Mary Vore, and Daret K. St. Clair

Abstract Chemotherapy has played a critical role in increasing the number of cancer survivors. The success rate of chemotherapy is contributed, in part, by the development of novel agents and more aggressive treatment strategies. However, the effect that chemotherapeutic drugs have on normal tissue can create serious dose-limiting and quality-of-life issues. The majority of the known side effects of chemotherapy are related to proliferating tissues, which can be renewed and replaced. However, the effect of chemotherapeutic agents on tissues with limited renewal capability, such as cardiac and neuronal tissues, is of serious concern. The anticancer effect of the current FDA approved chemotherapeutic agents can be classified into multiple groups based on their predicted mechanisms of toxicity, and generation of reactive oxygen species (ROS) in normal tissue is implicated in a large number of these agents. However, how tissue pathology is induced by ROS-generating chemotherapeutic agents is unclear. Furthermore, the underlying mechanisms by which these agents affect a selected target tissue are relatively unexplored. This review briefly discusses these unexplored areas focusing on cardiac and neuronal tissues. The goal is to provide the basis for future development of anticancer drugs with reduced normal tissue injury to improve the quality of life for the ever-increasing number of cancer survivors.

6.1 Introduction

Chemotherapy provides one of the most successful approaches in controlling tumor growth. However, the uses of chemotherapeutic agents are often limited by their adverse effects on normal tissues. In general, the tissues affected by chemotherapeutic

P. Jungsuwadee, PhD • M. Vore, PhD • D.K. St. Clair, PhD (✉)
Graduate Center for Toxicology and Markey Cancer Center, University of Kentucky,
458 HSRB, Lexington, KY 40536, USA
e-mail: DSTCL00@UKY.EDU

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agents are those that have rapidly regenerating capacities such as hair follicles, mucosal tissues, and the bone marrow. However, while occurring with less frequency, toxicities to heart and brain caused by chemotherapy are more serious in that these tissues are less likely to regenerate. Thus, in this chapter, we focus on cardiac and neuronal tissues with an emphasis on understanding the underlying mechanisms associated with chemotherapeutic agent-induced injury, the goal being to improve the quality of life of the cancer patient.

6.2 Classification of Chemotherapeutic Agents

Chemotherapeutic agents can be classified according to their mechanisms of action. The main mechanism of action of chemotherapeutic agents is to interfere with cell growth and cell division. Different classes of drugs may act on different molecular targets (Fig. 6.1).

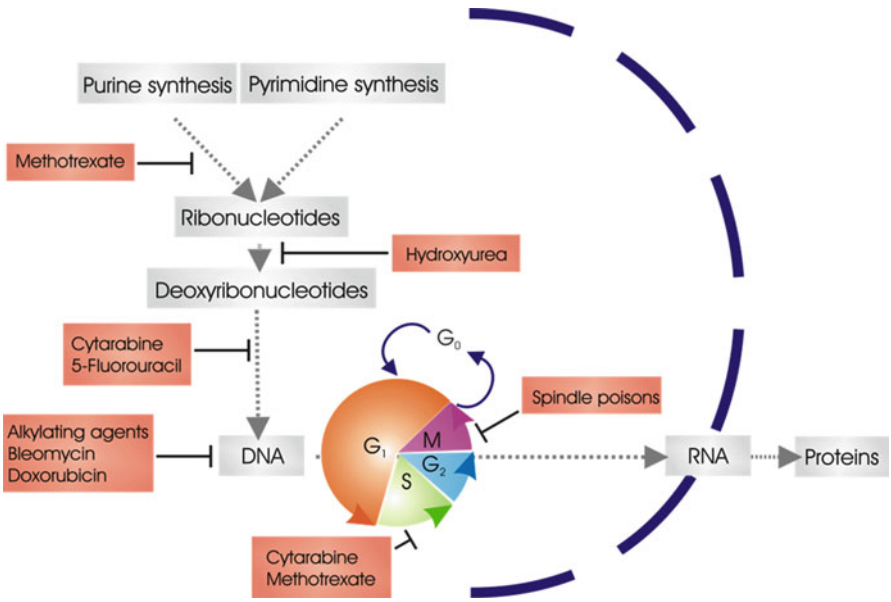

Fig. 6.1 Sites of action of major chemotherapeutic agents

6.2.1 *Alkylating Agents (Busulfan, Carmustine, Chlorambucil, Cisplatin, Cyclophosphamide, Melphalan, Procarbazine, Thiotepa, and Treosulfan)*

Alkylating agents add alkyl groups to intracellular electronegative groups. They cross-link guanine bases in DNA at the N-7 position during cell division and produce double-helix strands so that the strands cannot uncoil and separate. In addition, alkylation of DNA bases results in DNA fragmentation due to the attempts of DNA repair enzymes to replace the alkylated bases. Alkylating agents are not cell cycle specific.

6.2.2 *Antimetabolites (5-Fluorouracil, Cytarabine, Dactinomycin, Methotrexate, and Thioguanine)*

Antimetabolites masquerade as purine or pyrimidine bases, thereby competing with normal metabolites and preventing these normal metabolites from becoming incorporated into DNA during the S phase of the cell cycle. Methotrexate, a folic acid antagonist, competitively inhibits dihydrofolate reductase (DHFR), preventing the synthesis of tetrahydrofolic acid (coenzymes needed for one-carbon transfer reactions) necessary for the synthesis of thymidylate, which is important in the synthesis of nucleic acids. Purine analogs (azathioprine, mercaptopurines) and pyrimidine analogs (cytarabine, 5-FU) also work in a similar manner as methotrexate, i.e., to deprive cells of normal metabolites essential for DNA synthesis. Pyrimidine analogs inhibit the thymidylate synthetase-mediated conversion of deoxyuridylic acid to thymidylic acid, leading to inhibition of DNA and RNA synthesis.

6.2.3 *Antibiotics (Bleomycin, Dactinomycin, Doxorubicin, Mitoxantrone, Plicamycin, and Streptozotocin)*

These antibiotics interfere with DNA and/or RNA synthesis in a variety of different ways that include intercalation, DNA strand breakage, and inhibition of the topoisomerase II enzyme. Doxorubicin forms complexes with DNA by intercalating between base pairs and also inhibits topoisomerase II activity by stabilizing the DNA-topoisomerase II complex. This prevents the ligation–religation reaction that occurs during the uncoiling and repair of damaged DNA. Dactinomycin binds strongly to DNA and interferes with RNA synthesis. Bleomycin is also known to interfere with DNA synthesis; oxidative stress is another possible mechanism utilized to kill tumor cells [52], however, the exact mechanism remains unclear.

Mitoxantrone intercalates into DNA through hydrogen bonding and causes DNA strand breaks. Mitoxantrone also interferes with RNA and is a potent inhibitor of topoisomerase II. It is cell cycle nonspecific. Streptozocin interferes with biochemical reactions that involve NAD and NADH, and inhibits enzymes involved in gluconeogenesis. Its activity appears to occur as a result of formation of methylcarbonium ions, which alkylate or bind to many intracellular molecular structures including nucleic acids.

6.2.4 *Hormonal Agents (Aminoglutethimide, Cyproterone, Flutamide, Leuprolide, Prednisolone, Stilbestrol, and Tamoxifen)*

This class of drugs is used in the treatment of tumor types that are hormone-dependent, such as breast cancer, prostate cancer, and endometrial cancer. The main mechanism of action is to interfere with hormones specific to the target tissue. Aminoglutethimide inhibits enzymes in the steroid synthesis pathway that convert cholesterol to D5-pregnenolone, resulting in a decrease in the production of androgens, estrogens, adrenal glucocorticoids, and mineralocorticoids. Cyproterone inhibits the binding of dihydrotestosterone to the androgen receptor that is specific in the prostatic carcinoma cell. Bicalutamide and hydroxyflutamide, the active metabolite of flutamide, are also androgen receptor antagonists. They also act as potent inhibitors of testosterone-stimulated prostatic DNA synthesis [7]. Tamoxifen is a selective estrogen modulator (SERM) widely used in estrogen receptor (ER) positive breast cancer. Tamoxifen is metabolized by CYP2D6 and is 50- to 100-fold more potent than 4-hydroxytamoxifen and endoxifen, which act by competitively inhibiting the binding of estrogen to ER in breast tissue.

6.2.5 *Signaling Targeted Agents (Erlotinib, Gefitinib, Imatinib/Gleevec, Interferons, and Trastuzumab)*

This class of chemotherapeutic agents modulates immune responses through different biological targets. Erlotinib and gefitinib are human epidermal growth factor receptor type 1/epidermal growth factor receptor (HER1/EGFR) tyrosine kinase inhibitors. They inhibit the intracellular phosphorylation of tyrosine kinase associated with the epidermal growth factor receptor (EGFR). While EGFR is expressed on the cell surface of both normal and cancer cells, it is overexpressed in certain types of human carcinomas, e.g., breast cancers. Overexpression of EGFR leads to an improper activation of the Ras signal transduction cascade, leading to uncontrolled cell proliferation. Gefitinib binds to the adenosine triphosphate (ATP)-binding

site of the EGFR tyrosine kinase and inhibits the Ras signal transduction cascade. Imatinib is a 2-phenylaminopyrimidine derivative that is used to treat chronic myeloid leukemia (CML). It inhibits a number of tyrosine kinase enzymes, including receptor tyrosine kinases for the platelet derived growth factor (PDGF) and the Bcr (breakpoint cluster region)–Abl fusion protein. Binding of Imatinib to the enzyme decreases Bcr–Abl tyrosine kinase activity, thereby inducing apoptosis in Bcr–Abl positive cells. Trastuzumab is a humanized monoclonal antibody that acts on the human transmembrane orphan receptor (ErbB2), which is the product of the HER2/neu gene. Neuregulin activates HER2 by inducing its phosphorylation. The binding of Trastuzumab to the HER2 (or c-erbB2) proto-oncogene leads to antibody-mediated cellular cytotoxicity of the HER2 positive cells in that the binding of the antibody to HER2 overexpressing cells leads to preferential cell death. Interferon-α binds to the conventional type I interferon receptor comprises IFNAR1 and IFNAR2c, a long transmembrane isoform of IFNAR2, which upon dimerization, activates two Jak (Janus kinase) tyrosine kinases – Tyk2 and Jak1 that associate with IFNAR1 and IFNAR2c, respectively. These transphosphorylate each other and phosphorylate the receptors. The phosphorylated INFAR receptors then bind to signal transducers and activators of the transcription (STAT) 1 and 2 proteins, which in turn become phosphorylated, dissociate, dimerize, and subsequently translocate to the nucleus [69]. Interferons exert a wide range of biological activity. They stimulate cytotoxic activity of immune cells and increase the expression of tumor-associated surface antigens. This amplifies the recognition of transformed cells by immune effectors.

6.2.6 Topoisomerase Inhibitors (Epipodophyllotoxins: Etoposide, Tenoposide, and Irinotecan)

Etoposide is an epipodophyllotoxin, a semisynthetic derivative of the podophyllotoxins. It inhibits DNA topoisomerase II and DNA synthesis. Etoposide is cell cycle-dependent and phase-specific, affecting mainly the S and G2 phases. Two different dose-dependent responses are seen. At high concentrations (10 μg/mL or more), lysis of cells entering mitosis is observed. At low concentrations (0.3–10 μg/mL), cells are inhibited from entering prophase. Etoposide does not interfere with microtubule assembly. The predominant macromolecular effect of etoposide appears to be the induction of DNA strand breaks by an interaction with DNA-topoisomerase II or the formation of free radicals. Irinotecan is used in the treatment of colorectal cancer. Irinotecan is a derivative of camptothecin. Irinotecan and its active metabolite SN-38 bind to the topoisomerase I-DNA complex and prevent religation of these single-strand breaks and cause double-strand DNA breakage. Irinotecan is cell cycle phase-specific (S phase). Topotecan, similar to irinotecan, binds to the topoisomerase I-DNA complex and prevents religation of these single strand breaks.

6.2.7 Spindle Poisons (Taxanes: Docetaxel, and Paclitaxel; Vinca Alkaloids: Vinblastine and Vincristine)

This class of drugs acts through a unique mechanism by promoting the assembly of microtubules and stabilizing them against depolymerization. Paclitaxel is a first-line therapy for the treatment of advanced carcinoma of the ovary and breast cancer. Paclitaxel (and Docetaxel) binds to the β subunit of tubulin and promotes the assembly of microtubules from tubulin dimers and stabilizes microtubules by preventing depolymerization, which results in the inhibition of the normal dynamic reorganization of the microtubule network that is essential for vital interphase and mitotic cellular functions. This adversely affects cell function because the shortening and lengthening of microtubules (termed dynamic instability) is necessary for their function as a transportation highway for the cell. Further research has indicated that paclitaxel induces programmed cell death (apoptosis) in cancer cells by binding to and inhibiting the function of an apoptotic inhibitory protein called Bcl-2 (B-cell leukemia 2).

6.2.8 Miscellaneous Antineoplastic Agents (Hydroxyurea, L-Asparaginase, Mitotane, Procarbazine)

Hydroxyurea is used in the treatment of myeloproliferative disorders, in particular chronic myelogenous leukemia and polycythemia rubra vera. It has been extensively used as a radiation sensitizer, as it can synchronize cells in a radiation-sensitive phase of the cell cycle and can also inhibit the repair of radiation-induced DNA damage. In vivo, hydroxyurea is converted to the free radical nitric oxide (NO) and diffuses into cells where it quenches the tyrosyl free radical at the active site of the M2 protein subunit of ribonucleotide reductase, inactivating the enzyme. The entire replicase complex, including ribonucleotide reductase, is inactivated, and DNA synthesis is selectively inhibited producing cell death in S phase and synchronization of the fraction of cells that survive.

Asparaginase converts asparagine to aspartic acid and ammonia. It facilitates production of oxaloacetate, which is needed for general cellular metabolism. L-Asparaginase starves malignant cells that are dependent on an exogenous source of asparagine for survival by competitively inhibiting asparaginase, which results in asparagine deprivation. Its use is almost confined to acute lymphoblastic leukemia. Mitotane is an oral chemotherapeutic agent indicated for the treatment of inoperable adrenal cortical carcinoma. Mitotane is an adrenal cytotoxic agent; it alters the extraadrenal metabolism of cortisol, leading to a reduction in 17-hydroxy corticosteroids. Procarbazine can be classified as an alkylating agent. Procarbazine is cell-phase specific for the S phase of cell division. Procarbazine may directly damage DNA; its precise mode of cytotoxic action has not been clearly defined.

6.3 Toxicity

Cytotoxicity is the main mechanism of chemotherapy. Unfortunately, similar mechanisms collaterally damage normal tissues, which are the main focus of this chapter. Generally, drug toxicity can be classified as acute or delayed toxicity relative to time of exposure.

Acute toxicity: An onset of acute toxicity can occur in minutes, hours or days. Chemotherapeutic agents that are associated with acute toxicities often include gastrointestinal tract complications such as anorexia, nausea, vomiting, and diarrhea. Cisplatin, doxorubicin, and mustines are known to strongly induce nausea and vomiting.

Delayed toxicity: In contrast to acute toxicity, delayed toxicity occurs in months or years after an exposure to chemotherapeutic agents. Chemotherapy usually is a common cause of chronic delayed toxicity, especially for bone-marrow suppression.

Of note, several chemotherapeutic agents are more toxic to particular organs. For example, doxorubicin is more toxic to the heart, and cisplatin is more toxic to the kidney. Underlying mechanisms of such toxicity may be varied; however, many of these mechanisms are associated with induction of oxidative stress in the tissues. Table 6.1 summarizes specific adverse effects of commonly used chemotherapeutic agents and their association with oxidative stress.

Table 6.1 Chemotherapeutic agents and their specific adverse effects that are associated with oxidative stress

Drug	Adverse effect associated with oxidative stress
Alkylating agents	
Carmustine	Pulmonary fibrosis [46]
Cisplatin, carboplatin, oxaliplatin	Nephrotoxicity [20, 123]
	Peripheral neuropathy [61]
	Cardiac toxicity [19]
Cyclophosphamide, ifosfamide	Haemorrhagic cystitis [2, 128]
Antimetabolites	
Cytarabine	Neurotoxicity [40]
Methotrexate	Hepatic damage [15]
	Nephrotoxicity [104]
Antineoplastic antibiotics	
Bleomycin	Pulmonary fibrosis [106, 143]
Doxorubicin, epirubicin	Cardiac toxicity [29, 54, 155]
Hormonal agents	
Tamoxifen	Nephrotoxicity [139]
Immunotherapies	
Trastuzumab	Cardiac toxicity [112]
Spindle poisons	
Docetaxel, paclitaxel	Peripheral neuropathy [99]
	Cardiac toxicity [112]

6.4 Normal Tissue Targets of Chemotherapeutic Agents

It is not a surprise that inhibition of cell growth and cell division is one of the most common mechanisms of chemotherapeutic agent-induced normal tissue injury. As summarized in Table 6.1, the incidence of a particular toxicity is higher in one agent than another, and oxidative stress appears to be a common basic mechanism for many tissue toxicities causes by chemotherapeutic agents. Oxidative stress is a condition where there is an imbalance of prooxidant/antioxidant in steady state that could lead to cell injury. Prooxidants include (1) reactive oxygen species (ROS), e.g., superoxide radicals ($O_2^{\cdot -}$), hydroxyl radicals ($HO^{\cdot}$), (2) reactive nitrogen species (RNS), e.g., nitric oxide (NO), and peroxynitrite ($ONOOH/ONOO^-$), (3) alkoxyl/peroxyl radicals ($RO^{\cdot}/ROO^{\cdot}$), and nonradicals, e.g., hydrogen peroxide (H_2O_2). Antioxidants include small molecule antioxidants (e.g., vitamin C, vitamin E, glutathione) and antioxidant enzymes (e.g. superoxide dismutase, catalase, glutathione peroxidase). The uncontrolled ROS/RNS oxidize biological molecules such as membrane phospholipids, nucleic acids, and proteins, and result in failures of normal cellular functions and cell death. Multiple tissues are affected by the unintended side effects of chemotherapy; we focus our discussion on the heart and brain as cardiomyocytes and neurons are postmitotic cells, and therefore, the adverse effects of chemotherapy are more severe and difficult to manage. Damage to these cells is usually irreversible and conceivably will affect the functions of the heart and brain throughout the rest of the life of the patient who has been on chemotherapeutic agents.

6.4.1 Cardiac Toxicity

Cardiac toxicity of anthracyclines is well recognized [132] and is dose-dependent [96]. The cardiac toxicity is manifested as electrophysiological abnormalities (e.g., arrhythmia), dilative cardiomyopathy, and congestive heart failure (CHF). Clinical signs of cardiac abnormalities related to anthracyclines can be a reduction of left ventricular ejection fraction, left ventricular dysfunction, diastolic dysfunction, and diastolic heart failure. A measure of systolic function is the percentage of blood expelled from the left ventricle with each systolic contraction, termed left ventricular ejection fraction (LVEF). Normal values of VLEF are ≥50%. If there are signs of left ventricular dysfunction, but the LVEF value is within normal limits, the condition is termed "diastolic dysfunction." Diastolic dysfunction that presents concomitantly with dyspnea, fatigue and fluid retention is termed "diastolic heart failure." Anthracyclines and Trastuzumab are both associated with cardiac toxicity; the mechanism by which they cause toxicity, however, is different. Anthracyclines are believed to cause damage to cardiomyocytes by free-radicals-mediated reactions, since expression of the antioxidant enzyme manganese superoxide dismutase (MnSOD) is protective against doxorubicin (DOX)-induced cardiac injury. However, the cardiac toxicity caused by Trastuzumab remains largely unclear. Histological

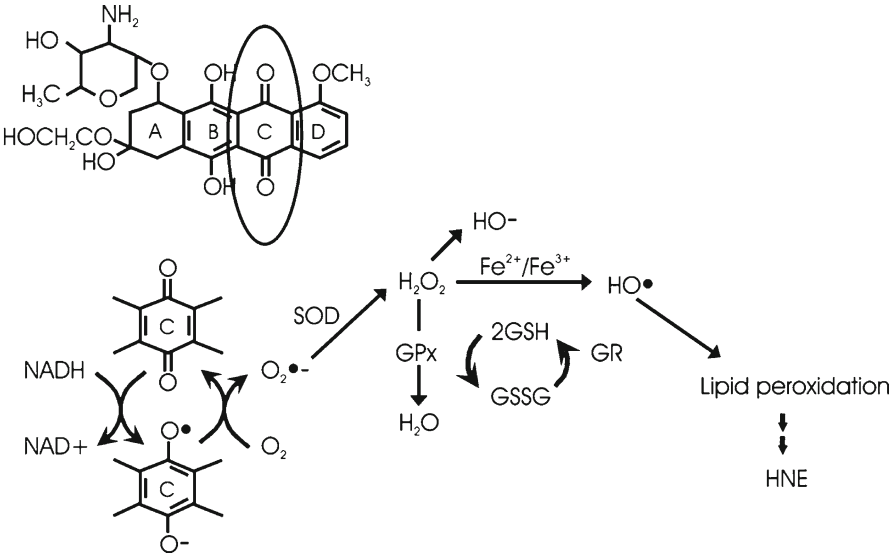

Fig. 6.2 Quinone and semiquinone redox cycling of doxorubicin

studies have revealed that DOX causes myofibril disruption, cytoplasmic vacuolization, and mitochondrial membrane disruption. Microarray analysis has shown that doxorubicin affects expression of cardiac proteins related to energy production and cell growth and division. Among these proteins, enzymes involved in bioenergetic and metabolic pathways are of particular importance because the heart is a high-energy demand organ [18, 82, 145]. Abnormalities in the metabolic pathway and energy production result in a mismatch between energy supply and demand and contribute to cardiac dysfunction.

Doxorubicin induces significant amounts of ROS, which can initiate lipid peroxidation. The ability of doxorubicin to generate substantial amounts of ROS is due to the quinone moiety in its chemical structure. The quinone ring can be reduced to a semiquinone in the presence of NADH via one-electron reduction by complex I of the electron transport chain (ETC) [88]. Oxygen can oxidize the semiquinone back to the quinone form with a superoxide radical ($O_2^{\cdot-}$) as a byproduct (Fig. 6.2). The redox cycling between the quinone and semiquinone forms of doxorubicin generates a large amount of $O_2^{\cdot-}$, which further gives rise to ROS and RNS that include H_2O_2, ·OH, and $ONOO^-$ [33, 152]. Proteomic analysis of cardiac proteins from mice treated with doxorubicin indicates elevated oxidative modifications of cardiac proteins such as triose phosphate isomerase (TPI), β-enolase, and electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) [18]. TPI and β-enolase are two enzymes in the glycolytic pathway and ETF-QO is an iron–sulfur ([4Fe–4S]) flavoprotein located in the inner mitochondrial membrane that catalyzes electron transfer from ETF to coenzyme Q. Oxidative modifications of these proteins interfere with their functions and therefore, compromise ATP production in cardiac mitochondria.

Cardiac mitochondria have been estimated to occupy approximately 40% of the total intracellular volume of cardiomyocytes [44]. Doxorubicin is effectively accumulated in the mitochondria [6], and the concentration in mitochondria is 100-fold higher than in plasma [124]. This mitochondrial accumulation of doxorubicin may be due to its high affinity to cardiolipin, a negatively charged phospholipid abundant in the mitochondrial inner membrane [108]. In addition, mitochondria are also the most reducing subcellular organelles in the heart, making the mitochondria a vulnerable target for oxidative damage. It is conceivable that cardiac toxicity induced by doxorubicin is due to this perfect combination of events, which appear to be selectively targeted to cardiac mitochondria.

It has been demonstrated that doxorubicin-induced oxidative stress causes formation of 8-hydroxydeoxyguanosine (8OHdG) [102], augments lipid peroxidation, induces mtDNA mutations, and decreases mtDNA content [77, 78]. Termination of doxorubicin treatment does not lead to decreased levels of 8OHdG following chronic administration of doxorubicin, indicating that the damage to mtDNA is persistent [129]. This persistent mitochondrial damage could provide an explanation for delayed adverse cardiac events observed in patients with a history of doxorubicin exposure.

In clinical practice, dexrazoxane, an iron-chelator and a free-radical scavenger, is used to prevent or reduce cardiac injury associated with the use of doxorubicin. The rationale for this comes from the fact that doxorubicin interferes with the cellular metabolic processes of iron, a redox active transition metal, leading to ROS generation [17]. Doxorubicin and $O_2^{\cdot-}$ can trigger the release of iron from ferritin, an iron storage protein [96]. $O_2^{\cdot-}$ and H_2O_2, produced by the redox cycling of doxorubicin, can also release iron from cytoplasmic aconitase, which contains a [4Fe–4S] cluster. Loss of the [4Fe–4S] cluster converts cytoplasmic aconitase into an iron regulatory protein (IRP)-1. IRP-1 binds with high affinity to conserved iron-responsive elements (IRE) in the untranslated regions of transferrin receptor (TfR) and ferritin mRNAs, stabilizing TfR mRNA but destabilizing ferritin mRNA [25, 97]. As a consequence, iron uptake exceeds iron sequestration. The increased cellular level of free iron leads to production of ·OH via Fenton chemistry, which contributes to oxidative stress and lipid peroxidation (Fig. 6.3). Doxorubicin metabolites are another factor leading to ROS generation. Secondary alcohols are one form of DOX metabolites, which are formed via the conversion of the carbonyl group at C13 to a hydroxyl group by aldo/keto-reductases [96]. The secondary alcohols of DOX are more reactive than $O_2^{\cdot-}$ or H_2O_2 in releasing iron from the [4Fe–4S] cluster of cytoplasmic aconitase [97, 98]. This results in further disturbance of iron metabolism and further progression of oxidative stress [71]. Doxorubicin and its metabolites and the ROS/RNS generated by doxorubicin lead to free iron overload. Hence, chelation of iron is the rationale for administration of dexrazoxane to prevent cardiac injury. Meta-analysis of cardioprotective interventions for cancer patients receiving anthracyclines indicates that dexrazoxane prevents heart damage without interfering with the antitumor effects of anthracycline treatment. Abnormal white cell count in the dexrazoxane-treated group was identified [151].

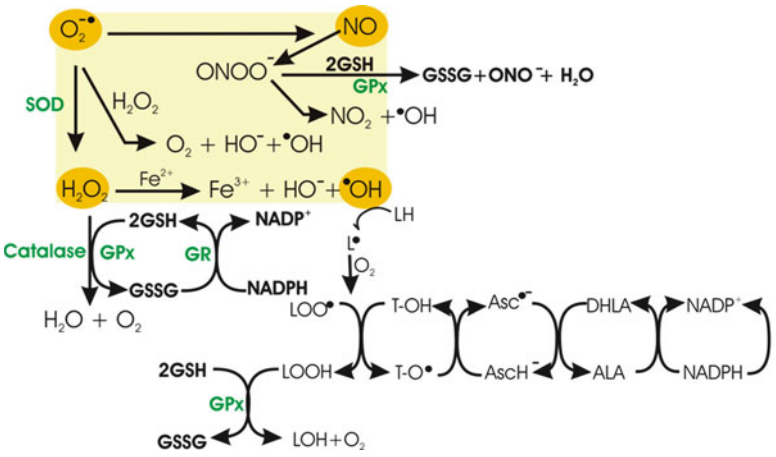

Fig. 6.3 Regulation of prooxidants. Reactive oxygen species, reactive nitrogen species, and nonradical molecules are tightly regulated by antioxidant enzymes and endogenous antioxidant small molecules. *T-OH* tocopherol, *ALA* alpha-lipoic acid, *Asc*$^{\bullet-}$ ascorbyl radical, *AscH*$^{-}$ ascorbate anion *DHLA* dihydrolipoic acid

6.4.2 Nephrotoxicity

Tamoxifen treatment results in a significant increase in lipid peroxidation in kidney tissues as compared to control [109]. Tamoxifen also decreases the hexose monophosphate shunt, a process that serves to generate NADPH, which might lead to an inability of kidney cells to maintain intracellular glutathione levels thereby amplifying oxidative stress that leads to renal injury. Cyclophosphamide and ifosfamide cause hemorrhagic cystitis due to the formation of a toxic metabolite, acrolein (Fig. 6.4), which can be inactivated in the urinary tract by mesna (sodium 2-sulfanylethanesulfonate). Mesna binds acrolein through its sulfhydryl-moieties, and the conjugation is excreted via urine. Therefore, mesna is used to prevent hemorrhagic cystitis in cyclophosphamide and ifosfamide chemotherapy. Finally, doxorubicin also causes oxidative stress-mediated kidney injury [131]. Thus, Mesna may also be effective in preventing doxorubicin-induced renal injury.

6.4.3 Neurotoxicity

Central nervous system (CNS) toxicities are frequently caused by chemotherapy regimens and manifest as encephalopathy or seizure. Most common chemotherapeutic agents that are associated with CNS toxicities include vincristine, cytarabine, and cisplatin. Seizures and neurotubular dissociation following vincristine treatment have been reported [53]. Neurological complications including papilledema with

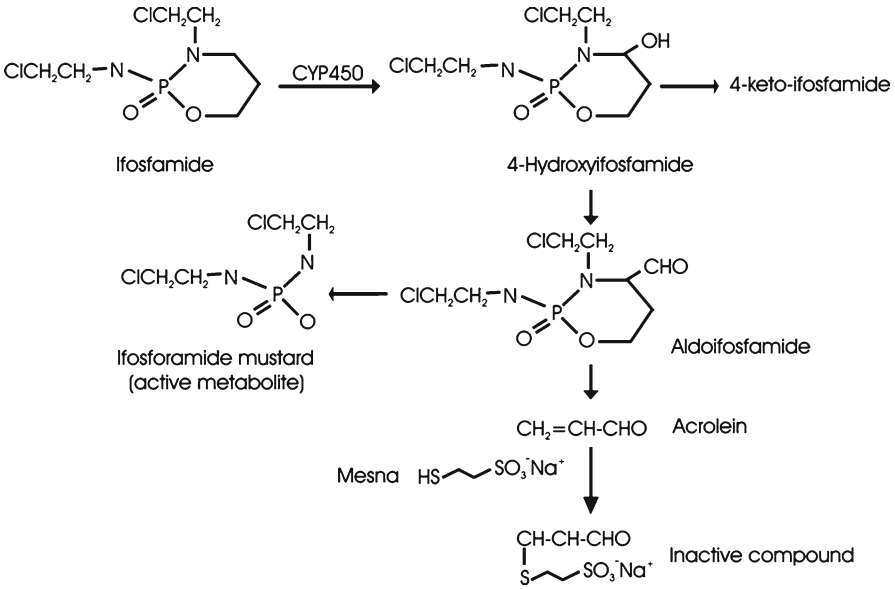

Fig. 6.4 Oxazaphosphorine alkylating agent ifosfamide and biotransformation pathways. Ifosfamide, a prodrug, is metabolized into the active metabolite, isophosphoramide mustard, and toxic metabolites, i.e., acrolein that causes hemorrhagic cystitis. Mesna forms a complex with acrolein to form an inactive compound

blindness, increased intracranial pressure, cauda equina syndrome, and seizure associated with intrathecal liposomal cytarabine have been reported [58]. While cisplatin may be associated with CNS toxicities such as cerebrovascular complications and leukoencephalopathy [32, 57], its neurotoxicity is more peripheral. Cisplatin preferentially forms DNA–platinum adducts in dorsal root ganglia (DRG) neurons rather than in neuron-like dividing PC12 cell line [91]. Additionally, a subset of DRG neurons express the copper transporter 1 (CTR1) that mediates the uptake of platinum compounds [84]. The toxicity of cisplatin is likely attributable to the highly reactivity of cisplatin with nucleophiles, which results from the highly reactive leaving chloro groups of cisplatin. Together, these findings may explain the sensory neuropathy of cisplatin. In addition, vincristine and taxane treatment has also been associated with peripheral nervous system injuries that manifest as peripheral neuropathy. In a rat model of peripheral neuropathy induced by administration of vincristine sulfate (50 μg/kg i.p.) for 10 consecutive days, the levels of reduced glutathione in the sciatic nerve was significantly decreased, as opposed to the rise in thiobarbituric acid reactive substances [100]. This result suggests that oxidative stress is involved in pathogenesis of peripheral neuropathy.

Interestingly, persistent changes in cognitive function, including memory loss, distractibility and difficulty in performing multiple tasks, have been observed in breast cancer survivors after treatment with chemotherapeutic agents, such as

doxorubicin, that do not directly affect the brain tissue [3, 4, 142]. It has been recognized that the short term loss of memory, also known as "chemobrain," is associated with chemotherapy [59, 125]. Magnetic resonance imaging (MRI) studies have revealed that chemotherapy decreases the volumes of brain gray and white matter in various brain structures including prefrontal, parahippocampal gyrus, and precuneus [13, 55, 135]. In addition, positron emission tomography (PET) studies have shown that the resting metabolism in the area of the brain involved in working memory (e.g., basal ganglia) was impaired in chemotherapy-treated subjects and was associated with breast cancer survivors at 5–10 years after chemotherapy [26, 136]. While the molecular basis for "chemobrain" remains elusive, oxidative stress has been thought to play a mechanistic role [3, 62, 63, 140, 141]. As detailed below, our recent studies have shown that neurotoxicity of doxorubicin is likely mediated by tumor necrosis factor-α (TNF-α), since pretreatment with an anti-TNF antibody attenuates neurotoxicity [141]. Unlike the heart, much less is known about the mechanism of ROS/RNS generation in the brain resulting from systemic doxorubicin administration [3, 63, 141]. The chemical properties of doxorubicin suggest that doxorubicin cannot pass through the blood–brain barrier (BBB), and doxorubicin has not been detected in the areas protected by BBB such as the cortex and the hippocampus [12, 141]. Moreover, ATP-dependent transporters Pgp [103] and MRP1 are localized basolateral of the choroid plexus epithelium and at the apical surface of the capillary endothelium of the BBB [93, 115]. These transporters function actively as efflux pumps, providing a major mechanism for the restriction of uptake of doxorubicin into the brain (Fig. 6.5). However, we have found an increased level of TNF-α in the cortex and the hippocampus after treatment of mice with doxorubicin [141]. Moreover, we found increased levels of oxidative-stress markers in the brains of doxorubicin-treated mice, including protein carbonyl, protein-conjugated 4-hydroxy-2-nonenal (4-HNE, a product of lipid peroxidation) and protein nitrotyrosine [63]. It is, therefore, likely that oxidative stress in the brain results from the indirect effects of doxorubicin with TNF-α being a potential mediator of the doxorubicin-induced ROS/RNS. Administration of doxorubicin has been shown to increase the circulating levels of TNF-α [139, 141, 150]. Circulating TNF-α can pass the BBB [105] and activate glial cells to initiate the local production of TNF-α [138], which in turn induces nitric oxide synthase leading to the generation of RNS (Fig. 6.5) [139]. The role of nitric oxide synthase in RNS production in the brain during doxorubicin treatment is strongly supported by two independent investigations in rat models. In one study, daunorubicin, an analog of doxorubicin, increased the level of nitric oxide synthase in the brain [64]. A second study reported that inhibition of nitric oxide synthase by aminoguanidine ameliorated oxidative stress in the brain upon doxorubicin treatment [1]. The role of circulating TNF-α in mediating ROS/RNS is supported by the fact that coadministration of doxorubicin and an anti-TNF antibody completely abolished the TNF-α increase in the brain and mitigated doxorubicin-induced brain injury [141].

Currently, there is no direct evidence of mtDNA damage in doxorubicin treatment. However, research on age-related neurodegenerative diseases such as Parkinson disease (PD) and amyotrophic lateral sclerosis (ALS) has demonstrated a strong

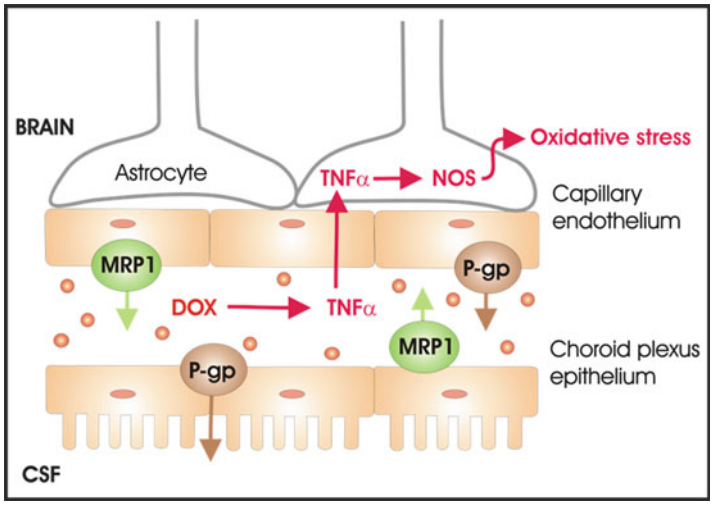

Fig. 6.5 The proposed mechanism of doxorubicin-mediated neurotoxicity via TNF-α activation. Doxorubicin leads to increased levels of circulating TNF-α, which can pass the blood–brain barrier and trigger the local production of TNF-α in the brain. Increased levels of TNF-α may, in turn, induce the expression of nitric oxide synthases leading to oxidative stress. P-glycoprotein (Pgp) may play a role in preventing uptake of doxorubicin into the brain, and multidrug-resistant associated protein 1 (MRP1) may play a role in the efflux of glutathione conjugated lipid adducts. *NOS* nitric oxide synthase, *CSF* cerebrospinal fluid

correlation between mtDNA oxidative damage and dysfunction of neurons [89]. It is tempting to speculate that mtDNA oxidative damage may play a vital role in mediating the cytotoxicity of doxorubicin in the brain. In spite of the side effects to normal tissues, doxorubicin remains an important component in most chemotherapeutic regimens due to its efficacy in treating a broad spectrum of cancers.

6.5 ROS/RNS Detoxification Mechanisms

Cellular antioxidants and antioxidant enzymes play a vital role in scavenging ROS/RNS and maintaining a balanced cellular redox status. The chemotherapeutic agent-induced ROS/RNS consume cellular antioxidants and lead to oxidative modification of antioxidant enzymes that results in the inhibition of their activity. This cascade of events further pushes the cellular redox status toward the direction of oxidative stress.

Glutathione (GSH), a ubiquitous thiol-containing tripeptide, functions directly as an antioxidant in vivo to remove ROS in reactions catalyzed by GSH-dependent enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione *S*-transferase (GST) [30]. GSH reduces H_2O_2 to H_2O and lipid peroxides

to their corresponding alcohols and results in the formation of glutathione disulfide (GSSG) with the catalysis by GPx. GSSG is converted to GSH, a reaction that is catalyzed by GR, using NADPH as the cofactor (Fig. 6.3). In addition, the nucleophilic thiol group (–SH) in glutathione can conjugate with lipid oxidation products or xenobiotics and form GSH thioethers via the GST-catalyzed reaction to initiate detoxification and excretion of these toxic substances. Chemotherapeutic agents including doxorubicin elicit ROS, which decrease the cellular GSH levels [1] and escalate oxidative stress [72, 121]. In addition, doxorubicin administration leads to a dose-dependent decrease in cardiac GPx activity [34] and brain GST activity [62], which probably results from doxorubicin-induced oxidative stress. In the brain tissue of doxorubicin-treated mice, manganese superoxide dismutase (MnSOD), a mitochondrial antioxidant enzyme essential for aerobic life has been found to be inhibited by nitration of tyrosines [139]. Tyr 34 is vital for MnSOD activity, and its nitration impedes substrate access and binding to MnSOD [113].

6.5.1 Oxidative Stress Protecting Systems

Comparing the potency of free radicals, superoxide anion ($O_2^{\cdot-}$) is weakly reactive compared to a hydroxyl radical. $O_2^{\cdot-}$ is protonated to yield a hydroperoxyl radical ($HO_2^{\cdot}$), which is more reactive than $O_2^{\cdot-}$. $HO_2^{\cdot}$ can serve as an initiating radical $HO_2^{\cdot} + LH \rightarrow H_2O_2 + L^{\cdot}$ due to its lipophilicity and redox potential [90]. At physiological pH, there is little $HO_2^{\cdot}$ relative to $O_2^{\cdot-}$; however, at the membrane surface, the pH is slightly acidic, which promotes protonation of $O_2^{\cdot-}$ to yield more $HO_2^{\cdot}$. $HO_2^{\cdot}$ can withdraw a hydrogen atom or proton from macromolecules or allylic compounds such as membrane lipids and has been proposed to cause membrane damage by initiating lipid peroxidation. Hydrogen peroxide is not very reactive at physiological pH but can directly inhibit some enzymes. For example, H_2O_2 oxidizes a histidine 118 residue in CuZuSOD that binds to Cu^{2+} at the substrate binding site [149].

Antioxidants have become increasingly recognized as potential weapons to control oxidative imbalance. However, spatial distributions and localized concentration of pro- and antioxidants can determine the outcome of these antioxidant defense mechanisms. There are two main categories of antioxidants (a) enzymatic and (b) nonenzymatic antioxidants.

6.5.1.1 Enzymatic Antioxidants

Superoxide dismutase (SOD): SOD can be categorized as metalloproteins, based on the metal molecule contained in its structure, and exhibits copper, zinc (Cu, ZnSOD), manganese (MnSOD), and iron (FeSOD) forms. MnSOD is mainly generated in the matrix of the mitochondria and is essential for the survival of aerobic organisms. Increases in MnSOD activity may provide increased protection against oxidative stress as demonstrated by several disease models.

Recent studies have shown that the SOD mimetic, Mn(III) meso-tetrakis (*N*-ethylpyridinium-2-yl)porphyrin, MnIIITE-2-PyP^{5+}, is able to dismute superoxide anion ($O_2^{\cdot-}$) and reduce peroxynitrite ($ONOO^-$) and $CO_3^{\cdot-}$. This activity is considered as a major mechanism of action of this SOD mimetic to protect normal tissue from oxidative stress-induced toxicity. In fact, MnIIITE-2-PyP^{5+} has been used to demonstrate its protective ability against peroxynitrite-mediated damage of electron transport chain components in submitochondrial particles (SMP) [37].

Catalase (CAT): Catalase is localized in peroxisomes and contains four ferriprotoporphyrins. Because catalase reduces two molecules of H_2O_2 to O_2 and H_2O in the reaction [$2H_2O_2 + \text{catalase} \rightarrow 2H_2O + O_2$], it is effective in the removal of hydrogen peroxide when the level of H_2O_2 is high.

Glutathione peroxidase (GPx): GPx is a selenium-dependent enzyme that is highly specific for GSH and thioredoxin. In the presence of glutathione (GSH), GPx reduces hydrogen peroxide and hydroperoxide to the corresponding water and alcohol. GPx also terminates the Fenton reaction as H_2O_2 is eliminated. There are at least six GPx isoforms – GPx1 (cGPx), GPx2 (GSHPx-GI), GPx3 (pGPx), GPx4 (PHGPx), GPx5 (eGPx), and GPx6 (embryonic tissue-specific GPx) [146]. GPx1 is found in the cytosol and mitochondria and reduces lipid hydroperoxide and hydrogen peroxide. GPx2 has shown to be associated with the gastrointestinal tract and is found in the cytosol [21]. GPx3 is a secreted protein and is found in plasma [156]. GPx4 or phospholipid hydroperoxide GPx is found in the cytosol and plasma membranes and reduces phospholipid hydroperoxide, membrane-bound lipid hydroperoxide and cholesterol peroxide. GPx4 is highly expressed in the testis, playing a crucial structural role in spermatogenesis [86]. GPx5 is expressed in the epididymis and plays a partial role in protecting spermatozoa from oxidative injuries that could compromise their integrity and, consequently, embryo viability [16].

In conjunction with GPx, GSH reductase (GR) plays a central role in the biochemistry of GSH as it keeps GSH in its reduced state thereby maintaining an adequate GSH/GSSG ratio. The reaction occurs in two steps. The first is the reduction of GSH reductase by NADPH. Electrons are channeled through the enzyme via FAD and subsequently, through a redox-active protein disulfide bond to GSSG. GR is inactivated upon reduction by its own electron donor, NADPH, and is reactivated by GSSG (Fig. 6.3). In the presence of GR inhibitors, such as 1,3-bis-(2-chloroehyl)-1-nitrosourea (BCNU) or doxorubicin, a decrease in the GSH/GSSG ratio is expected. Thus, GR is an important enzyme in maintaining GSH and GSSG levels and preventing oxidative stress.

Thioredoxin (Trx): Thioredoxin was originally purified from *Escherichia coli* and it functions as a hydrogen donor for ribonucleotide reductase, an enzyme essential for the synthesis of deoxyribonucleotides from ribonucleotides [50, 75]. Trx is a 12-kDa protein that contains an active catalytic sequence Trp–Cys–Gly–Pro–Cys–Lys (WCGPCK motif) [110]. $Trx(SH)_2$ converts Protein–S–S–Protein disulfide bonds to their respective reduced forms while $Trx(SH)_2$ itself gets oxidized to $TrxS_2$. The $TrxS_2$ is recycled back to $Trx(SH)_2$, a reaction that is catalyzed by thioredoxin reductase (TrxR) using electrons from NADPH (Fig. 6.6). There are two Trx systems in mammalian cells, the cytosolic Trx1/TrxR1 and the mitochondrial

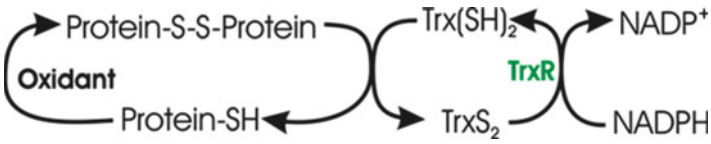

Fig. 6.6 Scheme of reactions catalyzed by thioredoxin–thioredoxin reductase system

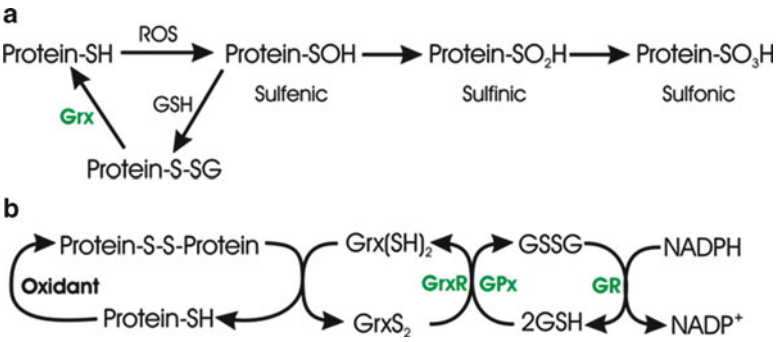

Fig. 6.7 (**a**) Thiol-containing proteins are susceptible to oxidative modifications. Oxidation of the thiol group may lead to the formation of sulfenic, sulfinic, and sulfonic acid. Only the formation of sulfenic acid can be reversed, for example by GSH and Grx. (**b**) Scheme of reactions catalyzed by the glutaredoxin-dependent system

Trx2/TrxR2 [11]. Both Trx isoforms share antioxidant capability to repair the catalytic activity of peroxiredoxins and glutathione peroxidases, decomposing hydroperoxides and H_2O_2. In addition, both Trx1 and Trx2 can scavenge HO [27, 68].

Glutaredoxin (Grx): Grx, similar to Trx, was first discovered as a hydrogen donor for *Escherichia coli* ribonucleotide reductase [49]. The structure of dithiol Grx, $Grx(SH)_2$, contains three important features (1) the Cys–X–X–Cys active motif, (2) certain hydrophobic regions, and (3) a GSH binding site [68]. Three Grx isoforms have been found in mammalians, cytosolic Grx1, mitochondrial Grx2 and Grx5. Glutaredoxins convert protein–S–S–protein to their respective reduced forms (Fig. 6.7). The known functions of Grxs are to maintain and regulate the cellular redox state and redox-dependent signaling pathways. Grx1 is involved in general disulfide–dithiol exchanges, cell differentiation, and regulation of transcription factors, and apoptosis [11]. Grx2 and Grx5 localize to the mitochondria of cardiomyocytes. Grx2 differs from the more abundant cytosolic Grx1 by its higher affinity toward *S*-glutathionylated proteins and by being a substrate for thioredoxin reductase [60]. Silencing of Grx2 increases the sensitivity of HeLa cells toward doxorubicin [83]. By contrast, overexpression of Grx2 prevents 2-deoxy-D-glucose- or doxorubicin-induced loss of cardiolipin in HeLa cells [36]. These results indicate a crucial role of Grx2 in the regulation of the mitochondrial redox status, which may be important in oxidative stress-induced tissue injuries.

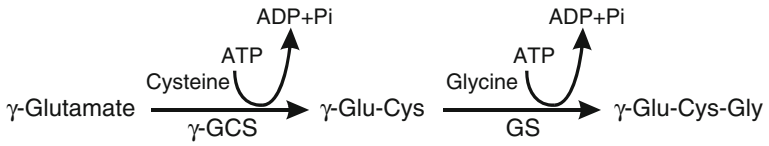

Fig. 6.8 Schematic of glutathione synthesis. Ligation between glutamate and cysteine by γ-GCS is the rate-limiting step. Amongst the three amino acids, cysteine is the rate limiting substrate for GSH synthesis

6.5.1.2 Nonenzymatic Antioxidants

1. Vitamin C: Vitamin C (ascorbic acid) is a water-soluble molecule that converts the vitamin E radical back to vitamin E. At physiological pH, most ascorbic acid will be in the dehydro-ascorbate ($AscH^-$) form. Therefore, it can donate 1 electron and yield $AscH^{\cdot -}$, which has a pKa of −0.86. Since the pKa is lower than physiological pH, no protonation takes place. However, $AscH^{\cdot -}$ donates a proton yielding ascorbyl radical ($Asc^{\cdot -}$), which is not a reactive molecule. The net reaction will eliminate $T–O^{\cdot}$ and yield T–OH (Fig. 6.3).
2. Vitamin E: Vitamin E is a lipid-soluble molecule. Among its 8 isomers, α-Tocopherol (T–OH) is the most potent form. It provides a hydrogen atom to a lipid radical ($L^{\cdot}$) or lipid peroxyl radical ($LOO^{\cdot}$) and becomes vitamin E radical ($T–O^{\cdot}$). The $T–O^{\cdot}$ is reduced by dehydro-ascorbate to form T–OH for reuse (Fig. 6.3).
3. Glutathione: Glutathione (GSH) is an endogenous tripeptidyl peptide synthesized by cells and is the most abundant endogenous antioxidant. It plays a key role in cellular antioxidant defenses by scavenging reactive oxygen species and reducing lipid peroxides, making it unique in protecting cells. Glutathione is present in either the reduced (GSH) or the glutathione disulfide (GSSG) forms. Intracellular GSH concentrations are tightly regulated by two main mechanisms: (a) de novo synthesis of GSH, and (b) uptake and efflux of GSH and GSSG.

   (a) The de novo synthesis of GSH from its building blocks (Cys, Glu, and Gly) is catalyzed by γ-glutamylcysteine synthetase (γ-GCS), which results in formation of a γ-linkage between glutamate and cysteine complexed to glutathione synthetase (GS) (Fig. 6.8); this linkage makes GSH resistant to hydrolysis by peptidases which hydrolyze the α-linkage of peptide bonds. γ-GCS is sensitive to the cellular redox status and the GSH concentration and is feedback-inhibited by GSH. Availability of GSH precursors also plays an important role in de novo synthesis of GSH. The GSH precursor L-cystine is taken up via the cystine/glutamate antiporter (system x_c^-), which can be induced by oxidative stress [133]. GSH can also be regenerated from GSSG by redox cycling, in which GSSG gains electrons from NADPH catalyzed by glutathione reductase (GR). This pathway serves as a salvage pathway that replenishes GSH.
   (b) Transport of GSH. Transporter proteins that have been demonstrated to transport GSH include organic anion transporting peptide 1 (oatp1), oatp2,

OATP8, multidrug resistance-associated protein 1 (MRP1), MRP2, MRP4, MRP5, and cystic fibrosis transmembrane conductance regulator (CFTR) [8]. GSH plays a pivotal role in multiple biochemical processes including protection against reactive intermediates such as free radicals and electrophiles, storage and transport of cysteine, and synthesis of leukotrienes and prostaglandins. Furthermore, export of GSH allows the cell to protect its membrane against oxidative or other forms of damage by keeping thiol groups and membrane components (e.g., vitamin E) in their reduced forms. It is generally thought that the export of GSSG serves as a protective mechanism for the cell to eliminate formation of GSH protein mixed disulfides and assists in the maintenance of thiol redox status or cystine–cysteine status. Thus, transport of GSH becomes significant as GSH is being made available for those processes.

In the biotransformation and detoxification processes of ROS/RNS, GSH participates in two major steps: (1) it functions as a substrate for GSH peroxidase (GPx), which mediates the reduction of oxygen free radicals and (2) it forms conjugates during the biotransformation of electrophilic exogenous compounds catalyzed by GST.

As mentioned above, several transporter proteins that have been demonstrated to efflux GSH include MRP1, MRP2, MRP4 and CFTR [51, 70, 118]. In addition, MRP1 appears to be a major mediator of both basal and apoptotic glutathione release [87]; Furthermore, MRP1 also appears to mediate GSH export and homeostasis with the capacity to directly regulate the cellular thiol-redox status. Thus, MRP1 can influence signaling pathways involved in apoptosis, cell proliferation, and cell differentiation [9]. The transport mechanisms of GSH and glutathione conjugates appear to be distinct [122]. MRP1 is also closely associated with GSH and GS-conjugates in the heart more so than others efflux transport such as MRP4, MRP5, and ABCG2. We discuss further about the biotransformation of GSH conjugates and the efflux transporters that are necessary for their elimination.

6.5.2 Phase II Biotransformation

Phase II drug metabolizing enzymes such as GST, sulfotransferase (SULT), and UDP-glucuronosyltransferase (UGT) usually detoxify toxins by increasing their solubility and converting the parent compounds into substrates of efflux transporters. This action decreases the total amount of the chemotherapeutic agents from the system.

6.5.2.1 Glutathione *S*-Transferases

GSTs are a family of isoenzymes that catalyze the conjugation of the intracellular nucleophile, glutathione (GSH), to electrophilic molecules such as HNE, chlorambucil, melphalan and doxorubicin. These enzymes have been discussed with respect

to drug resistance in cancer therapy [147]. GSTs also contribute to the biosynthesis of steroid hormones and eicosanoids [45]. GSTs have peroxidase and isomerase activities and can inhibit the Jun N-terminal kinase (Pi class), which protects cells against H_2O_2-induced cell death. GSTs are, therefore, believed to play a role in preventing oxidative stress [130].

GSTs are present as soluble and membrane-bound forms and are found in highest concentrations in the heart, lung, liver, kidney, and intestine. They can also be found in the nucleus and mitochondria. There are seven classes of cytosolic GSTs-alpha, mu, pi, sigma, theta-, zeta, and omega, one class of mitochondrial GST-kappa, and four subgroups (I–IV) of unique members of the GST superfamily designated as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) [45]. Cytosolic and mitochondrial GSTs are dimeric proteins composed of two homologous subunits. Pi and theta classes contain homodimers, while alpha and mu classes are more complex and display multiplicity of homodimeric and heterodimeric isoenzyme forms. The subunits have a catalytic center with two binding sites: a highly specific GSH binding site (G-site) and a hydrophobic site (H-site) where acceptor substrates can be accommodated. Structural differences in the H-site confer a certain degree of substrate selectivity [107].

In the human liver, alpha and pi classes of GSTs were isolated and identified from mitochondria [39]. COS cells treated with HNE resulted in increased mitochondrial localization of GSTA4-4 [117]. An additional class of GST that has been identified in mitochondria is the κ-class enzyme (GSTK1-1), which is expressed at relatively high levels in the murine liver and stomach and in moderate levels in the heart, lung, and kidney, which suggests its role as an antioxidant enzyme [111, 144].

Mitochondrial GSH is believed to be a reservoir for GSH. Mitochondria cannot synthesize GSH, but a complete redox cycle has been shown to exist in liver mitochondria, containing reduced GSH, GPx, GR, and NADPH; therefore, detoxification of hydrogen peroxide is largely dependent on GPx. The existence of mitochondrial GSTs suggests that GSH conjugates are formed in the mitochondria. At least two main questions remain unanswered. First, how is GSH transported into the mitochondria? Second, how are the GSH-conjugates effluxed out of the mitochondria? Meredith and Reed [95] made the original observation that the onset of chemically induced cell injury correlated with the depletion of mitochondrial rather than cytosolic GSH. Depletion of cytosolic GSH had no observable effect on cell viability, while the depletion of mitochondrial GSH caused cell injury. Cell injury occurred when the total liver GSH was depleted to 10–15% of normal levels. They also showed a half-life of 2 h for cytosolic GSH versus 30 h for mitochondrial GSH, and that the mitochondrial GSH concentration was higher than that in the cytosol (10 vs. 7 mM, respectively) [94] further suggesting the importance of mitochondrial GSH. The ratio of GSH/GSSG in mitochondria under normal conditions is about 18:1. Accumulation of GSSG leads to oxidation of thiols in mitochondrial proteins. Many mitochondrial proteins, including Ca^{2+}-dependent ATPase, metabolic carriers, and proteins controlling permeability changes in the inner mitochondrial membrane [119], are highly sensitive to changes in their cellular thiol status.

This may explain the observation that loss of mitochondrial GSH, rather than cytosolic GSH, correlates with cell injury.

Glutathione conjugates undergo a series of reactions that generally result in mercapturic acid formation in which GSTs catalyze the initial reaction in the biosynthesis of the mercapturic acids. The first step in the catabolism of GSH conjugates is the cleavage of the γ-glutamyl moiety catalyzed by γ-glutamyl transpeptidase (GGT). The next step is the cleavage of glycine followed by N-acetylation of the resulting cysteine conjugate, which can be subsequently taken up by the cell for further metabolism. As accumulation of GSH conjugates leads to inhibition of GSTs, as well as GST reductase, their disposal is of absolute necessity. Transporter proteins that likely to play the most significant role in the efflux of GSH conjugates are the broad specificity ATP-binding cassette (ABC) transporters that have been shown to exist in different organs and cell types.

6.5.3 Efflux Transporters

ATP-dependent transporter proteins such as multi-drug resistance-associated proteins (MRPs) also play important roles in maintaining the balance of cellular redox status by transporting GS-conjugated lipid oxidation products out of cells [73]. We found that in the heart tissue of DOX-treated mice, 4-HNE modified Mrp-1 and inhibited its activity, thus exacerbating DOX-induced oxidative stress [66]. The levels of Mrp-1 were elevated in brain of DOX-treated mice [63]. Given that MRPs are intrinsically expressed at the BBB, together with P-glycoprotein (MDR1), to regulate drug distribution and efflux [134], these findings prompt an intriguing question as to whether inactivation of ATP-dependent transporter proteins (via a similar mechanism) will compromise the integrity of the BBB, leading to entry of chemotherapeutics into the brain due to their insufficient efflux, and thus contributing to further oxidative stress. While MRPs family members (MRP1–MRP9) are capable of transporting GS-conjugates, MRP1 and MRP2 transporters have relatively higher affinities for GS-conjugates than other MRP members. However, MRP2 is localized primarily in the liver and gastrointestinal tract rather than in the heart and the brain. Therefore, we focus on MRP1 as the efflux transporter that functions to limit the efflux transport of oxidative compounds, such as GSSG and GS-conjugates of chemotherapeutic agents, thereby helping to protecting heart and brain tissues from oxidative stress-induced injury.

6.5.3.1 ABC Structure and Function

The discovery of the histidine transporter in *Salmonella typhimurium* [48] and the maltose-maltodextrin transporter in *Escherichia coli* [43] marked the early era of the ABC-mediated transporter protein field. Early during their discovery, it was suggested that one of the functions of these transporters was to uptake nutrients for cells.

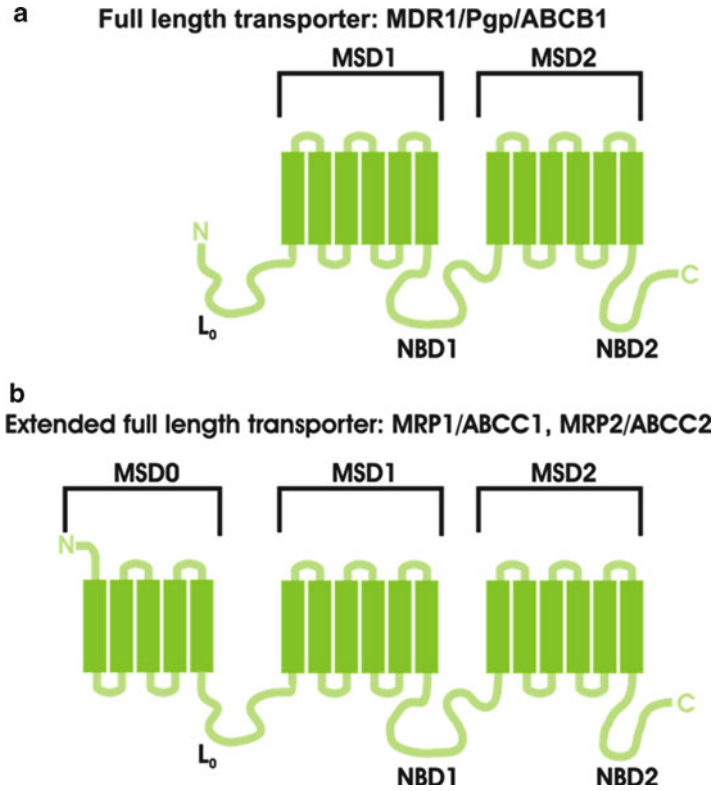

Fig. 6.9 Structural topology of ABC transporters. *MSD* membrane spanning domain; L_0 linker region 0; *NBD* nucleotide binding domain

Later, ABC proteins in prokaryotes had been subsequently described as import transporters, e.g., OppABCDF, DciA, BtuCDE, and PstABC, or as export transporters, e.g., HlyB, LtkB, CvaB, and CrtCD [10, 47]. The discovery of the export transporters then set the stage for the identification of the first eukaryotic transporter, human P-glycoprotein (Pgp, MDR1). It was predicted as an ATP-dependent efflux transporter based on the similarity of its ATP binding domains to that of bacterial HlyB [42].

ABC transporters are expressed in all cells of all organisms and couple the hydrolysis of ATP to the movement of a transport substrate across cell membranes. The core structure of ABC transporters comprises of two membrane spanning domains (MSDs – MDS1 and MSD2) and two cytoplasmic nucleotide binding domains (NBDs – NBD1 and NBD2) (Fig. 6.9). Transporters, such as P-glycoprotein (Pgp), that contain this core structure are known as full-length ABC transporters. MSDs usually consist of 6 transmembrane (TM) helices, bind to substrate, and provide specificity and a passageway for its movement. NBDs bind and hydrolyze ATP and thus drive translocation across the lipid bilayer. Members of the other group of ABC

transporters have an additional membrane spanning domain – MSD0 and are known as extended full-length transporters. Members of the extended full-length transporter group include MRP1 and MRP2 (Fig. 6.9).

6.5.3.2 P-glycoprotein

P-glycoproteins (Pgp, ABCB1, MDR1) are ATP-dependent efflux transporters that were first discovered in colchicine-resistant Chinese hamster ovary cells [65]. This glycoprotein appears to be unique to mutant cells that display altered drug permeabilities compared to their parental control cells and so it was designated as P glycoprotein. Pgp is a 170-kDa transmembrane transporter. It is constitutively expressed on the apical membrane of hepatocytes, renal proximal tubule cells and enterocytes, the epithelium of the choroid plexus, and the luminal surface of blood capillaries in the brain, placenta, ovaries, and testes. Localization at epithelial membrane barriers coupled with its efflux function have led to the concept that MDR1 is important in the absorption, distribution, metabolism, excretion and toxicity (ADMET) of drugs and xenobiotics. MDR1 clearly functions as a protective barrier by minimizing accumulation of drugs and toxins in sensitive tissues such as the brain and bone marrow. When the antihelmintic drug, Ivermectin, was administered to $Mdr1a^{-/-}$ mice, which do not express Pgp, the mice develop marked neurotoxicity. This finding was led to the serendipitous discovery of the role of Mdr1a at the blood–brain barrier [127].

Pgp is known to transport several chemotherapeutic agents including doxorubicin and taxol and to confer resistance to cancer chemotherapy [92]. Because these drugs are associated with oxidative stress induced tissue injury (Table 6.1), restriction of the uptake of these lipophilic drugs, i.e., doxorubicin by Pgp, particularly into the brain, would be an essential mechanism in protecting cells from oxidative damage induced by such drug [62, 63].

6.5.3.3 MRP1

Multidrug resistance-associated protein 1 (MRP1) was first discovered in a small cell lung cancer cell line (H69AR) that was resistant to doxorubicin [22]. The MRP1/ABCC1 gene, located on chromosome 16p13.1, encodes a 1531-amino acid protein that belongs to the ATP-binding cassette (ABC) transporter protein subfamily C [22]. The most recognized function of ABCC1 is to efflux its substrates from cells/tissues, and this attribute confers resistance to various chemotherapeutic and cytotoxic agents, e.g., epipodophyllotoxins, Vinka alkaloids, anthracyclines, mitoxantrone, and several other compounds overlapping with MDR1 substrates [74]. ABCC1 also transports drugs that are amphiphilic, anionic organic compounds, and drugs conjugated to glutathione, glucuronide, and sulfate [22, 23, 79, 157].

Constitutive ABCC1 is expressed predominantly in the heart, lung, brain capillary endothelia, and small intestine [38, 101, 137] where it mediates efflux of a broad range of substrates into the interstitial space or blood. Intracellular accumulation of MRP1

has also observed in subcellular organelles, including endocytic vesicles [5], lysosomes located near the nucleus [114], trans-Golgi vesicles [41], and the mitochondria [67] where its role is uncertain. To date, the most recognized physiological role of MRP1 is to protect cells/organs from endo- and xenobiotics resulting from biotransformation processes. While Abcc1$^{-/-}$ mice show no phenotype, it appears that highly proliferative cells such as Sertoli cells in the seminiferous tubules of the testis, the oropharyngeal mucosa, bone marrow cells and urinary collecting tubules in the kidney are adversely affected by chemotherapeutic agents such as etoposide [153, 154]. Mrp1$^{-/-}$ mice exposed to the pesticide methoxychlor were shown to develop spermatocyte and spermatid damage [148]. These mice are also hypersensitive to sodium arsenite, sodium arsenate, and antimony potassium tartrate [80, 116]. On the contrary, there was no difference between wild-type and Mrp1$^{-/-}$ mice in tumor incidence induced by chronic exposure to aflatoxin B1 [85]. This implies a redundancy of the transporter proteins, which is suggested by their overlapping substrate specificities.

Glutathione (GSH) appears to be a key factor that influences MRP1 ATP-dependent transport activity, either by its direct conjugation to the substrates or by binding to MRP1 and stimulating transport. The relationship between GSH and MRP1 function is complex, but it is apparent that MRP1 functions in part to efflux GSH. The GSH content in cells overexpressing MRP1 is much lower than that of parental control cells [24, 76, 126], and the baseline level of GSH export in Mrp1$^{-/-}$ mice stem cell is approximately one half of that observed in wild-type cells [116]. The higher GSH levels in Mrp1$^{-/-}$ mice tissues appears to be associated with decreased GSH efflux since γ-glutamylcysteine synthethase activity in Mrp1$^{-/-}$ mice is the same as wild-type mice [116]. The importance of effluxing GSH into the interstitium or blood vessels and whether this plays a role in protection against oxidative stress are not known. In mice treated with doxorubicin, Mrp1 expression in cardiac tissue is increased, as is adduction of proteins (including Mrp1) with the highly reactive product of oxidative stress, 4-hydroxyl-2,3-nonenal (HNE) [66]. MRP1 transports the cytotoxic GSH conjugate of HNE implying that its efflux serves to protect the cell [120]. Because MRP1 mediates ATP-dependent efflux of GSH and GSH-conjugates, it is likely to play a role in the protection against oxidative stress-mediated tissue injury.

6.5.3.4 MRP2

MRP2 is the second member identified in the ABCC subfamily of ABC transporters [28]. MRP2 is localized to the apical membrane surface of various polarized epithelial cells, primarily in the liver, but also in kidney, proximal small intestine, and placenta; it was initially identified as the transporter that mediates the biliary excretion of numerous drugs and their metabolites. Rodents that are deficient in MRP2 have a hyperbilirubinemic phenotype [14, 56]. In humans, the absence of a functional MRP2 leads to conjugated hyperbilirubinemia in the hereditary disorder described by Dubin and Johnson [35].

The efflux activity of MRP2 can either protect against or induce toxicity. Like MRP1, MRP2 also mediates efficient transport of the glucuronide conjugate of the tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, thus providing a protective role and ensuring its efficient excretion in bile or urine [81]. MRP2 contributes to the toxicity of arsenite, which forms a reversible complex with GSH in the hepatocyte and is subsequently effluxed into the bile. The GSH-arsenite complex dissociates in the bile, which allows for the reabsorption of arsenite into the hepatocyte, thus depleting the hepatocyte of GSH while delivering high concentrations of the arsenite to the biliary tree, its primary site of toxicity [31]. Altered MRP2 function influences the clearance of many clinically important chemotherapeutic agents such as irinotecan, methotrexate, and vinblastine. Despite its high substrate specificity for GS-conjugates, MRP2 is primarily expressed in the liver and the gastrointestinal tract. Therefore, compared to Pgp and MRP1, its role in protecting oxidative stress-induced the heart and brain injury is likely diminished.

6.6 Summary and Future Directions

Therapy-induced side effects of chemotherapeutic agents on normal tissues can limit the success of cancer therapy and/or reduce the quality of life after cancer therapy. Normal tissue tolerance for a drug frequently imposes a limit on the dose of the anticancer agent that can be given safely to the patient, which, in turn, determines the probabilities of cure. Even when cancer therapy has been successful, the cancer survivor may suffer unanticipated side effects that reduce the patient's quality of life. Many observed side effects on normal tissues are often not those predicted for the particular chemotherapeutic effects on cancer cells. It is now well documented that generation of ROS is a cause of normal tissue injury for a large number of chemotherapeutic agents. Modulation of ROS by Dexrazoxane (Zinecard), an iron-chelation therapeutic, has been in clinical use for the prevention of cardiac injury after doxorubicin treatment in pediatric oncology and in women with metastatic breast cancer. However, the potential for toxicity related to iron-chelation in normal tissues and the potential for causing secondary malignancies are causes for concern regarding the iron-chelation approach. In fact, a recent report on the use of Dexrazoxane as a cardiopulmonary protectant during treatment for Hodgkin's disease revealed an unexpected risk for acute myeloid leukemia/myelodysplastic syndrome and other secondary malignancies. Thus, antioxidants that do not involve potential prooxidant production would represent a safe alternative to Dexrazoxane.

To our knowledge, current ongoing investigations to minimize side effects of many anticancer drugs including doxorubicin have not considered the consequences of the drug on CNS. Thus, future studies to improve the quality of life for cancer patient should include consideration of the effects of interventions for the brain even when the drugs used may not directly enter the brain. Furthermore, development of any new anticancer agents should emphasize its potential effect on normal tissues at the early stage of drug development.

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Chapter 7
Tetrahydrobiopterin and Endothelial Nitric Oxide Synthase: Implications for Radiation-Induced Endothelial Dysfunction and Normal Tissue Radiation Injury

Maaike Berbée, Qiang Fu, K. Sree Kumar, and Martin Hauer-Jensen

Abstract Radiation-induced microvascular injury plays an important role in the mechanisms of acute, as well as chronic normal tissue radiation toxicities. There is currently a growing body of evidence suggesting that depletion of the nitric oxide synthase (NOS) cofactor, 5,6,7,8-tetrahydrobiopterin (BH4) is involved in the pathogenesis of endothelial dysfunction in many disorders. BH4 is an essential cofactor for all NOS enzymes, which, in the presence of adequate amounts of BH4, produce mainly nitric oxide (NO). Under conditions of BH4 deficiency, however, NOS is in the "uncoupled" state and production shifts to highly reactive oxygen radicals, superoxide, and peroxynitrite, at the expense of NO. Excessive oxidative stress, which occurs after exposure to ionizing radiation, reduces the bioavailability of BH4 because of rapid oxidation to 7,8-dihydrobiopterin (7,8-BH2). Free radical-induced BH4 insufficiency may thus further increase oxidative stress locally, inhibit beneficial NO-dependent endothelial processes, and contribute to the development of endothelial dysfunction. Given that BH4 depletion and subsequent endothelial NOS uncoupling appear to play a major role in the pathogenesis of endothelial dysfunction in a number of disease processes, there is substantial reason to believe that

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M. Berbée, MD
Department of Radiation Oncology (Maastro), Maastricht University Medical Center, 6229 ET Maastricht, The Netherlands

Q. Fu, MD, PhD • M. Hauer-Jensen, MD, PhD (✉)
Division of Radiation Health, University of Arkansas for Medical Sciences, 4301 West Markham, Slot 522-10, Little Rock, AR 72205, USA
e-mail: mhjensen@life.uams.edu

K.S. Kumar, PhD
Armed Forces Radiobiology Research Institute, Uniformed Services University of the Health Sciences, Bethesda, MD 20889, USA

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improving postirradiation BH4 bioavailability, either by exogenous supplementation of BH4 or by modulating BH4 metabolism, might be a novel strategy by which radiation-induced endothelial dysfunction and subsequent tissue injury could be reduced. This chapter presents evidence to support the therapeutic potential of BH4 as a biological modulator of radiation toxicity.

7.1 Introduction

Normal tissue radiation injury may occur in the clinical as well as in the nonclinical setting. In patients undergoing radiotherapy of cancer, injury to surrounding normal structures remains the most important limiting factor for cancer cure and a major cause of early and delayed toxicities. Moreover, in the nonclinical setting, exposure to ionizing radiation in the context of radiological/nuclear accidents or terrorist attacks may result in substantial lethality.

Approaches to prevent, mitigate, or treat, normal tissue radiation toxicity are still an unmet need. Over the years, many different agents, including antioxidants, free radical scavengers, steroids, thiols, and cytokines, have undergone investigations as potential radioprotective agents [1–3]. For example, the thiol-containing compound, amifostine, is currently the only compound approved for this specific use in humans under clinical conditions, but its use for nonclinical settings as described above is hampered by a narrow therapeutic window and severe, performance degrading side effects [4]. As a consequence, amifostine has only been approved for use in select groups of patients undergoing radiotherapy, and it is not at all useful as a medical countermeasure in radiological emergencies.

It was previously believed that the severity of normal tissue toxicity after radiation exposure was solely dependent on the extent of death of the so-called "target cells." This view has now been firmly supplanted by the recognition that radiation-induced changes in cellular function and alterations secondary to cell death contribute substantially to the development of pathophysiological manifestations of normal tissue radiation injury. Notably, an increasing body of evidence indicates that endothelial dysfunction and injury of the microvasculature play particularly important roles in the development of early and delayed radiation responses in many normal tissues. In other words, an increasing body of evidence suggests that radiation-induced microvascular endothelial dysfunction is not merely a characteristic of radiation-injury, but is in fact a crucial contributor to the pathogenesis of radiation-induced tissue injury in various organ systems [5–13]. It follows, as a logical consequence, that interventions that prevent or reduce radiation-induced endothelial dysfunction have the potential to reduce clinical toxicity and improve outcome after radiation exposure.

Radiation exposure elicits both structural and functional changes in the microvascular endothelium. These changes include among others, apoptosis, increased endothelial permeability and loss of thromboresistance. Altered expression and/or activity of endothelial nitric oxide synthase (eNOS) are believed to play a key role

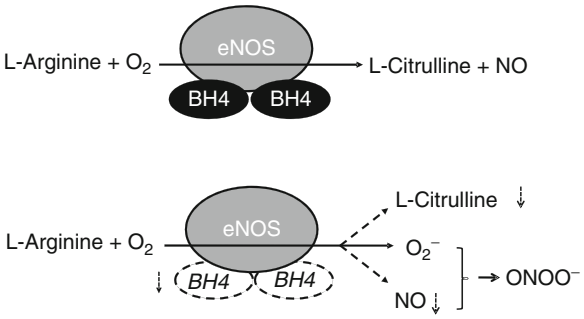

Fig. 7.1 *Schematic representation of NOS uncoupling.* Under normal conditions, NOS catalyzes the conversion of L-arginine and O_2 to L-citrulline and NO. When uncoupled, NOS produces O_2^- at the cost of NO. Decreased availability of the NOS cofactor BH4 is an important cause of NOS uncoupling

in the development of endothelial dysfunction, and both eNOS expression and the endothelial nitric oxide (NO) production are impaired after radiation exposure.

This chapter focuses on the role of the NOS-cofactor 5,6,7,8-tetrahydrobiopterin (BH4) in the development of postirradiation endothelial dysfunction. As a cofactor, BH4 is a critical determinant of eNOS function. To be functional the highly redox-sensitive BH4 should be in its fully reduced form. Conversely, when oxidized to 7,8-dihydrobiopterin (7,8-BH2), its activity as an eNOS cofactor is lost. Inadequate bioavailability of BH4, resulting in impaired NOS enzyme function (NOS uncoupling), plays an important role in a number of pathological conditions characterized by endothelial dysfunction and increased oxidative stress, such as hypertension, diabetes, hypercholesterolemia, and cardiac ischemia [14–20]. Radiation-induced free radicals may also oxidize BH4 to 7,8 BH2, thereby inducing eNOS malfunction and subsequent endothelial dysfunction (Figs. 7.1 and 7.2).

7.2 Radiation-Induced NO-Dependent Endothelial Dysfunction

NO, produced by eNOS, plays a critical role in the regulation of endothelial function. Inadequate NO production is believed to be one of the most important causes of endothelial dysfunction [21–24].

Several studies have investigated the effect of radiation exposure on endothelial NO production and eNOS-dependent vascular function. Endothelial NO is a strong vasodilator and one of the key regulators of vascular tonus. Hence, eNOS-dependent vascular function can be assessed by measuring the vascular relaxation response to substances that stimulate endothelial NO production. Vascular relaxation responses to substances such as acetylcholine are considered to be reliable measures of postirradiation NO-dependent vascular function.

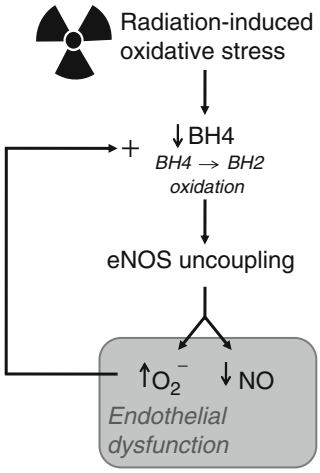

Fig. 7.2 *Model of postirradiation endothelial dysfunction.* Radiation-induced free radicals oxidize the eNOS cofactor BH4. Decreased bioavailability of BH4 results in eNOS uncoupling, thereby reducing the synthesis of NO and increasing the production of $O_2^{\cdot-}$. $O_2^{\cdot-}$ produced by eNOS may reduce the availability of BH4 even further

Both animal and human tissue samples have been used to study the effect of ionizing radiation on eNOS-dependent vascular function [25–31]. Most studies have shown that radiation exposure impairs NO-dependent vascular relaxation responses.

Different animal models have been used to study the effects of both low- and high-dose irradiation as well as of single fraction and fractionated irradiation on postirradiation vascular function.

Impaired vascular relaxation of the rabbit central ear artery was measured at different time points after irradiation with a single dose of 10–45 Gy [25, 26, 28]. The effects of high-dose irradiation on vascular relaxation are dose dependent (i.e., more severe after exposure to higher doses).

The effects of low-dose irradiation on the occurrence of NO-dependent endothelial dysfunction are less clear. Suvorava et al. measured the aortic relaxation response after exposure to a total radiation dose of 1 Gy [32]. In their experiments, rats were either chronically exposed to radiation, i.e., irradiated with a low-dose rate and an overall exposure time of about 6 weeks, or exposed to a similar total radiation dose in an acute manner. Only chronic exposure resulted in impaired endothelium-dependent (NO dependent) relaxation. In this particular study, acute radiation exposure did not affect vascular relaxation.

Hatoum et al. investigated the effects of fractionated irradiation on NO-dependent vascular relaxation [29]. Rats were exposed to total body irradiation with nine equal fractions of 2.5 Gy to a total dose of 22.5 Gy. From the third fraction onward, relaxation of intestinal microvasculature in the submucosa was found to be impaired. Moreover, the authors showed that vascular ROS production was elevated from fraction 3 on and that incubation with SOD mimetics improved postirradiation

vascular relaxation and reversed the decrease in intestinal endothelial NO levels. These findings strongly suggest that radiation-induced free radical production may play an important role in the development of postirradiation impaired eNOS function and consequential endothelial dysfunction.

When Sugihara et al. studied the effect of radiation exposure on endothelial function in human tissue samples, similar result as in the above-mentioned animal studies were found [27]. Impaired NO-dependent vascular relaxation was measured in tissue specimens from patients who had received preoperative fractionated radiotherapy with total doses from 40 to 65 Gy as compared to the vascular response in operation specimens from patients who had not undergone radiotherapy. Notably, the vascular relaxation response was impaired without significant changes in endothelial morphology.

The precise mechanism by which radiation exposure impairs eNOS-dependent vascular relaxation remains to be determined. A radiation-induced decrease in eNOS expression might play a role in the impairment of eNOS function. Decreased levels of endothelial eNOS mRNA and protein have been reported by various authors [26, 28]. However, decreased eNOS expression might not be the sole cause of the reduction in endothelial NO production, which to a large extent depends on the *activity* of eNOS. Hence, it is likely that postradiation ROS production reduces endothelial BH4 bioavailability because of the rapid oxidation of BH4 to BH2. As a result, eNOS uncoupling occurs, thereby impairing the local production of NO and increasing the production of O_2^-. Accumulating experimental evidence suggests that postirradiation eNOS uncoupling, due to increased oxidation of BH4, may be an important factor in radiation-induced eNOS malfunction, endothelial dysfunction, and normal tissue radiation injury (Fig. 7.2).

7.3 BH4 and eNOS Function

The unconjugated pterin analog BH4 is a known cofactor for a number of different enzymes. Aromatic amino acid hydroxylases, i.e., tyrosine, tryptophan, and phenylalanine hydroxylase, and glyceryl-ethermonoxygenase were the first enzymes recognized to depend on BH4 as a cofactor. Later, soon after the discovery of NO and the NOS enzymes, BH4 was discovered to be important for NOS enzyme function as well [33].

During the last few years, significant progress has been made in our understanding of how BH4 regulates NOS enzyme function. BH4 is a critical regulator of the catalytic activity of all three NOS isoforms: the constitutively expressed isoforms, endothelial NOS and neuronal NOS, as well as the inducible isoform iNOS. Several reviews have focused on the role of BH4 in the regulation of NOS function [34–36].

NOS enzymes catalyze the conversion of L-arginine into NO and L-citrulline [37]. Two moles of oxygen and 1.5 mol of NADPH are used per mole of L-citrulline formed. A functional NOS enzyme consists of two identical monomers containing a C-terminal reductase domain and an N-terminal oxygenase domain. At the C

terminal, there are binding sites for flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) as well as for NADPH. The N terminal contains binding sites for BH4, heme, and L-arginine. The process of NO biosynthesis can be divided into two successive steps. In the first step, L-arginine is hydroxylated to *N*-hydroxy-L-arginine (NHA). During this process, one molecule of O_2 and two electrons are consumed per molecule of L-arginine. The second step is an oxidation step. NHA is converted to L-citrulline and NO, using one extra molecule of O_2 and one electron. Adequate amounts of BH4 are required for the conversion of L-ariginine to NHA as well as for the formation of L-citrulline and NO from NHA.

BH4 affects NOS activity on multiple levels. It increases NOS activity by shifting the heme iron from a low to a high spin state [38, 39]. BH4 also enhances NOS substrate affinity and improves NOS synthetic activity by stabilizing the NOS dimer structure [40, 41]. Moreover, BH4 is known to play an important role in NOS electron transfer by donating an electron to the heme group [42]. Finally, BH4 may scavenge NOS-derived free radicals and thereby modulate NOS output [43, 44].

Insufficient availability of BH4 can cause so-called "uncoupling" of the NOS enzyme. NOS uncoupling refers to a form of NOS malfunction in which NADPH oxidation and O_2 reduction are uncoupled from the hydroxylation of L-arginine and the production of NO. In an uncoupled state, NOS enzymes will produce O_2^- anions at the cost of NO. Thus, NO production appears to be intricately associated with superoxide formation from irradiation, and superoxide formation in turn depends on the oxic–hypoxic status of the tissue. The dependency of superoxide formation on the equilibrium of oxic–hypoxic status suggests a close relationship to the efficacy of therapeutic radiation of cancer. Cancers, particularly those with necrotic lesions, are known to have hypoxic core regions. How these hypoxic regions respond to radiation vis-à-vis NO formation is still enigmatic.

7.4 BH4 Biosynthesis

BH4 synthesis occurs by two distinct pathways [45]. The de novo pathway synthesizes BH4 from the precursor guanidine triphosphate (GTP), whereas the salvage pathway regenerates BH4 from its oxidized forms (Fig. 7.3).

The rate of production via the de novo pathway is controlled by its first enzymatic conversion step. In this rate-limiting step, the formation of dihydroneopterin triphosphate from GTP is catalyzed by GTP cyclohydrolase I (GTPCH) in a zinc, magnesium and NAPH-dependent manner. Two additional steps are needed for the formation of BH4 from dihydroneopterin triphosphate. First, 6-pyruvol-tetrahydrobiopterin synthase catalyzes the conversion of dihydroneopterin triphosphate to pyruvol-tetrahydrobiopterin. In the last step of the de novo pathway, pyruvol-tetrahydrobiopterin is converted to BH4 by the action of sepiapterin reductase.

The rate of de novo BH4 synthesis depends on the activity of the rate-limiting enzyme GTPCH. The activity of GTPCH can be regulated in several ways. Hence, CTPCH activity is known to be modulated both by transcriptional and posttranscriptional changes, as well as by protein–protein interaction.

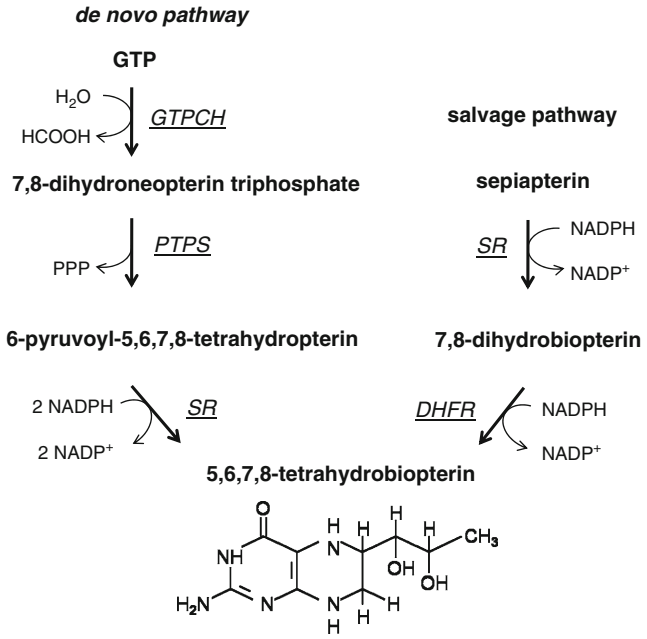

Fig. 7.3 *BH4 synthesis.* The synthesis of BH4 occurs by two distinct pathways. In the de novo pathway, BH4 is synthesized from GTP. GTPCH is the rate-limiting enzyme of de novo BH4 synthesis. BH4 is regenerated from its oxidized forms by the salvage pathway. Moreover, the salvage pathway is used to generate BH4 from exogenous sepiapterin. *GTPCH* GTP cyclohydrolase I, *PTPS* 6-pyruvoyl-tetrahydropterin synthase, *SR* sepiapterin reductase, *DHFR* dihydrofolate reductase

The CRE response element in the GTPCH gene is believed to play a crucial role in the regulation of GTPCH transcription. In vitro studies using cell cultures of hepatocytes, endothelial cells, and inflammatory cells have shown that GTPCH expression, as well as the production of BH4 increase after treatment with insulin, various cytokines, lipopolysaccharide (LPS), and hydrogen peroxide [45]. In vivo experiments have confirmed that LPS treatment has a similar effect in vivo [46]. After administration of LPS, increased GTPCH mRNA levels were found in rat tissue. Further research is needed to determine which stimuli are most important in the regulation of GTPCH expression and to elucidate the exact mechanism by which they exert their effect.

Protein phosphorylation appears to be another important mechanism of post-translational regulation of GTPCH. Protein kinase CK2 can increase GTPCH activity without increasing GTPCH levels by phosphorylation of the GTPCH protein [47, 48]. Widder et al. showed that laminar shear stress increases endothelial GTPCH activity and BH4 levels by stimulating the phosphorylation of GTPCH serine 81 [47].

Notably, the activity of GTPCH is controlled by the interaction of GTPCH with other proteins. GTPCH activity can be either stimulated or inhibited by the interaction between GTPCH and the GTPCH feedback regulatory protein (GFRP) [49–51].

GFRP–GTPCH binding enables end-product feedback inhibition by BH4. On the other hand, the binding of GFRP to GTPCH may enable phenylalanine to stimulate the enzymatic activity of GTPCH, although this process may be most relevant in the liver.

The salvage pathway regenerates BH4 from its oxidized forms by the action of sepiapterin reductase and dihydrofolate reductase.

Dihydrofolate reductase, an enzyme which also plays an important role in folate metabolism, produces BH4 by the reduction of inactive 7,8-BH2 to BH4. Cai et al. have demonstrated the importance of the salvage pathway in regulating endothelial BH4 bioavailability both in vitro and in vivo [52, 53]. They showed that reduced expression of dihydrofolate reductase decreases endothelial BH4 levels. Conversely, when the activity of dihydrofolate reductase is stimulated, BH4 levels as well as NO production increase, while the production of superoxide decreases.

Even though sepiapterin is not considered to be a physiological precursor of BH4 in mammals, it can be used as an exogenous source to increase BH4 bioavailability. Sepiapterin can be converted to BH4 by the action of the salvage pathway. Sepiapterin reductase reduces sepiapterin to 7,8-BH2. Subsequently, the conversion of 7,8-BH2 to BH4 is catalyzed by dihydrofolate reductase.

7.5 Pharmacological Approaches to Increase Postirradiation BH4 Levels

Recent studies performed in our laboratory have demonstrated, in vivo, that radiation causes a temporary reduction in tissue BH4 levels [54] and that exogenous administration of BH4 reverses some of the effects of radiation [55]. Taken together, these studies suggest that that radiation-induced decreased BH4 supplies and the resulting eNOS uncoupling play role in the pathogenesis of postirradiation endothelial dysfunction and tissue injury. Therefore, it might be possible to reduce radiation injury by restoring BH4 levels or preventing BH4 deficiency in order to maintain adequate BH4 levels.

It is possible to increase intracellular BH4 levels either directly, by supplementation of BH4 itself or its precursor sepiapterin, or indirectly, by targeting BH4 metabolism. Several pharmacological compounds have been proven to increase BH4 stability or to change the expression and/or activity levels of the key regulatory proteins involved in BH4 metabolism. Notably, some of the agents which are known to affect BH4 metabolism have already been shown to reduce the development of radiation injury [56, 57].

7.5.1 BH4/Sepiapterin

Intracellular BH4 availability can be increased by administration of BH4. For example, in vivo studies in animal models of diabetes, hypertension, hypercholesterolemia, and organ transplantation have shown that BH4 administration decreases eNOS

uncoupling as well as endothelial dysfunction [17, 58–60]. BH4 supplementation has also been proven to be effective in human subjects. Hence, clinical studies have shown beneficial effects of BH4 administration in patients suffering from diabetes, hypertension, hypercholesterolemia, as well as after ischemia–reperfusion [19, 61–65].

BH4 can be administered via multiple routes. In human subjects, BH4 is mainly administered by mouth. When administered orally, the small intestine is the main site of absorption and peak plasma levels are reached after 1–4 h. The elimination half life of BH4 is about 3–5 h [66].

Exogenously administered BH4 only reaches the intracellular compartment after several conversion steps [67, 68]. Before being taken up by tissues, BH4 is rapidly oxidized to 7,8-BH2. It is this oxidized form, 7,8-BH2, which is taken up by tissues and then converted back to BH4 once it has reached the intracellular compartment. Therefore, the efficacy of administered BH4 may be determined by the activity of the salvage pathway.

The salvage pathway also plays an essential role in the manner by which the BH4 precursor sepiapterin increases intracellular BH4 levels. Sepiapterin has been shown to improve endothelial functions both in vitro and vivo. In vitro, sepiapaterin reduces endothelial dysfunction in atherosclerotic coronary arteries [69]. In vivo, sepiapterin improves endothelial dysfunction in animal models of cardiac ischemia and diabetes [15, 70].

It needs to be noted that little is known about the possible negative effects of long-term and/or high dose administration of sepiapterin. Several authors have raised the concern that sepiapterin might compete with BH4 for eNOS binding and thereby actually worsen eNOS uncoupling [71, 72].

Additional research is clearly needed to fully elucidate the effects of BH4 and/or sepiapterin administration on various aspects of normal tissue radiation injury.

7.5.2 HMG-CoA Reductase Inhibitors

HMG-CoA reductase inhibitors, the so-called statins, have been shown to augment endothelial BH4 levels both in vitro and in vivo [73, 74]. With their in vitro experiments, Hattori et al. demonstrated that statin treatment increases de novo BH4 synthesis by increasing the transcription of the rate-limiting enzyme GTPCH. Moreover, they showed that statin treatment increases intracellular BH4 levels and NO production. This indicates that statin-induced upregulation of GTPCH may result in enhanced eNOS function because of improved BH4 bioavailability and decreased enzymatic uncoupling.

Later on, statins were shown to improve the availability of BH4 in vivo as well. Statin treatment was shown to augment the expression of GTPCH, to improve the availability of BH4, and to reduce vascular oxidative stress in a diabetic rat model.

We and others have shown that statins reduce the severity of radiation injury in several organs systems in vivo. Hence, animal studies haves shown that statins ameliorate the development of radiation-induced injury to the intestine, lung, and

skin [56, 57, 75–77]. In general, the beneficial effect of statins on radiation injury is believed to depend on the anti-inflammatory, antifibrotic, and vasculo-protective properties of statins. Using wild-type and eNOS deficient mice, Holler et al. showed that statin treatment improves postirradation vascular function in an eNOS-dependent manner [78]. Unfortunately, this study did not assess the effect of statins on postirradiation endothelial BH4 levels.

Further research is needed to determine the importance of endothelial BH4 and eNOS function in mechanisms by which statins ameliorate radiation injury. Moreover, the equilibrium between BH2 and BH4 may determine the response of tumor tissue versus normal tissue toward radiation. It can be surmised that if the equilibrium is tilted toward BH2 formation by oxidation of BH4, it may enhance the susceptibility of tumor toward radiation. If on the other hand, the equilibrium is shifted more toward BH4 formation, for example, with statins, normal tissues may be protected. This is consistent with findings by us and others that statins indeed protect normal tissues from radiation.

7.5.3 Ascorbic Acid

Ascorbic acid increases endothelial BH4 bioavailability as well as endothelial production of NO. For long, ascorbic acid was believed to protect BH4 against oxidation, thereby increasing its stability. However, evidence has emerged suggesting that that ascorbic acid does not prevent oxidation, but in fact facilitates the reduction of the intermediate radical BH3– back to BH4 [79, 80]. In vivo studies in mice demonstrate that long-term treatment with ascorbic acid reduces vascular endothelial dysfunction due to improved BH4 bioavailability and eNOS function [81].

Over the years, several studies have been conducted to investigate the effectiveness of ascorbic acid as a radioprotector [82–84]. In these studies, ascorbic acid was either given alone or in combination with other antioxidants. The results from these studies have been inconclusive and contradicting. Until now, the usefulness of ascorbic acid as a radioprotectant remains unclear.

7.5.4 Folates

Folic acid and its active metabolite 5-methyltetrahydrofolate have been shown to improve endothelial function in various diseases characterized by endothelial dysfunction, such as hypercholesterolemia and hyperhomocysteinemia [85–87]. These effects appear to be mediated by improvement in eNOS function and not to depend on folic acid-dependent remethylation of homocysteine to methionine.

BH4 plays an important role in the mechanism by which folic acid and 5-methyl tetrahydrofolate improve eNOS function. Folic acid and 5-methylhydrofolate prevent eNOS uncoupling by (1) direct interaction with eNOS, (2) enhanced BH4–eNOS

binding, (3) chemical stabilization of the BH4 molecule, and (4) increased conversion of inactive BH2 to BH4. The latter was shown to depend on upregulation of dihydrofolate reductase, an enzyme which plays an important role in both folate metabolism and the BH4 salvage pathway [52, 53].

Currently, little is known about the possible usefulness of folates as radioprophylactic or mitigating agents. Further research is needed to determine the effects of folates on radiation injury.

7.5.5 Angiotensin II Type I Receptor Blockers

Angiotensin II type I receptor antagonists are known to protect against radiation-induced pulmonary and renal injury. Animal experiments have shown that the angiotensin II type I receptor antagonist L158,809 may prevent and/or ameliorate postirradiation renal injury when started both before and after irradiation [88, 89]. L158,809 also protect against radiation-induced lung injury [90, 91]. Recent neurological studies have shown that angiotenstin II type I receptor blockers are also effective in reducing cognitive impairment after whole brain irradiation in rats [92].

The exact mechanisms by which angiotensin II type I receptor blockers confer radioprotection remains to be elucidated, but recent evidence suggests that modulation of BH4 metabolism might be important. Hence, angiotensin II type I receptor antagonist have been shown to increase GTPCH protein levels and BH4 availability and thereby prevent eNOS uncoupling in an animal model of diabetic nephropathy [93].

7.5.6 γ-Tocotrienol

The vitamin E analog γ-tocotrienol has potent radioprotective properties. A single injection of γ-tocotrienol 24 h before radiation exposure greatly ameliorates radiation injury and subsequent mortality in mice [94, 95]. γ-Tocotrienol reduces intestinal and vascular radiation injury and stimulates the recovery of the hematopoietic system after total body irradiation. The radioprotective effects of γ-tocotrienol are not solely mediated by its antioxidant properties, but also by inhibition of HMG-CoA reductase. Where statins directly inhibit HMG-COA reductase activity, γ-tocotrienol reduces enzyme activity by stimulating the proteasomal degradation of HMG-CoA reductase [96, 97].

As discussed above, inhibition of HMG-CoA reductase has been shown to have a beneficial effect on the availability of BH4 due to stimulation of de novo BH4 production. γ-Tocotrienol reduces postirradiation vascular peroxynitrite production in an HMG-CoA reducatase-dependent manner. Hence, it is likely that γ-tocotrienol ameliorate radiation-induced endothelial free radical production by increasing BH4 availability and prevention of eNOS uncoupling.

7.6 Conclusions

Inadequate availability of the NOS cofactor BH4 is believed to play a critical role in the development of various diseases characterized by increased oxidative stress. Adequate amounts of BH4 are essential to prevent NOS uncoupling, a state in which the NOS enzymes produce O_2^- at the cost of NO.

Radiation-induced free radicals may decrease endothelial bioavailability of BH4 due to rapid oxidation of BH4 to BH2, thereby prolonging and amplifying the postradiation oxidative burden. Radiation-induced decreases in BH4 may thus be important for the development of postirradiation endothelial dysfunction and tissue injury.

Further research is needed to determine the exact role of BH4 insufficiency and eNOS uncoupling in the pathogenesis of both acute and delayed radiation toxicity. Yet, it seems to be reasonable to believe that increasing BH4 levels could be a novel approach to prevent or reduce radiation injury. BH4 bioavailability can be augmented either by exogenous administration of BH4 or its precursor sepiapterin, or by modulating BH4 metabolism. Studies that determine the efficacy of BH4, sepiapterin, and various drugs known to regulate BH4 metabolism, such as statins, folates, ascorbic acid, angiotensin I type II receptor antagonists, and γ-tocotrienol in preventing or reducing radiation injury and whether their effect is BH4 dependent should be performed. The knowledge of the mechanisms of actions of these agents, particularly statins and γ-tocotrienol, may help devise strategies that provide concomitant protection of normal tissues and sensitization of tumors with the result of increasing the therapeutic ratio of radiation.

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Chapter 8
Amifostine and the Endogenous Cellular Antioxidant Enzyme Manganese Superoxide Dismutase in Radioprotection

Jeffrey S. Murley, Yasushi Kataoka, and David J. Grdina

Abstract Amifostine is currently the only drug approved by the US Food and Drug Administration for use as a radiation protector in the clinical treatment of cancer. While its approved use is limited to reducing the incidence of moderate to severe xerostomia in patients undergoing postoperative radiation treatment for head and neck cancer, where a substantial portion of the parotid glands are in the radiation field, the drug has been used in a number of off-label applications. Amifostine's effectiveness as a radioprotector is due to a number of factors that include the following: a polyamine-like structure that facilitates its concentration within mitochondria and the microenvironment of chromatin; a free thiol group in its active form that participates directly in free radical scavenging processes; and through its reductive properties, the ability to activate a nuclear transcription factor κB signaling pathway that culminates in an inductive, antioxidant-mediated protective process resulting from the enhanced transcription and subsequent elevation in activity of the potent mitochondrial antioxidant enzyme manganese superoxide dismutase. This latter effect is a thiol-mediated adaptive response that is the basis for a newly identified phenomenon described as a delayed radioprotective effect. If limited to only normal tissues, this phenomenon could be exploited to enhance amifostine's overall usefulness as a radiation protector. Concomitant effects on malignant tissues would, however, severely compromise therapeutic gain and limit its usefulness as a radiation protector in cancer treatment.

J.S. Murley, PhD • Y. Kataoka, PhD • D.J. Grdina, PhD (✉)
Department of Radiation and Cellular Oncology, The University of Chicago,
5841 S. Maryland Ave., MC1105, Chicago, IL 60637, USA
e-mail: dgrdina@uchicago.edu

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8.1 Introduction

Amifostine (*S*-2-[3-aminopropylamino]ethylphosphorothioic acid) is a radioprotective drug from the class of drugs known as phophorothioates developed by the Antiradiation Drug Development Program of the US Army [1]. From the initial observation published in 1949 demonstrating that the sulfhydryl-containing amino acid cysteine was capable of protecting cells against the lethal effects of radiation [2], a number of factors were identified as important in the development of a very effective class of radioprotector drugs. First, a relatively little appreciated but very important property of an effective chemical radioprotector was that it is positively charged to facilitate its ability to concentrate within the microenvironment of negatively charged mitochondrial and nuclear DNA. This was accomplished through the synthesis of drugs having positively charged amine groups similar in structure to that of the naturally occurring polyamines. Second, the presence of a free sulfhydryl group attached to these molecules, e.g., forming a resultant aminothiol compound, to facilitate the ability to scavenge free radicals offered the design for an effective radioprotective compound [1]. Third, the interaction of two such free sulfhydryl molecules to form a disulfide structure significantly diminishes the free radical scavenging efficacy of the drug. Fourth, because the underlying mechanism of action of these agents involves the scavenging of highly reactive and short-lived free radical species, they are required to be present during their formation as they are produced by exposure to ionizing radiation [3]. Finally, the magnitude of protection that can be achieved by this class of drugs is proportional to the amount of drug that can be effectively delivered prior to irradiation. While adequately describing the relevant factors underlying the effectiveness of aminothiol drugs as free radical scavengers and potent radioprotectors, an additional important property of this class of agents remained unknown until a report in 1995 that described the ability of certain free thiol-containing drugs to initiate a nuclear transcription factor κB (NFκB)-mediated signaling pathway that resulted in the increased transcription and subsequent elevated activity 24 h later of the mitochondrial antioxidant enzyme manganese superoxide dismutase (SOD2) [4]. Cells exposed to a 2 Gy dose of ionizing radiation at that time exhibited not only an elevation in survival [5–8], but also a reduction in the frequencies of radiation-induced DNA hyperrecombination and micronuclei formation [9].

8.2 Physicochemical Mechanisms of Action

8.2.1 Free Radical Scavenging

Amifostine is a prodrug in which its sulfhydryl group is shielded by a phosphate group which makes the drug less toxic but relatively ineffective as a radioprotector. In order to become an effective radioprotector, amifostine, having the chemical structure $H_2N\text{-}(CH_2)_3\text{-}(CH_2)_2\text{-}S\text{-}PO_3H_2$, must first be dephosphorylated by alkaline phosphatase to expose the active SH group [10, 11]. This active form of amifostine,

routinely referred to as WR1065, has the structure H_2N-$(CH_2)_3$-NH-$(CH_2)_2$-SH in which the sulfhydryl group, a highly reactive moiety, is available to participate in the scavenging of free radicals. In this manner, WR1065 actively binds to and removes highly reactive products of water radiolysis before these free radicals can react with and damage molecules of biological importance, such as mitochondrial and nuclear DNA. Furthermore, sulfhydryl-containing radioprotective drugs, such as amifostine, can form reversible disulfide bonds as a result of the interaction of two thiol groups. When this occurs between a WR1065 molecule and a thiol group of a protein, a mixed disulfide is formed that can affect both a protein's structure and function. However, such an interaction between two WR1065 molecules gives rise to the disulfide form of amifostine having the chemical structure H_2N-$(CH_2)_3$-NH-$(CH_2)_2$-*S*-*S*-$(CH_2)_2$-NH-$(CH_2)_3$-NH_2. Both the structure and biological properties of the symmetrical disulfide form of amifostine (i.e., WR33278) mimic those of the polyamine spermine, H_2N-$(CH_2)_3$-NH-$(CH_2)_2$-$(CH_2)_2$-NH-$(CH_2)_3$-NH_2, and both of these molecules can participate in the removal of free radicals [13, 14]. Since the free radical-generated indirect effect of ionizing radiation accounts for approximately 75–80% of the damage to DNA, the ability to scavenge water-derived free radicals is an important component of amifostine's radioprotective effectiveness.

8.2.2 Hydrogen Atom Donation

A second radioprotective mechanism of action attributed to amifostine is the ability of its free thiol form to participate in the direct chemical repair of a biological lesion through a direct hydrogen atom donation process [12]. The loss of a hydrogen atom in an important biological molecule, such as DNA, as a result of either the direct absorption of radiant energy or indirectly through a reaction with free radicals can be compensated through the donation of a hydrogen atom by a sulfhydryl group. Thus, hydrogen atom donation has the potential of repairing molecular lesions after they are produced by free radical damage. Consequently, the sulfhydryl group on the active free thiol from of amifostine has the potential to affect molecular damage induced by both the indirect and direct effects of ionizing radiation.

8.2.3 Intracellular Hypoxia via Auto-Oxidation

The role of oxygen in radiation sensitivity is well-known. Removal of oxygen results in a hypoxic environment. Hypoxic cells exposed to ionizing radiation exhibit a two- to three-fold elevation in radiation resistance as evidenced by increased survival. Thus, any agent that can reduce the degree of oxygenation in a tissue or cell system can also evoke a radioprotective effect. Such a radioprotective mechanism is known to be associated with thiols. In the presence of heavy metals, the rapid oxidation of thiols by oxygen can result in the removal of oxygen and result in a transient and localized hypoxic microenvironment [12]. Hypoxia induction is, therefore, a third potential mechanism that has been attributed to the radioprotective effectiveness of amifostine [15].

8.3 Role of Polyamine Structure in Radiation Protection

8.3.1 Drug Delivery

The disulfide form of amifostine is structurally similar to the polyamine spermine (see above). The polyamines are ubiquitous polycationic molecules that participate in numerous cellular functions, including maintenance of chromatin stability, effects on DNA synthesis, modulation of kinase activities, effects on gene expression, and changes in protein confirmation [16]. The close structural similarity between amifostine's disulfide form and spermine is also correlated with many similar functional properties. Examples of this include the ability of amifostine to inhibit putrescine uptake into the lung tissues of rats [17] and to serve as a substrate for polyamine oxidase [18]. While amifostine's free thiol form, WR1065, has been extensively studied and found to be taken up by cells primarily through a passive diffusion process, the disulfide form (WR33278) is transported actively into cells via the polyamine transporter system [13]. Transport of WR33278 by this process occurs at the same velocity as spermidine and can effectively compete with and inhibit its incorporation into cells. The cellular uptake of both WR33278 and spermidine are equally inhibited under conditions that also inhibit the endogenous cellular polyamine transporter system, such as through the use of either inhibitory agents or site-directed mutagenesis [13].

Intracellular redox-mediated reactions can significantly affect the interconversion between the thiol and disulfide forms of amifostine. Following exposure of cells to millimolar concentrations of WR1065, the intracellular ratio of thiol to disulfide molecules is 10 to 1 if the measurements are based on total cellular volume [19]. The disulfide form can concentrate over ten-fold within the nucleus as compared to the cytoplasm. Thus, if measurements are made only within the nucleus rather than the entire cellular volume following WR1065 exposure, the ratio of the thiol form to the disulfide form is 1 to 1.2 [20]. These findings have been interpreted as being the consequence of an active sequestration of the disulfide form of amifostine within the nuclear volume of cells.

8.3.2 DNA Binding: Localizing the Radical Scavenger to the Molecular Target

It is well-accepted in radiation biology that DNA is the most important intracellular molecular target for radiation damage. Over 75% of low LET radiation-induced DNA damage is due to free radical formation, e.g., the indirect effect. In the case of thiol-based radioprotectors, it has been determined that the net positive charge of the thiol compound is a major determinant of its ability to protect against radiation-induced free radical damage since DNA is negatively charged [19, 21, 22]. The free thiol and disulfide forms of amifostine have positive net charges of +2 and +4,

respectively [22]. Polyamines are also positively charged molecules. Like polyamines of similar structure and net charge, the thiol and disulfide forms of amifostine exhibit the same propensity toward electrostatic binding to the minor grove of DNA [23–25]. The disulfide form, WR33278, occupies a binding site of approximately ten nucleotides while the free thiol, WR1065, binds to only five nucleotides [26]. Each form of amifostine competes equally with its respective polyamine counterpart exhibiting the same net-positive electrostatic charge for the same DNA-binding sites [23– 26]. In this manner, locally high concentrations of amifostine's free thiol and disulfide forms can be achieved in the microenvironment of critically important biological targets, such as nuclear and mitochondrial DNA, to enhance their associated free radical scavenging mechanisms that lead to radiation protection. Furthermore, such binding to DNA can alter and stabilize chromatin structure [27–29] that in turn can affect gene expression [30, 31], DNA repair [32, 33], enzymatic activity, and cell cycle progression [34, 35]. Each of these properties can contribute, therefore, to overall protection against radiation-induced free radical damage in cells. The timing of radioprotector administration is also critical. Empirical data have demonstrated that intracellular levels of free thiol, WR1065, and disulfide, WR33278, forms of amifostine reach their maximum concentrations approximately 30 min after administration and fall to background levels 1 h later [20]. Thus, their direct protective effects are transitory and require their administration within a very limited window of time prior to radiation exposure. Furthermore, radiolytic products produced by radiation occur within 10^{-12} s following exposure. By 10^{-9} s, free hydroxyl radicals are produced in high concentrations and by 10^{-3} s radiation-induced free radical damage is essentially completed [36]. The usefulness of amifostine as a radioprotector has, therefore, been limited to a finite temporal interval of approximately 1 h following drug administration and ending immediately after radiation exposure.

8.4 Thiols, NFκB, and SOD2

8.4.1 Thiol Activation of NFκB

The historical focus of studies on free radical scavenger-based radioprotective chemicals is well-reflected in the discovery and development of amifostine. Specifically, thiol-containing compounds having positively charged amine groups to facilitate their sequestration to microenvironments surrounding negatively charged mitochondrial and nuclear DNA formed the basis for the extensive radioprotector development program conducted by the Antiradiation Drug Development of the US Army Medical Research and Development Command [1]. Little appreciation was given, however, to an additional component of the overall free radical scavenging property of these agents, e.g., their ability to induce in cells endogenous antioxidant enzymes that could prolong the effective window for the scavenging of free radicals. A seminal observation was reported in 1995 in which it was reported

that thiol-reducing agents could initiate a signaling process through the activation of the redox-sensitive transcription factor NFκB that culminated in the enhanced expression of the *SOD2* gene and a concomitant elevation of mitochondrial-localized enzyme activity [4]. *N*-acetylcysteine, dithiothreitol, and 2-mercaptoethanol were all observed to be potent inducers of this process. These observations were later both confirmed and expanded to include the thiol-containing drugs oltipraz [37], mesna, and captopril and the free thiol form of amifostine [38]. Using electrophoretic mobility supershift assays, it was demonstrated that both the free thiol (WR1065) and disulfide (WR33278) forms of amifostine at micromolar concentrations were effective in inducing elevated levels of the heterodimer form of NFκB composed of the p50 and p65 subunits. Under these conditions, neither the p52 nor c-Rel subunits of NFκB were affected [38]. The alteration of the redox state of cysteine residues in the p50 and p65 subunits of NFκB has been proposed as the underlying mechanism of action of this process [39]. Interestingly, activation of NFκB by these drugs did not lead to altered expression of inflammatory genes or their proteins, such as intercellular adhesion molecule-1, tumor necrosis factor-α, macrophage inflammatory protein 1α, and interleukins 1, 6, 8, 11, and 12, as determined by RT-PCR [30, 31, 38], cDNA expression arrays containing genes having NFκB-responsive elements [31], or ELISA assays [40].

8.4.2 *NFκB and Elevated SOD2 Gene Expression and Enzymatic Activity*

SOD2 is an important nuclear-encoded mitochondrial enzyme that scavenges superoxide radicals in the mitochondrial matrix and is known to be highly protective against radiation-induced reactive oxygen species (ROS) damage that can lead to cell lethality and genomic instability [41–43]. While the *SOD2* gene contains binding motifs for a number of transcription factors other than NFκB, thiol exposure was found not to affect their activation. These include activator proteins 1 (AP1) and 2 (AP2), specificity protein 1 (Sp1), adenosine 3′,5′-cyclic monophosphate-regulator element-binding factor (CREB) [7], and Forkhead box 03A (FOXO3a) (unpublished data). Further evidence supporting the unique involvement of NFκB in the thiol-initiated SOD2 signaling pathway is the demonstration that treatment of cells with specific NFκB inhibitors like BAY 11-7082 just prior to thiol exposure not only inhibits activation of this transcription factor, but also prevents the elevation of SOD2 protein levels as determined by Western blotting [6]. Thiol-induced activation of NFκB in both human and rodent cells appears to be maximal at about 1 h following a 30-min exposure of cells to WR1065 [38]. A representative example of subsequent changes in the levels of SOD2 protein in the RKO36 human colon carcinoma cell line determined by Western blotting as a function of time following WR1065 exposure is presented in Fig. 8.1. SOD2 activity was also measured at 24 h post thiol exposure and was found to increase 6.2-fold over background. This kinetic pattern describing

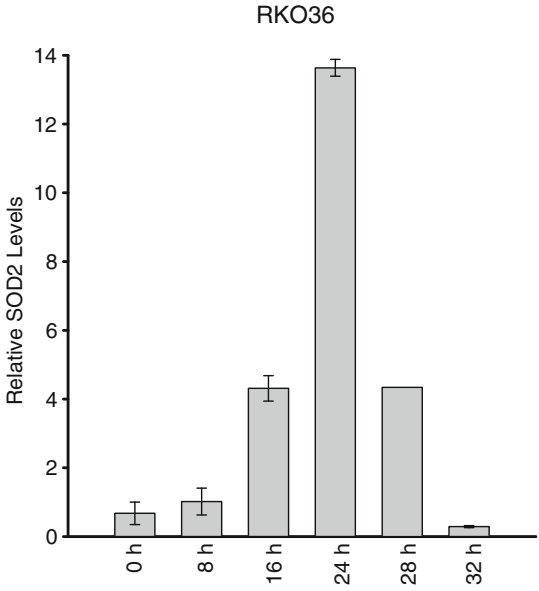

Fig. 8.1 Time course showing the kinetics of change in SOD2 protein levels after a 30-min exposure to WR1065, the free thiol form of amifostine

the peak elevation of SOD2 protein level and activity at 24 h followed by a return to baseline at 32 h is also consistent with that reported for both human microvascular endothelial cells (HMEC) [38] and mouse sarcoma cells (SA-NH) grown under similar in vitro conditions [5].

SOD2 is localized within the mitochondria, where it efficiently converts superoxide anion ($O_2^{-\bullet}$) to hydrogen peroxide (H_2O_2) which in turn is converted to water and oxygen through the action of catalase or glutathione peroxidase. Superoxide anion is an important ROS species that is generated normally within the mitochondria as the result of a one-electron reduction of molecular oxygen. As a result of cellular exposure to stress-inducing agents, superoxide anion generation within the mitochondria can initiate a ROS cascade culminating in the production of damaging molecules, such as hydrogen peroxide, peroxynitrite, and hydroxyl radicals [44, 45]. This process in turn can lead to an ROS-induced ROS release (RIRR) capable of intra- and extracellular propagation resulting in damage to additional mitochondria within the same as well as adjacent cells leading to cell death [46–48]. A model describing this process is presented in Fig. 8.2. SOD2 plays a pivotal role in the modulation of this process. Studies have demonstrated that mitochondrial localization of SOD is required for protection against radiation-induced cellular damage [49]. Specifically, mitochondrial-localized SOD2 activity is required to exert a protective effect against radiation-induced ROS while elevation of extramitochondrial or extracellular SOD activities is relatively ineffective in enhancing radiation resistance [43, 49]. These results are consistent with the RIRR model and $O_2^{-\bullet}$ as the initiating ROS. Presumably, modulation of $O_2^{-\bullet}$ production within the mitochondria

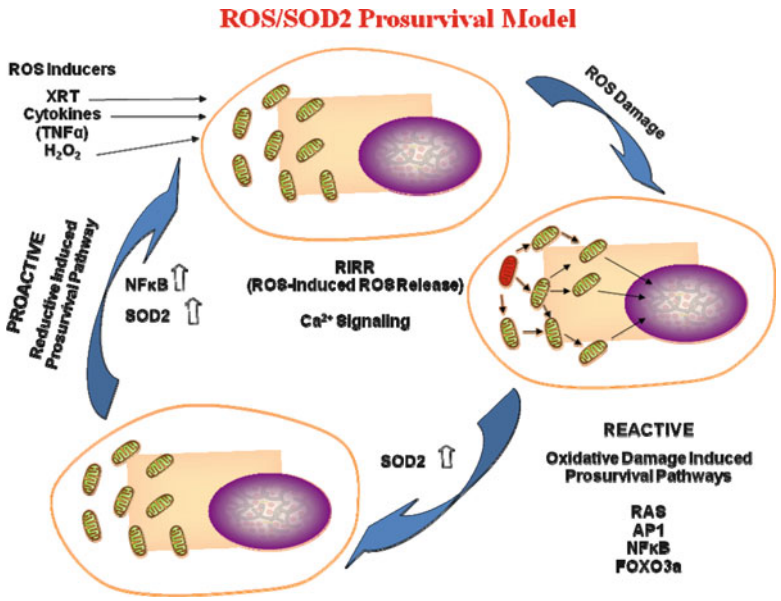

Fig. 8.2 Proposed model describing both reactive and proactive processes in cells leading to SOD2-mediated survival protection following the induction of damage by ROS

by SOD2 prevents the initiation of the ROS cascade and the subsequent RIRR effect. However, if such an ROS cascade was initiated, the range of different damaging ROS produced and released from the mitochondria in the RIRR process would limit the effectiveness of any cytoplasmic or extracellular-localized SOD since their only function would be limited to the dismutation of $O_2^{-\bullet}$ and not the removal or scavenging of other ROS, such as peroxynitrite or hydroxyl radicals.

8.5 The Thiol-Induced Adaptive/Delayed Radioprotective Effect

8.5.1 The Delayed/Adaptive Radioprotective Effect

The effectiveness of amifostine and its free thiol and disulfide metabolites as free radical scavengers and radioprotectors is well-known. However, radiation protection has routinely been demonstrated within the context of the "immediate" effects of these agents. That is, the drugs must be administered within minutes to an hour before radiation exposure to insure that sufficient concentrations of the protective agents are present during irradiation to facilitate the effective reduction

of ROS-mediated damage. What has not been appreciated heretofore, however, was the ability of thiol drugs in general, and amifostine in particular, to be effective as inducers of a prosurvival cell signaling pathway resulting from their ability to activate the redox-sensitive NFκB transcription factor and to subsequently induce *SOD2* expression culminating in the significant elevation of SOD2 enzymatic activity at a later time [4– 8]. SOD2 is a known endogenous cellular antioxidant enzyme that exhibits radioprotective properties [43–49]. Since the time duration required for significant elevation of SOD2 following thiol exposure is about 24 h, radioprotection exhibited at this time can be considered as a "delayed" or "adaptive" radioprotective effect.

8.5.2 *Role of NFκB in the Delayed/Adaptive Radioprotective Effect*

The importance of NFκB activation by thiols in the delayed radioprotective effect was demonstrated using SA-NH mouse sarcoma cells that were stably transfected with a mutant IκBα gene under the control of a CMV promoter in which series 32 and 36 were mutated. These mutations abolish the inducible phosphorylation of these residues, thus preventing the subsequent ligand-induced degradation that leads to activation of NFκB [50]. A stable clone, designated SA-NH + mIκBα1, was isolated and used in studies along with the parent SA-NH cell system to assess the effects of amifostine's free thiol, WR1065, on inducing in them a delayed/adaptive radioprotective effect [6]. Both wild-type and mIκBα transfected cells were exposed to a 4-mM concentration of WR1065 for 30 min and then irradiated immediately afterward (I) or after they were washed free of drug and grown in fresh medium for an additional 24 h to allow for a maximal elevation of SOD2 activity (D) [4–8]. Wild-type and SA-NH + mIκBα1 cells were then exposed to a single 2 Gy dose of X-rays. Surviving fractions were determined for cells exposed under these conditions and were compared to the survival of cells exposed to 2 Gy only. Representative data are presented in Fig. 8.3. Wild-type SA-NH and SA-NH + mIκBα1 exhibited similar sensitivities to 2 Gy of radiation. Furthermore, both wild-type and transfected cells were equally protected if they were irradiated immediately following a 30-min exposure to 4 mM of WR1065. In contrast, only the nontransfected, wild-type SA-NH cells exhibited an enhanced cell survival when they were irradiated 24 h following thiol exposure. No effect of thiol exposure on radiation response was observed in the SA-NH + mIκBα1 cells. Furthermore, accompanying gel shifts and Western blots run on cells from each experimental group failed to show any evidence of NFκB activation or subsequent elevation of SOD2 protein levels in the SA-NH + mIκBα1 as compared to wild-type SA-NH cells, demonstrating that NFκB activation following thiol exposure is a critical requirement for induction of the delayed/adaptive radioprotective effect [6].

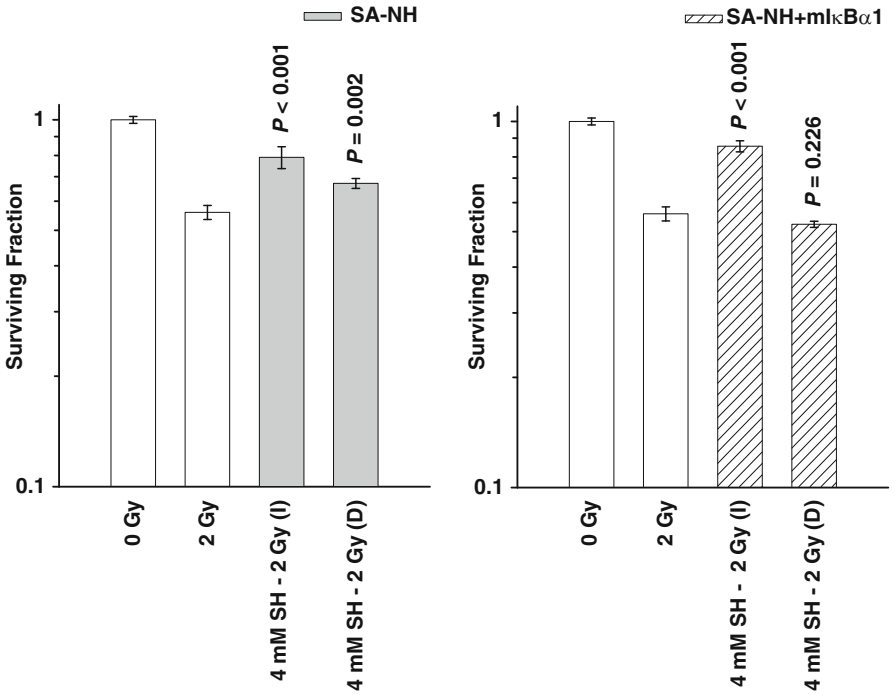

Fig. 8.3 Survival of SA-NH and SA-NH+mIκBα1 cells exposed to 2 Gy of 250 kVp X-rays immediately after (I) or 24 h after (D) a 30-min treatment with 4 mM WR1065 (SH). Each *bar* represents the mean ± SEM of three separate experiments

8.5.3 The Role of SOD2 in the Delayed/Adaptive Radioprotective Effect

SOD2 is a metalloprotein that, while localized in the mitochondria, is known to be effective in protecting against radiation-induced toxicity. To assess its role in the thiol-induced delayed/adaptive radioprotective effect, SA-NH cells were transfected using Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions with either SOD2 antisense oligonucleotide obtained from Oligos etc. (Bethel, ME) having the sequence 5′ ACACTGCCCGGCTCAACATG 3′ or scrambled sequence (Scram) oligonucleotide from the same company. Nontransfected control SA-NH cells and cells transfected with either antisense or Scram oligonucleotides were then characterized with respect to their particular plating efficiencies, response to a 2 Gy dose of radiation, and a 30-min treatment with either a 4 mM or 40 μM concentration of WR1065 followed 24 h later with an exposure to a 2 Gy dose of radiation. The results of the average of three separate

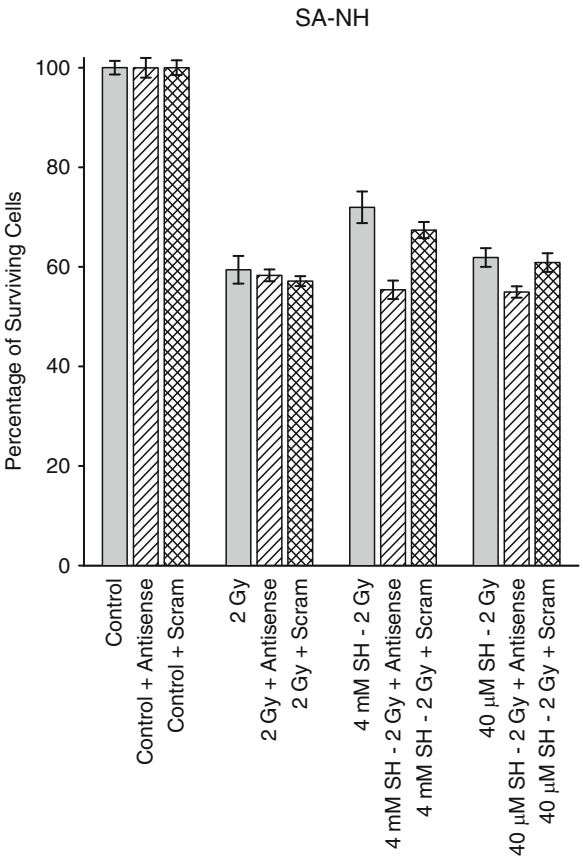

Fig. 8.4 Survival of SA-NH cells transfected with SOD2 antisense or scrambled-sequence (Scram) oligonucleotide and exposed to 2 Gy 250 kVp X-rays 24 h after a 30-min treatment with 4 mM or 40 μM WR1065 (SH) relative to untransfected cells

experiments are presented in Fig. 8.4 as bar graphs. Transfection with either antisense or Scram oligonucleotides had no effect on the radiation response of non-thiol-treated cells. Nontransfected control cells or cells transfected with Scram oligonucleotide both exhibited an elevated surviving fraction to 2 Gy regardless if they were treated with 4 mM or 40 μM of WR1065 24 h prior to irradiation. In contrast, neither concentration of WR1065 was effective in inducing a delayed/adaptive radiation response in cells transfected with SOD2 antisense oligonucleotide. These data are in agreement with data published from studies using RKO36 [8] and HMEC [51] cell systems in which cells transfected with *SOD2 siRNA* failed to exhibit either an elevation in SOD2 activity or a delayed/adaptive elevation in radiation resistance following exposure 24 h earlier to WR1065.

8.5.4 Implications of SOD2 as an Effector Molecule in the Delayed/Adaptive Radiation Response

SOD2, in contrast to extracellular (SOD3) or cytoplasmic CuZnSOD (SOD1) superoxide dismutases, has been identified in numerous cell systems as being an effective mediator of radiation-induced ROS damage leading to cell death and genomic instability [9, 43, 49, 52]. Since SOD2 is only localized with the mitochondrion, a subcellular organelle that has been implicated in a cytoplasmic amplification mechanism for the generation of ROS following exposure to ionizing radiation that leads to cell death and genomic instability [53, 54], it is reasonable to suggest that SOD2's effectiveness lies in its ability to prevent the initiation of a mitochondrial-localized ROS cascade process previously identified as RIRR [46–48]. Superoxide anion is the product of a one-electron reduction of oxygen, precursor of most ROS, and a mediator of oxidative chain reactions. Dismutation of $O_2^{-\bullet}$ produces hydrogen peroxide, which in turn can be converted into water and oxygen by catalase and glutathione peroxidase or partially reduced to the highly reactive hydroxyl radical in the presence of transition metals. Hydroxyl radicals can further be re-reduced by $O_2^{-\bullet}$ leading to a propagation of this process [44, 45, 55]. Superoxide can also react with nitric oxide to form the highly reactive and damaging molecule peroxynitrite [45, 55]. Thus, if sufficient enzymatically active SOD2 is present within the mitochondria at the time of irradiation, $O_2^{-\bullet}$ production could be effectively inhibited through the SOD2-mediated dismutation process preventing the initiation and downstream production of a highly reactive but diverse ROS cascade. SOD1 would be relatively ineffective in modulating such an ROS cascade once released from the mitochondria since its antioxidant property would be limited only to the dismutation of $O_2^{-\bullet}$. An elegant demonstration of the requirement for the localization of SOD within the mitochondria to facilitate protection against radiation-induced cellular damage and cytotoxicity has been described in detail elsewhere using various *SOD* transgenes and mitochondrial localization leader sequences to evaluate relative SOD protective properties as a function of subcellular localization at the time of irradiation [49].

The effectiveness of SOD2 as an endogenous antioxidant capable of modulating stress-induced RIRR has been well-characterized in numerous cell models, including human and mouse tumors, and nonmalignant human microvascular endothelial cells. The SOD2-mediated delayed/adaptive response can be initiated not only by thiols, but also following initiation by low-dose ionizing radiation exposure and/or exposure to the cytokine tumor necrosis factor-α (TNFα) [5–8, 43, 51, 52, 56]. Three models of such adaptive responses are presented in Fig. 8.5. The unique difference between these three models is in the mechanism of their respective induction. The adaptive responses initiated by ionizing radiation and TNFα are the result of a cellular response to stress-induced, ROS-mediated damage resulting in the activation of redox-sensitive transcription factors, such as NFκB and FOXO3a, that lead to subsequent elevated transcription of *SOD2* [43, 57–59]. In contrast, the thiol-induced adaptive response is initiated through the reductive action of SH- groups on cysteine residues in the

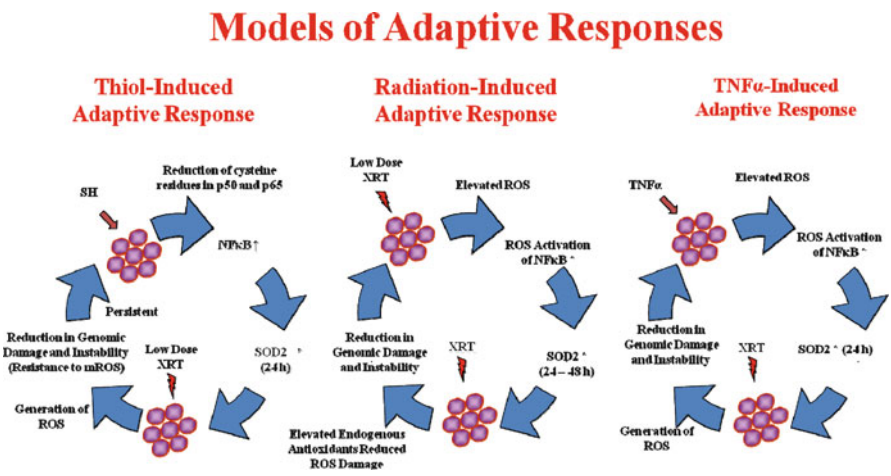

Fig. 8.5 Proposed models describing the SOD2-mediated adaptive responses induced in cells following exposure to thiols, radiation, or TNFα

p50/p65 NFκB subunits that results in its activation and subsequent elevation of *SOD2* gene expression. The thiol-induced adaptive response can, therefore, be considered a proactive rather than a reactive prosurvival response that is induced by oxidative insult. The important implication of this difference between reductive vs. oxidative initiation of adaptive responses is that thiols have the potential to be used therapeutically to both elevate endogenous antioxidant defenses with chronic dosing, as well as to facilitate the mitigation of oxidative damage induced by RIRR processes. Daily or once every other day dosing of HMEC and SA-NH cells with 40 μM WR1065, for example, was sufficient to chronically elevate their respective SOD2 enzymatic activities by five- to eight-fold over background levels [51]. These elevated activities were maintained for up to 2 weeks and resulted in a 20–30% increase in overall radiation resistance as evidenced by increased survival values following challenge with a 2 Gy dose of ionizing radiation. It is important to note that chronic low-dose administration was effective in elevating SOD2 activity and enhancing corresponding radiation resistance in both the nonmalignant and malignant cell lines.

8.6 Radiation Response Implications of Thiol-Induced Elevated SOD2 Levels in Normal and Tumor Tissues

8.6.1 Elevated SOD2 Levels In Vivo

The ability of thiol-containing drugs, such as amifostine, to induce elevated levels of active SOD2 enzyme under in vivo conditions would have important implications not only in the field of radioprotection in general, but also in regard to their safety

profile for use in cancer therapy. Clearly, if significant elevation of SOD2 could be achieved in normal tissues without associated detrimental effects, such as a toxic buildup of H_2O_2 following chronic low dosing of thiol-containing drugs, it could be possible to devise dietary regimens that would elevate the inherent radiation resistance of high-risk individuals, such as those involved as first responders to radiation accidents or terrorist events, cleanup workers participating in radiation decontamination activities, or individuals required to work in a radiation environment, such as astronauts and international flight crews and pilots. However, if such a buildup of SOD2 activity induced by amifostine occurred in tumors of cancer patients undergoing radiation therapy, the potential for tumor protection and a compromise of therapeutic outcomes could occur. To test for this possibility, a C3H mouse model system was used. SA-NH tumor-bearing mice were injected intraperitoneally with 400 mg/kg of amifostine when tumors were 8 mm in diameter. At selected times following amifostine injection, animals, three per experimental group, were sacrificed and small intestine, pancreas, lung, liver, spleen, and SA-NH tumor tissues were removed and flash frozen. Samples were coded and sent to the Radiation and Free Radical Research Core Laboratory at the University of Iowa Holden Comprehensive Cancer Center for analysis of changes in SOD2 activity. These data are presented in Fig. 8.6. While no significant change in activity was observed in liver tissue, SOD2 activity significantly rose over corresponding background levels in small intestine, pancreas, spleen, and tumor tissues. An increase in SOD2 activity approached significance in lung tissue, with maximal levels being reached at 24 or 32 h in the various tissues sampled following amifostine treatment. Of potential concern is the significant elevation of SOD2 activity observed at 24 h in SA-NH tumors. Presumably, patients undergoing radiation treatment with amifostine would have their treatments on a daily basis five times a week with each successive treatment occurring approximately 24 h following the previous exposure. An elevation in SOD2 activity in the tumor could have a significant impact on their therapeutic outcome.

8.6.2 Radiation Response of SA-NH

The SA-NH tumor system was chosen for this study not only because of the large database that has been generated regarding the thiol-induced delayed/adaptive response [5, 6, 51], but also because of the ability of its cells to be easily grown under in vitro conditions following their dissociation and isolation from excised solid tumor tissue to assess clonogenic responses to previous in vivo treatments. Using this approach, the surviving fraction of SA-NH tumor cells irradiated with 2 Gy was determined as a function of time following amifostine exposure. Radiation resistance was elevated 27% ($P = 0.001$), as measured by an in vitro colony-forming assay, in SA-NH cells irradiated 24 h following their removal from amifostine-treated animals [60]. The increase in radiation resistance correlated with the increased SOD2 activity in SA-NH cells measured at this time, indicating that a thiol-induced delayed/adaptive response can also occur under in vivo exposure conditions.

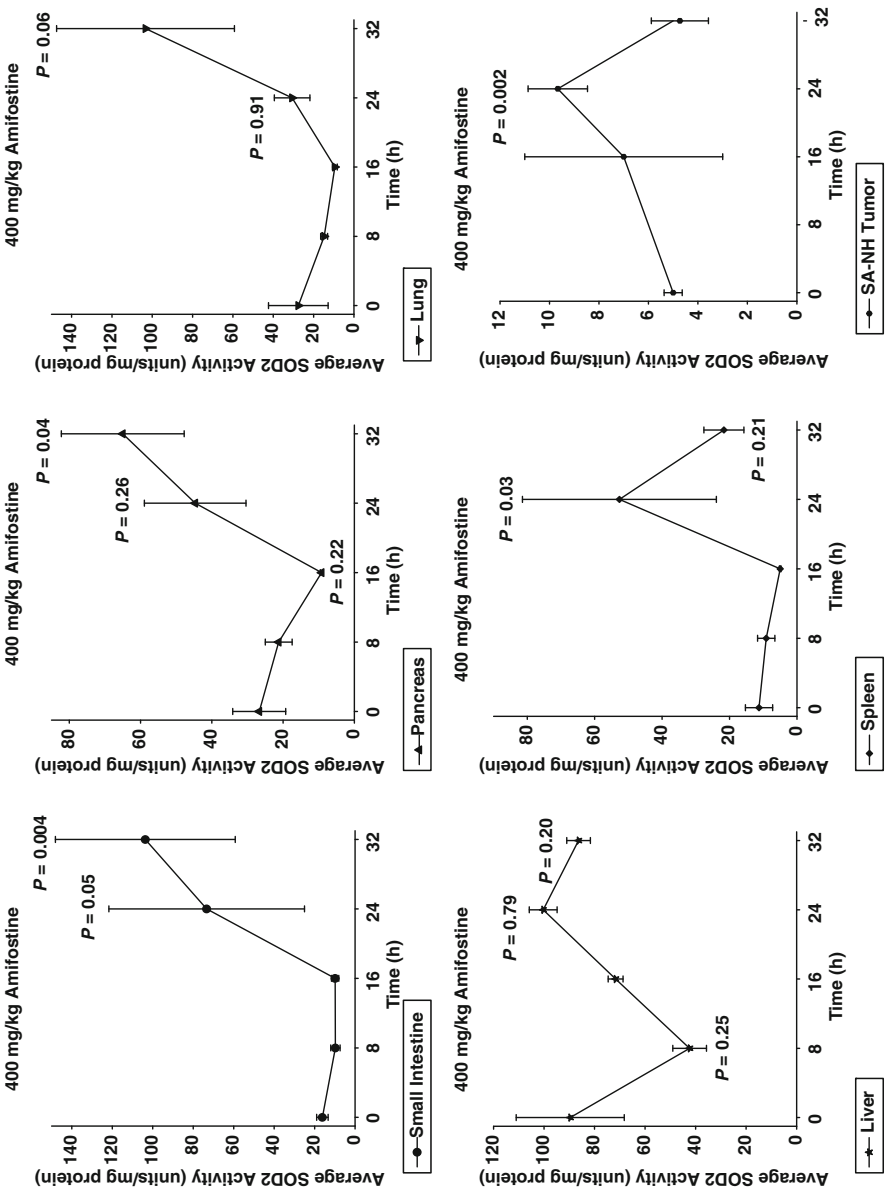

Fig. 8.6 Kinetics of effects of amifostine on SOD2 activity in normal mouse tissues and SA-NH tumor

The potential for tumor protection by amifostine has long been recognized and debated. Yuhas addressed the issue of tumor protection in 1983 in which he noted that the preponderance of data from mouse studies indicated that solid tumors are either not or only marginally protected by amifostine, and that tumor protection can be demonstrated for well-oxygenated ascetic tumors or leukemias [61]. Other investigators at the time reported tumor protection factors ranging from 1.1 to 2.5 using such end points as cell survival, growth delay, latency, and tumor control dose 50 in a range of mouse tumors [62–64]. It is possible that amifostine's ability to elevate SOD2 levels in tumors undergoing fractionated treatments might have contributed in part to these elevated protection factors.

The issue of tumor protection by amifostine continues to be controversial, evoking strong responses from both sides of the debate [65, 66]. Amifostine is currently the only radiation protector approved by the US Food and Drug Administration for use against radiation therapy-induced moderate-to-severe xerostomia in patients undergoing postoperative radiation therapy for head and neck cancer [67]. However, from its original approval by the FDA to the present, there has been a lack of clinical studies designed to be robust enough to evaluate the influence of amifostine on the therapeutic index [68]. For example, to identify a reduction in patient survival from 45 to 40%, e.g., a 5% increase in failure rate, with a significance of 0.05 and an 80% power, a total of 1,200 patients per study arm would be required [68]. An alternative to performing such a comprehensive study has been the performance of meta-analyses on completed randomized studies [66]. However, these studies suffer from the vagaries and variances in protocol design, implementation, and data analysis that come with multi-institutional studies. As an example, a meta-analysis on the effect of amifostine on the response rates in patients with locally advanced non-small-cell lung cancer was completed using data from 7 randomized trials and 600 patients. The conclusion reached from this analysis was that amifostine had no affect on tumor response. However, the data sets used in this meta-analysis came from studies that used five different dosing regimens, two different routes of administration, and three studies in which amifostine exhibited no evidence of even normal tissue protection [66]. Presumably, amifostine protection of tumors is not an issue under those circumstances, where it cannot be demonstrated that even normal tissues are not protected. The ability of amifostine to induce a delayed/adaptive response in tumors as a result of elevating intratumor SOD2 levels gives rise to an additional concern for its use as a cytoprotector in radiation therapy.

8.7 Summary and Conclusions

Amifostine is the only FDA-approved radioprotector drug for use in radiation therapy. Its usefulness as a radioprotector stems from its well-characterized cytoprotective properties that include its abilities to: (a) scavenge free radical species (ROS), (b) induce intracellular hypoxia as a result of auto-oxidation, (c) donate hydrogen atoms to facilitate direct chemical repair of DNA lesions, (d) mimic

polyamine properties related to stabilization of DNA and its damaged sites and facilitate error-free repair, (e) delay progression of cells through the cell cycle, (f) inhibit apoptosis, and (g) induce a delayed/adaptive response through its reductive activation of NFκB and subsequent induction of *SOD2* gene expression and elevation of mitochondrial-localized active enzymatic levels. All of these properties contribute to amifostine's overall effectiveness as a radioprotector. It is the ability of this drug, along with thiol-containing drugs in general, to induce a prosurvival response through a reductive rather than oxidative damage process that is the most recent discovery. The implications of this can have profound consequences in the development of novel antioxidant paradigms for use both in environmental protection of at-risk radiation workers and clinically relevant therapeutic applications to mitigate against stress-induced, ROS-associated pathologies caused by the RIRR phenomenon.

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Chapter 9
Redox Regulation of Stem Cell Compartments: The Convergence of Radiation-Induced Normal Tissue Damage and Oxidative Stress

Ruth K. Globus, Vincent Caiozzo, Munjal Acharya, John R. Fike, and Charles Limoli

Abstract The redox environment impacts normal stem cell niches throughout the body. Hematopoietic, muscle, and neural stem cell compartments respond to changes in reactive oxygen (ROS) and nitrogen (RNS) species by triggering signaling networks that impact cellular proliferation, survival, and differentiation. Work from many labs including our own has found that irradiation can trigger acute and chronic increases in oxidative stress. Low dose and/or protracted dose rates can elicit radioadaptive changes that have beneficial effects on proliferation and survival while influencing the development lineage-specific cell fates. Higher doses and dose rates have been found to impede the regeneration of irradiated tissues, through the depletion and/or damage of endogenous stem cell pools, and by promoting the onset and persistence of secondary reactive processes involving oxidative stress and inflammatory cytokines. Increasing evidence suggests that these important stem cell pools are differentially protected from DNA damaging agents compared to their immediate progeny (i.e., precursor/progenitor cells) due to enhanced DNA repair, antioxidant status, and reduced cell cycle activity. Thus, many of the adverse effects of irradiation on normal tissue are the consequence of damage to the rapidly

R.K. Globus, PhD
Space Biosciences Division, NASA Ames Research Center,
Moffett Field, CA 94035-1000, USA

V. Caiozzo, PhD
Department of Orthopaedic Surgery, University of California,
Irvine, CA 92697-5399, USA

M. Acharya, PhD • C. Limoli, PhD (✉)
Department of Radiation Oncology, University of California,
Irvine, CA 92697-2695, USA

J.R. Fike, PhD
Departments of Neurological Surgery and Radiation Oncology,
Brain and Spinal Injury Center, University of California,
San Francisco, CA 94110-0899, USA

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expanding pool of precursor cells derived from asymmetric cell division. Irradiation of the bone marrow impairs the health of bone by promoting osteoclastogenesis (osteoclast-mediated bone resorption) and inhibiting osteoblastogenesis (osteoblast-mediated bone formation), with the net effect of reducing bone mass and structural integrity. Irradiation of the skeletal musculature impairs myogenesis (formation of muscle tissue) by damaging satellite cells (i.e., muscle stem cells) and reducing proproliferative levels of nitric oxide. In the brain, irradiation depletes neural stem and precursor cells and leads to persistent increases in ROS/RNS and inflammatory cytokines that inhibit neurogenesis (formation of new neurons and glia) and adversely impact cognition. In each of these foregoing cases, interventions targeted to reduce specific reactive species can attenuate the adverse effects of radiation exposure and point to the importance of understanding the interplay between endogenous stem cell niches and the microenvironmental redox state.

9.1 Introduction

Throughout life, stem cells play critical roles in the development and maintenance of health. Their capability to continually regenerate proliferative, multipotent progeny provide the tissues of the body the means to counteract exposure to damaging agents, disease, and aging. While the mechanisms regulating the responses of tissue-specific stem cells and their immediate progeny to stress are diverse, underlying themes are emerging that suggest changes in redox state are critical. Alterations in oxidative stress prime stem cell pools for the adaptation and remodeling of the irradiated tissues in which they reside. Evidence from the CNS, hematopoietic, and musculoskeletal organ systems is highlighted that emphasizes the importance of redox state to the stress response of their representative stem cells.

In the CNS, irradiation of multipotent neural stem and precursor cells has been shown to cause a persistent oxidative stress that impacts radiosensitivity, mitochondrial function, and cell fate. The nature, magnitude, and duration of reactive species dictate whether these radiation-induced changes are harmful or beneficial to a variety of in vitro and in vivo end points of viability and function. Animals existing under elevated oxidative stress caused by superoxide dismutase (SOD) deficiency exhibit reduced levels of neurogenesis. This is also found in WT animals after irradiation. However, the inhibition of neurogenesis caused by relatively moderate doses (5 Gy) of irradiation in WT animals can be ameliorated in animals deficient for any of the three SOD isoforms, suggesting that there is a common pathway dictating how neurogenesis is affected by ionizing irradiation.

The bone marrow compartment contains the stem and progenitor cells required to reconstitute the entire hematopoietic system, in addition to the osteoblasts and osteoclasts that possess critical bone formation and resorption activities, respectively. In the hematopoietic system, the LD_{50} of irradiated mice can be significantly raised by strategies designed to protect stem cells from the direct and indirect oxidative changes caused by irradiation. Irradiation of adult mice causes a very rapid loss of bone tissue from high-turnover skeletal sites. In mesenchymal-lineage cells from

bone marrow, irradiation stimulates generation of reactive oxygen species and can inhibit differentiation into mature, bone-forming osteoblasts. By contrast, irradiation of hematopoietic-lineage cells from bone marrow can stimulate differentiation, transforming these cells into mature, bone-resorbing osteoclasts. The final outcome of these differential radiation responses of discrete cell lineages, i.e., bone loss, can be mitigated by treatment with an antioxidant, and indicates a role for oxidative stress in mediating radiation-induced acute bone loss.

While additional work has shown that the systemic stress of musculoskeletal disuse may further impair differentiation of osteoblast progenitors, it may well prime the myogenic precursor cells, known as satellite cells, which are responsible for maintaining the health of skeletal muscle. Although skeletal muscle is thought to be radioresistant, very little is known about the acute and long-term effects of irradiation on satellite cell proliferation and differentiation. Recent findings have demonstrated that irradiation also produces a persistent oxidative stress in satellite cells. Radiation impairs the capability of muscle to undergo compensatory hypertrophy, and in mouse models deficient for antioxidant enzymes, elevated muscle wasting and impaired growth are evident. Very recent data suggest that a certain fraction of radiation effects on muscle are mediated by pathways sensitive to changes in the level of nitric oxide (NO), a molecule that has a key role in promoting satellite cell proliferation.

The foregoing is the focus of this chapter: emphasizing the central importance of redox state to the stress response of stem cells. Evidence is presented that demonstrates the selective and opposing effects of irradiation on many stem cell compartments, and highlights the importance of understanding the details of the redox microenvironment and stem cell niches.

9.2 How Does the Interplay Between Ionizing Radiation and Oxidative Stress Impact the Functionality of Stem/Precursor Cells in the Brain?

Stem/precursor cells in the brain contribute to the generation of new brain cells throughout the life of mammalian organisms. These multipotent cells are located in discrete cellular layers in the subventricular zone lining the lateral ventricles, and in the subgranular zone of the dentate gyrus within the hippocampal formation [1, 2]. Neural stem cells and their progeny precursor cells retain the capability to generate new neurons, astrocytes, and oligodendrocytes, which represent the three principal cell lineages of the brain. The capability of multipotent neural cells to develop into mature neurons (defined here as neurogenesis) has been hypothesized to contribute to new memory formation, and changes in the extent of neurogenesis have been linked the alterations in specific learning behaviors involving the hippocampus [1, 2]. Given the intimate link between stem cell function, neurogenesis, and cognition, understanding what factors impact these processes becomes critical to those individuals subjected to conditions that might adversely impact the cellular processes responsible for maintaining cognitive health.

In this light, one of the stresses known to adversely impact stem cell function, neurogenesis, and cognition is exposure to ionizing radiation. The principal scenario in which individuals incur radiation exposure is during radiotherapy, used routinely for the clinical management of cancer. For patients afflicted with primary or metastatic tumors in the brain, incremental doses delivered over the course of 6–8 weeks to control tumor growth also elicits normal tissue damage that often manifests as various degrees of cognitive impairment [3–7]. Risks for impaired cognition also exist for those exposed to lower doses occupationally and for astronauts exposed to the unique radiation fields in space composed of highly energetic protons and fully ionized atomic nuclei [8, 9].

9.3 Radiation-Induced Oxidative Stress

Significant work from our laboratories has now found that one of the major consequences of exposure to ionizing radiation is the development of an acute and persistent oxidative stress [10–14]. This is particularly pronounced for neural stem and precursor cells, although certainly not limited to these cell lineages (as discussed further below), and is an effect that can be demonstrated in vitro and in vivo. Exposure of rodent neural stem cells leads to an increase in reactive oxygen (ROS) and nitrogen (RNS) species that can elicit damage throughout any subcellular compartment, in a manner dictated by their intrinsic reactivity and corresponding half-lives. Increased oxidative stress can be found within hours after irradiation in neural stem and precursor cells, and can persist from days to weeks to months (Fig. 9.1). Typical assays for ROS and RNS involve incubating cells at some specified time postirradiation with fluorogenic dye precursors that become fluorescent upon entry and oxidation within the cell [1, 2]. Depending on the specificity of the selected dye for a particular reactive species, and on the level of intracellular peroxidases that catalyze the reactions [15], a reliable assessment of the state of global oxidative stress can be made when cells are quantified for fluorescent yield and compared against the appropriate controls. For the measurements described here cells were treated with the ROS/RNS sensitive dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), and assayed 1 and 7 days postirradiation for changes in oxidative stress. Radiation-induced oxidative stress has been demonstrated for both sparsely and densely ionizing radiations (Fig. 9.2) and generally increases with dose and the LET of the radiation when compared to sham irradiated controls [10, 12, 13]. Pluripotent human embryonic stem cells and multipotent human neural stem cells also exhibit qualitatively similar increases in oxidative stress postirradiation (Fig. 9.1), suggesting that this may constitute a general and/or global response of stem cells to irradiation. To the extent that the foregoing is true, then it follows that changes in oxidative stress following various insults should also be important in mediating physiologic changes that impact stem cells and their specialized microenvironmental niches.

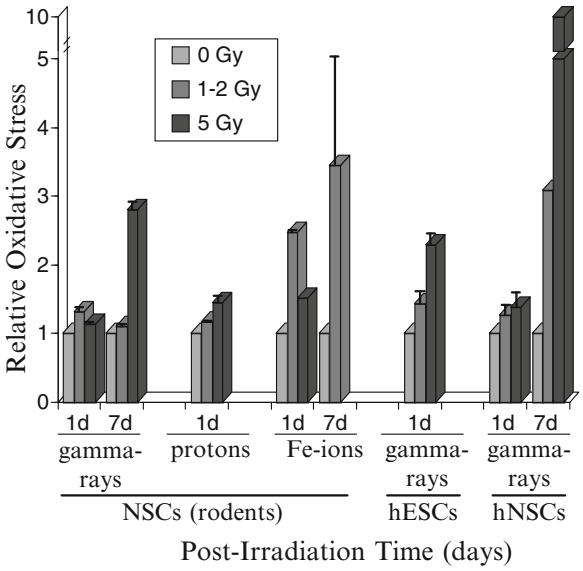

Fig. 9.1 Radiation-induced oxidative stress in stem cells. Multipotent neural stem cells (NSCs) from rodents and humans (hNSCs) and pluripotent human embryonic stem cells (hESCs) were exposed to various doses of gamma-rays, protons, and heavy ions (^{56}Fe) and analyzed for increased ROS/RNS by fluorescence-activated cell sorting (FACS) using the fluorogenic dye CM-H_2DCFDA (5 μM, 60 min). Evident from the data is that all cells exhibited an increase in oxidative stress following irradiation that was evident at day 1 and 7. Values reflect triplicate measurements (±SEM) and were normalized against sham-irradiated controls arbitrarily set to unity

9.4 Functional Consequences of Oxidative Stress: The Good, the Bad, and the Not so Ugly

Significant effort has now been undertaken in our laboratories to analyze the functional consequences of oxidative injury to stem/precursor cells. The persisting nature of oxidative stress found in vitro has been demonstrated in vivo and leads to increased oxidative damage to DNA, proteins and lipids [13, 14, 16, 17]. Radiation-induced oxidative stress in neural stem cells and the microenvironment of the CNS is likely to play a significant role in the inhibition of neurogenesis found postirradiation (discussed further below) [17–20]. Oxidative stress is a complicated biochemical imbalance between the antioxidant and prooxidant forces within and outside the cell. Many regulatory pathways have evolved to generate and regulate various types of reactive species, and the precise nature of the reactive species and the persistence of those species are now being recognized as increasingly critical to the systemic response to irradiation as well as many other injury paradigms [1, 2, 21–25].

The onset of oxidative stress triggered by irradiation is, in part, mitochondrially derived [26], but other redox-sensitive regulatory hubs such as the NADPH oxidases are likely to contribute to the rise in ROS observed after irradiation [27]. The relatively

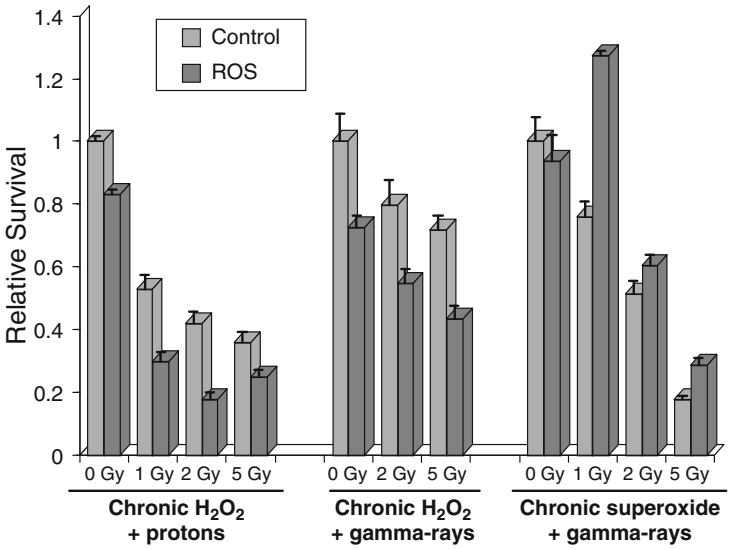

Fig. 9.2 Opposing effects of oxidative stress on the radioresponse of neural stem cells. Cells were subjected to chronic oxidative stress through the daily treatment (3–5 days) of cultures with either hydrogen peroxide or superoxide. One day following the final oxidative treatment, cells were irradiated and plated for the assessment of survival by XTT assays and cell counts 5 days later. Data show that cells subjected to hydrogen peroxide were sensitized to irradiation, while those exposed to superoxide were protected. Data reflect triplicate measurements (±SEM) and were normalized to untreated and unirradiated controls set to unity

benign superoxide anion derived from leaky electron transport chain complexes in the mitochondria can play significant roles in altering stem cell survival and fate (differentiation) in the CNS, as well as other stem cell compartments (a topic that is addressed in detail below). Superoxide can react with nitric oxide to produce the damaging peroxynitrite species ($ONOO^-$) [28] or be converted to hydrogen peroxide by any of the three isoforms of superoxide dismutase [29]. Hydrogen peroxide escaping the surveillance (detoxification) of/by glutathione peroxidase or catalase is more freely diffusible than superoxide and can traverse cellular compartments where it can react with transition metals such as iron [30]. Participation of hydrogen peroxide in such redox-mediated Fenton chemistry can generate the highly reactive hydroxyl radical [31], capable of producing toxic genetic lesions in the form of DNA double-strand breaks (DSBs). Cells comprising the CNS are generally sensitive to various forms of reactive species [32–34], and chronic exposure to low level hydrogen peroxide sensitizes neural stem/precursor cells to the action of ionizing radiation (Fig. 9.2). Chronic exposures (3–5 days) to relatively nontoxic levels (~20% induced cell kill) of this prooxidant were found to enhance the sensitivity of cells by ~20–40%, when subjected to various doses of sparsely ionizing radiation (gamma rays and protons) (Fig. 9.2). In contrast to hydrogen peroxide, chronic exposure to superoxide was found to have an opposing effect. In this instance, cells subjected to nontoxic levels of superoxide over 6 days prior to irradiation

were found to be protected by ~20–60% (Fig. 9.2). These data highlight the complexity of the redox response of neural stem and precursor cells, where depending on the particular species involved, cells may exhibit opposite responses. Thus, oxidative stress may not always be harmful, but may actually be beneficial to cells under certain circumstances.

9.5 Oxidative Stress as a Biochemical Mechanism Underlying Radioadaptive Changes

While detrimental under many circumstances, the idea that oxidative stress may be beneficial might appear contradictory. A number of reports have documented the mitogenic role for oxidative stress in various cells systems [35, 36], and it's importance for cell cycle progression [37, 38]. Cells have evolved an integrated system of redox-sensitive signaling cascades designed to tailor cellular responses to the dynamic changes occurring within the tissue microenvironment. These changes are pronounced following normal tissue damage, and in the case of the irradiated tissue bed, remodeling proceeds for weeks to months to years, as a complex series of secondary reactive processes is superimposed on tissue repair and regenerative mechanisms [39]. Tissues can also adapt to prior stress, where subsequent cellular challenges are met with markedly different responses than those observed after acute insults. Adaptive responses have been described for a wide-range of stress-responsive end points [40–43], and are generally found to ameliorate the impact of secondary or subsequent exposures to irradiation [44]. Increased activation and/or efficiency of DNA damage responsive pathways are believed to underlie the mechanisms that promote proliferation and survival and reduce genomic instability (chromosome aberrations, mutations) and cell transformation [45–48]. Radioadaptation has been demonstrated under a variety of dosing paradigms that include multiple exposure scenarios involving dose fractionation, temporal separation of priming and challenge doses, and chronic exposures using low dose rates [47, 48].

Data from us and others suggest that one of the underlying mechanisms driving radioadaptive effects may be oxidative stress [49–54]. Radiation-induced oxidative stress has been shown to activate signaling cascades that are dose modifying. Subsequent exposures are therefore not as deleterious. In support of these ideas, a pilot study was undertaken to analyze the effects dose fractionation on the induction of oxidative stress in neural stem and precursor cells. Acute exposures were selected to match the individual and total doses used for subsequent dose fractionation. Cells subjected to acute, low dose irradiation (10–60 cGy) with heavy ions showed increased (≥2-fold higher) oxidative stress compared to sham irradiated controls (Fig. 9.3). These observed increases were found 1 day after acute exposure, and were not dissimilar from many of our prior findings [10, 12, 13]. Cells were then subjected to a fractionation regime involving three fractions of 10 cGy (3 × 10) or 20 cGy (3 × 20) given every other day to yield total final doses of 30 or 60 cGy. Following the final dose, cells were then assessed 1 day later for relative changes in

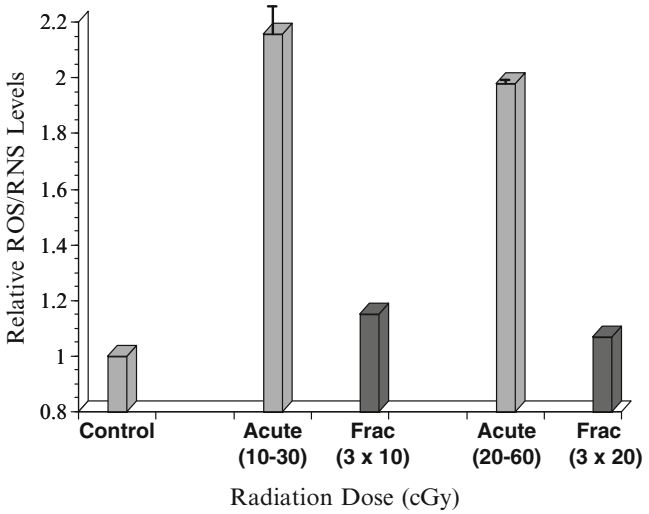

Fig. 9.3 Radioadaptation in neural stem cells. Neural stem and precursor cells were exposed to low dose heavy ion exposure (600 MeV ^{56}Fe ions) either acutely or following a fractionation regime using three separate doses interspersed by a day. One day after the final dose cells were assessed for oxidative stress by FACS analysis using the fluorogenic dye CM-H_2DCFDA (5 μM, 60 min). While cells exposed acutely demonstrated increased oxidative stress (twofold), those that were subjected to dose fractionation did not, showing ROS/RNS levels similar to controls. Data reflect triplicate measurements (acute, ±SEM) or duplicate measurements (fractionation) and were normalized to sham-irradiated controls set to unity

oxidative stress. Cells treated under dose fractionation showed a muted increase in oxidative stress that was not significantly different from unirradiated controls (Fig. 9.3). These data demonstrate that prior exposure attenuated the increase in oxidative stress normally evident in cells exposed acutely and suggest that radiation-induced oxidative stress may trigger a protective radioadaptive response in these cells.

9.6 Radioadaptation and Cellular Antioxidant Capacity

The idea that oxidative stress serves as a biochemical mechanism regulating adaptive changes in cells implies that insults capable of causing oxidative stress trigger compensatory changes in cells that render them more "fit" to deal with subsequent insults, such as irradiation. Cells can alter their gene expression profiles to adapt to more "hostile" environments [21, 51], and one strategy employed to provide protection against radiation-induced sequelae is to boost the antioxidant capacity [55–58]. Evidence for this can be demonstrated in neural stem and precursor cells exposed to dose fractionation or protracted exposures at very low dose rates (Fig. 9.4). Cells subjected to single daily exposures of gamma rays over the course of 10 days showed increased levels of the mitochondrial isoform of superoxide dismutase

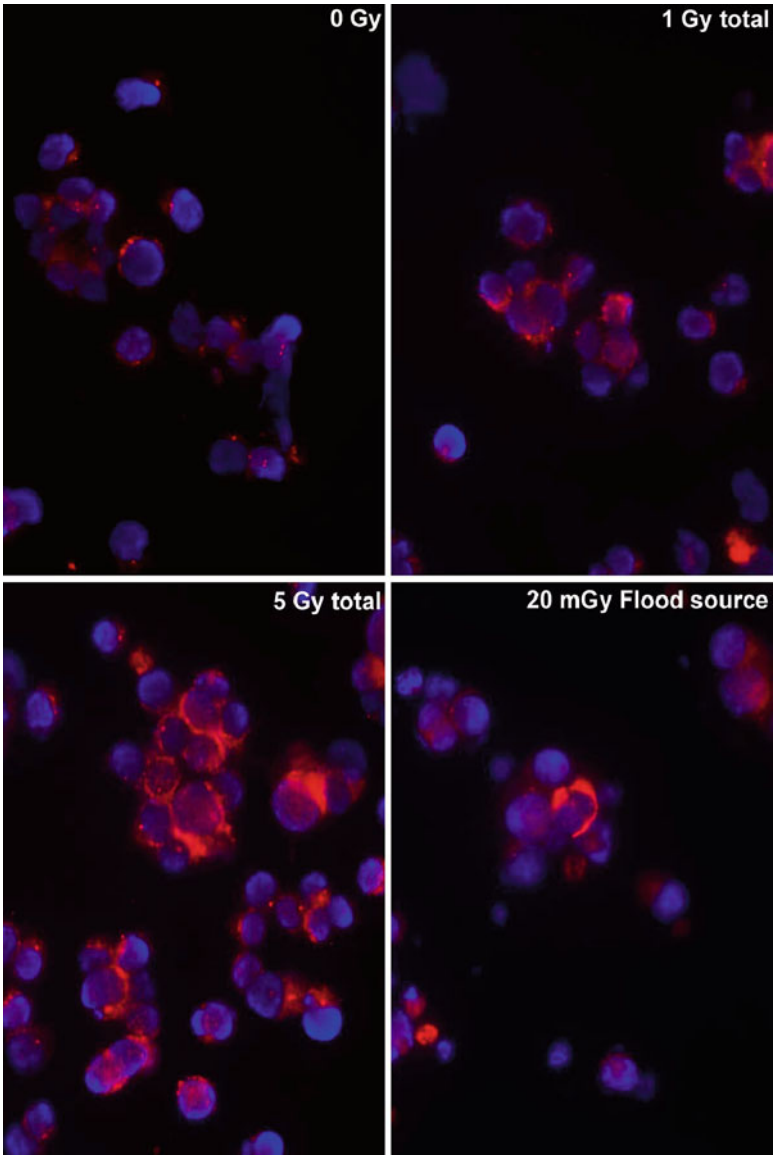

Fig. 9.4 Upregulation of antioxidant capacity in neural stem cells subjected to chronic irradiation. Neural stem/precursor cells were subjected to dose fractionation at high dose rates (2 Gy/min) involving ten total doses (0.1 or 0.5 Gy/day) delivered once daily, yielding total doses of 1 or 5 Gy. Alternatively, cells were subjected to continuous low dose rate (1 cGy/day) exposure using a ^{57}Co-flood source to a total dose of 20 cGy. Compared to unirradiated controls, cells exposed to these dosing paradigms showed an upregulation of the mitochondrial isoform of superoxide dismutase (MnSOD). *Representative images* show cells immunostained for MnSOD (*red*) against a nuclear DAPI counterstain (*blue*)

(MnSOD) (Fig. 9.4, upper right and lower left). When neural stem/precursor cells were exposed to continuous low dose rate (1 cGy/day) irradiation using a flood source containing the gamma-emitting ^{57}Co, similar increases in MnSOD were found (Fig. 9.4, lower right). Given the elevated immunostaining for MnSOD in neural stem and precursor cells subjected to repeated or protracted exposure scenarios, it seems probable that one mechanism of protective radioadaptation involves an upregulation of cellular antioxidant capacity.

The foregoing section has highlighted some of the interesting aspects one needs to consider when assessing if and how oxidative stress might impact stem cell function in the CNS before and after irradiation. The capacity of irradiation to elicit acute and chronic increases in oxidative stress in neural stem and precursor cells is now well established [10, 12, 13]. The persistence of this effect suggests a possible mechanism for the prolonged inhibition of neurogenesis following irradiation, and suggests that strategies targeted to reduce the persistent oxidative stress in the irradiated brain might improve the formation of new neurons. Unfortunately, this interpretation is likely to be over simplistic, as the response of multipotent cells in the CNS can vary depending on the nature of the oxidative species and the frequency and nature of pre-existing stressors. In vitro data suggest that while elevated hydrogen peroxide is sensitizing, elevated superoxide may have the opposite effect. In vivo this scenario may also be true (see below), and may be related to the capability of cells within the CNS to undergo adaptive changes that are neuroprotective. These ideas are explored in more detail in the following sections and provide both compelling and fascinating examples of how various tissue compartments respond to changes in redox state to regulate the function of their respective stem cell pools in efforts to manage the repair and regeneration of radiation-induced tissue injury.

9.7 Oxidative Stress and Preconditioning in the Irradiated Brain: The Role of Superoxide Dismutase

As outlined above, one of the pathways involved in oxidative stress involves the antioxidant enzyme, superoxide dismutase (SOD), which exists as three genetically and geographically distinct isoenzymes [59]. The three different SOD isoforms catalyze the same chemical reaction, but have different enzymatic properties and distinct subcellular localizations. Therefore, deficiency in SOD, regardless of location, should result in relatively higher levels of ROS and altered redox state, which will induce a state of persistent oxidative stress. As documented previously, ROS have often been considered to be hostile or destructive entities, but data also exist showing that ROS can have beneficial effects [60, 61], and in the brain, they are critically involved in a number of important processes, particularly those involved in learning and memory formation [62–64]. One way to characterize the extent to which ROS affects processes relevant to cognition, and if they are affected by ionizing irradiation, is to use mutant mice that lack specific antioxidant molecules such as SOD.

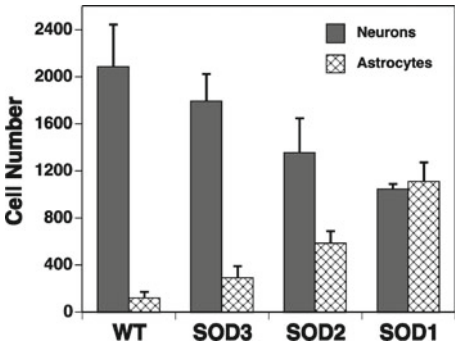

Fig. 9.5 Fate of neural precursor cells in vivo depends on superoxide dismutase (SOD). Before irradiation, SOD isoform-dependent reductions in the numbers of newly born neurons in the dentate subgranular zone contrasted with isoform-dependent increases in the numbers of newly born astrocytes. These data suggest that an SOD-deficient environment favors differentiation toward an astrocytic lineage. Newly generated neurons or astrocytes were detected using bromodeoxuridine to label newly born cells and an antibody against NeuN or GFAP to label mature neurons or astrocytes respectively. Each *bar* represents a mean of 4–5 mice; *error bars* are SEM

We have recently assessed how irradiation affected dentate neurogenesis in mice deficient in extracellular isoform of SOD (EC-SOD, SOD3) [17]. This study showed that a persistent level of oxidative stress in EC-SOD knockout (KO) mice was associated with a lower baseline level of neurogenesis relative to wild-type (WT) mice (Figs. 9.5 and 9.6). However, when those same mice were subjected to a modest dose of X-rays (5 Gy), there was no effect on neurogenesis in KO mice but a highly significant reduction in WT animals [17] (Fig. 9.6). Furthermore, those effects were not coupled with any obvious compensatory changes in other antioxidant enzymes. Another recent study showed that in contrast to what happened in wild-type mice, irradiation enhanced hippocampus dependent cognitive measures in mice that lacked EC-SOD [65], and that outcome was associated with genotype-dependent effects on measures of oxidative stress. Thus, in terms of neurogenesis and cognitive function, both negative and positive (no apparent effect of irradiation) effects were observed in the EC-SOD-deficient mice, presumably as a result of yet to be identified mechanisms associated with redox balance. These paradoxical effects highlight the importance of better understanding the delicate balance in redox homeostasis [61], and how that may ultimately affect cell/tissue function.

Given the EC-SOD data described above, another study was initiated to determine if the site of SOD deficiency influenced how irradiation impacted neurogenesis [18]. In that study, neurogenesis was assessed after irradiation in mice deficient in the other SOD isoforms [59]: CuZn SOD (SOD1), which is generally localized in the cytoplasm, and MnSOD (SOD2), which is localized in the mitochondria. The results from that study showed that a partial depletion (~50%) of the SOD1 and SOD2 isoforms was associated with reduced baseline levels of neurogenesis, but was also associated with a "protective" or adaptive response to irradiation (Fig. 9.6). Those effects were not coupled with any obvious compensatory changes in

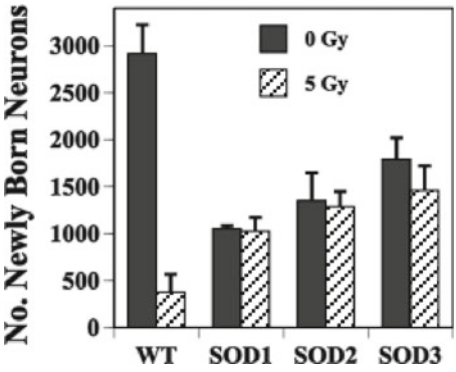

Fig. 9.6 Neurogenesis before (*solid bars*) and after (*hatched bars*) irradiation is affected by SOD deficiency. Regardless of SOD isoform, under baseline conditions the number of newly generated neurons in the dentate subgranular zone is significantly reduced relative to wild-type (WT) controls. After a single dose of 5 Gy, the numbers of newly born neurons produced in WT mice was reduced by about 85%, but irradiation had no effect in animals deficient in any of the SOD isoforms. These data show paradoxical effects of SOD deficiency, because both negative (baseline neurogenesis) and positive (no apparent effect of irradiation) effects were observed. Newly generated neurons were detected using bromodeoxuridine to label newly born cells and an antibody against NeuN to label mature neurons. Each *bar* represents a mean of 4–5 mice; *error bars* are SEM

other antioxidant enzymes although it is possible that circulating antioxidants, including ascorbic acid, tocopherol, uric acid, bilirubin, proteins, and other compounds, could be increased as a compensatory mechanism and could conceivably be involved. The fact that a partial deficiency in the SOD1 and SOD2 isoforms [18], as well as a full deficiency in SOD3 [17], all imparted a common protective effect in the hippocampus, suggests that there are common effectors throughout the cell capable of engaging prosurvival or differentiation pathways. The precise mechanism(s) responsible for this type of response is not yet known, but in a general sense this effect resembles a preconditioning [66], adaptive (reviewed in Yu et al. [67]), or inducible-like radioprotective response [68], where a sublethal or potentially injurious stimulus (i.e., oxidative stress) induces tolerance to a subsequent and potentially more damaging insult (irradiation). In the CNS, this could involve specific trophic factors and signaling molecules that favor differentiation and long-term survival of newly generated neurons which are upregulated or activated in the brains of irradiated SOD KO mice. Such factors could include brain derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and nitric oxide (NO), all of which have been shown to favor differentiation and survival of neurons [69–72]. Regardless of the mechanism involved, these findings clearly suggest that SOD-deficient mice develop a resistance to radiation-induced inhibition of neurogenesis that may involve some type of adaptation within the microenvironment, without any obvious compensatory changes in other major intracellular antioxidants.

Astrocytes are now recognized as dynamic regulators of a variety of neuron-related functions, including neurogenesis [73–75]. In fact, it has been suggested that the astrocytes within the neurogenic niche are highly specialized and contribute to the regulation of proliferation and fate specification of neural precursor cells.

It is particularly interesting to note that prior to irradiation, the average number of newly born cells that differentiated into astrocytes in SOD1 mice (1,112 ± 157) was higher than that seen in SOD2 (586 ± 102), which in turn was higher than the value for SOD3 (291 ± 98) [17, 18] (Fig. 9.5). Additionally, when the number of newly born cells that differentiate into neurons prior to irradiation was analyzed between the different SOD isoforms, the opposite trend was found, where SOD1, SOD2, and SOD3-deficient mice have an average of 1,048 ± 40, 1,355 ± 290, and 1,793 ± 226 new neurons, respectively [17, 18] (Fig. 9.5). These trends are provocative and it may be that different cellular compartments from which the SOD isoforms evolved may contain redox-sensitive factors that play a deciding role in the lineage commitment during precursor cell differentiation [76]. Given the supportive role of astrocytes in neurogenesis [73–75], the relatively higher numbers of the newly generated astrocytes in the KO mice may have promoted the survival of newly born neurons after irradiation, although at this time, such an idea is only speculative.

9.7.1 What Is the Radiosensitivity of Myogenic Precursor Cells?

Skeletal muscles possess a remarkable regenerative capacity that is due largely, if not exclusively, to the presence of myogenic stem cells known more commonly as satellite cells. Satellite cells were first described by Mauro [77] and their role in muscle growth and regeneration has subsequently been demonstrated by a large number of studies [78–83]. During development, satellite cells play a critical role in regulating the growth of skeletal muscle. In the adult state, satellite cells are relatively quiescent with a very low level of proliferation. However, their proliferation can be dramatically increased by a number of altered physiological conditions some of which includes the following: (1) increased loading of the muscle as might occur during resistance training, (2) trauma, and (3) degeneration induced by neuromuscular diseases such as Duchene's muscular dystrophy. In essence, satellite cells are responsible for maintaining the health of skeletal muscles, and from this, it follows that any factor that negatively influences their proliferation and differentiation will negatively impact the functional capacity of skeletal muscle.

While skeletal muscle is thought to be radioresistant, it is well known that radiation can negatively impact the proliferation of stem cells via direct damage of DNA and other mechanisms such as oxidative stress. In this context, skeletal muscle and, as a consequence, satellite cells are commonly exposed to radiation during diagnostic screening and radiotherapy of tumors [84–91]. Additionally, skeletal muscles are irradiated (7 Gy is a typical dose) for conditions such as ectopic bone formation that often occurs following total joint replacements [84–86, 91]. Given this background and the importance of satellite cells in promoting both the growth and health of skeletal muscle, it is somewhat surprising that the radiosensitivity of satellite cells has been so poorly studied. Indeed, this important observation has stimulated our interest in exploring the effects of γ-irradiation on satellite cell proliferation and differentiation, with a special focus on the role of oxidative stress in mediating such radiosensitivity.

9.7.2 *Nitric Oxide Plays a Central Role in Promoting Satellite Cell Proliferation: What Is the Effect of γ-Irradiation on NO Levels?*

It is well known that one of the consequences of oxidative stress is the production of reactive nitrogen species, and, in particular, the production of peroxynitrite via a reaction between nitric oxide (NO) and the superoxide anion ($O_2^{\bullet -}$). Importantly, the production of $O_2^{\bullet -}$ via oxidative stress may represent an important mechanism by which NO levels are quenched, and, in satellite cells this would be highly significant given that NO seems to play a central role in satellite cell proliferation. As shown in Fig. 9.7, NO has been shown to control the proliferation of satellite cells by acting through the NO/MMP2/HGF/c-met pathway [82, 92–95]. Stretch of satellite cells as might occur in vivo is thought to lead to an elevation of NO levels, which subsequently acts on proMMP2 converting it into MMP2. This produces a conformational shift in MMP2, which leads to the release of hepatocyte growth factor (HGF). The release of HGF from MMP2 allows HGF to bind to c-met and promote the proliferation of satellite cells.

With respect to oxidative stress and the central role of NO in promoting satellite cell proliferation, we have found that clinically relevant doses of γ-irradiation (e.g., 2 and 5 Gy) not only reduce satellite cell proliferation but also reduce NO levels. Hence, exposure to γ-irradiation may depress satellite cell proliferation by negatively impacting one of the initial steps in the NO/MMP2/HGF/c-met pathway. Given the scheme outlined in Fig. 9.7, it seems logical to propose that the effects of γ-irradiation on satellite cell proliferation might be mitigated using pharmacological interventions that (1) elevate NO levels (i.e., NO donors), (2) modulate the binding of HGF to pro-MMP2 or elevate HGF levels, and (3) modulate the phosphorylation of c-met. In this context, we have recently observed that the use of NO donors such a sodium nitroprusside (SNP) is effective in rescuing satellite cell proliferation following exposure to γ-irradiation (doses of ≤5 Gy).

9.8 What Is Radiosensitivity of Osteoblast and Osteoclast Precursors?

Irradiation with moderate or high doses (>2 Gy) is applied therapeutically for malignancies or to sites of ectopic mineralization that can arise after complex orthopedic surgery or traumatic wounds. Therapeutic irradiation sometimes leads to a later increase in incidence of fractures [96], and therefore, it is a late effect that is clinically relevant. Although low doses of radiation (<2 Gy) have possible occupational relevance (e.g., astronauts and radiation workers), the impact of such low doses on skeletal health is unknown. A principal concern of irradiation in the context of mineralized tissue is that it may accelerate or worsen skeletal diseases such as osteoporosis.

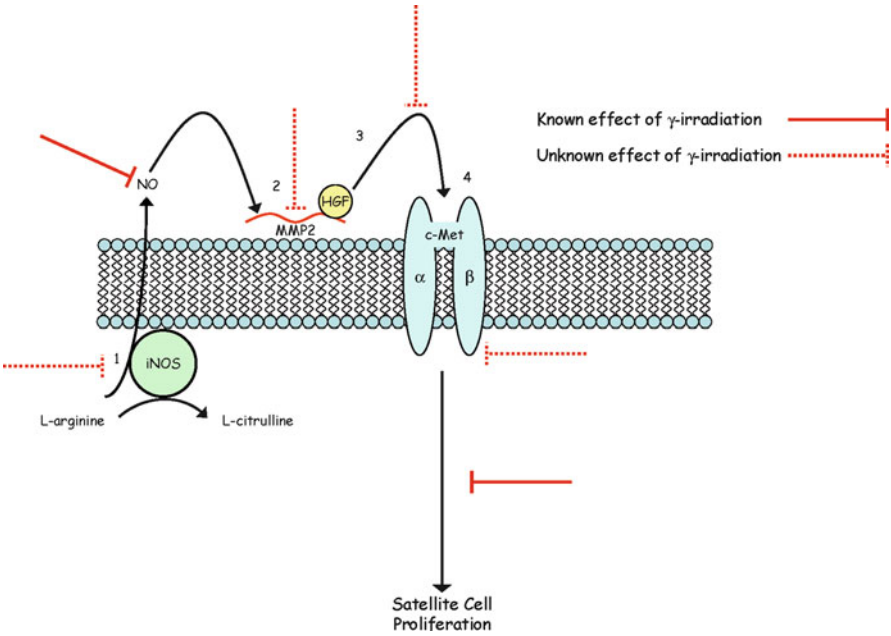

Fig. 9.7 NO/MMP2/HGF/c-met pathway. Based on our initial work, we currently know that exposure of satellite cells to clinically relevant doses (≤5 Gy) of γ-irradiation produce a profound decrease in satellite cell proliferation. Additionally, initial studies also demonstrate that such doses of γ-irradiation also produce significant reductions in NO levels. This gives rise to the intriguing possibility that γ-irradiation reduces satellite cell proliferation, at least in part, by negatively impacting a key pathway thought to play an essential role in satellite cell proliferation

Skeletal remodeling occurs throughout life and relies on a continuous supply of stem, progenitor and precursor cells to replenish the differentiated cells that are responsible for maintaining bone structure and function. The tightly regulated activities of fully differentiated, bone-forming osteoblasts and bone-resorbing osteoclasts define bone structure (summarized in Fig. 9.8). Osteoblasts arise from the proliferation and progressive differentiation of pluripotent stem cells and progenitors in the mesenchymal lineage, whereas osteoclasts arise via monocytes/macrophages in the hematopoietic lineage [97, 98]. Extensive communication between cells in the two lineages regulates bone forming and resorbing activities. Osteoblasts can produce various factors, including cytokines and reactive oxygen species (ROS), which stimulate bone resorption by increasing the numbers and activity of terminally differentiated osteoclasts [99]. Conversely, bone formation is normally coupled to bone resorption, thus preserving a net balance in healthy tissue. A key function of osteoblasts is to contribute to niches that form at the endosteal interface between marrow and bone. Niches created by osteoblasts and other cells are responsible for supporting both hematopoietic stem cells and quiescent osteoclast precursors [100, 101]. Changes in stem, progenitor, and precursor skeletal cell

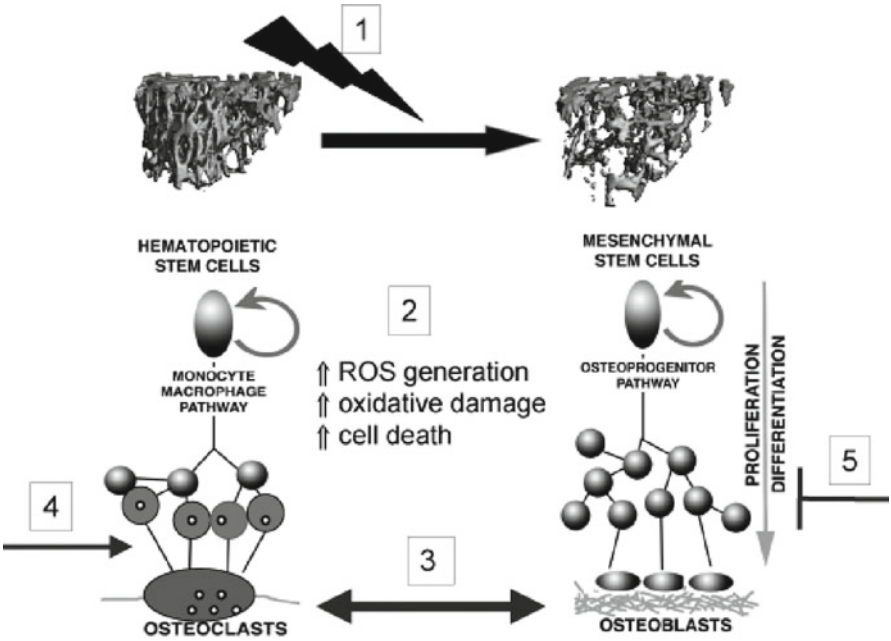

Fig. 9.8 Influence of irradiation and oxidative stress on bone cell lineages and skeletal tissue. Therapeutic radiation can cause bone loss and increase fracture incidence. (*1*) Similarly, total body irradiation of adult mice causes rapid loss of high turnover, cancellous tissue adjacent to the marrow. (*2*) Irradiation causes bone marrow cells to increase generation of ROS, resulting in oxidative damage to both marrow and mineralized tissue. Oxidative stress appears to mediate the rapid adverse radiation effects because treatment with an antioxidant blocks the bone loss. (*3*) Differentiated progeny from two discrete cell lineages, osteoclasts (hematopoietic) and osteoblasts (mesenchymal), resorb or form bone, respectively. Extensive communication between osteoblasts and osteoclasts regulates skeletal remodeling, and osteoblasts residing at the marrow–bone interface contribute to niches that support both hematopoietic stem cells and postmitotic osteoclast precursors. (*4*) Irradiation increases the generation of mature osteoclasts both in vivo and in vitro, resulting in acute bone loss. (*5*) Irradiation also can inhibit the progressive proliferation and differentiation of osteoblast lineage cells, including mesenchymal stem cells; this is likely to be important in late radiation effects with subsequent skeletal aging

populations appear to contribute to the pathogenesis of bone disease caused by aging and sex hormone withdrawal [102, 103], and also are relevant to the skeletal damage induced by radiation [104].

Stem, progenitor and precursor cells for both skeletal cell lineages reside within the marrow, which is exquisitely radiosensitive [105, 106]. Irradiation of human bone marrow-derived mesenchymal stem cells (MSC) in vitro leads to altered gene expression, impaired proliferation and osteoblast differentiation, and increased senescence [107, 108]. In osteoblast progenitors and precursors, irradiation can inhibit proliferation and differentiation [109, 110], but in more mature osteoblasts, irradiation can stimulate the expression of osteoblast markers [111]. Consistent with

in vitro results showing differentiation-dependent radiosensitivity of the osteoblast lineage, we find that irradiation (2 Gy) of skeletally mature mice in vivo suppresses indices of osteoblast number but stimulates indices of osteoblast activity, yielding no net effect on bone formation rate within cancellous tissue [112]. Irradiation also rapidly increases the number of cancellous osteoclasts, accounting for the rapid net loss of bone tissue observed in adult mice [112, 113]. The rise in numbers of osteoclasts following irradiation may be mediated by increased production of osteoclastogenic or survival factors by osteoblast lineage cells, or by radiation-induced fusion and terminal differentiation of osteoclast precursors. Consistent with both of these possibilities, we find that irradiation in vitro of primary bone marrow cells or the monocyte/macrophage cell line, RAW264.7, stimulates osteoclast formation, but only in the presence of submaximal concentrations of the obligate osteoclastogenic factor, Receptor Activator of NFκB Ligand (RANKL) that is produced by osteoblasts [112], unpublished). Long-term effects of radiation on the mesenchymal stem cell lineage also may account for the persistent decrement in bone density observed in adults. Thus, both precursors and progenitors of osteoblasts and osteoclasts contribute to the radiosensitivity of mineralized tissue.

9.9 What Is the Role of Oxidative Stress in Skeletal Radioresponses?

Oxidative pathways are important both for mediating skeletal remodeling [114] and for radiation responses. During progressive osteoblast differentiation in the mesenchymal lineage, cells switch from relying mainly on glycolysis in the undifferentiated state, to relying mainly on aerobic metabolism [87, 115]. Mitochondria also undergo morphological changes, mitochondrial transmembrane potential rises [115], and expression of the antioxidant enzymes MnSOD and catalase are upregulated during osteogenic differentiation [87], presumably serving to protect cells from oxidative damage given their greater metabolic activity.

Cellular redox also plays a key role in the hematopoietic lineage during osteoclast differentiation. Hematopoietic stem cells are maintained within hypoxic niches at the earliest stage [116]. At later stages of differentiation, ROS or Reactive Nitrogen Species (RNS) stimulate osteoclast formation from monocyte/macrophage precursors [117] and can display biphasic effects [118]. Furthermore, ROS are produced intracellularly by the mitochondria-rich osteoclasts during bone degradation of the bone matrix [119]. Elevated ROS in cultures of osteoblasts or mixed bone marrow stromal cells stimulates the production of the key osteoclastogenic factor, RANKL [120], providing yet another mechanism whereby oxidative stress can lead to enhanced bone resorption.

Based on these findings, we hypothesize that irradiation may increase ROS generation by cells within bone marrow, leading to an increase in terminal osteoclast differentiation and activity and subsequent loss of cancellous bone tissue. A single

dose of low LET radiation (1 or 2 Gy) to adult mice increases ROS generation by bone marrow cells, induces extensive marrow cell death, and causes oxidative damage to lipids distributed throughout the marrow and mineralized tissue [106]. Treatment of irradiated mice with a potent antioxidant, α-lipoic acid, mitigates the acute loss of cell viability observed in the marrow and inhibits the net loss of cancellous bone tissue, showing that oxidative stress mediates acute cancellous bone loss [106]. We propose radiation-induced release of ROS by osteoblasts within niches formed at the endosteal interface between marrow and bone is responsible for both damaging hematopoietic stem cells and activating the quiescent, osteoclast precursors to fuse and terminally differentiate into active, bone resorbing osteoclasts (Fig. 9.1).

9.10 Concluding Remarks

Historically, the adverse effects of ionizing irradiation have generally been considered in terms of cell survival. While an increased understanding of cell and molecular biology, along with new and sophisticated technologies, has facilitated a much more mechanistic appraisal of biochemical pathways and cell signaling elements, this new understanding is still often focused on cell death or recovery processes. More recently, and particularly in the context of normal cells and tissues, investigators are beginning to focus not so much of cell death per se, but on the ability of surviving cells to function, and what factors may be involved. The microenvironment is now recognized as having a profound effect on the phenotypic fate and the ability of an irradiated cell to function normally. In the case of stem/precursor cells, which have been identified in a number of critical normal tissues, the composition and biochemical nature of the microenvironmental niche is being shown to be critical in this context. One important microenvironmental influence after irradiation is oxidative metabolism, and it has been suggested that ROS may constitute environmental cues that are critical in the control of stem/precursor cell survival and differentiation. While the mechanisms responsible for such control are not well defined at this point, the data discussed above for the CNS, bone and muscle suggest that oxidative stress and the maintenance of redox homeostasis may play an important role. Understanding such processes may ultimately contribute to the development of strategies or approaches to manage potentially serious complications associated with ionizing irradiation, and the survival and function of stem/precursor cell populations in normal tissues.

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Part III
Imaging Redox Changes and Therapeutic Response (Preclinical and Clinical)

Chapter 10
Functional Imaging in the Assessment of Normal Tissue Injury Following Radiotherapy

Kenneth J. Dornfeld and Yusuf Menda

Abstract Functional imaging after radiotherapy is an increasingly powerful tool to assess tumor response to treatment. Functional imaging may also provide an opportunity for surveillance not only for tumor recurrence but also for treatment-related toxicity. Persistently elevated glucose avidity in normal tissues seen on 18F-fluorodeoxyglucose (FDG) PET scans after radiotherapy for head and neck cancer is associated with increased toxicity and decreased quality of life. Increased FDG avidity in normal lung after radiotherapy can also help predict which patients will subsequently develop radiation pneumonitis. These findings may also provide insight into the metabolic alterations that are associated with radiotherapy-induced toxicity. More options for functional imaging are being developed, for example, 18F-fluorothymidine and magnetic resonance spectroscopy. As these agents are used for tumor surveillance, exciting possibilities will also be created for monitoring, predicting, and understanding normal tissue toxicity.

10.1 Introduction

Radiotherapy imparts a strong, brief oxidative stress to treated tissues. The consequences of this stress are multiple and incompletely understood. Detailed molecular studies of irradiated cells in culture provide the opportunity to define relevant biochemical changes and relevant pathways. Functional imaging provides a different

K.J. Dornfeld, MD, PhD (✉)
Department of Biochemistry and Molecular Biology, University of Minnesota,
400 East Third Street, Duluth, MN 55805, USA

Duluth Clinic Radiation Oncology, Duluth, MN, USA
e-mail: kdornfeld@smdc.org

Y. Menda, MD
Department of Radiology, University of Iowa, Iowa, IA, USA

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perspective, allowing analysis of irradiated tissues in situ. Functional imaging has also the advantage of assessing the changes imparted by radiotherapy with minimal disruption and impact on the tissues studied. Insight gained from in vitro studies may serve as a rational basis to guide interpretation and development of functional imaging.

The most widely used functional imaging techniques currently include 18F-fluorodeoxyglucose positron emission tomography (FDG-PET), magnetic resonance spectroscopy (MRS) and Tc-99m Sestamibi scans. The primary use of these studies to date has been to characterize cancer in terms of stage and prognosis prior to treatment and response after therapy. However, the goal of cancer therapy is not just annihilation of cancer tissue. Successful cancer treatment must also preserve function of normal tissue adjacent to cancer. Achieving the highest therapeutic ratio possible, total death of cancer tissue and no collateral damage to host tissue, is the goal of therapy. Widespread use of functional imaging, especially via FDG-PET provides the opportunity to also assess the effects of treatment on normal tissue. In addition, functional imaging may also provide mechanistic clues to the response of normal tissues to irradiation. This chapter provides some observations on the use of functional imaging to assess response of normal tissues to radiotherapy.

10.2 FDG-PET as an Assessment of Toxicity Following Radiotherapy for Head and Neck Cancer

Radiotherapy can be quite toxic when used to treat head and neck cancer. Recent developments in the field of radiation oncology allow greater precision in delivery of high-dose radiotherapy to target tissues. Intensity-modulated radiation therapy (IMRT) is a radiotherapy planning and delivery approach that allows specific targeting of high-dose radiotherapy to cancer tissues while minimizing doses to sensitive adjacent normal structures with a low risk of harboring cancer cells [1]. IMRT treatment allows for heterogeneous radiation dose delivery to tissues within the head and neck. A current central challenge for IMRT and head and neck cancer is identifying which structures should be protected, how much protection different tissues require, and the optimal way to protect those structures. One example is salivary gland function. Parotid glands are responsible for a significant amount of saliva production. They are very sensitive and appear to decrease saliva output after receiving radiation doses in the mid-20 Gy range. While parotid glands are adjacent to lymph node regions commonly harboring metastatic deposits of cancer, the parotid glands themselves only rarely contain metastatic deposits. Landmark studies by Eisbruch and colleagues at the University of Michigan [2] showed that salivary flow could be maintained after radiotherapy using IMRT if certain dose constraints were respected. Specifically, maintaining the mean parotid dose below 25 Gy allows virtually complete recovery of salivary production by 24 months after radiotherapy [2].

Increasing the intensity of therapy increases the cure rates for head and neck cancer. Higher doses of radiation [3] or concurrent delivery of chemotherapy [4, 5] with radiotherapy both improve locoregional control over head and neck cancer.

However, increasing the intensity of therapy can also increase complication rates. For example, two large randomized studies recently showed improved locoregional control and disease-free survival with concurrent chemotherapy and conventional radiotherapy after surgical resection for patients at high risk of recurrence. Unfortunately, these studies also showed an increase in treatment-related deaths in the cohort of subjects treated with combined chemotherapy and radiation therapy after surgical resection [6, 7].

The promise of IMRT is to provide a means to selectively escalate the intensity of radiotherapy in tissues harboring cancer while minimizing exposure of sensitive critical normal structures. Swallow function after radiotherapy is often severely affected [8]. Many if not most patients undergoing treatment currently require feeding tubes because they cannot swallow adequate fluids or calories to meet their needs. Long-term swallowing deficits resulting from treatment can continue to pose a threat to adequate nutrition, increase the risk of aspiration pneumonia and delay healing. Eating and swallowing difficulties can also decrease quality of life (QoL) and social interactions [9].

To preserve function after treatment for head and neck cancer, the appropriate anatomic structures essential to that function must be protected from radiation doses above their tolerance. Radiation doses to key anatomic structures necessary for swallow function must be kept low enough that they can still perform their normal function. Optimal use of IMRT requires specific information regarding what structures are necessary for swallowing and their respective dose limitations [10]. As an initial step to understand key anatomic determinants of swallowing, Dornfeld et al. examined a group of 27 patients with head and neck cancer treated with chemoradiotherapy and followed for both objective endpoints such as PEG feeding tube dependence and weight loss as well as subjective endpoints assessed with self-reported QoL questionnaire [11]. These 27 patients all had stage III or IV squamous cell cancer of the head and neck, including sites within the oropharynx, hypopharynx, and larynx. These patients were treated with chemotherapy and radiation therapy. The IMRT delivered daily doses of 1.6–2.0 Gy to regions at risk of harboring microscopic disease or gross disease respectively over a median of 35 fractions as described previously [1]. None received surgery as initial treatment. All subjects were followed using clinical exam and CT-based imaging for at least 1 year without recurrence. Fifteen points were chosen at sites along the mucosal surface of the upper aerodigestive tract, including the base of tongue, posterior pharyngeal wall, and supraglottic and glottic larynx (Fig. 10.1). The radiation dose delivered to each of these points was determined. The points were selected in conjunction with a speech pathologist as sites likely to play a critical role in swallowing.

The dose delivered to each of the 15 points were then correlated with a variety of outcome measures including type of diet tolerated (such as unrestricted, liquid only, or feeding tube dependent), weight loss, and persistence of PEG feeding tube at 1 year. A validated questionnaire was used to assess self-reported overall QoL and QoL in the domains of speech, eating, social interactions, and esthetics [12]. High doses of radiation or chemoradiation to the larynx (both glottic and supraglottic) are correlated with a more restrictive diet, persistence of PEG tube dependence at

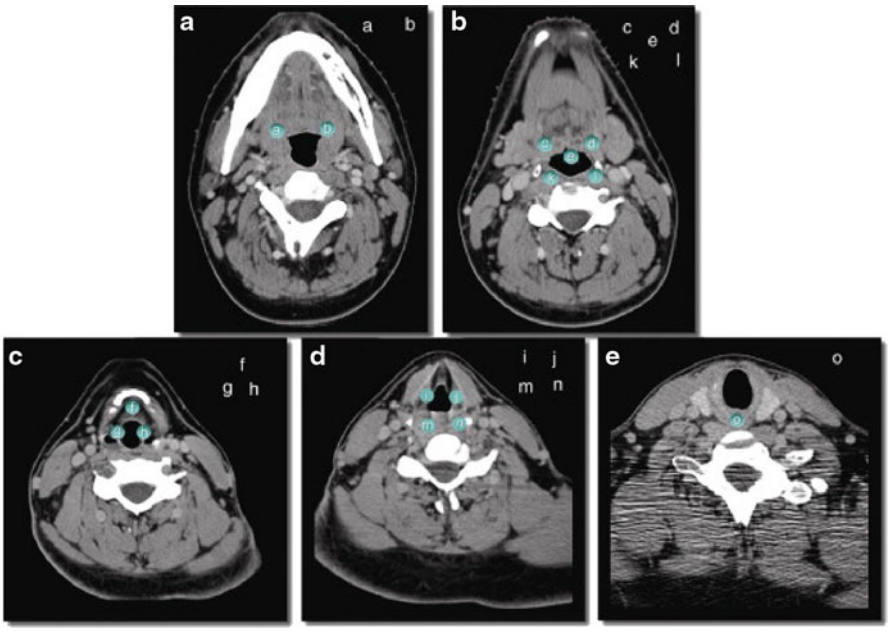

Fig. 10.1 Representative CT images used to define the various dosimetric points were examined. The dose points analyzed were the right (**a**) and left (**b**) superior base of tongue, the right (**c**) and left (**d**) inferior base of tongue, the epiglottis (**e**), the right (**k**) and left (**l**) lateral pharyngeal walls at the level of the inferior base of tongue, the pre-epiglottic space (**f**), the right (**g**) and left (**h**) aryepiglottic folds, the right (**i**) and left (**j**) false vocal cords, the right (**m**) and left (**n**) lateral pharyngeal walls at the level of the false vocal cords, and the upper esophageal sphincter (**o**)

1 year, decreased QoL and greater weight loss after treatment [11]. Patients who experienced higher doses of radiation to sites within and adjacent to the glottic and supraglottic larynx had a worse functional outcome. Higher doses to these structures (i, j, m, n, o in Fig. 10.1) had a more restrictive diet 1 year after treatment and were more likely to require a feeding tube 1 year after treatment (Fig. 10.2). Speech-related QoL for these patients, as determined by a self-reported questionnaire was also inversely correlated with dose to the supraglottic and glottic larynx (Fig. 10.3). A significant increase in toxicity appeared to occur with radiation doses greater than 50 Gy. Eisbruch et al. [13] and Allen et al. [14] have also identified the larynx and supraglottic larynx as important structural determinants of swallowing function.

Surveillance with FDG-PET is useful to characterize response to treatment and potentially detect recurrence [15] after nonsurgical management of head and neck cancer. FDG-PET scans are performed by injecting patients with an analog of glucose, where the hydroxyl group in the 2 position is replaced by a positron emitter, Fluoride-18. Inside the cell FDG is phosphorylated by hexokinase but is not further catabolized [16]. Patients are imaged after injection with FDG using a PET camera, which detects the 511 keV annihilation photons that result from positron decay.

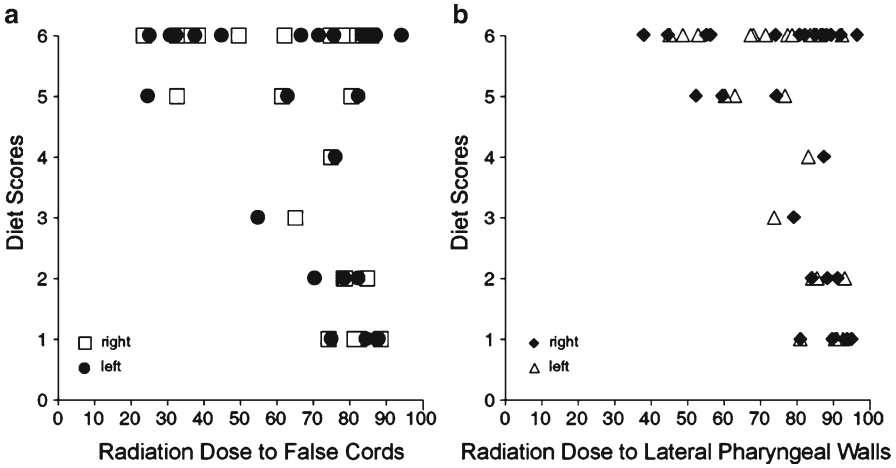

Fig. 10.2 Dose–response relationships between radiation dose and diet scores and radiation dose delivered to the false vocal cords (**a**) and lateral pharyngeal walls at the level of the false cords (**b**). Radiation doses were measured at points *i* and *j* (**a**) and *m* and *n* (**b**). Scores are defined in the text. Briefly nothing by mouth is 1, soft mechanical is 4, and unrestricted is 6

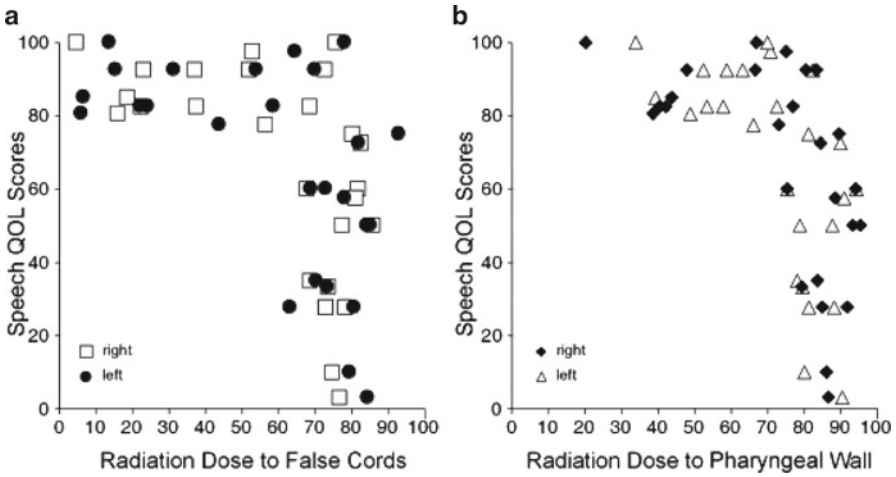

Fig. 10.3 Dose–response relationships between radiation dose and speech-related quality of life (QoL) for radiation doses delivered to false vocal cords (**a**) and lateral pharyngeal walls at the level of the false cords (**b**). Doses for both right and left structures are included. The dosimetric points for these graphs are *i* and *j* (**a**) and *m* and *n* (**b**)

Sites of altered glucose metabolism will have altered signals on the FDG-PET scan. Several reports have documented the utility of FDG-PET scans obtained after (chemo)radiotherapy in predicting long-term control over head and neck cancer [17, 18]. FDG-PET scans may also provide a means to assess changes imparted to adjacent normal tissue by radiotherapy. Given the central importance of glucose

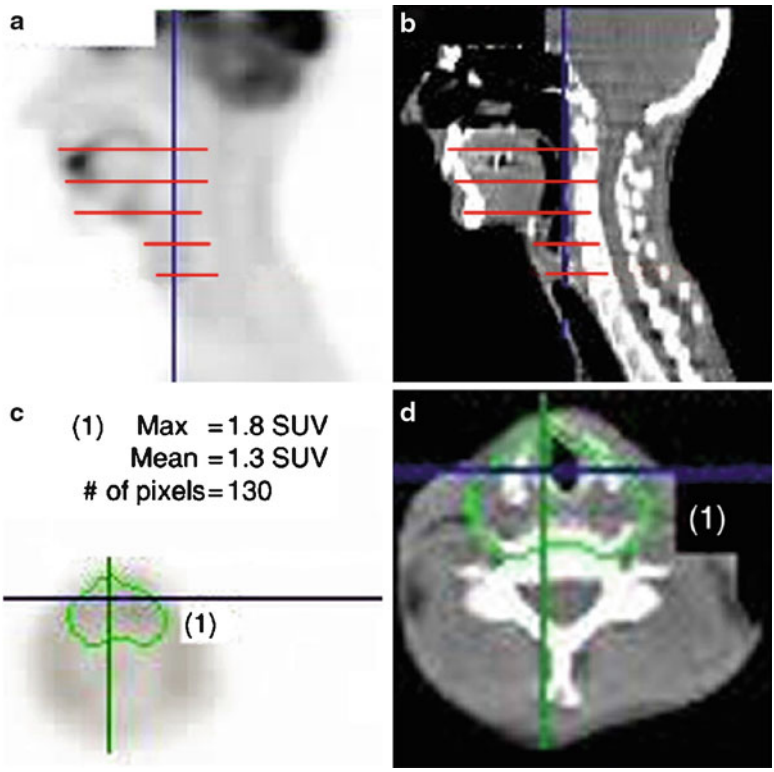

Fig. 10.4 Determining FDG SUV in irradiated tissues. Sagittal views of PET (**a**) and CT (**b**) showing the five axial planes for which FDG SUVs were determined. At each level, a region of interest was defined to include all mucosal surfaces and surrounding soft tissue. An example is shown for the laryngeal axial plane with both PET (**c**) and CT (**d**) images

utilization in cell and molecular biology and the significant functional changes in normal tissue function after radiotherapy, changes in FDG uptake in normal tissues after radiotherapy seemed likely.

To address this possibility, Dornfeld and coworkers examined FDG-PET scans at 3 and 12 months after treatment in patients with head and neck cancer receiving chemoradiotherapy without surgery [19]. Eighteen subjects were analyzed. These subjects were a subset of the 27 patients described in the above study showing increasing dose of radiotherapy to the larynx correlated with decreased subjective and objective functional outcome. These 18 were chosen because they had FDG-PET scans performed at 3 and 12 months after treatment. To assess the degree of FDG uptake in normal tissue, five axial planes were chosen, roughly corresponding to the levels of dose points analyzed in the study described above (Fig. 10.4). In each of the five levels, a region anterior to the spine encompassing the aerodigestive tract and adjacent soft tissue was defined as a region of interest. The FDG uptake within the region was quantified using maximum pixel standardized uptake value (SUV).

Table 10.1 PET SUV outcomes

	PET 1		PET 2		SUV change[a]	
	Average	Range	Average	Range	Increase[b]	Decrease[b]
Top of tongue	3.0	1.4–4.7	2.8	1.6–3.5	4	7
Mid-tongue base	3.1	2.3–4.7	3.0	1.7–5.9	3	8
Vallecula	2.8	1.4–5.0	3.0	1.8–5.7	7	5
Supraglottic larynx	2.8	1.9–5.1	2.7	1.6–4.2	4	5
Glottic larynx	3.4	2.2–5.4	3.4	1.8–6.6	5	9

SUV standardized uptake value

[a] Change in SUV, either increasing or decreasing, was defined as greater than 12%

[b] Number of patients with noted change (increase or decrease) between the two PET scans

The SUV numbers were analyzed for correlations between type of diet tolerated, degree of weight loss, persistence of PEG tube 1 year after treatment, and self-reported QoL in eating, speaking, social, and esthetic domains as determined by a validated questionnaire.

The results from analyzing posttreatment FDG-PET scans showed a variable degree of FDG uptake in normal tissue in the head and neck region. The upper aerodigestive tract shows a high degree of variation in FDG avidity even in normal subjects, particularly in the oropharynx and larynx. In comparing FDG uptake in soft tissues in five axial planes extending from the superior aspect of the tongue to the larynx, roughly one quarter (17–40%, depending on the particular level) of subjects showed an increase in FDG avidity in normal tissues between 3 and 12 months after chemoradiotherapy (Table 10.1). This degree of increase in FDG avidity is greater than that can be explained by the innate 12% variation in SUVs seen in FDG-PET scanning. The biochemical basis for the FDG elevation is unclear. All subjects have been followed for at least 1 year after the 12-month PET scan with no clinical recurrence, suggesting the rise in FDG avidity was likely not due to cancer.

Subjective outcome measures were also associated with elevated FDG uptake. Questionnaires were used to collect patient reported QoL in domains relevant to head and neck cancer, such as social interactions, eating, and speaking. An inverse correlation exists between the intensity of FDG in the larynx and supraglottic larynx and speech and eating-related QoL. The inverse correlation between FDG avidity and QoL was seen with the 12-month FDG-PET scan, but not with the 3-month scan (Figs. 10.5 and 10.6). The correlations between the level of FDG uptake (quantified using SUV) and outcome measures were stronger than correlations between dose delivered to the regions of interest and subsequent SUVs. The stronger correlation between FDG uptake and toxicity rather than radiotherapy dose and FDG uptake suggests an individual's reaction to the radiation, and not just the radiation dose itself, is important in determining the altered metabolism seen on FDG- PET. These findings suggest metabolic changes imparted to normal tissues following radiotherapy are active for prolonged periods of time after treatment, and indeed may progress with time. While the patient numbers are small and clearly need more rigorous prospective corroboration, the findings detailed above provide compelling

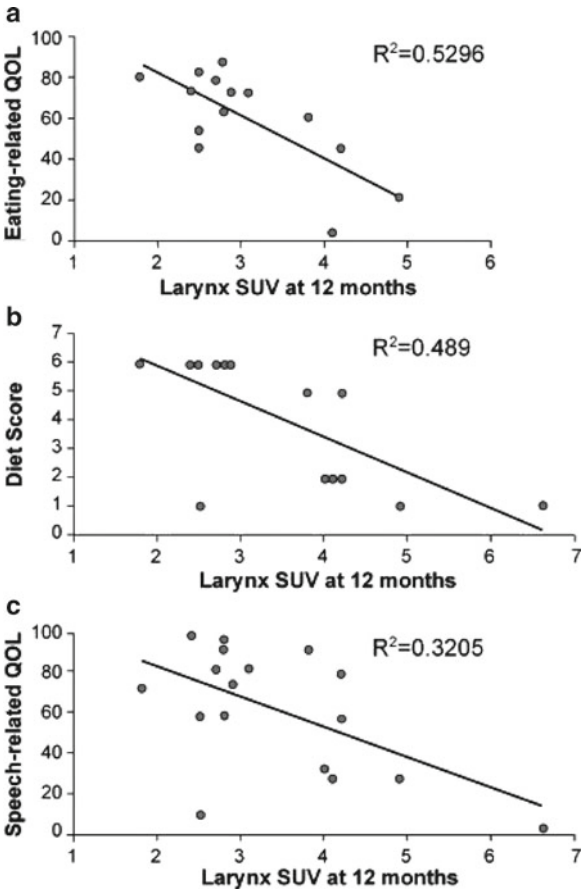

Fig. 10.5 Higher fluorodeoxyglucose standardized uptake value (FDG SUVs) in the glottic larynx 12 months after treatment are correlated with a worse functional outcome. (**a**) Normalized eating-related QoL scores measured at 12 months posttreatment from the Head and Neck Cancer Inventory (HNCI) are shown as a function of normal tissue FDG uptake at the level of the glottic larynx (see Fig. 10.4) from a PET scan also obtained 12 months after therapy. (**b**) Diet scores ranging from 1 for no oral intake to 6 for full, unrestricted diet determined 12 months after treatment are shown as a function of normal-tissue FDG uptake at the level of the glottic larynx (see Fig. 10.4) from a PET scan also obtained 12 months after therapy. (**c**) Normalized speech-related QoL scores measured at 12 months posttreatment using the HNCI are shown as a function of normal tissue FDG uptake at the level of the glottic larynx (see Fig. 10.4) from a PET scan also obtained 12 months after therapy. In each graph, there is a significant association showing that higher FDG uptake is associated with increased toxicity 12 months after therapy

evidence that functional imaging can be used to characterize treatment effects on normal tissues, not just cancer tissue. The FDG-PET studies were able to provide meaningful information regarding the therapeutic ratio of radiotherapy, in terms of both tumor and normal tissue response.

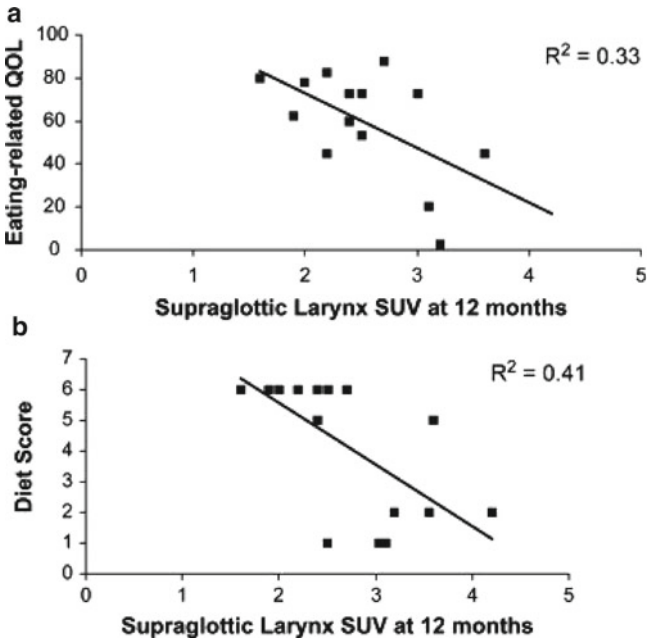

Fig. 10.6 Higher fluorodeoxyglucose standardized uptake value (FDG SUVs) in the supraglottic larynx 12 months after treatment are correlated with a worse functional outcome. (**a**) Normalized eating-related QoL scores measured at 12 months posttreatment from the Head and Neck Cancer Inventory are shown as a function of normal tissue FDG uptake at the level of the supraglottic larynx (see Fig. 10.4) from a PET scan also obtained 12 months after therapy. (**b**) Diet scores ranging from 1 for no oral intake to 6 for full, unrestricted diet determined 12 months after treatment are shown as a function of normal tissue FDG uptake at the level of the supraglottic larynx (see Fig. 10.4) from a PET scan also obtained 12 months after therapy. In each graph, there is a significant association showing that higher FDG uptake is associated with increased toxicity 12 months after therapy

The elevated FDG avidity in normal tissues after treatment may signify a metabolic derangement, either due to changes within the irradiated tissues or secondarily due to inflammation. In either situation, the elevated FDG avidity may reflect on-going injury. Within this population of 18 subjects, both objective and subjective measures of outcome were worse in patients with elevated FDG avidity in the 12-month posttreatment scan. The type of diet tolerated, such as a full or soft diet, liquid only or gastrostomy tube-dependent diet was correlated with elevated FDG uptake in the larynx such that subjects with greater FDG uptake were more likely to have a more restrictive diet. Virtually all domains of QoL were negatively correlated with posttreatment FDG avidity on PET scans obtained 1 year after therapy (Table 10.2).

Table 10.2 Summary of correlations between fluorodeoxyglucose standardized uptake value and functional outcomes

	Quality of life				
Maximum SUV	Diet	Eating	Speech	Esthetic	Social function
Level of supraglottic larynx	–0.64[a]	–0.57[b]	n.s.	–0.62[a]	–0.44[b]
Level of glottic larynx	–0.70[a]	–0.73[a]	–0.57[a]	–0.53[b]	–0.45[b]

n.s. not significant, *SUV* standardized uptake value
[a] Significant at $p < 0.05$
[b] Significant at $p < 0.01$

10.3 Abnormal FDG After Radiotherapy for Other Cancers

Postirradiation FDG-PET scans have also been studied following radiotherapy to the chest for esophageal and lung cancer. A substantial dose of radiation can be delivered to normal lung as radiation treatment beams pass through the chest to treat thoracic tumors. Measuring tumor response has been the main focus of FDG-PET scans obtained after (chemo) radiotherapy for esophageal and nonsmall cell lung cancer. However, some investigators have examined the impact of irradiation on FDG avidity in normal lung adjacent to the tumor, exposed to radiation, but not involved with cancer. The majority of these studies in thoracic cancers have shown results similar to those described above for head and neck cancer. Specifically, these studies have also reported prolonged elevation of FDG uptake and retention in a substantial minority of patients following treatment.

Guerrero et al. examined effects of radiation treatment on irradiated lung delivered as treatment for esophageal cancer [20]. Since the esophagus is a deep thoracic structure, radiation beams targeting the esophagus pass through a significant portion of normal lung. Although the dose delivered to normal lung is not the full dose delivered to tumor, given the sensitivity of lung to radiation, even partial dose can result in normal lung injury. Guerrero and colleagues at MD Anderson examined the dose–response relationship in 36 patients after treatment for esophageal cancer. These investigators registered CT scans with radiation dose information with FDG-PET scans obtained 24–78 days after completing radiotherapy. The median dose delivered to the esophagus was 50.4 Gy, with a range of doses delivered to normal lung. There is a linear relationship between increasing radiation dose and FDG uptake in the lung [20]. The slope describing the relationship varied significantly from patient to patient, suggesting a wide variation in normal lung tissue response to radiotherapy. For each patient, however FDG localization in the lung increased as radiation dose increased.

This same group extended these findings by including lung toxicity in their analysis. In their report by Hart et al., 101 patients treated with conventional thoracic irradiation as part of their treatment for esophageal cancer were studied [21]. These subjects underwent an FDG-PET scan an average of 6 weeks after radiotherapy. Sixty-six patients experienced symptomatic pneumonitis. Radiation pneumonitis is a form of lung injury clinically characterized by dry cough, shortness

of breath, and low grade fever. It often precedes anatomic imaging changes on computerized tomography (CT) scans, such as fibrosis, that occur after radiotherapy to the lung. The volume of lung irradiated and the dose delivered to normal lung both determine the risk of developing radiation pneumonitis. Even with precise knowledge of dose and volume of lung irradiated, it is difficult to predict which patients will develop radiation pneumonitis. FDG uptake and retention in normal, irradiated lung was assessed using FDG-PET scans in these patients. The maximum SUV in lung distinct from the cancer site was significantly greater in patients with pneumonitis symptoms than those without. Their findings again suggest that FDG localizes in irradiated tissue and the degree of FDG uptake and retention may reflect the degree of injury and toxicity.

Several well-established dosimetric parameters have been identified that are correlated with the risk of developing pneumonitis. For example, the mean lung dose and the volume of lung receiving more than 20 Gy are both correlated with increasing risk of developing pneumonitis. However, dosimetric parameters alone provide an imperfect estimate of radiation pneumonitis risk. Hart et al. [21] used information from the posttreatment FDG-PET scan in combination with these dosimetric parameters and increased the accuracy in predicting which patients would develop pneumonitis. Whether the ability to identify patients at risk can be used for earlier intervention and decreased overall pneumonitis symptoms remains to be seen. Taken together, these data show that radiation to normal lung causes an increase in FDG uptake and retention. The degree of FDG uptake varies significantly from patient to patient. The specific association between FDG uptake and radiation dose is useful in predicting lung toxicity following chest irradiation. These overall findings are consistent with the findings described above for head and neck cancer.

Persistent elevated FDG uptake and retention has also been described in a minority of patients treated with stereotactic radiosurgery for nonsmall cell lung cancer. In this treatment, a very high dose of radiation is delivered over 3–5 sessions to a discrete target, usually less than 4 cm in diameter. The large doses used in radiosurgery are able to provide a high chance of local control, but also carry the potential for significant tissue damage in normal tissue immediately adjacent to the tumor. Hoopes et al. described their experience with 28 patients treated with stereotactic radiosurgery to the lung who have been followed with FDG-PET [22]. Four of the 28 patients (14%) had persistently elevated, or even increasing FDG uptake within the treated volume on PET scans obtained 22–26 months following radiosurgery. These patients were followed for an additional 20–26 months without clinical or radiographic evidence of recurrence, suggesting that the elevated FDG uptake was a response to the radiation treatment in normal lung and not recurrence of cancer.

Similar findings were reported by Ishimori et al. in 2004 [23]. This group used both C-11 methionine (the uptake of which reflects amino acid metabolism) and 18F FDG-PET scans prior to radiosurgery and in subsequent follow-up to gauge response to treatment. They found FDG and C-11 methionine scans showed concordant results. Three of nine patients showed a delayed elevation in FDG uptake and retention several months after treatment. Based on CT image analysis and clinical

follow-up, the rise in FDG uptake was felt to be located normal lung tissue adjacent to the irradiated tumor tissue. None of the nine subjects suffered a relapse. The authors concluded that the increased FDG and C-11 methionine uptake was due to pneumonitis and not tumor recurrence.

FDG-PET may also provide evidence of myocardial injury. Jingu et al. [24] examined FDG-PET scans at least 5 months after completing chemoradiotherapy for esophageal cancer. They found 13 of 64 patients showed increased FDG uptake and retention in the irradiated myocardium in FDG-PET scans performed 5–65 months after irradiation. In each case, the region of elevated uptake was localized to an area of the heart that received 60 Gy, the full prescription dose. Eight of the 13 underwent further study with SPECT scans using the fatty acid analog 123I-methyl-iodo-pentadecanoic acid (123I-BMIPP) and thallium-201 to assess fatty acid metabolism and perfusion, respectively. Five of the eight showed decreased uptake and retention of the fatty acid analog, suggesting a primary defect in energy production and/or mitochondrial function.

The studies described above consistently highlight increased uptake of glucose after irradiation for months, if not years, after treatment. Patients with persistently elevated glucose uptake may also be those with higher side effects or greater tissue damage. The increased uptake and retention of FDG appears to add information about toxicity risk beyond traditional risk factors such as dose and volume. FDG-PET may provide a functional assessment of toxicity based on individual variation in radiation response.

10.4 Potential Biochemical Explanation of Increased FDG Uptake After Radiotherapy

The glucose utilization by tissues contributes to many critical biochemical functions. Glucose has a central role in biosynthesis since it serves as a precursor for a variety of necessary cellular components, including nucleotides, amino acids and other metabolites. Glucose also plays a key role in both aerobic and anaerobic energy production through respiration and glycolysis. Glucose metabolism through the pentose phosphate pathway provides a major source of NADPH used for both biosynthesis and as a cofactor for several antioxidant enzymes. The increased uptake and retention of glucose analogs in normal tissues following radiotherapy may be due to an increased demand for glucose by any or all of these processes.

Inflammation can clearly lead to increased local consumption of glucose. For example, sites of infection due to a variety of agents may cause increased FDG uptake [25]. A variety of drugs, including chemotherapeutic agents, carry a risk of causing lung inflammation. These include rituximab, bleomycin, and others. Increased FDG uptake and retention in lung is seen with these drug reactions [26, 27]. Even hip replacement may lead to increased FDG uptake and retention in the treated joint following the surgery [28, 29].

Alternatively, radiation may affect mitochondrial structure and/or function [30]. Radiation exposure does alter mitochondrial membrane potential (see discussion of sestamibi below). If mitochondria are damaged such that they become inefficient at respiration, glucose uptake could be affected in two main ways. First, damaged, poorly functioning mitochondria may produce less ATP by oxidative phosphorylation. Increased glucose consumption may be required to produce energy by alternative means such as glycolysis. Inefficient respiration by damaged mitochondrial may also lead to chronic elevation in the production of reactive oxygen species (ROS). Damage to the electron transport chain may cause electrons to react with oxygen prematurely. Transfer of four electrons to oxygen in the terminal step of electron transport creates water. Transfer of less than four electrons to oxygen creates unstable and ROS such as peroxides and superoxide. If radiotherapy alters mitochondrial efficiency, increased ROS production would lead to increased glucose consumption to create the NADPH needed as a cofactor for several types of enzymes acting to detoxify the ROS. Further discussions regarding mitochondrial function and oxidative stress in radiation and cancer biology have been reviewed previously [31]. A more detailed understanding of why tissues take up and retain glucose, and whether this uptake is increased in tissues experiencing greater toxicity remain topics for further investigation. Understanding these connections may allow clinical tools already in use, such as FDG-PET, to recognize radiotherapy toxicity, possibly allowing earlier and more effective amelioration.

10.5 Other PET Tracers

FDG is by far the most commonly used PET imaging agent. However, multiple other agents are being developed. The potential list of agents is virtually endless. For cancer staging and prognosis pretreatment, any biochemical pathway or chemical difference between normal and cancer tissue can provide a foundation for novel PET imaging agents. For normal tissue injury, ideal PET agents would involve pathways responsible for greater tissue damage and dysfunction. Hypoxic markers or clotting cascade participants may provide benefit. Since the mechanisms of tissue injury following irradiation are incompletely understood, novel PET agents may also provide an insight into which biochemical processes are most important in determining toxicity. For this promise to be realized, investigators developing novel PET agents must examine normal tissue uptake and correlate uptake with normal tissue toxicity as well as tumor response.

One potential new imaging agent is 18F-fluorothymidine (FLT) [32]. Like FDG, FLT contains an 18F isotope and lacks a hydroxyl group in a key position (3′ on the deoxy ribose moiety). FLT is imported into cells similarly to thymidine, phosphorylated and retained inside the cell. Due to the lack of the 3′ hydroxyl, FLT is catabolized more slowly than thymidine. FLT uptake may be more related to DNA synthesis and therefore may have a preferential uptake in cancer tissue. FLT is being developed to image a number of different cancers based on their avidity for thymidine and may serve as an excellent complement to FDG-PET.

Pretreatment proliferation rates and other tumor cell cycle kinetic markers have been investigated as potential means to individualize cancer treatment [33]. For example, more rapidly dividing tumors may benefit most from shortening the overall treatment time using accelerated fractionation. Unfortunately, pretreatment cell cycle kinetics have not been able to accurately identify which patients will benefit from more aggressive treatment [34]. Changes in proliferation and cell cycle kinetics following partial delivery of radiotherapy may be more informative. Ki67 is a cellular protein expressed almost exclusively during S-phase and is used as a marker of DNA replication. Valente et al. examined Ki67 in tumor biopsies before treatment and after 10 Gy (5 treatments of 2 Gy each) and correlated Ki67 levels with response to radiotherapy in 31 patients with locoregionally advanced head and neck cancer treated with radiotherapy [35]. There was no correlation with pretreatment Ki67 levels, but there was a significant association between changes in Ki67 and response to therapy. Greater decreases in Ki67 were associated with a higher likelihood of achieving a complete response. Zackrisson used in vivo cell cycle kinetic measures to determine changes following four treatments with radiotherapy in 33 head and neck cancer patients [36]. Subjects were infused with IdUdr and tumor biopsies taken 4–8.2 h after infusion and prior to treatment. Tumor biopsies were subjected to flow cytometry using labeled antibodies against IdUdr to determine the degree of IdUdr uptake into DNA to determine cell cycle kinetics. BrdUdr was infused and biopsies were taken before the fifth radiation treatment and again tumor biopsies obtained and analyzed for cell cycle kinetic parameters. Tumor biopsies showing decreases in the labeling index, a cell cycle parameter determined by the portion of tumor cells incorporating halogenated pyrimidine, of greater than 10% showed a statistically better survival than those with less change in the labeling index. These two studies suggest changes in cell cycle kinetics induced by radiotherapy may be predictive of long-term outcome and thereby serve as a means to individualize treatment, either intensifying therapy for poor responders or decreasing intensity for favorable responders.

Unfortunately, techniques to measure potential doubling times or tumor proliferation are cumbersome and have not gained widespread use. Imaging studies capable of providing tumor proliferation estimates may be more clinically useful. Changes in tumor proliferation rates in response to radiotherapy may also have prognostic value for the likelihood of achieving local tumor control. In addition, tumor response following treatment may be seen more quickly using FLT-PET than FDG-PET since DNA synthesis and replication may be more susceptible and respond more rapidly than glucose metabolism. Animal xenograft tumor models using both FDG and FLT to assess response to radiotherapy indeed show a more rapid change in FLT avidity than FDG avidity [37]. In other words, changes in tumor SUV are more rapid with FLT as the imaging agent than with FDG.

Menda et al. have examined changes in FLT uptake and retention using 18F-FLT PET in a group of eight patients with squamous cell cancer of the head and neck prior to therapy and after 1 week (of a 7-week course) of chemoradiotherapy. Tumor FLT uptake and retention measured both with Patlak analysis and SUV methods showed a significant decrease in both primary and nodal sites of disease.

The mean pretreatment SUV in cancer was 3.5 ± 0.5. The SUV was reduced to 1.8 ± 1.1 following five daily radiation treatments of 2 Gy each for a total of 10 Gy. Patients also received one cycle of cisplatin chemotherapy. Retention appeared especially affected by radiotherapy since the second scan showed much greater efflux of FLT from tumor tissue than seen on the pretreatment scan. Further follow-up is necessary to determine if the degree of change in SUV following radiation predicts overall outcome of treatment [38].

Very few reports have investigated the effects of irradiation on FLT uptake and retention in normal tissues. The utility of FLT in assessing and predicting normal tissue injury warrants future investigation. As FLT is developed as a cancer imaging agent, the opportunity for use of FLT as a predictor and measure of normal tissue damage should also be assessed.

10.6 Normal Tissue Effects Seen by Techniques Other than PET

10.6.1 Magnetic Resonance Spectroscopy

Brain irradiation is useful in many settings, including treatment of primary and metastatic cancers as well as prophylactic treatment. Significant concern has been raised regarding the potential toxicity of brain radiotherapy and the potential loss in cognitive function [39]. Understanding the exact nature of the injury inflicted to normal brain following radiotherapy is essential to minimizing the consequences of brain radiotherapy. MRS holds great promise as a method able to provide information on the relative abundance of common biochemicals such as glutamine [40], glutathione [41, 42], choline, and creatine [43]. MRS is limited to detect biochemicals with relatively large, millimolar, concentrations. MRS is performed with the same equipment used to obtain magnetic resonance imaging. MRI and MRS have been used to distinguish between cancer recurrence and tumor necrosis after treatment of brain cancers [44]. The chemical signature provided by MRS adds predictive value to MRI.

MRS has also been applied to understanding changes in normal brain after radiotherapy. Atwood and coworkers have used MRS to analyze the effects of whole brain radiotherapy on rats 1 year after treatment [45]. They described several chemical changes. The most prominent findings were increases in glutamate + glutamine as well as γ-aminobutyric acid in irradiated brain tissue. Myoinositol levels were reduced in irradiated brains relative to nonirradiated controls. Significant functional changes in cognition may also be monitored by MRS. Using similar techniques, Atwood et al. again found biochemical changes 1 year after brain irradiation in rats [46]. Cognition was also altered in irradiated but not in nonirradiated rats. Imaging and cognitive testing were similar between irradiated and nonirradiated groups 12 weeks after treatment. However, significant differences were seen in the irradiated

group both by imaging and cognitive testing at 52 weeks, suggesting a temporal relationship between biochemical changes and cognitive function after brain irradiation. MRS may provide a powerful tool to explore these connections.

99-mTc sestamibi SPECT imaging: Tc-99m Sestamibi (MIBI) is a lipophilic cation used for SPECT imaging. Cellular uptake and of MIBI appears to depend on multiple factors, including extracellular concentration and both cellular and mitochondrial membrane potentials [47]. Cancer cell retention of MIBI can be lowered by export via multidrug resistance protein P-glycoprotein. MIBI is useful in assessing perfusion because better perfusion delivers a greater amount of MIBI to tissues, resulting in greater uptake. Historically, MIBI has been widely used to assess myocardial perfusion and health. However, since both heart disease and cancer are common diseases, the utility of MIBI in detecting cancer became apparent as cancers were found incidentally in patients undergoing MIBI scans for cardiac monitoring.

Several studies have confirmed the ability of MIBI to detect a variety of cancers. Indeed, MIBI is becoming a powerful technique to detect and stage breast cancer [48]. MIBI may also be useful in detecting response of a variety of cancers to treatment, allowing comparisons of pre- and posttreatment imaging. Khawar et al. described changes in MIBI imaging both within head and neck cancer and in adjacent normal parotid tissue after irradiation [49]. Shortly after a course of radiotherapy, tumor uptake and signal is significantly reduced in most but not all cancers. Further study is required to determine the relationship between MIBI changes and prognosis for head and neck cancer. Changes in MIBI uptake may also be useful in predicting the degree of injury to normal tissues such as parotid glands. MIBI has also been shown to reliably predict recurrence of nonsmall cell lung cancer following radiotherapy. Furuta et al. examined 18 patients with nonsmall cell lung cancer treated with radiotherapy [50]. All 18 patients were free from local failure as determined by chest radiographs at 1 year follow-up. One year after completing treatment, these patients underwent a sestamibi SPECT scan. Uptake of MIBI in the area irradiated lung compared to a contralateral region of normal lung was determined. The group of 18 was followed for another 18 months to determine clinical outcome. Nine of the 18 developed local recurrence after the MIBI scan. Eight of nine patients who ultimately developed a local recurrence had a treated to untreated lung ratio of MIBI uptake greater than 1.6. Eight of nine patients who maintained local control had a treated to untreated lung ratio of less than 1.5, suggesting MIBI may be very useful in predicting local failure following irradiation. MIBI has also been used to assess for radiation effects versus recurrence in brain tumors [51]. The use of MIBI in cancer imaging is significant, and a thorough review of is beyond the scope of this chapter. However, the potential utility of this agent to assess normal tissue injury resulting from irradiation will be briefly discussed.

Cardiac toxicity occasionally results from radiotherapy for breast cancer to the left breast or chest wall. Radiotherapy to the left chest is associated with altered cardiac perfusion using MIBI and similar compounds. Prosnitz et al. examined 160 patients treated with radiotherapy for left-sided breast cancer using MIBI SPECT [52]. Forty-four patients underwent scans 3–6 years after treatment, providing a large group of long-term survivors. Among the 44 patients with long-term follow-up,

over half developed perfusion deficits; 52% at 3 years and 67% at 5 years. The risk of developing perfusion deficits was statistically associated with the volume of left ventricle irradiated and the location of the perfusion deficit was anatomically coincident with the irradiation portals for most patients. The incidence of wall motion deficits and significant decreases in ejection fraction was very small and was not associated with perfusion deficits. Similar changes in cardiac perfusion have been described for patients undergoing radiotherapy for esophageal cancer [53]. These studies highlight the utility of monitoring normal tissue toxicity, at least for the heart, with MIBI SPECT scans. MIBI may therefore be useful to both monitor for tumor recurrences and cardiac toxicity following radiotherapy to the chest.

The biochemical basis for MIBI uptake and retention is related, at least in part to mitochondrial function, number, and membrane potential. Furata has shown that irradiating lung cancer cells in vitro causes a dose-dependent increase in mitochondrial membrane potential 1 h after treatment using both MIBI and DiOC6, a fluorescent dye specific for mitochondrial membrane potential [54]. The uptake of DiOC6 and MIBI both decreased by 8 h after treatment, suggesting initial mitochondrial injury followed by dysfunction. How normal tissues other than heart respond to radiotherapy remains an open question. If MIBI SPECT imaging is able to provide information on mitochondrial number and function, it may provide complementary data in combination with FDG-PET. For example, the increased glucose uptake seen by FDG-PET in tissues suffering greater damage following radiotherapy may have damaged mitochondria. The damaged mitochondria would be expected to show decreased MIBI uptake relative to comparable unirradiated normal tissue. In light of the mitochondrial changes described with MIBI, the changes in glucose utilization associated with toxicity as seen by FDG-PET, it may be worthwhile to more fully characterize normal tissue changes following radiation with MIBI.

10.7 Summary

Radiotherapy is based on generating high doses of ROS in treated tissue. It has been used for over 100 years to treat cancer. Until recently, assessing the effects of radiotherapy has been almost exclusively limited to changes in size of the treated tumor based on either physical exam or anatomic imaging. With the advent of functional imaging techniques such as fluorodeoxyglucose (FDG) PET treatment response can now be assessed in terms of biochemical changes resulting from irradiation. Changes in metabolism as seen via functional imaging are indeed associated with toxicity of radiotherapy.

The primary concern of imaging after treatment is to determine the effects of irradiation on the cancer. However, these functional imaging techniques also provide the opportunity assess changes imparted to adjacent normal tissue. It is imperative that as new imaging techniques are developed, attention is paid not only to treatment effects on tumor, but also on normal tissue. In this way, we may best be able to develop more selective and effective treatments for our patients, increasing cure and QoL while decreasing toxicity.

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Part IV
Oxidative Stress in Tumor Response (Clinical)

Chapter 11
Histone Deacetylase Inhibitors, Oxidative Stress, and Multiple Myeloma Therapy

Rentian Feng and Suzanne Lentzsch

Abstract Multiple myeloma (MM) is a hematologic malignancy characterized by dysregulated proliferation of plasma cells. Histone deacetylase (HDAC) inhibition results in the accumulation of acetylated nucleosomal histones and induces differentiation and apoptosis in transformed cells. HDAC inhibitors (HDACi) induce oxidative stress, DNA damage, and mitochondrial damage/dysfunction in myeloma cells. The release of apoptogenic factors from mitochondria leads to cell death. Synergistic antimyeloma activity of a combination regimen of HDACi with other agents was observed, in which increased oxidative stress plays a major role. A preclinical animal model also showed a strong inhibition of myeloma tumor growth and prolonged survival by KD5170 monotherapy. These findings indicate HDACi are promising anti-MM agents, mediated by gene modulation and induction of oxidative stress and subsequent apoptosis.

Abbreviations

ATM	Ataxia telangiectasia mutated
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitors

R. Feng, PhD
Department of Pharmaceutical Sciences and Drug Discovery Institute,
University of Pittsburgh School of Pharmacy, 3501 Terrace Street, Salk Hall, 541A,
Pittsburgh, PA 15261, USA

Division of Hematology/Oncology, University of Pittsburgh Cancer Institute,
Pittsburgh, PA, USA

S. Lentzsch, MD, PhD (✉)
UPMC Cancer Pavilion, #568, 5150 Centre Avenue, Pittsburgh, PA 15232, USA

Division of Hematology/Oncology, University of Pittsburgh Cancer Institute,
Pittsburgh, PA, USA
e-mail: lentzschs@upmc.edu

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JNK	c-Jun NH2-terminal kinase
MM	Multiple myeloma
Mn-TBAP	Manganese(III) meso-tetrakis (4-benzoic acid)porphyrin
NAC	*N*-Acetyl-cysteine (NAC)
ROS	Reactive oxygen species

11.1 Introduction

Multiple myeloma (MM) is the second most prevalent hematological malignancy, characterized by aberrant proliferation of terminally differentiated plasma cells and impairment in apoptosis capacity. While recent advances in the knowledge and treatment of MM have been made, it is still an incurable disease with a median survival of 3–5 years [10]. Over the last several years, a diverse spectrum of novel agents has shown therapeutic potential in myeloma including thalidomide, lenalidomide, arsenic trioxide, and bortezomib, but high relapse rates, drug toxicity, and resistance continue to call for novel effective compounds with unique targets in the treatment of MM. HDAC inhibitors (HDACi) represents a novel therapeutic strategy for MM treatment. This review focuses on the role of oxidative stress induced by HDACi resulting in MM cell death.

Histone deacetylase (HDAC) are divided into several classes, among which class I (HDAC1-3 and HDAC8) and II (HDAC4-7 and HDAC9-10) stereostructurally have a catalytic pocket containing a zinc ion required for the deacetylation reaction. The sides of the pocket are more hydrophobic, allowing the insertion of a lysine side chain which is a substrate for acetylation. Aberrant altered activities of HDAC are associated with human hematologic malignancies [14, 27, 55]. A comprehensive screen of histone H4 modifications in human cancer cells revealed a global loss of both H4K16 monoacetylation and H4K20 trimethylation associated with the hypomethylation of DNA repetitive sequences, which appeared early and accumulated during the tumorigenic process. This was postulated to contribute to genomic instability frequently observed in tumor cells [14]. Potent HDACi block access to the catalytic pocket thereby inhibiting deacetylation. High levels of acetylated nucleosomal histones may facilitate to keep "open" structures in the transcription sites and therefore regulate the accessibility of transcription factors to gene regulatory sequences. HDACi have pleotropic effects on a variety of nongenomic targets such as tubulin and heat shock proteins and facilitate the production of potentially toxic reactive oxygen species (ROS). Thus, ROS-induced oxidative stress represents not the whole but one extremely important mechanism of action of HDACi in the treatment of cancers.

Suberoylanilide hydroxamic acid (SAHA) is the first FDA-approved HDACi and is successfully used in the treatment of cutaneous lymphoma [34]. Several other novel HDACi such as LBH589 are in different phases of clinical trials [16]. Most of them show good tolerance and safety profiles in the phase I and II trials [58].

To identify HDACi, Feng et al. used an unbiased ultrahigh throughput screening biochemical screen of 600,000 compounds and identified a structurally novel mercaptoketone series of HDACi, among which the lead compound KD5170 was preclinically evaluated [11].

11.2 HDACi-Induced Oxidative Stress and Cell Death

11.2.1 Oxidative Stress and MM Cell Death

An excess of ROS occurs in the mitochondria if the cell is subjected to external stress such as chemical agents or a dysfunctional complex within the electron transport chain. While ROS undoubtedly can cause much general damage to the mitochondrial components, ROS may also have specific targets in cell death signaling pathways. Mounting evidence suggests that excess of ROS facilitates the detachment of cytochrome *c* from its membrane-anchoring cardiolipin and increases the mobilized pool of cytochrome *c*, which is a prerequisite for its release into the cytoplasm through the pores, created by proapoptotic Bcl-2 family members such as Bax. While the exact mechanism is not yet known, it was proposed that cardiolipin peroxidation induced by oxidative stress might have a critical role in this process [36]. In addition, ROS may also play an important role in the permeabilization of the membrane. On the other hand, the link between ROS production and the extrinsic death receptor pathway implies that chemical-induced intracellular ROS may in turn contribute to Fas receptor aggregation, which is critical for cell death (Fig. 11.1) [19].

Cellular oxidative stress induced by either endogenous ROS production via therapeutic/radiation treatment or exogenous imposition (such as addition of oxidant into the cell culture) can lead to MM cell death [20, 35]. Of particular note is that human myeloma cells ($CD138^+$) are more sensitive to peroxide or chemotherapy-induced oxidative stress than their normal marrow leukocyte counterparts ($CD138^-$) [11, 13, 20, 37]. This may represent a universal phenomenon for the sensitivities of transformed cells and their normal counterparts to oxidative injury-induced cellular component damage and cell death [12, 29, 49, 53, 56].

Different levels of thioredoxin and response to ROS in transformed and normal cells may partly explain this effect (see Sect. 11.2.3). Therefore, the induction of oxidative stress might be an effective and selective therapeutic strategy in MM. The observed selective cytotoxicity of HDACi in myeloma cells appears, at least in part, to be attributable to a generally heightened susceptibility of myeloma cells to HDACi-induced oxidative stressors [4, 5, 11, 13, 37].

11.2.2 HDACi-Induced Oxidative Stress

ROS accumulation has been described in malignant cells exposed to structurally diverse HDACi, including vorinostat, trichostatin A, sodium butyrate, MS275, and LAQ-824, and has been proposed as an underlying mechanism of HDACi-induced cell death. Feng et al. showed that ROS production as well as downstream events, including mitochondrial dysfunction and ceramide induction, caspase activation and apoptosis, were significantly diminished by the free radical scavengers such as *N*-acetyl-cysteine (NAC), indicating that oxidative damage represents a primary

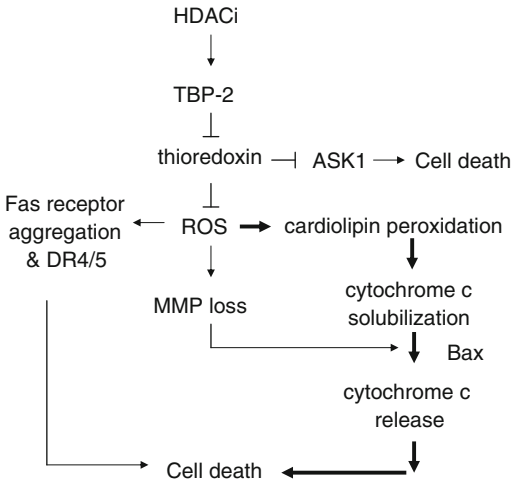

Fig. 11.1 HDACi induced cell death through ROS production. One possible mechanism by which HDACi causes MM cell apoptosis is shown. HDACi enhances excess of ROS formation through inhibiting thioredoxin redox system by upregulation of TBP-2, which is an endogenous inhibitor of thioredoxin system. Excess of ROS may facilitate the detachment of cytochrome *c* from its membrane-anchoring cardiolipin via peroxidation of cardiolipin and increases the mobilized pool of cytochrome *c*, which is a prerequisite for its release into the cytoplasm through the pores created by proapoptotic Bcl-2 family members such as Bax. In addition, ROS may also damage mitochondrial membrane and induce membrane potential loss (MMP) that favors cytochrome *c* release. Death receptor aggregation may also result from ROS production and induce cell death through a different pathway

event in the induction of the apoptotic cascade. Furthermore, combination regimens of HDACi with other agents (such as doxorubicin, TRAIL, and bortezomib) also showed that oxidative stress plays a primary role in the synergistic or additive activity of these combinations against hematologic malignant cells (Fig. 11.2) [7, 11, 13, 25, 31, 44]. In contrast, some researchers proposed that the functional significance of HDACi-induced ROS production in human tumor cells may vary, and oxidative injury is necessary but not sufficient to trigger apoptosis in different cell types. Down-regulation of antiapoptotic molecules and/or up-regulation of proapoptotic signals are involved in ROS-induced cell death [45].

HDACi-induced ROS causes oxidative DNA damage. Levels of phosphorylated histone H2AX (γ-H2AX) and ataxia telangiectasia mutated (ATM), early markers of DNA damage, significantly increase as early as 0.5 h after the administration of HDACi and continue increasing for 24 h and beyond (Fig. 11.3a), but are markedly diminished by antioxidants NAC or manganese(III) meso-tetrakis (4-benzoic acid) porphyrin (Mn-TBAP) [11, 44]. It was reported that cytoplasmic translocation of histone H1.2 and activation of caspase-2 possesses important roles in nuclear DNA damage responses [23, 24]. Rosato et al. proposed that a low dose of HDACi LAQ-824 and fludarabine dramatically induced histone H1.2 release from the nucleus and cleavage of procaspase-2. This effect was largely abrogated by co-treatment of cells

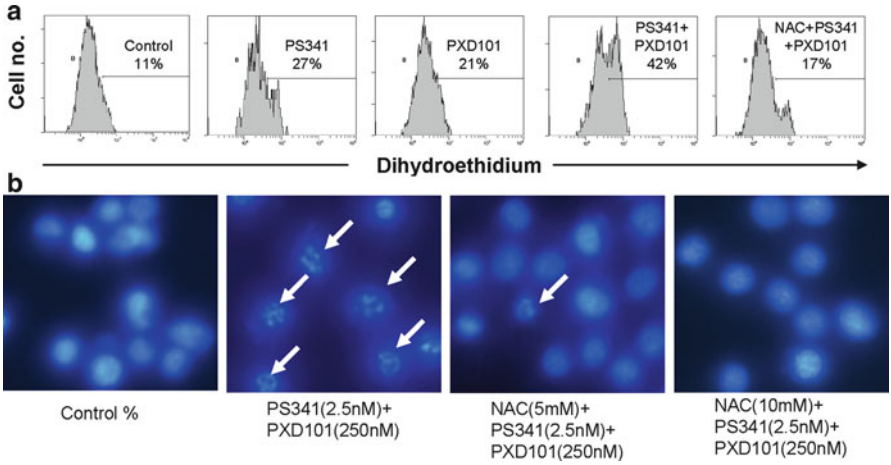

Fig. 11.2 Intracellular generation of reactive oxygen species contributes to HDACi PXD101 and bortezomib (PS341)-mediated apoptosis in MM cells. (**a**) After preincubation with or without 5 mM *N*-acetyl-L-cysteine (NAC) for 3 h, MM cells MM.1S were treated with 2 nM bortezomib, 250 nM PXD101, combination, or combination along with 5 nmol/l NAC for another 15 h. The cells displaying increased ROS production were determined by monitoring dihydroethidium staining analyzed by flow cytometry. (**b**) MM.1S cells were treated by combination of 2.5 nM bortezomib and 250 nM PXD101 in the absence or presence of NAC (5 or 10 mM) for 17 h. Cells exhibiting apoptotic morphology were determined by evaluating bis-benzamide Hoechst 33258-stained for nuclear fragmentation/condensation (as the *arrows* indicate). The data shown are representative of three independent experiments

with antioxidants, suggesting a critical role of HDACi-mediated ROS production in DNA damage through modulation of histone H1.2 release and caspase-2 activation [44]. More importantly, Gaymes et al. found that HDACi-induced DNA damage and apoptosis is significantly increased in hematologic malignant cells but not in normal cells such as peripheral blood lymphocytes [15], which is consistent with the production levels of ROS induced by HDACi in the neoplastic and normal cells [54].

Louis et al. described that HDACi-induced oxidative stress also results from pronounced depletion of glutathione that is associated with cell apoptosis [54]. Heme oxygenase-1, the inducible enzyme for degradation of heme to biliverdin in the cells, is a ubiquitous and redox-sensitive inducible stress protein. We found heme oxygenase-1 is induced by exposure to HDACi KD5170 in as little as 1 h and maintained thereafter for 16 h in U266 cells (Fig. 11.3b) [11].

Loss of mitochondrial membrane potential is the downstream event of increased oxidative stress in HDACi-treated cells, and often reflects increases in mitochondrial outer membrane permeability, which results in the release of proteins that trigger cell death (Fig. 11.3c) [11]. One of these proteins is cytochrome *c*, which together with caspase-9 and Apaf-1 constitutes the apoptosome, a macromolecular structure that causes activation of the effector caspase-3. By using the mitochondria-sensitive dye, JC-1, which probes the changes of mitochondrial membrane potential,

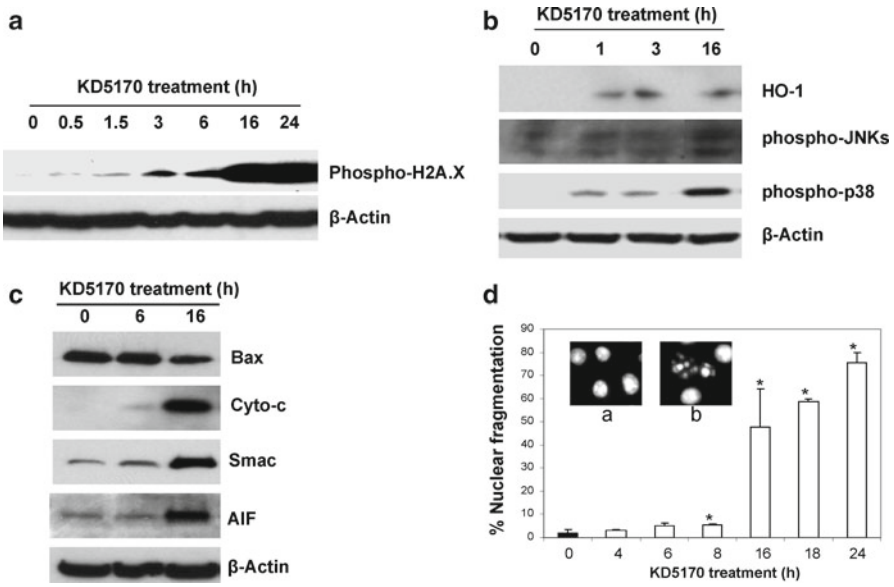

Fig. 11.3 HDACi induces cell death through mitochondrial pathway by oxidative stress-induced MAPK activation and DNA damage in human MM cells. U266 cells were treated with KD5170 (0.75 μM) for the indicated time, whole cell lysates (**a**, **b**) or cytoplasmic fractions (**c**) were subjected to immunoblot assay and detected with indicated antibodies. β-Actin was used as loading control. (**d**) U266 cells were exposed to KD5170 (1 μM) for different hours. Percentages of the apoptotic cells with typical apoptotic nuclear morphology were determined by staining the cells with 10 μM Hoechst 33258 fluorochrome for 20 min. Nuclear morphologic changes of U266 cells before and after treatment (16 h) with KD5170 were shown in the *small windows*. $*P<0.05$, with respect to vehicle control

Feng et al. showed that KD5170 treatment caused a loss of mitochondrial membrane potential in MM cell line U266 in a time-dependent manner (Fig. 11.4). The decline in membrane potential was detected as early as 6 h after treatment. Consistent with the lack of effect on apoptosis and caspase activation is the observation that KD5170 failed to induce mitochondrial depolarization in the HDACi-resistant cell line MM.1S (Fig. 11.4) [11]. This suggests that KD5170-induced loss of mitochondrial membrane potential may be an essential event for induction of cell death resulting in the release of the apoptogenic factors that reside in the mitochondria.

Feng et al. further described that HDACi-induced cell apoptosis occurs between 6 and 16 h after treatment (Fig. 11.3d), whereas ROS production occurred as early as 0.5 h and DNA damage at 2 h followed by mitochondrial membrane potential disruption. This suggests that HDACi-mediated ROS induction and DNA damage represented a cause rather than a consequence of mitochondrial membrane potential loss and cell death [11, 44].

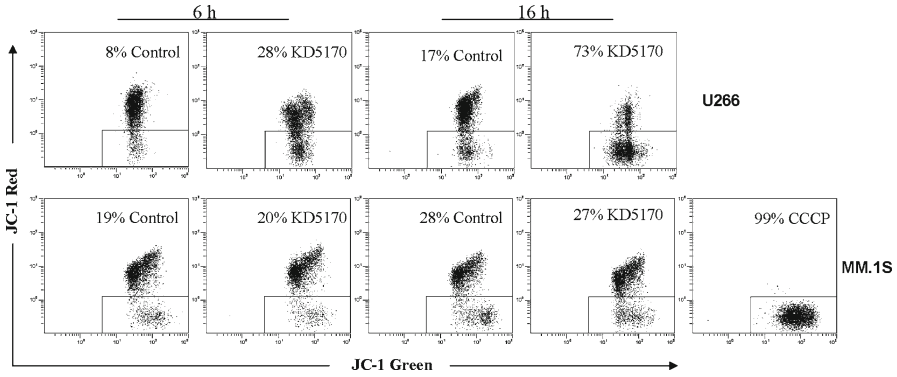

Fig. 11.4 KD5170 induces loss of mitochondrial membrane potential. After exposure to KD5170 (0.75 μM) for either 6 or 16 h, human multiple myeloma cell lines U266 (sensitive) and MM.1S cells (resistant) were stained with JC-1 (Molecular Probes) before flow cytometry analysis. Data analyses were done with CellQuest software by measuring both green (530F 15 nm; FL-1) and red (585 F 21 nm; FL-2) JC-1 fluorescence. Mitochondrial membrane potential loss was observed as the shift to lower JC-1 red fluorescence and/or an increase in JC-1 green fluorescence. At least 10,000 events were collected and counted per sample. The mitochondrial membrane potential disruptor, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), was used as a positive control and followed the same staining procedures. Representative histograms

11.2.3 Regulation of Oxidative Stress-Related Gene Expression by HDACi

Besides DNA damage induction, Fas receptor aggregation, and mitochondrial membrane potential dissipation, ROS also causes an imbalance in the intracellular redox state, disrupting the antioxidant defense mechanisms that usually control the ROS levels. As discussed above, HDACi regulate global gene expression. Proapoptotic proteins (Bax, Bak, Bim, TRAIL, etc.) and redox components (thioredoxin, thioredoxin-binding protein [TBP], glutaredoxin, etc.) are induced by HDACi, whereas antiapoptotic genes and angiogenic factors are repressed by treatment with SAHA [28]. Among those genes, the oxidative stress-related gene, thioredoxin, plays a critical role in HDACi-induced ROS production as well as cell death.

Thioredoxin, a small redox-active multifunctional protein, acts as a potent antioxidant and a redox regulator in cellular signal transduction. The thioredoxin system is a major antioxidant system integral to maintaining the intracellular redox state via thiol redox control. Thioredoxin protein regulates the activity of oxidative stress-related enzymes, counteracts oxidative stress by scavenging ROS, and directly inhibits proapoptotic proteins such as apoptosis signal-regulating kinase 1 [22, 46]. Cytoplasm and mitochondria contain equivalent thioredoxin systems and inhibition of either system can lead to activation of apoptotic signaling pathways. It was reported that HDACi SAHA may indirectly act as a thioredoxin system inhibitor.

Studies in gene expression revealed that HDACi specifically transactivated TBP-2 (an inhibitor of the thioredoxin system) in various cancer cell types [3]. As a result, increased ROS is found in several cancer cell types treated with HDACi. Nonmalignant cells appear to compensate by over-expression of thioredoxin in response to the HDACi-induced ROS, but cancer cells do not. Thus, thioredoxin, independent of the caspase apoptotic pathway, is an important determinant of resistance of cells to HDACi-induced cell death [3, 44, 52]. Interestingly, like normal cells, many chemotherapy-resistant cancer cells also constitutionally express low levels of TBP-2 and high levels of thioredoxin, thereby reducing thioredoxin expression which could increase the sensitivity of the resistant cells to ROS-generating agents, such as cisplatin and doxorubicin, as well as hydrogen peroxide and UV irradiation. Cellular levels of thioredoxin thus appear to limit sensitivity to various ROS-generating anticancer drugs in cancer cells [1, 3, 39]. Therefore, thioredoxin/TBP-2 represents a novel redox target pathway for the development of drugs to treat therapy-resistant cancer types.

Preclinical studies show that MM and leukemia cells that are resistant to established cytotoxic agents are sensitive to HDACi treatment [5, 26, 37, 54, 59], and patients resistant to traditional therapies experienced clinical remission and symptomatic relief after receiving a combined regimen with HDACi [34, 57]. Excess of ROS formation to overcome drug-resistance in MM was also observed with other ROS-producing agents such as adaphostin and chaetocin [6, 20]. Although HDACi-induced oxidative stress and TBP/thioredoxin modulation may partially explain the synergistic effects of HDACi and other agents in the drug-resistant MM cells, it should be noted that other factors such as proapoptotic genes and repressed anti-apoptotic proteins are also critical elements to facilitate the recovery of sensitivity of tumor cells to chemotherapy. In response to oxidative stress, MAPK members and p53/p21WAF1 pathway are usually activated [30, 48]. MAPK activation was also linked to the thioredoxin/TBP system [47]. Feng et al. investigated the effect of HDACi on downstream kinases of ROS-induced activation cascade. They found that MAPK p38 and c-Jun NH2-terminal kinase (JNK) are markedly phosphorylated as early as 1 h after HDACi treatment (Fig. 11.3b). As negative regulators of cell cycle progression, p53 and p21WAF1 are essential in oxidative stress-induced DNA damage and cell cycle arrest. Feng et al. also found that DNA damage-induced augmentation of p53–p21 signaling, as evidenced by p53 phosphorylation (Ser15) and up regulated p21, further contributes to PXD101/bortezomib-mediated cell cycle arrest and subsequent apoptosis [13]. These results are in accordance with other reports in which epigenetic regulation of p21WAF1 promoter was highly affected by HDACi [21, 32, 37, 42, 43]. HDACi-induced ROS might play a critical role in MAPK activation and p53/p21 upregulation [17]. On the other hand, p53 activation-induced gene spectrum revealed that redox-related genes, ROS formation, and oxidation degradation of mitochondrial components are the three-steps necessary to culminate in cell death [38].

11.3 Clinical and Translational Leads

MM is an incurable cancer of the plasma cell with high relapsed rate, which is associated with development of drug resistance in advanced stages. There are approximately 60,424 people in the USA living with MM and an estimated 19,900 new cases of MM will be diagnosed in the USA in 2007 (Facts 2007–2008, The Leukemia & Lymphoma Society). Though FDA-approved HDAC inhibitor SAHA shows anti-MM activity preclinically, and in clinical trials, concerns of poor pharmacokinetics and toxicity have been proposed [51]. Novel HDACi or combination regimens with other chemotherapeutic agents have been demonstrated to be an effective and selective approach to induce MM cell death, overcome drug resistance and prolong survival time in patients. Due to the promising preclinical activity of HDACi, numerous clinical trials have been initiated [41].

Phase I investigation with the combination of SAHA (vorinostat) and bortezomib in MM patients has been completed, with primary objectives of determination of the maximum tolerated dose of vorinostat and toxicity of the regimen when given together with bortezomib. Treatment repeated every 21 days for up to eight courses in the absence of disease progression or unacceptable toxicity. Recently, a Phase IIb study testing vorinostat in combination with bortezomib in MM treatment was initiated within the Multiple Myeloma Research Consortium. The study will assess the objective response rate as well as progression-free survival, overall survival, time to disease progression, and tolerability of the combination. Researchers predicted that vorinostat and bortezomib may help to overcome prior resistance of MM patients to bortezomib (http://www.themmrc.org/news_press/6.02.34.php; http://www.cancer.gov/clinicaltrials/MSGCC-GCC-0514).

Romidepsin (FK228) is another HDACi with novel cyclic peptide and is under investigation for the treatment of hematologic malignancies. An early phase I study demonstrated its efficacy and some durable responses with acceptable toxicity when combined with bortezomib [40]. The developer, Gloucester Pharmaceuticals, recently announced at the 50th ASH Annual Meeting the positive results from a phase I/II MM clinical trial using a combination of romidepsin, bortezomib, and dexamethasone in patients with relapsed or refractory MM. Combination treatment had an overall response rate of 85% (17/20). Four (20%) patients achieved a complete response (CR), two (10%) a very good partial response, six (30%) a partial response (PR), and five (25%) a minor response (MR). Five of six patients previously exposed to bortezomib responded in this study. The median time to progression for all patients was 7 months [18].

Another HDACi, LBH589 (panobinostat), has significant activity against MM cell lines, exerting cytotoxicity at <10 nM in MM cell lines resistant to conventional therapies. LBH589 causes cell apoptosis through the aggresome pathway [4]. A phase II study at a conservative dose (20 mg per day, thrice weekly) provided encouraging results with one durable VGPR and 3/38 disease stabilization, good tolerance and safety profiles, and warrant further clinical investigation [58].

11.4 Conclusions and Future Challenges

As an anticancer strategy, induction of ROS represents a promising therapeutic approach in cancer therapy [2, 8, 9, 33, 50]. An important attribute of HDACi is their ability to induce cancer cell death at concentrations at which normal cells are relatively unaffected. The unique modulation mechanism of the TBP-2/thioredoxin system in normal and tumor cells warrants more attention for the study of novel HDACi alone and in combination with other agents. Based on the knowledge of epigenetic mechanisms, the potential application of HDACi and demethylating agents should also be investigated.

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Chapter 12
Curcumin, Oxidative Stress, and Cancer Therapy

Heather C. Hatcher, Frank M. Torti, and Suzy V. Torti

Abstract Curcumin is the active ingredient in turmeric, a traditional herbal remedy and dietary spice. Curcumin has numerous beneficial medicinal properties, including cancer chemopreventive and cancer chemotherapeutic activity. Curcumin displays complex redox activity and functions as both pro- and antioxidant. These opposing activities are observed in cell-free systems, cultured cells, and in intact organisms. The redox properties of curcumin are a key feature of its activity and are due to the inherent chemical activities of the molecule, as well as its ability to induce multiple signaling pathways. This chapter provides an overview of the in vitro and in vivo studies that links curcumin's redox activity to its chemopreventive and chemotherapeutic effects. It also highlights the need for caution in combining curcumin with certain chemotherapies or in the setting of selected preexisting conditions.

H.C. Hatcher, PhD
Department of Cancer Biology, Wake Forest School of Medicine,
Winston-Salem, NC 27157, USA

F.M. Torti, MD, MPH
Department of Cancer Biology, Wake Forest School of Medicine,
Winston-Salem, NC 27157, USA

Comprehensive Cancer Center, Wake Forest School of Medicine,
Winston-Salem, NC 27157, USA

S.V. Torti, PhD (✉)
Comprehensive Cancer Center, Wake Forest School of Medicine,
Winston-Salem, NC 27157, USA

Department of Biochemistry, Wake Forest School of Medicine,
Winston-Salem, NC 27157, USA
e-mail: storti@wakehealth.edu

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Abbreviations

AhR	Aryl hydrocarbon receptor
AP-1	Activator protein-1
ARE	Antioxidant response elements
BaP	Benz-*a*-pyrene
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CAT	Catalase
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase-2
CuZnSOD	Copper zinc superoxide dismutase
CYPs	Cytochromes P450
DHC	Dihydrocurcumin
DMBA	Dimethylbenzanthracene
EPR	Electroparamagnetic spectroscopy
EpRE	Electrophile response element
ERK/MAPK	Extracellular signal-related kinase/mitogen-activated protein kinase
Fe-NTA	Ferric nitriloacetic acid
GPx	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione *S*-transferase
HO-1	Heme oxygenase-1
HNE	4-Hydroxy-2-nonenal
iNOS	Inducible form of nitric oxide synthase
Jak	Janus family of kinase
Keap1	Kelch-like ECH associating protein 1
LOOH	Lipid hydroperoxide
MDA	Malondialdehyde
MMPs	Matrix metalloproteinase
MnSOD	Manganese-containing superoxide dismutase
mTOR	Mammalian target of rapamycin
NAC	*N*-acetyl-cysteine
NFκβ	Factor kappa B
Nrf2	Nuclear factor-erythroid 2-related factor 2
NQO1	NAD(P)H:quinone oxidoreductase-1
ODC	Ornithine decarboxylase
8-OHdG	8-Hydroxy-2′-deoxyguanosine
PKC	Protein kinase C
PI3K	Phosphatidylinositol 3-kinase
ROS	Reactive oxygen species
STAT	Signal transducer and activator of transcription
THC	Tetrahydrocurcumin
TNF-α	Tumor necrosis factor-alpha
TRAIL	TNF-related apoptosis inducing ligand

12.1 Introduction

In healthy oxygen-consuming organisms, a delicate balance exists between the production of reactive oxygen species (ROS) and the antioxidant defense systems that control levels of ROS to minimize damage while still allowing essential oxidation reactions to remain functioning [1]. Chronic disturbance of this balance can contribute to the onset of cardiovascular, neurodegenerative, and respiratory diseases in addition to cancer and stroke. The social and economic impacts of these diseases are devastating as millions of people worldwide are affected each year [2].

Oxidative stress is defined as a state of diminished antioxidants and/or increased production of ROS. Oxidative stress is characteristic of many cancer cells [3]and fosters cancer development. For example, oxidative stress leads to direct oxidative modification of proteins, lipids, and nucleic acids [1], these damaged species may initiate malignant change. In addition, ROS act as second messengers in intracellular signaling cascades that can induce and maintain the oncogenic phenotype of cancer cells [1, 3–7]. To prevent redox imbalance, cellular defense mechanisms have evolved to manage exposure to ROS. These include antioxidant enzymes, such as the mitochondrial manganese-containing superoxide dismutase (MnSOD), cytosolic copper zinc superoxide dismutase (CuZnSOD), glutathione peroxidase (GPx), and catalase (CAT). Nonenzymatic dietary antioxidants including ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, and flavonoids [6] also contribute to maintenance of redox balance.

Curcumin, a polyphenol and the active ingredient in the dietary spice turmeric (*Curcuma longa* Linn) (Fig. 12.1), is an ancient herbal remedy that is well documented for its medicinal properties in traditional medicines of China and India. As biomedical research has focused greater attention on natural and complementary medicines, numerous beneficial properties, including anti-inflammatory, antioxidant, antidiabetic, antiangiogenic, cancer chemopreventive and cancer chemotherapeutic activity, have been linked to curcumin [8, 9]. Curcumin exhibits anticancer activity in vitro and in vivo, and is currently being tested in human clinical trials.

Curcumin displays complex redox activity and functions as both pro- and antioxidant in biological systems. These opposing activities are observed both in cell-free and in cell systems and are due to the inherent chemical activities of the molecule, as well as its ability to induce multiple signaling pathways. This chapter provides an overview of the extensive published literature that links curcumin's redox activity to its chemopreventive and chemotherapeutic effects. It also highlights the need for caution in combining curcumin with certain chemotherapies or in the setting of selected preexisting conditions.

12.2 Redox Activity of Curcumin in Cell-Free Systems

Early studies revealed that curcumin functions as an antioxidant. Liposomes were prepared and oxidized by iron/ascorbic acid [10]. Addition of curcumin reduced lipid peroxidation by 85%. In this assay, curcumin was as effective as BHA (butylated

Fig. 12.1 *Curcuma longa* (from Koehler's Medicinal-Plants)

hydroxyanisole, a common food preservative) and was more effective than equimolar concentrations of beta carotene or alpha tocopherol. Like alpha tocopherol, curcumin effectively scavenges peroxyl radicals, a property that would make it a likely candidate as chain breaking antioxidant [11]. Subsequent work confirmed these results [12] and further showed that curcumin inhibited formation of superoxide anion and hydroxyl radicals [13]. Similarly, using electroparamagnetic (EPR) spectroscopic techniques, curcumin was shown to be a potent singlet oxygen quencher at physiological or pharmacological concentration (2.75 μM) in aqueous systems [14]. Pulse radiolysis studies, which offer a unique system to study reactions of short-lived free radicals in a micro to millisecond time scale, demonstrated that curcumin's free radical scavenging is preserved in liposomes and protects cell membranes from lipid peroxidation [11, 15]. Being more polar than curcumin, the phenoxyl radical may "travel" to the surface of the membrane, where it may be repaired by any water-soluble antioxidant [11, 16].

Typical extracts of *Curcuma longa* L. contain the structures I (curcumin), II (demethoxycurcumin), and III (Bis-demethoxycurcumin) (Fig. 12.2), of which I is the most common [17]. Reports conflict as to whether I or III is the most potent as

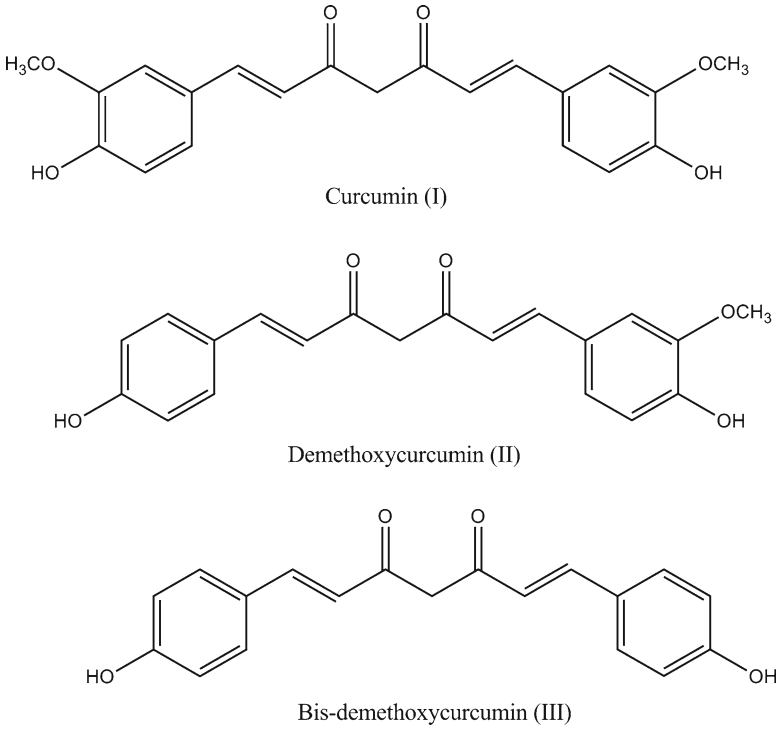

Fig. 12.2 Curcumin I, II, and III (curcumin, demethoxycurcumin, and bisdemethyoxy curcumin) and keto-enol tautomers of curcumin

an antioxidant and antitumor agent [9, 17, 18] (please see refs. [9, 19] for comprehensive curcumin chemistry). However, studies of the comparative antioxidant activity of curcumins I–III in reducing lipid peroxidation revealed that their order of potency was I>II>III [17].

Curcumin not only scavenges radicals but also directly inhibits enzymes that catalyze prooxidant pathways. For example, curcumins I–III inhibited the enzymatic activity of purified cyclooxygenase (COX) I by ~30%, and COX II by 80% [17], with all forms exhibiting approximately equivalent activity. By contrast, curcumin irreversibly inactivates thioredoxin reductase [20], which is an enzyme that catalyzes NADPH-dependent reduction of thioredoxins and is essential in substrate reduction, defense against oxidative stress, and redox regulation. Curcumin was found to bind to thioredoxin reductase and alkylate a critical cysteine residue, thus converting the activity of the enzyme to NADPH oxidase [20]. The authors postulated that this ability of curcumin to shift the activity of thioredoxin reductase from anti- to prooxidant may contribute to curcumin's anticancer activity, given the high expression of thioredoxin reductase in cancer and the enhanced sensitivity of cancer cells to oxidants.

Identification of the chemical moiety responsible for curcumin's redox activity has been the subject of active investigation. The phenolic hydroxy groups of curcumin have been proposed to play a significant role in curcumin's diverse antioxidant activity [21].

Jovanovic et al. [16, 22] indicated that hydrogen abstraction from the central CH_2 group also contributes to the remarkable antioxidant activity of curcumin. However, Barclay et al. suggested that the H atom from the phenolic OH is primarily responsible for curcumin's antioxidant activity, as results from their studies showed that the reaction medium is critical in determinations of antioxidant activities of phenols and that a relatively nonpolar medium such as styrene/chlorobenzene is preferable for the measurement of such antioxidant activities [23]; therefore, they and others conclude that the phenolic groups play the predominant role in the antioxidant activity of curcumin [23–25].

Metabolites of curcumin may contribute to curcumin's antioxidant activity. Pharmacologic studies of intestinal metabolites in humans and rats demonstrated that, following oral dosing, curcumin is transformed to curcumin glucuronide and curcumin sulfate as well as reduced to dihydrocurcumin (DHC), tetrahydrocurcumin (THC), hexahydrocurcumin, octahydrocurcumin, and hexahydrocurcuminol [26–30]; curcumin, DHC, and THC can be further converted in monoglucuronide conjugates [30, 31]. Since some of these metabolites, such as THC, possess anti-inflammatory [32] and antioxidant [33, 34] activity, they may be relevant to curcumin's biological activity [9].

12.3 Redox Activity of Curcumin in Cellular Systems and Animal Models

Considerable evidence exists to suggest that the cancer-related activities of curcumin may be linked to its known antioxidant and prooxidant properties. With respect to its antioxidant activity, curcumin has been reported to be a potent inhibitor of ROS formation in vivo as well as in cell-free systems [35–40]. For example, treatment of mice with 1% curcumin in the diet almost completely inhibited Fe-NTA (ferric nitriloacetic acid)-induced protein oxidation as monitored by the formation of protein reactive carbonyl contents in the kidney [41]. Similarly, pretreatment of rats with curcumin at a dose of 200 mg/kg/day orally attenuated gentamicin-induced increases in both plasma and kidney malondialdehyde (MDA), as well as lipid hydroperoxide (LOOH) formation [36]. Curcumin's antioxidant activity seems to be mediated by an ability to both scavenge ROS [11, 14, 39, 42–45] and activate endogenous antioxidant mechanisms that reduce the cellular levels of ROS [35–38, 46–50].

It is also well described that curcumin can act as a prooxidant to increase cellular levels of ROS [37, 51–63]. Curcumin induced apoptosis through the generation of ROS in a rat histiocytoma AK-5 [55] and human renal carcinoma Caki cells [64], as well as in human tumor cell lines established from malignancies such as leukemia, breast, colon, hepatocellular, and ovarian carcinomas, whereas cell lines

from lung, kidney, prostate, cervix, central nervous system malignancies, and melanomas showed resistance to the cytotoxic effects of curcumin [65]. In another study using cultured human leukemia cells, HL60, curcumin was shown to act as a prooxidant by forming ROS through the reduction of copper; moreover, the ensuing curcumin-mediated apoptosis was closely related to the increase in the concentrations of ROS in cells [54]. Interestingly, nontransformed cell lines were unaffected by curcumin treatment and showed neither ROS generation nor the induction of a stress response [65].

A number of in vivo studies have indicated that the antioxidant effects of curcumin are preceded by an oxidative stimulus, which is dose and time dependent [36, 66–68]. For example, exposure of myelomonocytic U937 cells to curcumin resulted in a time- and dose-dependent increase in ROS and decreased cell viability [69]. In another study, Kang et al. observed a significant decrease in the levels of ROS in human hepatoma Hep3B cells treated for 8 h with curcumin at concentrations of 10 and 20 μM; however, at 25, 50, and 100 μM, curcumin induced a significant increase in the cellular levels of ROS, which was dose- and time-dependent [57]. Therefore, experimental data would suggest curcumin at low concentrations will exert antioxidant activity, while higher curcumin concentrations may produce prooxidant effects.

The ability of curcumin to increase cellular levels of ROS may be related to its ability to selectively target cancer cells [61, 70–73]. Cancer cells produce higher levels of hydrogen peroxide compared to nonmalignant cells; thus, cancer cells are constantly under oxidative stress [74, 75]. Increasing hydrogen peroxide beyond the threshold would become cytotoxic to cancer cells but not normal cells [5, 76, 77]. Therefore, specific concentrations of curcumin could yield increased cellular levels of hydrogen peroxide and produce selective killing of cancer cells [46] and spare normal cells which are less sensitive to curcumin-induced ROS.

Several studies have shown that the prooxidant activity of curcumin facilitates the anticancer effects of radiotherapy and chemotherapy [78–90]. Recently, curcumin has been shown to be a potent radiosensitizer in two cervical tumor cell lines, HeLa and SiHa, while having no effect in normal cells [79]. Pretreatment with 10 μM curcumin before radiation dramatically increased ROS levels (approximately sixfold) compared with radiation alone (approximately threefold) [79]. Curcumin may also function as a chemosensitizer, enhancing the activity of other antineoplastic agents, in part by inhibiting pathways that lead to treatment resistance [88]. For example, curcumin-induced ROS was shown to be critical in mediating the upregulation of death receptor 5 (DR5) to render human prostate tumor cells more sensitive to the cytotoxic activities of TRAIL (TNF-related apoptosis inducing ligand) [91]. Concentrations of curcumin that induce an elevation in the cellular levels of ROS facilitate the anticancer effects of radiotherapy and chemotherapy and lead to cellular damage and ultimately cause cell death.

In noncancer cells, curcumin exhibits an opposite effect, reducing the activity of radiation and several chemotherapeutic agents [92–100]. This dual activity may contribute to curcumin's beneficial effects in vivo. For example, when cultured human lymphocytes were pretreated with curcumin prior to γ-irradiation, there was a significant decrease in lipid peroxidation and improved antioxidant status preventing

damage to lymphocytes [98]. The authors suggested that curcumin administration prior to radiation therapy may be useful to cancer patients to prevent normal cell damage. Thus, the studies described in this section illustrate that curcumin can behave as both a prooxidant and antioxidant depending on its concentration and cellular redox status.

12.4 Modulation of Cellular Redox Status Through Effects on Signaling Pathways

Considerable evidence suggests that the anticancer activities associated with curcumin relate to its ability to suppress signaling pathways important in acute and chronic inflammation [101] as well as its prooxidant properties [46]. Chronic inflammation is widely accepted as a risk factor for cancer development with evidence supporting the occurrence of elevated DNA damage by ROS in inflammation-related cancer [4]. Moreover, a tumor itself often induces an inflammatory response, which may or may not promote tumor development depending on tumor type [102]. ROS have been shown to activate numerous cellular targets and pathways including nuclear factor kappa B (NFκβ) [103], activator protein-1 (AP-1) [104], matrix metalloproteinase (MMPs) [105], tumor necrosis factor-alpha (TNF-α) [106], Akt [107, 108], the oncogenes ras, src, and myc [109–114], and the extracellular signal-related kinase/mitogen-activated protein kinase (ERK/MAPK), PI3K/Akt, and janus family of kinase (Jak)-signal transducer and activator of transcription (STAT) pathways [108, 115, 116]. As a modulator of cellular levels of ROS, curcumin has been shown to disrupt these cellular targets and signaling pathways [9, 117, 118]. The following section highlights several studies which link the chemotherapeutic properties of curcumin with specific signaling pathways mediated by oxidant stress.

12.4.1 NFκB

Curcumin modulates the expression of NFκB and NFκB-downstream targets including the inflammatory proteins cyclooxygenase-2 (COX-2) and inducible form of nitric oxide synthase (iNOS), MMPs, and TNF-α [119]. NFκB plays a critical role in signal transduction pathways involved in chronic and acute inflammatory diseases and various cancers [120–123]. The NFκB proteins reside in the cytoplasm in an inactive state and are translocated to the nucleus following activation, which is dependent upon the activation of various kinases and the phosphorylation and degradation of IκBα, the NFκB cytoplasmic inhibitor [124]. In vitro studies have shown that curcumin reduces COX-2 expression by inhibiting TNF-α-induced NFκB activation in human colon epithelial cells [125], and inhibits TNF-dependent NFκB activation in human myeloid ML-1a cells [126], as well as activation induced by various other agents including phorbol ester and hydrogen

peroxide. Treatment of human cervical carcinoma cells for 24 h with curcumin at doses of 50–100 μM downregulated COX-2 and iNOS [127]. Curcumin has been shown to suppress NF-κB activation in human leukemia cells [128, 129], human head and neck squamous cell carcinoma cells [130, 131], human colorectal adenocarcinoma cells [124]; human pancreatic cells [132], and human melanoma cells [133] by inhibiting the phosphorylation and subsequent degradation of IκBα. Suppression of NF-κB activation by curcumin could be reversed by reducing agents [119]; thus, it is possible that curcumin's strong antioxidant activity inhibits NF-κB activation by scavenging ROS, thereby altering the redox status of cancer cells.

12.4.2 Akt/mTOR

Several recent studies have shown that curcumin targeting of the mTOR (mammalian target of rapamycin) pathway represents a potential mechanism of its anticancer activity [81, 134–137]. The phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathway plays a central role in regulation of multiple critical cellular functions including stress responses, cell growth and survival, and metabolism [138]. Interestingly, a number of proteins regulated by mTOR, such as cyclin D1, ornithine decarboxylase (ODC), c-myc, NFκB, Akt, and protein kinase C (PKC), are also targeted by curcumin [134]. In human renal carcinoma cells (Caki), the expression and phosphorylation of Akt in Caki cells were significantly decreased in response to curcumin [62]. Treatment with an antioxidant, *N*-acetyl-cysteine (NAC), inhibited curcumin-induced apoptosis and prevented the release of cytochrome *c* from the mitochondria, suggesting a role for ROS in this process [62]. Recently, curcumin has been shown to inhibit phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR (mammalian target of rapamycin) signaling in various tumor cells [139]. Curcumin inhibited IGF-I-stimulated phosphorylation of S6K1 and 4E-BP1, the two best characterized downstream effector molecules of mTOR, in a dose-dependent manner, starting at 200 nM in human rhabdomyosarcoma cells [134]. This inhibition of cell proliferation occurred through a p53-independent mechanism; thus, by primarily targeting mTOR signaling pathways, curcumin may have potential applications as a chemotherapeutic agent against p53 mutant tumor cells, which are resistant to irradiation therapy or other chemotherapies [134].

12.4.3 Nrf2

A potential molecular target for curcumin's chemopreventive activity is nuclear factor-erythroid 2-related factor 2 (Nrf2), a key transcription regulator for antioxidant and detoxification enzymes, including heme oxygenase-1 (HO-1) [140]. Nrf2 is sequestered in the cytoplasm by an actin-binding protein, Kelch-like ECH associating protein 1 (Keap1), and upon exposure of cells to oxidative stress and certain

chemopreventive agents, such as curcumin, Nrf2 dissociates from Keap1, translocates to the nucleus, binds to antioxidant response elements (ARE), and transactivates phase II detoxifying and antioxidant genes [141, 142]. Curcumin has been reported to induce (HO-1), a ubiquitous and redox-sensitive inducible stress protein [49, 143] and a potential therapeutic target in a variety of oxidant- and inflammatory-mediated diseases [143]. HO-1 protein expression as well as activity was upregulated via induction of Nrf2 following orally administered curcumin in rats challenged with the hepatocarcinogen dimethylnitrosamine (DMN) [140]. Additionally, curcumin treatment led to an increase in nuclear accumulation of Nrf2, Nrf2–ARE binding and increased activities of phase II detoxifying enzymes such as glutathione *S*-transferase (GST) and NAD(P)H:quinone oxidoreductase-1 (NQO1) in liver and lungs of mice exposed to the carcinogen benz-*a*-pyrene (B*a*P) [144]. Curcumin administration induced Nrf2-ARE/EpRE (electrophile response element) signaling by stimulating the upstream kinases through phosphorylation or oxidizing the cysteine thiol of Keap1 [140]. Because curcumin bears two α,β-unsaturated carbonyl moieties, it can act as a Michael reaction acceptor; therefore, it is possible that the presence of these electrophilic carbonyl moieties could directly interact with a critical cysteine thiol of Keap1, lowering its affinity for Nrf2, releasing Nrf2 for nuclear translocation [140]. The results of these studies imply that dietary curcumin induces phase II detoxifying enzymes involved in the detoxification of carcinogens.

12.5 Oxidant Stress and the Anticancer Activity of Curcumin

An individual's sensitivity to environmental carcinogens is in part due to differences in the relative levels of their phase I and phase II xenobiotic metabolizing enzymes [1]. Phase I reactions will make the toxin metabolically active, often done by cytochromes P450 (CYPs), which add polar functional groups onto the toxin [1]. Phase II reactions involve conjugation of the phase I reaction product or the xenobiotic directly, for example, the conjugation of glutathione (GSH) catalyzed by glutathione *S*-transferases (GSTs) [1] to protect cells from stress by detoxifying carcinogens or reducing oxidant stress [145]. Curcumin has been shown to inhibit procarcinogen activating phase I enzymes, such as cytochrome P4501A1 [146]. Curcumin blocked the carcinogen activation pathway mediated by the aryl hydrocarbon receptor (AhR) in MCF-7 mammary epithelial carcinoma cells and appears to be a natural, dietary ligand of the AhR [146]. Curcumin inhibited the activation of the mammary carcinogen dimethylbenzanthracene (DMBA), both by competing with DMBA for the Ah receptor (involved in expression of CYP1A1, the gene that encodes the phase I enzyme cytochrome P450 1A) and by competitively inhibiting cytochrome P4501A enzymatic activity [146]. In a separate study, curcumin inhibited benzo(*a*)pyrene (BaP)-induced forestomach cancer in mice [147]. The mechanism proposed again involved activity of hepatic CYP 1A1, which activates BaP to the DNA-reactive diol epoxide. Curcumin increased in GSTs and epoxide hydrolase which are important in detoxifying the BP diol epoxide [147]. According to some investigators, both

hydroxyl and β-diketone groups of curcumin are involved in curcumin-mediated induction of phase II detoxification enzymes [148]. Thus, the chemopreventive properties of curcumin may be linked to its ability to both inhibit phase I carcinogen activation and stimulate phase II detoxification activation pathways [146].

12.6 Inhibition of ROS by Metal Chelation

Curcumin also binds metals, and this can contribute to both its pro- and antioxidant activity. Curcumin binds Fe-III [149], and the redox cycling of bound iron may foster Fenton chemistry [51, 52, 150, 151]. Curcumin has been shown to act as an iron chelator in vivo [152, 153], and to the extent that it can reduce systemic iron, may also mitigate iron-catalyzed formation of ROS. In cultured rat liver epithelial cells, curcumin reduced iron-dependent oxidative stress and iron toxicity without blocking iron uptake or bioavailability [154]. The chemical properties of curcumin are consistent with iron chelator activity [149], and our laboratory has recently observed that liver cells treated with curcumin exhibit hallmarks of iron depletion, including decreases in the iron storage protein ferritin, increases in TfR1, and activation of iron regulatory proteins [152]. Curcumin also acts as an iron chelator in vivo, particularly in the setting of mild iron deficiency [152, 153]. Under these conditions, dietary curcumin exerted profound effects on systemic iron, inducing a decline in hematocrit, hemoglobin, serum iron and transferrin saturation, the appearance of hypochromic red blood cells, and decreases in spleen and liver iron content [153]. Curcumin also repressed synthesis of hepcidin, a peptide that plays a central role in regulation of systemic iron balance [153]. Consistent with these reports, curcumin reduced non-transferrin-bound iron in a mouse model of β-thallassemia [155]. In another animal study, curcumin was shown to attenuate iron-induced oxidative damage caused by Fe-NTA, a kidney-specific carcinogen [41]. Feeding mice a 1.0% curcumin diet for 4 weeks abolished the formation of 4-hydroxy-2-nonenal (HNE)-modified protein adducts, 8-hydroxy-2′-deoxyguanosine (8-OHdG), and protein reactive carbonyl in renal proximal tubules of Fe-NTA-treated animals [41]. Both antioxidant and iron chelating properties may underlie the protective effect of curcumin against these early markers of renal carcinogenesis, and support its potential as a cancer chemopreventive agent [41].

12.7 Curcumin as an Anticancer Agent in Clinical Trials

Despite curcumin's relatively short biological half-life and low bioavailability after oral administration, multiple clinical trials investigating its potential chemotherapeutic activities are currently underway. To date, these studies have largely focused on pancreatic cancer, multiple myeloma, and colorectal cancer (Table 12.1). Centuries of use have proven that curcumin is remarkably well tolerated without apparent

Table 12.1 Clinical trials of curcumin and cancer

Trial	Status of trial	Site	Disease target	Objective	Clinical trials.gov identifier or reference
Curcumin in preventing colorectal cancer in patients undergoing colorectal endoscopy or colorectal surgery	Recruiting	University Hospitals, Leicester	Colorectal cancer	Phase I: prevention-determining levels of curcumin in colorectal tissue, blood, and urine	NCT00973869
Curcumin with preoperative capecitabine and radiation therapy followed by surgery for rectal cancer	Recruiting	M.D. Anderson Cancer Center	Rectal cancer	Phase II: clinical benefit of curcumin with standard radiation therapy and capecitabine	NCT00745134
Curcumin in preventing colon cancer in smokers with aberrant crypt foci	Active, not recruiting	Chao Family Comprehensive Cancer Center; National Cancer Institute (NCI)	Colorectal cancer	Phase II: prevention – effect of curcumin on biomarkers of colon cancer	NCT00365209
Trial of curcumin in advanced pancreatic cancer	Recruiting	M.D. Anderson Cancer Center	Pancreatic cancer	Phase II: clinical benefit of curcumin (8 g/day)	NCT00094445
Gemcitabine with curcumin for pancreatic cancer	Recruiting	Rambam Health Care Campus	Pancreatic cancer	Phase II: clinical benefit of curcumin plus gemcitabine	NCT00192842
Phase III trial of gemcitabine, curcumin and celebrex in patients with metastatic colon cancer	Not yet recruiting	Tel-Aviv Sourasky Medical Center, Israel	Colon neoplasm	Phase III: clinical benefits of gemcitabine plus curcumin and celebrex	NCT00295035
Curcumin for the prevention of colon cancer	Completed	University of Michigan Cancer Center; National Cancer Institute (NCI)	Colorectal cancer	Phase I: pharmacokinetics, MTD in healthy subjects	NCT00027495

Phase III trial of gemcitabine, curcumin and celebrex in patients with advance or inoperable pancreatic cancer	Recruiting	Tel-Aviv Sourasky Medical Center	Pancreatic cancer	Phase III: clinical benefits of gemcitabine plus curcumin curcumin and celebrex in pancreatic cancer	NCT00486460
Use of curcumin for treatment of intestinal adenomas in familial adenomatous polyposis (FAP)	Recruiting	University of Puerto Rico	Familial adenomatous polyposis and colorectal cancer	Effect of curcumin on formation of multiple adenomatous colorectal polyps	NCT00927485
Sulindac and plant compounds in preventing colon cancer	Suspended	Rockefeller University	Colorectal cancer	Prevention – effect of curcumin on biomarkers of colon epithelial cell turnover	NCT00003365
Curcumin (diferuloylmethane derivative) with or without bioperine in patients with multiple myeloma	Completed	M.D. Anderson Cancer Center	Multiple myeloma	Clinical benefit of curcumin alone or combined (2 g po bid) with bioperine	NCT00113841
Curcumin for the chemoprevention of colorectal cancer	Recruiting	University of Pennsylvania	Colorectal cancer	Phase II: effect of curcumin (4 g po daily) on cell proliferation in colorectal mucosa of subjects with previously resected adenomatous colonic polyps	NCT00118989
Trial of curcumin in cutaneous T-cell lymphoma patients	Not yet recruiting	M.D. Anderson Cancer Center	Cutaneous T-cell lymphoma	Phase II: safety and efficacy of curcumin (~8 g/day, orally) to decrease the size of lesions and/or decrease itching in patients with cutaneous T-cell lymphoma	NCT00969085

(continued)

Table 12.1 (continued)

Trial	Status of trial	Site	Disease target	Objective	Clinical trials.gov identifier or reference
Pilot study of curcumin formulation and ashwagandha extract in advanced osteosarcoma	Recruiting	Tata Memorial Hospital, India	Osteosarcoma	Phase I/II: pharmacokinetics	NCT00689195
Curcumin for prevention of oral mucositis in children chemotherapy	Recruiting	Hadassah Medical Organization, Israel	Chemotherapy induced mucositis	Phase III: effect of curcumin mouthwash in prevention and reduction of mucosal injury	NCT00475683

toxicity in animals [156] or humans [157], even at high doses. Phase I studies have shown that curcumin can be administered safely at oral doses of up to 8 g/day [14, 15, 158, 159]; furthermore, curcumin administered to patients with high risk or premalignant lesions showed improvement in some cases, including one patient with bladder cancer, two patients with intestinal metaplasia of the stomach, one patient with uterine cervical intraepithelial neoplasm (CIN) and two patients with Bowen's disease, a neoplastic skin disease [158].

An independent dose-escalation study on 15 patients with advanced colorectal cancer was conducted in the UK [160] in which patients consumed a single daily dose of 440–2,200 mg of curcuma long extract, equivalent to 36–180 mg curcumin, for up to 4 months. The treatment was well tolerated and there was no dose-limiting toxicity. Consistent with earlier reports, neither curcumin nor its metabolites were detected in the plasma, blood cells or blood lipoproteins after a month of daily treatment. Curcumin was not detected in the urine, but both curcumin and curcumin sulfate were present in feces. Stable disease was observed in five patients receiving 2–4 months of therapy.

Importantly, several effects of curcumin on redox-sensitive pathways observed in vitro have been recapitulated in these human trials, underscoring the critical role of these pathways in curcumin's biological activity. For example, a phase II study in patients with advanced pancreatic cancer again showed that despite its limited absorption, curcumin had biological activity as evidenced by the antitumor effects noted in two patients and by effects on cytokine levels and on NF-κB, COX-2, and pSTAT3 [161]. The majority of the patients showed downregulation of NF-κB and COX-2 after treatment with curcumin [161], consistent with earlier preclinical studies [126, 129, 162]. In most patients, curcumin treatment also led to a decrease in constitutive pSTAT3 activation, in agreement with earlier studies in human multiple myeloma cells showing that curcumin can modulate pSTAT3 activation [163].

12.8 Detrimental Effects of Curcumin Redox Activity

Although the redox activity of curcumin is linked to many of its beneficial cancer preventative and cancer therapeutic effects [8, 54, 68], recent evidence suggests that the redox activity of curcumin can also be detrimental in selected tissue environments.

For example, in a transgenic mouse model of lung cancer that expresses the human Ki-*ras*^{G12C} allele, treatment with the lung tumor promoter butylated hydroxytoluene (BHT) and dietary curcumin caused a statistically significant increase in tumor multiplicity when compared to BHT alone [164]. Enhanced oxidative damage was observed in the lung tissue after only a week of curcumin administration in the diet [164]. Therefore, the early prooxidant effect may account for the tumor-promoting effects of curcumin in lung tissue [164]. Curcumin may also inhibit the activity of chemotherapeutic agents, as it was shown that it inhibited camptothecin-induced death of cultured breast cancer cells through inhibition of ROS generation, and attenuated cyclophosphamide-induced breast tumor regression in

nude mice [99]. Thus, screening patients for future trials of curcumin-based chemoprevention trials may warrant the exclusion of those patients who have a history of smoking [164] as well as exclusion of breast cancer patients receiving selected chemotherapeutic agents [99].

12.9 Conclusions

Curcumin exhibits complex redox activity and can act as both a pro-and antioxidant. These effects derive from inherent properties of the molecule itself, as well as its ability to affect multiple signaling pathways that respond to or mediate redox balance. Both pro- and antioxidant activity contribute to the salutatory effects of curcumin as a chemopreventive and chemotherapeutic agent. For example, the ability of curcumin to active ARE/EpRE-driven expression of cytoprotective phase 2 enzymes may contribute to its chemopreventive effect; conversely, curcumin's ability to exacerbate oxidative stress in cancer cells, which are inherently more oxidatively stressed than normal cells, may contribute to curcumin's activity as an anticancer agent.

Initial results demonstrating activation of redox-modulated pathways such as NF-kB in patients treated with curcumin [162] indicate that redox activities of curcumin and its metabolites are a central aspect of its activity in vivo, and are not just an in vitro curiosity. Future studies will be required to test the relationship between clinical outcome and expression of markers such as NF-kB, pSTAT3, and COX-2 in patients treated with curcumin. It may also be interesting to test whether NF-kB-dependent induction of systemic cytokines can be used as a surrogate marker of curcumin chemopreventive activity in longer-term chemoprevention trials. Finally, despite the intense current interest in curcumin as a well-tolerated chemotherapeutic agent, studies suggesting that the redox activity of curcumin can be detrimental as well as beneficial, and in oxygen-rich tissues may lead to the promotion rather than prevention of malignant change, should not be ignored. The redox properties of curcumin are a key feature of its activity and should be borne in mind when designing clinical trials of this promising anticancer agent.

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Chapter 13
Oxidative Stress and Pancreatic Cancer

Joseph J. Cullen

Abstract Pancreatic cancer is the fourth most common cause of cancer death in the USA with greater than 33,000 fatal cases annually in the USA alone, and an overall 5-year survival rate of less than 5%. The relationship between reactive oxygen species (ROS), antioxidants, and possible treatments for pancreatic cancer is discussed in this review. ROS are generated during normal aerobic metabolism, and are produced at increased levels during various forms of oxidative stress. Our laboratory studies have demonstrated that in pancreatic cancer, ROS at high concentrations are cytotoxic, and at low concentrations are involved in the regulation of several key physiological processes such as cell proliferation. As seen in other cancers, enforced expression of antioxidant enzymes that scavenge ROS have profound effects in altering the malignant phenotype of pancreatic cancer both in vitro and in vivo. In addition to the major antioxidant enzymes, food-derived polyphenols with antioxidant properties can also inhibit pancreatic cancer growth. The opposite is also true; ROS at high concentrations are cytotoxic to pancreatic cancer cells and this mechanism forms the basis of recent potential treatments.

J.J. Cullen, MD (✉)
Department of Surgery, University of Iowa College of Medicine,
Iowa City, IA 52242, USA

Department of Radiation Oncology, University of Iowa College of Medicine,
Iowa City, IA 52242, USA

The Holden Comprehensive Cancer Center, and Veterans Affairs
Medical Center, Iowa City, IA, USA

University of Iowa Hospitals and Clinics, 4605 JCP, Iowa City, IA 52242, USA
e-mail: joseph-cullen@uiowa.edu

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Abbreviations

2-DG	2-Deoxy-D-glucose
CuZnSOD	Copper- and zinc-containing superoxide dismutase
EcSOD	Extracellular SOD
GPx	Glutathione peroxidase
GPx	Cytosolic glutathione peroxidase
LOX	Lipoxygenase
METC	Mitochondrial electron transport chain
MnSOD	Manganese superoxide dismutase
NOX	NADPH oxidase
NQO1, DT-diaphorase, EC 1.6.99.2	NADPH:quinone oxidoreductase
PanIN	Pancreatic intraepithelial neoplasia
ROS	Reactive oxygen species
SOD	Superoxide dismutase

13.1 Unique Features of Pancreatic Cancer

MacMillan-Crow and colleagues have demonstrated nearly 100-fold increases in nitrotyrosine, a footprint of peroxynitrite which is formed by the reaction of $O_2^{\cdot-}$ with $NO^{\cdot}$, in human pancreatic cancer specimens compared to normal pancreas [1]. Our recent findings add to this by demonstrating that pancreatic cancer cell lines have increased intracellular $O_2^{\cdot-}$ compared to other cell lines, as measured by hydroethidine fluorescence [2]. Therefore, increased oxidative stress may exert harmful effects including damage to DNA and cell membranes, leading to carcinogenesis and tumor progression. The increased oxidative stress associated with the induction of pancreatic cancer correlates well with other studies in various model systems that demonstrate that ROS can initiate and promote carcinogenesis as well as findings that antioxidants in general inhibit malignant transformation [3, 4].

Although the majority of pancreatic cancers are not found in the setting of chronic pancreatitis, chronic pancreatitis is one of the most significant risk factors for pancreatic cancer yet identified [5–7]. Pancreatic adenocarcinoma in the setting of chronic pancreatitis is usually characterized by marked fibrosis and high expression of extracellular matrix proteins [8, 9]. The extracellular matrix proteins, fibronectin and laminin, have been shown to stimulate NADPH oxidase activity and increase intracellular ROS in pancreatic cancer cells [10]. Activation of the Nox4 isoform in pancreatic cancer cells to increase ROS levels is a common mechanism by which both extracellular matrix proteins and growth factors stimulate growth.

Another unique feature of pancreatic cancer is the presence of *K-ras* mutations which have been identified in up to 95% of pancreatic cancers, implying their critical role in their molecular pathogenesis [11, 12]. Recently, Qian and colleagues have

shown that the expression of *K-ras* in an immortalized human pancreatic ductal epithelial cell line originally derived from normal pancreatic ductal epithelium induced the formation of carcinoma in mice [13]. These cells also showed increased activation of the mitogen-activated protein kinase, AKT, and NF-κB pathways [13].

Increases in intracellular production of ROS in pancreatic cancers, due to local oxidative stress from chronic pancreatitis, extracellular matrix proteins, or *K-ras* mutations, may influence downstream propagation of mitogenic signaling. Recent studies demonstrate that fibroblasts transfected with the viral *ras* oncogene have increased superoxide ($O_2^{\cdot-}$) production, and the generated $O_2^{\cdot-}$ may act as a second messenger molecule to promote cell proliferation [14]. Based on these observations, it is hypothesized that *ras* activates the NADPH oxidase (NOX) system to produce ROS that leads to cell proliferation. This hypothesis is strengthened by studies of Vaquero and colleagues using pancreatic cancer cells deficient in mitochondrial electron transport chains (METCs) [15]. Their studies demonstrated that pancreatic cancer cells express many nonphagocytic NOX isoforms and that ROS generated by activation of these nonmitochondrial NOX are prosurvival, antiapoptotic factors [15]. Additionally, NOX4 antisense or inhibiting ROS by other approaches stimulates apoptosis in pancreatic cancer cells [15]. Similar results have been found in *ras*-transformed human keratinocytes [16] where increased $O_2^{\cdot-}$ levels were seen. The increased levels of $O_2^{\cdot-}$ could be blocked efficiently by superoxide dismutase (SOD). Most interestingly, these results showed that SOD was enough to kill *ras*-transformed cells, while it did not kill any of the other cancer types examined [16]. As mentioned, K-ras may activate NADPH oxidase, which may be a Rac1-dependent mechanism. This Rac1 activation of NADPH oxidase is a key source of superoxide. DNA microarray analysis and RT-PCR have demonstrated that Rac1 is also upregulated in pancreatic cancer [17]. Using a chemical rac inhibitor, NSC23766 [18], human pancreatic cancer cells with mutant K-*ras* (MIA PaCa-2), decreased superoxide levels, and inhibited in vitro growth (Fig. 13.1). These results suggest that the activation of Rac1-dependent superoxide generation leads to pancreatic cancer cell proliferation. In pancreatic cancer, inhibition of Rac1 may be a potential therapeutic target.

13.2 Scavenging ROS with Antioxidants Inhibits Pancreatic Cancer Growth

There are three major types of primary intracellular antioxidant enzymes in mammalian cells – SOD, catalase, and peroxidase, of which glutathione peroxidase (GPx) is the most prominent [19]. The SODs convert $O_2^{\cdot-}$ into H_2O_2, while the catalases and peroxidases convert H_2O_2 into water. In this way, two toxic species, $O_2^{\cdot-}$ and H_2O_2, are converted to the harmless product water. An important feature of these enzymes is that they are highly compartmentalized. Extracellular SOD (EcSOD) is the only isoform of SOD that is expressed extracellularly, while manganese superoxide dismutase (MnSOD) is localized in the mitochondria, copper- and zinc-containing

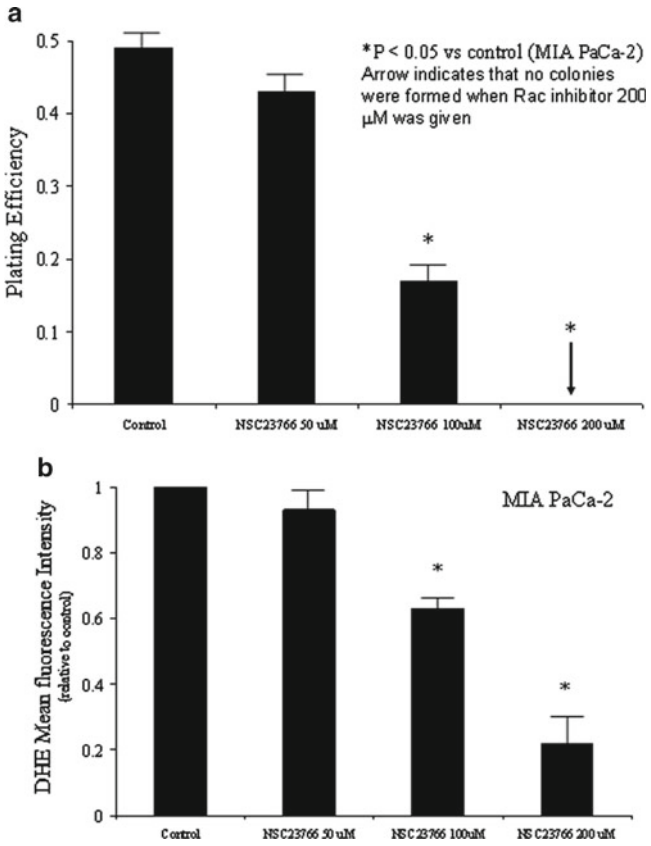

Fig. 13.1 (**a**) Plating efficiency was decreased with NSC23766 in MIAPaCa-2 cells. MIAPaCa-2 cells were seeded at 5×10^5 cells/plate overnight until attached, treated with the Rac chemical inhibitor and were then trypsinized and plated for clonogenic survival. Chemical inhibition of rac resulted in a dose-dependent decrease in clonogenic survival. Each point represents the mean values, $n = 3$. $^*P < 0.05$ vs. controls (no treatment). (**b**) Intracellular hydroethidine fluorescence decreased in MIA PaCa-2 cells after treatment with NSC23766. Intracellular superoxide levels as measured by DHE decreased significantly 48 h after treatment with the chemical rac inhibitor. $^*P < 0.05$ vs. control (no treatment) means ± SEM, $n = 3$

superoxide dismutase (CuZnSOD) in the cytoplasm and nucleus, catalase in peroxisomes and cytoplasm, and GPx in many subcellular compartments.

As with most other solid tumors, human pancreatic cancer has low levels of antioxidant enzymes [20–22]. Immunohistochemistry demonstrated that MnSOD, CuZnSOD, catalase, and GPx protein are decreased in human pancreatic carcinoma specimens when compared to normal human pancreas and MnSOD protein in pancreatic cancer was further decreased compared to chronic pancreatitis. In addition, Western blots, enzyme activity, and enzyme activity gels for MnSOD, CuZnSOD, catalase, and GPx in normal human pancreas and in various human pancreatic

cancer cell lines demonstrated decreased levels of MnSOD immunoreactive protein as well as activity compared to normal human pancreas [20–22]. The metastatic pancreatic cancer cell line had increased levels of immunoreactive protein and activity compared to normal human pancreas. MnSOD levels correlated well with increased rates of tumor cell proliferation as determined by cell-doubling time. Thus, as with most other solid tumors, pancreatic cancer and primary pancreatic cancer cell lines have lowered levels of MnSOD compared to normal pancreas, and this lowered MnSOD correlated well with increased cell proliferation.

As mentioned previously, the levels of antioxidant enzymes in metastatic pancreatic cancer may differ when compared to the primary tumor [20–22]. MnSOD, CuZnSOD, and GPx protein and activity were increased in pancreatic cancer ascites and metastatic pancreatic tumor cells compared to the primary pancreatic cancer cell line [23]. The ascites and metastatic tumor cells had decreased cell growth, plating efficiency, and growth in soft agar when compared to the primary pancreatic cancer cells, but the pancreatic cancer ascites cells had increased cell growth in 4% and 1% O_2 concentrations in vitro and more rapid growth in vivo. Thus, metastatic pancreatic cancer is associated with changes in the content and activity of antioxidant enzymes with an associated change in growth characteristics depending on the O_2 concentrations.

If SOD is important in pancreatic cancer, then normalization of the levels of SOD could result in reversal of at least part of the cancer cell phenotype. Enforced expression of MnSOD in pancreatic cancer cells was accomplished by infection of an adenoviral vector construct containing the cDNA for MnSOD [20–22, 24]; MnSOD overexpression was shown to suppress the in vitro malignant phenotype of human pancreatic cancer cells. In addition, an inverse relationship was observed, as cell growth and plating efficiency decreased with increasing amounts of the Ad*MnSOD* construct. In addition, intratumoral injections of the same adenoviral vector containing the cDNA for *MnSOD* suppress growth in established pancreatic tumors [24]. Multiple injections of the adenoviral *MnSOD* construct further inhibited tumor cell growth and extended survival.

Overexpression of SOD in other various forms may also reverse the pancreatic cancer cell phenotype. CuZnSOD levels are also lowered [20–22], while EcSOD is undetectable in pancreatic cancer [25]. Overexpression of MnSOD, CuZnSOD, and EcSOD was accomplished by infecting pancreatic cancer cells with adenoviral vectors containing the cDNA for the individual SODs. As predicted, increased SOD activity decreased superoxide levels and increased hydrogen peroxide levels. In vitro cell growth characteristics of pancreatic cancer were reversed with CuZnSOD overexpression having the greatest effect, while inhibiting endogenous SOD with siRNA increased superoxide levels and promoted tumor growth [25]. Of the three SODs, tumors grew the slowest and survival was increased the greatest in nude mice injected with the Ad*EcSOD* construct.

These findings are consistent with the hypothesis that *ras* activates the NADPH oxidase system to produce ROS that leads to cell proliferation. Thus, nonmitochondrial sources of superoxide may be prosurvival in pancreatic cancer, and scavenging plasma membrane-generated superoxide with EcSOD and CuZnSOD may prove

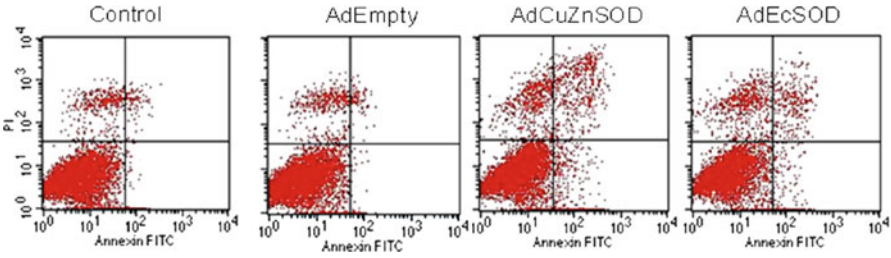

Fig. 13.2 Representative plots of the analysis are shown with necrotic cells in the *upper left quadrant*, late apoptotic/necrotic cells in the *upper right quadrant*, viable cells in the *lower left quadrant* and apoptotic cells in the *lower right quadrant*. MIA PaCa-2 cells (controls) were infected with Adempty, Ad*CuZnSOD*, and Ad*EcSOD* at 100 MOI and Annexin/PI and flow cytometry performed 96 h later

beneficial for suppression of pancreatic cancer growth. To evaluate whether scavenging superoxide from the plasma membrane inhibits tumor growth by inducing cell death, we performed flow cytometry on MIA PaCa-2 cells infected with the AdEmpty, Ad*CuZnSOD*, or Ad*EcSOD* vectors and then stained with AnnexinV-FITC and propidium iodide. Representative data plots are shown in Fig. 13.2. In control cells at 96 h, the apoptotic fraction was 2.6 ± 0.2%, 1.5 ± 0.2% with AdEmpty, 4.6 ± 0.8% with Ad*CuZnSOD*, and 4.4 ± 1.3% with Ad*EcSOD*. The results from the Annexin/PI studies suggest that apoptotic cell death may not play a significant role in the growth inhibition seen with SOD overexpression.

The effectors of MnSOD pancreatic tumor suppression could potentially be O_2^- and H_2O_2. These molecules are logical since they are the substrate and product of SOD. In addition, previous work in a breast cancer cell line had demonstrated a reversal of the tumor suppressive effect of MnSOD by overexpression of GPx [26]. Since GPx can also remove lipid hydroperoxides, it was unclear if the effect on GPx reversing the MnSOD-induced tumor suppression was due to H_2O_2 or other hydroperoxides. To gain further insight into MnSOD inhibition of pancreatic tumor growth, the effects of increasing GPx on the pancreatic cancer phenotype were investigated. Cytosolic glutathione peroxidase (GPx) is a selenoprotein that converts H_2O_2 into water and requires several secondary enzymes and cofactors to function at high efficiency.

As stated before, reduction in antioxidant enzymes may also lead to malignant transformation. As mentioned previously, cytoplasmic values of GPx1 protein in human pancreatic cancers were decreased when compared to normal pancreas [20] as was GPx activity [21, 22]. Although overexpression of GPx reversed the tumor suppressive effect of MnSOD in breast cancer [26] (presumably by removal of H_2O_2), infection with the combination of the Ad*GPx* and Ad*MnSOD* vectors had the greatest effect on growth inhibition both in vitro and in vivo and also increased animal survival [27]. To study the converse, antisense oligonucleotides for GPx was used. 20-mer sequences were synthesized with the start codon of the gene in question in the center of the oligo. Oligos were then made by shifting the start sequence 5′

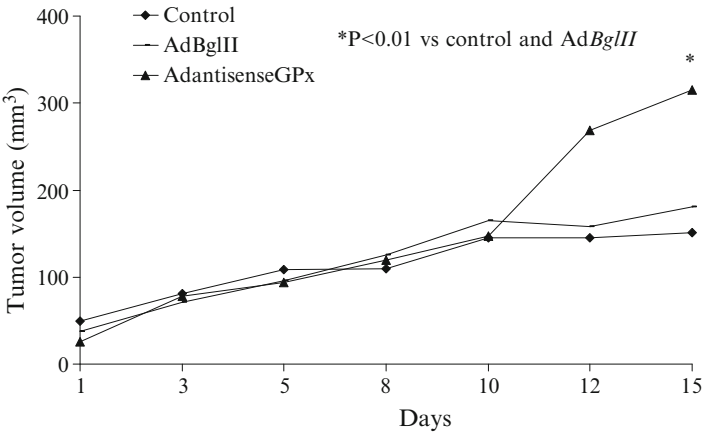

Fig. 13.3 Ad*antisenseGPx* injections increased MIA PaCa-2 tumor growth in nude mice when studied out to 15 days postinjection. The Ad*antisenseGPx* group had significantly faster tumor growth when compared to the parental cell line or mice treated with Ad*BglII* ($P<0.05$, $n=5–8$/group). Approximately 1×10^9 plaque-forming units (PFU) (100 μL) of the Ad*antisenseGPx* or Ad*BglII* constructs were delivered through two injection sites in the tumor by means of a 25-gauge needle attached to a 1-cm^3 tuberculin syringe. This was defined as day 1 of the experiment

and then 3′ from the original sequence. For all oligos, the first five and last five bases were phosphothiolated for stability. The oligos were demonstrated to inhibited GPx immunoreactive protein activity and were then placed in an adenovirus construct for delivery to established human pancreatic tumors grown in nude mice. Tumor volume was compared among three groups using the data from days 1 through 15, and the linear mixed model suggested that the interaction between day and group was highly significant ($P<0.0001$), that is, there is a difference among the groups in the slopes for tumor volume over time (Fig. 13.3). Animals with tumors treated with the Ad*antisenseGPx* vector had the fastest in vivo growth when compared to the controls and Ad*BglII* groups ($P<0.05$).

There are at least five GPx isoenzymes found in mammals with two members of this family, GPx1 and PhGPx, widely studied in relation to antioxidant cytoprotection and lipoxygenase regulation. GPx1 and PhGPx both contain the rare amino acid selenium (Se)-cysteine at the active site and the active selenocysteine residue participates in the two-electron reduction of peroxides to alcohols. However, the two enzymes differ in functional size, subcellular distribution, and amino acid sequence [28]. They also exhibit differences in peroxide reactivity with GPx1, a major enzyme responsible for removing H_2O_2 and organic hydroperoxides while PhGPx is the only known intracellular antioxidant enzyme that can directly reduce peroxidized phospholipid and cholesterol in membranes. Therefore, PhGPx can reduce lipid hydroperoxides and is thought to contribute to the enzymatic defenses against oxidative damage to the mitochondrial membrane [29]. PhGPx is synthesized as a long form, highly expressed in mitochondria, and a short form that is highly expressed in nuclei, endoplasmic reticulum, and cytosol, but not in mitochondria [30]. Pancreatic cancer

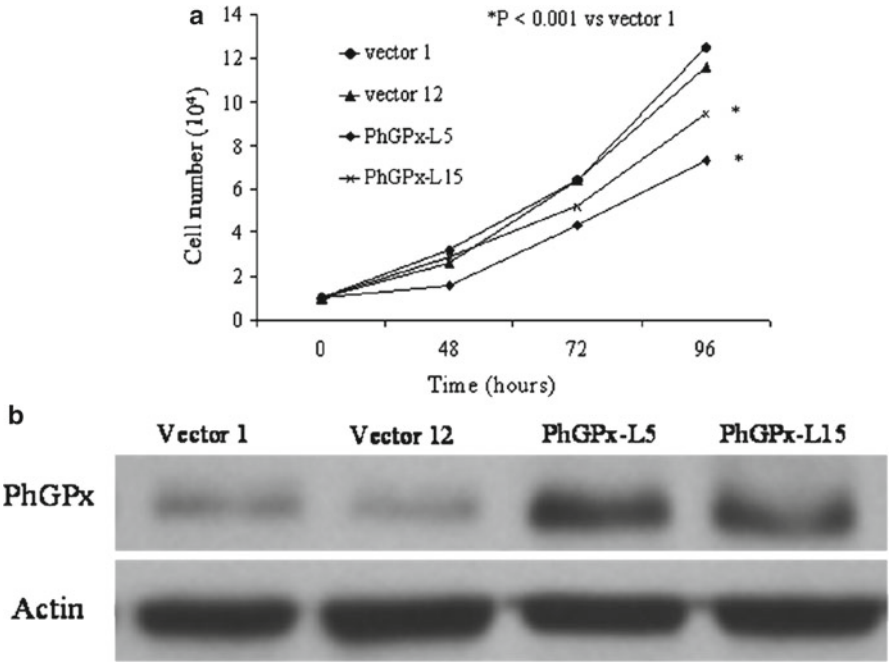

Fig. 13.4 Stable transfection of PhGPx-L form inhibits in vitro growth. (**a**) Cell growth. MIA PaCa-2 cells stably transfected with *PhGPx* demonstrate reductions in cell growth. No significant changes were seen with vector 1 and vector 12 compared with parental cells (wild-type). Mean in vitro cell growth is shown. Each point was determined in triplicate. $^*P < 0.01$ vs. vector 1. (**b**) Western blotting analysis for PhGPx. Total protein was electrophoresed in a 12.5% SDS-polyacrylamide running gel and a 5% stacking gel. After blocking in 20% fetal bovine serum for 1 h, the sheets were washed and then treated with polyclonal rabbit-anti-human antibodies to PhGPx. MIA PaCa-2 cells were transfected with the pcDNA3 plasmid containing a sense human PhGPx cDNA that was cloned into the Kpn1 and EcoR1 site and controlled by the human CMV promoter. The transfection was performed with Lipofectamine

cell lines were found to have diminished levels of PhGPx-immunoreactive protein compared with normal human pancreas [31]. Overexpression of various adenoviral PhGPx vectors resulted in 80–95% in vitro growth inhibition, while the PhGPx adenoviral vector targeted to the mitochondria significantly inhibited pancreatic cancer tumor growth [31]. Ad*PhGPx-L* form injected into preestablished pancreatic tumors in nude mice inhibited growth and increased survival [31]. In addition, pancreatic cancer cells stably transfected with *PhGPx-L form* demonstrated increased PhGPx protein and slower in vitro growth (Fig. 13.4). Thus, removal of lipid hydroperoxides had a beneficial effect in inhibiting growth of pancreatic cancer.

The work with antioxidant enzymes that scavenge lipid hydroperoxides added additional insight into pancreatic carcinogenesis. It has been known since the 1940s that dietary fat influences the postinitiation phase of carcinogenesis [32]. In human epidemiological studies, protective factors, such as vitamins C and E, β-carotene,

glutathione, selenium, and high-fiber diets, have been found to be associated with a lower risk of pancreatic cancer. Epidemiologic and animal studies have linked pancreatic cancer growth with high fat intake, particularly unsaturated fats [33]. Unsaturated fats in particular enhance carcinogen-induced pancreatic carcinogenesis in both hamsters and rats [34]. In rats given multiple doses of azaserine, a carcinogen used to produce pancreatic tumors in rats, highly unsaturated fatty acids significantly increased the pancreatic neoplasm incidence during postinitiation stage [35]. Similar findings were reported in hamsters when a semisynthetic diet, high in corn oil, significantly increased the incidence and number of pancreatic tumors induced by *N*-nitroso-bis (2-oxopropyl) amine [36]. After initiation of this protocol, hamsters fed a high-fat diet significantly enhanced pancreatic carcinogenesis. It has been postulated that the underlying mechanism might be related to increased intermediates of lipid peroxidation, which can increase the susceptibility of cellular DNA damage by certain carcinogens. Oxidation of unsaturated fatty acids is the result of normal metabolism, physical activity, ionizing irradiation, and metabolites of various chemicals, drugs, and foods. It is well known that reactive oxygen species (ROS) are generated during lipid peroxidation processes or arise directly from arachidonic or linoleic acid hydroperoxide decomposition. Furthermore, alkoxyl and peroxyl radicals, as well as reactive aldehydes, can be formed from polyunsaturated fatty acid hydroperoxides. Damage to DNA by ROS is frequently postulated to cause mutations that are associated with the initiation and progression of human cancers. Lipid hydroperoxides play a dual role in peroxidation since they are both products of peroxidation and substrates for further initiation reactions. The cleavage of the O–O bond of lipid peroxides by ferrous iron in a Fenton-like reaction generates alkoxyl radicals ($LO^{\cdot}$), which are able to start new peroxidative chains. In fact, following the formation of some hydroperoxides, peroxidation proceeds at a faster rate by secondary initiation or branching, as long as iron, reducing equivalents, oxygen, and polyunsaturated lipids are available. These chain reactions then subside via several termination reactions.

In addition to lipid peroxidation, arachidonic acid release occurs in many kinds of oxidative stress and has been shown to be a mitogenic signal in pancreatic cancer. The two ω-6 polyunsaturated essential fatty acids, arachidonic and linoleic acid are substrates for the lipoxygenase (LOX) enzymatic pathway. If arachidonic acid release is important in pancreatic cancer cell proliferation, exogenous addition of arachidonic acid should stimulate growth. MIA PaCa-2 human pancreatic cancer cells incubated with MiaPaCa2 cells (1×10^4/well) were grown in DMEM supplemented with 10% FBS modified by different concentrations of arachidonic acid (0, 1, 4, 8, 16, and 32 μM) for 4 days and growth was determined. There was a dose-dependent increase in growth at 4 days with increased concentrations of arachidonic acid (Fig. 13.5a). As mentioned, arachidonic acid is a substrate for the LOX pathway. Lipoxygenases produce 5(S)-, 12(S)-, and 15(S)-HPETE and then 5(S)-, 12(S)-, and 15(S)-HETE, respectively. Thus, the addition of arachidonic acid should lead to increases in HETEs, which stimulate pancreatic cancer cell proliferation. In addition to the increase in growth with the addition of arachidonic acid at 4 days, there was a concomitant increase in 12(S)-HETE as measured by enzyme immunoassay

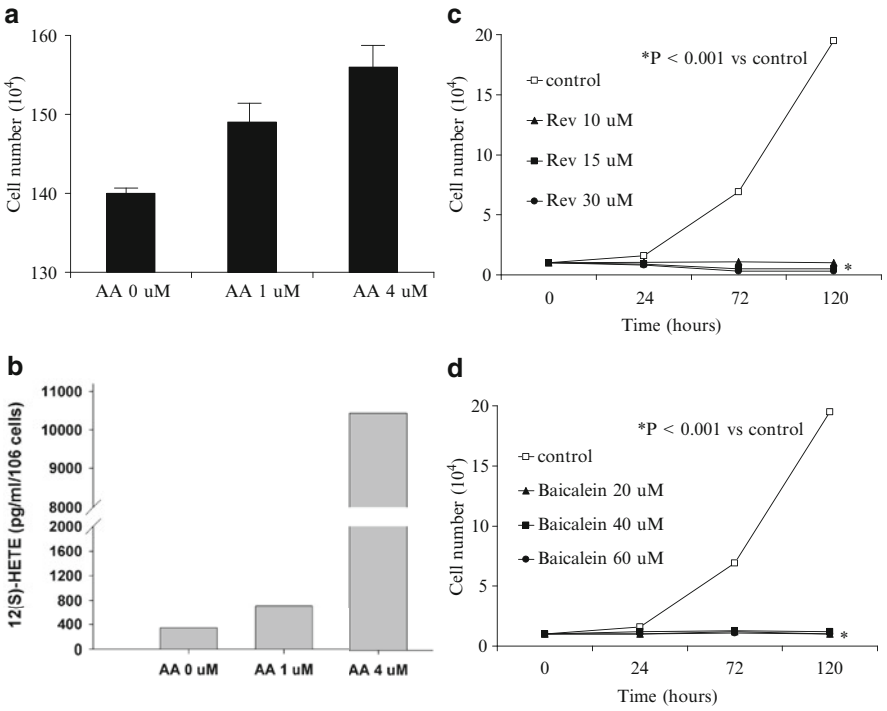

Fig. 13.5 (**a**) MIA PaCa-2 cells incubated with arachidonic acid (AA) (0, 1, and 4 μM) demonstrated significant dose-dependent increases in growth. Mean in vitro cell growth of MIA PaCa-2 cells are shown. Each point represents the mean values, $N=3$. (**b**) Stimulation of 12(S)-HETE production by arachidonic acid. MIA PaCa-2 cells were incubated with arachidonic acid (AA) (0, 1, and 4 μM) for 4 days and 12(S)-HETE measured by enzyme immunoassay. 12(S)-HETE production increased over 25-fold with the addition of 4 μM arachidonic acid. Values are mean 12(S)-HETE in pg/ml/10^6 cells. (**c**) The 5-LOX inhibitor Rev-5901 (1.0–7.5 μM) inhibits proliferation of MIA PaCa-2 human pancreatic cancer cell. (**d**) The 12-LOX inhibitor baicalein (1.0–7.5 μM) inhibits proliferation of MIA PaCa-2 pancreatic cancer cells. Mean in vitro cell growth of MIA PaCa-2 cells is shown. Each point represents the mean values, $N=3$. $^*P<0.001$ vs. control

(Fig. 13.5b). Furthermore, the LOX metabolites 5-HETE and 12-HETE stimulate cancer growth through activation of p44/42 mitogen-activated protein kinase and PI3/Akt kinase pathways [37]. Several studies demonstrate the importance of LOX in regulating human pancreatic cancer development and growth. 5-LOX and 12-LOX mRNA and proteins are expressed in all human pancreatic cancer cell lines but not in normal pancreatic ductal cells [38]. If the LOX pathway is important for pancreatic cancer cell proliferation, then inhibition of these pathways should inhibit growth. Preliminary studies demonstrate that Rev-5901, a 5-LOX inhibitor, and baicalein, a 12-LOX inhibitor, have dramatic effects on inhibiting proliferation of MIA PaCa-2 human pancreatic cancer cells (Fig. 13.5c, d). In addition, the 12-LOX inhibitor decreased the production of 12(S)-HETE as measured by enzyme immunoassay.

In untreated MIA PaCa-2 pancreatic cancer cells, 12(S)-HETE production was measured at 1,746 pg/ml/10^6 cells. Treatment with baicalein (10 μM) decreased 12(S)-HETE levels to 814 pg/ml/10^6 cells. This decrease in 12(S)-HETE was reversed when cells were treated with both baicalein and arachidonic acid (1 μM) whereupon 12(S)-HETE levels increased to 1,737 pg/ml/10^6 cells.

Polyphenolic compounds, found in some fruits and vegetables, may also have beneficial effects in pancreatic cancer. These compounds possess antioxidant, anti-inflammatory, cytotoxic, and antimutagenic properties both in vitro and in vivo. The flavonoid quercetin decreased primary tumor growth, increased apoptosis, and prevented metastases in a mouse model of pancreatic cancer [39]. Quercitin and the nonflavonoid *trans*-resveratrol markedly enhanced apoptosis, mitochondrial depolarization, and cytochrome *c* release. NF-κB activity was also inhibited by both quercetin and *trans*-resveratrol [39]. Further studies on the antioxidant mechanisms of these compounds in pancreatic cancer could have important implications for dietary therapy in pancreatic cancer prevention.

13.3 Increases in ROS Induce Cytotoxicity in Pancreatic Cancer

So far our studies demonstrate that antioxidants induce a nonapoptotic inhibition of cell proliferation in pancreatic cancer (Fig. 13.2). Pancreatic cancer is an aggressive disease and numerous strategies to increase ROS levels to induce cell killing without normal tissue damage seem to offer the most promise.

One of the potential strategies is to exploit the increased levels of NAD(P)H:Quinone oxidoreductase (NQO1) in pancreatic cancer. NADPH:quinone oxidoreductase (NQO1, DT-diaphorase, EC 1.6.99.2), a homodimeric, ubiquitous, cytosolic, and membrane flavoprotein, is considered to be a deactivation enzyme because it catalyzes the two-electron reduction of quinones, including membrane ubiquinone [40]. This reaction prevents the one-electron reduction of quinones by cytochrome P450 reductase and other flavoproteins that would redox cycle with molecular oxygen to generate $O_2^{\cdot-}$. NQO1 has been shown to redox couple with and reduce membrane ubiquinone and also plays a role as an antioxidant enzyme and generates antioxidant forms of ubiquinone and α-tocopherol during oxidative stress [41, 42]. Microarrays have demonstrated that there is a tenfold upregulation of NQO1 in pancreatic cancer when compared to normal pancreas [43]. Immunohistochemistry of resected pancreatic specimens demonstrated an increased immunoreactivity for NQO1 in pancreatic cancer and pancreatic intraepithelial neoplasia (PanIN) specimens vs. normal human pancreas [44]. Immunocytochemistry and western immunoblots demonstrated increased immunoreactivity in pancreatic cancer cells when compared to a near normal immortalized human pancreatic ductal epithelial cell line and a colonic epithelial cell line [44]. The NQO1 properties of catalyzing bioactivation of antitumor quinones and high expression in pancreatic cancer make it a principal target in therapeutic strategies to design chemotherapeutic agents. Streptonigrin,

a compound known to cause redox cycling in the presence of NQO1, decreased clonogenic survival and decreased anchorage-independent growth in soft agar [44]. Streptonigrin had little effect on cell lines with absent or reduced levels of NQO1. The effects of streptonigrin were reversed in pancreatic cancer cells pretreated with dicumarol, a known inhibitor of NQO1.

Another potential strategy to exploit in pancreatic cancer is the fact that cancer cells demonstrate increased intracellular hydroperoxide production [45]. The increased intracellular hydroperoxide production may be caused by a defect in mitochondrial respiration. Therapeutic interventions designed to inhibit glucose metabolism and hydroperoxide detoxification combined with manipulations that increase METC-mediated oxidative energy metabolism and prooxidant production could hypothetically preferentially kill pancreatic tumor cells via metabolic oxidative stress.

Although it is not possible to deprive cells of glucose in vivo, it is possible to treat tumor-bearing animals and humans with 2-deoxy-D-glucose (2-DG), a relatively nontoxic analog of glucose that competes with glucose for uptake via the glucose transporters as well as being phosphorylated by hexokinase at the entry point to glycolysis.

In human pancreatic cancer cells, 2-DG and glucose deprivation induced cytotoxicity and disruptions in thiol metabolism in a time-dependent manner [46]. In nude mice with heterotopic pancreatic tumors, the combination of 2-DG and irradiation resulted in the greatest inhibition of tumor growth and extended survival. These results support the hypothesis that 2-DG exposure causes cytotoxicity in human pancreatic cancer cells via disruptions in thiol metabolism resulting from metabolic oxidative stress.

Therapeutic interventions designed to increase METC-mediated oxidative energy metabolism and prooxidant production will also preferentially kill pancreatic tumor cells via metabolic oxidative stress. Dicumarol is a naturally occurring anticoagulant derived from coumarin that induces cytotoxicty and oxidative stress in human pancreatic cancer cells [20–22]. Although dicumarol has been used as an inhibitor of the two-electron reductase NAD(P)H:quinone oxidoreductase (NQO1), dicumarol is also thought to affect quinone-mediated electron transfer reactions in the mitochondria, leading to the production of superoxide and hydrogen peroxide [47]. Dicumarol, with the addition of METC blockers, decreased clonogenic cell survival in human pancreatic cancer cells and increased superoxide levels. Dicumarol with the METC blocker antimycin A decreased clonogenic survival and increased superoxide levels in cells with functional mitochondria but had little effect on cancer cells without functional mitochondria. Overexpression of MnSOD and mitochondrial-targeted catalase with adenoviral vectors reversed the dicumarol-induced cytotoxicity and reversed fluorescence of the oxidation-sensitive probe [47]. Thus, therapeutic interventions that increase METC-mediated oxidative energy metabolism and prooxidant production preferentially killed pancreatic tumor cells.

Our laboratory then combined therapeutic interventions designed to inhibit glucose metabolism and hydroperoxide detoxification (2-DG) with manipulations that increase METC oxidative stress (dicumarol) in pancreatic tumor cells. We hypothesized that dicumarol and 2-DG would act additively by increasing cytotoxicity and

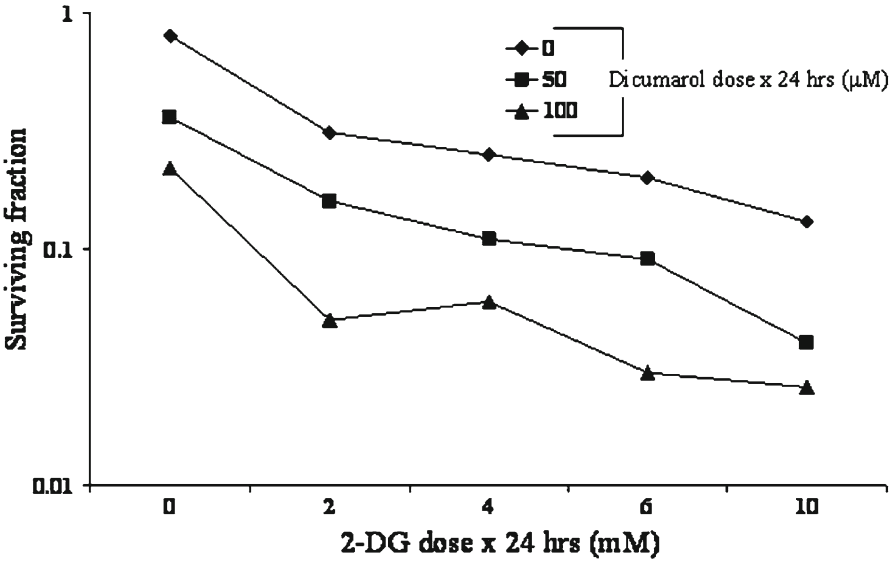

Fig. 13.6 Dicumarol acts additively with 2-DG by decreasing pancreatic cancer clonogenic cell survival in a time-dependent and dose-dependent manner. A clonogenic assay was performed on MIA-PaCa-2 cells treated with dicumarol (0, 50, and 100 mM) for 24 h with and without 2-DG (0, 2, 4, 6, and 10 mM)

oxidative stress of pancreatic cancer cells. In the human pancreatic cancer cell line MIA PaCa-2, dicumarol (50 and 100 μM), and 2-DG (4, 6, and 10 mM) decreased clonogenic survival 30–99%, and resulted in increased total glutathione as well as glutathione disulfide. In fact, dicumarol acts additively with 2-DG by decreasing pancreatic cancer clonogenic cell survival in a time-dependent and dose-dependent manner (Fig. 13.6). Clonogenic assays on MIA-PaCa-2 cells treated with dicumarol (0, 50, and 100 μM) for 24 h with and without 2-DG (0, 2, 4, 6, and 10 mM) once again demonstrated decreased survival when cells were treated with either dicumarol or 2-DG alone. However, the combination of dicumarol and 2-DG further increased cytotoxicity resulting in decreased clonogenic survival in all groups. The combination of dicumarol and 2-DG also increased total glutathione content as well as GSSG. In established orthotopic pancreatic tumors in nude mice, intratumoral injections of dicumarol (100 μM) alone and 2-DG (i.p., 0.5 g/kg q.o.d×2 weeks) alone slowed tumor growth and extended survival, while the combination of dicumarol and 2-DG had the greatest effect in inhibiting tumor growth and increasing survival (Fig. 13.7).

These data support the hypothesis that dicumarol and 2-DG act additively to induce cell killing via a mechanism involving oxidative stress.

Ascorbate (vitamin C, $AscH^-$) is one of the early unorthodox therapies for cancer. The evidence for use of ascorbate in cancer treatment falls into two categories: clinical data on dose concentration relationships and laboratory data describing potential cell toxicity with high concentrations of ascorbate in vitro. Clinical data show that

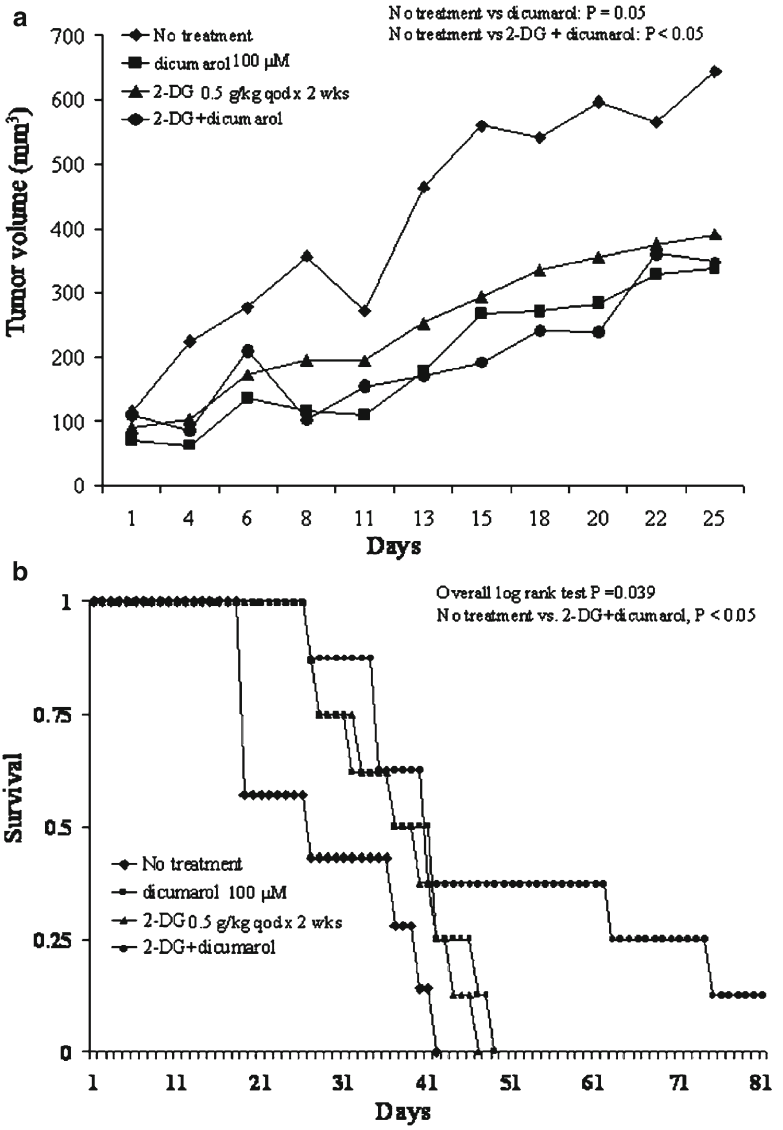

Fig. 13.7 (**a**) Single intratumoral injections of dicumarol (100 mM) + 2-DG (0.5 g/kg q.o.d × 2 weeks) decreased MIA-PaCa-2 tumor growth in nude mice when studied out to 25 days postinjection. Dicumarol alone treated group (100 mM) decreased tumor volume ($P = 0.05$) compared to no treatment. Mice receiving both dicumarol and 2-DG exhibited a 1.8-fold decrease in tumor growth over no treatment. Day 25: Median tumor volume 644 mm3 in control tumors vs. 347 mm^3 in tumors with one intratumoral injection of dicumarol (100 μM) and six every other day i.p. injections of 2-DG (0.5 g/kg). Means, $N = 8$/group. (**b**). Dicumarol + 2-DG increased survival in pancreatic tumor xenografts. Kaplan–Meier plots of estimated survival after injection of MIA PaCa-2 tumors in nude mice. Single dose intratumoral injections of dicumarol (100 μM) + 2-DG (0.5 g/kg i.p. × 6 doses) resulted in increased time to sacrifice when compared with control group ($P < 0.05$)

when ascorbate is given orally, fasting plasma concentrations are tightly controlled at <100 μM [48]. In contrast, when 1.25 g of ascorbate are administered intravenously, concentrations as high as 1 mM are achieved. Some clinicians have infused more than 10 g of ascorbate in cancer patients and achieved plasma concentrations of 1–5 mM [49]. Thus, it is clear that intravenous administration of ascorbate can yield very high plasma levels, while oral treatment does not.

Pharmacologic ascorbic acid concentrations have been shown to selectively kill some cancer cell types. Chen et al. measured cell death in ten cancer and four normal cell types using 1-h exposures to pharmacological ascorbate [50, 51]. Normal cells were unaffected by 20 mM ascorbate whereas five cancer cell lines had EC_{50} values of <4 mM, a concentration achievable by intravenous administration. In addition, cell death was independent of metal chelators, but dependent on formation of H_2O_2. H_2O_2 generation was dependent on ascorbate concentration, incubation time; [H_2O_2] displayed a linear increase with [$AscH^-$] and it increased as a quadratic function of ascorbate radical, ascorbate being an electron donor to O_2 to form H_2O_2 [52].

To determine if ascorbate would inhibit pancreatic cancer in vivo growth, MIA PaCa-2 tumor cells (2×10^6) were delivered subcutaneously into the flank region of nude mice and allowed to grow until they reached 3 mm in greatest dimension (~10 days), at which time they were randomly assigned to a treatment group. This was defined as day 1 of the experiment. The animals were randomized to receive either ascorbate (4 g/kg) or osmotically equivalent i.p. saline as a control (1 M) given to mice i.p. every day for 2 weeks. 4 g/kg i.p. ascorbate resulted in blood concentration from baseline of 40 μM to peaks of 40 mM while tumor extracellular fluid increased to peaks of 20 mM for up to 3 h [52]. The primary outcomes of interest were tumor growth over time. Tumor size (mm^3) was periodically measured throughout the experiments, resulting in repeated measurements across time for each mouse. Linear mixed effects regression models were used to estimate and compare the group-specific tumor growth curves. The observed tumor volumes for all mice are plotted over time in Fig. 13.8. This work has been confirmed by Chen et al. demonstrating that pancreatic carcinoma (Pan02) cells had decreased growth rates in mice treated with ascorbate 4 g/kg twice daily [51]. In addition, a phase 1 clinical trial of intravenous ascorbic acid in advanced malignancy demonstrated that high dose ascorbic acid was well tolerated in this patient population [53]. Thus, high dose ascorbate therapy may be another therapeutic strategy to increase prooxidant production to preferentially kill pancreatic tumor cells via metabolic oxidative stress.

13.4 Summary

The prognosis of pancreatic cancer is grim. Surgical resection of the primary tumor remains the only potentially curative treatment for pancreatic cancer with dismal 5-year survival rates of <5% [54]. In pancreatic cancer, ROS are generated by activation of membrane nonmitochondrial NADPH oxidase. ROS at low levels are pro-survival factors that are necessary for pancreatic cancer cell proliferation. Inhibiting the production of ROS or scavenging ROS by enforced expression of antioxidant

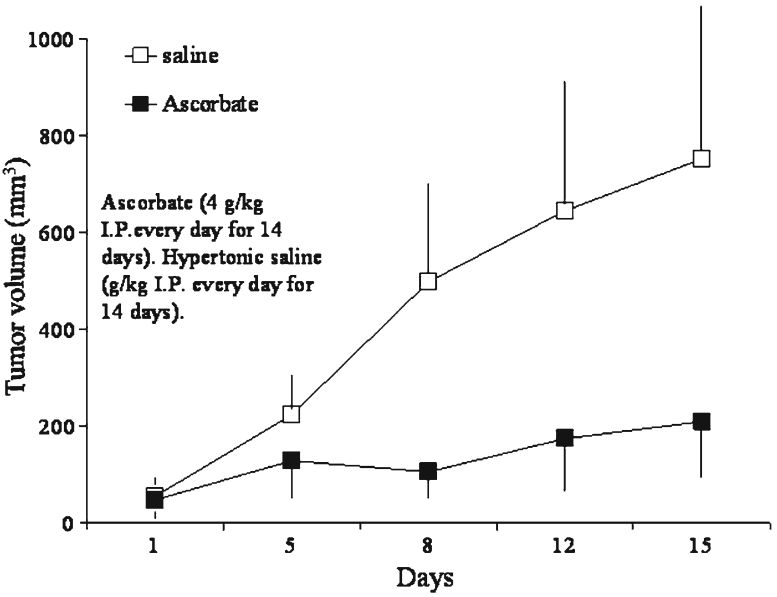

Fig. 13.8 Ascorbate decreased tumor growth in nude mice. The group of animals that received ascorbate (4 g/kg/day, i.p.) had significantly slower rate of tumor growth when compared to the controls receiving osmotically equivalent saline ($P<0.0001$, $n=5–8$/group). Ascorbate (4 g/kg, i.p.) was given every day for 14 days beginning on day 1 of the experiment. On day 15 there was a 3.5-fold decrease in tumor size in animals receiving ascorbate when compared to controls

enzymes have profound effects in altering the malignant phenotype of pancreatic cancer both in vitro and in vivo. ROS at high concentrations are cytotoxic to pancreatic cancer cells. Thus, a more effective strategy to exploit in pancreatic cancer is the fact that increased intracellular hydroperoxide production may be caused by a defect in mitochondrial respiration. Therapeutic interventions designed to increase prooxidant production could preferentially kill pancreatic tumor cells via metabolic oxidative stress. With the lack of treatment options for pancreatic cancer, clinical application of increasing levels of prooxidant may offer benefits to these patients where survival is measured in months.

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Chapter 14
Oxidative Stress and Photodynamic Therapy for Prostate Cancer

Kevin L. Du, Jarod C. Finlay, Timothy C. Zhu, and Theresa M. Busch

Abstract Photodynamic therapy (PDT) takes advantage of the generation of reactive oxygen species and subsequent oxidative effects to generate focal tissue damage. It involves the local activation of a chemical photosensitizer by a specific light wavelength corresponding to the absorption characteristics of the photosensitizer. The excited photosensitizer then transfers its energy to oxygen within the tissue, thereby creating the cytotoxic oxygen species. PDT is an established treatment modality for cancer, and its feasibility has been demonstrated for a variety of cancer types. Here, we briefly detail the known mechanisms of the photodynamic reaction, with particular attention to the role of reactive oxygen species in PDT-mediated cell death. We then describe the application of PDT in recent clinical studies for the treatment of prostate cancer.

14.1 Oxidative Stress in Photodynamic Therapy

The generation of reactive oxygen species occurs as a result of many biological processes, primarily related to aerobic metabolism, and these reactive species are incorporated into regulatory processes including redox signaling and immunologic response. These processes are important for the maintenance of homeostasis. Oxidative stress, which is the excess production of reactive oxygen species, has been demonstrated to cause cell death via apoptotic and necrotic mechanisms. Photodynamic therapy (PDT) utilizes an oxygen-dependent photochemical reaction to generate singlet oxygen, thereby taking advantage of the damaging properties

K.L. Du, MD, PhD • J.C. Finlay, PhD • T.C. Zhu, PhD • T.M. Busch, PhD (✉)
Department of Radiation Oncology, Hospital of the University of Pennsylvania,
University of Pennsylvania School of Medicine,
Philadelphia, PA 19104, USA
e-mail: buschtm@mail.med.upenn.edu

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of reactive oxygen species to induce localized cell death. This oxidative effect of PDT has been demonstrated to be effective for the treatment of a variety of malignant and premalignant lesions [1, 2]. The first step of PDT is the activation of a chemical photosensitizer by a specific light wavelength corresponding to the absorption characteristics of the photosensitizer. This results in the excitation of the photosensitizer from a ground state to an excited singlet state. This then undergoes a transition to an excited triplet state that interacts with molecular oxygen ($^3O_2$), generating singlet oxygen ($^1O_2$) that reacts with nearby molecules. Since the radius of interaction for singlet oxygen is <0.2 μm and the half-life is short, <0.04 μs [3], only cells that are in the immediate vicinity of the area of activated photosensitizer are damaged. Therefore, the region of tissue damage induced by PDT is determined primarily by the distribution of light and photosensitizer in the tissue, and the transport and diffusion of singlet oxygen plays a negligible role.

PDT induces tumor damage via cellular, vascular and immunological mechanisms. Intracellular cytotoxic mechanisms include stress responses, apoptotic, necrotic, and/or autophagic cell death [4]. Vascular damage can induce vasoocclusion and hypoxia, subsequently leading to tumor cell death [5–8], as well as can cooperate with other aspects of host response to trigger activation of innate and/or adaptive immune reactions [9]. Accordingly, tumor cell death may result from the cytotoxic action of singlet oxygen directly on tumor cells during illumination, and/or as an indirect effect consequent to vascular occlusion, inflammation, and activation of host immune response. As mentioned above, because of the localized and short effect of singlet oxygen, the type of damage mediated by PDT is largely dependent on the location of the photosensitizer at the time of excitation. Factors affecting drug location include the time interval between drug administration and initiation of light delivery, as well as properties of the photosensitizer itself, e.g. lipophilicity and charge, which can determine how the photosensitizer distributes among compartments of the cell or within the vasculature. More recent efforts have been made to customize drug delivery systems using liposomal, antibody, peptide, or nanoparticle strategies for targeted localization of the photosensitizer [10–13]. In all, the nature of the cytotoxic effects of PDT are, therefore, dependent on the photosensitizer used and its dose, the drug–light interval, the total dose of light administered, the light fluence rate, and levels of preexisting and treatment-induced tumor cell hypoxia.

14.2 Direct Cytotoxicity

14.2.1 Considerations: Effects of the Local Microenvironment

In PDT, direct cell cytotoxicity to tumor cells and host tissue in the treatment field is mediated by the action of reactive oxygen species produced by the photochemical reaction. The extent and distribution of damage is necessarily, therefore, a function of the local distributions of photosensitizer, oxygen and light, the presence of all of which are necessary to produce singlet oxygen. However, the distribution of all of

these factors can be highly heterogeneous within and between tumors [14–17]. Furthermore, PDT will affect local concentrations. Photosensitizer itself can be destroyed by reactive oxygen species (photobleaching), reducing its concentration as treatment progresses. The photochemical reaction can consume oxygen in the tissues being treated, thereby limiting the continued production of singlet oxygen. Moreover, an interdependence exists among these factors, and, for example, changes in the oxygenation status of a tumor has the potential to affect light distribution at specific wavelengths because oxygenated hemoglobin absorbs less light at 630 nm than does deoxygenated hemoglobin [18].

In order to increase the direct cytotoxicity of PDT, including its damage of tumor and vascular cells, various approaches have been studied to compensate for the heterogeneities and dynamics in key PDT components. Adjusting the delivered light dose based on individualized assessment of photosensitizer distributions within a tumor serves to improve the consistency in response [19]. Oxygen levels during PDT can be better maintained if the partial pressure of oxygen in the treated tissues is increased through hyperoxia or hyperbaric oxygen breathing [20, 21] or if the fluence rate of illumination is lowered [15, 22], allowing greater opportunity for replenishment of tissue oxygen through the blood supply to keep pace with its consumption by the photochemical process. Alternatively, fractionation of the treatment light into rapid on/off cycles can also allow for recovery of tumor oxygenation during treatment due to the intermittent nature of light delivery [23, 24]. In an effort to improve the individualized delivery of PDT, approaches toward measuring local concentrations of photosensitizer or oxygen during PDT, as well as tissue optical properties, for the purpose of real-time, adaptable treatment dosimetry are in various stages of development [25].

Even when considering the direct cellular target of PDT-created damage some heterogeneity exists. Within the cell itself, the localization pattern of photosensitizer will determine subcellular targets and can include various cellular structures. Cytotoxicity can result from loss of cellular, mitochondrial, endoplasmic reticulum, Golgi apparatus, or lysosomal membrane integrity [26–28], depending on photosensitizer localization, although DNA damage induced by PDT is limited because most photosensitizers do not demonstrate significant localization to the nucleus [29]. In addition to inflicting direct cytotoxicity on a specific target, oxidative stress can also initiate a series of cellular mechanisms and signaling pathways [30, 31] that the cell induces in an attempt to recover from cellular damage, and which can ultimately lead to cell death. The mechanisms of cell death that are induced by PDT can include both apoptosis and necrosis, and PDT can also trigger autophagy as a cytotoxic event.

14.2.2 Apoptosis

Previous studies have demonstrated that photosensitizers that localize to the mitochondria are very potent inducers of apoptosis [28, 32]. Photosensitizers exhibiting mitochondrial localization include those that are hydrophobic or negatively charged,

such as silicon phthalocyanine Pc4 [33], benzoporphyrin derivative monoacid ring A (BPD) [34], 2-devinyl-2-(1-hexyloxyethyl)pyropheophorbide (HPPH) [35], and Photofrin [36], as well as aminolevulinic (ALA)-induced protoporphyrin IX (PpIX) [37]. The apoptotic pathway is initiated by the permeabilization of the mitochondrial membrane, either by direct interaction with the membrane, or by activation of upstream mediators. The mechanisms for this have primarily been demonstrated by in vitro experiments in cell culture. Photodynamic treatment of cells with photosensitizers that localize to the mitochondria can result in a rapid release of cytochrome c into the cytoplasm [38–40]. Upon its release into the cytoplasm, cytochrome c interacts with apoptotic protease activating factor 1 (APAF1) and dATP in the formation of the apoptosome, subsequently leading to generation of caspase 9, which cleaves procaspase 3 to caspase 3. Caspase 3 triggers a cascade of caspases that cleave proteins such as poly ADP-ribose polymerase (PARP), DNA-dependent protein kinase (DNA-PK), and inhibitor of caspase-activated DNase (ICAD), leading to DNA fragmentation [4, 41].

Independent of direct PDT-induced mitochondrial damage, activation of apoptosis via an extrinsic pathway can also occur. In the latter pathway, the apoptotic signal initiates from the cell surface when death ligands such as Fas ligand (FasL), tumor necrosis factor α (TNF-α), and TNF-related apoptosis-inducing ligand (TRAIL) bind to their receptors. This ultimately leads to the formation and activation of a dimer of procaspase-8 and procaspase-10, which then triggers the caspase cascade through caspases 3 and 7 [29]. Examples of PDT-mediated activation of this pathway include apoptosis induced by Rose Bengal in HL-60 cells [42] and by Photofrin in NR-S1 tumors [43].

The role of Bcl-2 family members of pro- and antiapoptotic genes has been examined in PDT by several investigators. In particular, transfection of the antiapoptotic genes Bcl-2 [44, 45] or Bcl-X_L [46] into cell lines were demonstrated in in vitro experiments to inhibit PDT-mediated apoptosis, although this finding may depend upon the conditions employed for PDT photosensitization [44]. It has also been reported that Bcl-2 transfection can lead to increases in the stability of bax, a proapoptotic Bcl-2 family member, which contributes to a finding that apoptosis increased, not decreased, after PDT of Bcl-2-transfected cells [47]. However, a protective role for Bcl-2 in PDT-mediated responses has been confirmed through in vitro investigations with a Bcl-2 antagonist. In these studies, PDT-mediated apoptosis increased and cell viability decreased when PDT was performed of cells preincubated in HA14-1, a small molecule Bcl-2 antagonist [48].

While it has clearly been demonstrated that apoptotic pathways are induced by PDT, inhibition of these pathways may, at least in some instances, be compensated for by alternative mechanisms of cell death. Xue et al. reported that caspase 3 deficient breast cancer cells failed to demonstrate PARP cleavage and DNA fragmentation after PDT [49]. The apoptotic response could be recovered with the reintroduction of caspase 3 into this cell line; however, the extent of PDT-induced cell death was similar between the caspase 3 negative and caspase 3 positive cell lines regardless of the absence or recovery of the apoptotic response. Thus, the data suggest a role for cell death pathways other than apoptosis after PDT of these cell lines.

14.2.3 Autophagy

There have been recent reports that PDT may induce autophagy as a mechanism of cell death [50]. In particular, Buytaert et al. evaluated PDT in wild-type and apoptosis-deficient Bax/Bak knockout cells [27]. The apoptosis deficient cells underwent nonapoptotic cell death displaying characteristics of autophagy. The inhibition of autophagy by wortmannin reduced PDT-mediated cell death in these knockout cells, demonstrating that autophagy did function as a prodeath mechanism. Similarly, Xue et al. demonstrated autophagy to occur after PDT with Bax deficient and caspase-3 deficient cell lines [51]. Bcl-2 overexpression was able to protect neither against the development of autophagy nor against cell death in these cells, suggesting that cells deficient in apoptosis may alternatively die via an autophagic response.

14.2.4 Necrosis

Cell death by necrosis can be induced by PDT-generated reactive species, leading to respiratory catastrophe, ATP depletion, and loss of plasma membrane integrity [29]. This process is also accompanied by cell swelling and loss of mitochondrial membrane potential [52]. Taken together, current data suggest that cell type, photosensitizer localization, and light and photosensitizer doses all can affect the prevalence of cell death by necrosis [53–55]. For example, short incubation times that favor drug localization to the plasma membrane [52], as well as increasing light dose [56] led to necrotic as opposed to other mechanisms of cell death.

14.3 Tumor Vascular Targeting

Vascular damage occurs in cases where photosensitizer activation occurs within the endothelial cells or vascular space, resulting in direct vascular endothelial cell destruction. Vascular damage can accompany direct tumor cell damage, for example such as that found after typical Photofrin-PDT protocols [57]. Alternatively, PDT can be designed to specifically target the blood vessels by initiating illumination shortly after i.v. injection of photosensitizer, for example the approach used in PDT of age-related macular degeneration [58]. Observable effects of PDT-induced vascular damage can include thrombus formation, vasospasm, vascular leakage and occlusion, but the specific nature of these effects is dependent on the light and photosensitizer used and their respective doses [59].

Among others, Visudyne (Verteporfin; BPD) and Tookad (WST09) are photosensitizers that have been used as vascular-targeting agents in conjunction with PDT. Verteporfin-PDT is FDA-approved for treatment of age-related macular degeneration and commonly involves illumination (690 nm light) at 5 min after 10 min of drug infusion [60, 61]. Using similar protocols, Verteporfin has been

applied in vascular-targeting PDT of rodent tumors. Chen et al. [62] demonstrated a primarily intravascular localization of Verteporfin in a rat prostate model at 15 min after its intravenous administration. Light delivery at this time resulted in substantial decreases in tumor perfusion measured immediately after PDT that endured throughout the monitoring period of 60 min and led to prominent increases in tumor hypoxia. Tookad-mediated PDT of tumor vasculature was evaluated by Huang et al. [63, 64] using interstitial delivery of 763 nm light to the canine prostate simultaneous with drug infusion. One week after PDT, histopathologic sections of the prostate gland were examined. Lesions were characterized by hemorrhagic necrosis with necrotic debris at the center of the exposed regions. Remaining blood vessels exhibited fibrinous necrosis and thrombosis. Interestingly, there were some regions of PDT-induced damage that extended beyond the predicted depth of light penetration; this was speculated to be due to vasculature ablation within the primary lesions resulting in downstream hypoxia and necrosis in distal tissues.

PDT-created vascular damage can create a hypoxic tumor environment that compliments the effects of direct cell damage in causing tumor cell death, but also contributes to upregulation of cell stress proteins and angiogenic factors. For example, PDT in vitro or in vivo can induce expression of heat shock proteins [65–68] that act as chaperone proteins to sequester damaged components of the cell, glucose regulated proteins [69–71] that play a role in calcium homeostasis, and heme oxygenase proteins [72, 73] that can regulate levels of certain antioxidants. More recently, focus has been placed on PDT-induced increases in the expression of proteins associated with angiogenesis, such as vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMP), and cyclooxygenase-2 (COX-2) [74]. Combining PDT with pharmaceuticals that inhibit these molecules can improve PDT responses. For example, Avastin (bevacizumab), a humanized monoclonal antibody against VEGF, and Prinomastat, an MMP inhibitor, improved the PDT response of human tumor xenografts and murine tumors, respectively [75–77]. Celecoxib, a COX-2 inhibitor, increased PDT cytotoxicity both in vitro and in vivo. The combination of PDT and Celecoxib was associated with increases in tumor cell apoptosis in vitro, suggesting that Celecoxib could augment the direct cell effects of PDT induced by oxidative stress, while in vivo, a role for Celecoxib in decreasing PDT-induced expression of angiogenic and inflammatory mediators was found [78].

14.4 Immunogenic Response

The immune response against cancer cells has been extensively studied and is recognized to play a key role in the progression of cancer [79, 80]. Immunotherapy has become an attractive emerging paradigm for the treatment of cancer, as it would allow not only the destruction of cancer cells at the primary site, but also a systemic response that would theoretically destroy local–regional cancer cell metastases as well as distant metastases. This enhanced tumoricidal effect may assist with long-term tumor control. PDT has been demonstrated to exert a potential stimulatory effect on the immune system, which can significantly contribute to tumor

responses [9]. This stimulatory effect can be accompanied a local suppressive effect due to PDT damage of immune cells in the treatment field, but much data supports a role for PDT-induced antitumor immunity in achieving tumor control [81].

PDT results in a rapid inflammatory response at the site of treatment. This is associated with the mobilization of inflammatory and immune factors including interleukin 6 (IL-6), macrophage inflammatory protein 1/2 (MIP1/2), IL1beta, IL8, and IL10 [82–84]. There is a systemic increase and localized infiltration of neutrophils and macrophages following PDT [82, 85–88] that is essential to PDT response to certain illumination conditions [84, 89]. Studies examining the effects of depletion of inflammatory factors after PDT confirm their contribution to the PDT effect. For example, therapeutic effect was abrogated in vivo when PDT was combined with a molecule that interferes with IL-6 signaling [90].

One compelling demonstration of the importance of the immune response to the effectiveness of PDT came from mouse studies in which it was observed that immunodeficient mice implanted with a mammary sarcoma had a higher rate of tumor recurrence after PDT than did tumor-bearing immunocompetent mice treated on the same PDT protocol [91]. The rate of tumor recurrence after PDT was decreased by reconstituting the immunodeficient mice with bone marrow from immunocompetent mice. Conversely, there was an increased rate of tumor recurrence in immunocompetent mice whose bone marrow was reconstituted from the bone marrow of immunocompromised mice. Thus, these studies suggested that lymphocytes played a role in immune response to PDT.

Others have more specifically examined the role of T-cells in PDT responses. Kabingu et al. showed that local treatment with PDT could suppress the growth of tumors outside of the treatment field and that this antitumor immunity was dependent on the presence of cytotoxic (CD8+) T-cells [92]. In animals bearing multiple tumors, PDT of one tumor led to an influx of cytotoxic T cells into an untreated tumor, and the adaptive transfer of these T cells into naïve animals enabled the animals to suppress the growth of a subsequent tumor challenge. In another study, Castano et al. [93] demonstrated that memory immunity after PDT was potentiated by low dose cyclophosphamide that reduces the number of regulatory T-cells. Regulatory T-cells can suppress cytotoxic T-cells. Their findings showed that when combined with PDT low dose cyclophosphamide increased the cytolytic activity of tumor-specific T cells in the spleen and improved tumor cure rates. Furthermore, when cured animals were reinjected with tumor cells, the cells were rejected in the majority of mice and did not form tumors.

A link between PDT-induced innate cell infiltration and antitumor immunity has also been established. In a study by Kousis et al, PDT-induced cytotoxic T-cell responses were examined in animals that were chemically depleted of neutrophil activity or exhibited impaired neutrophil migration due to genetic alteration. When neutrophil involvement in PDT response was abrogated through these means, a decrease was found in T-cell infiltration of PDT-treated murine tumors, as well as in the proliferation of these T-cells [94]. It was possible to transfer to naïve mice a durable antitumor immune memory induced by PDT conditions that caused high, but not low, levels of neutrophil influx into the treated field.

14.5 PDT for Prostate Cancer

14.5.1 The Principles of Prostate PDT

There are clear advantages of PDT over other therapies for localized disease such as surgery or radiation therapy. PDT does not appear to target DNA, and has not been demonstrated to have the risk of secondary malignancies or late tissue side effects. Therefore, it is likely that repeated PDT sessions could be given for increased efficacy or for treatment of recurrent disease, with a lower likelihood of long-term morbidities as one might observe with radiation therapy. PDT, if appropriately applied, also appears to specifically damage the glandular epithelium of target tissues and therefore maintains the stromal architecture of the organ, again minimizing the risk of secondary morbidities [95, 96]. With respect to cancer treatment, PDT is an attractive therapy because there is little cross-resistance with chemotherapy or radiation therapy mechanisms [97]; thus, PDT can be considered for patients who have failed or received maximum doses of these other modalities.

The effective illumination of large tissue volumes for PDT requires use of interstitial optical fibers. Extensive experience in the treatment of prostate cancer with interstitial brachytherapy makes this disease an excellent model for the study of interstitial light delivery, which can benefit from existing techniques for placement of brachytherapy implants within solid organs. Additionally, the prostate is an attractive target for interstitial PDT due to its size, which accommodates comprehensive treatment of the entire organ. Prostate cancer is the most common visceral cancer in men in the USA [98]. Current treatments for early stage prostate cancer are radical prostatectomy, external beam radiation therapy, brachytherapy, and androgen deprivation therapy. For patients who present with recurrence or disease after radiation therapy, salvage options are limited and include radical prostatectomy [99], cryosurgery [100], or an additional course of radiation therapy. These salvage therapies carry an increased risk of morbidities [101–103], and therefore novel modalities for the treatment of recurrent prostate cancer would be of considerable benefit to expanding patient treatment options and increasing quality of life [104]. Several principles are important to be considered in the design and optimization of PDT for prostate cancer:

1. Prostate cancer is a multifocal disease [105, 106] and current imaging techniques cannot detect microscopic disease in the entire gland. Therefore, it is essential that any interstitial PDT approach provide comprehensive coverage throughout the entire gland.
2. The PDT dose must be controlled so that there is damage to the prostate epithelium but sparing of the underlying stroma, therefore maintaining the structural integrity of the prostate gland [107].
3. Normal tissues, particularly the bladder, rectum, and neurovascular bundle must be spared significant damage from the PDT dose [63].

4. An ideal salvage therapy would allow for repeated treatments with minimal additional morbidity. Therefore, a safe and minimally invasive approach for drug and light delivery must be established to conveniently retreat the prostate with PDT [64].

The effectiveness of PDT depends on the spatial and temporal interactions between photosensitizer, light and oxygen. As already described, these variables include photosensitizer type, concentration, biodistribution, the wavelength, total light dose, fluence rate, and drug-to-light interval of light delivered, tissue optical properties, and tumor oxygenation state [15, 107–122]. We, and others, have shown substantial intra- and interpatient heterogeneity in these variables in prostate glands from patients with prostate cancer [123, 124], which points to the need for individual dosimetry. The optimization of these variables is critical for the design of future PDT studies.

14.5.2 Recent Clinical Trials for Prostate PDT

Recently, several groups have published their experience with PDT for prostate cancer. Moore et al. [125], reported a series of six patients treated with *meso*-tetrahydroxyphenylchlorin (mTHPC). These patients had prostate cancer that was confined to the organ, Gleason scores were all 3 + 3, and PSA ranged from 1.9 to 15. These patients had not received prior definitive treatment for their cancer. mTHPC was administered and between 2 and 5 days later, the photosensitizer was activated by light delivered via brachytherapy catheters. Treatment was only limited to the regions of the prostate where there was biopsy proven cancer, and there was no attempt to treat the gland comprehensively. Four of the six patients had a repeat PDT session, following the finding of biopsy proven persistent disease at 3 month follow-up. PDT was well-tolerated in this study; however, one patient developed a gram-negative sepsis requiring antibiotic treatment. PSA response was variable in this study, with 8 of 10 PDT treatments resulting in PSA response range from a reduction of 67% to an increase of 133% at the post treatment nadir, which occurred between 1 and 10 months after PDT. In all patients, while necrosis and fibrosis was seen in post-PDT biopsy, there was also residual disease identified in all patients. Nevertheless, this study does demonstrate the feasibility of this approach, with demonstrated treatment effect.

Trachtenberg et al. [126], recently reported their experience using Tookad (WST09) mediated PDT in a Phase II trial for salvage therapy of recurrent prostate cancer after external beam radiation therapy. Tookad is a photosensitizer that has been demonstrated to primarily localize to the vasculature and is activated at a long wavelength (763 nm), providing greater depth of light penetration. This trial attempted to treat the entire gland via a vascular ablation approach. Twenty-eight patients were enrolled in this study, which incorporated escalating light doses of delivery. As the light dose increased, there was a greater vascular response as

measured by MRI and less residual cancer as determined by biopsy at 6 months. For patients who had large avascular lesions, which the authors defined as >60% of the prostate, all had negative biopsies at follow-up. In addition, there were patients who had much smaller avascular lesions who were also complete responders. Toxicities were significant, with two patients developing urethrorectal fisulas. PSA levels decreased initially at 1 month after PDT and, in patients with low baseline PSA, remained undetectable at 6 months, while in patients with higher baseline PSA, there was a subsequent increase in PSA by 6 months. This study demonstrated the potential for pathologic complete response over a short-term follow up (6 months) and emphasizes the need for longer term studies.

Our group at the University of Pennsylvania has performed extensive studies using Motexafin Lutetium (MLu) as a photosensitizer for prostate PDT. Motexafin Lutetium is a pentadentate aromatic metallotexaphyrin that has an absorption peak at 732 nm [127–129]. We have completed a Phase I trial of Motexafin Lutetium-mediated PDT in patients with locally recurrent prostate adenocarcinoma who have previously been treated with definitive radiotherapy. The primary end point of this trial was to determine the maximally tolerated dose and dose limiting toxicities. Secondary end points were (1) to measure Motexafin Lutetium levels in needle biopsies of the prostate pre- and post-PDT. (2) To use optical methods to measure Motexafin Lutetium fluorescence in situ in the prostate pre- and post-PDT and to correlate these results with the direct tissue measurements made in biopsies following tissue solubilization. Optical spectroscopic methods were also used to measure the optical properties and the absorption spectrum of the prostate pre- and post-PDT. (3) To calculate the percent change in Motexafin Lutetium concentration after treatment. (4) To measure clinical outcome from time of PDT salvage therapy; including clinical response, progression-free survival, and time to biochemical relapse, after Motexafin Lutetium-mediated PDT in patients with recurrent prostate cancer. (5) To use a multimodality optical instrument to monitor tissue optical properties, photosensitizer concentration, tissue blood oxygenation, and blood flow throughout treatment at a single position in the prostate. While these measurements provided some overlap with the pre- and post-PDT spectroscopic measurements, their purpose was to monitor the dynamic changes in blood flow and oxygenation continuously throughout treatment.

We enrolled 18 patients in this trial with localized recurrent prostate adenocarcinoma [130]. Nine patients had been previously treated with interstitial brachytherapy, and 8 had been treated with external beam irradiation; 1 patient was excluded after enrollment based on extracapsular extension. At the time of recurrence, the median PSA was 7.4 ng/mL (range of >0.2–13.6 ng/mL) with Gleason scores of 6 in 3 patients, 7 in 8 patients, 8 in 2 patients, 9 in 3 patients, and one patient whose biopsy specimen was too small to score. Treatment was tolerated well, although one patient at the highest light dose level developed a urethrorectal fistula. PSA levels were followed at each patient visit with the finding that at 1 day after PDT PSA levels significantly increased relative to baseline values [131], likely due to treatment induced prostatic tissue damage. This was typically followed by a rapid return of

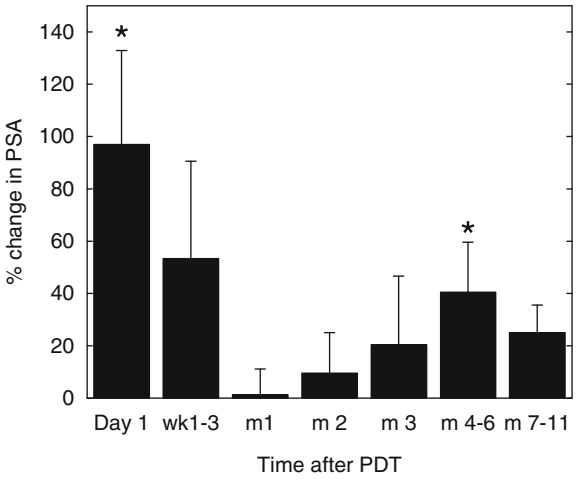

Fig. 14.1 Time course of PSA response to comprehensive treatment of the prostate with Motexafin Lutetium-mediated PDT. The percent change (mean ± SE) in PSA was calculated as $(100*(PSA_{after\ PDT} - PSA_{before\ PDT})/PSA_{before\ PDT})$ for each patient using the pre-PDT PSA value closest to the time of treatment and the post-PDT PSA value in the time frame of interest. Wk=week; m=month; "*" = $p<0.05$ for Wilcoxon signed rank test comparing post-PDT PSA values to baseline measurement in the same patient. Reprinted with permission from Clin Cancer Res. 2008 Aug 1;14(15):4869– 4876

elevated PSA to pre-PDT levels within 1–2 months, followed by a maintenance phase of stable or gradually increasing PSA values (Fig. 14.1). Yet, ultimately all patients did experience biochemical failure. In this study, the treatment-induced change in PSA level was analyzed relative to PDT dose, with PDT dose defined as the product of prostate photosensitizer concentration and the in situ measured light dose. Patients who received a high PDT dose had an increase in PSA concentration at day 1 that was ~50% greater than that found in patients treated with a low PDT dose. Patients who received high PDT dose also demonstrated a more durable PSA response. In these patients, the length of time between PDT and a nonreversible increase in PSA to a value greater than or equal to baseline was a median of 82 days, versus 43 days in patients who received lower PDT doses.

Altogether, these initial clinical studies have demonstrated that PDT for prostate cancer is feasible and that there is a treatment response. However, they have also clearly demonstrated that further refinement is required for this therapy to be consistently reliable. Our experience suggests that consistent treatment will largely be dependent on accurate understanding of dosimetric issues – in particular the interaction between photosensitizer, light and oxygen distributions, and how they affect the overall PDT dose to the prostate gland. The next section details some of our observations on this and outlines our current approach toward optimizing PDT dosimetry.

14.5.3 Optical Properties of the Prostate

Optical properties were measured in 14 of the patients that were treated in our Phase I study [123, 124], using spherical light detectors placed at fixed distances from the light source. Light delivery to one quadrant of the prostate was found to result in delivery of a measurable fluence to other quadrants as well. The dominant absorbers in the wavelength range of measurements are oxygenated and deoxygenated hemoglobin, Motexafin Lutetium, and water. At 732 nm, the absorption coefficient (μ_a) varied from 0.07 to 1.62 cm^{-1} (mean 0.37 ± 0.24 cm^{-1}) and the reduced scattering coefficient (μ_s') varied from 1.1 to 44 cm^{-1} (mean 14 ± 11 cm^{-1}). The effective attenuation coefficient (μ_{eff}), defined as $(3\mu_a \mu_s')½$ [132] varied between 0.91 and 6.7 cm^{-1} (mean 2.9 ± 0.7 cm^{-1}), corresponding to an optical penetration depth of 0.1–1.1 cm (mean 0.4 ± 0.1 cm). The maximum variation of optical properties within the same patient was larger than the standard variation of the mean in all patients. The variations within a single patient were as large as 300% for μ_a and 1,000% for μ_s'. Therefore, the optical penetration depth varied by 290% within a single patient's prostate.

Clearly, there is a significant heterogeneity of optical properties throughout the prostate gland, at this wavelength, which can contribute to variable fluence rates at any given location, and lead to inaccuracy in PDT dose deposition. This can cause overdosing, which can result in increased toxicities, and this can also cause underdosing, which can result in untreated regions of the prostate. Therefore, there is a need for an in situ dosimetry system to ensure accurate dosing of PDT in prostate, and several groups are presently working on developing such a system [16, 133–135]. Light measurements are required to determine the actual light dose deposited within the prostate gland, which includes both incident and scattered light. Modeling algorithms for PDT treatment planning should take this variation into account and, in theory, should correct for it in real-time to allow for a consistent deposition of light dose throughout the prostate.

Our group has developed a treatment planning system for an optically heterogeneous prostate gland based on the Cimmino feasibility algorithm. For this optimization, a light-fluence matrix is calculated for a heterogeneous tissue domain for each linear source at each location [16]. The initial treatment plan is calculated based on the average optical properties of our previously treated patients, assuming an optically homogeneous prostate. During treatment, this plan is modified based on real time in vivo measurements of light-fluence rate at various locations within the target tissue. The treatment time at each source point is modified based on the ratio of the prescribed light fluence and half of the maximum fluence rate measured in each quadrant of the prostate (the approximate mean fluence rate of the quadrant). The time required for this optimization for a 12 light source plan is 37 s, and for a 35 source plan is 137–148 s, and efforts are underway to shorten this calculation time. A dose–volume histogram of PDT dose demonstrates that there are significant differences between the dose distributions of the initial and optimized treatment plans, indicating the importance of accurate measurement of the optical properties

of the prostate [136]. Furthermore, recent developments demonstrate the potential to directly optimize PDT dose distribution by incorporating the heterogeneous distribution of photosensitizer and optical properties [137].

14.5.4 Photosensitizer Biodistribution Within the Prostate

Motexafin Lutetium levels in prostate biopsies were measured by fluorescence spectroscopy. In general, drug levels increased with increasing injected drug dose or decreasing drug–light interval [138]. The measurement was performed with a single optical fiber beveled to emit light at a right angle to the fiber axis. This fiber acted as the source of 465-nm excitation light and as the collector of fluorescence light emitted by the sensitizer [138]. In addition, diffuse transmission spectroscopy measurements, in which a broadband light-emitting fiber was placed in one catheter in the prostate and an isotropic detection fiber connected to a spectrograph was scanned along a parallel catheter, were used to detect the characteristic absorption of the drug [124]. Again, variability was apparent between patients at the same drug dose level, and between biopsy locations in the same prostate. There was an overall decrease in post-PDT drug levels, perhaps due to treatment-related photobleaching. When comparing in situ optical measurements of Motexafin Lutetium with direct tissue spectroscopy measurements, a linear relationship was noted between the absorption coefficient and the drug concentration in the prostate [124].

Another interesting feature is that Motexafin Lutetium levels varied by as much as fivefold over a distance of less than 1 cm within a single prostate (Fig. 14.2) [124, 138]. Others have also reported on intraprostate heterogeneity in photosensitizer distribution [17]. The degree of intraprostate variation may be, at least in part, a result of the heterogeneity of the tissue itself, which includes both normal prostate and foci of cancer. There is also possibly a vascular component, with variations in vascular density or perfusion that may limit the delivery of blood and photosensitizer to regions of the gland. Whatever the underlying cause of the variations in optical property and sensitizer heterogeneity, it is important to take them into account in treatment planning.

14.5.5 Prostate Oxygenation and Blood Flow

Tissue oxygen concentrations during PDT are influenced by blood flow and blood oxygenation, among other factors. The destruction of tumor vasculature by PDT leads to oxygen and nutrient deprivation of tumor cells and therefore can benefit response. However, vascular occlusion during PDT may also lead to tumor hypoxia during treatment, resulting in decreased efficacy. In preclinical models, both blood flow and hemoglobin oxygen saturation have demonstrated therapeutic significance for PDT [139–142]. Monitoring of blood flow and oxygenation status during

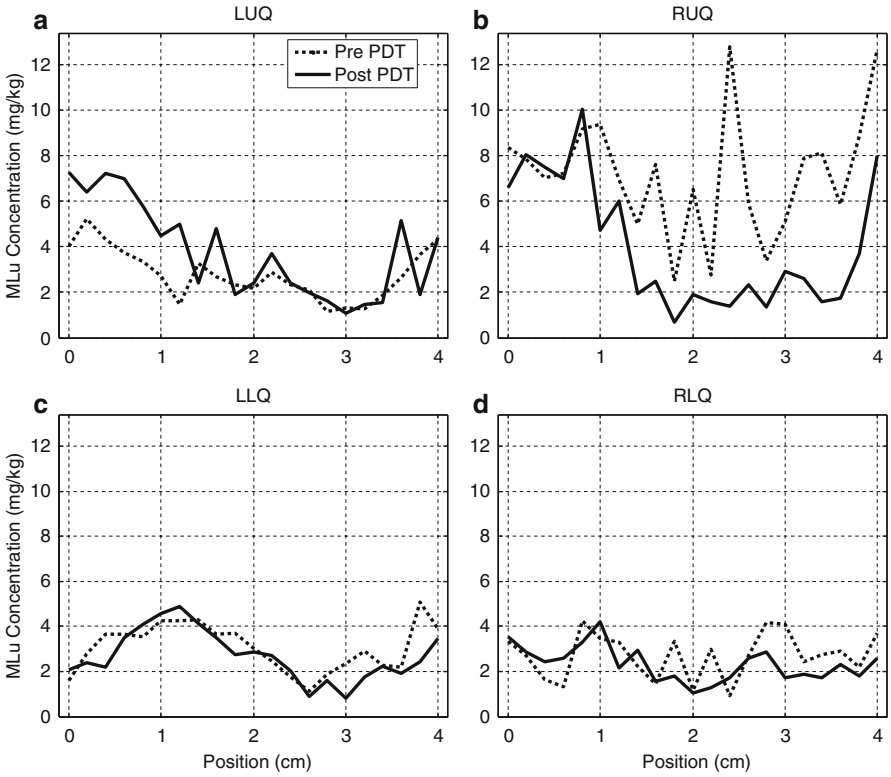

Fig. 14.2 Fluorescence profiles of the distribution of Motexafin Lutetium in the four quadrants of the prostate of a representative patient on a clinical trial of Motexafin Lutetium-PDT for prostate cancer. Plots indicate Motexafin Lutetium concentration before (dashed lines) and after (solid lines) illumination for PDT. Frames (**a**) through (**d**) depict the results from the right upper, left upper, left lower and right lower quadrants, respectively, as indicated in the panel titles. Data were corrected for optical properties as described in the source publication. Reprinted with permission from Photochemistry and Photobiology, 2006, 82:1270–1278

PDT is, therefore, of interest in our clinical trial. Using a near-infrared diffuse optical instrument combining diffuse reflectance spectroscopy (DRS), for the measurement of hemoglobin oxygenation and total hemoglobin concentration, with diffuse correlation spectroscopy (DCS), for measurement of tissue blood flow [140, 143–147], we have measured hemodynamic responses to PDT in 3 of our Phase I patients. A fiber-optic probe containing a source and detector was placed into a brachytherapy catheter in the center of each quadrant of the prostate gland, and remained in place throughout the treatment. All 3 patients demonstrated similar blood flow responses. Average post-PDT blood flow and total hemoglobin both decreased, and blood oxygen saturation remained relatively constant (Fig. 14.3). The decrease in blood flow and total hemoglobin concentration indicates a

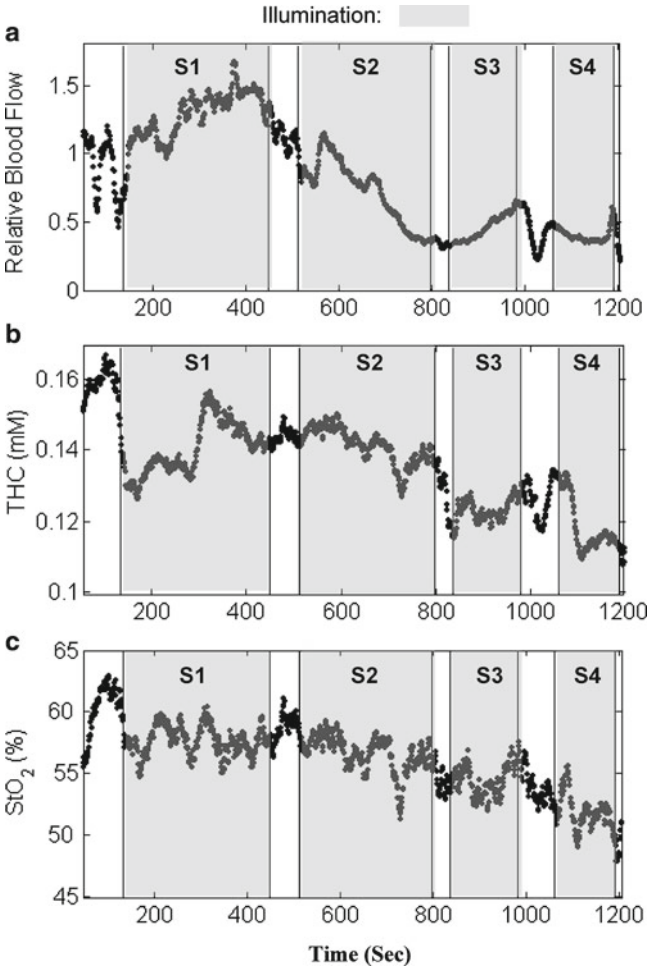

Fig. 14.3 Time course of changes in prostate hemodynamic properties during PDT of a representative patient with prostate cancer. Individual panels from top to bottom demonstrate, respectively, the dynamic changes in relative blood flow (**a**), total hemoglobin concentration (THC; **b**), and tissue blood oxygen saturation (StO_2; **c**). The patient was illuminated sequentially in four sessions (S1 to S4) until the entire gland was treated. The illumination periods are presented as shaded bars. Reprinted with permission from Photochemistry and Photobiology, 2006, 82:1279–1284

perfusion decrease, likely due to vascular destruction mediated by PDT. Intriguingly, the relatively unchanged hemoglobin oxygen saturation suggests that oxygen consumption during illumination may have been small at the site of the vasculature or balanced by other PDT-induced effects. For example, PDT-mediated cell death could potentially reduce metabolic tissue oxygen consumption. Further study will be done to relate hemodynamic responses to clinical outcome.

14.5.6 New Directions in Prostate-PDT

Overall, our initial clinical studies, as well as those of other groups, have demonstrated the potential to safely deliver PDT to the prostate gland at doses of photosensitizer and light sufficient to induce cell death within the prostate. A significant finding of these studies is the intrapatient and interpatient heterogeneity in light, photosensitizer, oxygen, and tissue optical properties within the prostate. This heterogeneity is not surprising and has been observed in other human studies of PDT that have been performed [148–152]. However, it does raise the concern that needle placement in any one individual may not correspond with consistent dose delivery and may lead to "skip" areas within the prostate gland. Given this possibility, it may be beneficial to deliver PDT to the gland in a two-phase or fractionated schedule to help ensure a uniform delivery of dose to the prostate, although to date no clinical trials have been published that test this approach.

Another potentially new direction for PDT of the prostate is focal treatment of the gland, which is an active area of investigation in cancer sites including breast cancer and central nervous system cancers. A contemporary controversy is the possibility of treating focal regions of cancer within the prostate rather than treating the gland comprehensively [153]. This is largely driven by the desire to spare patients the increased morbidity, primarily urinary dysfunction and sexual dysfunction resulting from treatment of the entire gland. Classically, this has not been an acceptable treatment for prostate cancer. Prostate cancer is frequently multifocal, and the current commonly employed diagnostic methods of biopsy and radiologic evaluation are not sensitive enough to reliably detect and map multifocal disease within the prostate. Therefore, the current standard of care for prostate cancer is the treatment of the entire prostate, with the primary goal to maximize the chances of cure. However, there is a current debate on whether focal treatment may safely be considered in carefully selected patients. In particular, these patients would ideally have biologically indolent cancers that are likely to be well-localized and have a low risk for metastasis. They would have a more extensive workup to characterize the focality of their cancer including a more comprehensive prostate biopsy and endorectal coil MRI. They would also have to accept the increased risk of a subcurative treatment and would have to follow a very close surveillance schedule to monitor for progression of their cancer.

PDT may be a suitable modality for focal treatment of prostate cancer [154, 155] as it would be readily repeatable with minimal additional morbidity, and as the initial treatment of prostate cancer with PDT would not preclude management with either surgery or radiation therapy at a later time. However, the focal treatment of prostate cancer remains an area of intense controversy.

The treatment of prostate cancer using PDT presents significant challenges, whether the treatment of focal disease of the ablation of the entire gland is considered. In both cases, the problem of delivering a predictable (and possibly nonuniform) photodynamic dose to an optically nonuniform (and unpredictable) organ requires the ability to deliver treatment tailored to the individual patient's prostate at the time of treatment. Our efforts so far have focused on tailoring the treatment to the patient's

optical properties, ensuring a consistent light dose. The cytotoxic effect, however, depends on the distributions of available sensitizer and oxygen as well. An area of ongoing research in our laboratory and others is the development of the capability to perform real-time optical measurements to determine the spatial distributions of sensitizer, oxygen (via hemoglobin saturation), and optical properties. Given this information, it should be possible to optimize not just the light dose and the photodynamic dose, but the deposited singlet oxygen dose as well. It is expected that a singlet oxygen dose-optimized treatment will lead to better outcomes, especially in patients with highly heterogeneous sensitizer distributions or variable tissue oxygenation.

14.6 Conclusions

Interstitial PDT holds an enormous potential for treating locally confined prostate cancer. Especially attractive is the potential to treat repeatedly with only mild additional morbidities. Overall, the results from early clinical trials have been encouraging. They have demonstrated that photosensitizer and light can be successfully delivered, with only mild and transient toxicities. Moreover, there is a significant clinical response with prostatic tissue inflammation and damage, and short-term biochemical responses. It remains to be seen, however, whether comprehensive treatment of the gland is clinically achievable and, of course, whether long-term PSA responses and clinical benefits are possible.

The heterogeneity observed in optical properties, drug distribution, and tissue oxygenation underscores the need for continued detailed study of the parameters that affect PDT response. Ideally, PDT will be administered via a real-time system that accounts for variations in intraprostate conditions and optimizes light delivery to specific areas of the gland.

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Chapter 15
Oxidative Stress in Prostate Cancer

Weihua Shan, Weixiong Zhong, Jamie Swanlund, and Terry D. Oberley

Abstract Prostate cancer primarily affects older men and is the second leading cause of cancer deaths in males in the USA. Known risk factors for prostate carcinogenesis include age, race, and family history, while possible risk factors include diet, lifestyle, androgens, and inflammation. At least three of these known or potential risk factors (androgens, inflammation, and age) are indirectly linked by the fact that each may result in and/or be derived from oxidative stress. Low levels of reactive oxygen species (ROS) participate in many important physiological and pathological processes in the cell, including proliferation, cell cycle progression, migration,

W. Shan, PhD
Molecular and Environmental Toxicology Center, University of Wisconsin School of Medicine and Public Health, Madison, WI USA

W. Zhong, MD, PhD
Department of Pathology and Laboratory Medicine, University of Wisconsin School of Medicine and Public Health, Madison, WI USA

Pathology and Laboratory Medicine Service, William S. Middleton Memorial Veterans Hospital , Room A-35, 2500 Overlook Terrace, Madison, WI 53705, USA

J. Swanlund, BS
Pathology and Laboratory Medicine Service, William S. Middleton Memorial Veterans Hospital, Room A-35, 2500 Overlook Terrace, Madison, WI 53705, USA

T.D. Oberley, MD, PhD (✉)
Molecular and Environmental Toxicology Center, University of Wisconsin School of Medicine and Public Health, Madison, WI USA

Department of Pathology and Laboratory Medicine, University of Wisconsin School of Medicine and Public Health , Madison, WI USA

Pathology and Laboratory Medicine Service, William S. Middleton Memorial Veterans Hospital Room A-35, 2500 Overlook Terrace, Madison, WI 53705, USA
e-mail: toberley@wisc.edu

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angiogenesis, invasion/metastasis, and inhibition of apoptosis, features that cancer cells might regulate to facilitate progression to a more aggressive phenotype. High levels of ROS/oxidative stress are capable of causing damage to various cellular constituents, including DNA, proteins, and lipids. Elevated levels of ROS, due to increased ROS production or impaired antioxidant defense systems, have been implicated in prostate carcinogenesis. Specific redox compartmental oxidation, which is believed to function as a mechanism for specificity in redox signaling and oxidative stress, is also manifested in prostate cancer. In this chapter, we present and analyze recent findings and ideas relating oxidative stress to prostate cancer and discuss their implications for future studies.

Abbreviations

ADAM	A disintegrin and metalloproteases
AE	Antioxidant enzyme
Anti-CTLA-4	Anticytotoxic T-lymphocyte-associated antigen 4
AP-1	Activator protein 1
AR	Androgen receptor
CAT	Catalase
CDCFDA	5-(and-6)-Carboxy-2′,7′-dichlorofluorescein diacetate
COX	Cyclooxygenase
CuZnSOD	Copper zinc SOD
DHE	Dihydroethidium
DPI	Diphenyliodonium
ECSOD	Extracellular superoxide dismutase
EGF	Epidermal growth factor
ERK	Extracellular regulated kinase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH/GSSG	Glutathione–glutathione disulfide couple
GST	Glutathione *S*-transferase
GSTP1	Glutathione *S*-transferase pi
H_2DCFDA	2′,7′-Dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
HGPIN	High grade prostatic intraepithelial neoplasia
HIF	Hypoxia-inducible factor
4HNE	4-Hydroxy-nonenal
$HO^{\cdot}$	Hydroxyl radical
IL1RN	Interleukin 1 receptor antagonist
MAPK	Mitogen-activated protein kinase
MEK	Extracellular signal regulated kinase
MIC1	Macrophage inhibitory cytokine-1
MMP	Matrix metalloproteinases
MnSOD/SOD2	Manganese superoxide dismutase
MnTBAP	Mn 5, 10, 15, 10-tetrakis (4-benzoic acid) porphyrin

mtDNA	Mitochondrial DNA
NAC	*N*-acetyl-cysteine
NF-κB	Nuclear factor-kappa B
$^{\cdot}NO$	Nitric oxide
NOX	NADPH oxidase
Nrf2	NF-E2-related factor 2
3NT	3-Nitro-L-tyrosine
$O_2^{\cdot-}$	Superoxide
ODP	Oxidative damage products
8OHdG	8-Hydroxy-2′-deoxyguanosine
$ONOO^-$	Peroxynitrite
PIA	Proliferative inflammatory atrophy
Prdx	Peroxiredoxin
PTEN	Phosphatase and tensin homologue
R1881	Methyltrienolone
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TLR	Toll-like receptor
TRAMP	Transgenic adenocarcinoma of the mouse prostate
Trx	Thioredoxin
TrxR	Thioredoxin reductase
VEGF	Vascular endothelial growth factor
VP	Ventral prostate

15.1 Introduction

Prostate cancer is a significant cause of death among males in the USA. Known risk factors for development of prostate cancer include age, race, and family history, while possible risk factors include diet, lifestyle, androgens, and inflammation; however, possible etiologies linking these factors are still uncertain. Oxidative stress has been proposed as an important factor in the pathogenesis of prostate cancer and several studies are consistent with this hypothesis. Additional studies are needed to mechanistically prove the role of ROS/oxidative stress in prostate cancer.

15.2 Prostate Cancer Incidence, Mortality, and Treatment

Prostate cancer is a major health problem in Western countries. This cancer is the most commonly diagnosed and second leading cause of cancer deaths in men in North America. The American Cancer Society estimated that there would be about 186,320 new cases of prostate cancer in the USA in 2008, with approximately 28,660 men potentially dying from this disease [1]. One in six men will get prostate cancer during his lifetime, and one in 35 men will die of this disease. Treatment for prostate cancer depends upon several factors including the stage and grade of the

cancer, the age of the patient, the presence of other health problems, the treatment preferred by the patient, and the doctors' recommendations [2]. Early confined prostate cancer can be treated by radical prostatectomy, transurethral resection of the prostate, radiation therapy, or in certain situations, watchful waiting. Androgen deprivation is the preferred treatment for more advanced prostate cancer, especially when initial findings signify a poor prognosis in which no other therapeutic modalities have proven to be successful. Despite the initial success of androgen deprivation therapy in controlling advanced prostate cancer, hormone refractory aggressive metastatic cancer will eventually evolve, which almost always results in patient death. Chemotherapy is occasionally used if prostate cancer has spread outside of the prostate gland and hormone-deprivation therapy has not been effective. However, to date chemotherapy has not been effective in curing the disease because of treatment-related toxicity and poor responses to drugs currently available [3]. Although the etiology and pathogenesis of prostate cancer are not well understood, certain risk factors have been implicated in the development and progression of prostate cancer. Known risk factors include age, race, and family history, while possible risk factors include lifestyle, androgens, infection and inflammation of the prostate, diet, and environmental factors [4]. Some of the risk factors (e.g., age, androgens, and inflammation) are possibly linked by the fact that each may result in and/or be derived from oxidative stress.

15.3 Redox States and Redox Systems

Reactive oxygen species (ROS) are generated endogenously from normal metabolic processes and exogenously from the cellular microenvironment. Mitochondrial bioenergetics is the predominant source of ROS; other endogenous systems that can generate ROS include the NADPH oxidase (NOX) complex, cytochrome P-450, lipoxygenase, cyclooxygenase (COX), xanthine oxidase, and select enzymes present in peroxisomes [5]. To protect against elevated ROS levels and subsequent oxidative stress, cells are equipped with intricately regulated antioxidant defense systems [6, 7]. The major antioxidant systems within cells include low-molecular-weight antioxidant compounds such as vitamins E and C, multiple antioxidant enzymes (AEs) including superoxide dismutases (SODs), catalase (CAT), glutathione peroxidases (GPxs), peroxiredoxins (Prdxs), thioredoxin reductases (TrxRs), and reducing buffer systems such as the glutathione, glutaredoxin, and thioredoxin (Trx) systems [6–9]. ROS can also be regulated by the interaction of nitric oxide ($^{\cdot}NO$) with ROS [10]. DNA repair enzymes may be considered to be a part of the antioxidant system because of their potential to decrease levels of oxidative DNA damage [6]. From an even broader standpoint of biological processes, proteins that participate in modulation of reduction–oxidation (redox) state in response to oxidative stress, such as the tumor suppressor protein p53, heat shock proteins, and hypoxia-inducible factor (HIF), should also be included as important components of the antioxidant defense system [11–14]. To prevent transition metal-mediated oxidative stress, cells

have also evolved elaborate systems for copper and iron storage and transport that deliver copper and iron to metalloenzymes and proteins (e.g., ceruloplasmin, Fet3 multicopper ferroxidase, and transferrin) [15, 16]. These proteins work together to help maintain the intracellular homeostasis of redox states, the balance of reducing and oxidizing equivalents within cells. More importantly, the specific subcellular distribution of each enzyme ensures the specificity of redox signaling [8].

15.4 The Role of ROS/Oxidative Stress in Cancer

ROS at low levels have physiological functions, while at high levels, ROS are toxic to cells. Physiological levels of ROS regulate many cellular functions such as proliferation, cell cycle progression, migration, and metastasis; these biological processes may modulate cancer progression and could possibly be the result of oxidative modifications of redox sensitive transcription factors and signal transduction molecules. Low doses of hydrogen peroxide (H_2O_2) have been demonstrated to stimulate prostate cancer cell growth and migration by modulation of heparin affin regulatory peptide [17, 18]. Oxidative signals in the cytoplasm are essential to initiate signaling for transcriptional activation of activator protein 1 (AP-1), nuclear factor-kappa B (NF-κB), and NF-E2-related factor 2 (Nrf2) via phosphorylation of Jun or dissociation of NF-κB or Nrf2 from inhibitory protein complexes [8]. Epidermal growth factor (EGF)-induced H_2O_2 in human ovarian cancer cells can stimulate hypoxia-inducible factor alpha (HIF-1α) expression followed by subsequent transcriptional activation of vascular endothelial growth factor (VEGF) through activation of phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) pathways, resulting in enhanced angiogenesis and vascular permeability [5]. In human prostate cancer, insulin-stimulated ROS can activate the extracellular signal regulated kinase/extracellular regulated kinase (MEK/ERK) and PI3K/Akt signaling pathways and promote cancer progression and metastasis through HIF-1α-induced VEGF expression [5, 19]. ROS-mediated induction of cell adhesion molecules of the A disintegrin and metalloproteases (ADAMs) transmembrane protein family via p38 mitogen-activated protein kinase (MAPK) has been associated with prostate carcinogenesis and chemotherapeutic resistance [20, 21]. Matrix metalloproteinases (MMPs), enzymes that facilitate degradation of the extracellular matrix and thus may enhance cancer cell invasion/metastasis, can be activated by ROS, and this effect is attenuated by blockage of ROS production through inhibition of NOX activity and overexpression of extracellular superoxide dismutase (ECSOD) in prostate cancer cells [22, 23].

When ROS exceed physiological levels, oxidative stress ensues. Oxidative stress is capable of causing damage to various cellular constituents, including DNA, proteins, and lipids [24]. Oxidative stress can produce oxidized DNA bases [e.g., 8-hydroxy-2′-deoxyguanosine (8OHdG)], initiate mutations, result in the formation of DNA adducts, and produce DNA strand breaks [25, 26]. ROS can also cause lipid peroxidation [27]. Lipid peroxidation in cellular membranes generates a variety of aldehydes and alkenals such as malonaldehyde and 4-hydroxy-2-nonenal (4HNE).

4HNE can readily react with key proteins in signal transduction pathways to form secondary adducts, leading to cellular dysfunction or cell death [27–29]. Lipid peroxidation in cell membranes also results in the formation of prostaglandins, important mediators of inflammation [30].

Oxidative stress has been shown to be involved in the inactivation of several key proteins, including those involved in DNA repair, apoptosis, cell signaling, and essential enzymatic pathways [31]. Elevated ROS/reactive nitrogen species (RNS) can inactivate caspases, phosphatases, and phosphatase and tensin homologue (PTEN), and inhibit p53 binding to gene promoters, leading to decreased apoptosis, increased cell survival and gene damage [18, 32–36]. The accumulation of genetic and epigenetic instability and protein dysfunction as a result of sustained oxidative stress in prostate cells may be a factor in carcinogenesis [31]. Excessive oxidative stress, however, has been shown to cause cancer cell death. Indeed, a reduction of antioxidant defenses, elevation of ROS/RNS-generating enzymes, increased ROS/RNS levels, and accumulation of oxidative damage products (ODPs) have all been observed with the progression of malignancy [24, 37]. Attenuation of intrinsically higher levels of oxidative stress with antioxidants or use of chemotherapeutic agents to further increase oxidative stress beyond the tolerance of cancer cells have been demonstrated in several studies to inhibit prostate cancer growth and invasion or induce apoptosis both in vitro and in vivo [38–40].

15.5 Detection of ROS/Oxidative Stress in Cancer

Historically, the detection of ROS has been difficult due to their relatively short life and high reactivity. Direct identification of free radicals can be achieved using electron spin resonance with spin traps or by detection of fluorescence or chemiluminescence signals using redox-sensitive dyes or lucigenin reagents. The major advantage of electron spin resonance is its high specificity, but it is limited in widespread use by the requirement for expensive equipment [6, 41]. Because of high sensitivity, wide availability of instruments, and the capability to detect ROS in living cells, detection of fluorescence signals using flow cytometry or confocal microscopy has become one of the most commonly utilized methods, despite the relative lack of specificity. The widely used cell-permeant redox-sensitive fluorescence dyes are 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and its derivatives. H_2DCFDA fluoresces in the presence of hydroxyl radicals ($HO^{\cdot}$), H_2O_2 in the presence of peroxidases, and peroxynitrite [42]. Since the intensity of H_2DCFDA fluorescence is also affected by the rate of influx and efflux of the dye and activities of nonspecific esterases, the signal must be normalized with a redox-insensitive dye such as 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDCFDA). Dihydroethidium (DHE) is often used to detect superoxide ($O_2^{\cdot-}$ levels with flow cytometry or confocal microscopy) [43], but since the spectrum of $O_2^{\cdot-}$ and DNA overlap, it is not highly specific.

Another method used to measure levels of oxidative stress is to analyze ODP using biochemical, immunologic, or immunohistochemistry assays [6]. For example,

oxidized DNA (e.g., 8OHdG) can be measured using biochemical techniques (high performance liquid chromatography), immunologic techniques (ELISA assay), or in tissue sections with immunohistochemistry techniques using specific antibodies to 8OHdG [44]. Immunohistochemistry techniques allow for identification of ODP in specific subcellular compartments, and are especially useful in tissue sections where other methods normally used to detect ROS are not available or difficult to perform. Antibodies to oxidative or nitrative damage products such as 4HNE-modified protein adducts, 8OHdG, and 3-nitro-L-tyrosine (3NT) are frequently used in these studies [6, 24, 27, 29, 37]. These antibodies have also been widely used in western blot analyses. Oxidative stress or ODP can also be measured with biochemical assays of whole cell lysates when localization of specific ROS/RNS markers to subcellular compartments is not necessary. These biochemical assays include glutathione assays and other AE assays. Levels of AEs, either increased or decreased, are often indicators of oxidative stress since such stress may result from the reduction or induction of antioxidant defenses. The glutathione–glutathione disulfide couple (GSH/GSSG) frequently serves as an important indicator of overall cell or tissue redox state. New emerging redox western analysis and redox-sensitive green fluorescence proteins have provided powerful new tools to quantify thiol/disulfide redox changes in specific subcellular compartments [8].

15.6 Prostate Cancer Risk Factors and Their Links to Oxidative Stress

Important prostate cancer risk factors include age, race, and family history. Possible risk factors include lifestyle, androgens, infection and inflammation of the prostate, and diet. Some of these risk factors are linked by the fact that they may result in and/or be derived from oxidative stress. In order to understand the relationship between oxidative stress and prostate cancer, we briefly review risk factors possibly related to oxidative stress and prostate cancer (aging, androgens, inflammation, and diet).

15.6.1 Androgens

The importance of androgens in prostate carcinogenesis is suggested by observations that prostate cancer rarely occurs in males castrated at a young age or in men with a deficiency in 5-alpha-reductase, an enzyme responsible for converting testosterone to its more active form, 5-alpha-dihydrotestosterone [45]. Most prostate cancers are androgen-dependent at initial diagnosis [45]. It is well known that androgens are capable of altering the expression of a number of genes involved in cell survival and cell growth by binding to specific receptors and inducing subsequent transcriptional activation. Some studies suggest that androgens might, at least in part, exert their effects via oxidative stress mechanisms. Ripple et al. [46] demonstrated that

physiological levels of androgens can alter the prooxidant–antioxidant balance of the androgen-responsive LNCaP human prostate cancer cell line. Using the synthetic androgen methyltrienolone (R1881), this group found decreased ROS levels at a dose range (0.025–0.1 nM R1881) that stimulated cell growth while levels of oxidative stress dramatically increased at a higher dose range (1.0–5.0 nM R1881) that inhibited cell growth. Consistent with these results, Shigemura et al. [47] suggested that ROS are common downstream mediators of androgen-induced ADAM9 protein expression in prostate cancer cells because the addition of H_2O_2 or the introduction of CAT either enhanced or abolished ADAM9 protein expression, respectively. Some mechanistic studies indicated that the transcription factor JunD is an essential mediator of the androgen-induced increase in ROS levels [48], while others emphasized the involvement of mitochondrial redox regulation of p66Shc in LNCaP cells [49].

Different races have different incidences of prostate cancer (255.5, 161.4, and 96.5 per 100,000 population for African-Americans, white Americans, and Asian Americans, respectively, from the years 2000 to 2004) [1]. Differences in prostate cancer incidences between different races could possibly be explained by differences in testosterone levels and gene activities that regulate biosynthesis and metabolism of androgens [45]. For instance, testosterone levels are 19% higher in African American men in Los Angeles than their white counterparts [50]. Levels of 3α, 17β androstanediol glucuronide and androsterone glucuronide, two indices of 5α-reductase activity, are higher in white and black men than that in Japanese men (31 and 25% higher for 3α, 17β androstanediol glucuronide, and 50 and 41% higher for androsterone glucuronide, respectively) [51, 52].

The association of androgens with ROS/oxidative stress is further emphasized by other studies. Sharifi et al. [53] examined the possible role of downregulation of manganese SOD (MnSOD or *SOD2*) in the function of androgen receptor (AR) using SOD knockdown technology. They found that *SOD2* knockdown resulted in the upregulation of androgen-regulated gene expression and induction of AR DNA binding and transcriptional activity in an *N*-acetyl-L-cysteine (NAC)-reversible manner. This group concluded that downregulation of *SOD2* is responsible for AR reactivation in hormone-refractory AR positive prostate cancer, and thus AR reactivation is ROS dependent. Given that a large majority of human hormone refractory prostate cancers do express AR, and that androgen insensitive pathways still rely on active signaling through AR in most cases [54], such discoveries are very promising for prostate cancer treatment because they suggest a means to silence AR reactivation. However, it has not been demonstrated that AR reactivation is a specific consequence of *SOD2* downregulation in human prostate cancers. Persistent oxidative stress caused by any number of other risk factors may instead be responsible for AR reactivation.

One study has addressed the role of ROS/oxidative stress in prostate tissues in vivo. Neville et al. [24] provided the first evidence of androgen regulation of redox status in normal rat ventral prostate (VP). In this study, castration clearly induced an elevated prooxidant state in the regressing VP, while testosterone replacement in castrated rats only partially reduced oxidative stress levels in the VP.

Higher levels of oxidative damage were documented in the VP of testosterone-treated castrates than that present in intact rats; this mild state of oxidative stress was suggested to contribute to cell proliferation, differentiation, and tissue remodeling observed in the regeneration of the VP. Androgen-induced prooxidant shift observed in LNCaP cells in vitro is in contrast to the in vivo observation that androgen can partially reduce oxidative stress in castrated normal prostate. Determining the actual levels of androgens in normal and prostate cancer tissues is crucial in interpreting these seemingly contradictory data. Dose–response studies are required in which androgen and ROS levels are directly measured in prostate tissues in in vivo studies. Specifically, measurement of androgen concentrations in normal human and prostate cancer tissues, as well as levels of ROS/RNS changes and/or ODP in animal models of androgen-dependent prostate cancer following castration or androgen replacement would be useful to determine ROS/oxidative stress responses to androgens directly in prostate tissues. Methodological caution is also needed in such studies, since redox status is a prooxidant–antioxidant balance. For example, instead of measuring GSH concentrations alone, measurement of the GSH/GSSG ratio should be performed. However, the available data support the occurrence of androgen-induced prooxidant and antioxidant shifts in normal prostate and prostate cancer.

15.6.2 Inflammation

Chronic inflammation and proliferative inflammatory atrophy (PIA) are often found adjacent to high-grade prostatic intraepithelial neoplasia (HGPIN), the most likely precancerous lesion of prostate in humans [55, 56]. Pathological observations suggested that HGPIN may stem from PIA and then progress to prostate cancer [55, 56]. Components of several key molecular pathways involved in prostate cancer such as NKX3.1, CDKN1B, and PTEN have been shown to be altered in PIA or HGPIN lesions [55, 57]. Epidemiologic studies found an increased relative risk of prostate cancer in men with a prior history of certain sexually transmitted infections or prostatitis, while the use of nonsteroidal anti-inflammatory drugs that inhibit COX enzymes was linked to reduced prostate cancer risk. The evidence linking clinical prostatitis and prostate cancer is often contradictory [30, 31]. Identification of several genes involved in inflammation-related pathways associated with prostate cancer risk, including macrophage inhibitory cytokine-1 (MIC1), interleukin 1 receptor antagonist (IL1RN) and members of the Toll-like receptor (TLR) family, further suggests an association between inflammation-related processes and prostate cancer development [55, 58–64]. Immunotherapy consisting of irradiated tumor cell vaccine and anticytotoxic T-lymphocyte-associated antigen 4 (anti-CTLA-4) antibodies to attenuate T cell activity has been shown to markedly decrease the incidence of prostate tumors by 60% (15% vs. 75% in controls) in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model [65]. Gupta et al. [66] have

demonstrated a remarkable reduction in the rate of prostate tumorigenesis and metastasis in TRAMP mice fed with the COX-2 inhibitor celecoxib (100% vs. 25% tumorigenesis and 65% vs. 0% metastasis, respectively). These findings suggest a possible role of inflammation and the proinflammatory enzyme COX-2 in prostate cancer development and progression [66].

Primary mediators of the nonspecific host immune defense system include free radicals, predominantly ROS and RNS. Examples of ROS and RNS include H_2O_2, $O_2^{\cdot-}$, $HO^{\cdot}$, peroxynitrite ($ONOO^-$), and $^{\cdot}NO$, all of which cause deleterious effects associated with inflammation [31]. The proposed "injury and regeneration" hypothesis highlights the importance of inflammation-induced oxidative/nitrative stress in prostate cancer. In this model, oxidative stress secondary to ROS/RNS production by activated inflammatory cells and/or secreted inflammatory cytokines causes repeated cellular injury and accumulation of genomic damage, promoting cellular replacement and creating a tissue microenvironment rich in cytokines and growth factors capable of enhancing cell replication, angiogenesis, and tissue repair. Tissue regeneration may also hold potential for the generation of undifferentiated precursors, in which gene mutation may occur [31, 55]. Indeed, this hypothesis is, at least in part, supported by evidence that several inflammatory conditions in humans are accompanied by increased levels of oxidative (and sometimes nitrative) DNA damage [67]. Additional evidence linking chronic inflammation-induced ROS to carcinogenesis is provided by the fact that mice lacking phagocyte NOX showed less metastasis after injection of fibrosarcoma cells [18, 68]. The proinflammatory enzyme COX-2 has recently been recognized as a potential mediator for the development of human cancers in organs such as the colon and stomach [69–71]. COX-2 catalyzes the synthesis of prostaglandins, whose interactions with their receptors promote cell survival and stimulate angiogenesis, processes that have been proposed as primary molecular mechanisms underlying the procarcinogenic functions of COX-2 [69–71]. COX-2 can also function through ROS production, leading to cellular and genomic damage [72, 73]. However, a consensus has not been reached regarding the role of COX-2 in prostate cancer. Some investigators have reported elevated levels of COX-2 mRNA and protein levels in prostate cancer tissues [74, 75], while others failed to detect this upregulation [76, 77]. Other data suggest extensive silencing of COX-2 in prostate cancers via CpG island promoter hypermethylation [78]. Data from transgenic *K14.COX2* mice suggest that COX-2 overexpression may actually inhibit carcinogenesis [79, 80].

Several suggestions have been made for future studies of inflammation and prostate cancer. For example, study of the role of inflammation in prostate carcinogenesis via inducible modulation (knockdown or overexpression) of chemokine/cytokine receptors in prostate tissues in genetically altered animal models, such as *Pten* or *Nkx3.1* mutant mouse models, may provide valuable insights [55]. Modulation of ROS/RNS using overexpression or knockdown of prooxidant or antioxidant enzymes could also be utilized to study the role of ROS/RNS in inflammation-induced prostate carcinogenesis.

15.6.3 Aging

Aging as a risk factor is shown by the direct relationship between development of prostate cancer and advancing age. The odds of developing prostate cancer is rare in men younger than 40, while it is common in men greater than 80 years old. Several studies suggest that 42–80% of men will develop prostate cancer in their eighth decade [45]. It has been proposed that age-related prostate cancer is largely due to a lifetime exposure to oxidative stress, which is generated endogenously as byproducts of normal metabolic processes and exogenously by environmental exposures to toxic substances. Indeed, progressive accumulation of DNA strand breaks, DNA adducts and oxidative modification of enzymes for DNA repair have been documented to be increased, while antioxidant defenses including ROS detoxification enzyme activities, declined with age [81].

ROS toxicity is a limiting factor for life span in mammals. Mice with genetically inactivated MnSOD died at a mean age of 8 days from dilated cardiomyopathy; however, they can be rescued from their cardiomyopathy by treatment with the SOD mimetic MnTBAP [Mn 5, 10, 15, 10-tetrakis(4-benzoic acid) porphyrin] [82]. Overexpression of human CAT in the mitochondria of transgenic mice protected against mitochondrial oxidation and mitochondrial DNA (mtDNA) mutations and extended life span [81, 83, 84]. With respect to cancer, the association of increased levels of 8OHdG and age-dependent cancer incidence has been observed in mouse models [85, 86]. Mice lacking MutT homolog protein 1 enzyme, an enzyme that hydrolyzes 8OHdGTP, 8- and 2-OHdATPs, and 8-chlorodGTP, showed increased rates of spontaneous tumorigenesis with age, especially in the lung, stomach, and liver [18, 85, 86]. Although it is unclear why aging preferentially causes some types of tumors but not others, lack of appropriate animal models is clearly an obstacle to study age-related oxidative stress in prostate cancer. However, some insights may be obtained by comparing ODP in prostate tissues from men of different ages in relation to prostate cancer incidence.

15.6.4 Other Factors

In addition to androgens, inflammation, and aging, other factors such as diet and environmental exposure may also contribute to overall prostate cancer risk. A correlation to oxidative stress has been demonstrated in numerous studies. Examples include several antioxidants (e.g., vitamin E, selenium, lycopene, and epigallocatechin gallate) that reduce prostate cancer risk, while higher fat diets and exposure to ROS-producing carcinogens such as cadmium are correlated to increased prostate cancer risk [18, 45, 87–90]. However, there exists the possibility that some or even all of these risk factors could be interconnected to each other. For instance, excessive consumption of certain dietary fats may contribute to prostate carcinogenesis via oxidative stress with subsequent lipid peroxidation; high dietary fats may also be associated with chronically high levels of androgens [45]. Thus, the coupling of

oxidative stress with one or multiple prostate cancer risk factors could provide a general mechanism for prostate carcinogenesis. Identification of these mechanisms may assist in the development of better strategies for the prevention of prostate cancer.

15.7 Causes of Redox Imbalance/Oxidative Stress in Prostate Cancer

Redox imbalance/oxidative stress could result from increased ROS/RNS production, a decrease in antioxidant defenses, or a combination of both [18].

15.7.1 Increased ROS Production

15.7.1.1 Altered Mitochondrial Bioenergetics

One of the major sources for increased ROS generation is believed to be altered mitochondrial bioenergetics. Metabolic transformation is a common feature of tumors, including prostate cancer [91]. Normal prostate glandular epithelial cells, as a result of high concentrations of accumulated zinc, have a truncated Krebs cycle, low rate of respiration, are energy inefficient, and presumably generate less ROS. By contrast, malignant prostate cells, which are virtually always associated with decreased concentrations of zinc, allow complete metabolism of citrate through the Krebs cycle and thus are bioenergetically more efficient cells. On the one hand, this metabolic transformation provides efficient energy and lipid biosynthesis from citrate to facilitate the malignant process; on the other hand, such a transformation might generate excess ROS and consequently increased mitogenic activity and mutation potential [92, 93].

Mutation rates in mtDNA are high in prostate cancer presumably because mtDNA is surrounded by high concentrations of ROS, lacks protection from histones, and has inadequate DNA proofreading and repair mechanisms [92]. Accelerated mtDNA mutations may further increase ROS production and oxidative stress within cells. Indeed, frequent mtDNA mutations have been identified in human prostate cancer, and these mutations, in most cases, involved patients with Gleason grades 5–7 by pathologic analysis, the most frequent clinical grade observed at clinical presentation [94]. More importantly, Petros et al. [95] studied the role of mtDNA mutation-induced oxidative stress in tumor growth. They prepared transmitochondrial cybrids by introducing cytoplasts from the same patients harboring the homoplasmic mtDNA mutant (T8993G) or homoplasmic mtDNA wild-type (T8993T) into rhodamine 6-G treated prostate cancer PC3 cells, and found that injection of cybrids into nude mice that harbored a homoplasmic mtDNA mutation (T8993G) known to cause increased mitochondrial ROS production resulted in more than seven times greater tumor growth, demonstrating the potential involvement of ROS in prostate tumorigenesis.

In addition to mtDNA mutations, alterations in mitochondrial enzymes have also been demonstrated in prostate carcinogenesis. For example, elevated ROS generation by increased expression of mitochondrial glycerophosphate dehydrogenase was demonstrated to contribute to prostate cancer progression [96]. Important questions concerning the role of mtDNA mutations in prostate cancer remain: (1) Are there any specific mutations associated with prostate cancer? (2) Are there any specific mutations related to increased oxidative stress? and (3) Can inhibition of mitochondrial-generated oxidative stress prevent prostate carcinogenesis?

15.7.1.2 Upregvulation of NADPH Oxidases

Upregulation of plasma membrane-bound NOXs is another potential source of increased ROS production. NOX is an enzyme that catalyzes the production of $O_2^{\cdot-}$ utilizing oxygen as a substrate and NADPH as a cofactor. The catalytic subunits of NOX constitute a family of NOX enzymes (NOX1–5 and Duox) that are homologues of gp91phox of the phagocytic oxidase [97, 98]. Some studies have indicated the presence of NOX1 and NOX2 in human prostate [99], while others suggested the presence of additional NOX4 in the rat prostate [24]. Interestingly, all three NOX enzymes in the rat prostate were upregulated in response to castration, indicating the possible hormonal control of NOX expression [24]. Studies on the importance of the involvement of the NOX system in cancer have largely focused on NOX1, driven by the observation that NOX1 overexpression transformed NIH 3T3 cells and increased $O_2^{\cdot-}$ production in oncogenic-*ras* transformed cells [100, 101]. In human prostate cancer, it has been demonstrated that NOX5 levels were elevated in DU145 human prostate cancer cells and downregulation using antisense oligonucleotides for *NOX5* or inhibition of NOX with diphenyliodonium (DPI) inhibited ROS production, leading to decreased cell proliferation and increased apoptosis [102]. In human prostate cancer tissues and cancer cell lines, increased NOX1 protein and mRNA levels were correlated with elevated H_2O_2 [103]. Recent studies relating NOX levels in normal and prostate cancer cells of various degrees of aggressiveness showed very different NOX expression profiles [22]. Results showed that NOX2, NOX4, and NOX5 mRNA were present in prostate cancer cell lines, but not in normal prostate cells, while NOX1 and NOX3 were absent in both cancer and normal cells. This study used DPI to inhibit NOX and decrease ROS generation, resulting in inhibition of the malignant phenotype and leading the investigators to suggest a role of NOX in prostate carcinogenesis. However, studies such as these have failed to measure NOX enzyme activity, so whether or not the NOX family of enzymes is actually modulated in human prostate cancer remains unclear. Since NOX enzyme activities are usually regulated by cytosolic proteins such as p47phox and p67phox, increased protein or mRNA levels do not necessarily result in increased enzyme activity with resultant increased $O_2^{\cdot-}$ production [18, 97]. In addition, many of these studies utilized the NOX inhibitor DPI. DPI is a nonspecific inhibitor of many different electron transporters and inhibits not only all of the NOX isoforms but also nitric oxide synthase, xanthine oxidase, mitochondrial complex I, and cytochrome P-450 reductase [97].

15.7.1.3 Hypoxia

Tumor hypoxia is considered to be a hallmark characteristic of locally advanced solid tumors including prostate cancer. Intracellular ROS increase under hypoxia conditions via the transfer of electrons from ubisemiquinone to molecular oxygen at the Q_0 site of complex III of the mitochondrial electron transport chain [104]. Hypoxia-induced ROS can activate numerous signaling components, and HIF-1 acts as a key mediator of hypoxia response. HIF-1 controls over 70 genes that are of pivotal importance in regulating cellular metabolism, survival, cell cycle progression, angiogenesis, and inhibition of apoptosis [5]. Hypoxia-induced ROS generation participates in the stabilization of HIF-1α through activation of PI3K and p38 MAPK [105–107]. Studies have shown that under hypoxia conditions, human prostate cancer cells have increased ROS levels and HIF-1α upregulation. HIF-1α stabilization is ROS dependent, as the ROS scavenger NAC prevented HIF-1α accumulation. Mechanistic studies demonstrated that p38 is activated by hypoxia-induced mitochondrial ROS and contributes to HIF-1α activation by inhibiting its hydroxylation by prolyl and asparaginyl hydroxylases [105]. ROS may also be involved in hypoxia-induced resistance to apoptotic agents by inhibiting mitochondria-induced apoptosis through downregulation of the mitochondrial outer membrane permeabilization process [20, 108, 109].

15.7.2 Impaired Antioxidant Defenses

15.7.2.1 Altered Antioxidant Enzymes

Several lines of evidence implicate a relationship between changes in antioxidant defense systems and malignant transformation. First, transformation is usually accompanied by lower levels of MnSOD in the malignant tissue of primary cancers compared to corresponding nontumor tissue [37, 81]. Second, the relationship between altered protection against ROS and carcinogenesis is highlighted by deregulation of antioxidant defense systems in several kinds of cancers in animal models. It has been demonstrated that copper zinc SOD (CuZnSOD) knockout mice develop liver cancer later in life [18, 110]. Loss of function of GPx1 and GPx2 in mutant mice results in increased susceptibility to inflammation and cancer in the intestine [111, 112]. Mice lacking either Prdx1 or Prdx6 display increased levels of ROS, elevated oxidative damage, and increased propensity for tumor formation including hepatocellular carcinoma, fibrosarcoma, osteosarcoma, islet cell adenomas, and adenocarcinomas of the lung and breast [18, 112–114]. Finally, increased cellular ROS have been demonstrated in carcinogenesis, while antioxidants prevent malignant transformation in some systems both in vivo and in vitro [115, 116]. Elevation of various isoforms of SOD (MnSOD, CuZnSOD and ECSOD), GPx1 or CAT by overexpression or addition of exogenous liposomal SOD decreases ROS levels, followed by suppression of cancer cell growth in vitro and in vivo [115, 116].

Altered antioxidant defenses have been observed in human prostate cancer. Studies on AEs in malignant prostate cell lines (LNCaP, DU145, and PC3) and in primary cultured prostate cells showed cancer cell lines have higher SOD, CAT, and glutathione reductase (GR), but lower glutathione *S*-transferase (GST) and GPx activities than in the primary cell cultures [117]. In LNCaP cells CAT and GR were significantly higher, while by contrast, GST was significantly lower than levels found in PC3 and DU145 cells [117]. High levels of GST activity in PC3 in comparison to LNCaP cells was observed by Chaiswing et al. [118] who compared redox states of two cancer cell lines (LNCaP and PC3) with different degrees of aggressiveness. In human prostate cancer tissues, Baker et al. [119] found lower levels of CAT, MnSOD, and CuZnSOD in most prostatic adenocarcinomas in comparison to corresponding nontumor tissues. Our laboratory has demonstrated low immunoreactive protein levels of MnSOD in human primary prostate cancer tissues, but a significantly greater level of MnSOD in metastatic tissues in comparison to normal tissues [37]. In addition, Bostwick et al. [81] showed lower expression of MnSOD, CuZnSOD, and CAT in human HGPIN and prostate adenocarcinomas compared to that observed in benign epithelium, indicating a shift in the balance of antioxidant defense systems in prostate cancer. However, there are only rare studies employing analyses of enzyme activities in the prostate tissue. Data from Bostwick et al. [81] suggest statistically unchanged but widely variable enzyme activities. The major concern in the latter study is the possible cross-contamination of cancer tissues by surrounding normal tissues of both stroma and epithelium.

Genetic analysis suggests that the Ala variant of *SOD2* is associated with a moderately increased risk of prostate cancer, particularly among men with lower intakes of dietary and supplemental vitamin E [120]. Future studies will be necessary to assess the role of polymorphisms of other AEs in prostate cancer risk.

Mechanisms resulting in alteration of AE activities may be complex, but two general explanations are usually involved: an increase in AEs may be a compensatory regulation in response to oxidative stress, while a decrease in AEs may lead to elevated levels of oxidative stress in cancers. However, variations in AEs levels during cancer progression may be caused by cancer cell heterogeneity, including the possible emergence of cancer stem cells.

Additional findings implicate ROS in prostatic carcinogenesis, possibly due to impairment of other antioxidant defense systems. Hypermethylation with resultant inactivation of the glutathione *S*-transferase pi (*GSTP1*) gene is a common feature found during prostate carcinogenesis. *GSTP1* plays an important role in the detoxification of electrophilic compounds such as carcinogens and cytotoxic drugs by glutathione conjugation. Inactivation of *GSTP1* may impair cellular processing of mutagens and allow ROS to damage DNA. *GSTP1* gene methylation has been detected in 50–70% of PIN lesions, and in 70–95% of prostate cancers [121–124]. Patients with the highest percentage of GSTP1 immunoreactive protein in the cells of benign epithelium had a better overall survival [124].

Despite the frequent downregulation of antioxidants in most primary cancers, a few cancers have demonstrated an upregulated antioxidant defense system. One example, as demonstrated above, is higher levels of MnSOD in more advanced

human prostate cancer tissues in comparison to normal prostate tissues [37]. Prdxs were also increased in some malignant cells [125, 126]. One possible reason to explain these findings is that high levels of oxidative stress in primary cancer select for those cells with high antioxidant defense capability. Another possibility is that ROS production is also increased, and thus the overall oxidative stress is elevated. In addition, protein levels are not always equivalent to activity since AEs are often inactivated by ROS/RNS. Experiments to evaluate enzyme activity in the context of oxidative damage to biomolecules will help to clarify mechanisms responsible for AE changes. Indeed, our laboratory has already demonstrated higher levels of oxidative damage products (4HNE-modified protein adducts and 8OHdG) in metastatic human prostate cancer tissues in comparison to normal prostate tissue [37]. This signifies an overall imbalance of ROS production and detoxification in prostate cancer [18].

Another question that needs to be addressed is whether or not the overall antioxidant defense capacity is related to dose-dependent effects of antioxidants. At low concentrations and under physiological conditions, vitamin C is an antioxidant. However, it has been shown that vitamin C under conditions of stress can oxidize DNA [127]. MnSOD at physiological levels functions as a protective antioxidant, but at high levels of expression kills cells [128]. H_2O_2 at low concentrations causes cell proliferation, but at higher concentrations is cytotoxic [17]. In vivo, selenium and vitamin E, two low molecular antioxidants, show a negative association with prostate cancer in some experimental models, but these results are not reproducible to date in clinical studies [129]. Although the exact reason for the above effects is not clear thus far, one possibility is failure to reach sufficient antioxidant concentrations in target tissues at the doses administered, or perhaps lower doses of antioxidants are also protective for already transformed cancer cells by relieving oxidative stress. The uneven distribution or distinct ability of antioxidants to reduce oxidative stress in specific subcellular compartments may also account for these paradoxical effects. Further studies involving the dose–response effects of antioxidants on cancer, and even more detailed studies such as redox regulation in specific subcellular compartments during the progression of cancer, need to be conducted.

15.7.2.2 Transcriptional Factors Modulated by Redox

The most compelling argument for a role of redox regulation/dysregulation in cancer is that transcriptional factors modulated by redox have frequently been found to be altered in cancers, and these in turn could also regulate redox states. Such transcription factors include p53, Nrf2, AP-1, and NF-κB [8, 12]. It has become clear that the tumor suppressor gene *p53* is especially important since it is mutated in many human cancers, and has been shown to regulate oxidative stress and oxidative metabolism [12, 130]. p53 is now known to have both prosurvival and proapoptotic effects related to prooxidant and antioxidant functions. *p53* mutations are uncommon in primary prostate cancers, but mutation or loss of expression has been associated with prostate cancer progression [131–133]. In collaboration with Dr. Daret St. Clair, our

laboratories have shown that p53 binds directly to MnSOD (a prosurvival protein), with subsequent decrease in MnSOD activity [134]. Dr. St. Clair has also shown that *SOD2* transcription is directly regulated by *p53* [135]. Under conditions of severe oxidative stress, *p53* changes to proapoptotic function, and selected downstream genes (e.g., *puma* and *bax*) are upregulated. Products of such gene upregulation potentially generate ROS and presumably contribute to p53-mediated cell death [136]. Zhao et al. [137] have shown that selenite-induced superoxide can activate *p53* and cause p53 mitochondrial translocation. Activation of *p53* by selenite in turn synergistically enhances superoxide production and apoptosis in human prostate cancer cells. These combined results suggest an intimate relationship between redox-controlled transcriptional and posttranslational events in cancer.

Other examples of redox-sensitive transcription factors modulated during prostate cancer cell progression are Nrf2, AP-1, and NF-κB. In turn, each of these transcription factors regulates downstream genes that affect cell or organelle redox state. Nrf2 is known to regulate several antioxidant and detoxification enzymes such as NAD(P)H:quinone oxidoreductase, heme oxygenase, thioredoxin reductase 1, glutamate–cysteine ligase modifier subunit, glutamate–cysteine ligase catalytic subunit, and specific GST family members [138–140]. A study by Frohlich et al. [141] found that Nrf2 was downregulated in human prostate cancer and that loss of Nrf2 function reduced both expression and activity of specific GST enzymes and increased ROS and DNA damage; such damage may promote prostate tumorigenesis. There is also increasing evidence that inflammation plays a role in prostate carcinogenesis with studies of several other tissue types indicating Nrf2 may play a role in the severity of inflammation. However, the role of Nrf2 in prostatic inflammation has yet to be specifically investigated. AP-1 and NF-κB are nuclear transcription factors that regulate the expression of genes involved in several processes including proliferation, apoptosis, and angiogenesis [142–144]. Both AP-1 and NF-κB have been shown to be constitutively activated in prostatic malignancies and increases in such activation are believed to contribute to malignant transformation and progression of the prostate cancer phenotype [142, 145]. Conversely, suppression of AP-1 and NF-κB activities has been shown to reduce or inhibit the invasive and metastatic properties of several cancer cell types [146, 147]. There have also been recent studies that demonstrated that constitutive activation of NF-κB and members of the AP-1 family (e.g., c-Jun, JunD, and Fra-1) was associated with prostate cancer progression toward an hormone refractory phenotype [145, 148].

15.8 Recent Evidence of Redox Imbalance in Prostate Cancer

Over the past several years, a significant body of evidence, as discussed above, strongly implicated ROS or oxidative stress in prostate carcinogenesis. Owing to space limitations, we only examine and summarize major findings from the most important recent studies, as well as discuss issues relating to methodology and study design that may lead to discrepant results.

15.8.1 Evidence from In Vitro Cell Culture Models

In analyzing the redox profiles of two different human prostate carcinoma cell lines (LNCaP vs. PC3) with varying degrees of invasiveness, Chaiswing et al. [118] recently showed that in comparison to the hormone-dependent cancer cell line LNCaP, the more metastatic PC3 cells, in general, had a more reducing redox state, a conclusion obtained following analysis of AE levels and enzyme activities, ODP levels, and responses to ROS/RNS-generating compounds. High levels of ROS/RNS in S and G_2/M phases of the cell cycle in LNCaP cells were observed; these levels were found to be constitutively lower in PC3 cells in all phases of the cell cycle. PC3 cells had higher immunoreactive protein levels of ECSOD, TrxR1, and several glutathione-related proteins such as glutamylcysteine synthase, glucose-6-phosphate dehydrogenase, and GSTP1, as well as higher GPx and GST enzyme activities than LNCaP cells; these results correlated with lower levels of both intracellular and extracellular ROS. The reducing state of PC3 cells was also supported by higher levels of both intra- and extracellular GSH/GSSG ratios and lower levels of lipid peroxidation, although DNA damage as indicated by 8OHdG was found to be higher than that of LNCaP cells. It was hypothesized that LNCaP cells require a prooxidant state for cell proliferation, whereas PC3 cell growth may not depend on a prooxidant state. LNCaP cells had higher ATP levels as well as increased ROS-generating NOX1 protein levels and a corresponding greater sensitivity to growth inhibition by the commonly used NOX inhibitor DPI. By contrast, PC3 cells were more sensitive to menadione, a compound that produces superoxide and depletes glutathione pools within cells, suggesting distinct redox profiles between these two cell lines. A major advantage of this study is extensive examination of redox profiles using three different methods (AE protein levels and activities, analysis of ODP, and response to ROS-generating compounds), but the study is limited by a lack of analysis of normal prostate epithelial cells as a control, which makes it difficult to evaluate redox state changes during prostate cancer tumorigenesis.

Kumar et al. [22] characterized oxidative stress in three different prostate cancer cell lines with various degrees of aggressiveness (LNCaP, DU145, and PC3) and normal and immortalized prostate cells in culture (WPMY1, RWPE1, and primary cultures of normal epithelial cells). This group observed higher levels of ROS in PC3 cells compared to DU145 and LNCaP cells. Expression of NOX2, NOX4, and NOX5 mRNA was higher in the cancer cell lines, but not detectable in normal prostate epithelial cells. Contrary to data from other studies, they also reported that NOX1 and NOX3 were absent in all cell lines tested. By using DPI as an inhibitor of NOX, Kumar et al. [22] suggested the essential role of ROS production by the extramitochondrial NOX system in prostate cancer. They also found that inhibition of NOX with DPI resulted in more effective reduction of malignant potential as demonstrated by inhibition of growth and proliferation, decrease in clonogenic activity, cell migration, cell invasion and cell cycle arrest in G_2-M phase of the cell cycle as well as loss of mitochondrial membrane potential in comparison to effects of neutralization of ROS by NAC. They concluded that active ROS generation by

the NOX system rather than ROS accumulation might be more important in prostate cancer cell malignancy. Although the measurement of multiple changes in cell behavior and bio-functional markers, as well as the inclusion of normal cell controls were important in these studies, several methodological problems need to be carefully examined before final conclusions are warranted.

Discrepancies exist between the above two studies. Chaiswing et al. [118] showed a more reducing redox state in more aggressive PC3 cells than less aggressive LNCaP cells, while Kumar et al. [22] observed higher ROS levels in PC3 cells in comparison to both DU145 and LNCaP cells. Although such a difference may result from different experimental conditions or the sensitivity of the analytic methods, the lack of redox-insensitive fluorescence control dyes in the latter study may account for this major discrepancy. Indeed, our laboratory has demonstrated high levels of redox-insensitive CDCFDA fluorescence in PC3 cells indicating that the results reported by Kumar et al. [22] were not related to ROS. H_2DCF fluorescence intensity is a function of ROS levels, DCF influx and efflux rates, and esterase cleavage of the fluorescence dyes. Thus, the use of the redox-sensitive fluorescence dye alone is not sufficient for analysis of ROS levels. It is noteworthy that the metabolic switch from aerobic to anaerobic pathways concurrent with the progression of cancer also points to the possibility of less ROS production from the mitochondrial respiration chain, which may result in a more reducing environment in more aggressive cancer cells. However, more studies are needed to clarify this issue simply because any comparison between cell lines of very different genetic backgrounds may introduce a great deal of complexity because of differing AE gene regulation. More importantly, measurements of redox state in animal models or in human tissues are essential due to the possible involvement of artificial ROS generation under conditions used in cell culture.

Another discrepancy between the two studies comes from the study of NOXs. Chaiswing et al. [118] showed expression of NOX1 protein in both PC3 and LNCaP cells, which is consistent with previous observations [103]. Conversely, Kumar et al. [22] reported the presence of other NOX mRNAs, but NOX1 and NOX3 mRNA were absent in these cells. Irrespective of which NOX isoforms are present in prostate cancer, both of these studies used DPI as an NOX inhibitor to postulate the possible role of NOX in ROS production and prostate cancer. DPI is not a specific inhibitor of NOX, and thus the above conclusions are questionable. Neither mRNA nor protein levels may accurately represent the actual enzyme activity of NOX. Downregulation of specific NOX using siRNA or shRNA and the correlation of protein or mRNA expression with NOX activity assay are necessary to more definitively determine the role of NOX in prostate cancer. It is also interesting to note the apparent ineffectiveness of NAC in the inhibition of prostate cancer malignancy in the studies of Kumar et al. [22], which is contradictory to many other cancer prevention studies [149–151]. Indeed, several instances of this antioxidant paradox have been observed (e.g., vitamin E and selenium studies). Again, studies of antioxidant dose–response, analysis of redox states in specific subcellular compartments, and documentation of redox changes during cancer progression may be more important in understanding the role of redox status in cancer than analysis of individual antioxidant molecules in cell lysates.

A subsequent study by Chaiswing et al. [23] demonstrated a higher extracellular GSH/GSSG ratio in prostate cancer cells in comparison with immortalized parental cells. By using adenovirus-mediated *ECSOD* gene transduction to modulate redox states in several prostate cancer cell lines (e.g., WPE1-NB26, DU145, and PC3 cells), they showed that overexpressed ECSOD was primarily localized in the extracellular space. Addition of heparin to the culture media, which resulted in overexpression of ECSOD in the media, significantly decreased membrane type 1-matrix metalloprotease and MMP2 activities, as well as cell invasion capability. Conversely, overexpression of ECSOD without the addition of heparin to the media, which resulted in intracellular overexpression of ECSOD, did not show significant changes in these biologic and biochemical parameters. This study suggested the importance of extracellular membrane-localized superoxide generation in the modulation of cancer cell behavior. Although this study postulates that NOX1 may play a role in prostate carcinogenesis because of its specific membrane localization as well as the high expression and activity of NOXs in WPE-NB26 cancer cells, the role of NOX1 is still not conclusive since specific NOX1 knockdown studies were not performed. Together, the data suggest changes in both intracellular and extracellular redox states in prostate cancer cell culture models.

15.8.2 Evidence from Animal Models

One animal model provides direct evidence for the role of oxidative stress in prostate cancer. Ouyang et al. [112] have provided evidence for loss of protection against oxidative stress in mice with a loss of function of *Nkx3.1*, a homeobox gene that is known to be required for prostate epithelial differentiation and suppression of prostate cancer. Using gene expression profiling, it was demonstrated that *Nkx3.1* mutant mice had altered expression of several antioxidant and prooxidant enzymes, including Gpx2 and Gpx3, Prdx6, and sulfhydryl oxidase Q6. Mice with a loss of *Nkx3.1* function developed HGPIN but not cancer, and HGPIN was associated with oxidative damage to DNA. However, compound mutant mice lacking both *Nkx3.1* and *Pten* tumor suppressor genes progressed to adenocarcinoma. These compound mutant mice displayed further decreases in antioxidants, especially lack of expression of the SODs (CuZnSOD, located in the cytoplasm, nucleus, and mitochondrial intermembrane space, and MnSOD, located in the mitochondrial matrix), with more profound oxidative damage to DNA and protein observed. Therefore, these studies are believed to directly link oxidative stress and prostate cancer development and progression.

Studies have shown that increased oxidative damage correlated with tumor progression in three different mouse models of prostate tumorigenesis [27, 112]. As described previously, Ouyang et al. [112] have shown that DNA and protein were increasingly damaged by oxidation during tumor progression in *Nkx3.1*$^{-/-}$ and *Nkx3.1*$^{-/-}$*Pten*$^{-/-}$ mice. Additionally, Tam et al. [27] demonstrated similar results in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model of prostate carcinoma, where increased ROS and RNS damage was demonstrated

in the prostate gland during tumorigenesis. Tam et al. [29] have also shown that administration of testosterone plus 17β-estradiol for 16 weeks induced dysplasia and stromal inflammation in the lateral prostate, but not in the ventral prostate in Noble rats. This combined hormone regimen, which with additional time resulted in prostate cancer, increased the expression of mRNA of specific members of the NOX family (NOX1, NOX-2, and NOX-4), inducible NOS, endothelial NOS, and COX-2 in the lateral prostate epithelium and/or its adjacent inflammatory stroma. There was also accumulation of 8OHdG, 4HNE protein adducts, and 3NT primarily in the lateral prostate epithelium, suggesting that NOX, NOS, and COX may mediate hormone-induced oxidative/nitrative stress in prostate epithelium. Damage resulting from oxidative/nitrative stress as a result of the combined hormone therapy was concluded as from stromal inflammatory lesions.

The major criticism of these studies to date is that no mechanistic studies were performed. In order to clarify the role of oxidative stress present in these animal models, it is necessary to study carcinogenesis by modulation of redox state using overexpression or knockdown of antioxidant or prooxidant enzymes in the prostates of these genetically altered animal models.

15.8.3 Evidence from Human Tissues

Using specific antibodies against ROS/RNS damage products, our laboratory demonstrated changes of redox state with progression of human prostate cancer [37]. Primary prostate cancer showed low levels of 4HNE-modified proteins, but high levels of 3NT and 8OHdG compared to normal prostate epithelium. By contrast, metastatic prostate cancer had higher levels of ROS (4HNE-modified proteins and 8OHdG) and RNS (3NT) damage products than either primary cancer or normal prostate epithelium. The shift of redox state seems to have a specific subcellular distribution; the oxidative damage product 4HNE-modified proteins was predominantly localized in the cytoplasm but also in the nucleus, the DNA oxidation product 8OHdG was present predominantly in a diffuse nuclear distribution, and the RNS damage product 3NT was found predominantly in the cell cytoplasm. A shift in AEs was also detected, with generally lower levels of AEs in prostatic adenocarcinoma tissues compared to normal epithelium, but higher levels of MnSOD in metastatic prostate cancer compared to primary tumors [37]. These results are consistent with studies by Bostwick et al. [81], who compared oxidative stress in benign prostate epithelium, HGPIN, and prostate cancer. This group found decreased expression of MnSOD, CuZnSOD, and CAT in HGPIN and prostate carcinoma compared with benign epithelium [81]. It is also noteworthy that Baker et al. [119] also found lower levels of CAT, MnSOD, and CuZnSOD in most prostatic adenocarcinomas in comparison to corresponding nontumor tissues. Although additional quantitative biochemical activity studies need to be performed, these results confirmed abnormalities in ROS and RNS metabolism in human prostate cancer.

15.9 Compartmental Oxidative Stress and Specificity in Redox Control and Signaling

ROS and oxidative stress are not evenly distributed within cells. ROS generation occurs in specific locations and antioxidant defenses are localized in specific subcellular compartments [6]. For example, mitochondria are the primary location for ROS production, while NADPH oxidase generates ROS primarily at the cell membrane. MnSOD is distributed exclusively in mitochondria; CuZnSOD is present in the cytoplasm, nucleus, and mitochondrial intermembrane space, and ECSOD is specifically localized in the extracellular space. Under physiological conditions, the relative redox states differ in different compartments. From more reducing to more oxidizing redox environment as ascertained by levels of biologic redox couples is mitochondria > nucleus > cytoplasm > endoplasmic reticulum > extracellular space [8]. It has been demonstrated that ROS can function as second messengers in signal transduction, and redox compartmentalization functions as a mechanism for specificity in redox signaling and oxidative stress [8]. Three major redox couples, namely GSH/GSSG, reduced thioredoxin [Trx-$(SH)_2$]/oxidized thioredoxin [Trx-SS] and cysteine/cystine, are not in redox equilibrium and therefore could function as control nodes for many different redox-sensitive processes [8]. Specific compartmental oxidation in controlling signaling transduction is illustrated by Halvey and Jones [152], who studied compartmental oxidation following EGF treatment. This group showed that ROS appeared to be a critical component for growth factor signal transduction, and ROS generation following EGF treatment resulted in specific cytopasmic oxidation of Trx1, but not nuclear Trx1, mitochondrial Trx2, and cellular GSH. Nuclear and cytoplasmic compartmentalization of redox processes has been demonstrated to regulate transcription factors [8, 153]. AP-1, NF-κB, and Nrf2 require an oxidative signal in the cytoplasm to initiate signaling for activation. After activation and translocation into the nucleus, reduction of cysteine residues within the DNA binding domain by Trx1 and redox factor-1 (Ref-1) of each transcription factor is a prerequisite for transcription factor binding to DNA and subsequent gene activation [8, 153].

In most cancers, including prostate cancer, oxidative damage also showed specific subcellular distribution [37]. Alteration of extracellular redox state in prostate cancer by overexpression of ECSOD in the extracellular space decreased cell invasion, whereas overexpression of ECSOD intracellularly had no significant effect [23]. This latter study implicated the importance of membrane-localized superoxide generation in modulation of prostate cancer cell behavior. However, specific compartmental redox states in prostate cancer have not been precisely defined. It would be of great interest and importance to understand how redox states in specific compartments change during the progression of prostate cancer, how they change in response to various intracellular and extracellular stimuli, and how these changes are related to cancer cell behavior. Thus, selective manipulation of redox state in specific compartments may provide powerful tools in the management of prostate cancer.

15.10 Summary and Conclusions

Current data support the role of ROS/oxidative stress in prostate carcinogenesis. ROS can be generated endogenously or exogenously. When more ROS are produced and/or antioxidant defenses impaired, redox imbalance/oxidative stress then occurs. Physiological levels of ROS participate in many cellular processes such as proliferation, migration, and metastasis, while high levels of oxidative stress are capable of causing damage to various subcellular constituents, including DNA, proteins, and lipids. The accumulation of genetic and epigenetic damage as well as protein dysfunction resulting from sustained oxidative stress in prostate cells may contribute to prostate carcinogenesis. Some prostate cancer risk factors including androgens, advancing age, and inflammation are associated with oxidative stress. However, current knowledge is not sufficient enough to ascertain the precise role of ROS/oxidative stress in prostate carcinogenesis, specifically whether or not ROS/RNS are a significant cause or just a secondary effect of cancer. If ROS/oxidative stress is higher in prostate cancer, why have antioxidants thus far appeared to be ineffective in the treatment of prostate cancer? Since ROS and RNS are somehow interconnected to each other, how could we distinguish their role in prostate cancer initiation and progression? Numerous signaling pathways are affected by ROS, but we are still not certain as to which specific pathways are most important in prostate cancer.

15.11 Future Directions

In view of so many questions regarding the role of ROS/RNS in prostate cancer, we would like to make several suggestions for future studies of oxidative stress in prostate cancer. (1) When evaluating oxidative stress, it is important to measure ODP and perform a total antioxidant assay rather than detecting ROS and antioxidant defenses alone. (2) Enzymatic activity assays should be included in evaluating prooxidant or antioxidant protein levels. (3) To define whether ROS/oxidative stress is a cause and not a secondary effect of prostate cancer, overexpression or knockdown of specific enzymes or proteins should be performed in transgenic animal models. (4) Specific subcellular compartmental oxidation rather than whole cell lysates should be analyzed. This would help to design strategies to modify specific compartmental redox states for possible therapeutic intervention. (5) ROS and RNS are somehow interconnected to each other, and they are both implicated in prostate carcinogenesis; therefore, low-molecular-weight compounds or overexpression or knockdown technologies to specifically modulate ROS or RNS should be used to differentiate their effects. (6) Oxidative stress may potentiate carcinogenesis via oxidative modifications of redox sensitive transcriptional factors and signaling molecules; however, the precise modification profile is thus far poorly defined. Future studies on specific modifications to key proteins (such as ˙NO- or glutathionyl-cysteine modifications) will open another avenue for understanding the role of ROS/RNS in prostate cancer.

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Chapter 16
The Role of Vitamin E in Prostate Cancer

William L. Stone, Sharon E. Campbell, and Koyamangalath Krishnan

Abstract This chapter reviews the current literature linking vitamin E to prostate cancer with the overall goal of providing a rationale for the design of potential future large-scale clinical chemoprevention studies. Vitamin E is not a single organic compound and refers to at least four tocopherols (alpha, beta, gamma, and delta) and four corresponding tocotrienols. Much of the literature linking vitamin E with cancer does not distinguish between these various isoforms and has primarily focused on alpha-tocopherol which is the primary vitamin E isoform found in plasma from fasting individuals and in most dietary supplements. Considerable evidence now supports the view that the various isoforms of vitamin E (and their chemical derivatives) have distinct biochemical properties and distinct abilities to modulate oxidative stress, signal transduction pathways, and pathophysiological processes important in carcinogenesis (e.g., apoptosis and angiogenesis). This chapter reviews the recent clinical trials as well as the in vitro and in vivo evidence connecting the various isoforms of vitamin E with prostate cancer. Particular emphasis is placed on gamma-tocopherol, the primary dietary isoform of vitamin E. A major conclusion is that some non-alpha-tocopherol forms of vitamin E hold considerable promise for both the chemoprevention and chemotherapy of prostate cancer.

W.L. Stone, PhD
Department of Pediatrics, East Tennessee State University,
Johnson City, TN, USA

S.E. Campbell, PhD
Department of Biochemistry and Molecular Biology,
East Tennessee State University, Johnson City, TN, USA

K. Krishnan, MD (✉)
Department of Internal Medicine, East Tennessee State University,
Box 70622, Johnson City, TN 37614, USA
e-mail: krishnak@etsu.edu

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16.1 Introduction

The clinical rationale for attempting to identify and test potential antioxidant/anti-inflammatory nutraceuticals with chemopreventive properties against prostate cancer is well justified despite the spate of recent conflicting or negative clinical trials that have primarily focused on alpha-tocopherol (RRR-alpha-tocopherol or all-racemic-alpha-tocopherol), the isomer almost universally present in vitamin E supplements. We review the results of those clinical trials in which prostate cancer was a primary or secondary endpoint. Given that hindsight is 20/20, composing a critical review of these previous clinical studies, without suggesting a viable alternative, is not particularly constructive. Nevertheless, it is valuable for the design of future studies to point out the importance of robust descriptive epidemiology, extensive in vitro and in vivo work in model systems, sound nutritional biochemistry and a firm mechanistic foundation. The review provided below explores these critical factors with specific emphasis on vitamin E and prostate cancer. A major conclusion is that future clinical trials should consider non-alpha-tocopherol forms, such as gamma-tocopherol or delta-tocotrienol, as potential chemopreventive agents for prostate cancer.

It is possible, however, that a prostate cancer chemopreventive dietary factor could only be effective when "delivered" in the form of a whole food or a natural diet (e.g., the Southern Mediterranean) rather than as a purified nutraceutical in the form of an oral supplement. It is also conceivable that an interaction between a natural diet and life style factors (e.g., daily exercise and nonsmoking) could be critical in slowing the progression of prostate cancer. Unesterified vitamin E contained in foods prevents in vitro oxidation during the cooking process and thereby potentially helps to reduce the in vitro production of carcinogens generated by high heat [1]. By contrast, an oral vitamin E supplement containing esterified alpha-tocopherol (e.g., all-racemic alpha-tocopheryl acetate) would have no such effect, since this form is inactive as an antioxidant until hydrolyzed during intestinal absorption. Finally, it is possible that advanced drug design, aided by bioinformatics technology, could produce xenobiotic chemoprevention agents that are nontoxic when taken over long time periods, inexpensive to manufacture, and more effective than any potential nutraceutical chemopreventive agent.

16.2 The Importance of Chemopreventive Antioxidants/Anti-inflammatory Nutrients in Prostate Cancer

Prostate cancer is the most frequently diagnosed cancer in American men and the second leading cause of cancer: it is a multifactorial disease with age, family history, hormonal levels, race, and diet being important factors. Over one million American men over the age of 50 will eventually die of prostate cancer unless new treatments are developed. Prostate cancer is somewhat unique in having a long latency period during which cancer growth is slow and can cause few symptoms.

Prostate cancer is often preceded by prostatic intraepithelial neoplasia (PIN) characterized by an increased proliferative ability of prostate gland cells and morphological alterations. Chemopreventive agents are particularly well suited for decreasing prostate cancer mortality by extending the time during which PIN progresses to prostate cancer.

While the incidence of latent prostate cancer (microfocal tumors) is similar throughout the world, the incidence of clinically evident prostate cancer has the greatest country-to-country variation (as much as 20-fold) of any cancer type [2, 3]. These data suggest the potential importance of both nutritional and environmental factors. This notion is supported by data showing that native Japanese have the lowest risk of clinical prostate cancer, whereas first-generation Japanese-American immigrants have an intermediate risk factor and subsequent generations have a risk similar to the general US population [4]. It should be noted, however, that not only do US immigrants adopt a Western diet, but also a Western lifestyle characterized by lack of exercise and a marked tendency toward obesity. There is now good evidence showing that obesity increases the risk of aggressive prostate cancer while reducing the risk of low-grade, nonaggressive cancer [5]. Similarly, there is evidence suggesting that regular vigorous activity can slow the progression of prostate cancer [6].

It has been estimated that 35% of cancer deaths in the USA are attributable to dietary factors [7]. Identifying the specific nutraceuticals that could act as potential chemoprevention factors for prostate cancer is clearly a well-justified goal: such agents are more likely to be inexpensive and nontoxic (over a long time span) compared with xenobiotics. Vitamin E, the major lipid-soluble antioxidant in human plasma and tissues, has long been considered a cancer chemopreventive agent with great potential. RRR-alpha-tocopherol and RRR-gamma-tocopherol are the primary isoforms (see below) of vitamin E found in human plasma and tissues.

Vitamin E is best know for inhibiting the process of lipid peroxidation which occurs in the biological membranes of subcellular organelles and other lipid–protein complexes such as lipoproteins. The process of lipid peroxidation yields peroxy radicals, lipid hydroperoxides and reactive aldehydes all of which are genotoxic. Malondialdehyde, for example, is a major aldehyde by-product of lipid peroxidation and it forms adducts with deoxyguanosine, deoxyadenosine, and deoxycytidine nucleosides that can be mutagenic [8]. By diminishing lipid peroxidation, vitamin E could potentially reduce endogenous DNA damage and thereby reduce cancer in general. A recent large-scale prospective study has shown that “dietary vitamin E” is associated with reduced risk of clinically relevant prostate cancer [9]. As discussed below, we carefully use the phrase “dietary vitamin E” to distinguish it from “supplemental vitamin E”: a major goal of this review is to stress the importance of this distinction.

Cigarette smoking is perhaps the most well documented and potent source of oxidative stress and it markedly increases the risk of prostate cancer death [10]. Bruno et al. [11] have extensively reviewed the effects of cigarette smoking on oxidative stress and vitamin E biokinetics. In human subjects, smoking has been found to markedly increase the utilization of plasma RRR-alpha- and RRR-gamma-tocopherol

as well as increase the nitration of gamma-tocopherol resulting in the formation of 5-nitro-gamma-tocopherol [11].

There is a large body of literature linking prostate cancer with chronic inflammation and inflammation is always associated with an increased level of oxidative stress [12, 13]. De Marzo et al. [13] and Bardia et al. [12] have elegantly reviewed the data supporting the view that environmental factors (e.g., infectious agents, carcinogens in the diet or from cigarette smoke) cause prostate injury and an accompanying chronic inflammation referred to as proliferative inflammatory atrophy (PIA) which can then progress to prostatic intraepithelial neoplasia (PIN) and prostate cancer. This etiological schema is significant to vitamin E since gamma-tocopherol has unique properties as an anti-inflammatory factor (as discussed below in more detail).

16.3 Intervention Trials

16.3.1 Prostate Cancer Chemoprevention Trials with Supplemental Vitamin E

The Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study was a randomized, large-scale (29,133 men), double blind, placebo-controlled, primary prevention trial to determine if daily supplementation with 50 mg/day of all-racemic-alpha-tocopheryl acetate, all-trans-beta-carotene (20 mg/day), or both would reduce the incidence of lung cancer or other cancers among male smokers in Finland [14]. The ATBC study found that men taking beta-carotene or both vitamin E and beta-carotene had an increased incidence of lung cancers, while vitamin E alone had no effect on lung cancer [14]. Quite remarkably, men receiving vitamin E had a 31% decrease in the incidence of prostate cancer and a 41% lower prostate cancer mortality lower compared to men not receiving vitamin E [15]. For men receiving beta-carotene the incidence of prostate cancer incidence was 23% higher and mortality was 15% higher (95% CI = 30–89%) compared with men not receiving beta-carotene [15]. It needs to be emphasized that the men in this study were smokers and, therefore, subjected to a very potent oxidative stress affecting a major alteration in vitamin E utilization [11].

The Beta-Carotene and Retinol Efficacy Trial (CARET) is also relevant here: this study tested the combination of 30 mg beta-carotene/day and 25,000 IU retinyl palmitate/day (vitamin A) taken daily against placebo in men and women at high risk of developing lung cancer, i.e., either smokers or asbestos workers. The CARET study found an increased incidence of lung cancer and mortality in the subjects taking beta-carotene and vitamin A: results consistent with the ATBC [16, 17]. Although prostate cancer was a secondary outcome in the CARET study, recent results suggest that men taking the high-dose beta-carotene plus and vitamin A supplement with at least one other dietary supplement could have an increased risk of aggressive prostate cancer [18]. The potential procarcinogenic effect of beta-carotene remains

uncertain but recent work by van Helden et al. [19] shows that beta-carotene inhibits myeloperoxidase, an H_2O_2-utilizing enzyme released by neutrophils, and increases the formation of genotoxic hydroxyl radicals ($^{\cdot}OH$) from H_2O_2 via the Fenton reaction.

The Selenium and Vitamin E Cancer Prevention Trial (SELECT) was undertaken based on the ATBC trial as well as another secondary trial providing evidence for a preventive role of selenium in preventing prostate cancer [20]. The SELECT study had an optimal study design: it was a large-scale (35,533 relatively healthy men), randomized, double blind, placebo-controlled trial conducted at 427 sites in the USA with a mean follow-up of 5.46 years. The results were unambiguous; supplementation with 400 IU/day of all racemic alpha-tocopheryl acetate or with selenium (200 μg/day from L-selenomethionine) or both did not prevent prostate cancer in a population of healthy men and, moreover, did not protect against lung or colorectal cancer [20]. The primary conclusion of the SELECT trial was that "selenium or vitamin E, alone or in combination at the doses and formulations used, did not prevent prostate cancer in this population of relatively healthy men." Moreover, a subgroup analyses of the SELECT population did not show a selective protective effect in current and former smokers [20].

The Physicians' Health Study II was a randomized, double blind, placebo-controlled factorial trial of all-racemic-alpha-tocopherol (400 IU/day) and vitamin C. This large-scale study enrolled 14,641 US male physicians initially aged 50 years or older, with a very low incidence of smoking. The conclusion, after 8 years (mean) of treatment, was that "neither vitamin E nor C supplementation reduced the risk of prostate or total cancer" but that neither agent showed any discernable harm [21].

The HOPE-TOO trial extension was designed to test the hypothesis that long-term supplementation with RRR-alpha-tocopheryl acetate at 400 IU/day could decrease the risk of cancer, cancer deaths and major cardiovascular disease outcomes. As detailed below, RRR-alpha-tocopheryl acetate is an isoform of vitamin E with "natural" stereochemistry. This was a long-term (7 years) randomized, double blind, placebo-controlled, international study in subjects with vascular disease or diabetes mellitus. There was no evidence that RRR-alpha-tocopheryl acetate supplementation reduced the incidence of prostate, lung, oral and pharyngeal, colorectal, breast, or melanoma cancers. The study did, however, raise a concern about an increased risk of heart failure related to RRR-alpha-tocopheryl acetate supplementation. The conclusion from this study was that "In patients with vascular disease or diabetes mellitus, long-term vitamin E supplementation does not prevent cancer or major cardiovascular events and may increase the risk for heart failure."

Despite the predominantly negative results for the clinical interventions studies reviewed above, it may be premature to generalize a finding made with all racemic alpha-tocopheryl acetate or RRR-alpha-tocopherol (supplemental vitamin E) to "dietary vitamin E," which is at least eight different isoforms having unique chemistries and biological affects. Equating vitamin E only with alpha-tocopherol is not justified biochemically (see below) and is likely to create obstacles to exploring other useful "non-alpha-tocopherol" isoforms of vitamin E with potentially powerful chemopreventive effects.

16.4 Prospective Studies

The Vitamins and lifestyle (VITAL) study utilized a prospective design which recruited 35,242 men from western Washington State who completed a questionnaire, including detailed questions about vitamin E and selenium supplement intake during the past 10 years [22]. This study found no association between long-term supplemental intake of vitamin E and selenium and prostate cancer risk overall but did note that the risk of clinically relevant advanced prostate cancer (regionally invasive or distant metastatic) was reduced with greater long-term vitamin E supplementation [22].

The Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial was a large-scale clinical trial whose goal was to determine the efficacy of some cancer screening tests in reducing the number of deaths from cancer. This study also evaluated the potential association between intake of a variety of antioxidants from foods and supplements and the risk of prostate cancer [23]. No overall association was found between prostate cancer risk and dietary or supplemental intake of vitamin E, beta-carotene, or vitamin C but among current and recent smokers there was a decreased risk of advanced prostate cancer associated with increasing dose and duration of supplemental vitamin E [23]. Kirsh et al. [23] noted that the lack of association between the estimated levels of dietary beta-, gamma-, and delta-tocopherols and prostate cancer could be due to limitations inherent in using food frequency questionnaires. In particular, the food frequency questionnaires used did not capture nut intake or the types of vegetable oils consumed, which are major sources of both alpha-tocopherol and non-alpha-tocopherols as discussed below [23]. This factor is a shortcoming in many studies and may play a role in masking the potential importance of dietary vitamin E in reducing the risk of prostate cancer.

A recent analysis of the very large scale (295,344 men) NIH-AARP Diet and Health Study showed that supplemental vitamin E (alpha-tocopherol) intake was not related to prostate cancer risk, whereas dietary gamma-tocopherol was associated with a reduced risk of advanced prostate cancer [9]. The men in this prospective study were 50–71 years old and cancer free at enrollment: there was a 5-year follow-up during which 10,241 cases of prostate cancer were identified [9]. Although prospective studies have limitations, this study had a robust number of prostate cancer cases and the database and methodology used to estimate the intake of individual vitamin E isoforms from a dietary questionnaire was quite comprehensive [9]. The conclusion from this study was that "The potential benefit of gamma-tocopherol for prostate cancer prevention deserves further attention".

Helzlsouer et al. [24], in a much smaller observational study (the CLUE II Study), looked at the association between alpha-tocopherol, gamma-tocopherol, selenium, and the subsequent development of prostate cancer. In this study, however, the plasma levels of tocopherols were measured at enrollment using a blood sample from apparently nonfasting men: the distinction between fasting and nonfasting blood samples is important with respect to gamma-tocopherol (see below). A protective effect for plasma alpha-tocopherol was only evident when plasma

gamma-tocopherol levels were high [24]. Men in the highest quintile of plasma gamma-tocopherol had a remarkable fivefold reduction in the risk of developing prostate cancer compared to men in the lowest quintile suggesting that a combined supplement with both and alpha- and gamma-tocopherol would be optimal for reducing the risk of prostate cancer [24].

If an interaction (as yet to be defined) between alpha- and gamma-tocopherols were required to reduce prostate cancer risk, then a very high dose of only alpha-tocopherol would not be beneficial since it would (as detailed below) reduce both plasma and tissue levels of gamma-tocopherol. This could explain why 50 mg/day of all-racemic-alpha-tocopherol was effective in the ATBC study, whereas the 400 mg/day used in the SELECT study and the Physicians' Health Study II was not, i.e., 400 mg of all-rac-alpha-tocopherol would be very effective at reducing plasma and tissue levels of gamma-tocopherol compared to 50 mg/day. It could also be that high plasma levels of gamma-tocopherol are a biomarker for vegetable fat consumption, which could be much less procarcinogenic than the consumption of animal fat (see below).

16.5 Vitamin E Biochemistry and Nutrition

All of the studies reviewed above need to be placed in the context of vitamin E biochemistry and nutrition to be usefully evaluated. The term "Vitamin E" does not refer to a single unique organic compound but rather to all tocopherols and tocotrienols and their chemical derivatives [25]. Naturally occurring dietary vitamin E is primarily four tocopherols and four tocotrienols with specific chirality. As shown in Fig. 16.1, the number and positions of the methyl groups on the chromanol rings defines alpha-, beta-, gamma-, and delta-tocopherols as well as the corresponding four tocotrienols. Tocopherols have a saturated phytyl side chain at the 2-position of the chromanol ring, whereas tocotrienols have a farnesyl side chain with three double bonds at this position. Tocopherols have three asymmetric carbons (at the 2, 4′ and 8′ positions) and tocotrienols have one asymmetric center at the 2-position (see Fig. 16.1). Naturally occurring tocopherols and tocotrienols all have the R-configuration at the chiral carbons.

All forms of vitamin E are fat-soluble antioxidants that inhibit in vivo peroxidation. In addition to preventing lipid peroxidation, vitamin E can modulate signal transduction pathways [25–28]. Since redox inactive derivatives of vitamin E, such as alpha-tocopheryl succinate, can also modulate signal transduction pathways, not all biologically relevant actions of vitamin E can be explained solely on the basis of antioxidant activity [27, 29–32].

All-racemic-alpha-tocopherol is an equimolar mixture of eight stereoisomers (an R- or S-configuration at the three asymmetric carbons). Only one-eighth of all-racemic-alpha-tocopherol is the naturally occurring RRR-alpha-tocopherol. This is an important point since tocopherols could modulate signal transduction pathways by binding to proteins such as tocopherol associated proteins [33]. Most binding

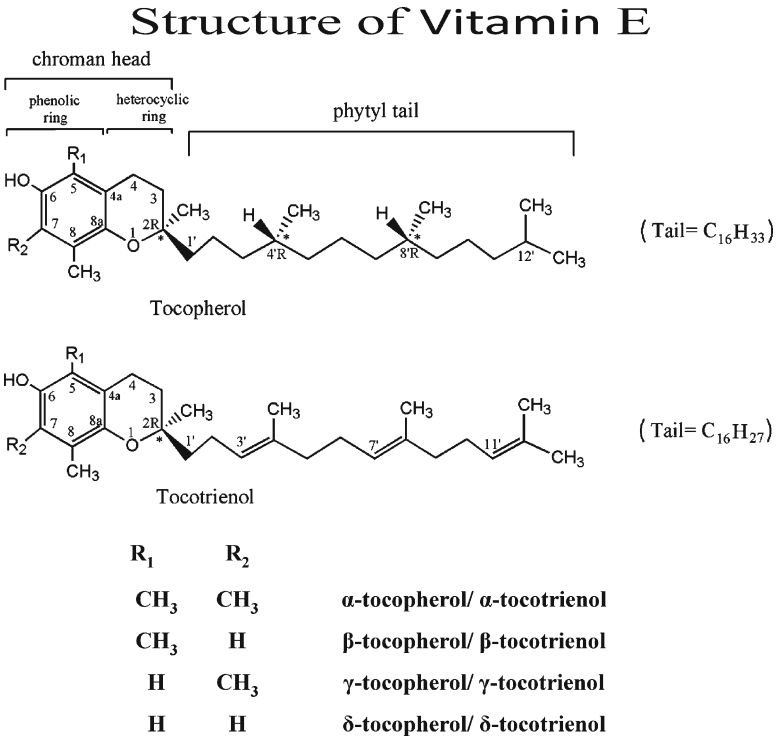

Fig. 16.1 Structure of vitamin E

sites on proteins exhibit stereoselectivity: proteins modulated by RRR-alpha-tocopherol may not be responsive to other alpha-tocopherol stereoisomers and, conversely, proteins normally not modulated by RRR-alpha-tocopherol could be modulated by the other alpha-tocopherol stereoisomers (with potentially deleterious effects).

16.6 The Unique Biochemistry and Anti-inflammatory Properties of Gamma-Tocopherol

The only structural difference between gamma-tocopherol and alpha-tocopherol is the lack of a methyl group at position 5 of the chromanol ring for gamma-tocopherol (see Fig. 16.1). Nevertheless, this subtle difference has profound biochemical and physiological consequences as detailed by the excellent review of Hensley et al. [34]. When oxidized, beta-, gamma-, or delta-tocopherol form partially substituted quinones that can function as an arylating species, whereas the fully substituted

alpha-tocopheryl quinone is not an arylating species [35]. All quinones are highly reactive redox cycling agents that can generate ROS in vivo and the partially substituted quinones also function as arylating agents that can react with cellular nucleophiles such as thiols. This leads to profound differences in the biochemistry of alpha-tocopherol compared to beta-, gamma-, and delta-tocopherols [35].

Another major distinction in the biochemistries of gamma- versus alpha-tocopherol is illustrated in their reactions with reactive nitrogen oxide species (RNOS) such as peroxynitrite (ONOO–) and nitrogen dioxide (NO_2) [36–39]. Alpha-tocopheryl quinone is the major product formed by the reaction between alpha-tocopherol and peroxynitrite [40], whereas 5-NO_2-gamma-tocopherol is formed by the reaction of gamma-tocopherol and peroxynitrite [36, 37, 41]. The in vivo formation of peroxynitrite and protein nitration species is thought to occur during many inflammatory processes and may contribute to carcinogenesis [42].

Using an animal model, Jiang et al. [43] demonstrated that supplementation with gamma-tocopherol inhibits protein nitration (i.e., 3-nitro-tyrosine formation) and ascorbate oxidation in rats with inflammation. These data suggest that gamma-tocopherol could act in concert with alpha-tocopherol to protect macromolecules from oxidative damage by trapping reactive nitrogen oxide species [43]. Surprisingly, there is little information on 3-nitro-tyrosine as a biomarker for prostate cancer: recent data suggests, however, that increased prostate levels of 3-nitrotyrosine are associated with prostate cancer but not benign prostatic hyperplasia [44].

In contrast to alpha-tocopherol, gamma-tocopherol and its water-soluble catabolite gamma-CEHC are inhibitors of cyclooxygenase-2 (COX-2) activity in macrophages and epithelial cells [45]. COX-2 catalyzes the synthesis of prostaglandin E_2 which is a well-characterized mediator of inflammation and regulates tumor angiogenesis in prostate cancer [46].

16.7 Vitamin E in Animal Fat Versus Vegetable Fat

Dietary fat has often been associated with an increased risk of prostate cancer. Giovannucci et al. [47] have provided evidence showing that total fat consumption is related to the risk of advanced prostate cancer and this association is primarily due to animal fat rather than vegetable fat. Animal fat contains primarily RRR-alpha-tocopherol tocopherol (or more precisely 2R, 4′R, 8′R-alpha-tocopherol) and, in general, contains much less total vitamin E than vegetable fat, which often contains high levels of “non-alpha-tocopherol” isomers. Butter, for example, contains 2 mg of RRR-alpha-tocopherol per 100 g of fat with negligible amounts of other vitamin E isomers [25]. By contrast, dietary vegetable oils typically contain high levels of RRR-alpha-tocopherol and often contain even higher levels of non-alpha-tocopherols [25]. Corn oil, for example, contains 10 mg RRR-alpha-tocopherol per 100 g fat and 60 mg of RRR-gamma-tocopherol per 100 g fat [25]. The typical USA diet contains about 2–4 times more RRR-gamma-tocopherol than RRR-alpha-tocopherol [48, 49], i.e., a ratio of 2–4:1, but in Europe this ratio is about 1:2 [49].

16.8 Supplemental Vitamin E Generally Does Not Mimic Dietary Vitamin E

Despite the fact that the primary form of dietary vitamin E in the US diet is RRR-gamma-tocopherol most clinical research (see above) has been done with supplements containing only all-racemic-alpha-tocopherol (or all-racemic-alpha-tocopheryl acetate) or RRR-alpha-tocopherol. The primary rationale for using alpha-tocopherol in clinical interventions studies lies in the fact that this isomer is present in plasma from fasting individuals at levels five- to tenfold higher than gamma-tocopherol. The fasting state, may not, however, be an accurate representation of circulating tocopherols in the nonfasting normal physiological state of most Westerners. All forms of dietary vitamin E isomers are absorbed equally well by the intestine, packaged into chylomicrons and secreted into circulation where they are acted upon by lipoprotein lipases and converted into smaller chylomicrons remnants which are rapidly taken up by the liver along with most of the dietary vitamin E [50]. These observations suggest that postprandial levels of gamma-tocopherol could be considerably higher than observed in the fasting state when triglyceride-rich chylomicron levels are very low. Meydani et al. [51] have, indeed, found that the plasma levels of gamma-tocopherol and alpha-tocopherol were 13 and 21 μM, respectively, 9 h after a fat-rich meal, whereas they were 6.7 and 21 μM, respectively, in the initial fasting state. The fat-rich meal contained gamma- to alpha-tocopherol in a 2.8:1 ratio [51]. These data indicate the level of gamma-tocopherol under normal nonfasting conditions is likely to be at least twice that observed in the fasting state. Similarly, tocotrienols, which are not normally detected in plasma from fasting individuals, can reach 10 μM in postprandial plasma 6 h after consuming a tocotrienol-rich palm oil supplement [52].

The molecular events resulting in the selective retention of RRR-alpha-tocopherol in plasma from fasting individuals have, for the most part, been well characterized. The liver contains an alpha-tocopherol transfer protein (alpha-TTP), which selectively transfers RRR-alpha-tocopherol into very low-density lipoprotein (VLDL), which is then secreted into circulation [53–55]. The mechanism whereby alpha-TTP transfers alpha-tocopherol from a membrane to VLDL is thought to involve the formation of an alpha-TTP-bilayer complex [56].

It was initially suggested that the gamma-tocopherol not packaged into VLDL could be eliminated from the liver via excretion into bile [53–55]. Work by Yamashita et al. [57] has shown, however, that secretion into bile is not a major route for the elimination of either gamma- or alpha-tocopherol. Early work by Dr. John Bieri and his coworkers showed that radiolabeled alpha- and gamma-tocopherols (intraperitoneally injected) are taken up equally well by various rat tissues but that gamma-tocopherol is subsequently metabolized more rapidly [58]. As detailed below, the fate of gamma-tocopherol may be particularly relevant to prostate cancer. Gysin et al. [59], for example, have found that gamma-tocopherol is much more effective than alpha-tocopherol at inhibiting the growth of both androgen-dependent and androgen-independent prostate cancer cell lines by the downregulation of cyclins.

16.9 The Fate of Gamma-Tocopherol

Work by Swanson et al. [60] suggests that differences in the rate of catabolism between alpha- and gamma-tocopherol could contribute to the higher levels of alpha-tocopherol in human plasma. Both alpha- and gamma-tocopherols are catabolized via oxidation of their phytyl side chain resulting in water-soluble carboxyethyl-hydroxychroman (CEHC) metabolites. The Parker group has elegantly described the cytochrome P450-mediated omega-hydroxylation of the tocopherol phytyl side chain followed by stepwise removal of two- or three-carbon moieties [61, 62]. Gamma-tocopherol is, more specifically, catabolized into 2,7,8-trimethyl-2-(beta-carboxyethyl)-6-hydroxychroman (or gamma-CEHC as shown in Fig. 16.2) [63, 64]. Swanson et al. [60] found that urinary excretion of gamma-CEHC was a major route of elimination of gamma-tocopherol in humans. Human studies using deuterium labeled alpha- and gamma-tocopherol have clearly demonstrated that gamma-tocopherol disappears from plasma more rapidly and shows a much higher rate of conversion into the CEHC metabolite than does alpha-tocopherol [65]. As would be expected, plasma levels of gamma-CEHC are much higher than that of alpha-CEHC [66, 67]. Galli et al. [66] for example found that although plasma levels of alpha-tocopherol were 15-fold higher than gamma-tocopherol, the plasma levels of gamma-CEHC were 12-fold higher than that alpha-CEHC. The urine data are not as consistent: Galli et al. have found a threefold higher levels of alpha-CEHC than gamma-CEHC, whereas Devaraj et al. [67] found similar urinary levels of alpha- and gamma-CEHC. These differences could be due to analytical issues [68].

It is of great interest that alpha-CEHC and gamma-CEHC (water-soluble) have both antioxidant [34, 69, 70] and anti-inflammatory properties[71]. Galli et al. [72] compared the effects of alpha-tocopherol and gamma-tocopherol, and their

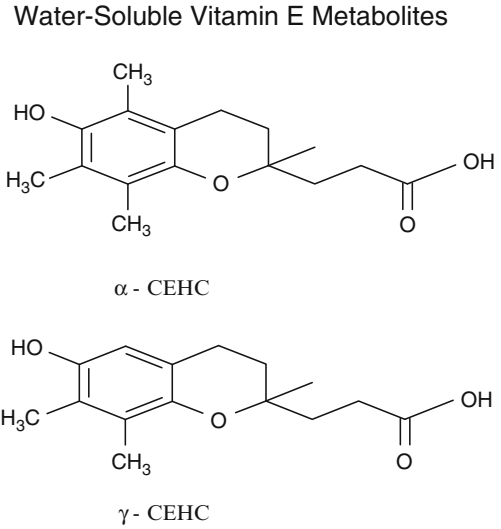

Fig. 16.2 Water-soluble vitamin E metabolites

corresponding CEHC metabolites, on the proliferation and expression of cyclin D1 in the PC-3 prostate cancer cell line. These investigators found that gamma-tocopherol and gamma-CEHC were the most effective (even at 1 μM) at inhibiting PC-3 cell growth and the expression of cyclin D1. Moreover, the inhibition of cyclin D1 expression by gamma-CEHC was competed for by alpha-CEHC [72]. These data clearly demonstrate that non-alpha-tocopherol forms of vitamin E may play important roles in preventing prostate cancer. The fact that water-soluble metabolites of vitamin E have anticancer activities also suggests that we must broaden our thinking with respect to vitamin E being relevant only to the lipid phase of cells and tissues.

16.10 The Cellular Uptake of Gamma-Tocopherol and Tocotrienols is Faster than that of Alpha-Tocopherol

For any vitamin E isoform to impact carcinogenesis it must be taken up by tissues where it could potentially protect DNA from oxidative stress induced damage and/or modulate signal transduction pathways relevant to carcinogenesis. Plasma vitamin E levels are, therefore, less relevant than intracellular vitamin E levels (and intracellular vitamin E metabolites). Surprisingly, there are only a few studies focusing on the uptake of vitamin E isoforms. Campbell et al. [73] determined that more than fivefold higher concentrations of gamma-tocopherol entered colon cancer cells compared to alpha-tocopherol at the same treatment concentration. Similarly, Gao et al. [74] studied the uptake of tocopherols by RAW 264.7 macrophages and found that gamma-tocopherol was taken up more rapidly than alpha-tocopherol with uptake being defined as the net difference between tocopherol transported into the cells and loss due to catabolism and/or in vitro oxidation. After 6 h, the cellular uptake of RRR-gamma-tocopherol was at least sixfold higher than that of RRR- alpha-tocopherol. Surprisingly, these investigators found that the presence of gamma-tocopherol promoted, by at least fivefold, the cellular uptake of alpha-tocopherol. If these results could be extrapolated to in vivo conditions they suggest that gamma-tocopherol is selectively taken up by cells and removed from plasma more rapidly than alpha-tocopherol and that dietary gamma-tocopherol could increase the tissue uptake of alpha-tocopherol. It is interesting, that in an animal model, graded levels of dietary gamma-tocopherol in a diet containing a constant level of alpha-tocopherol were found to increase the levels of alpha-tocopherol in serum and most tissues [75]. This remarkable result has yet to be reproduced in humans but certainly suggests an interaction between dietary gamma-tocopherol and alpha-tocopherol similar to that reported by Gao et al. [74] at the cellular level.

Zuo [76] has compared the uptake (after 4 h of incubation) of RRR-gamma-tocopherol and R-gamma-tocotrienol in the PC-3 prostate cancer cell line. The uptake of both gamma-tocopherol and gamma-tocotrienol by the PC-3 cells were dose-dependent (0–1.0 μM), but R-gamma-tocotrienol was taken up at levels fourfold higher than that of RRR-gamma-tocopherol. Similarly, Zuo [76] compared the PC-3 uptake of R-alpha-, R-gamma-, and R-delta-tocotrienols and found that

R-alpha- and R-gamma-tocotrienol were taken up at similar levels but the uptake of R-delta-tocotrienol was at least twofold higher. These data suggest that PC3 prostate cancer cells take up tocotrienols (particularly R-delta-tocotrienol) more rapidly than tocopherols.

16.11 Supplemental Vitamin E Decreases Plasma and Tissue Gamma-Tocopherol Levels

Handelman et al. [77] were the first to make the critical observation that supplemental alpha-tocopherol decreases plasma levels of gamma-tocopherol to about 30–50% of their initial values. Work by Traber et al. [78], using animal models, supports the view that dietary alpha-tocopherol supplementation increases the expression of hepatic cytochrome P450 Cyp3a and the tissue catabolism of gamma-tocopherol to gamma-CEHC. These results may also help explain some of the clinical data reported above. If gamma-tocopherol were a key anticancer chemopreventive nutraceutical, then supplemental alpha-tocopherol would decrease the plasma and tissue levels of gamma-tocopherol (by increasing its catabolism) and thereby negate its potential anticancer effect. As detailed below, the evidence supporting a key role for gamma-tocopherol in preventing prostate cancer is substantial.

16.12 Aging, Vitamin E, and Prostate Cancer

A primary risk factor for prostate cancer is aging with men over the age of 65 at highest risk. The dramatic increase in the incidence of prostate cancer with age is a hallmark of prostate cancer (http://seer.cancer.gov/). It is interesting, therefore, that plasma gamma-tocopherol levels appears to be the primary form of tocopherol depleted with age. Ford et al. [79] demonstrated that serum alpha-tocopherol concentrations increase with age in a population of more than 4,000 participants of the National Health and Nutrition Examination Survey, while plasma gamma-tocopherol levels decrease with age. These studies suggest that additional gamma-tocopherol rather than alpha-tocopherol might be more appropriate in aging men.

16.13 In Vitro Mechanistic Evidence Suggests that Gamma-Tocopherol is more Effective than Alpha-Tocopherol in Preventing Prostate Cancer

Early work by Moyad et al. [80] found that RRR-gamma-tocopherol was superior to all-racemic-alpha-tocopherol at inhibiting the growth of the LNCaP prostate cancer cell line. While gamma-tocopherol was effective at reducing cell growth at 0.75 μM,

alpha-tocopherol was not effective at levels lower than 250 μM (not physiological). Gunawardena et al. [81] investigated the role of all-rac-alpha-tocopherol to inhibit cell growth and induce apoptosis in a variety of prostate cancer lines but only tested very high (nonphysiological) levels of all-rac-alpha-tocopherol, i.e., 180–581 μM. They found that only a modest effect on cell growth inhibition and attributed this to apoptosis [81]. Many human prostate cancers arise because these cancerous cells have escaped apoptotic cell death. A key strategy in cancer prevention is, therefore, to promote apoptotic cell death in cancerous cells.

Gysin et al. [59] were among the first to provide some mechanistic insight into how gamma-tocopherol inhibits the growth of prostate cancer cells. These investigators found that gamma-tocopherol (24-h treatment) was more effective than alpha-tocopherol at inhibiting prostate cancer cell growth (LNCaP and DU-145 cell lines): a physiological level of 25 μM was used for both tocopherols. [59]. Flow cytometry data showed that the gamma-tocopherol treated DU-145 prostate cancer cells had a decreased progression into the S-phase associated with reduced DNA synthesis and decreased expression of cyclin D1 and cyclin E. Gysin et al. [59] reasonably assert that a nonantioxidant mechanism is likely, since gamma- and alpha-tocopherol have a similar antioxidant capacity. Gysin et al. [59] did not observe any increased apoptosis in prostate cancer cells treated with 25 μM gamma-tocopherol compared to untreated cells. However, cyclin D1, in addition to regulating cell proliferation, also plays a key role in mediating resistance to apoptosis by upregulating molecular chaperones and the subsequent redistribution of cell death regulators [82]. None of the researchers referred to in this paragraph compared the results observed with prostate cancer lines to normal or nontumorigenic cell prostate epithelial cells. This is an important control because it distinguishes a general affect of gamma-tocopherol on all prostate epithelial cells from a specific affect on prostate cancer cells.

Jiang et al. [83] made a major advance in further understanding the underlying molecular events giving rise to the inhibition of prostate cancer cell growth caused by gamma-tocopherol. These investigators found that gamma-tocopherol, but not alpha-tocopherol, inhibited the proliferation of PC3 and LNCaP prostate cancer cells but had no affect on normal prostate epithelial cells. Gamma-tocopherol (50 μM) but not alpha-tocopherol was found to induce apoptosis in the LNCaP cell line after 3 days of treatment. The higher concentration of gamma-tocopherol and longer duration of treatment used by Jiang et al. [83] most likely accounts for their positive apoptosis finding with LNCaP cells compared with the lack of apoptosis reported by Gysin et al. [59]. Jiang et al. [83] found that LNCaP cells treated with gamma-tocopherol released cytochrome *c*, activated caspase 9 and caspase 3, and cleaved poly-ADP-ribose polymerase (PARP): evidence was also presented for the involvement of caspase-independent pathways. A key observation was that LNCaP cells treated with gamma-tocopherol rapidly accumulated dihydroceramide and dihydrosphingosine, whereas ceramide and sphingosine levels did not increase until day 3, when substantial cell death took place [83]. The authors concluded that LNCaP prostate cancer cell death caused by gamma-tocopherol is linked to the de novo synthesis of sphingolipids [83].

Ceramides are neutral sphingolipids produced from either by the hydrolysis of sphingomyelin or by de novo synthesis from serine and palmitate. Considerable evidence supports the view that ceramide accumulation in biological membranes promotes the formation of specialized lipid domains called lipid rafts which, in turn, activate several downstream phosphorylation cascades important in promoting apoptosis [84–86]. It is increasingly apparent that some chemotherapeutic agents are proapoptotic by activating ceramide synthase and increasing membrane pools of ceramide [84, 85]. It is generally accepted that dihydroceramide, the precursor of ceramide, is not proapoptotic [87]. Stiban et al. [88] have suggested that dihydroceramides inhibit ceramide channel formation in the outer mitochondrial membrane thereby blocking the release of small proteins such as cytochrome *c* and preventing apoptosis. Jiang et al. [83] suggest, however, that long-chain dihydroceramides could play a role in apoptosis.

Jiang et al. [83] also addressed the possibility that cyclooxygenases and/or lipoxygenases could mediate the anticancer affects of gamma-tocopherol since these enzymes have abnormal levels or activities in many types of cancers. These enzymes utilize arachidonic acid and linoleic acid as their substrates. The addition of either arachidonic acid or linoleic acid to the cell culture media was found to partially block the inhibitory affect of gamma-tocopherol on the growth of PC3 or LNCaP prostate cancer cells Jiang et al. [83]. These authors could not, however, verify the presence of cyclooxygenases 2 or lipoxygenase in the prostate cancer cell lines by Western blots.

More recently, Campbell et al. [89] looked at the potential role of gamma-tocopherol in modulating lipoxygenases in prostate cancer cells. These authors noted that gamma-tocopherol has a strong structural similarity to troglitazone which is a ligand activating the γ-subtype of peroxisome-proliferator activated receptor (PPARγ). Troglitazone belongs to the thiazolidinedione class of drugs used to treat type-2 diabetes but has also been shown to suppress tumor development by mechanisms involving apoptosis induction, cell cycle arrest, and differentiation. PPARs are ligand-activated nuclear proteins and members of the nuclear receptor superfamily that regulate gene expression by binding to DNA as heterodimers with the retinoic acid receptor (RXR).

In agreement with the in vitro data presented above, Campbell et al. [89] found that gamma-tocopherol at physiological levels selectively inhibited the growth of PC-3 compared to normal prostate epithelial cells. Moreover, gamma-tocopherol was found to upregulate the mRNA and protein expression of PPARγ. By transfecting PC-3 cells with a dominant negative vector inhibiting PPARγ activity, the growth arrest caused by gamma-tocopherol was almost fully blocked: a result that was confirmed by the use of a chemical inhibitor of PPARγ. These results strongly suggest that gamma-tocopherol exerts its anticancer affect through a PPARγ dependent mechanism. Nevertheless, Campbell et al. [89] found that gamma-tocopherol was not a direct PPARγ ligand and reasoned that gamma-tocopherol might increase the production of an endogenous PPARγ ligand such as 15-S-HETE, which is known to suppress prostate cancer cell growth [90]. Lipoxygenases can insert oxygen atoms at various carbons on arachidonate with 5-, 8-, 12-, 15-lipoxygenase (5-LOX,

8-LOX, 12-LOX, and 15-LOX) inserting oxygen at carbon 5, 8, 12, or 15 yielding 5S-, 12S-, or 15S-hydroperoxyeicosatetraenoic acid (5-, 8-, 12-, or 15-HPETE) which can be reduced by glutathione peroxidase to the hydroxy forms, i.e., 5-, 8-, 12-, 15-HETE, respectively. Campbell et al. [89] found that gamma-tocopherol (40 μM) did, indeed, increase the production of 15-S-HETE as well as 15-LOX-2 mRNA and protein levels. The gamma-tocopherol-induced increase in 15-LOX-2 levels is particularly significant since tissue expression of this lipoxygenase is limited to prostate, lung, skin and corneal tissues, and prostate cancer has a decreased protein expression and a decreased enzymatic activity of 15-LOX-2 [90, 91].

The downstream targets regulated by PPARγ, relevant to cancer, include cyclin D1, cyclin D3, bcl-2, and NFkappaB. Campbell et al. [89] found that gamma-tocopherol treatment of PC-3 prostate cancer cells resulted in a downregulation of cyclin D1 and D3, which would stop the cell cycle and potentiate cell death. The phosphorylation of NFkappaB was also inhibited by gamma-tocopherol treatment and the constitutive activation of this nuclear transcription factor is thought to provide an antiapoptotic mechanism for many cancer cells [89]. The inhibition of NFkappaB would be predicted to result in the downregulation of bcl-2, a key antiapoptotic factor: Campbell et al. [89] did, in fact, find that gamma-tocopherol treatment downregulated bcl-2 levels.

The mechanistic results reviewed above suggest that gamma-tocopherol can inhibit prostate cancer growth by modulating ceramide metabolism [83] or indirectly by modulating the activity of PPARγ via 15-LOX-2 [89]. It is interesting, therefore, that recent work by Wang et al. [92] shows that C2-ceramide can modulate PPARγ expression level and its transcriptional activity resulting in apoptosis in cancer cells. The finding that C2-ceramide induces apoptosis via a PPARγ-dependent pathway may be the "missing link" between the work of Jiang et al. [83] and Campbell et al. [89].

16.14 Gamma-Tocopherol in Animal Models of Prostate Cancer

A major challenge for testing any prostate cancer chemopreventive agent, under well-controlled laboratory conditions, is the lack of animal models that mimic all aspects of human prostate cancer [93]. Dogs are the only large nonhuman species in which prostate cancer occurs spontaneously and with a high frequency [93, 94]. Moreover, dogs have a single lobed prostate gland like humans, whereas mice and rats have four distinct lobes [93]. Bone metastasis occurs in both human and dog prostate cancer [93]. Given the superiority of the dog model, it is surprising that male pet dogs have not been extensively utilized in testing the efficacy of dietary chemopreventive agents to inhibit spontaneous prostate cancer. Transgenic mouse or rat models of prostate cancer are widely used to test chemopreventive agents but have intrinsic limitations [93].

The transgenic adenocarcinoma of the mouse prostate (TRAMP) model is useful since these male mice develop progressive prostate cancer with a histology and pathology that resembles the human disease. Barve et al. [95] recently found that 0.1% (or 1,000 mg/kg diet) dietary mixed tocopherols, enriched with gamma-tocopherol, significantly suppressed PIN and tumor development and also upregulated the expression of Nrf2 compared to TRAMP mice fed a AIN-76A diet. Nrf2 is a redox-sensitive transcription factor that regulates the expression of a variety phase II detoxifying and antioxidant enzymes. No tocopherol analyses of mice tissues, plasma, or diets were provided in this study. Takahashi et al. [96] also studied the effects of gamma-tocopherol on prostate cancer suppression but used transgenic rat for adenocarcinoma of prostate (TRAP) model. These investigators found that dietary gamma-tocopherol significantly suppressed the sequential progression from PIN to adenocarcinoma in a dose-dependent manner as well as activating caspases 3 and 7 in the ventral lobe. In this study, dietary gamma-tocopherol was used at levels from 50 to 200 mg/kg diet, whereas alpha-tocopherol was used only at 50 mg/kg diet: this makes it difficult to compare the tumor suppressor effectiveness of the two isoforms.

16.15 Conclusions and Future Directions

The clinical intervention studies reviewed above provide the unambiguous conclusion that dietary supplementation with alpha-tocopherol alone is not effective for the chemoprevention of prostate cancer in nonsmokers. Nevertheless, a variety of prospective epidemiological studies strongly implicate gamma-tocopherol, but not alpha-tocopherol as a possible prostate cancer chemopreventive agent. Supporting studies in animal and cellular models further strengthen the unique role of gamma-tocopherol: detailed mechanistic studies have also identified important signal transduction pathways relevant to prostate cancer. It is a major mistake to equate vitamin E with only one of its isoforms, i.e., alpha-tocopherol. There is now sufficient evidence to warrant a large-scale clinical intervention trial looking at the potential role of gamma-tocopherol supplements as an effective chemoprevention agent for prostate cancer.

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Part V
Oxidative Stress in Normal Tissue Response (Clinical)

Chapter 17
Pentoxifylline, Vitamin E, and Modification of Radiation-Induced Fibrosis

Geraldine Jacobson

Abstract Radiation-induced fibrosis (RIF) in the breast, skin, and head and neck sites can result in pain, poor cosmesis, and functional limitations. In addition, RIF in the lung, kidney, and bowel, can result in functional loss that can be extremely morbid or life threatening. Studies with pentoxifylline (PTX) have demonstrated the ability of PTX to prevent or improve chronic RIF. Endogenous tocopherol (vitamin E) protects cell membranes against lipid peroxidation. The majority of clinical studies involving PTX and/or vitamin E have investigated their potential role in reversing late radiation changes while only a few studies have focused on their possible role in prevention of delayed radiation effects. The extensive clinical trial experience from 1989 to the present suggests that PTX with or without vitamin E is effective at both preventing and reversing some manifestations of RIF. Mechanisms may include reversal of local hypoxia, inhibition of fibroblast proliferation, and reduction of oxidative stress via multiple pathways. The change in perception of RIF as fixed and irreversible to the realization that it represents a dynamic and continuous process invites us to continue to explore the mechanisms involved and to develop appropriate treatments.

17.1 Introduction

Our knowledge of normal tissue tolerance provides dose constraints for therapeutic radiation. The radiation dose that can be delivered to an anatomic site with an acceptable risk of late complications has been derived from clinical observations.

G. Jacobson, MD, MPH, MBA (✉)
Department of Radiation Oncology, University of Iowa Hospitals and Clinics,
200 Hawkins Drive, Iowa City, IA 52242, USA
e-mail: geraldine-jacobson@uiowa.edu

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Tolerance is site specific, and is modified by volume, fraction size, total dose, and possibly by dose rate. The ideal radiation treatment would deliver the total dose within the tumor and no dose in healthy tissue. Despite technological progress in radiation therapy, internal organ motion and precise definition of tumor boundaries make this unlikely in the foreseeable future. In addition, our clinical experience of healthy tissue tolerance is based on the radiation practice of past decades. The last decade has seen a proliferation of treatment planning software and delivery, accompanied by new approaches and fractionation schedules. These may present as yet unforeseen late radiation toxicity in the future.

Most patients treated with radiation will develop some degree of fibrosis or atrophy in the irradiated normal tissue. This varies in clinical significance depending on site, dose, and individual tolerance. Fibrosis predominates in breast, skin, small bowel, lung, kidney, and liver. Atrophy and necrosis can occur following radiation and surgery, or radiation and local trauma to bone, nerve, and brain [1].

Radiation-induced fibrosis (RIF) in the breast, skin, and head and neck sites can result in pain, poor cosmesis, and functional limitations. In lung, kidney, and bowel, associated functional loss can be extremely morbid or life threatening.

Our conception of RIF has progressed from considering it as a static irreversible condition to thinking of radiation injury to normal tissues as a dynamic process, mediated by modifiable molecular pathways, and potentially reversible. Radiation-induced injury response and repair is a multicellular process driven by intercellular communications via cytokines and growth factors.

Early radiation damage in mammalian tissue is characterized by inflammatory changes and vascular permeability, both of which are characterized by acute oxidative stress [1–5]. Acute vascular changes within 24 h are characterized by radiation-induced apoptotic death of endothelial cells [6]. Late vascular changes include capillary collapse, basement membrane thickening, and loss of clonogenic capacity [7]. Capillary endothelium responds to radiation with edema, leukocyte attachment, and increased capillary permeability. These changes activate cytokines and growth factors. Within hours after radiation exposure, an increased expression of gene coding for growth factors occurs, including interleukin-1, tumor necrosis factor alpha (TNF-α), and transforming growth factor beta (TGF-β). TGF-β promotes progenitor fibroblasts, which differentiate into myofibroblasts and promote deposition of extracellular matrix. In normal wound healing, myofibroblast activation is transient, but in radiation fibrosis, the chronic activation of these cells leads to long-term deposition and remodeling of scar tissue. The fibrotic process can continue for many years and appears to be a dynamic process involving acute and chronic oxidative stress. In this regard, RIF may be considered a chronic inflammatory condition. Hypoxia is known to generate reactive oxygen species (ROS), promote inflammation, vascular permeability, and activate profibrotic cytokines. Radiation injury differs from surgical or traumatic injury in that it is not a single event, but, in fractionated radiation, is caused by a series of radiation events. The process described above occurs multiple times in an environment that is already responding to injury. Another critical difference between normal wound healing and the fibrotic process following radiation injury may be the hypoxic environment created by radiation-induced vascular injury and self-perpetuating oxidative stress.

The biologic response to radiation begins with the generation of ROS. The effects of ROS are rapidly amplified by their interactions with lipids and oxygen. In addition, ROS probably mediate change in the microenvironment because many proteins have built-in sensors for oxidative stress [8]. In the extracellular compartment, ROS affect extracellular matrix degradation, leukocyte chemotaxis and phagocytosis, thrombomodulin, and fibroblast activation [9]. Within the cell, adaptive reactions to oxidative stress trigger DNA repair, cell cycle arrest, and the secretion of growth factors such as TNF-α and IL-1. In addition, ROS interferes with biologic membranes. There is evidence that increased ROS can persist for days in irradiated cells in culture [10]. Patients treated with total body irradiation (TBI) have shown evidence of increased markers for lipid peroxidation in blood that lasts for several days [11]. Robbins also found evidence of oxidative damage to DNA in irradiated kidneys that persisted for up to 24 weeks [12]. Evidence of oxidative damage to lipids in the lung has been observed months after irradiation in patients developing radiation pneumonitis [13]. Tofilon and Fike [14] found increased expression of Hmox-1, a marker of oxidative stress, in CNS 5–6 months following irradiation.

Postradiation hypoxia is thought to contribute to the persistence of oxidative stress following irradiation. Hypoxia is known to generate ROS, promote inflammation and vascular permeability, activate TGF-β, and promote collagen formation [15, 16]. A growing body of evidence supports the hypothesis that chronic oxidative stress contributes to radiation-induced late normal tissue effects and promotes progressive fibrosis and tissue injury [10–14, 17].

If chronic oxidative stress, local tissue hypoxia, and aberrant cytokine-mediated wound healing processes contribute to late radiation effects and RIF, therapy directed to these conditions can potentially improve or ameliorate this pathology.

Preclinical and clinical studies have demonstrated the ability of pentoxifylline (PTX), with or without vitamin E, to prevent or improve chronic RIF. Multiple mechanisms are probably involved, including the impact of PTX/vitamin E on improving tissue oxygenation and reducing the chronic oxidative stress contributing to RIF.

17.2 Preclinical Studies of PTX

PTX is a methylxanthine derivative that was developed and indicated for conditions involving impaired microcirculation [18]. PTX improves blood perfusion by multiple processes. It inhibits cyclic adenosine monophosphate (cAMP) phosphodiesterase, increasing cAMP and adenosine-5′-triphosphate in erythrocytes, and increasing RBC deformability. PTX minimizes leukocyte adherence to endothelial cells, increases prostacylin production, and inhibits platelet aggregation. These effects lead to capillary dilatation. Together, these effects decrease blood viscosity and improve peripheral blood flow [19, 20].

Studies in the late 1980s demonstrated that PTX inhibits the proliferation and biosynthetic activities of fibroblasts derived from normal skin. The addition of PTX to fibroblast cultures resulted in a dose-dependent reduction in collagen, fibronectin,

and glycosaminoglycan production [21]. More recent work showed that PTX inhibits human mesothelial cell growth and collagen synthesis and that PTX inhibited TFG-β-induced nRNA expression in collagen I and III [22, 23]. Lin et al. demonstrated that PTX inhibits connective tissue growth factor (CTGF) expression by interfering with Smad 3/4-dependent CTGF transcription through protein kinase A and blocks the profibrogenic effects of CTGF on renal cells [24]. PTX has been shown to reduce fibrosis in multiple organ systems from stimuli other than radiation [22–25].

Thrombomodulin (TM) is an endothelial cell membrane glycoprotein that plays a major role in intravascular coagulation by preventing thrombus formation. PTX has been shown to increase thrombomodulin (TM) expression in endothelial cells and to prevent tumor necrosis factor (TNF)-induced suppression of TM expression. Seigneur et al. demonstrated that endothelial membrane TM levels are decreased during hypoxia and are restored by PTX, suggesting that PTX may protect endothelial cells against the prothrombotic tendency induced by hypoxia [26].

PTX has been shown to down-regulate the production of proinflammatory cytokines, particularly TNF-α. It reduces the synthesis of TNF-α by blocking transcription activation [27]. A study of radiation-induced lung injury in mice compared the effects of a single 12 Gy dose to the thorax in mice with and without PTX treatment. The study demonstrated a significant radiation-induced increase in TNF-α on the mRNA and protein level in lung tissue during the pneumonic phase, with predominant localization of TNF-α in inflammatory infiltrates. There was a marked reduction of TNF-α mRNA and protein production in the group that received PTX compared to the radiation-only group, demonstrating that PTX-α down-regulates the TNF-α and protein production in the lung induced by radiation [28].

Boerma et al. used a rat model of fractionated local heart irradiation to assess the effects of PTX/Vit E on cardiac radiation injury [29]. Rats were treated with sham radiation or 5 × 9 Gy to the heart. Both groups were divided to receive normal chow, PTX/Vit E enhanced chow starting 1 week prior to and continuing 6 months postradiation, or PTX/Vit E enhanced chow from 3 to 6 months postradiation.

Radiation induced significant increases in deposition of collagen type I and type II. PTX/Vit E significantly reduced both types of collagen in the irradiated animals. No differences were found between early and late treatment with PTX/Vit E. Both PTX/Vit E protocols induced a significant improvement in radiation-induced myocardial fibrosis and left ventricular diastolic dysfunction, but did not improve reduced heart/body weight ratio or myocardial degeneration.

Lefaix et al. studied the effects of postradiation treatment with PTX, and without α tocopherol (Vit E), in an experimental pig model of localized radiation overexposure [30]. Three groups of five pigs were irradiated using a collimated Iridium 192 source to deliver 160 Gy onto the skin surface of the outer thigh. The groups were divided into controls, PTX added to feed for 26 weeks, and PTX/Vit E added to feed for 26 weeks. Following radiation a well-defined volume of necrosis developed within a few weeks which healed at 26 weeks to leave a block of subcutaneous fibrosis involving skin and skeletal muscle. The limits of the fibrotic block were measured; the depth was determined by ultrasound. No changes in the fibrotic scar were observed in pigs treated with PTX alone. Significant softening and shrinking

of the fibrotic block was found in pigs treated with PTX/Vit E. Postautopsy histological examination showed normal muscle and subcutaneous tissue surrounding the residual scar tissue in the PTX/Vit E-treated pigs. Immunolocalization of TNF-α was similar in all three groups of pigs. Immunostaining of TGF-β diminished more in the residual scar tissue of the PTX/Vit E dosed pigs than in the two other groups. This was the first study to demonstrate that the PTX/Vit E combination, given in vivo following high-dose gamma radiation, could reverse measurable RIF. Previously, RIF was considered an irreversible condition.

17.3 Tocopherol and Fibrosis

ROS are generated during inflammatory reactions and induced by radiation injury. If these are not efficiently scavenged, the resulting oxidative stress can lead to cell necrosis or apoptosis. Endogenous tocopherol (vitamin E) scavenges ROS generated during oxidative stress and protects cell membranes against lipid peroxidation. It has been reported to have other critical functions, such as maintenance of energy metabolism and protection of cell membranes. The antioxidant properties of vitamin E have been demonstrated in vivo and in vitro [31].

Vitamin E has been shown to protect gastrointestinal mucosa against radiation injury in mice by its antioxidant properties [32]. Bese et al. demonstrated that vitamin E protected rats against radiation-induced pulmonary fibrosis immediately after irradiation [33].

A preliminary clinical study of vitamin E given to 53 patients with superficial RIF following breast cancer irradiation showed a mean linear regression of 20% after 4 months [34]. A more recent randomized trial showed identical response in patients treated with placebo or vitamin E [35]. Given prophylactically, a randomized trial of rinsing the oral cavity with a solution containing vitamin E was more effective than placebo in reducing radiation-induced mucositis in head and neck cancer patients [36].

The safety of high-dose vitamin E was raised by two studies showing that daily ingestion of vitamin E, greater than 400 IU, was associated with increases in all causes of mortality [37, 38]. A later meta-analysis showed that vitamin E is unlikely to affect mortality, regardless of dose [39].

17.4 Clinical Studies of Pentoxifylline in Irradiated Patients

Some of the early preclinical and clinical studies of PTX in irradiated tissue were conducted at the University of Iowa [40, 41].

Two theories regarding late normal tissue damage were predominant at that time. The first was that late radiation damage resulted from depletion of parenchymal and stromal cells [42]. The second theory attributed late changes to vascular injury and damage to endothelial cells [43, 44]. When PTX became available for the treatment

of vasculo-occlusive disorders; it seemed reasonable to investigate its action in treating or preventing normal tissue radiation damage. A study of the prophylactic effect of PTX in irradiated mice showed no effect on acute radiation reactions, but was shown to reduce the incidence and severity of late radiation injury [41].

Based on this research, a pilot study was conducted to evaluate the management of soft tissue radionecrosis [40]. Twelve patients with 15 sites of late radiation fibrosis were treated. These included four sites in oral mucosa, four sites in female genitalia, and seven skin sites. PTX 400 mg was given orally three times daily. One patient had the dose raised to 400 mg four times daily because of minimum response at 3 months. The protocol specified that the drug be continued for 6 months regardless of healing. Two patients discontinued the drug at 1 month, one because of diarrhea and the other because of surgical intervention. These patients were excluded from analysis. At the time of analyses, 13/15 of the necroses were completely healed and one was partially healed. The average time required for healing was 9 weeks, compared to an average duration of nonhealing of 30.5 weeks. All patients had pain relief. Eight patients who required narcotic pain relief prior to the study were able to discontinue this at completion. This study demonstrated that PTX contributed to a decrease in pain and healing of soft tissue necrosis associated with late radiation injury. Since PTX improved tissue perfusion, the authors concluded that this study supported the theory that late radiation damage is at least partly caused by vascular injury.

Following this report, other groups began to investigate the role of PTX or the combination PTX/Vit E in reducing late radiotherapy effects. Delanian et al. published results of 43 patients with chronic radiotherapy damage treated with 400 mg PTX and 500 IU vitamin E twice daily for at least 6 months and as long as continued regression occurred [45]. The combination of PTX and vitamin E was chosen because the investigators earlier work with induced radiation injury in pigs showed a benefit from PTX/Vit E but not from PTX alone [30]. Patients in this trial had been treated for head and neck cancer or breast cancer for a mean period of 8.5 ± 6.5 years earlier. Clinical regression of fibrosis and functional improvement was noted in all accessible areas. All fibrotic areas improved rapidly with regard to local pain.

These investigators continued their clinical exploration of this drug in combination with a randomized study of breast cancer patients with clinically measurable RIF [35]. Twenty-four eligible patients were randomized to one of four groups: PTX/Vit E, PTX/placebo, placebo/Vit E, and placebo/placebo. Treatment continued daily for 6 months. PTX dose was 400 mg twice daily. Vitamin E was taken 500 IU twice daily. The PTX/Vit E group showed significant reduction in measurable RIF. A limitation of the study was the small sample size, with only six patients in each treatment group.

A review of patients treated for RIF with PTX/Vit E over a 15-year period (1990–2005) demonstrated significant regression of RIF with a maximum response at 2 years. The study included 44 women with 55 RIF zones [46]. The original treatment protocol was for only 6 months, but with increasing experience, patients were treated for a more extended duration. In the earlier group of patients they noted a rebound effect of recurring fibrosis in patients that stopped treatment after 6–12 months. Thirty-seven patients were treated until fibrosis regression peaked,

a mean of 36 months. Acute tolerance was satisfactory and none of the patients discontinued the treatment because of an adverse event. Results were based on measurement of RIF area and symptom severity. The mean estimated maximal treatment effect was 68% RIF surface area regression.

In addition to RIF, these investigators tested the combination of PTX/Vit E, sometimes with other medications, to treat a variety of radiation-induced sequelae. An example is given of a patient who developed extensive fibrosis, skin ulceration and, a fistula track 30 years following postmastectomy radiation [47]. MRI showed osteoradionecrosis with bone destruction. The patient was started on 800 mg PTX and 1,000 IU vitamin E daily. Two months later clodronate 1,600 mg/day, 5 days a week was added. After 3 years, the clinical response was complete with total regression of clinical fibrosis, a closed fistula and the disappearance of localized inflammation. Brachial plexopathy persisted. Medication was continued for 1 year following this complete response.

A recent publication describes two case reports of patients with progressive lower limb polyradiculopathy following lumbar irradiation [48]. The treatment combined 800 mg PTX, 100 IU vitamin E, and 1,600 mg clodronate 5 days per week alternating with prednisone 20 mg 2 days per week. After 6 months of treatment, patients had clinical improvement of their neurological sensorimotor symptoms. The PTX/Vit E combination was also tested and found to be effective in increasing endometrial thickness and improving pregnancy rate in young women, some of whom had received prior radiation [49, 50].

Other investigators have studied the potential benefit of PTX in reducing symptoms of radiation late effects. A pilot study in the treatment of radiation-induced trismus enrolled 20 patients with symptomatic, measurable radiation-induced trismus. Sixteen completed an 8-week course of PTX 400 mg two to three times daily. This study showed a modest improvement in mean dental gap. The investigators noted the small sample size and the lack of a randomized trial. In light of the experience of Delanian et al. [46], 8 weeks was probably too short interval to assess the potential response to this medication.

Okunieff et al. studied the effects of oral PTX (400 mg orally for 8 weeks) in patients with measurable functional impairment due to radiation fibrosis [51]. Patients were 1–29 years posttherapy (mean, 10.1 years). The primary outcome was a change in physical impairment due to radiation including active and passive range of motion (AROM and PROM), muscle strength, limb edema, and pain. Plasma levels of TNF-α and fibroblast growth factor 2 (FGF2) were measured. After 8 weeks, 20/23 patients with impaired AROM and 19/22 patients with impaired PROM improved; 11/19 patients with muscle weakness showed improved motor strength; 5/7 patients with edema had decreased limb girth; and 9/20 patients had decreased pain. Assessments were made at the completion of 8 weeks of therapy and at week 16 (8 weeks off drug). For some patients, initial improvements regressed during the 8 weeks off treatment. This is not surprising, considering the experience of other investigators [46]. The pretreatment circulating plasma levels of TNF-α were not elevated, so posttreatment changes were difficult to assess. Pretreatment FGF2 levels dropped significantly by 8 weeks of treatment, and were associated with reversal of the delayed radiation effects.

A group in Tehran treated 29 patients with 34 RIF superficial lesions with PTX/Vit E for 3 months. The mean surface area decreased to almost half in 3 months. A subgroup of patients received the medication for 6 months with significant surface area regression of RIF [52].

A retrospective study of patients with radiation proctitis/enteritis reviewed the experience of 30 patients selected by their physicians for treatment with PTX/Vit E or supportive care [53]. The combination treatment appeared to improve symptoms. Patients who experienced symptom relief took the drug combination for an average of 9 months. Patients with no improvement took the medication for an average of 5 months.

The previously discussed clinical trials showed a beneficial effect of PTX in treating postradiotherapy fibrosis or fibrosis-related side effects. In contrast to these, a British placebo-controlled randomized trial of vitamin E and PTX showed no improvement in patients with arm edema and fibrosis following breast cancer surgery and radiation [54]. This trial treated patients with a minimum 20% increase in arm volume at a median of 15.5 years (range 2–41) with 500 IU vitamin E twice a day and PTX twice a day for 6 months versus placebo for 6 months. The primary endpoint was ipsilateral limb volume measured opto-electronically using a perometer and expressed as a percentage of the contra lateral limb volume. At 12 months there was no significant difference between the treatment and control groups in terms of arm volume. Nor was there a significant difference in secondary endpoints including tissue induration, patient self-assessment of function, or quality of life. The authors were confident about the measurements of arm volume. They expressed less confidence about the reliability of the physician assessments of RIF. Multiple physicians scored patients, and when a single observer scored a patient at different time intervals, significant variation was noted in the placebo scores.

A phase II study of patients treated with pelvic radiation with a minimum grade 3 or 4 disability (LENT-SOMA) assigned patients to be treated with 6 months of PTX/Vit E [55]. Late changes were evaluated by LENT-SOMA at baseline, 6 and 12 months. Twenty-three of 27 volunteers completed 6 months of therapy, follow-up assessments, and self-assessment questionnaires. Changes in LENT-SOMA scores suggested beneficial effects. Magnetic resonant imaging (MRI) was obtained at baseline and 6 months after therapy in 13/23 evaluable patients. No significant changes were reported on MRI images at 6 months from baseline. No significant changes were seen over the study period in quality of life.

The majority of clinical studies involving PTX have investigated its potential role in reversing late radiation changes while only a few studies have focused on its possible role in prevention of late radiation effects.

Ozturk et al. conducted a randomized double-blind trial of PTX versus placebo in patients receiving radiation for breast or lung cancer to determine if the drug could prevent early or late normal lung tissue damage [56]. Patients were randomized to take PTX 400 mg orally three times daily or a placebo during the entire period of radiotherapy. Analytic tests were obtained at baseline, and at 3 months, and 6 months follow-up. These included pulmonary function tests (PFTs), diffusing capacity of carbon dioxide (DLCO), chest X-ray, CT lung, and ventilation perfusion (V/Q) studies. Patients were also evaluated by LENT-SOMA scores and by common

toxicity criteria [56–58]. A statistically significant difference was found between the LENT scores of the two groups, in favor of PTX. During the 6 months of follow up, clinically impaired lung function was observed in 30% of the placebo group versus 5% in the study group. This finding was borderline significant. A comparison of the DLCO between the two groups at 3 and 6 months showed a significant difference in favor of the PTX group. The use of PTX showed a noticeable reduction in the degree of lung injury detected radiologically. Side effects during treatment were not different in the two groups.

A study of patients referred for postoperative irradiation of squamous cell carcinoma of the head and neck, randomized patients to PTX 400 mg three times daily or control [59]. The drug was given during 6 weeks of radiation until 2 weeks after treatment completion. The differences in acute skin reactions between the two groups were not significant. Late changes appeared later in the PTX group although late skin changes and soft tissue injury were more severe in the control patients. This study also showed a prophylactic effect of concurrent PTX on late radiation complications.

The studies just discussed evaluated the benefit of PTX in prevention of late effects by treatment during radiation therapy. PTX/Vit E can also be used in a prophylactic manner by treating patients following radiation, before the development of late fibrotic effects.

A randomized placebo-controlled, double blind parallel group trial was performed in breast cancer patients following radiation to investigate whether the PTX/Vit E drug combination could prevent late effects [60]. Inclusion criteria were segmental resection or mastectomy and axillary dissection followed by radiation to breast/chest wall and adjacent nodal areas. Treatment was PTX 400 mg and vitamin E 100 IU mg thrice daily for 12 months starting 1–3 months following completion of radiation therapy. Controls received placebo and vitamin E over the same period. Study end points consisted of changes of passive abduction of the shoulder, changes in arm volume, LENT-SOMA, and pain measurement by the Visual Analog Scale (VAS) [61]. At inclusion both groups had impaired passive abduction of the shoulder. This improved in both groups during therapy and the difference between groups was not significant. Arm volumes increased over time in the placebo group but not in the treatment group. No difference in VAS for subjective pain was noted between the groups. The VAS score for pain described as stiffness in the skin significantly decreased in the PTX group during the treatment time. In this study, PTX/Vit E was effective in preventing arm edema when given prior to its development. This contrasts with the Gothard study [55], which showed no benefit to PTX/Vit E in reversing longstanding arm edema in breast cancer patients. This is consistent with other clinical observations that arm edema can be prevented, but rarely reversed.

At the University of Iowa we have completed accrual of a randomized study of postradiation PTX/Vit E in breast cancer patients. The study was originally designed to include both breast and head and neck cancer patients since both groups are at risk for visible RIF. However, accrual of this latter group was limited by lack of a liquid oral formulation of PTX and only breast cancer patients are evaluated. Subjects were randomized to receive oral PTX 400 mg three times daily

and vitamin E 400 IU daily for 6 months to be started within a week of completing radiation. Evaluation includes RTOG acute side effects at baseline, LENT-SOMA at 3, 9, 12, and 18 months; pain by VAS, and measure of tissue compliance in the treated and untreated side by tissue compliance meter at 12 and 18 months. An interim analysis [62] showed significant difference in tissue compliance between treated and untreated breast/chest walls in the controls compared to study subjects, demonstrating decreased fibrosis in the PTX/Vit E group. For the most part the drug combination was well tolerated. One of 57 patients developed a skin rash, which resolved when the drug was discontinued. One patient discontinued the drug because of side effects.

17.5 The Role of Pentoxifylline-Based Therapy in Radiation-Induced Fibrosis: Prevention and Treatment

The extensive clinical trial experience from 1989 to the present suggests that PTX with or without vitamin E is effective at both preventing and reversing some manifestations of RIF. The majority of studies that were directed to improvement of existing fibrosis were positive. The drug combination showed some protective effect against development of lymphedema [60], but was not shown to be effective in reversing lymphedema [54]. The benefit following pelvic radiation was ambiguous, with improvement in quality of life measures, but no measurable improvement on imaging studies. A randomized trial of PTX during radiation for lung or breast cancer showed a decrease in late lung toxicity [56]. The medication has not been shown to prevent acute effects, even if given prior to or during radiation [29, 56, 59].

A positive aspect of working with PTX is that the drug has been in widespread use for peripheral vascular disease for several decades with a well-known safety profile. Potential side effects include hot flushes, nausea, headache, and dizziness. These are usually transient and can sometimes be avoided by phasing in the dose from once daily to three times a day. In the studies described, long-term tolerance was excellent and no deaths were attributed to the drug. In addition, this drug is available in generic formulation and the cost of treatment is modest.

However, many questions about the role of PTX in treatment or modification of RIF remain.

The first is whether PTX alone or PTX with vitamin E is more effective. The data regarding this are conflicting. Some studies using PTX alone have shown a beneficial effect [40, 41, 56]. The majority of the studies cited, including the most robust randomized trials, have used PTX in conjunction with vitamin E [19, 29, 30, 35, 45, 47–50, 52, 54]. Since the addition of vitamin E adds minimal cost and minimal potential toxicity, it is reasonable to plan future studies with this combination.

A second issue is the effective duration of treatment when PTX/Vit E is used to reverse established radiation fibrosis. Based on the work of investigators with the longest experience using this combination [46], treatment should probably be given

until a maximal response has been obtained and reached a plateau. For patients with longstanding RIF this may be 2–3 years.

A third question is the timing of the intervention. Should PTX/Vit E be given during radiation or after radiation, but before development of RIF in high risk patients? Or should treatment only be indicated for patients who actually develop RIF or fibrosis-related complications?

A small group of trials suggests that treatment during radiation may prevent late effects in head and neck and lung, without reducing acute effects [59]. There may be additional therapeutic benefits to this strategy, since some preclinical and clinical studies have suggested that PTX, by improving tissue oxygenation and sensitizing p53 mutant tumors, may function as a radiation sensitizer [63–65].

When PTX is used prophylactically, either during radiation or started shortly after radiation completion, some caution is warranted. The effects that occur in normal tissue following radiation may have some benefit in tumor control. Residual cancer cells have been observed in irradiated tissues but do not always form tumors [66]. The therapeutic effects of radiation may be partially due to formation of a microenvironment that impedes tumor regrowth, as well as cell killing [67].

Despite this concern, the randomized studies discussed have not shown increased local or metastatic relapse in patients treated with PTX [35, 54, 56, 60].

Clinical studies have shown both a protective effect if given before RIF and a beneficial effect if used to treat established RIF.

While prevention of RIF seems preferable to patients developing late toxicity, there are drawbacks to treating patients who may not develop the condition, including cost and potential side effects. If patients could be identified as high risk for developing late effects based on site, total dose, known radiation sensitivity, or cytokine assay, preventive treatment could be initiated in those patients. In the case of patients receiving significant radiation to the lung, including lung cancer, esophageal cancer, and breast cancer with nodal irradiation, prophylactic PTX may prevent irreversible decrease in pulmonary function. Prevention of RIF in head and neck cancer patients, where associated morbidity is common and involves multiple functions, is a setting where prophylactic treatment with an antifibrosis regimen might be warranted. Postmastectomy radiation, with the risks of arm edema and pulmonary function impairment, is another candidate for preventative treatment.

Clinical experience has demonstrated that patients with similar indications and treatments may have widely variable late normal tissue reactions. Ideally, dependable predictive assays or markers would be clinically available to determine risk for individual patients.

The possibility of identifying serum markers has been studied by several groups. A multinational prospective trial sponsored by Radiation Therapy Oncology group (RTOG) showed that elevated levels of IL-6 after 10 Gy of lung irradiation appeared to predict lung toxicity [68]. Zhao et al. studied the predictive role of plasma TGF-β in radiation-induced lung toxicity (RILT) in nonsmall cell lung cancer patients [69]. They found that plasma TFG-β levels at 4 weeks of radiation was predictive of RILT. They noted that other studies of this marker had conflicting results, and described the numerous factors that could contribute to inaccurate values of plasma

TGF-β. Microarray assays, single nucleotide polymorphisms, and genome wide association studies have been conducted to identify predictive tests. But at this time, there are no tests available to routinely predict normal tissue reactions following therapeutic radiation [70].

In addition, the benefit gained from PTX/Vit E is quite variable. We have used PTX/Vit E at our institution since the 1990s to treat patients with severe late radiation effects with a wide variety of manifestations. The majority of patients receive some measurable benefit. In some cases the results are striking, but there are also cases with no discernible improvement.

17.6 Potential Mechanism of Fibrosis Reduction by Pentoxifylline

While hypoxia and tissue perfusion may be important factors in radiation repair, the reversal of fibrosis seen in many of these clinical studies is probably caused by a combination of improved tissue oxygenation and other mechanisms. A major aspect of chronic RIF is the abnormal proliferation of fibroblasts. PTX has been shown to inhibit proliferation and biosynthetic activities of fibroblasts. Current research indicates that cytokines such as tumor necrosis factor, TFG-β, and macrophage-derived growth factor may play important roles in RIF [21, 71–73]. PTX has been shown to down regulate TNF α [27, 32]. PTX has also been shown to inhibit TGF-β-induced mRNA expression in collagen I and III.

If one considers RIF as a dynamic biologic process involving injury, hypoxia, hypoxia-induced oxidative stress, and profibrogenic and proangiogenic cytokines; it is likely that PTX, with or without vitamin E, impacts multiple components of this cycle.

Just as cancer is not a single disease, RIF is probably not a single pathologic entity. Although there are common pathways involved, the specific form RIF takes may depend on the host genetics and the specific tissue microenvironment in which it occurs, with different interactions occurring in skin, heart, lung, bowel, and other organs [22, 29, 63, 74].

The change in perception of RIF as fixed and irreversible to the realization that it represents a dynamic and continuous process invites us to continue to explore the mechanisms involved and to develop appropriate treatments.

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Chapter 18
Antioxidants, Anorexia/Cachexia, and Oxidative Stress in Patients with Advanced-Stage Cancer

Giovanni Mantovani, Clelia Madeddu, and Antonio Macciò

Abstract Cancer cachexia is increasingly becoming a critical component in the comprehensive approach to cancer patients influencing morbidity, mortality, and quality of life. Therefore, its pathophysiology and the main contributing factors have been investigated with the aim of developing effective therapies. Reported findings indicate that increased production of reactive oxygen species and reduced activity of antioxidant enzymes contribute to development of anorexia and cachexia in cancer. Oxidative stress, almost always accompanying cancer cachexia, may be counteracted by effective antioxidant treatments: in this review, the most relevant recent clinical approaches addressing this target are reported. Fairly advanced clinical data on efficacy of and antioxidants in advanced cancer patients are promising, but the best way to administer and combine them with other agents, the optimal dose, and timing remain uncertain. However, because cachexia is a multifactorial syndrome, therapeutic approaches targeting a single contributing factor may be inadequate: targeting oxidative stress only one determinant is addressed, thereby limiting clinical efficacy. Therefore, antioxidants should be included in developing a therapeutic approach for cachectic cancer patients, however, they cannot encompass all symptoms of cancer cachexia. Recent evidence seems to confirm that the treatment of cancer cachexia, a multifactorial syndrome, is more likely to yield success with a multitargeted approach.

G. Mantovani, MD (✉) • C. Madeddu, MD • A. Macciò, MD
Department of Medical Oncology, University of Cagliari, SS 554,
km 4.500, 09042, Monserrato, Cagliari, Italy
e-mail: mantovan@medicina.unica.it

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18.1 Introduction

The presence of oxygen is a fundamental component of cellular metabolism. Aerobic energy metabolism, or oxidative phosphorylation, is a critical metabolic pathway within cells to provide energy. Inside the mitochondria, electron transport chain activity is responsible for a series of redox reactions which result in the synthesis of ATP. As the demand and subsequent flux for this process increase so does the chance that redox uncoupling will occur and increase the accumulation of free radicals throughout the cell [1, 2].

Any situation which results in an acute or chronic overconsumption of oxygen can lead to the production of the free radicals, which are more appropriately termed reactive oxygen species (ROS). A free radical is a molecule that contains at least one unpaired electron in its outer spin orbits. Superoxide radicals, hydroxyl radicals, hydrogen peroxide, nitric oxide, lipid alkoxyl, and peroxyl radicals are the most common ROS in living, aerobic systems. Normal human cells produce small amounts of ROS, which are reduced by antioxidant enzymes and low molecular weight radical scavenger. It is widely accepted that ROS play both positive and negative roles in vivo. The positive roles are those related to ROS involvement in energy production, phagocytosis, regulation of cell growth, intercellular signaling, and synthesis of biologically active compounds [3].

To protect against the deleterious effect of ROS, our body is provided with a complex system of endogenous antioxidant protection in the form of enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, as well as nonenzymatic defenses, such as GSH, the iron-binding protein transferrin, dihydrolipoic acid, and reduced CoQ10 [4]. In situations in which the production of prooxidant molecules increase to a point where the antioxidant system cannot effectively remove them, oxidative stress is known to occur. Indeed, oxidative stress is defined as the imbalance between oxidants and antioxidants in favor of the oxidants.

ROS, if not detoxified by the antioxidant system, exert a toxic action on circulating proteins, cell surface protein, lipids, enzymes, and nucleic acids (DNA). One of the most frequent targets is the polyunsaturated fatty acids that largely comprise the cell membranes. The systematic oxidation of these polyunsaturated fatty acids called lipid peroxidation is one of the primary means by which oxidative stress leads to an overall decrease of cellular functions. Thus, an adequate presence and functioning of antioxidant systems is paramount for cell activity. Vice versa, oxidative stress is implicated in a number of diseases which include atherosclerosis, pulmonary fibrosis, cancer, Parkinson's disease, multiple sclerosis, and aging [5].

Humans have evolved antioxidant systems to protect against free radicals. These systems include endogenous antioxidants (i.e., produced by the body) and exogenous antioxidants that are supplied by the diet. Endogenous antioxidants include enzymatic defenses, such as Se-dependent glutathione peroxidases (GPx), catalase, and superoxide dismutases (SOD), which metabolize superoxide, hydrogen peroxide, and lipid peroxides, thus preventing the formation of the toxic $^{\cdot}OH$, as well as nonenzymatic defenses, such as glutathione, histidine peptides, the iron-binding proteins transferrin

and ferritin, lipoic acid, reduced CoQ10, melatonin, urate, and plasma protein thiols. Urate and plasma protein thiols account for the major contribution to the radical-trapping capacity of plasma [6]. Nevertheless, oxidative stress may occur when the generation of ROS exceeds the system's ability to neutralize and eliminate them. The imbalance can result from an excessive production of ROS or from a lack of the system's antioxidant capacity.

18.2 Cachexia and Oxidative Stress

18.2.1 Cancer-Related Anorexia/Cachexia Syndrome

The progression of the neoplastic disease is characterized by specific alterations of energy metabolism and by symptoms such as fatigue, anorexia, nausea, anemia, and immunodepression, which finally result in a peculiar clinical picture known as cancer cachexia which, unless counteracted, can lead to patient's death.

Cachexia is a multifactorial syndrome characterized by tissue wasting, loss of body weight, particularly of lean body (muscle) mass (LBM) and, to a lesser extent, adipose tissue, metabolic alterations, fatigue, reduced performance status, very often accompanied by anorexia leading to a reduced food intake. Cachexia accompanies the end stage of many chronic diseases including cancer which is termed "cancer-related anorexia/cachexia syndrome" (CACS) [7–10]. Key features of CACS are increased resting energy expenditure (REE), increased levels of circulating factors produced by the host immune system in response to the tumor, such as proinflammatory cytokines, or by the tumor itself, such as proteolysis-inducing factor [11]. At the time of cancer diagnosis, 80% of patients with upper gastrointestinal cancers and 60% with lung cancer have already had substantial weight loss. The prevalence of cachexia increases from 50% to 80% before patients' death [12].

Proinflammatory cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α) play a central role in the pathophysiology of CACS through a long-term inhibition of feeding by negatively acting on hypothalamic orexigenic peptides such as neuropeptide Y (NPY) and agouti-related protein (AGRP), and/or positively acting on anorexigenic peptides (proopiomelanocortin, POMC, and cocaine- and amphetamine-related transcripts, CART), respectively. There is evidence that a chronic, low-grade, tumor-induced activation of the host immune system, which shares several characteristics with the "acute-phase response" found after major traumatic events and septic shock, is involved in CACS [13].

18.2.2 Oxidative Stress and Cancer Cachexia/Anorexia

Although a widely accepted definition of cancer cachexia does not yet exist, muscle wasting is among the major features of this syndrome. Experimental and human

data consistently indicate that muscle wasting results both from increased protein degradation, mediated by the ubiquitin–proteasome system, and reduced protein synthesis.

Proinflammatory cytokines have been shown to upregulate the ubiquitin–proteasome system, but recent data suggest that oxidative stress may also contribute to protein degradation [14]. Consistent evidence indicates that increased oxidative stress is involved in experimental models of cancer cachexia [14]. It was demonstrated that mild oxidative stress increased protein degradation in skeletal muscle by causing greater expression of the major components of the ubiquitin–proteasome pathway [15]. Barreiro et al. [16] showed that in the muscles of tumor-bearing animals, oxidative stress was increased and, moreover, protein levels of the antioxidant enzymes were similar in the muscles of tumor-bearing rats and control animals, suggesting that oxidative stress results from increased ROS production and inefficient antioxidant activity [16]. Also, oxidative stress promotes skeletal muscle apoptosis, as documented in several experimental models of cancer cachexia. In particular, it appears that nitric oxide formation and thus nitrosative stress-inducing DNA fragmentation and muscle apoptosis during tumor growth are related [17]. Increased oxidative and nitrosative stress influence muscle protein degradation via specific molecular effects. ROS upregulate the ubiquitin–proteasome system, via nuclear factor-κb (NFκB) activation, which accelerates wasting [15]. On the other hand, nitration of tyrosine residues triggers increased degradation by the proteasome of the modified proteins [18].

Recent experimental studies have shown that both proteolysis-inducing factor and angiotensin II induced a rapid and transient increase in ROS formation in murine myotubes, which appear to be important in muscle atrophy in cancer cachexia, because treatment of weight-losing mice bearing the MAC-16 tumor with D-alpha-tocopherol attenuated protein degradation and increased protein synthesis in skeletal muscle [19]. Moreover, both ROS and TNF-α activate many catabolic elements in skeletal muscle, including NFκB and mitogen-activated kinase, p38 MAPK. In concert with increased degradation, ROS-regulated catabolic signals may reduce protein synthesis. ROS have been shown to reduce translational activity in Chinese hamster ovary cells as well as decrease phosphorylated p70S6k levels and protein synthesis [20].

18.2.3 Pathogenesis of Oxidative Stress in Cancer Cachexia

Several mechanisms may lead to oxidative stress in cancer patients [21]. First of all, altered energy metabolism, which may be secondary to symptoms such as anorexia/cachexia, nausea and vomiting, which prevent a normal nutrition and thereby a normal supply of nutrients such as glucose, proteins, and vitamins, eventually leads to the accumulation of free radicals. Indeed, in advanced cancer patients, the altered energy metabolism in addition to symptoms such as anorexia, nausea, and vomiting, do not allow an adequate synthesis of reducing compounds and a normal intake of

carbohydrates and dietary antioxidants, thus favoring the accumulation of ROS. Therefore, in advanced cancer patients oxidative stress may be considered a manifestation of reduced food intake and impaired glucose utilization [22].

The second mechanism results from chronic inflammation associated with a nonspecific activation of the immune system with an excessive production of proinflammatory cytokines, which, in turn, may increase the ROS production [23]. The third mechanism, favoring the development of oxidative stress in cancer patients, may result from the use of antineoplastic drugs: many of them, particularly alkylating agents and cisplatin, are able to produce an excess of ROS and, therefore, lead to oxidative stress [24]. Thus, it could be speculated that the body redox systems, which include antioxidant enzymes and low molecular weight antioxidants, might be dysregulated in cancer patients, and this imbalance might enhance disease progression.

In a clinical setting, an increased oxidative stress has been detected in advanced cancer patients: Mantovani et al. [25] enrolled 120 cancer patients and 60 healthy individuals and found that ROS levels were significantly higher and the activities of antioxidant enzymes, namely superoxide dismutase and GPx, were significantly lower in patients than in control individuals.

Moreover, in a series of our published studies [26–28] we have demonstrated that oxidative stress was associated with high levels of proinflammatoy cytokines IL-6 and TNF-α, and CPR, and low levels of leptin. In particular, the inverse correlation between leptin levels and the parameters of oxidative stress (ROS) strongly suggests that leptin is a signal of negative energy balance and low energy reserves and that oxidative stress is the consequence of the metabolic derangement, particularly of glucose metabolism. Therefore oxidative stress, consequent in advanced cancer patients to the low energy reserves and the inability to utilize efficiently the energy substrates, particularly glucose, may be considered the direct evidence of the metabolic impairment of which leptin is the most important parameter. Accordingly, in a recent paper [22] we demonstrated that in advanced ovarian cancer patients the lowest leptin levels and the highest IL-6 levels correlated with the highest levels of ROS and the lowest levels of GPx.

Moreover, the impairment of energy metabolism, by inducing oxidative stress, is responsible for defective immune functions shown in advanced cancer patients. Immunodepression is a key feature of patients with CACS and its severity is related to the stage of disease and severity of cachexia. Several of our studies have shown that PBMCs isolated from cancer patients shows an impaired blastic response to mitogens (such as PHA, anti-CD3 antibody, and recombinant IL-2). These defective functions correlate with the severity of disease and poor survival, and are actually considered a consequence of the oxidative stress, which in turn is an effect of the cell's impaired glucose metabolism. In advanced cancer patients, the altered energy metabolism and particularly the defective glucose utilization are responsible for the reduced synthesis of reducing compounds by the pentose–phosphate pathways. However, the correct immune cell functioning requires adequate concentrations of intracellular reducing compounds and particularly GSH.

18.3 Antioxidants

To counteract ROS and oxidative stress, several approaches have been tried both in experimental studies and in humans. Reduced glutathione (GSH), ALA, cysteine-containing compounds, amifostine, and vitamins are among the most used antioxidant agents [21].

18.3.1 The Glutathione Cycle

Glutathione is the most important cellular antioxidant. It is endogenously synthesized throughout the body and it exerts several essential functions such as antioxidant defense, detoxification of xenobiotics, modulation of redox regulated signal transduction, storage and transport of cysteine, regulation of cell proliferation, synthesis of deoxyribonucleotides, and regulation of immune responses. As for glutathione availability, one of the most important issues is to maintain the blood availability of cysteine as that it is known to be the rate-limiting substrate for glutathione resynthesis. Therefore, identifying ways to achieve an optimal availability of cysteine is a primary approach to maintain an adequate cell biosynthesis of reduced glutathione. Glutathione is a tripeptide, c-L-glutamyl-L-cysteinyl-glycine, found in all mammalian tissues and especially highly concentrated in the liver. Glutathione exists in a thiol-reduced (GSH) and a disulfide-oxidized (GSSG) form. GSH serves several vital functions [29–32] including

1. Detoxifying electrophiles
2. Scavenging free radicals
3. Maintaining the essential thiol status of proteins
4. Providing a reservoir for cysteine
5. Modulating critical cellular processes such as DNA synthesis, microtubular-related processes, and immune function

In addition, GSH has been shown to regulate the nitric oxide homeostasis [33], modulate the activity of proteins by post-translational modification (protein *S*-glutathionylation) [34], increase the neurotransmitter receptors activity [35] and the blastic response and function of T lymphocytes.

GSH, which is the dominant nonprotein thiol in mammalian cells, undergoes thiol-disulfide exchange in a reaction catalyzed by thiol-transferase as follows:

$$\text{Protein} - \text{SSG} + \text{GSH} \rightarrow \text{Protein} - \text{SH} + \text{GSSG}$$

Since this reaction is reversible, the equilibrium is determined by the cell redox state, which depends on the concentrations of GSH and GSSG [36].

Mitochondrial GSH is critical in defending against both physiologically and pathologically generated oxidative stress [37]. Severe oxidative stress can overcome the cell ability to reduce GSSG to GSH leading to the accumulation of GSSG.

To protect the cell from a shift in the redox equilibrium, GSSG can be actively exported out of the cell or react with a protein sulfhydryl group, such as cysteine, leading to the formation of a mixed disulfide. Thus, severe oxidative stress depletes cellular GSH.

GSH is a key molecule in the redox body homeostasis. Oxidative stress induces the transformation of GSH into oxidized glutathione (GSSG) by the action of the enzyme GPx. GSSG may in turn be transformed into glutathione protein mixed disulfide or reduced back to GSH by glutathione reductase. During cancer growth, the glutathione redox status (GSH/GSSG) decreases in the blood of tumor-bearing animals and humans. This effect is mainly due to an increase in GSSG levels [38].

18.3.2 Alpha Lipoic Acid

Alpha lipoic acid (ALA) has recently gained considerable attention as an antioxidant [39]. It is present in human cells in a bound lipoil-lysine form, and particularly in mitochondrial proteins, which play a central role in oxidative metabolism. It has been reported to have beneficial effects in disorders associated with oxidative stress, by inducing a significant increase in cellular GSH and restoring GSH-deficient cells [40]. Within drug-related antioxidant pharmacology, ALA is a model compound that improves the understanding of the mode of action of antioxidants in drug therapy.

18.3.3 Cysteine-Containing Compounds

Among the cysteine-containing compounds, the carbocysteine-lysine salt appears to be the most interesting one: cysteine is the precursor for GSH synthesis and has been shown to act on redox balance and significantly improve the antioxidant potential by elevating GSH levels [4]. Carbocysteine-lysine salt protects alpha-1-antitrypsin from inactivation by hypochlorous acid: indeed, its chemical structure, which is similar to that of methionine, competes with methionine against the oxidative activity of ROS. Amifostine, an analogue of cysteamine, is a phosphorylated aminothiol prodrug that is dephosphorylated at the tissue site by membrane-bound alkaline phosphatase to its active metabolite, the free thiol, WR-1065. WR-1065 is the form of the drug that is rapidly taken up into cells, and it is the major cytoprotective metabolite.

18.3.4 Antioxidant Vitamins

Antioxidant vitamins, which include vitamin A, vitamin C, and vitamin E, are thought to decrease cancer risk and prevent cancer progression by trapping organic free radicals or deactivating reactive oxygen molecules or both [41, 42]. Several

studies have been carried out in an attempt to demonstrate a preventive role for vitamins as antioxidant agents against cancer and other diseases. The discrepancies between the results of these studies may be explained by the type of population studied (general or high-risk patients), the different doses of supplementation (physiological or pharmacological levels), the number of antioxidants tested, and the type of administration (alone or in a balanced association). Thus, it appears that the preventive effect may be related to multiple nutrients consumed at physiological doses: the optimal effect may be expected when a combination of nutrients at levels similar to those found in a healthy diet is provided [43].

18.3.5 L-Carnitine

A potentially interesting compound is L-carnitine, which acts, at least in part, as an antioxidant by increasing the amount of acetyl-CoA and enhancing the citric acid cycle, inducing increased NADH/$FADH_2$ production, thus positively influencing cell redox status by decreasing ROS production and enhancing their scavenging effects. Reduced L-carnitine levels are associated with the development of cachexia [44].

18.4 Antioxidants and Cancer Cachexia: New Approach of Treatment

18.4.1 Preclinical Studies

Preliminary animal data suggest that antioxidant supplemented rat chow ameliorates the decrease in food intake in tumor-bearing rats only when this diet is given to the animals following tumor inoculation [45]. When antioxidant supplementation is started with the onset of anorexia, no improvement in food intake is observed.

Extremely interesting are preliminary data obtained in tumor-bearing rats receiving carnitine supplementation. When compared with rats receiving placebo, supplemented rats exhibited a marked improvement in anorexia, reduced LBM wasting and decreased systemic inflammation, as measured by circulating levels of proinflammatory cytokines [46].

Several studies have widely demonstrated that the supplementation of GSH to the medium of cultured T cells increases the IL-2 receptor expression as well as its internalization and degradation and ameliorates the blastic response of lymphocytes to PHA, anti-CD3, and recombinant IL-2. These hypotheses have been confirmed by several of our in vitro experiments [25, 47], which demonstrated that, by adding different thiol-containing antioxidants to the medium of cultured PBMCs isolated from advanced cancer patients significant functional defects of immune cells, such as response to mitogen (PHA) and antigens (anti CD3), the expression of surface activation markers (CD25 and CD95), and cell cycle progression (from G1 to S phase) are improved.

18.4.2 Clinical Trials

More human than animal data are available to assess the role of antioxidant therapy on anorexia and cachexia. In some studies on advanced-stage cancer patients, Mantovani et al. demonstrated that the antioxidant agents ALA and *N*-acetyl-cysteine (NAC) restore several important in vitro T-cell functions [25, 47]. Moreover, the same authors showed that different antioxidant agents, used alone or in combination, in a population of advanced cancer patients with tumor of different sites, were effective in reducing ROS and increasing GPx activity. The antioxidant treatment also reduced serum levels of IL-6 and TNF-α [48, 49].

Also clinical trials using L-carnitine have been consistently positive. In a study carried out on 50 cancer patients undergoing chemotherapy treatment with L-carnitine (4 g/day for 7 days) induced in 45 patients showed improvement of mood and quality of sleep and a significant reduction of fatigue [50]. A more recent study by Gramignano et al. demonstrated that cancer patients receiving 6 g/day of L-carnitine for 4 weeks had significantly decreased fatigue and increased LBM and appetite [51].

In the context of combined approaches, the most recent and intriguing were our phase II and phase III studies testing an integrated treatment based on diet, pharmaconutritional support, and drugs including antioxidants, in a population of cachectic advanced cancer patients.

The phase II study carried out by us [52] aimed to test the efficacy of an integrated approach to cancer cachexia, consisting of antioxidants, pharmaconutritional support, progestogen, and anticyclo-oxygenase-2 in improving nutritional, functional, laboratory, and quality of life variables in cachectic cancer patients. The integrated treatment included diet with high polyphenols content (300 mg/day) obtained by alimentary sources or orally supplemented with tablets, oral antioxidant treatment [alpha lipoic acid (ALA), 300 mg/day; carbocysteine lysine salt, 2.7 g/day; vitamin E, 400 mg/day; vitamin A, 30,000 IU/day; vitamin C, 500 mg/day], pharmaconutritional support [energy and protein dense nutritional supplement enriched with $n-3$ fatty acids, eicosapentaenoic acid (EPA, 1.1 g per can) and docosahexaenoic acid (0.46 g per can), 2 cans/day], orally administered progestogen (MPA, 500 mg/day), and selective COX-2 inhibitor (celecoxib 200 mg/day). The duration of the integrated treatment was 4 months. Thirty-nine patients completed the treatment: 22 of them responded to treatment (17 "responders" and 5 "high responders"), achieving a significant improvement of the endpoint variables, that is, a significant increase of LBM, a decrease of REE, an increase of appetite, a significant decrease of IL-6, TNF-α, fatigue, and a significant improvement of quality of life (EORTC QLQ-C30v3). Therefore, the treatment proved to be effective. As regards to safety, the treatment was well tolerated without any significant adverse event.

Based on the results of the phase II study, Mantovani et al.'s [53], which was the most effective and safest treatment to improve the primary endpoints of cancer cachexia: LBM, REE, fatigue; and relevant secondary endpoints: appetite, quality of life, grip strength, Glasgow Prognostic Score (GPS), and proinflammatory cytokines. Three hundred and thirty-two assessable patients with CACS were included. All patients received a "basic" antioxidant treatment consisting of polyphenols

(300 mg/day) obtained by alimentary sources or supplemented by tablets and antioxidant agents ALA 300 mg/day plus carbocysteine 2.7 g/day and vitamin E 400 mg/day, vitamin A 30 000 IU/day, and vitamin C 500 mg/day. Patients were then randomized to one of five treatment arms. Arm 1: MPA 500 mg/day; Arm 2: oral supplementation with EPA 2.2 g/day; Arm 3: L-carnitine 4 g/day; Arm 4: thalidomide 200 mg/day; Arm 5: a combination of all the above agents. The planned treatment duration was 4 months. According to the original intention, i.e., the comparison between arms, the ANOVA test showed a significant difference. The post hoc analysis showed a superiority of arm 5 versus the others as for all primary endpoints. Additionally, analysis of changes from baseline showed that LBM (DEXA) significantly increased in arm 5. L3-computerized tomography (25 patients): the estimated LBM (kg) improved in arm 5. REE decreased significantly and fatigue improved significantly in arm 5. Appetite increased significantly in arm 5. IL-6 decreased significantly in arms 5 and 4. GPS significantly decreased in arms 5, 4, and 3. Total daily physical activity showed that total energy and active energy expenditure increased significantly in arm 5. ECOG-PS significantly decreased in arms 5, 4, and 3. Toxicity was substantially negligible, comparable between treatment arms. In conclusion, the most effective treatment for all three primary efficacy endpoints as well as secondary endpoints appetite, IL-6, TNF-α, GPS, and ECOG PS was the combination regimen which included all selected agents [54].

18.5 Conclusions

So far, attempts at drug therapies for cachexia with a variety of agents have yielded limited success. The most widely used agents, megestrol/MPA, have been successful to a certain extent in reversing weight loss, although this occurs as a result of an increase in the fat mass and water retention rather than because of the preservation of LBM [55]. It is to be taken into account that cancer cachexia is a multifactorial syndrome to which many factors contribute, to a differing extent, including progression of the tumor per se, anorexia/malnutrition/reduction of food intake, oxidative stress, chronic inflammation, hypermetabolism with significant derangements of energy metabolism and metabolism of the specific nutrients such as carbohydrate, lipid, and protein, impaired or poor quality of life, and fatigue. These contributing factors are reciprocally correlated and have a different impact in the different stages of disease. Therefore, the attempt to counteract and/or correct one or more of the key contributing factors, that is, oxidative stress may be rationale. Indeed, as described in this chapter oxidative stress plays a significant role in inducing and worsening cancer cachexia. Consistent experimental and clinical data indicate that oxidative stress is involved in the pathogenesis of cancer cachexia, although the extent of its contributory role remains to be established. Therefore, oxidative stress should be addressed in developing a therapeutic approach for cachectic cancer patients. However, antioxidants cannot encompass all symptoms of cancer cachexia. In fact, the results of our phase III study showing the efficacy of a combined

treatment approach, seem to confirm the basic assumption that the treatment of cancer cachexia, a multifactorial syndrome, is more likely to yield success with a multitargeted approach.

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Chapter 19
Radiation Protection by MnSOD-Plasmid Liposome Gene Therapy

Joel S. Greenberger, Valerian E. Cagan, James Peterson, and Michael W. Epperly

Abstract Understanding the molecular mechanism of ionizing irradiation killing of normal tissues compared to tumor cells has uncovered potential therapeutic strategies for radiotherapeutic dose escalation in the treatment of solid tumors. One strategy exploits the difference in redox balance between normal tissues and solid tumors with organ-specific and systemic (intravenous) administration of plasmid liposomes containing the human manganese superoxide dismutase (MnSOD) transgene. When delivered, this transgene product produced significant protection against single fraction and fractionated radiation in organ-specific radioprotective gene therapy by each of several routes of administration (inhalation, swallowed, intravesicle, intra-intestinal), and this strategy was shown to avoid tumor tissue. Furthermore, systemic administration of MnSOD-PL also provides radioprotection with selective normal tissue reduction of apoptosis compared to tumor. The complex mechanism of selective normal tissue protection involves specific differences in redox balance, constitutive MnSOD expression, and compensatory metabolic changes, many of which can enhance the therapeutic effect.

J.S. Greenberger, MD (✉) • M.W. Epperly, PhD
Department of Radiation Oncology, University of Pittsburgh School of Medicine,
200 Lothrop Street, Pittsburgh, PA 15213, USA
e-mail: greenbergerjs@UPMC.EDU

V.E. Cagan, PhD
Center for Free Radical and Antioxidant Health, Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA, USA

J. Peterson, PhD
Department of Environmental and Occupational Health,
University of Pittsburgh, Pittsburgh, PA, USA

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19.1 Introduction

Improvement in clinical radiotherapy over the past several decades has relied heavily upon treatment planning techniques and radiotherapy delivery techniques that enhance tumor-specific killing and minimize normal tissue dose [1–4]. These advances include development of three-dimensional conformal radiotherapy techniques, based upon sophisticated CAT, MRI, PET, and combined imaging techniques [5]; Intensity Modulated Radiotherapy Treatment (IMRT) beams in which moving multileaf collimator components are used during treatment delivery [6]; alternative fractionation techniques; alterations in dose rate and beam energy [7]; the development of the Gamma Knife as well as advanced frameless stereotactic treatment devices, and brachytherapy techniques including radioactive seed and radioactive mesh implantation; and high dose rate brachytherapy [8–12]. Despite tremendous improvements in radiotherapy treatment planning and beam delivery, normal tissue toxicity remains dose limiting to most tumor targets [2–4, 13].

The therapeutic ratio (ratio of tumor killing over normal tissue toxicity) remains a key factor limiting the development of radiotherapy treatment plans [1]. Combining ionizing irradiation with new chemotherapeutic agents, many of which are normal tissue radiosensitizers, further limits radiotherapy dose escalation [14]. The development of tumor-specific radiosensitizers has been one approach toward increasing the therapeutic ratio [1]. This approach has been limited by the incomplete targeting through intra-arterial or intra-tumor injection of specific radiosensitizing agents such as bromadeoxyuridine (BUDR) [1] or in the case of p53-deficient tumors, p53-transgene therapy [1]. One successful approach has been the development of hypoxic cell cytotoxins. Tirapazamine relies upon the hypoxic environment in large epithelial tumors to provide activation of the pro-drug into a cytotoxic moiety [14]. The use of hypoxic cell cytotoxins has been particularly attractive in head and neck cancer and lung cancer where large hypoxic areas exist in rapidly growing tumors [15].

We have developed an alternative approach for improving the therapeutic ratio by decreasing normal tissue irradiation damage through a technique of antioxidant gene therapy [16–19]. Tissue culture laboratory and animal preclinical trials demonstrated the importance of mitochondrial targeting of the antioxidant therapeutic agent in optimizing therapeutic effect [20–24]. This chapter will review molecular biologic, biochemical, cell biologic, and the tissue- and organ-specific mechanisms of radioprotection by MnSOD transgene therapy and highlights areas for potential additional improvement of this clinical therapeutic strategy.

19.1.1 Antioxidant Gene Therapy Compared to Direct Delivery of Antioxidants

Initial discoveries in the fields of radiation chemistry identified cellular oxygen and water as primary targets for the creation of free radicals also called radical oxygen species (ROS) [1, 25–30]. Hydroxyl radical and superoxide were identified as active

moieties that combine to purine and pyrimidine bases in nuclear DNA. Alkaline sucrose gradient biochemistry techniques analyzing removed nuclei from irradiated cells identified single- and double-strand breaks in DNA and first linked the irradiation chemistry events of ionizing irradiation with the molecular biologic changes in irradiated cells [1]. The kinetics of restoration of DNA integrity after these single- and double-strand breaks led the way toward identification of DNA repair enzymes [31–35] and repair enzyme deficient conditions that were associated with increased radiosensitivity of cells in culture [36–44]. These initial studies pointed the way toward development of radioprotective agents by suggesting the possibility that immediate capture, neutralization, or inactivation of irradiation-induced free radicals might result in significant radioprotection [1, 45]. The development of WR2721 (ethyol, Amifostine), a normal tissue radioprotector, and N-acetylcystine (a sulfhydryl compound) both of which act though reactive oxygen species (ROS) or free radical scavenging [46] serve as two examples of the applications of free radical scavenging [47].

During the development of radioprotective free radical scavengers, and identifying the mechanism of action of these compounds, it was quickly appreciated that levels of total cellular antioxidant capacity dropped rapidly after exposure of cells to ionizing irradiation [1, 48–50]. It was learned that preservation of the total antioxidant cellular levels could be radioprotective [49, 50]. Paramount in the list of early identified antioxidant compounds was glutathione itself, a free radical scavenger [1, 51]. Under conditions of lowered glutathione levels, cells were more radiosensitive [1, 51]. It followed that by adding new free radical scavengers, the consumption of glutathione from the antioxidant pool would be reduced and this would render cells more capable of neutralizing additional irradiation-produced free radicals [48].

Radiation biologists studied the kinetics of production of free radicals following irradiation of cells in culture and defined several parameters of cellular, tissue, and organ response, which further complicated the strategy of using antioxidant drugs as radioprotectors [1]. First, irradiation induction of free radicals was found to continue long after irradiation [1, 16–18, 52, 53], and secondly ROS production could be detected outside the irradiated target volume [52, 53]. These data uncovered the phenomena of irradiation-induced production of cytokines [54, 55], which contributed to the delayed toxicity in adjacent or distant sites. The data also defined the phenomenon of the "bystander effect," which included both cell contact and humoral transmission of irradiation damage and free radical induction (ROS) to other cells [56]. Finally, the mechanism of action of irradiation induced ROS was not limited to nuclear DNA strand breaks and included the oxidation of lipids in cell membranes, most prominently those in the mitochondria [49, 50].

Basic radiation chemistry also revealed other natural cellular antioxidant defense mechanisms separate from the antioxidant pool of free radical scavenging small molecules. These studies identified natural radioprotective enzymes including glutathione peroxidase, gamma glutamyltranspeptidase, catalase, and the three forms of superoxide dismutases (SODs) [26, 27]. One form of SOD (MnSOD or SOD2) contains a specific 23-amino acid leader sequence that facilitated mitochondrial localization [21, 49]. In radiation chemistry reactions, superoxide is dismutated by SOD to hydrogen peroxide [25], another potent oxidative chemical which itself is

acted upon by catalase to produce benign substances oxygen and water [25]. The combination of superoxide with nitric oxide, another active free radical, induced in mammalian cells by each of three nitric oxide synthase isoforms, produces the potent free radical peroxynitrite [49, 50]. Peroxynitrite is one of the most potent inducers of lipid oxidation associated with the formation of the mitochondrial specific oxidized lipids [48]. Conclusions from these observations led to methods by which to reduce the production of peroxynitrite [49]. The consensus was that reducing levels of peroxynitrite might also decrease ionizing irradiation-induced normal tissue damage [48–50, 57].

Initial translational research experiments by which to adapt these radiation chemistry and cell biology experiments to normal tissue radioprotection in vivo, were largely ineffective. Attempts to deliver MnSOD protein or nitric oxide synthase inhibitor drugs systemically required administration of very high levels to achieve therapeutic effect at the level of the mitochondria [58]. The therapeutic antioxidant, Tempol, which counteracts both hydroxyl radical and superoxide was only marginally effective in experimental animal studies due to the very high drug dose requirements to gain therapeutic effect [59, 60]. This data led to development of techniques to mitochondrially target Tempol and such research not only overcame some of these drug dose problems but further substantiated the importance of the mitochondrial membrane as a key target in prevention of ionizing irradiation-induced apoptosis of normal cells [61, 62].

Mitochondrial targeting of MnSOD, through its naturally occurring 23-amino acid mitochondrial localization sequence, combined with the knowledge that very high levels of protein were required to obtain mitochondrial specific expression posed the question of whether transgene delivery of the MnSOD cDNA sequence directly to cell nuclei, with a strong promoter to facilitate gene transcription and translation, might be a way to provide adequate mitochondrial targeted levels of protein delivered as such from the “inside out” [58]. Initial experiments with MnSOD-plasmid liposomes, using a radiosensitive murine hematopoietic progenitor cell line, 32D cl 3, first demonstrated the effectiveness of this approach and also confirmed the importance of mitochondrial targeting [20, 21]. Attaching the 23AA mitochondrial localization signal to CU/ZnSOD, a cytoplasmic form of SOD, which itself is non-radioprotective [21], had the same effect as MnSOD in radioprotection in vitro [21]. Conversely, removing the mitochondrial localization sequence from MnSOD produced a non-radioprotective cytoplasmic moiety [21]. In these experiments, mitochondrial targeting of SOD provided significant radioprotection. These experiments were translated in vivo and showed organ-specific radioprotection against single fraction and fractionated irradiation [21, 63].

19.1.2 The Biochemistry of MnSOD-PL Radioprotective Gene Therapy

Basic biochemistry and radiation chemistry experiments carried out on cells in culture first revealed that 32D cl 3 murine, Interleukin-3 dependent hematopoietic progenitor cells, transfected with the MnSOD transgene were significantly

radioresistant [20, 21]. The mechanism of this radioresistance was linked to the stabilization of a high level of antioxidant capacity in the cells compared to non-transfected, non-MnSOD-overexpressing cells [48]. In particular, intracellular levels of glutathione and other radical scavenging antioxidants were shown to persist at higher levels and for longer duration after the cells were exposed to radiation compared to parent control cells [48].

Cell lines overexpressing MnSOD were compared to parent cell lines with respect to radiation-induced oxidized lipids [22, 23]. It was discovered that specific oxidized lipids, notably phosphatidyl serine and cardiolipin were localized to the mitochondrial membrane in irradiated cells [22, 23] and the magnitude of lipid oxidation was decreased by MnSOD overexpression of clonal cell lines [22, 23].

The data on oxidized lipids that demonstrated an increased antioxidant pool size, and decreased susceptibility to specific PS and CL lipid oxidation was also detected in experiments in irradiated mouse esophagus [64]. Esophagus was removed at serial time points after irradiation from mice that received intraesophageal administration of MnSOD-PL, control liposomes, or no gene therapy. Tissues from animals that received intraesophageal injection of MnSOD-PL showed higher levels of antioxidant pool size and decreased lipid oxidation [64].

A separate series of experiments demonstrated that treatments that decreased phospholipid peroxidation of the mitochondria also stabilized cytochrome C binding to cardiolipin [49, 50].

Cytochrome C, which is a natural and intrinsic component of the mitochondrial respiratory cascade, is nearly 70% bound to cardiolipin in the inner mitochondrial membrane [49]. Thirty percent of cytochrome C is free, but is maintained inside the mitochondria by an intact mitochondrial membrane. During ionizing irradiation exposure of cells, mitochondrial membrane depolarization is detected, and cytochrome C leaks from the mitochondria into the cytoplasm where it activates caspase-3 leading to cleavage of PARP and subsequent nuclear DNA fragmentation resulting in apoptosis [20, 21].

Initial experiments demonstrated that MnSOD-PL treatment prevented cytochrome C leakage out of the mitochondria, but the mechanism was unknown. A separate series of experiments demonstrated that maintaining the antioxidant pool within the mitochondria stabilized cardiolipin binding to cytochrome C and contributed significantly to a decrease in cytochrome C leakage [48]. Furthermore, it was demonstrated that consumption of antioxidants in the mitochondria led to deleterious effects on one of the most potent enzyme antioxidants, namely MnSOD [48]. Following ionizing irradiation, MnSOD was demonstrated to be both oxidized and nitrated [65]. Oxidized MnSOD still retained superoxide dismutation activity, while nitrated MnSOD became less capable of functioning as a superoxide dismutase. Rather, it became a peroxidase causing lipid damage in the mitochondria and leading to cytochrome C leakage and apoptosis [65].

In experiments, both in cell lines in vitro and in tissues in vivo including intestine [66–68], ionizing irradiation caused specific mitochondrial phospholipid oxidation that was a signature for the oxidative stress induced by ionizing irradiation. MnSOD-PL overexpression in cells and tissues prior to irradiation preserved the antioxidant pool and in some experiments led to a decreased apoptotic response [64].

19.2 The Cell Biology of MnSOD-Radioprotective Gene Therapy

Initial experiments were carried out in several preclinical rodent models of irradiation-induced toxicity: esophagitis [69], pneumonitis [70], cystitis [71], intestinal damage [72], oral cavity [73], and bone marrow failure [74]. In these models ionizing irradiation was observed to reduce cell numbers, produce apoptosis and ulceration in tissues (in the case of the esophagus), and lead to functional organ inactivation. Irradiation effects on tissues were both direct on cycling cell populations [75] and indirect via cytokine production [76, 77]. MnSOD-PL therapy reduced the magnitude of the direct effect by stabilizing the cycling cells. However, it also became evident that irradiation in vivo affected the microenvironment in which proliferating cell populations resided and cytokines were released.

Apoptosis induced by irradiation is known to be more dominant in rapidly cycling cells [1]. In experiments comparing 32D cl 3 hematopoietic progenitor cells in liquid culture with those in cell pellets, (simulating the hypoxic, contact-inhibited microenvironment in tissues), irradiation-induced apoptosis was decreased in pelleted cell populations [78]. Other studies demonstrated that hematopoietic cells in contact with cells of the hematopoietic microenvironment (bone marrow stromal cells) showed reduced ionizing irradiation-induced toxicity both when hematopoietic cells and stromal cells were irradiated together and in experiments in which unirradiated hematopoietic cells were engrafted onto irradiated bone marrow stromal cells [79]. In both the model of irradiation exposure of both populations and the model of co-cultivation of cell populations with only the stromal cells irradiated, there was decreased toxicity to cycling hematopoietic cell population if MnSOD-PL was added and the total antioxidant pool size was increased [48, 80]. Therefore, MnSOD-PL treatment of the microenvironment into which restorative and repopulating cells migrate facilitates engraftment [80, 81]. In recent experiments with chimeric mice having bone marrow replaced with specifically marked donor cell populations, it was demonstrated that MnSOD-PL treatment of an epithelial organ (esophagus or lung) facilitated better engraftment of cells of bone marrow origin coming in from outside the irradiation field compared to non-MnSOD-PL treated mice [80–82].

In vitro experiments demonstrated that production of ROS by plateau phase stromal cells increased with irradiation dose [79]. Antioxidant treatment of irradiated stromal cells decreases ROS production and facilitates improved attachment of engrafted cells in vivo [80, 81].

Apoptosis of cells exposed to ionizing irradiation has been shown to be similar to that which occurs in cells deprived of specific required growth factors such as IL-3 [83]. MnSOD-PL overexpression by transgene introduction into IL-3 dependent cell facilitated longer duration survival in the absence of IL-3 compared to non-MnSOD overexpressing cells [83]. These data provide evidence that growth supporting cytokine withdrawal as well as ionizing irradiation induce oxidative stress and depletion of the antioxidant pool in both non-cycling and cycling cells [48]. Among the non-cycling cells are those of the tissue-specific microenvironment.

The data also demonstrate that non-cycling cells, exposed to irradiation, produce increased levels of ROS and inflammatory cytokines, such that these cells can induce a toxic indirect effect on homing and engrafting cells. The overexpression of MnSOD in stromal cells prior to irradiation facilitates a reduced depletion of antioxidant stores and reduced toxicity of the microenvironment. The data suggest that MnSOD-PL therapy both in vitro and in vivo with respect to stromal cells reduces the irradiated cellular capacity to induce the bystander effect.

19.2.1 MnSOD Radioprotective Gene Therapy Tissue Effects

Initial studies in irradiated esophagus quantitated the number of apoptotic bodies and ulcerated areas in the esophagus at serial time points after exposure to single fraction 29 Gy [69]. In C57BL/6J mice, by 12–14 days after irradiation, significant esophagitis prevented adequate nutrition and animals were observed to be dehydrated and significantly losing weight. Histopathologic evaluation showed increased apoptotic bodies, microulceration, and significant tissue damage (Figs. 19.1 and 19.2). Mice given a single intraesophageal administration of MnSOD-PL, but not empty liposomes, or control LAC-Z transgene-PL showed significant reduction in irradiation-induced apoptosis, improved survival, and the animals showed less dehydration and better weight gain (Fig. 19.3; Photographs from [69] reproduced with permission from Wiley, Inc. *Radiation Oncology Investigations*). In addition, apoptotic bodies and microulcerations induced by irradiation were significantly decreased in MnSOD-PL pretreated versus control irradiated tissues [80, 81].

These studies in the esophagus demonstrated that MnSOD-PL administration in a single dose or multiple doses during fractionated irradiation resulted in stabilization of the esophageal tissue response, namely expression of cytokine mRNA and elaboration of humoral cytokines [77]. The secondary effects of ionizing irradiation on tissues (cytokine release) were also decreased by MnSOD-PL mediated overexpression of this antioxidant transgene [77].

Other tissue-specific experiments were carried out with animal models of oral cavity irradiation. In both single fraction and fractionated irradiation to the head and neck region, mice demonstrated significant tongue ulceration, apoptotic bodies in the oral cavity, significant dehydration, and weight loss [73]. Intraoral administration of MnSOD-PL was demonstrated to introduce transgene deep within the oral cavity tissues [73]. An epitope-tagged hemagglutinin-tagged MnSOD was utilized to facilitate histochemistry. Histochemical staining showed that cells deep in the tissue going down to the stem cell or basal cell layer were reached by intraoral MnSOD-PL gene therapy [75].

In a murine lung irradiation model, intratracheal injection of MnSOD-PL in single and multiple fraction irradiation experiments demonstrated reduction of irradiation-induced acute lung damage [70, 76]. Inhalation delivery of MnSOD-PL made it easier to facilitate multifraction irradiation experiments since the animals did not suffer trauma of multiple surgeries to the trachea for injection [84]. MnSOD-PL was

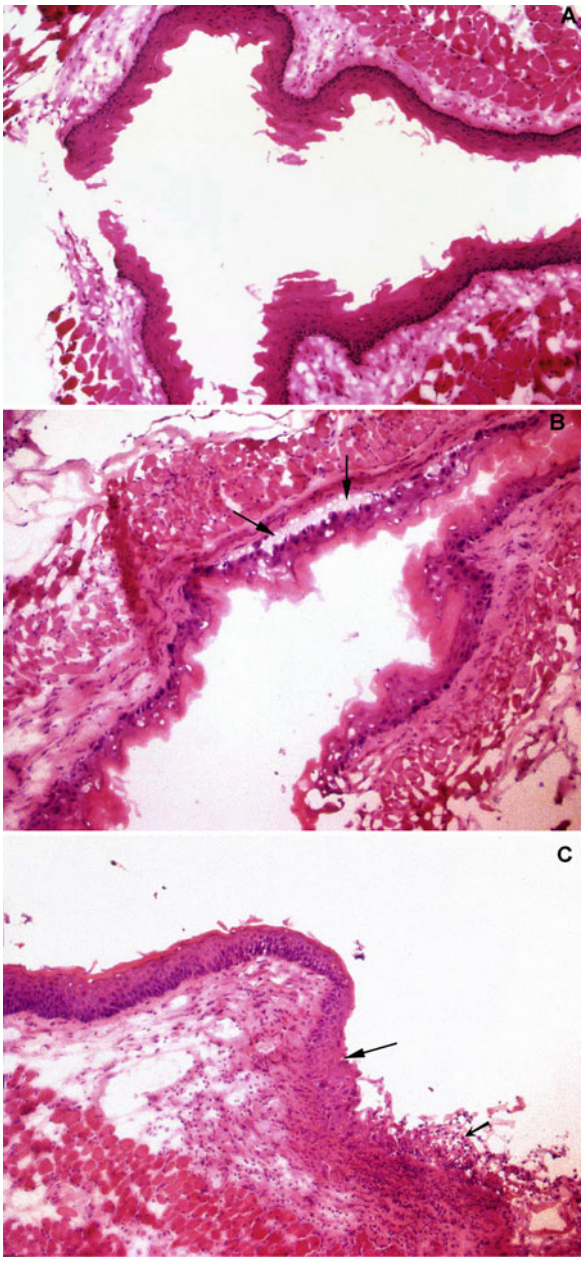

Fig. 19.1 Pathogenesis of irradiation-induced esophagitis. C3H/HeNsd mice were irradiated to 3,500 cGy and sacrificed at day 0, 5, or 7 after irradiation. The esophagus was excised, frozen in optimum cutting temperature (OCT) compound, sectioned, and hematoxylin and eosin (H&E) stained. Representative appearances of the esophagus from a mouse sacrificed on day 0 (before irradiation) (**a**), day 5 (**b**), and day 7 (**c**) are shown. Five days after irradiation, individual vesicles

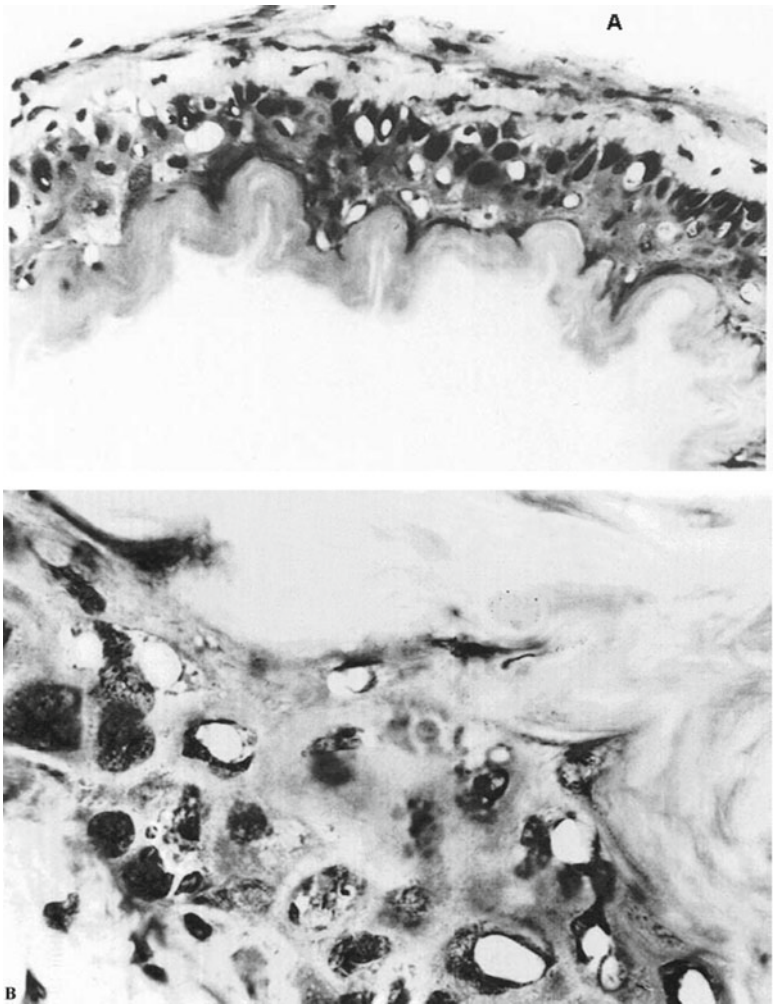

Fig. 19.2 Vacuole formation reflects esophageal lining cell degeneration. At higher magnification, (**a**) ×400 and (**b**) ×1,000, the vacuoles observed were identified as pyknotic and karyorexic nuclei with expansion of the cells caused by fluid or fat accumulation

Fig. 19.1 (continued) in squamous cells were detected (see *arrow* in **b**). The vesicles appeared fused to form vacuoles, leading to a separation of the squamous epithelial layer and the submucosal layer accompanied by ulceration (**c**). The *arrow* in C shows an early ulceration. Closer examination of the ulcer also revealed a clear inflammatory response within the muscle layer, as evidenced by the small *dark blue* cells that were identified as neutrophils and other inflammatory cells (*arrow*, **c**) (×40). Figures 19.1–19.3 reproduced with permission from Wiley, Publishers. Reprinted from [69]

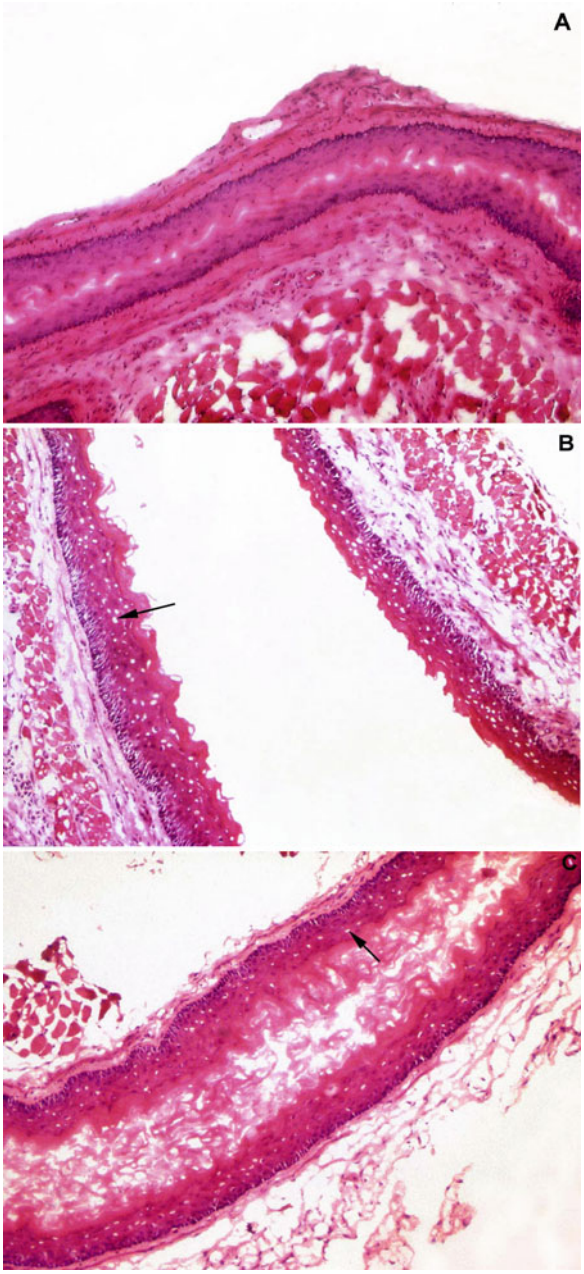

Fig. 19.3 Decreased vacuole formation in squamous lining cells of the irradiated esophagus from manganese superoxide dismutase (MnSOD) plasmid/liposome-injected mice. Mice were intraesophageally injected with liposomes with no inserts, LacZ (bacterial β-galactosidase gene) plasmid/liposome complex, or MnSOD plasmid/liposome complex and received 3,000 cGy to the thorax 24 h later. The mice were sacrificed at 0 or 15 days after irradiation, the esophagus was removed, frozen in optimum cutting temperature (OCT) compound, sectioned, and hematoxylin and eosin (H&E) stained. The sections were examined for vacuole formation. Control mice (**a**) were sacrificed

demonstrated to reach deep into the lung past terminal bronchials and into alveolar spaces, and the inhalation gene therapy route was shown to successfully deliver adequate transgene [84]. The lung irradiation damage model was different from the esophagus model in that esophageal toxicity was expressed primarily as acute esophagitis. Surviving animals did have a higher incidence of esophageal stricture (late effect) in the animals that did not receive MnSOD-PL, compared to MnSOD-PL-treated animals [77]. These experiments were confirmed in other studies with photodynamic therapy delivered damage to the pig esophagus [85]. Animals treated with MnSOD-PL intraesophageally demonstrated decreased ulceration of the esophagus caused by the photodynamic therapy. Thus, in the esophagus both acute esophagitis and the late effect of stricture whether caused by ionizing irradiation or PDT were decreased by MnSOD-PL.

The lung irradiation damage model in the C57BL/6J mouse was different in that damage was detected at 120–150 days after irradiation and was prominently displayed as the histopathologic picture of organizing alveolitis or fibrosis [70]. In this model system, the administration of MnSOD-PL prior to irradiation gave the best results with respect to reducing radiation toxicity, lethality, and decreasing the magnitude of organizing alveolitis in quantitative examination of lung sections. However, delivery of MnSOD-PL at serial time points after irradiation including at 100 days (immediately before appearance of the late lesion) did produce a detectable reduction in the magnitude of radiation damage [86]. These data were supported by other experiments in which fractionated delivery of MnSOD-PL by inhalation, and fractionated delivery by intraoral or intraesophageal administration during fractionated radiotherapy was also radioprotective.

Experiments in a rat model of bladder radiation induced cystitis demonstrated that intra-vesicle administration of MnSOD-PL resulted in significant uptake in the bladder epithelium and reduced both acute cystitis and bladder stricture. In this model physiological functioning of the bladder was preserved by MnSOD-PL gene therapy prior to irradiation. Animals treated with MnSOD-PL showed less bladder permeability with decreased movement of water and urea across the bladder membrane [71].

Experiments in intestinal radiation protection by exposing a loop of mouse intestine, and then administering either MnSOD-PL or control liposomes, and then delivery irradiation showed that MnSOD-PL preserved intestinal villus length and volume suggesting protection of these structures [72]. Total abdominal irradiation models have not been carried out, but the results are suggestive of significant intestinal protection.

Fig. 19.3 (continued) on day 0 and mice injected with liposomes with no insert (**b**), LacZ plasmid/liposome complex (**c**), and or MnSOD plasmid/liposome complex (**d**), were sacrificed on day 15. Irradiated mice injected with liposomes only (**b**) or LacZ plasmid/liposome complexes (**c**) had extensive vacuole formation. Mice injected with the MnSOD plasmid/liposome complexes (**d**) and control nonirradiated mice (**a**) had few vacuoles (×40)

19.2.2 Systemic Radioprotection by MnSOD-PL

Intravenous administration of MnSOD-PL prior to total body irradiation provided protection against both the 9.5- and 10.0-Gy doses. Systemic radioprotection did not induce increased tumorogenesis, and no increase in late effects or new tumors was detected [74]. MnSOD-PL was detected in tissues for 48–72 h, and expression was documented by RTPCR studies in both the lung and esophagus [63, 70, 77].

19.2.3 Combining MnSOD-PL with Other Transgene Therapies or Other Drugs

The radiation chemistry experiment that defines the effects of MnSOD suggested that its action would be to enhance and preserve dismutation of superoxide to hydrogen peroxide [25–27]. However, hydrogen peroxide itself is a toxic moiety and can cause cell lethality [25, 87]. Therefore, experiments were carried out to determine whether addition of another transgene for neutralization of hydrogen peroxide might add to radioprotection [88, 89]. The first experiments involved adding a transgene for glutathione peroxidase [63]. One publication demonstrated that glutathione peroxidase itself was radioprotective [90]. Other experimental data failed to confirm this observation [63].

To determine whether overexpression of another enzyme that neutralizes H_2O_2 catalase increased protection of cells already overexpressing MnSOD-PL, a mitochondrial targeted catalase transgene was compared to non-mitochondrial targeted catalase for radioprotective capacity in vitro and in vivo. 32D cl 3 hematopoietic progenitor cell lines or a subclone overexpressing MnSOD (2C6) were transfected with the transgene for either catalase or mitochondrial targeted catalase [88, 89]. These experiments demonstrated that mitochondrial targeting of catalase in cells transfected with mt-CAT were radioprotected [88]. The significant additional radioprotection observed in 2C6 cells overexpressing mt-CAT as well as MnSOD in this in vitro experiment showed that combining two transgenes in a clonal cell line appeared to be advantageous.

A potential problem with using two different transgenes on separate plasmids delivered by plasmid liposome is that of not adequately facilitating expression of both transgenes in the same cell. Previous studies have demonstrated that under optimal conditions, 20-50% of cells are transduced in the esophagus or lung by MnSOD-PL administration [91]. Therefore, further experiments will depend upon the ability to construct a single plasmid containing both MnSOD and catalase transgenes. Experiments with both transgenes on a single plasmid are being carried out to confirm whether this is additive or potentially synergistic in vivo.

Adding other drugs to MnSOD-PL gene therapy has been a subject of intense interest particularly with respect to orthotopic tumor models and hopeful translation of such results into clinical radiotherapy protocols.

In previous studies with C57BL/6J mice bearing orthotopic tumors, MnSOD-PL protected normal tissues while not protecting tumors from radiation injury [92–94]. In a recent series of experiments, nude mice bearing human orthotopic oral cavity tumors from the CAL33 cell line were treated with intraoral MnSOD-PL prior to a single fraction irradiation [95]. In the nu/nu model, CAL-33 cells of human origin were treated with Cetuximab, an antibody to the human epidermal growth factor receptor. Mice receiving both Cetuximab and MnSOD-PL demonstrated improved radiation responsiveness. In these studies, animals were examined by micro-PET scanning using a hypoxia imaging technique with F-misonidazole imaging. F-miso targeted hypoxic areas in the CAL-33 tumors and confirmed the capacity of F-miso to image hypoxic areas. The hypoxic cell cytotoxin, Tirapazamine, was therapeutic when delivered to these mice [95]. Combining two agents MnSOD-PL plus Cetuximab, MnSOD-PL plus Tirapazamine, or all three agents resulted in significant improvement in local tumor control [95]. These experiments suggest that MnSOD-PL protection of normal tissues can be supplemented with other therapies to facilitate improved therapeutic ratio and improved outcome.

19.2.4 Tumor Cell Redox Status Differs from Normal Tissues and Facilitates the Use of MnSOD PL Gene Therapy

Studies by St. Clair et al. [96] and Oberley et al. [26–30] as well as others [25] first demonstrated that many epithelial tumor cell types including those in lung and head and neck cancers have intrinsic reduction of levels of MnSOD activity. These observations suggested that the hypoxic microenvironment in tumors and reversion to anaerobic metabolism may have facilitated down regulation of MnSOD [94], which in oxidative metabolism is naturally utilized to counteract ROS production during the electron transport cascade in oxidative metabolism in the mitochondria [94]. The mechanism of MnSOD reduction in tumors was found to be more complex. Tumor cell lines were demonstrated to have decreased production of MnSOD through point mutations in the promoter of the genes [26, 27]. Furthermore, some tumor cell systems showed compensatory reduction in glutathione peroxidase such that re-introduction of elevated levels of MnSOD by transgene transfection resulted in sensitivity of those cells to hydrogen peroxide-induced injury. Transfecting into those cells with a transgene for glutathione peroxidase restored the capacity to catabolize hydrogen peroxide [90].

One of the prominent concerns in the use of MnSOD-Plasmid Liposome therapy for normal tissue protection was the possibility that transgene expression in tumor cells would also provide radioprotection thereby equalizing both sides of the therapeutic ratio at a higher level of protection and potentially compromising the effectiveness of radiotherapy [92–94]. While organ-specific gene therapy was designed to prevent this by physical means (for example, inhalation of MnSOD-PL resulted in normal bronchiolar and alveolar cell uptake of the transgene while not reaching tumor cells that were in a solid mass and not connected to the airway), the

data showing differences in redox balance between normal tissue and tumors suggested that systemic administration might actually be therapeutic.

The first studies testing this hypothesis involved transfection of epithelial tumor cell lines with MnSOD-PL in vitro [93]. Epithelial tumor cell lines of both mouse and human origin and of both pulmonary and oral cavity origin demonstrated radiosensitization by MnSOD-PL [93]. Cell lines derived from normal tissues of these same tumor sites (normal lung, normal oral cavity) demonstrated MnSOD-PL radioprotection. It appeared that differences in redox balance between normal tissues and tumors might facilitate improvement in therapeutic ratio combining radiosensitization of tumor tissue with radioprotection of normal tissues [94]. Orthotopic lung tumors at the carina of mice demonstrated that intra-tracheal injection of MnSOD-PL radiosensitized tumor cells while providing radioprotection of the lung [92–94]. In situ histochemistry identification of an epitope-tagged MnSOD transgene failed to identify significant levels of MnSOD in the tumor tissue [94], and the radiosensitization of tumor was concluded to be that of indirect effect, of unknown origin. Similar experiments with oral cavity, orthotopic tumors in the cheek pouch showed the same result. Intra-oral administration of MnSOD-PL was more tumor radiosensitizing than was intravenous administration while both techniques did provide some tumor cell radiosensitization [94].

At the present time, it appears that there are both direct MnSOD transgene-mediated tumor radiosensitizing effects and indirect effects through protection of normal tissue. Such indirect effects could include those of a physiological nature, such as normal tissue radioprotection of blood vessels including limitation of microvascular formation in the tumor, or even reduction of blood flow to tumor cells. Decrease in production of inflammatory cytokines by irradiated normal tissues through MnSOD-PL-mediated radioprotection might reduce the availability of such cytokines to growing tumor tissue. Tumor cells have been demonstrated to benefit from addition of growth factors including TGFβ, IL-1, TNFα, and others which are deleterious to normal tissue by the second wave of apoptosis [93].

19.2.5 MnSOD-PL Gene Therapy Radioprotection Strategies Lead the Way to Small Molecule Mitochondrial Targeted Radioprotective Agents

Experiments with MnSOD-PL have demonstrated the importance of mitochondrial localization for normal tissue radioprotection. They also identified critical redox differences between normal and tumor tissue, which can facilitate improved radiation protection to normal tissue with acceleration of radiation injury in tumors. Long-lived MnSOD-PL radiotherapeutic effects in vivo have demonstrated that survivors of intravenous MnSOD-PL administration prior to total body irradiation are not at increased risk for secondary cancers or additional irradiation-induced life shortening [74]. All these data suggested that substitution of a small molecule that is mitochondrial targeted and has MnSOD radioprotective capacity might be a more

efficient, less expensive, and more feasible approach toward achieving normal tissue radioprotection. In addition, concerns for the long-term consequences of gene therapy in humans, not the least of which is a potential for germ line integration of transgene, induction of mutations, or other side effects, made the search for small molecules a top priority.

These studies led to the recent development of a series of hemigramacidin-targeted nitroxide compounds, GS-nitroxides, with identification of several that have significant radioprotective capacity both in organ-specific and in systemic administration experiments [61, 62]. Long-term studies with GS-nitroxides have yet to be carried out and the feasibility of adaptation of this small molecule approach in the clinical setting has yet to be facilitated. However, the original studies with MnSOD-PL led the way toward logical development of small molecule alternatives for radioprotection of normal tissues.

19.3 Conclusions

MnSOD-PL gene therapy has demonstrated organ-specific and systemic radioprotective capacity. Success relies upon the observation that mitochondrial targeting of MnSOD activity is critical for a therapeutic effect and that the biochemical consequences of irradiation at the level of the mitochondrial membrane appear to be those critical for interruption of the apoptotic pathway. Clinical translation of the observation of the success of MnSOD-PL as a therapeutic radioprotector gene therapy must be demonstrated before this technique is validated and successful for routine clinical use.

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Chapter 20
Antioxidants and Inhibition of Cisplatin-Induced Kidney Injury: Role of Mitochondria

Neife Ap. Guinaim dos Santos and Antonio Cardozo dos Santos

Abstract Since its approval by the FDA in 1979, cisplatin (CP) has been widely employed in the treatment of several malignancies, including both solid and hematological tumors. Kidneys are particularly susceptible to the action of cisplatin, and despite the preventive measures currently applied, nephrotoxicity remains the most important dose-limiting factor in cisplatin chemotherapy. It has been suggested that mitochondrial oxidative stress and unbalance of the redox status play a key role in the mechanism of CP-induced nephrotoxicity. Therefore, as an attempt to counteract the renal damage associated with cisplatin chemotherapy, many antioxidants, both synthetic and natural, endogenous and exogenous, with distinct mechanisms of action, including free-radical scavengers, sulfhydryl donors and iron chelators, have had their potential as protective agents evaluated. Results are encouraging in the great majority of the studies and many different antioxidants have been presented as potential candidates to the adjuvant protective therapy. The most promising compounds, the conflicting findings and the remaining concerns are thoroughly discussed in this chapter.

20.1 Cisplatin Use and Limitations

Cisplatin (cis-diamminedichloroplatinum II) is one of the most effective chemotherapeutic agents, which has been used, alone or in combination with other agents, in the treatment of a wide range of tumors, including testicular cancer, metastatic ovarian cancer, head and neck cancer, bladder cancer, cervical cancer, small cell and non-small cell lung cancer, osteosarcoma, metastatic melanoma, penile cancer, adrenocorticol

N.A.G. dos Santos, PhD • A.C. dos Santos, PhD (✉)
Departamento de Análises Clínicas, Toxicológicas e Bromatológicas,
USP, Avenida do Café s/n, Ribeirão Preto, SP 14040-903, Brazil
e-mail: acsantos@fcfrp.usp.br

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carcinoma, and pancreatic cancer [1, 2]. Its effectiveness increases at higher doses, but dose escalation and, consequently, the achievement of its maximum antitumor effect are limited by its toxicity on healthy tissues, mainly on kidneys [3, 4].

20.2 The Susceptibility of Kidneys to Cisplatin

Some physiological and biochemical characteristics contribute to the vulnerability of kidneys to the toxicity induced by many xenobiotics. In the case of CP, two main factors contribute to nephrotoxicity: (1) kidneys constitute the main route of CP elimination and (2) CP is selectively accumulated in the renal epithelial cells. For these reasons, kidneys are exposed to higher levels of CP as compared to other organs such as liver, intestine, testicles, or spleen. The concentration of CP in the epithelial tubular cells reaches a level approximately five times higher as compared to the concentration in the plasma [5–9].

The renal clearance is responsible for more than 90% of CP elimination. Approximately 25% of the dose is excreted in urine within 24 h after the administration. The elimination is performed mainly via glomerular filtration; however, secretion and reabsorption of CP and its metabolites in the proximal tubules have also been suggested. Conversely, the intestinal or biliary excretion of CP seems to be minimal [5, 8, 10, 11].

The segments of the nephron are differently affected by CP. The most sensitive segment is the proximal tubule, mainly the S1 cells in the early portion and the S3 cells in the late portion, both with large concentration of renal mitochondria. Although less intensely, the cells of the distal convoluted tubule (DCT) seem to be also affected, as well the epithelial cells of the ascending limb of loop Henle, the epithelial glomerular cells, and the vascular endothelial cells [12–16, 144].

The transport mechanisms of renal cells probably contribute to CP accumulation in the proximal tubular segment. Besides the passive diffusion of CP initially proposed, carrier-mediated transport has also been suggested and some mechanisms have been recently described. The uptake of CP by the renal proximal tubular cells has been proposed to be mediated by organic cation transporters (OCTs), more precisely by the OCT2 isoform, mainly expressed in the basolateral membrane of proximal tubules. The copper transporter Ctr1 has also been suggested as a mediator in the cellular influx of CP. Since Ctr1 is abundant in renal tubular cells, it is possible that this transporter plays a role in CP nephrotoxicity [3, 5, 17–23, 145, 146].

The concentration of CP in the renal filtrate prior to elimination and the selective accumulation of CP in the epithelial cells of the proximal tubule, which in turn is favored by the transport systems of these cells, are all factors that probably account for the major role nephrotoxicity plays in CP chemotherapy.

In general, patients who have been treated with CP present a permanent decrease in their renal filtration capability and it is estimated that 28–36% of those patients

treated with an initial dose of 50–100 mg/m^2 develop acute renal failure [24, 25]. Clinical findings include a reduction of 20–40% in the glomerular filtration rate, increased blood urea nitrogen (BUN), increased serum creatinine levels as well as electrolyte abnormalities including hypomagnesemia, hypocalciuria, and hypokalemia [3, 26, 27].

20.3 The Nephrotoxicity Induced by Cisplatin

The toxic effects of CP have been associated with the metabolites produced by the intracellular hydrolysis of the drug. This hydrolysis occurs because of the high concentration of water and the low concentration of chloride in the interior of the cells, contrary to the exterior side; the concentration of chloride ions is approximately 145 mM outside and 3 mM inside the cells. Such differences lead to the replacement of one or two chloride ions of the molecule resulting in positively charged and highly reactive electrophilic products: (1) mono-aquo-cisplatin ($[Pt(NH_3)_2Cl(OH_2)]^+$) and (2) diaquo-cisplatin ($[Pt(NH_3)_2(OH_2)_2]^{+2}$). These electrophilic species can (1) affect subcellular organelles, mainly mitochondria, (2) react with glutathione (GSH) in the cytoplasm, and (3) react with DNA in the cellular nucleus. The platinum atom of the aquated CP metabolites covalently binds to nuclear DNA through inter-strand or intra-strand crosslinks, forming adducts CP- DNA. The formation of these adducts distorts the DNA double helix structure, leading to the inhibition of the (1) transcription and (2) replication; (3) arrest of cell cycle and (4) inhibition of cell proliferation. The S phase of the cell cycle seems to be the most affected; however, this may vary among cells from different tissues. These effects on DNA are more intense in tumor and other diving cells and have been associated with the antitumor effect of CP [28–35].

The renal damage caused by CP has been associated with different mechanisms and events: (1) cytotoxicity, (2) vasoconstriction of the renal microvasculature, and (3) pro-inflammatory effects [4, 17, 36, 147]. The cytotoxic effects of CP, on their turn, has been postulated to occur via several mechanisms, including mitochondrial injury and nuclear DNA damage, both leading eventually to the activation of the apoptotic cell death pathways [148]. In this chapter the discussion will be focused on the mitochondrial alterations, such as the mitochondrial dysfunction and the mitochondrial oxidative stress, both proposed as very early events in the nephrotoxicity induced by CP. Kidneys are particularly susceptible to mitochondrial toxicity, an event frequently involved in renal cells injury mechanism. Renal functions demand a lot of energy, which is almost entirely provided by the mitochondrial oxidative phosphorylation. Decreased ATP production by renal mitochondria impairs (1) proximal tubular sodium/water absorption; (2) proximal tubular reabsorption (S3 portion); (3) glomerular filtration; (4) secretion; (5) active transport; (6) biosynthesis; and (7) metabolism processes [14, 37–39].

20.4 Preventive Measures in Clinical Practice

Intensive hydration; osmotic diuresis; pharmacological diuresis; and administration of chelating agents, sulfhydryl donors, and antioxidants are some approaches that have been suggested as possible strategies to reduce CP nephrotoxicity [3, 40]. Despite these prophylactic measures, irreversible renal damage still occurs in approximately 30% of the patients under CP treatment [41–44].

The currently most employed measures in clinical practice include vigorous hydration with saline, diuresis with mannitol or furosemide, and administration of the FDA-approved agent amifostine, an organic thiol-phosphate, whose action mechanism is discussed later in this chapter.

20.5 The Oxidative Stress Induced by Cisplatin: Role of Mitochondria

The mitochondrial respiratory chain is the main source of reactive oxygen species (ROS), which are physiologically and continuously generated. Approximately 2% of the electrons which flow along the respiratory chain escape from it and react with molecular oxygen, which is partially reduced originating superoxide anion ($O_2^{-\bullet}$), the precursor of most of the ROS generated in mitochondria [45, 46]. The balance between ROS generation and detoxification is guaranteed by an efficient mitochondrial antioxidant defense system. This mitochondrial system is constituted by (1) enzymatic compounds such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione redutase (GRd) and (2) non-enzymatic compounds such as the reduced form of GSH and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). Under normal conditions the $O_2^{-\bullet}$ produced is converted by the action of SOD into H_2O_2, which in turn is fully reduced to water by the action of GPx, at the expenses of the oxidation of GSH to its disulfide form (GSSG). GSH is maintained in its reduced form by the action of the enzyme GRd, at the expenses of NADPH, which is then oxidized to $NADP^+$ [47–49]. Xenobiotics might unbalance the oxidant–antioxidant ratio, condition known as oxidative stress, by augmenting ROS generation and/or depleting the antioxidant defense system.

Oxidative stress and unbalance of the redox status play a key role in CP-induced nephrotoxicity [50, 51]. One possible explanation for the oxidative stress induced by CP is the direct inhibition of the individual complexes I, II, III, and IV embedded in the inner mitochondrial membrane, leading to the inhibition of electron transport along the respiratory chain [52, 53]. This electron flow blockade favors the leak of electrons mainly from Complexes I, II, and III of the respiratory chain, and therefore, the formation of ROS via the partial reduction of oxygen to superoxide anion ($O_2^{-\bullet}$), which originates hydrogen peroxide (H_2O_2) in a reaction catalyzed by SOD. The hydrogen peroxide can be fully reduced to water or instead originate the highly reactive and most toxic oxidant species, hydroxyl radical ($OH^{\bullet}$), which damages

macromolecules such as lipids, proteins, and DNA. The formation of $OH^{\bullet}$ from H_2O_2 is catalyzed by transition metals, mainly iron salts, via Fenton/Haber-Weiss reaction [47, 54, 55, 149]. The formation of $OH^{\bullet}$ and its involvement in the toxic effects of CP have been indirectly demonstrated in rat kidney mitochondria via the administration of dimethylthiourea (DMTU), a scavenger of $OH^{\bullet}$. The oxidative damage to macromolecules induced by CP in kidney has also been demonstrated. There is evidence of CP-induced lipid peroxidation, an important marker of oxidative damage and moreover, oxidation of cardiolipin, an anionic phospholipid present almost exclusively in the inner mitochondrial membrane (IMM), to which it confers stability and fluidity [50]. Cardiolipin plays important structural functions in the IMM such as (1) holding together the components of the respiratory chain, especially Complexes III and IV; (2) interacting with adenine nucleotide translocator (ANT); and (3) anchoring cytochrome c, a pro-apoptotic factor. Therefore, the oxidation of cardiolipin has been associated (1) with mitochondrial dysfunction as a result of impaired oxidative phosphorylation, increased rigidity of the IMM and decreased ANT activity as well as (2) with apoptotic cell death as a consequence of permeability transition pores (PTP) opening and cytochrome c release to cytosol [51, 56–61, 150]. Mitochondrial ROS induced by CP has also been demonstrated to oxidize the sulfhydryl groups of mitochondrial proteins and enzymes as well as the $[4Fe–4S]^{+2}$ cluster of the enzyme aconitase, which participates in the citric acid cycle and therefore, is essential for ATP production and renal cell viability [51]. The oxidation of aconitase leads to Fe^{+2} release and favors the Fenton/Waber-Heiss reation, leading to $OH^{\bullet}$ formation and amplification of the oxidative damage [146]. The oxidation of sulfhydryl proteins has been associated with Ca^{+2}-induced PTP opening and apoptosis [62–64]. Cisplatin-induced apoptosis has been demonstrated in renal tissue of rats by the increased activity of the final executioner caspase-3 [51].

Besides favoring electrons leak and ROS formation, CP also depletes the mitochondrial antioxidant defense system, both as a consequence of the increased ROS formation itself and by a direct inhibition of the mitochondrial antioxidant enzymes, including SOD, glutathione peroxidase, and glutathione reductase, as well as the renal glucose-6-phosphatase (non-mitochondrial) responsible for the conversion of $NADP^{+}$ back to NADPH [32, 44, 65–67]. The formation of a conjugate CP-GSH [2], could also account for the mitochondrial GSH depletion and eventually for the alteration of the redox status in kidney mitochondria. The main effects of CP on the mitochondrial antioxidant defense system are depicted in Fig. 20.1.

20.6 Antioxidants as Protective Agents During Cisplatin Chemotherapy

The denomination “antioxidants” refers to a large variety of natural and synthetic compounds, which, by means of distinct mechanisms, are able to detoxify or inhibit the formation of ROS and therefore, prevent the oxidative damage to cell constituents.

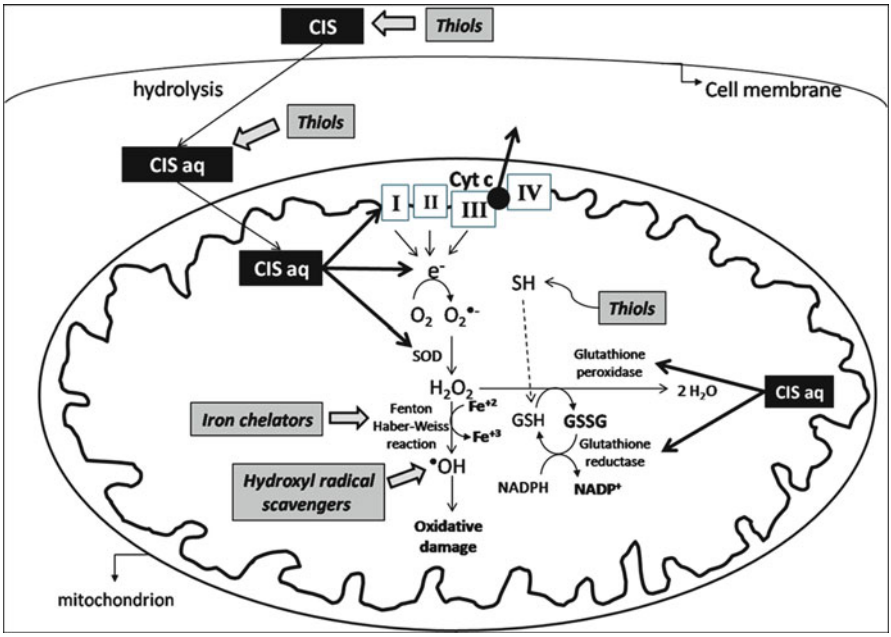

Fig. 20.1 Mitochondrial targets of cisplatin and antioxidants. Cisplatin (CIS) undergoes intracellular hydrolysis, with the formation of electrophilic aquated compounds (CIS aq), which probably cause the following sequential events: direct inhibition of electron transport through complexes I, II, III, and IV; electrons leak (mainly from Complexes I, II, and III); partial reduction of oxygen to superoxide anion radical ($O_2^{-\bullet}$), which is converted by SOD to hydrogen peroxide (H_2O_2), which, in turn, might originate the most toxic oxidant species, hydroxyl radicals ($OH^{\bullet}$), via iron catalyzed reaction (Fenton/Haber-Weiss reaction). The oxidative damage caused by these oxygen species leads to structural damage to the inner membrane, PTP opening, and cytochrome c release. On the other hand, antioxidants are able to counteract most of these alterations acting on distinct targets: *thiols*: prevent the absorption of CP by the renal cells, form inactive compounds with aquated CP, replenish the stores of sulfhydryl groups, scavenge free radicals; *iron chelators*: prevent the formation of $OH^{\bullet}$ by sequestering iron; *hydroxyl radical scavengers*: sequester the $OH^{\bullet}$ formed, preventing the oxidative damage

Antioxidants have been proposed as adjuvant agents in chemotherapy in order to increase the therapeutic index of anticancer agents. Ideally, the antioxidant should (1) be able to selectively counteract the toxic effects on healthy tissues; (2) not to decrease the antitumor effect and (3) not presenting themselves any pro-oxidative effect or other serious side effects. As a consequence, the adjuvant therapy with antioxidants would increase the efficacy of the chemotherapeutic agent by minimizing its toxicity on normal cells and therefore enabling dose escalation. Additionally, tumor resistance and unresponsiveness related to small cell lung cancer, ovarian cancer, colon, and bladder cancer [34, 68] might be overcome if higher doses could be applied safely.

In general, the mechanism of action of the chemotherapeutic agent is a factor that determines whether or not the antioxidant therapy will interfere in the antitumor effect. When generation of ROS is involved in the antineoplastic action, the antioxidant therapy might impair the antitumor efficiency. On the other hand, if the anticancer activity does not depend or depends in very low proportion on the formation of ROS and if this event is crucial for the adverse effects on healthy tissues, then the antioxidant therapy might be useful [45, 69]. In the specific case of CP, antioxidants are not expected to decrease its effectiveness, since distinct mechanisms seem to be involved. The anticancer activity of CP has been attributed mainly to the formation of ciplatin-DNA adducts, which occurs after the uptake of the drug into the nucleus of cells, and which can cause various cellular responses, culminating in apoptosis [34]. The rapidly dividing cells of tumors are supposedly more susceptible than the normal cells to the distortion of DNA duplex and all of its implications on cell cycle. Such differences in susceptibility might confer to this mechanism certain level of selectivity towards tumor cells. On the other hand, several lines of evidence suggest that ROS, mainly generated in mitochondria, play a central role in CP-induced renal injury [37, 44, 70]. If each mechanism really determines each event, namely the anticancer activity and the nephrotoxicity, then the antioxidant therapy might be a promising strategy to counteract this adverse effect of CP. Many natural and synthetic antioxidants have been studied as possible candidates to protect against CP-induced nephrotoxicity. Some of the most common ones are discussed in this chapter, grouped according to their origin or mechanism of action. The mitochondrial targets of these antioxidants are depicted in Fig. 20.1.

20.6.1 Dietary Antioxidants

The protective effects of compounds such as selenium, some vitamins, and some flavonoids have been studied (Table 20.1).

Vitamin C and vitamin E have antioxidant properties and their protective role against the renal damage caused by CP in animal models, mainly rats and mice, have been reported [71, 74, 90]. Vitamin E scavenges peroxyl radicals and interrupts the chain reactions involved in lipid peroxidation; while vitamin C scavenges superoxide, hydrogen peroxide, hydroxyl radical, hypochlorous acid, aqueous peroxyl radicals, and singlet oxygen [91, 93]. Experimental studies suggest that the combination of both vitamins, C and E, leads to a better protection against the renal damage than the isolated administration of each one [71, 75]. It seems to happen because of a regeneration process in which the tocopherol radical, formed during the trap of peroxyl radicals by vitamin E, is scavenged by vitamin C, resulting in the regeneration of vitamin E [91]. The protective role of vitamin A against CP-induced oxidative damage in rat kidney has also been demonstrated [76].

Besides the antioxidant effect, vitamins may also be pro-oxidants, which could exacerbate the oxidative stress induced by CP on healthy tissues. Vitamin C induces free radical production when coupled with a transition metal and some in vitro studies

Table 20.1 Dietary antioxidants evaluated as possible protector agents against CP-induced nephrotoxicity

Antioxidants	Experimental model	Effect	References
Vit C/Vit E			
	Rats	Protection	[65, 71–73]
	Mice	Protection	[74]
	Guinea pig	Protection	[75]
	Rats	Pro-oxidant	[66]
Vitamin A	Rats	Protection	[76]
Selenium			
(+ vit C + vit E)	Humans	No protection	[77]
	Rats	Partial protection	[78]
(+ vit E)	Rats	Protection	[79]
	Rats	Partial protection	[80]
	Rats	No protection	[81]
	Human	Protection	[82]
	Rats/mice	Protection	[83]
Flavonoids			
Licochalcone A	Mice	Protection	[84]
Quercetin	Rats	Protection	[85, 86]
Quercetin	Mice	Increased toxicity	[87]
Silibinin	Rats	Protection	[88, 89]

Protection refers to cisplatin-induced nephrotoxicity; *vit* vitamin

have shown that vitamin C increases the cytotoxic effects of CP [92–95]. Vitamin E might also exert pro-oxidant effects, depending on the concentration, the time of administration, reaction conditions, and available substrates; it has been shown that vitamin E-mediated pro-oxidation can be interrupted by vitamin C [91, 93]. It has also been shown that vitamin A [96] increases CP cytotoxicity.

Supplementation with selenium (Se) is supposed to increase the activity of the Se-dependent glutathione peroxidase enzyme and, therefore, to protect against oxidative stress. Partial protection against CP-induced nephrotoxicity has been observed in rats [78–80] and it seems that the association of Se with vitamin E enhances the protection [82]. Studies in humans are less frequent and the effectiveness of dietary antioxidants against CP toxicities, including nephrotoxicity, remains unclear. A study performed with 41 patients demonstrated the protective role of selenium against nephrotoxicity [82]. On the other hand, another study in humans demonstrated that supplementation with combined antioxidant micronutrients (vitamin C, vitamin E, and selenium) was ineffective [77]. Such unsuccessful outcomes related to dietary supplementation in humans have been mainly attributed to inadequate dosage and patients' noncompliance [77, 93].

Flavonoids are a group of naturally occurring phenolic compounds, which have antioxidant properties, being able to chelate iron and scavenge free-radicals such as hydroxyl, superoxide, peroxyl, and alkoxyl [97]. Additionally, in vitro studies have shown that dietary phytochemicals have antiproliferative activity against several types of cancer [98, 99]. The flavonoid quercetin, for instance, has been shown to

Table 20.2 Free radical scavengers evaluated as possible protector agents against CP-induced nephrotoxicity

Antioxidants	Experimental model	Effect on kidney	References
DMTU	NRK52E cells, rats	Protection	[100]
	Rabbits, *RTE cells	Protection	[101]
	Rats	Protection	[50]
DMSO	Rats	Protection	[102]
Edaravone	Rats	Partial protection	[103]
	Rats, murine proximal tubular cell (PTC)	Protection	[104]
	Rats	Protection	[105]

*RTE cells, renal tubular epithelial cells

(1) protect against the nephrotoxicity induced by CP, by preventing the oxidative stress in rat kidneys [85, 86], and to (2) enhance the chemoresponse to CP in human lung cancer [99] and in human head and neck squamous cell carcinoma [151]. This synergistic action of quercetin (or other phytochemicals) and CP might potentiate the anticancer activity of lower doses of CP and therefore indirectly contribute to reduce the toxicities induced by CP chemotherapy. However, data are conflicting. Although confirming the augmentation of the antiproliferative activity of CP by quercetin, a study also reports the higher toxicity in animals treated with both CP and quercetin, as compared to those treated with CP alone [87]. Another flavonoid, silibinin, has been demonstrated to protect against CP-induced nephrotoxicity in rats, without interfering with the antitumor activity in human testicular cancer cell lines [88, 89].

20.6.2 Free Radical Scavengers

DMTU, DMSO, and edaravone are free radical scavengers, which have been studied as possible renal cytoprotectors during CP chemotherapy (Table 20.2). Dimethylthiourea (DMTU) is a classical hydroxyl radical scavenger whose beneficial effect against CP-induced nephrotoxicity has been demonstrated both in vivo and in vitro. [50, 100, 101]. Besides scavenging hydroxyl radicals, DMTU is also a potent sulfhydryl donor and it has been reported to enhance renal GSH metabolism, which might contribute to its cytoprotective activity [106]. It has been suggested that DMTU does not interact with CP to form an adducted compound [100], which could limit the access of the drug to the tumor cells and consequently decrease its anticancer activity. Despite that, studies on tumor-bearing animals are necessary to determine whether or not DMTU affects the antineoplastic efficacy of CP. Although DMTU is the least toxic compound among the alkylthioureas [107], its use in humans should be carefully investigated, since there are reports of toxicity in rats; however, these effects, namely fetotoxicity and lung damage, seem only to occur at high doses [108, 152, 153]. Another aspect to consider is that the metabolism of DMTU in vivo has not been characterized and studies in vitro have shown that

thioureas are oxidized to their corresponding S-oxides, which seem to be more toxic than the parent compounds [108, 109, 152]. Therefore, further investigations on DMTU metabolism and on the effects of the derived metabolites should be conducted in order to safely propose its clinical application. However, considering the effectiveness of DMTU in inhibiting renal mitochondrial damage and protecting against CP-induced nephrotoxicity, it has been proposed as a model for the development of novel antioxidants for clinical application [50]. Similar to DMTU, dimethyl sulfoxide (DMSO) is a sulfur-containing compound with hydroxyl radical scavenging activity whose protective effect against CP-induced nephrotoxicity has also been reported [102]. The same study also reported that DMSO did not decrease the antitumor activity of CP against the Walker 256 carcinosarcoma in rats. Different from DMTU, DMSO has been suggested to be a cell differentiating agent with some antitumor activity, and because of that a synergistic action with CP has been proposed [110]. A recent study has demonstrated that, if combined before administration, CP and DMSO react rapidly forming an adducted compound that differs significantly from CP in terms of therapeutic and biological characteristics [111]. Therefore, even though the formation of CP-DMSO adduct might not occur in vivo it should be further investigated.

Edaravone (3-methyl-1-phenyl-pyrazolin-5-one; MCI 186) is a free radical scavenger, which has been used for neuroprotection in the treatment of acute cerebral infarction in Japan [112]. It has been demonstrated to be a potent scavenger of hydroxyl and peroxyl radicals, being also able to react with alkoxyl radicals [113–115]. The protection of edaravone against CP-induced nephrotoxicity has been demonstrated in vivo (rats) and in vitro [103–105]. The pointed advantages of edaravone are (1) the fact that it has already been used in clinical practice mostly without serious side effects at the currently applied doses [103]; (2) it is lipophilic and, therefore, readily accessible to tissues [104]; and (3) it preferentially accumulates in the kidneys [116]. This accumulation in the kidneys is desirable because it presumably confers some selectivity towards the healthy tissue and favors the nephroprotection; however, it also seems to be related to some cases of renal disorders in patients treated with edaravone, reported by Hishida [117]. Another problem related to edaravone is that the protection against CP-induced renal damage seems to be incomplete [113] and to occur only at higher doses as compared to those clinically tolerable (1.5 mg/kg) [103]. In fact, the three cited studies [103–105] report significant effects of edaravone only at doses above 3.0 mg/kg. Concerning the anticancer activity of CP, a study performed in AH-130 cells inoculated in the peritoneal cavity of rats showed that administration of edaravone did not cause any interference [105].

20.6.3 Thiol-Containing Compounds

Several experimental studies [118–122, 154] have demonstrated the protective effects of thiols against CP-induced nephrotoxicity (Table 20.3). The proposed mechanisms of their protective effects include the following: (1) reloading of the intracellular

Table 20.3 Thiol-containing compounds evaluated as possible protector agents against CP-induced nephrotoxicity

Antioxidants	Experimental model	Effect on nephrotoxicity	References
NAC	Rats	Protection	[118, 121, 123]
NAC	Humans	Reversal	[124, 125]
NAC + cysteine + STS + methionine	Renal epithelial cell lines (S_1, S_3 and DCT)	Protection	[120]
STS	Guinea pigs	Protection	[154]
STS	Rat	Protection	[122]
STS	Humans (Phase II trial)	Protection	[126]
Lipoic acid	Rats	Protection	[44]
Amifostine	Humans	Protection	[127, 128]
Amifostine	Mice	Protection	[129]
Amifostine	Humans	No-protection	[130–132]

NAC *N*-acetylcysteine, *STS* sodium thiosulphate

stores of sulfhydryl groups, with consequent restoration of thiol-containing enzymes function; (2) free radical scavenging activity; (3) formation of non-toxic compounds; (4) reduction of CP uptake by renal tubular cells; and (5) increase of the urinary excretion of CP [118, 120, 121, 123–125]. *N*-acetylcysteine (NAC) is a precursor of GSH, which has been used in clinical practice to treat acetaminophen overdose patients and for the prevention of contrast-induced nephropathy. The route of NAC administration seems to affect its renal protective effect; it has been suggested that, because NAC is highly metabolized in liver, both intravenous and intra-arterial routes are more effective as compared to intraperitoneal and oral routes [118]. Besides the preventive effect, it has also been suggested that NAC might be able to reverse CP-induced renal damage, and such beneficial effect might be related to the establishment of a reduced intracellular environment, which favors the processes of repair [124, 125]. On the other hand, a study reports that post-treatment with sodium thiosulphate (STS) showed no protection against CP-induced renal damage in rats [119]. Some strategies such as two-route administration have been proposed in order to administer CP and thiols simultaneously without compromising the antitumor effect of CP. A Phase II clinical trial in which STS was administered intravenously and CP was administered by the intra peritoneal route has been reported and results indicated the protection against renal toxicity [126].

The FDA-approved thiol amifostine is a prodrug that is dephosphorylated by a membrane-bound alkaline phosphatase to the active metabolite WR-1065, which is a free sulfhydryl compound. Because of the higher expression of alkaline phosphatase in normal cells, the decreased vascularity of tumors, and the pH dependence of WR-1065 uptake (pH is neutral in normal tissues and slightly acidic in tumors), amifostine is supposed to selectively protect normal tissues against CP toxicities, without affecting the antitumor effect. The suggested mechanisms of protection include (1) hydrogen donation by the thiol group and (2) nucleophilic attack to the charged molecule of CP with consequent inactivation [14, 24, 41, 133–135]. However, reports on amifostine efficacy are somewhat conflicting; while most studies indicate it reduces

CP renal toxicity [127–129], some indicate it does not [130–132]. Besides that, amifostine-induced side effects, especially transient hypotension, might be severe, and there is a reported case of death, which was attributed to amifostine [69].

20.6.4 Iron Chelators

Iron plays a crucial role in the generation of the highly reactive species, hydroxyl radicals, which are probably involved in CP-induced renal toxicity [50], therefore chelating of iron has been suggested as a protection mechanism [136]. Deferrioxamine (DFO) is an iron chelating agent already used in clinical practice for iron poisoning and iron-loading anemias, such as thalassemia, which has been reported to prevent CP-induced nephrotoxicity [32, 137, 138] however, an experimental study suggests that DFO aggravates cisplatin-induced nephrotoxicity [139]. Additionally, an important limitation factor of DFO therapy is that it has been associated with ototoxicity and high-frequency hearing loss. It has been suggested that the ototoxicity induced by DFO is dose dependent and might be related to individual susceptibility; therefore, audiometric monitoring and low dosages of DFO (below 50 mg/kg/day) have been recommended [140, 141]. The effect of DFO on CP antitumor activity was investigated in rats inoculated subcutaneously with squamous carcinoma cells (SCC-131) and in LLC-WRC 256 tumor cells line. According to both studies, DFO did not affect the antitumor activity of CP [142, 143].

20.7 Conclusions

The nephrotoxicity induced by cisplatin has been challenging physicians for decades. An extensive number of different antioxidants have been shown to be effective against CP-induced nephrotoxicity; however, there are still some concerns about the impairment of the antitumor activity of CP. Studies on the interference of antioxidants in the antitumor effect of CP are still a few, but results are encouraging. As soon as research advances and the remaining doubts fade out, the gap between the promising results from the experimental studies and the clinical application of the antioxidant protective therapy might be overcome.

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Chapter 21
Carrier-Mediated and Targeted Cancer Drug Delivery

William C. Zamboni and Ninh M. La-Beck

Abstract Major advances in the use of carrier vehicles delivering pharmacologic agents and enzymes to sites of disease have occurred in the past 10 years. This chapter focuses on the concepts and preclinical as well as clinical evaluation of carrier-mediated anticancer agents that are administered intravenously or orally. The primary types of carrier-mediated anticancer agents are nanoparticles, nanosomes, which are nanoparticle sized liposomes, and conjugated agents. Nanosomes are then subdivided into stabilized and nonstabilized or conventional nanosomes. The theoretical advantages of carrier-mediated drugs are increased solubility, prolonged duration of exposure, selective delivery of entrapped drug to the site of action, improved therapeutic index, and potential to overcome resistance to anticancer agents. The disposition of carrier-mediated agents depends on the physiochemical characteristics of the carrier, such as size, surface charge, membrane lipid packing, steric stabilization, dose, and route of administration. The primary sites of accumulation of carrier-mediated agents are the tumor, liver, and spleen compared to nonnanosomal formulations. The factors affecting the pharmacokinetic and pharmacodynamic variability of

W.C. Zamboni, Pharm D, PhD (✉)
Division of Pharmacotherapy and Experimental Therapeutics, School of Pharmacy, University of North Carolina, Genetic Medicine Building, Room 1013, CB# 7361, Chapel Hill, NC, 27599, USA

Molecular Therapeutics Program, UNC Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA

UNC Institute for Pharmacogenomics and Individualized Therapy, University of North Carolina, Chapel Hill, NC, USA

Carolina Center of Cancer Nanotechnology Excellence, University of North Carolina, Chapel Hill, NC, USA
e-mail: zamboni@unc.edu

N.M. La-Beck, Pharm D
Division of Pharmacotherapy and Experimental Therapeutics, School of Pharmacy, University of North Carolina, Genetic Medicine Building, Room 1013, CB# 7361, Chapel Hill, NC, 27599, USA

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these agents remain unclear, but most likely include the reticuloendothelial system (RES), which has also been called the mononuclear phagocyte system (MPS). As existing anticancer agents go off patent, these agents will most likely be evaluated in some type of carrier-mediated formulation. Future studies need to evaluate clearance mechanisms of carrier-mediated agents to identify the factors associated with pharmacokinetic and pharmacodynamic variability in patients and specifically in tumors. However, carrier-mediated anticancer agents represent a promising platform for the targeted delivery of antitumor therapy.

21.1 Cancer Problem and Potential: Issues Related to Drug Delivery in Solid Tumors

Major advances have been made in the use of cancer chemotherapy [53]. However, most patients, especially patients diagnosed with solid tumors, fail to respond to initial treatment or relapse after an initial response [53]. Thus, there is a need to identify factors associated with lack of response and to develop new treatment strategies that address those factors. The development of effective chemotherapeutic agents for the treatment of solid tumors depends, in part, on the ability of those agents to achieve cytotoxic drug concentrations or exposure within the tumor [65, 147].

It is currently unclear why within a patient with solid tumors there can be a reduction in the size of some tumors while other tumors can progress during or after treatment, even though the genetic composition of the tumors is similar [11]. Such variable antitumor responses within a single patient may be associated with inherent differences in tumor vascularity, capillary permeability, and/or tumor interstitial pressure that result in variable delivery of anticancer agents to different tumor sites [65, 147]. However, studies evaluating the intratumoral concentration of anticancer agents and factors affecting tumor exposure in preclinical models and patients are rare [14, 101, 147]. In addition, preclinical models evaluating tumor exposure of anticancer agents and factors affecting tumor exposure may not reflect the disposition of chemotherapeutic agents in patients with solid tumors due to differences in vascularity and lymphatic drainage [65, 147]. Moreover, it is logistically difficult to perform the extensive studies required to evaluate the tumor disposition of anticancer agents and factors that determine the disposition in patients with solid tumors, especially in tumors which are not easily accessible. Thus, there is impending need to develop and implement techniques and methodologies to evaluate the disposition and exposure of anticancer agents within the tumor matrix.

21.2 Carrier-Mediated and Artificial-Cell Formulations of Anticancer Agents for Targeted Cancer Drug Delivery

Major advances in the use of carrier vehicles delivering pharmacologic agents and enzymes to sites of disease have occurred in the past 10 years [1, 27, 35, 108, 111]. The primary types of carrier-mediated anticancer agents are liposomes, nanoparticles and conjugated agents (Table 21.1, Fig. 21.1). Liposomes that are nano-sized are

Table 21.1 Summary of carrier-mediated chemotherapeutic agents

Carrier-mediated chemotherapeutic agents				
Nanosomes				
Conventional (nonstabilized)	Stabilized nonpegylated pegylated		Nanoparticles	Conjugates and polymers
LE-SN38	Optisomal topotecan, TLI	Doxil (Caelyx)[a]	Abraxane (ABI-007) ABI-008 (docetaxel) ABI-009 (rapamycin)	Opaxio (Xyotax; PPX; CT-2103; paclitaxel)
Lurtotecan/OSI-211	Marqibo (Optisomal Vincristine)	S-CKD602 (AP-30)[a]	Paclimer (paclitaxel)	XMT-1001 (MER-1001; CPT)
9NC	Alocrest (Optisomal Vinorelbine)	IHL-305 (irinotecan)	TOCOSOL-Paclitaxel	IT-101 (CPT)
Irinophore-C Irinotecan	ATI-1123 (PSN-docetaxel)	Nanoliposomal CPT-11	APA-Thalidomide	Taxoprexin (DHA-paclitaxel)
LEP-ETU (paclitaxel)	ThermoDox (LTSL doxorubicin)	SPI-77	PX-NP (paclitaxel)	PEG-doxorubicin
Docetaxel	LTSL docetaxel	Dihydrotanshinone I	NK-012 (SN38)	PEG-methotrexate
Doxorubicin		Honokiol	NK-105 (paclitaxel)	PEG-interferon
Daunorubicin		SU1498	ANX-514 (docetaxel)	Pegamotecan (EZ-246, PEG-camptothecin)
Cytarabine		Vincristine	CNF1010 (17-AAG)	EZN-2208 (SN38) PEG-SN38
Topotecan		Vinorelbine	SN2310 (SN38)	NKTR-102 (irinotecan) NKTR-105 (docetaxel) PEG-irinotecan
FRL-Doxorubicin–vincristine		Vinblastine	MTX-HAS (methotrexate)	20-carbonate-CPT
CPX-351 (FRL-Daunorubicin–cytarabin)		Camptothecin	ABI-010 (tanespimycin; 17-AAG)	PL-ara-C
FRL-Floxuridine–irinotecan		Curcumin	CPT-HGC	PL-gemcitabine
FRL-Cisplatin–irinotecan		Doxorubicin/Verapamil	CDDP-HGC	CT-2106 (camptothecin)
Aroplatin (DACH-L-NDDP, AR-726, (oxaliplatin analogue)			Docetacel-HGC	AP-5346 DACH-HPMA (oxaliplatin analogue)

Table 21.1 (continued)

Carrier-mediated chemotherapeutic agents			
Nanosomes			
Conventional (nonstabilized)	Stabilized nonpegylated pegylated	Nanoparticles	Conjugates and polymers
MBP-426 (oxaliplatin)		Methotrexate PEPE drendrimer	ProLindac (AP-5280, cisplatin-analogue HPMA)
LEM (mitoxantrone)		Paclitaxel PEO-PbAE	PK1 (doxorubicin)
Annamycin		5-Fluorouracil Bola noisome	PNU-166148 (MAG-CPT)
Atragen (tretinoin)		Adriamycin PAMAM dendrimer	9-aminocamptothecin
GMC-5-193 (quinazolinone analogue)		Doxorubicin/Wortmaninn	aclarubicin (CBSA-NP-ACL)
		ABI-011 (microtubule + topoisomerase I inhibitor)	auristatin E (MMAE) auristatin F (MMAF)
		Paclitaxel/MDR-1 SiRNA	Paclitaxel-HA
		NC-6004 (cisplatin)	
		SN38-PNDS	

AP-5346 DACH-HPMA = an oxaliplatin analogue, bound to copolymer HPMA, *AP-5280* cisplatin-like analogue bound to copolymer HPMA, *APA* alginate-poly-lysine-alginate microcapsules, *Ara-C* cytosine arabinoside, *Bola* α,ω-hexadecyl-bis-(1-aza-18-crown-6), *CPT* camptothecin, *CT-2106* polyglutamated camptothecin, *DACH-L-NDDP* liposomal of DACH platinum, AR-726, an oxaliplatin analogue, *DHA* docosahexaenoic acid, *FRL* fixed ratio liposomes, *HA* hyaluronic acid, *HGC* hydrophobically modified glycol chitosan nanoparticle, *NK-012* micellar SN38, *PAMAM* poly(amidoamine), *PEG* polyethylene glycol, *PEO-PbAE* Poly(ethylene oxide)-modified poly(beta-amino ester), *PEPE* polyether-copolyester, *PL* phospholipids, *PSN* Protein Stabilized Nanoparticle for liposomes, *PX-NP* paclitaxel entrapped in cetyl alcohol/polysorbate nanoparticles

[a]Doxil (Caelyx) and S-CKD602 (AP-30) are pegylated liposomal agents

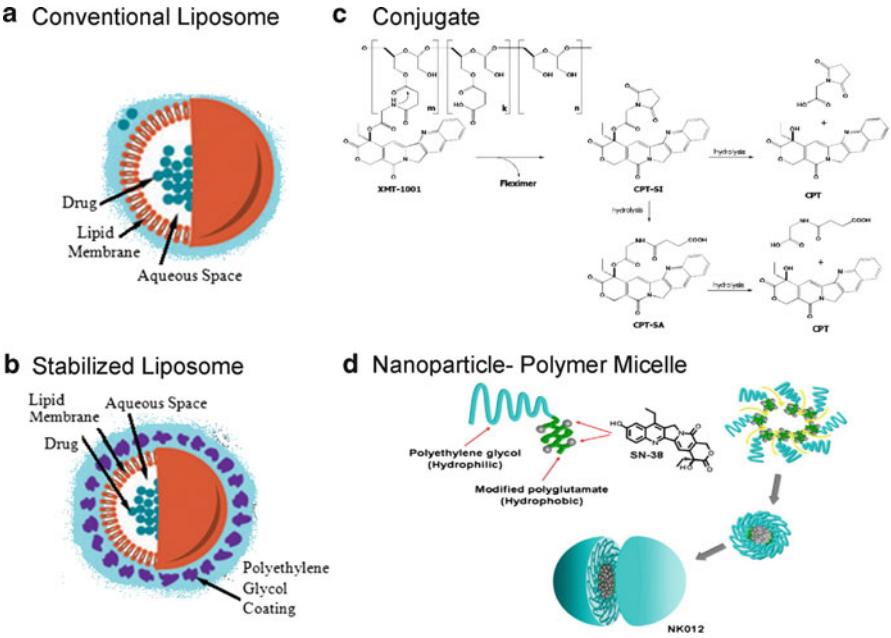

Fig. 21.1 Primary types of carrier-mediated anticancer agents: (**a**) conventional liposome; (**b**) stabilized liposome with polyethylene glycol coating; (**c**) XMT-1001, a polymer conjugated prodrug with dual phase release of camptothecin; (**d**) NK102, a micelle nanoparticle composed of SN-38 conjugated to polymer units

also referred to as nanosomes and can be subdivided into stabilized and nonstabilized (conventional) liposomes. Stabilized liposomes can also be subdivided into those that are stabilized by polyethylene glycol (PEG) or a non-PEG substitute such as sphingomyelin. Nanoparticles are subdivided into microspheres, which include polymer micelles, and dendrimers. Conjugate formulations consist of the drug linked to nano-sized PEG or non-PEG polymers [1, 27, 35, 108, 111]. The theoretical advantages of liposomal and nanoparticle encapsulated and carrier-mediated drugs are increased solubility, prolonged duration of exposure, selective delivery of entrapped drug to the site of action, improved therapeutic index, and potentially overcoming resistance associated with the noncarrier-mediated anticancer agent [35, 111]. The process by which these agents preferentially accumulate in tumor and tissues is called the enhanced permeation and retention effect [91]. Pegylated-liposomal doxorubicin (Doxil®, Caelyx®), liposomal daunorubicin (DaunoXome®), liposomal cytarabine (DepoCyt®), and paclitaxel albumin-bound particles (ABI007, Abraxane®) are the only members of this relatively new class of agents that are FDA approved [3, 28, 50, 80, 92, 34]. Myocet®, a nonpegylated liposomal formulation of doxorubicin, is approved in Europe for the treatment of breast cancer [8]. Doxil is FDA-approved for the treatment of refractory ovarian cancer and Kaposi's sarcoma [80, 92, 124]. DaunoXome is also approved for the treatment of advanced Kaposi's

Table 21.2 Nomenclature describing the pharmacologic forms of carrier-mediated drugs

Sum total drug		
Encapsulated or conjugated drug	Released drug	
	(Warhead)	
	(Naked drug)	
	(Legacy drug)	
	Protein bound	Protein unbound (Free)

sarcoma [28], Abraxane is approved for the treatment of refractory breast cancer [3] and DepoCyt is approved for the intrathecal treatment of lymphomatous meningitis. Although these are the only FDA-approved carrier-mediated chemotherapeutic agents, there are greater than 100 other agents that are in preclinical and clinical development. Newer generations of liposomes containing two anticancer agents within a single liposome and antibody-targeted liposomes which may improve selective toxicity are in preclinical development [2, 81, 115]. In addition, nanoparticle formulations such as microspheres, dendrimers, and conjugates provide a unique method to provide tumor-selective delivery of anticancer agents to tumors [121]. As more existing anticancer agents go off patent these agents will most likely be evaluated in some type of liposome or carrier-mediated formulation. In addition, antiangiogenesis agents, antisense oligonucleotides, and enzymes represent rational candidates for liposomal and nanoparticle formulations [115].

The pharmacokinetic disposition of these agents is dependent upon the carrier and not the parent-drug until the drug is released from the carrier [81]. Thus, the pharmacology and pharmacokinetics of these agents is complex, and detailed studies must be performed to evaluate the disposition of the encapsulated or conjugated form of the drug and the released active drug (Table 21.2) [150]. The factors affecting the pharmacokinetic and pharmacodynamic variability of these agents remain unclear, but most likely include the reticuloendothelial system (RES), which has also been called the mononuclear phagocyte system (MPS) [83, 89, 141].

21.3 Methods for Evaluation of Carrier Agents

The need to develop and readily gain information on the tumor disposition of agents may become more important with the increasing number of tumor targeting approaches, such as gene and antisense therapy, polyethylene glycol (PEG)-conjugated agents, and liposomal delivery [18, 148]. In addition, methodology and study designs used to develop classic cytotoxic anticancer agents, such as platinum, taxane, and camptothecin analogues, may not be appropriate for the new generations of anticancer therapy, such as angiogenesis inhibitors, antiproliferative agents, and signal transduction

inhibitors [18, 47]. Since these agents may not induce classic toxicities or any toxicity, it may be difficult to recommend a dose for future trials using the standard Phase I dose escalation methods and endpoints (i.e., maximum tolerable dose and dose limiting toxicities). As an alternative, defining the dose for Phase II studies could be based on the dose that achieves exposures associated with pharmacologic modulation, optimal biological exposure, or cytotoxicity results from in vitro studies [145, 147]. Historically, investigators have compared in vitro IC_{50} values with plasma concentrations in patients as a means to determine if sufficient exposure has been reached in clinical studies. However, the inherent tumor characteristics that influence tumor penetration and high intra- and intertumoral variability in tumor exposure make this comparison highly unreliable [16, 65, 147], especially when the ratio of tumor exposure to plasma exposure may be approximately 0.2–0.5 [37, 39, 102, 147, 149]. Thus, comparing the in vitro exposures and plasma exposures in patients result in an overestimation of drug exposure in the tumor extracellular fluid (ECF), and the estimated exposure for effect may in fact be insufficient. The use of methodologies, such as microdialysis, that measure the exposure of anticancer agents within the tumor may improve the level of information available to make informed decisions during the drug development process [150].

21.4 Liposomal Formulations of Anticancer Agents

Liposomes are microscopic vesicles composed of a phospholipid bilayer that are capable of encapsulating the active drug. However, conventional liposomes are opsonized by plasma proteins, quickly recognized as foreign bodies, and rapidly removed by the RES [7, 9, 35]. Depending on the size and composition of the liposome, RES uptake can occur within minutes after administration and remove the liposomes from the circulation. Studies evaluating the disposition and tumor penetration of liposomal and nonliposomal anticancer agents suggest liposomal agents have an extended systemic half-life ($t½$) and extravasate selectively into solid tumors through the capillaries of tumor neovasculature [7, 35]. The exact mechanism of liposomal clearance is currently unclear. The mechanisms by which liposomes enter tissue and tumors, and release drug are also not completely understood. In addition, liposomes can be engineered to produce a complete spectrum of drug release rates that need to be evaluated in in vivo systems [12, 82].

The development of pegylated liposomes, which contain lipids conjugated to polyethylene glycol (PEG), was based on the theory that incorporation of PEG-lipids into liposomes would allow the liposome to evade the immune system and prolong the duration of exposure (Fig. 21.2) [7, 111, 141]). pegylated liposomes have a lipid bilayer membrane like conventional liposomes, but the surface contains surface-grafted linear segments of PEG extending 5 nm from the surface [111, 141]. pegylated liposomes are relatively small, with an average diameter of approximately 100 nm. The size optimally balances drug-carrying capacity with circulation time, and allows extravasation through the endothelial gaps in the

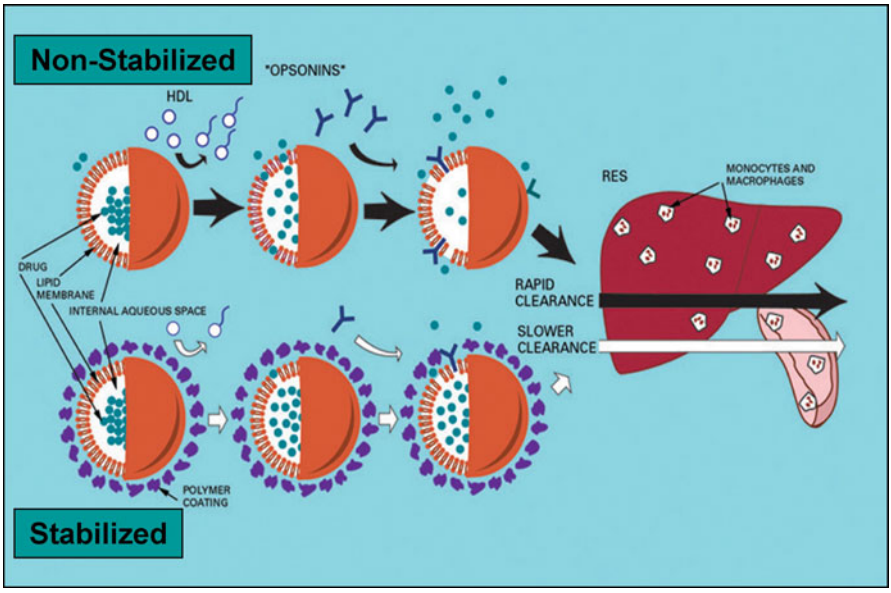

Fig. 21.2 Clearance of stabilized and nonstabilized nanosomes via the reticuloendothelial system (RES) in the liver and spleen

capillary bed of target tumors. Whether the drug is encapsulated in the core or in the bilayer of the liposome is dependent upon the characteristics of the drug and the encapsulation process. In general, water-soluble drugs are encapsulated within the central aqueous core, whereas lipid-soluble drugs are incorporated directly into the lipid membrane.

21.5 Systemic and Tissue Disposition of Liposomes

Liposomes can alter both the tissue distribution and the rate of clearance of the drug by making the drug take on the pharmacokinetic characteristics of the carrier [35, 111, 141]. Pharmacokinetic parameters of the liposomes depend on the physiochemical characteristics of the liposomes, such as size, surface charge, membrane lipid packing, steric stabilization, dose, and route of administration [35]. The primary sites of accumulation of conventional liposomes are the tumor, liver, and spleen compared to nonliposomal formulations [7, 35, 83, 89, 106, 142]. The development of pegylated liposomes was based on the discovery that incorporation of PEG-lipids into liposomes yields preparations with superior tumor delivery compared to conventional liposomes composed of natural phospholipids [7, 35]. Incorporation of PEG-lipids causes the liposome to remain in the blood circulation for extended periods of time (i.e., $t½ > 40$ h) and distribute throughout an organism relatively

evenly with most of the dose remaining in the central compartment (i.e., the blood) and only 10–15% of the dose being delivered to the liver [7, 106, 142]. This is a significant improvement over conventional liposomes where typically 80–90% of the liposomes deposit in the liver.

The clearance of conventional liposomes has been proposed to occur by uptake of the liposomes by the RES (Fig. 21.2) [7, 35]. The RES uptake of liposomes results in their rapid removal from the blood and accumulation in tissues involved in the RES, such as the liver and spleen. Uptake by the RES usually results in irreversible sequestering of the encapsulated drug in the RES, where it can be degraded. In addition, the uptake of the liposomes by the RES may result in acute impairment of the RES and toxicity. Sterically stabilized liposomes, such as pegylated liposomes, prolong the duration of exposure of the encapsulated drug in the systemic circulation [111, 141]. The presence of the PEG coating on the outside of the liposome does not prevent uptake by the RES, but simply reduces the rate of uptake (Fig. 21.2) [7]. The exact mechanism by which steric stabilization of liposomes decreases the rate of uptake by the RES is unclear [35, 97, 111, 141].

21.6 Tumor Delivery of Liposomal Agents

The development of effective chemotherapeutic agents for the treatment of solid tumors depends, in part, on the ability of those agents to achieve cytotoxic drug exposure within the tumor extracellular fluid [65, 147]. Solid tumors have several potential barriers to drug delivery that may limit drug penetration and provide inherent mechanisms of resistance [65]. Moreover, factors affecting drug exposure in tissue, such as alteration in the distribution of blood vessels, blood flow, capillary permeability, interstitial pressure, and lymphatic drainage, may be different in tumors as compared to the surrounding normal tissue [65].

The accumulation of liposomes or large macromolecules in tumors is a result of the extended duration of exposure in the systemic circulation as well as the leaky microvasculature and impaired lymphatics supporting the tumor area [35, 65, 106, 142]. Once in the tumor, the nontargeted pegylated liposomes are localized in the extracellular fluid (ECF) surrounding the tumor cell, but do not enter the cell [60, 61]. Thus, for the liposomes to deliver the active form of the anticancer agent, such as doxorubicin, the drug must be released from the liposome into the ECF and then diffuse into the cell [150]. As a result, the ability of the liposome to carry the anticancer agent to the tumor and release it into the ECF are equally important factors in determining the antitumor effect of liposomal encapsulated anticancer agents. In general, the kinetics of this local release is unknown, as it is difficult to differentiate between the liposomal-encapsulated and released forms of the drug in solid tissue, although with the development of microdialysis, this is becoming easier [150].

21.7 Modification of Toxicity with Liposomal Agents

Liposomal formulations can also modify the toxicity profile of a drug (e.g., Ambisome® [137]. This effect may be due to the alteration in tissue distribution associated with liposomal formulations [7, 106, 142, 150]. Anthracyclines, such as doxorubicin, are active against many tumor types, but cardiotoxicity related to the cumulative dose may limit their use [43]. Preclinical studies determined that liposomal anthracyclines reduced the incidence and severity of cumulative dose-related cardiomyopathy while preserving antitumor activity [43]. There is also clinical evidence suggesting that Doxil is less cardiotoxic than conventional doxorubicin [43, 107]. Direct comparisons between Doxil or Caelyx and conventional doxorubicin showed comparable efficacy but significantly lower risk of cardiotoxicity with the STEALH liposomal formulations of doxorubicin [43]. In addition, histologic examination of cardiac biopsies from patients who received cumulative doses of Doxil from 440 to 840 mg/m^2, and had no prior exposure to anthracyclines, revealed significantly less cardiac toxicity than in matched doxorubicin controls ($p < 0.001$) [13]. Administration of a drug in a liposome may also result in new toxicities [22, 124, 135]. The most common adverse event associated with Doxil is hand-foot syndrome (HFS, also known as palmar–plantar erythrodysesthesia) and stomatitis, which have not been reported with conventional doxorubicin [124]. The exact mechanisms associated with these toxicities are unknown, but are schedule and dose dependent. In general, Doxil is well tolerated and its side-effect profile compares favorably with other chemotherapy used in the treatment of refractory ovarian cancer. Proper dosing and monitoring may further enhance tolerability while preserving efficacy; however, there is still a need to identify factors associated with HFS, which can be dose limiting in some patients [124].

21.8 Current Preclinical and Clinical Evidence for Liposomal Agents

Pegylated (Doxil/Caelyx) and conventional liposomal formulations of doxorubicin (Myocet), daunorubicin (DaunoXome), and cytarabine (DepoCyt) are approved in the United States and Europe [80, 92, 124]). Some of the other liposomal anticancer agents that are currently in development include SN-38 (LE-SN38) [79, 87, 109, 155], lurtotecan (OSI-211) [30, 48, 49, 72], 9NC [74, 77, 138], irintotecan [36, 95], topotecan [154], pegylated liposomal CKD-602 (S-CKD602) [151], paclitaxel (LEP-ETU) [29], cisplatin [69], doxorubicin [94], vincristine [128], vinblastine [54], vinorelbine [63], and gemcitabine [23]. Liposomal encapsulation of camptothecins is an attractive formulation due to the solubility issues associated with most camptothecin analogues and the potential for prolonged exposure after administration of a single dose [30, 79, 151]. As compared to pegylated- or coated-liposomes, conventional liposomal formulations of camptothecin analogues, such as LE-SN38 and

OSI-211, may result in the rapid release of the drug from the liposome in blood and thus act more as an IV formulation as compared to a tumor targeting agent [30, 49, 79, 87, 109, 155]. However, studies evaluating encapsulated and released drug in plasma and tumor have not been reported [150].

A randomized phase II trial of OSI-211 in patients with relapsed ovarian cancer compared OSI-11 IV on days 1, 2, and 3 repeated every 3 weeks and OSI-11 IV on days 1 and 8 repeated every 3 weeks was performed [30]. OSI-211 daily for 3 days was declared the winner in terms of objective response. A phase I study of LE-SN38 was performed in which patients were prospectively assigned to cohorts based on UDP-glucuronosyltransferase 1A1 (UGT1A1) genotype [79]. The maximum tolerated dose (MTD) was not reached in the *28/*28 patients. The MTD of LE-SN38 in the WT/WT cohort was 35 mg/m^2 IV over 90 min every weeks. The pharmacokinetic disposition of SN-38 was similar in the WT/WT and WT/*28 cohorts. Interestingly, there were no reports of acute or delayed diarrhea even though the exposures of SN-38 were several fold higher after administration of LE-SN38 compared to irinotecan. Results of a phase I study of S-CKD602 administered IV over 1 h every 21 days reported that the $t_{1/2}$ was increased four- to eightfold and plasma exposure was increased approximately 50-fold higher after administration S-CKD602 compared to nonliposomal CKD602 [151]. The results of this study suggest that S-CKD602 exhibits the characteristics that are consistent with other pegylated liposomes and thus may have pharmacologic advantages over other liposomal formulations of camptothecin analogues [151]. In addition, S-CKD602 has produced responses in patients with platinum-refractory ovarian cancer [153]. Aerosolized administration of liposomal 9NC was found to be feasible and safe in patients with advanced pulmonary malignancies and 9NC was detected in plasma shortly after the start of treatment [74, 77, 138]. Liposomal formulations of irinotecan (nanosomal CPT-11, IHL-305) are currently in preclinical development and theoretically may provide targeted delivery of irinotecan to the tumor with subsequent conversion to SN-38 via tumor carboxyl esterase [36, 95]. IHL-305 is a peglyated liposomal formulation of irinotecan (CPT-11) that is currently in phase I studies. Preclinical studies of IHL-305 have reported greater tumor delivery and improved antitumor efficacy as compared with nonliposomal CPT-11 [144]. Liposomes can also be stabilized by non-PEG molecules. For example, TLI is a sphingomyelin-stabilized liposomal formulation of topotecan that is currently in phase I studies [154].

In a phase I study, the pharmacokinetic profile of paclitaxel was similar after administration of LEP-ETU and nonliposomal paclitaxel, suggesting that paclitaxel is immediately released from the liposome after LEP-ETU administration [29]. In addition, it is unclear if LEP-ETU has pharmacologic or cytotoxic advantages over paclitaxel albumin-bound particles [3, 29]. Conventional liposomal formulations of doxorubicin do not appear to have a pharmacologic or cytotoxic advantage over Doxil [43, 94, 107]. Liposomal vincristine (Onco-TSC) has been evaluated in relapsed non-Hodgkins lymphoma (NHL) as a way to overcome the toxicity limitations and required dose reductions associated with the use of vincristine in this setting [113]. Liposomal vincristine has a prolonged half-life and achieves higher exposures in tumors and lymph nodes compared with nerves. When administered at full doses liposomal vin-

cristine appears to be less neurotoxic and more active compared with nonliposomal vincristine in preclinical models and in patients. These data suggest a potential role for liposomal vincristine in the combination therapy for NHL.

In addition to improving the therapeutic index of traditional chemotherapeutics, the liposomal platform has enabled the development of novel anticancer agents that may otherwise have been abandoned due to physicochemical problems such as limited in vivo solubility or rapid inactivation. Examples include annamycin, a highly lipophilic doxorubicin analogue [15], and honokiol, a biphenolic compound isolated from the magnolia plant that has promising antitumor activity but has extremely poor water solubility [90].

The future generations of liposomes will include active targeting liposomes (e.g., immunoliposomes), triggered release liposomes (e.g., thermosensitive liposomes), single liposomes that contain multiple anticancer agents, and multimodality liposomes [2, 81, 94].

Active targeting can be achieved by surface modifying liposomes with antibodies or antibody fragments which target tumor surface antigens. These immunoliposomes combine antibody-mediated tumor recognition with liposomal delivery, which are designed for target cell internalization and intracellular drug release [115]. In addition to enabling targeted delivery of the liposome encapsulated drug, the antibody component may also have inherent antitumor effects. An example currently in preclinical development is anti-human epidermal growth factor receptor 2 (HER2) immunoliposomal doxorubicin [75, 114]. Anti-HER2 immunoliposomal doxorubicin was studied in various murine xenograft models of HER2 overexpressing tumors and found to have significantly increased antitumor effects as compared to free doxorubicin and Doxil either with or without trastuzumab, a well-established therapeutic monoclonal antibody targeting HER2. In addition, cure rates for anti-HER2 immunoliposomal doxorubicin in these studies ranged from 16 to 50% as compared to 0% for free doxorubicin and liposomal doxorubicin ($p<0.0001$ for both comparisons). Furthermore, the immunoliposome was found to have equivalent efficacy as the nontargeting doxorubicin liposome in non-HER2 overexpressing tumors, thus supporting the active targeting mechanism of the immunoliposome [114]. Immunoliposomes are also being studied which target epidermal growth factor receptor (EGFR) [116] and the B-lymphocyte antigen CD19 [24] among others [40].

Active tumor targeting can also be achieved by surface modifying the liposome with small molecules whose receptors are known to be overexpressed in tumor cells. This would also facilitate target cell internalization of the liposome and intracellular release of the encapsulated drug. Examples of this include surface modification with folic acid [96], mutant soluble B-cell activating factor [156], and many others.

Triggered release liposomes would utilize a stimulus to trigger release of the active drug form the liposome thus enabling controlled tumor specific release of the anticancer agent. The trigger can be applied via local hyperthermia (radiofrequency, microwave or ultrasound ablation), ultrasonic or photonic disruption of liposome membranes, changes in the pH, enzymatic cleavage, or even redox reactions that are intrinsic to the tumor tissue [119].

Thermosensitive liposomes may provide a means of improving the tumor-specific delivery of anticancer agents by rapidly releasing drug from the liposome when hyperthermia is applied to the tumor area [94]. An example is lysolipid thermally sensitive liposomal (LTSL) doxorubicin (ThermoDox) which is doxorubicin encapsulated within a thermosensitive liposome enabling release of the drug at temperatures greater than 39.5°C [98, 122]. LTSL doxorubicin liposomes contain lysolipids such as monostearoyl phosphatidylcholine in the lipid bilayer. At temperatures close to the liposome melting phase transition temperature, these lysolipids form stable pores in the lipid bilayer, which allow the rapid release of liposomal contents [119]. LTSL doxorubicin in combination with radiofrequency ablation is currently being studied for the treatment of unresectable primary and secondary liver tumors, prostate cancer and breast cancer with chest wall recurrence [119, 122, 140]. Early clinical trial results support the efficacy and tolerability of LTSL doxorubicin in patients with unresectable liver tumors and phase III trials are underway. In addition to triggering the release of drug from the liposome, local hyperthermia itself has antitumor effects and can increase drug penetration by altering tumor perfusion. One disadvantage of LTSL liposomes is an increased tendency for the drug to leak out of the liposome over time, as compared to stabilized nonthermosensitive liposomes [119].

Liposomes that are sensitive to pH have been studied for tumor specific triggered release. These liposomes are stable at physiologic pH but become leaky in more acidic environments. Tumor specific release is possible by targeting the slightly acidic tumor microenvironment or by targeting release via tumor endosomes [85]. These liposomes often incorporate poly(L-histidine) or poly sulfonamide in the lipid bilayer as these molecules are pH sensitive. Examples include pH sensitive cisplatin liposomes [68] and pH sensitive doxorubicin liposomes [85].

There are several liposomal formulations that contain fixed ratios of two anticancer agents, such as doxorubicin–vincristine, daunorubicin–cytarabine, cisplatin–irinotecan, and 5-fluorouracil–irinotecan that are currently in preclinical development [2, 67]. In addition to encapsulating multiple anticancer drugs, liposomes can encapsulate agents with different functions thus yielding multimodality liposomes. For example, stabilized liposomes containing both vinorelbine and indium-111 have been studied in mice with colorectal cancer xenografts. This approach combines chemotherapy with an antitumor and imaging radionuclide [25].

21.9 Nanoparticle Formulations: Characteristics, Development, and Current Evidence

Abraxane is the first protein-stabilized nanoparticle approved by the FDA [1, 3, 62]. Abraxane is an albumin-stabilized nanoparticle formulation of paclitaxel designed to overcome the solubility issues associated with paclitaxel that require the need for solvents such as cremophor, which have been associated with infusion related reactions and requires the need for premedication and other incompatibilities with

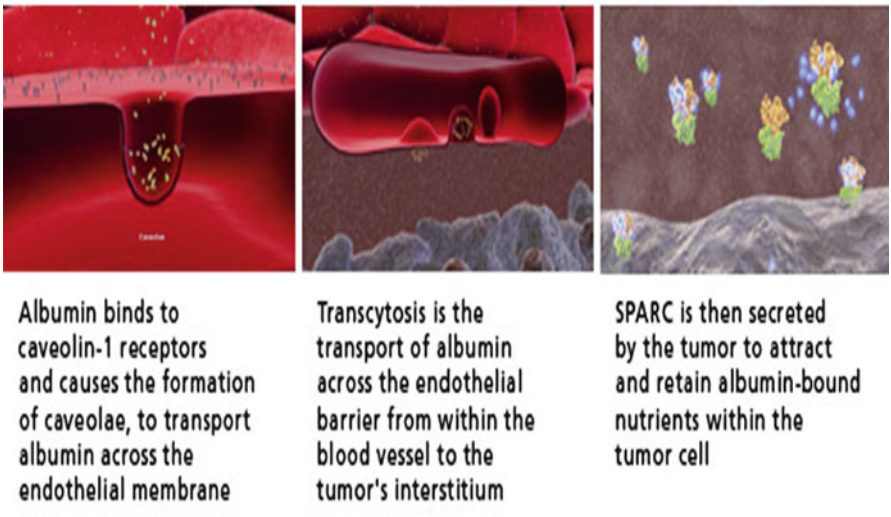

Fig. 21.3 Mechanism of albumin-bound paclitaxel transcytosis across blood vessels and accumulation of paclitaxel in tumor tissue via interactions with SPARC after administration of Abraxane

certain IV bags or tubing [1, 62]. The albumin-stabilized nanoparticle results in a more rapid distribution out of the vascular compartment and provides a tumor targeting mechanism. The albumin receptor-mediated transport on the endothelial cell wall within blood vessels facilitates the passage of Abraxane from the blood stream into the underlying tumor tissue [1, 62] (Fig. 21.3).

Similar to liposomal agents, the dosage of Abraxane is determined by the paclitaxel content of the formulation [1, 62]. The approved regimen for Abraxane is 260 mg/m^2 IV over 30 min every 3 weeks which is higher than the usual dose range for paclitaxel (i.e., 135–200 mg/m^2) [1, 3]. In addition there was a lower incidence of myelosuppression after administration of Abraxane than previously seen with similar doses of paclitaxel [62]. The other toxicities associated with Abraxane were similar to high-dose, short-infusion paclitaxel including sensory neuropathy and mucositis. Keratopathy, a relatively unique toxicity also associated with Abraxane, was reported [62]. Thus, as with liposomal formulations, administration of a drug in a nanoparticle formulation can alter the pharmacokinetic, tissue and tumor distribution, and toxicity pattern. Also similar to liposomal agents, the mechanism by which the albumin-stabilized nanoparticle is catabolized and paclitaxel is released is unclear.

Microencapsulation is an emerging technology in the development of bioartificial organs for drug, protein, and delivery systems [57]. Bioencapsulation technology offers several advantages and has shown promising results for the treatment of diseases. For all of these applications, appropriate performance of the microcapsules is critically dependent on the properties of the capsular membrane. Several studies have encapsulated bacterial cells for potential therapeutic applications

using oral administration, such as in kidney failure uremia, cancer therapy, diarrhea, cholesterolemia, and other diseases [108]. These microcapsule carriers may also be used for the oral administration of drugs and enzymes for cancer therapy. However, the success of microcapsule oral delivery depends on the suitability of the microcapsule membrane for oral delivery. The microcapsule can be disrupted by many different means during its intestinal passage and may be fractured by enzymatic action, chemical reactions, heat, pH, diffusion, mechanical pressure, and other related physiological and biochemical stresses. Microcapsule encapsulation of thalidomide allowed for the successful delivery of thalidomide in the gut and could prove beneficial in the treatment of Crohn's disease [96]. In addition, the use of microcapsules may be used for the delivery of anticancer agents in the treatment of gastric and colon cancer.

Patients with primary brain tumors or brain metastases have a very poor prognosis that is primarily attributed to the impermeability of the blood–brain barrier (BBB) to cytotoxic agents [78]. Paclitaxel has been shown to have activity against gliomas and other brain metastases; however, its use in the treatment of brain tumor is limited due to low BBB penetration and side effects associated with IV administration. The lack of BBB penetration is believed to be associated with the P-glycoprotein (P-gp) efflux transporter. To overcome these issues, a formulation of paclitaxel entrapped in novel cetyl alcohol/polysorbate nanoparticles (PX-NP) was developed [78]. PX-NP had reduced efflux by P-gp, increased brain exposure, and reduced toxicity compared with paclitaxel and thus may have potential in the treatment of brain tumors.

A dendrimer is a nanoparticle with a hydrophobic interior and hydrophilic exterior that act as a drug carrier [27]. Dendrimers are a class of different fractal polymers prepared by a set of iterative reactions attached to a central core. Each pair of iterations defines a generation, and while the diameter grows linearly, the number of surface functional groups grows geometrically. The interior host sites can shield the drug from the exterior biologic milieu and stabilize the drug. The exterior of the dendrimer can also be labeled with tumor specific-ligands, such as folate to provide tumor-selective delivery of anticancer agents to the tumor [133].

Several types of interactions between dendrimers and drugs have been evaluated, which can be broadly subdivided into the entrapment of drugs within the dendritic architecture (involving electrostatic, hydrophobic, and hydrogen bond interactions) and the interaction between a drug and surface of a dendrimer (electrostatic and covalent interactions) [27]. In addition, PEG has been added to dendrimers as a way to modify biocompatibility and biodistribution [55, 104, 110]. The applications of these systems have been used to enhance drug solubility and bioavailability, prolong circulation time, act as release modifiers, and drug targeting. For example, an indomethacin-loaded dendrimer has provided sustained release of drug over 30 h. Dendrimer carriers of anticancer agents are currently in preclinical development. Examples of these include methotrexate-loaded polyether-copolyester dendrimers, which are also surface modified with D-glucosamine to target the BBB [35], and adriamycin-loaded pegylated polyamidoamine dendrimers, which are also pH sensitive [76].

Drug-polymer conjugates that form nano-sized micelles appear to be effective carriers for anticancer therapy. This approach to conjugation would entail synthesis of amphipathic block copolymers with the capacity for self assembly via hydrophobic interactions, analogous to the self assembly of liposomes (Fig. 21.1). The resulting structure is a polymer micelle composed of individual units of drug-polymer conjugates which can be used as a drug carrier in a manner also similar to liposomes but with the drug conjugated to the micelle units rather than encapsulated freely within the micelle core or membrane [93]. An example is PEG-poly(glutamic acid) SN-38 (NK012) which is an SN38-releasing polymeric micelle [20, 70]. Preclinical studies have shown superior antitumor activity and less toxicity than CPT-11. In addition, it may be able to selectively accumulate in both hypervascular and hypovascular tumors with high interstitial pressure, with subsequent sustained release of SN-38, followed by SN-38 distribution throughout the tumor tissue [75, 103, 126]. Other examples of polymeric micellar nanoparticle agents include polyethylene glycol (PEG)-polyaspartate paclitaxel (NK105) [56, 64], PEG-poly(glutamic acid) cisplatin (NC-6004) [134], and SN38-PNDS [88]. SN38-PNDS is distinct in that it is an active camptothecin analogue that is orally administered via a pH sensitive polymer nano-delivery system (PNDS). Preclinical studies have demonstrated comparable efficacy and favorable toxicity and PK disposition compared with historical data for intravenously administered irinotecan (CPT-11) [88]. If successful in clinical trials, this would represent the first orally administered nanoparticle formulation of an anticancer agent.

Until recently, the synthesis of nanoparticles relied on “bottom up” techniques which are based on self assembly of the nanoparticles. Examples of these techniques include electrospray, inverse emulsions, ultrasonication, and supercritical solvent processes. Although there have been advances in these techniques, there remains several major disadvantages. The primary limitations are the lack of control over the shape and size of the particles as well as a tendency for the nanoparticles to aggregate [42, 73]. These limitations can be overcome by a technique called particle replication in nonwetting templates (PRINT) which relies on a “top down” approach to nanoparticle fabrication. PRINT is based on imprint lithography techniques adapted from the field of microelectronics. It enables the rapid and large scale production of organic nanoparticles with precise control over particle size, shape, and composition with little or no tendency for aggregation. PRINT particles can potentially be tailored to meet specific pharmacokinetic parameters such as bioavailability and circulation time by altering the particle matrix and surface composition. In addition, they can also be surface modified with functional moieties such as antibodies for targeting and polyethylene glycol for stabilization [51]. PRINT doxorubicin containing a reductively labile disulfide linker *N*,*N*′-cystaminebisacrylamide has been studied in vitro. The disulfide linker is destabilized in a reducing environment, such as within the cell cytosol, thus enabling triggered release of doxorubicin from the PRINT matrix intracellularly [117].

21.10 Conjugates and Polymers: Characteristics, Development, and Current Evidence

During the past 10 years there has been a renaissance in the field of PEG-conjugated anticancer agents [52]. This new development has been attributed to the use of higher molecular weight PEGs (>20,000) and especially with the use of PEG 40,000, which has an extended *t½* in plasma and potential selective distribution to solid tumors [52]. Various PEG-conjugates of anticancer agents, such as doxorubicin [10], methotrexate [123], interferon [21, 33], and camptothecin analogues [113, 125], are currently in development [10, 21, 33, 113, 125]. PEG- and 20-carbonate-conjugates of camptothecin analogues, are especially interesting as the conjugated-prodrug forms highly water-soluble agents and significantly extends the duration of exposure after a single dose [31, 113, 125]. EZN-2208 is a multi-PEG conjugate of SN-38. SN-38 in the lactone form is the active metabolite of camptothecin-11 (CPT-11). However, SN-38 itself has extremely poor bioavailability largely due to poor solubility and rapid in vivo hydrolysis of the lactone ring into the inactive carboxylate form of SN-38. Conjugation of SN-38 to multiple PEG molecules resulted in significantly increased solubility, longer half-life, higher exposure, and improved therapeutic index in xenograft models as compared with CPT-11 [127].

Hyaluronic acid conjugates of anticancer agents are also in development. Paclitaxel-hyaluronic acid conjugates in in vitro studies have been found to have a more pronounced cytotoxic effect on hyaluronic acid receptor overexpressing cells, suggesting a targeting mechanism [85]. Carrier-mediated conjugates of anticancer agents also have the same pharmacologic issues (the need to evaluate the pharmacokinetics of the prodrug conjugate and released drug) as liposomal and nanoparticle formulations and the overall clinical benefit of these agents remain unclear.

Additional conjugates-formulations of paclitaxel are in clinical and preclinical development. Paclitaxel poliglumex (PPX, Xyotax®), a macromolecular drug conjugate that links paclitaxel with a biodegradable polymer, poly-L-glutamic acid, has completed phase I studies [131]. PPX is a water-soluble formulation that also eliminates the need for cremophor in the formulation. Paclimer®, a microsphere formulation of paclitaxel is currently in preclinical development [34]. Paclimer microspheres contain paclitaxel in a polilactofate polymer microsphere and are designed to continuously deliver low dose paclitaxel. Previous conjugates of paclitaxel have been stopped in clinical development and have been associated with potential pharmacologic and pharmacokinetic problems [17, 139]. Docosahexaenoic acid (DHA)-paclitaxel, a novel conjugate formed by covalently linking the natural fatty acid DHA to paclitaxel, was designed as a prodrug targeting intratumoral activation [17]. At the MTD of DHA-paclitaxel (1,100 mg/m^2), paclitaxel represented only 0.06% of the DHA-paclitaxel plasma exposure [139]. However, the paclitaxel concentrations remained >0.01 μM for an average of 6–7 days and the paclitaxel AUC was correlated with neutropenia. The results of this study suggest that most of the drug remained in the

inactive-prodrug-conjugated form and that significant toxicity only occurred when released paclitaxel reached clinically relevant exposures. This depicts the need to perform detailed pharmacokinetic studies of conjugated and released drug in plasma and tumor.

Another example of a drug-conjugate nanoparticle is XMT-1001, which is a dual phase release prodrug conjugate of camptothecin. It consists of camptothecin (CPT) conjugated to a poly(L-hydroxymethylethylene hydroxyl-methyl formal) (PHF) backbone via a succinamido ester linker. This conjugate form of camptothecin has multiple advantages over free camptothecin and over camptothecin analogues such as irinotecan and topotecan including improved solubility, bioavailability, and decreased toxicity. The PHF backbone also increases circulation time thus enhancing tumor tissue accumulation presumably via the EPR effect. The PHF-CPT conjugate undergoes nonspecific hydrolysis and PHF is released while the succinamido linker undergoes cyclization forming a second prodrug, CPT-(O20)-succinimidoglycinate This second prodrug is then internalized and active CPT is released intracellularly [143]. XMT-1001 dosed at 45 mg/kg (5×q3d) was studied in athymic mice bearing HT-29 tumor xenografts and was found to have more antitumor effects and fewer toxicities as compared to free camptothecin at 22.5 mg/kg (5×q3d) [143]. A phase I study is currently underway in patients with solid tumors. Thus far, seven patients have been treated at three dose levels (highest dose level 3.1 mg CPT equivalents/m^2 once every 21 days) without any serious adverse events and the maximum tolerated dose has yet to be reached [130]. This supports the improved bioavailability and decreased toxicity associated with XMT-1001 and study accrual is ongoing.

Conjugates have also been developed to improve the oral bioavailability of drugs [5]. Phospholipid nucleoside conjugates and nucleosides with chemical additions to the amino moieties have been used since the 1970s to increase the biological activity of the parent compound. Synthetic phospholipid conjugates of cytosine arabinoside (ara-C) and gemcitabine have been developed [4, 6]. The novel ara-C conjugate has different systemic and cellular pharmacologic characteristics compared with the parent drug, such as decreased catabolism by cytidine deaminase, increased plasma half-life, penetrate the blood–brain barrier, and release of nucleoside monophosphate, a reaction that bypasses the rate limiting initial nucleoside phosphorylation [6]. In contrast to gemcitabine, the gemcitabine conjugate did not enter the cell via the human equilibrative nucleoside transporter (hENT1) and was not a substrate for the multidrug resistance efflux pump, MDR-1 [4]. These results suggest that the gemcitabine conjugate may be superior to gemcitabine due to the conjugate's ability to bypass three resistance mechanisms and can be administered orally. These phospholipid nucleoside conjugates posses the potential to have superior antineoplastic cytotoxicity profiles with less toxicity than the parent compound.

21.11 Summary, Conclusions, and Future Directions

Liposomes and nanoparticles may be effective carriers to deliver anticancer agents to tumors [7, 27, 35, 108, 111, 150]. However, for anticancer agents encapsulated or conjugated in liposomes or nanoparticles to be an effective treatment in patients with solid tumors, the active form of the anticancer agent must be released from the liposome into the tumor ECF or inside the cell [150]. As a result of this delivery process, new liposomal anticancer agents should be evaluated in preclinical models and early clinical trials to insure that adequate release of drug occurs at its site of action. It is unclear if drug conjugated to PEG or other carriers, or drugs encapsulated in microspheres or protein-stabilized nanoparticles must be released from the carrier to achieve cytotoxic effects. In addition, ligand labeled nanoparticles, such as dendrimers, may provide even greater tumor-selective delivery.

Future studies need to evaluate the mechanism of clearance of liposomes and nanoparticles and evaluate the factors associated with pharmacokinetic variability of these agents [30, 49, 79, 112, 142, 151]. The elimination of these agents may be similar to antibodies and proteins and most likely associated with the RES [7, 35]. In addition, it is currently unclear what is the most appropriate preclinical model for toxicity, efficacy, and pharmacokinetic studies [151, 152].

As more existing anticancer agents go off patent these agents will most likely be evaluated in some type of carrier-mediated formulation. Antiangiogenesis agents, antisense oligonucleotides, siRNA, and enzymes represent rational candidates for nanosomal and nanoparticle formulations. Anticancer agents that require continuous oral administration, such as tyrosine kinase inhibitors (e.g., imatinib, dasatinib, lapatinib, and sunitinib), may be candidates for nanoparticle carrier-formulations, which would provide prolonged drug exposure after administration of a single dose. Carrier-mediated anticancer agents represent a promising platform for the targeted delivery of antitumor therapy. This approach has the potential for improving therapeutic index and overcoming tumor resistance to traditional chemotherapy. However, further research is needed to optimize the formulation and clinical use of these agents.

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