

Redox Imbalance and Biochemical Changes in Cancer

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Abstract

For this article, we explore a hypothesis involving the possible role of reduction/oxidation (redox) state in cancer. We hypothesize that many modifications in cellular macromolecules, observed in cancer progression, may be caused by redox imbalance. Recent biochemical data suggest that human prostate cancer cell lines show a redox imbalance (oxidizing) compared with benign primary prostate epithelial cells; the degree of oxidation varied with aggressive behavior of each cell line. Our recent data suggest that human breast cancer tissues show a redox imbalance (reducing) compared with benign adjacent breast tissues. Accumulating data summarized in this article suggest that redox imbalance may regulate gene expression and alter protein stability by posttranslational modifications, in turn modulating existing cellular programs. Despite significant improvements in cancer therapeutics, resistance occurs, and redox imbalance may play a role in this process. Studies show that some cancer therapeutic agents increase generation of reactive oxygen/nitrogen species and antioxidant enzymes, which may alter total antioxidant capacity, cause cellular adaptation, and result in reduced effectiveness of treatment modalities. Approaches involving modulations of intra- and extracellular redox states, in combination with other therapies, may lead to new treatment options, especially for patients who are resistant to standard treatments. *Cancer Res*; 73(20); 6118–23. ©2013 AACR.

Introduction

Reduction/oxidation (redox) homeostasis is maintained by the net physiologic balance between reducing and oxidizing equivalents within subcellular compartments, with important components being reactive oxygen species and antioxidant enzymes (1). The free-radical theory of cancer traditionally encompassed the view that oxidative stress by reactive oxygen/nitrogen species (ROS/RNS) caused DNA damage and promoted genetic instability (1). However, ROS/RNS are now thought to be involved in both direct DNA damage and modulations of redox-regulated signaling pathways, which may be beneficial or detrimental in cancers. For example, data show that BCR/ABL oncogenes induce ROS, causing regulation of gene expression patterns of the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2), a major regulator of cellular redox homeostasis (2). Adaptations resulting from NRF2 activation may have beneficial effects under stress conditions through modulation of antioxidant pathways but may also contribute to cancer therapy resistance mechanisms (3). A recent study measured tissue redox activity in an animal model of neuroblastoma or glioma brain cancers using

nitroxide-enhanced MRI in living mice or electron paramagnetic resonance spectroscopy (EPRS) in excised organs (4). Brain tissues in mice with late-stage cancers differed in their reducing activity for the nitroxide radical measured by MRI or EPRS, tissue total antioxidant capacity, and plasma levels of matrix metalloproteinases (4), with combined results indicating that late-stage brain cancers were in an oxidized redox state. Furthermore, abundant data have shown that cancer cells showed altered epigenetic patterns relative to benign cells; epigenetic changes have been shown to be caused by redox imbalance in certain instances, resulting in alterations of gene expression patterns (5).

Several distinguishing hallmarks of cancer were originally proposed by Weinberg, with underlying genetic instability as a major feature of cancer (6). Currently, the role of changes in energy metabolism and interactions between tumor intra- and extracellular redox environments are being investigated. For example, as proposed by Warburg, tumor cells often exhibit increased aerobic glycolysis compared with benign cells. Recent data indicate that the mitochondrial antioxidant enzyme, manganese superoxide dismutase (MnSOD), may contribute to the Warburg effect by regulating redox flux and glucose consumption in transition from quiescence to proliferation in human breast cancer cells (7). In contrast, recently proposed as the reverse Warburg effect, tumor-associated stroma secrete energy-rich metabolites, which epithelial cancer cells take up and use in mitochondrial oxidative phosphorylation, promoting proliferation and tumor growth (8). Increased levels of glutamine and loss of Caveolin-1 (CAV-1) expression in human breast cancer-associated fibroblasts correlated with recurrence and poor patient prognosis, and, therefore, absence of CAV-1 may be a new biomarker for the reverse Warburg effect (8).

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While studies of genetic instability and metabolism were major milestones in our understanding of cancer, oxidative stress and redox imbalance have been suggested to be present in cancer tissues. Oxidative stress is often putatively identified by analysis of fluorescent dyes that are sensitive to ROS, whereas redox imbalance is usually measured by biochemical analysis of the stoichiometry of redox couples [NAD/NADH, NADP/NADPH, cysteine (Cys)/cystine (Cyss), glutathione (GSH)/glutathione disulfide, thioredoxin (TRX)/thioredoxin disulfide]. Unfortunately, analyses of ROS levels and/or redox imbalance do not provide mechanistic insights concerning cancer. What is missing is an understanding of how increased ROS and/or redox imbalance affect key macromolecules involved in cancer development and progression. Posttranslational modifications (PTM) involving redox-sensing Cys residues or addition/deletion of low-molecular-weight metabolites to macromolecules, induced by ROS and/or redox imbalance, need to be further investigated in cancer development and progression. Whereas PTMs regulated by redox are the subject of active investigation in cancer, the possible relationship of redox with other biochemical changes in cancer is less frequently studied. This article briefly summarizes links between redox imbalance and biochemical changes described in cancer.

Posttranslational Modifications

For this article, we focus on PTMs controlled by redox reactions, including the formation of intramolecular protein disulfide bonds between two closely spaced Cys residues, containing two paired sulfhydryl (-SH) groups, a reaction that may be reversed by reduction (9). Oxygen is required for cellular metabolism and ROS are produced as bioproducts (10). Physiologic (balanced) levels of oxidants and antioxidants are required for proper cellular functions, which are regulated by redox biochemistry and show alterations in cancers. Increased levels of oxidants for a required period of time may oxidize intramolecular closely spaced -SH groups to disulfides, potentially affecting protein function. For example, TRX is an antioxidant protein, which provides reducing equivalents to proteins involved in cancer, including ribonucleotide reductase and transcription factors. TRX may undergo reversible redox reactions involving Cys32 and Cys35 located within the active site, or other modifications involving Cys62, Cys69, and Cys73 outside of the active site (10). TRX with two -SH groups at Cys32, Cys35 and Cys62, Cys69 is enzymatically active, whereas protein with disulfides at these two positions is irreversibly inactivated. Oxidation in only the active-site cysteines is reversible by TRX reductase. Data in human prostate cell lines show increased TRX1 protein oxidation, with decreased enzyme activity in more invasive cancer cell lines (11). Studies showed increased TRX1 oxidation in human prostate cancer tissues with high Gleason scores in comparison with tissues with low Gleason scores (unpublished data). In contrast, recent preliminary studies in human breast cancer tissues using Western blot analysis of tissue lysates showed 1.5-fold increased levels of TRX1 in infiltrating ductal carcinomas compared with adjacent benign tissue (data not

shown). The reduced form of TRX1 was predominant over the oxidized, showing a 4.7-fold increase ($P \leq 0.04$; Fig. 1). Furthermore, TRX1 enzymatic activities were 2.1-fold increased (Fig. 2). Interestingly, these data suggest that while human prostate cancer cell lines may be in a state of redox imbalance, favoring an oxidizing environment and under oxidative stress, human breast cancer tissues may be in a state of imbalance, favoring a reducing environment and reductive stress. Further investigations in human breast tissues and mechanistic studies in a cell culture model of human breast cancer progression are in progress. It will be important to determine redox state in metastatic breast cancer tissues.

Redox state in extracellular spaces is largely regulated by the Cys/Cyss redox couple. Data in prostate cancer cell lines show that modulating the extracellular redox state by changing the concentration of Cys/Cyss in cell culture media altered prostate cancer invasiveness (12). It is likely that the Cys/Cyss redox couple regulates -SH to disulfide conversion in key proteins of the extracellular matrix and plasma membranes. Studies in head and neck squamous cell carcinoma cells suggest that activities of Cyss transporters control redox status and may be modulated to improve therapeutic efficacy (13). Studies have shown the importance of Cyss metabolism in extra- and intracellular interactions between stromal and chronic lymphocytic leukemia (CLL) cells, with modulations of redox state

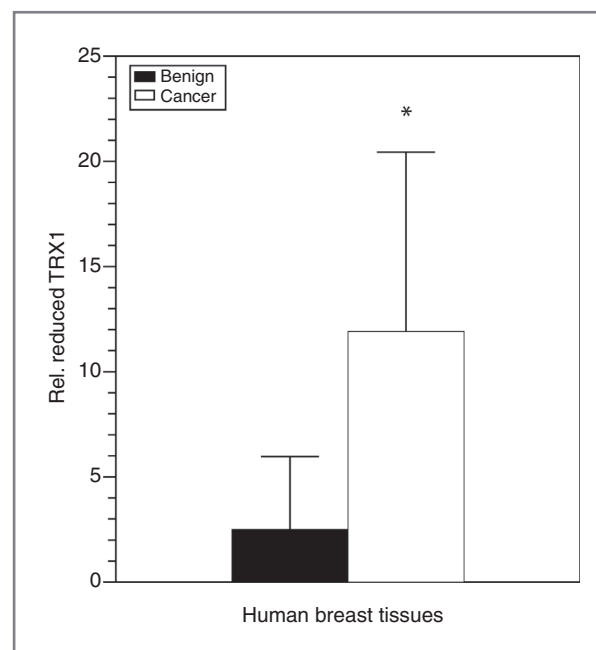


Figure 1. The reduced form of TRX1 was predominant in human breast cancer tissues. The redox state of TRX1 was expressed as the ratio of the reduced form of TRX1 to total TRX1. Cancer refers to infiltrating ductal carcinoma. Benign refers to the adjacent benign breast tissue. *, $P < 0.04$. Data from paired tissues obtained on biopsy or mastectomy were averaged from 2 patients from the University of Wisconsin School of Medicine and Public Health and 2 from Capital Biosciences. Redox Western blotting involves derivatization of proteins with a thiol-reactive reagent such as iodoacetic acid, followed by native gel electrophoresis and Western blotting.

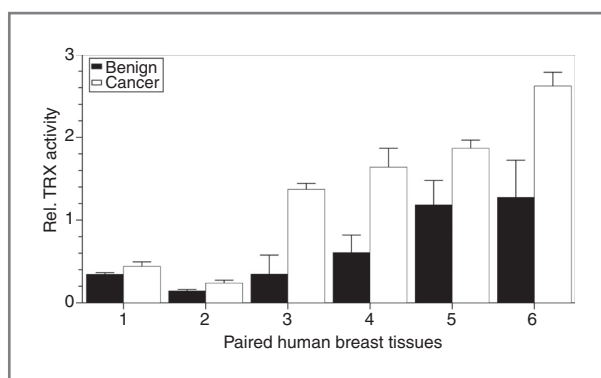


Figure 2. Human breast cancer tissues showed increased TRX activities. Data showed a 2.1-fold increased trend in TRX activities in infiltrating ductal carcinomas (cancer) versus benign/adjacent tissues (benign). Paired tissues obtained on biopsy/mastectomy from 6 patients were obtained from the University of Wisconsin School of Medicine and Public Health and Capital Biosciences. The insulin disulfide reduction assay was used to assess total TRX activity of 6 paired human tissues relative to GAPDH in triplicate. The assay measures the rate of which TRX (1×10^{-8} mol/L TRX) catalyzes reduction of insulin disulfides by dithiothreitol.

altering viability and drug sensitivity (14). Primary cells from patients with CLL expressed low levels of the active subunit of the Cyss/glutamate antiporter (xCT), resulting in limited ability to transport Cyss into the cell for GSH synthesis. Stromal bone marrow cells were shown to actively convert Cyss to Cys and release the molecule to the extracellular environment, which may then be taken up by CLL cells and result in

increased GSH (14). Increased GSH is thought to inhibit cell death and protect CLL cells from drug toxicity, a mechanism that could potentially be modulated as a therapeutic strategy to overcome resistance.

Several other important PTMs are being actively investigated in cancers. Examples of PTMs shown to be influenced by redox in cancer or under conditions causing oxidative/nitrosative stress are summarized in Table 1 and include phosphorylation (15), methylation (16), acetylation (17), SUMOylation (18, 19), nitrosylation (20, 21), nitration (22), and glutathionylation (23, 24). Potential important targets for PTMs in cancers include transcription factors, oncogenes, tumor suppressors, proteins involved in metabolism, and proteins controlling antioxidant/pro-oxidant balance. Further studies are needed to determine whether alterations of PTMs in cancers are a cause or consequence of changes in ROS, redox state, or redox imbalance. However, it has been shown that ataxia-telangiectasia mutated (ATM) protein kinase activation is regulated by ROS under oxidative stress (15). As mentioned above, BCR/ABL oncogenes have been shown to induce ROS, causing regulation of gene expression patterns of the transcription factor NRF2, a major regulator of cellular redox homeostasis (2).

Epigenetic and Transcriptional Changes

Epigenetics may be defined as heritable environmental or lifestyle dependent alterations to DNA or chromatin, acquired after birth (or *in utero*), without changes within the underlying

Table 1. Posttranslational modifications documented in cancer or oxidative/nitrosative stress

Modification	Addition	Cancer	Stress	Ref.
Phosphorylation	Phosphate to serine, threonine, tyrosine	ATM activation in response to double strand breaks; altered MnSOD (SOD2) activities	ATM activation under oxidative stress in absence of double strand breaks by oxidation of cysteine	15, 22
Methylation	Methyl to arginine, lysine	Altered MnSOD (SOD2) activities	Loss of MnSOD associated with chronic oxidative stress and aberrant epigenetic regulation	16, 22
Acetylation	Acetyl to lysine	Changes in HAT and HDAC activities modulate transcription; altered MnSOD (SOD2) activities	Under caloric restriction and resveratrol, SIRT1 and AMPK synergize to establish physiologic conditions; dysregulated MnSOD (SOD2) activities with oxidative stress	17, 22
Sumoylation	SUMO to lysine	eEF2 and SUMO-1 overexpression correlated with cisplatin resistance	Global effects involving inhibition of SUMO conjugating enzymes by oxidation of cysteine	18, 19
Nitrosylation	Nitric oxide to cysteine	Inflammatory mediators inhibit BRCA1	NRF2 activation by oxidation of cysteines within KEAP1	20, 21
Nitration	Nitric oxide to tyrosine	Altered levels of nitrated nucleotides, fatty acids, and proteins	Nitration of MnSOD results in loss of activity	22
Glutathionylation	Glutathione to cysteine	GST overexpression associated with drug resistance	Protection from irreversible oxidation	23, 24

Abbreviations: GST, glutathione S-transferase; HDAC, histone deacetylase.

DNA sequence; epigenetic changes may involve the reversible addition of methyl groups to DNA (methylation) or reversible PTMs involving addition of acetyl and/or methyl groups to histone proteins (acetylation/methylation). These reactions are catalyzed by DNA-histone acetyltransferases or methyltransferases (6). In addition, microRNAs bind to the 5' or 3' untranslated regions (UTR) of target mRNAs, which in turn modulate gene expression. Several effects on specific genes or subsets of genes have been characterized as being regulated by microRNA modifiers, including roles in cancer progression (6). For example, recent studies showed that repression of miR-200a in breast cancer cells, which targets Kelch-like ECH-associated protein 1 (Keap1) mRNA, subsequently leads to its degradation and contributes to dysregulation of NRF2 (25). The Keap1 levels were restored upon epigenetic treatment with a histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA). Recent literature suggests that epigenetic changes may be driven by redox imbalance through metabolic reprogramming of the existing cellular epigenetic program (5). Transcription may be modulated by redox imbalance through epigenetic mechanisms, which are linked to metabolism through variations of required intermediates, such as NAD⁺. For example, modulations of cellular processes may occur in any instance in which there is limited availability of enzymes required for modifications within subcellular compartments, such as class III histone deacetylases (HDAC), which are dependent on NAD⁺ [commonly referred to as sirtuins (SIRT); ref. 17]. Therefore, any alteration that depletes cellular NAD⁺ will affect the activity of SIRTs. In addition, xanthine oxidoreductase activity is increased in human serum obtained from patients with leukemia compared with healthy controls and is further increased in relapse (2). These data suggest a role for the xanthine oxidoreductase system in disease recurrence/resistance, of note because this system is known to use the cofactor NAD⁺ in drug metabolism with concomitant increased generation of ROS (2).

It has been suggested that an increased reducing environment provides conditions necessary for the optimal rate of electron transfer and enzymatic activity required for transcription factor binding to DNA. Transcription occurs within the nucleus of a cell and is a process by which nucleic acid sequences of DNA are copied to RNA (6). Transcription is dependent upon structural components and regulatory sequences located internally within the complete nucleic acid sequence. Splicing occurs concurrently or afterwards and is a process that removes introns and joins exons to produce mRNA, required for proper protein synthesis (6). Mutations in coding regions, gene promoters, and consensus binding sites are involved in regulation of gene expression, whereas modifications in splicing may affect protein function. Epigenetic modifications to DNA, histone modifications, and microRNA modifiers may modulate gene expression patterns (6).

Transcriptional regulation often involves redox-sensing Cys residues located within the region required for DNA binding of redox-regulated transcription factors. The promoter regions of redox-regulated genes contain an antioxidant responsive element (ARE), a cyclic AMP responsive element (CRE), and SP-1 sites required for activation of redox-regulated transcription

factors such as AP-1, NF- κ B, NRF2, tumor protein 53 (TP53), glucocorticoid, and other hormone receptors, among others (3, 10). The presence of AREs in so many transcription factors provides strong evidence that redox imbalance is a major factor in regulating cellular adaptation to redox injury. Reduced forms of TRX are active enzymatically, whereas partially oxidized forms are inactive and must be reduced by TRX reductases for activation (10). Therefore, modulations of nuclear compartment redox state may alter activity of transcription factors that are reduced by TRX and ultimately gene expression patterns. Interestingly, recent data suggest that the redox state of human TRX1 may be changed by treatment with the HDAC inhibitor SAHA in cell culture, and this compound is currently in clinical trials for treatment of acute myeloid leukemia (26).

Several examples document complex genetic and epigenetic influences in cancer progression. For example, mutational status of VHL in renal cell carcinoma was not correlated with clinical outcome, although alterations in DNA methylation were associated with metastasis (27). Monozygotic and dizygotic twin studies estimate the relative importance of complex genetic and epigenetic factors influencing disease phenotypes (28). One study comparing monozygotic twins revealed increased promoter methylation at specific CpG sites with no DNA mutations of the disease-associated genes (28). Another study showed that monozygotic twins, discordant for childhood leukemia (previously considered only a genetic disease), showed increased BRCA promoter methylation in somatic cells (fibroblasts of saliva) of the female diagnosed with childhood leukemia (12%), compared with her healthy sister (3%; ref. 29). Interestingly, mutations in IDH1 and IDH2 result in complex genetic and epigenetic cellular and biochemical changes such as increased hematopoietic stem cell progenitors, alterations in cell differentiation, and changes in DNA methylation and metabolism (30). Thus, single mutations affecting cell biochemistry can have complex effects on cell behavior, epigenetic changes, and metabolism.

Protein Stability

Changes in protein stability within subcellular compartments may influence protein levels and function. Protein stability is regulated by changes in protein folding/unfolding, conformational changes, hydrophobic interactions, hydrogen bonding, redox potential, pH, temperature, and metal binding (6). Oxidizing conditions favor unfolding of proteins and stabilization, with disulfide bond formation through oxidation of two Cys residues forming covalent sulfur-sulfur bonds (9). Reducing conditions favor highly reactive free -SH groups (9). Subcellular oxygenation levels and cellular responses to oxidative stress are important factors regulating protein stability, which are altered in cancers. For example, dysregulation of the transcription factors hypoxia-inducible factor α (HIF-1 α) and NRF2 have been identified in several cancers, and therapeutic approaches involving these transcription factors are being investigated (3, 6). Under normoxic conditions, HIF-1 α is ubiquitinated and subsequently degraded. However, under hypoxic conditions, it may be stabilized and may associate with the

VHL protein, which in turn activates HIF-1 α -regulated genes (6). Similar to HIF-1 α , under normoxic cellular conditions, NRF2 is ubiquitinated and subsequently degraded, potentially regulated by biochemical reactions involving Cys residues as redox sensors within the scaffold protein Keap1 (3). In response to oxidative/nitrosative stresses, NRF2 is stabilized, with subsequent activation of genes responsible for stimulating antioxidant genes and cytoprotective enzymes (3). Modulations to NRF2 compartmentalization and trafficking also occur, which play an important role in mediating cross-talk with other molecular signaling pathways, including NF- κ B, aryl hydrocarbon receptor, TP53, and notch homolog 1 (3). Importantly, high levels of NRF2 have been identified in several cancers, which result in constitutive activation of NRF2 and redox-regulated adaptations. The adaptations protect cells from cytotoxic effects of anticancer or chemoprevention compounds and cause resistance to chemo- and radiotherapy; therefore, controversy exists whether therapeutic approaches may be useful for activating or inhibiting NRF2 (3).

Conclusions and Discussion

It is undisputed that genetic and epigenetic factors influence cancer initiation and progression. It has been shown that oxidation may cause mutations and promote genetic instability. Despite an initial belief that rapid sequencing of DNA from cancer families and further extensive knowledge about the biochemistry of cancer would, perhaps, result in cancer cures, metastatic cancer remains a formidable challenge today. Two mechanistic reasons for failure are genomic instability and development of resistance to therapeutic agents. While the latter feature of the metastatic cancer phenotype may be related to genomic instability, it may also be caused by the other biochemical changes of cancer described in this article. Treatment failure may be due to a lack of understanding of an underlying feature that drives all of the biochemical changes described in cancer progression. This article provides beginning evidence that sequential changes in redox balance drive cancer development and progression. To prove this hypothesis, combined analyses of already described biochemical changes and biochemical and biomarker studies of redox imbalance need to be carried out in human benign and cancer tissues of varying degrees of progression. Subsequently, animal models will need to be developed in which key proteins regulating redox balance are mutated at active sites or overexpressed to determine effects on cancer formation.

Studies show that some cancer therapeutic agents increase generation of ROS/RNS and antioxidant enzymes, which may alter total antioxidant capacity and cause cellular adaptations. Adaptations resulting from HIF-1 α and NRF2 pathway activation may have beneficial effects through modulating anti-

oxidant pathways under stress conditions but may also contribute to cancer therapy resistance. Accumulating data show that oxidative stress and redox imbalance cause epigenetic changes. Regulation of gene expression is influenced by several factors, including posttranslational modifications and microRNA, which may in turn modulate existing cellular programs. Recent data suggest that cancers may be in a state of redox imbalance, which may increase with progression. Preliminary studies in our laboratory suggest that redox imbalance may be either oxidizing (prostate) or reducing (one pathologic type of breast cancer before metastasis). Many modifications in cellular macromolecules observed in cancer progression may be caused by redox imbalance. Proteomic studies are needed to identify protein modifications in cancer progression and will determine whether redox imbalance precedes or follows these modifications, or both, in a vicious cycle. Perhaps future cancer diagnostics may include an analysis of redox state with progression or provide a new method for monitoring response to therapy. New treatment options may involve modulating intra- or extracellular redox state of cancer cells at varying stages of progression, which may involve metabolic reprogramming. New clinical trials might involve attempts at modulating redox imbalance in cancers resistant to antihormonal treatments, radiation, or standard chemotherapy or sensitizing resistant cancers to drug treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed by the authors.

Authors' Contributions

Conception and design: T.C. Jorgenson, W. Zhong, T.D. Oberley
Acquisition of data (acquired and managed patients, provided facilities, etc.): T.D. Oberley
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.C. Jorgenson, W. Zhong, T.D. Oberley
Writing, review, and/or revision of the manuscript: T.C. Jorgenson, W. Zhong, T.D. Oberley
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.D. Oberley
Study supervision: W. Zhong, T.D. Oberley

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