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Original Contributions

Increasing discordant antioxidant protein levels and enzymatic activities contribute to increasing redox imbalance observed during human prostate cancer progression

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ABSTRACT

A metabolomics study demonstrated a decrease in glutathione and an increase in cysteine (Cys) levels in human prostate cancer (PCa) tissues as Gleason scores increased, indicating redox imbalance with PCa progression. These results were extended in the present study by analyzing the redox state of the protein thioredoxin 1 (Trx1) and sulfinylation (SO_3) of peroxiredoxins (Prxs) ($PrxSO_3$) in PCa tissues and cell lines. Lysates of paired human PCa tissues with varying degrees of aggressiveness and adjacent benign (BN) tissues were used for analysis. Redox Western blot analysis of Trx1 demonstrated low levels of reduced and high levels of oxidized Trx1 (functional and nonfunctional, respectively) in high-grade PCa (Gleason scores 4+4 to 4+5) in comparison to intermediate-grade PCa (Gleason scores 3+3 to 3+4) or BN tissues. $PrxSO_3$ were increased in high-grade PCa. Oxidized Trx1 and $PrxSO_3$ are indicators of oxidative stress. To study whether redox imbalance may potentially affect enzyme activities of antioxidant proteins (APs), we determined the levels of selected APs in PCa tissues by Western blot analysis and found that mitochondrial manganese superoxide dismutase (MnSOD), Prx3, and Trx1 were increased in high-grade PCa tissues compared with BN tissues. Enzyme activities of MnSOD in high-grade PCa tissues were significantly increased but at a lower magnitude compared with the levels of MnSOD protein (0.5-fold vs 2-fold increase). Trx1 activity was not changed in high-grade PCa tissues despite a large increase in Trx1 protein expression. Further studies demonstrated a significant increase in posttranslational modifications of tyrosine and lysine residues in MnSOD protein and oxidation of Cys at the active site (Cys32 and Cys35) and the regulatory site (Cys62 and Cys69) of Trx1 in high-grade PCa compared to BN tissues. These discordant changes between protein levels and enzyme activities are consistent with protein inactivation by redox imbalance and/or posttranslational modifications. In contrast, the protein level and activity of extracellular superoxide dismutase were significantly decreased in high-grade PCa compared with adjacent BN tissues. Results from cell lines mirror those from PCa tissues. Knowledge of redox-state profiles in specific cancers may help to predict the behavior and response of each cancer to chemotherapeutic drugs and radiation.

Alteration of redox status is an important feature of carcinogenesis and has been exploited therapeutically in the treatment of cancer. For example, cancer cells are frequently under oxidative stress conditions as demonstrated by higher levels of intracellular reactive oxygen species (ROS) and increased hydrogen peroxide (H_2O_2) release, as well as being susceptible to pro-oxidant-induced cell death [1–3]. Radiation and many other therapeutic agents such as doxorubicin or mitomycin C may bring about their cancer killing effects by increased ROS production, pushing already stressed cancer cells beyond their limits of tolerance [4,5]. On the other hand, upregulation of antioxidant enzymes has been frequently detected in cancers and has been suggested to confer resistance of cancer cells to ROS-generating therapeutic agents [6]. These contradictory results emphasize the importance of defining the redox state of each type of cancer.

Prostate cancer (PCa) is the most common cancer and second leading cause of cancer deaths of men in the United States. Previous studies in our laboratory have demonstrated an increase in oxidative/nitrative damage in human PCa tissues and cells [7,8], implicating a role of oxidative stress in cancer development. In addition, we demonstrated that intra- and extracellular redox states were altered in aggressive PCa cells [9,10] and subsequently regulated PCa cell

Abbreviations: 4HNE, 4-hydroxy-2-nonenal; 8OHdG, 8-hydroxy-2′-doxyguanosine; AP, antioxidant protein; DTT, dithiothreitol; ECSOD, extracellular superoxide dismutase; IAA, iodoacetic acid; MnSOD, manganese superoxide dismutase; MMP, matrix metalloproteinase; Nrf2, nuclear factor-erythroid 2-related factor 2; PCa, prostate cancer; Prx, peroxiredoxin; $PrxSO_3$, sulfinylated peroxiredoxin(s); redox, reduction/oxidation; ROS/RNS, reactive oxygen species/reactive nitrogen species; Trx1, thioredoxin 1; TR1, thioredoxin reductase 1; TXNIP, thioredoxin-interacting protein

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behavior [11]. It is increasingly recognized that subcellular redox systems have distinct functions and are subject to independent regulation [12]. The redox status of proteins involved in the transmission of redox signals in distinct suborganelles, such as thioredoxin 1 (Trx1), peroxiredoxin(s) (Prx), manganese superoxide dismutase (MnSOD), or extracellular superoxide dismutase (ECSOD), has not been previously studied in human PCa tissues.

Trx1 is a redox-modulating protein, providing reducing equivalents to numerous redox-sensitive proteins, such as ribonucleotide reductase [13] and redox-related transcriptional factors [6]. Trx1 contains five cysteine residues and reduces target proteins using its two vicinal cysteine (Cys) residues (Cys32 and Cys35) in the active site, where the molecule itself becomes oxidized, forming an intramolecular disulfide bond. Further oxidation of Trx1 results in the formation of a second intramolecular disulfide bond between Cys62 and Cys69 and then an intermolecular disulfide bond between Cys73 of two different Trx1 molecules [14]. Our previous study using PCa cell lines suggested that the Trx1 redox state may function as a biomarker of redox imbalance and play an important role in modulating the response of PCa cells to pro-oxidant treatments [15]. One of the Trx1-target proteins is the Prx family. Trx(s) reduce Prx(s) by transferring disulfide bonds to the Cys pair, and without Trx activity, Prx(s) will mainly be in the oxidized state [16]. Sulfinylation of peroxiredoxin ($PrxSO_3$ or hyperoxidized Prx) is one of oxidative stress indicators. Thus, Trx1 and $PrxSO_3$ are important cellular-redox-sensitive proteins.

MnSOD and ECSOD convert superoxide radical ($O_2^{\bullet -}$) to H_2O_2 and can have both antioxidant (reduction of $O_2^{\bullet -}$) and pro-oxidant (production of H_2O_2) effects. MnSOD is located in mitochondria and ECSOD is found in cell membranes/extracellular spaces. The ultimate effects of SODs on redox state depend on the cell milieu, environmental factors, reducing equivalents, and/or the balance of other antioxidant proteins (APs) inside/outside of the cells. Recent studies in our laboratory demonstrated that modulation of MnSOD inhibited PCa growth, possibly via H_2O_2 production [17], whereas modulation of extracellular redox state by overexpression of ECSOD resulted in inhibition of prostate cell invasion, possibly by inhibition of matrix metalloproteinase (MMP) and membrane type 1 (MT1)-MMP enzymatic activities [18].

Whereas many studies have focused on oxidative stress-mediated gene mutation, we hypothesize that redox imbalance-mediated alterations in protein function, specifically synthesis of inactive proteins or posttranslational modifications, may contribute to cancer progression. In the present study, Trx1, Prx(s), MnSOD, and ECSOD were analyzed using two systems: human PCa tissues with varying degrees of progression compared to their adjacent benign prostate tissues (BN) and normal prostate epithelial cells (PrEC) compared with androgen-responsive LNCaP and LNCaP-C4-2B and/or androgen-independent PC3 and PC3M prostate cancer cell lines.

Our results from both human PCa tissues and cell lines consistently demonstrated that redox imbalance-mediated posttranslational modifications are more extreme in highly aggressive PCa than in less aggressive cancer as demonstrated by oxidation of Trx1, sulfinylation of Prx(s), and nitration and methylation of MnSOD. These significant differences in cancer redox states provide new insights into the possible usefulness or dangers of pro-oxidant- or antioxidant-related cancer therapeutic drugs and radiation therapy.

Materials and methods

Chemicals and antibodies

Microspin G-25 columns were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Human purified Trx proteins, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-Prxs 1 to 4, anti-$PrxSO_3$, anti-Trx1, and anti-thioredoxin reductase 1 (TR1) antibodies were purchased from AbFrontier Co., Ltd (Geumcheon-gu, Seoul, Korea). Anti-thioredoxin-interacting protein (TXNIP) antibody was purchased from Invitrogen Corp. (Camarillo, CA, USA). Anti-CuZnSOD, anti-MnSOD, and anti-ECSOD antibodies were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Anti-3-nitrotyrosine antibody was purchased from EMD-Millipore (Darmstadt, Germany). Anti-methylated lysine antibody was purchased from Abcam (Cambridge, MA, USA). Anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). IRDye 800CW and 700CW goat anti-rabbit IgG antibodies were purchased from Li-Cor Biosciences (Lincoln, NE, USA). All other chemicals, antibodies, and reagents were purchased from Sigma Chemical Co., unless otherwise specified.

Human cancer tissue specimens

Paired human PCa and adjacent BN were purchased from the University of Wisconsin tissue bank (Madison, WI, USA) with approval of the University of Wisconsin Institutional Review Board (IRB). Both human PCa and BN tissues were composed of epithelial and stromal cells. Specimens were immediately frozen in liquid nitrogen and then stored at −80 °C for further use. Twenty-two paired human PCa tissues were included in the studies. Human prostate tissues in different stages of progression, Gleason scores 4+4 to 4+5 (10 pairs) and 3+3 to 3+4 (12 pairs), were used. The Gleason score represents the degree of deviation from normal behavior in tumors. Gleason scores 3+3 to 3+4 are intermediate PCa with a primary Gleason pattern 3 and a secondary Gleason pattern 3 or 4. Gleason scores 4+4 to 4+5 are high-grade PCa of a primary Gleason pattern 4 and a secondary Gleason pattern 4 or 5, with high metastatic potential. Cancer scoring was provided by board-certified pathologists. Details of each sample used in the experiment are shown in Supplementary Table 1.

Prostate cancer cells

LNCaP, PC3, and WPMY-1 cell lines were obtained from the ATCC (Manassas, VA, USA). LNCaP and PC3 cells were tested and confirmed for authenticity using short-tandem-repeat DNA typing by Biosynthesis Cell (Lewisville, TX, USA). LNCaP-C4-2B (C4-2B) was obtained from ViroMed Laboratories (Minnetonka, MN, USA). Benign prostate epithelial cells (PrEC) were obtained from Lifeline Cell Technology (Walkersville, MD, USA). PC3M cells were obtained from Dr. Ajit Verma (University of Wisconsin at Madison). LNCaP, C4-2B, PC3, PC3M, and WPMY-1 cells were cultured in RPMI 1640 medium supplemented with 5% serum (Hyclone Laboratories, Logan, UT, USA) and 100 mg/L kanamycin sulfate. PrEC was cultured in prostate epithelial growth medium (Lifeline Cell Technology). Supplies and reagents for cell cultures were purchased from BD Falcon (San Jose, CA, USA) and Thermo Fisher Scientific (Waltham, MA, USA), unless otherwise specified.

Western blotting and redox Western blotting analysis

Cell pellets were lysed with M-PER lysis buffer containing protease inhibitor cocktails (Pierce Biotechnology, Rockford, IL, USA) for 15 min on ice. Tissue specimens were cut into small pieces and then lysed with T-PER tissue protein extraction reagent containing protease inhibitor cocktails (Pierce Biotechnology) using a pellet pestle to homogenize tissues (Kimble Kontes, Vineland, NJ, USA). Crude supernatants were collected after centrifugation at 10,000 rpm, 4 °C, for 10 min. Protein concentrations were determined using the Bradford assay (Bio-Rad, Philadelphia, PA, USA). Equal amounts of proteins were loaded and electrophoresed in SDS–polyacrylamide gels and then transferred onto nitrocellulose