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Original Contribution

# Thioredoxin 1 as a subcellular biomarker of redox imbalance in human prostate cancer progression

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ABSTRACT

We determined protein levels and subcellular distribution of thioredoxin 1 (Trx1) in human prostate tissues using tissue microarrays and analyzed redox changes in Trx1 in the nucleus and cytoplasm in cell culture models with a redox Western blot technique. We demonstrated increased nuclear Trx1 levels in high- versus low-grade human prostate cancers. Despite increased protein levels, the oxidized forms of nuclear Trx1 were higher in prostate cancer cell lines compared to their benign counterparts, suggesting that nuclear redox imbalance occurred selectively in cancer cells. A growth-stimulating dose of androgen caused transient oxidation of Trx1 in androgen-responsive prostate cancer cells only, suggesting a loss of both androgen- and redox-signaling mechanisms during cancer progression. Androgen-independent PC3 cells showed a significant increase in nuclear and cytoplasmic Trx1 protein levels, but a significant decrease in total Trx activity. Trx1 redox state and activity correlated with the sensitivity of prostate cancer cells to pro-oxidant agents, and downregulation of Trx1 sensitized cancer cells to these agents. Our findings suggest that loss of Trx function because of oxidation and corresponding redox imbalance may play important roles in prostate cancer progression and response to therapies; and Trx1 may serve as a biomarker of subcellular redox imbalance in prostate cancer.

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Thioredoxin 1 (Trx1) is a redox-sensitive molecule that can be redox-modified during redox signaling or in response to cellular redox changes [1–3]. Trx1 redox modifications involve several steps. Initial oxidation of Trx1 results in the formation of an intramolecular disulfide bond between cysteines 32 and 35 in the active site. Trx1 has three additional cysteines: Cys62, Cys69, and Cys73. Further oxidation of Trx1 leads to the formation of a second intramolecular disulfide bond (S–S) between Cys62 and Cys69 and then to an intermolecular disulfide bond between Cys73 of two different Trx1 molecules, which ultimately leads to the formation of a dimer [3]. Trx1 in which all cysteine residues are oxidized is biochemically inactive. The redox state of Trx1 can be determined by the redox Western blot assay measuring the redox state of two pairs of cysteine residues (Cys32, Cys35 and Cys62, Cys69), with the possible results being all four cysteine residues reduced, one pair of cysteine residues oxidized in the active site (Cys32, Cys35) to form a disulfide bond, or oxidation of all four cysteine residues to form two pairs of disulfide bonds [4]. Analysis of Trx1 by the redox Western blot thus provides two important pieces of information: (i) the cellular redox status and (ii) the activity status of Trx1.

Although there are many redox couples in cells, such as NADH/NAD, glutathione/glutathione disulfide, and cysteine/cystine [5], Trx1 is unique because it has a specific role in modulation of redox signaling [4]. Trx1 also has distinct nuclear and cytoplasmic pools, each performing different functions. In the nucleus, Trx1 has been shown to interact with transcription factors such as p53, nuclear factor κB (NF-κB) and nuclear factor-like 2 (Nrf2), to regulate their binding to DNA [4,6–8]. In the cytoplasm, Trx1 can regulate apoptotic signal-regulating kinases [4,9]. Trx1 is also known to move from the cytoplasm to the nucleus in response to oxidative stress [4]. Selective oxidation of Trx1 can occur and has been detected in both the nucleus and the cytoplasm in response to cellular redox changes or during redox signaling in certain cell types [1,2,10,11]. However, subcellular redox changes in Trx1 in prostate cancer cells have not been reported to date.

*Abbreviations:* AR, androgen receptor; BCNU, carmustine; BSO, L-buthionine sulfoximine; CDCFDA, 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate; $H_2$DCFDA, dichlorofluorescein diacetate; IAA, iodoacetic acid; MnSOD, manganese superoxide dismutase; NF-κB, nuclear factor κB; Nrf2, nuclear factor-like 2; PrEC, benign prostate epithelial cells; PSA, prostate-specific antigen; ROS, reactive oxygen species; Trx, thioredoxin; TrxR, thioredoxin reductase; TXNIP, thioredoxin interacting protein.

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Increased Trx1 protein expression has been detected in multiple cancer tissues and cancer cell lines, and an increase in Trx1 expression was associated with higher tumor grade and has been implicated in the resistance of tumor cells to certain chemotherapies and ROS-generating agents, including doxorubicin, mitomycin C, etoposide, and ultraviolet radiation [10]. It has been suggested that Trx1 functions as a protective cellular antioxidant and its upregulation protects cancer cells from oxidative stress [10]. However, most of these studies mainly focus on Trx1 protein expression levels, with little attention being given to its subcellular redox states, a critical factor in understanding cancer cell biology and the functional implications of the role of Trx1 in cancer.

Prostate cancer tissues are in a state of redox imbalance, and the Trx/thioredoxin reductase (TrxR) system is frequently upregulated in prostate cancers [10,12]. To better understand the significance of Trx1 upregulation in prostate cancer, we utilized human tissue microarrays to determine protein levels and subcellular distribution of Trx1 and tissue culture models representing prostate cancer progression with the newly developed redox Western blot technique to analyze redox changes in Trx1 in the nucleus and cytoplasm during prostate cancer progression.

## Materials and methods

### *Chemicals and antibodies*

Methyltrienolone (R1881; a synthetic androgen) was purchased from PerkinElmer (Waltham, MA, USA). Microspin G-25 columns were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Dichlorofluorescein diacetate ($H_2$DCFDA) and 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDCFDA) were purchased from Molecular Probes (Eugene, OR, USA). Vectastain Universal Elite ABC kit was purchased from Vector Laboratories (Burlingame, CA, USA). Immunopure metal-enhanced DAB substrate kit was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Silencer Select Validated Trx1 small interfering RNA (siRNA; sequence 5′–3′: UGACUUCACACUCUGAAGCAA) was purchased from Ambion (Austin, TX, USA). This siRNA is commercial and has been experimentally validated by the company to reduce the expression of the target gene by 80%. Anti-Trx1 antibodies were purchased from AbFrontier Co. Ltd. (Geumcheon-gu, Seoul, Korea). IRDye 800CW goat anti-rabbit IgG was purchased from Li-COR Biosciences (Lincoln, NE, USA). Fetal bovine serum (FBS) was purchased from Tissue Culture Biologicals (Los Alamitos, CA, USA). Charcoal/dextran-treated FBS was purchased from Hyclone (Logan, UT, USA). All other chemicals and reagents were purchased from Sigma Chemical Co., unless otherwise specified.

### *Cell culture and treatment*

Benign human prostate epithelial cells (PrEC) were purchased from Lonza Walkersville (Walkersville, MA, USA). LNCaP (ATCC CRL-1740) and PC3 (ATCC CRL-1435) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Recent cultures of PC3 and LNCaP cells were analyzed using single-locus short-tandem-repeat DNA typing by Biosynthesis (Lewisville, TX, USA) to authenticate cell identity.

No contamination or misidentification was detected (data not shown). C4-2B cells were purchased from ViroMed Laboratories (Minnetonka, MN, USA). PrEC were grown in serum-free prostate epithelial growth medium (PrEGM; Lonza/Cambrex). LNCaP, C4-2B, and PC3 cells were maintained in RPMI 1640 supplemented with 5% FBS and 1% antibiotic–antimycotic (Life Technologies, Rockville, MD, USA) at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$, and cells grown for fewer than 30 passages were used. In experiments designed to assess the effects of androgen treatment, cells were seeded in androgen-depleted medium containing 5% charcoal/dextran-treated FBS and 1% antibiotic–antimycotic for 24 h before R1881 treatment. R1881 concentration used in this study was 1 nM, an optimal dose stimulating growth of LNCaP cells. Vehicle was 0.001% ethanol.

### *Tissue microarray construction and immunohistochemistry*

Paraffin-embedded tissue blocks of prostatectomies from 41 patients were obtained from the Department of Pathology and Laboratory Medicine, University of Wisconsin School of Medicine and Public Health. Approval for use of human prostate tissue was obtained from the University of Wisconsin Institutional Review Board. One-millimeter tissue cores containing cancerous tissues and/or adjacent benign epithelial tissues were used for construction of tissue microarrays with three to nine cores from each patient depending on the heterogeneity of each cancer. Five-micrometer paraffin sections on glass slides were deparaffinized in a 60 °C oven for 1 h and then rinsed in three changes of xylene for 10 min each. Slides were blocked for endogenous peroxidases with 0.3% methanol/hydrogen peroxide for 20 min and then rehydrated in 1-min changes each of 100, 95, 75, and 50% ethanol and then double-distilled $H_2O$. Heat-induced epitope retrieval was performed using a digital decloaking chamber (BioCare Medical, Concord, CA, USA) for 30 s at 120 °C in Tris urea buffer, pH 9.5. Slides were then blocked in 2.5% normal horse serum for 20 min at room temperature in a humidified chamber followed by incubation with primary antibodies (Trx1 1:400) overnight at 4 °C in a humidified chamber. The slides were then rinsed in three changes of 0.5 M Tris-buffered saline and incubated with the biotinylated universal secondary antibody (Vector Laboratories) for 30 min, followed by Immunopure metal-enhanced DAB substrate for 3 min. After being rinsed in distilled $H_2O$, the slides were counterstained with hematoxylin, dehydrated, and mounted with coverslips followed by microscopic analysis with digital image capture.

### *Western blot analysis*

Cell pellets were lysed with M-PER mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA), and protein concentrations were determined using the Bradford assay (Bio-Rad, Philadelphia, PA, USA). Equal amounts of proteins were electrophoresed in 8–15% SDS–polyacrylamide gels and then transferred onto nitrocellulose membranes. After incubation with primary antibodies overnight at 4 °C, immunoreactive proteins were detected with secondary antibodies and visualized on X-ray film.

### *Subcellular fractionation*

Nuclear and cytoplasmic fractions were prepared from cells as previously described [1,2]. Briefly, cells were washed once with ice-cold PBS and then were collected in 10 mM Hepes (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM NaF, and 0.2 mM $Na_3VO_4$ $6H_2O$ with protease inhibitors and 50 mM iodoacetic acid (IAA). Cell suspensions were then incubated on ice for 5 min, and NP-40 was added to a final concentration of 0.6%. After centrifugation at 12,000 *g* for 5 min, the nuclei were pelleted, and the supernatants were retained as the cytoplasmic fraction.

### *Redox Western blotting*

Analysis of the redox forms of Trx1 was performed as described previously [1,2,13]. The cytoplasmic and nuclear fractions were used to analyze redox forms of Trx1. After derivatization with 50 mM IAA, excess IAA was removed using microspin G-25 columns. Trx1 redox isoforms were separated by native polyacrylamide gels. For cytoplasmic Trx1, horseradish peroxidase-conjugated anti-rabbit secondary