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Original Contribution

Regulation of prostate cancer cell invasion by modulation of extra- and intracellular redox balance

Luksana Chaiswing [a], Weixiong Zhong [a,b], Yongliang Liang [c], Dean P. Jones [c], Terry D. Oberley [a,b,*]

[a] Department of Pathology and Laboratory Medicine, University of Wisconsin School of Medicine and Public Health, Madison, WI 53706, USA
[b] Pathology and Laboratory Medicine Service, William S. Middleton Memorial Veterans Hospital, Madison, WI 53705, USA
[c] Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, Emory University, Atlanta, GA 30322, USA

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ABSTRACT

Recent metabolic profiles of human prostate cancer tissues showed a significant increase in cysteine (Cys) and a significant decrease in reduced glutathione (GSH) during cancer progression from low- to high-grade Gleason scores. Cys is primarily localized extracellularly, whereas GSH is present mostly inside the cell. We hypothesized that extra- or intracellular redox state alterations differentially regulate cell invasion in PC3 prostate carcinoma cells versus PrEC normal prostate epithelial cells. Cells were exposed to media with calculated Cys/CySS redox potentials (E_hCySS) ranging from −60 to −180 mV. After 3 h exposure to a reducing extracellular redox state (E_hCySS = −180 mV), matrix metalloprotease (MMP), gelatinase, and NADPH oxidase activities increased, correlating with increases in cell invasion, cell migration, and extracellular hydrogen peroxide levels in PC3 cells but not PrECs. Knockdown of NADPH oxidase or MMP with silencing RNAs during cultivation with E_hCySS = −180 mV medium significantly decreased PC3 cell invasion. Modulation of extra- and intracellular redox states by exposure of PC3 cells to Cys/CySS-free medium (approx E_hCySS = −87 mV) containing 500 μM *N*-acetylcysteine resulted in a more reducing intracellular redox state and a significant decrease in cell invasive ability. The decrease in PC3 cell invasion induced by these conditions correlated with a decrease in MMP activity. Our studies demonstrated that an extracellular redox state that was more reducing than a physiologic microenvironment redox state increased PC3 cancer cell invasive ability, whereas an intracellular redox environmental that was more reducing than an intracellular physiologic redox state inhibited PC3 cell invasive ability.

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Intracellular and extracellular redox states are the result of the net balance of reducing and oxidizing equivalents inside and outside the cell, respectively. Additionally, each subcellular compartment of each distinct cell type has a unique redox state designed to allow optimal physiological functioning. Significant changes in redox state in specific subcellular compartments and/or extracellular spaces result in cell adaptation and/or cell dysfunction. It has been demonstrated by several investigators that intracellular redox state is altered during cancer progression in certain cell types [1–3], but little is known about possible changes in extracellular redox state. We hypothesize that cancer cells develop specific alterations in both intra- and extracellular redox states as cancer progresses to a more aggressive state, with these changes postulated to promote abnormal behavior of cancer cells.

Extracellular/microenvironmental redox state is determined at least in part by the following factors [4]: (1) redox-modulating proteins located in the plasma membrane, such as NADPH oxidase (NOX1), (2) redox-modulating proteins located outside of cells, such as extracellular superoxide dismutase, (3) thiol/disulfide couples such as cysteine (Cys)/cystine (CySS), (4) reactive oxygen species (ROS)/reactive nitrogen species (RNS) that are capable of traveling across cell membranes, such as hydrogen peroxide (H_2O_2), (5) extracellular free radical damage products, including protein carbonyls, and (6) extracellular repair systems, such as protein disulfide isomerase. These molecules are the machinery that maintains redox homeostasis in the extracellular space/microenvironment.

The major aim of this study was to examine the possible role of the microenvironmental redox state in the regulation of biology and biochemistry associated with prostate cancer cell behavior. We were particularly interested in the Cys/CySS redox couple because of its abundance in the extracellular space. Recent metabolic profiles of human prostate cancer tissues demonstrated a significant increase in Cys and a significant decrease in reduced glutathione (GSH) as cancer progresses in pathologic grade from low- to high-grade Gleason scores [5]. Moreover, it has been demonstrated that modifications of extracellular Cys/CySS could directly regulate cell proliferation by

Abbreviations: CySS, cystine; FITC, fluorescein isothiocyanate; DTT, dithiothreitol; GSH, glutathione; GSSG, glutathione disulfide; IAA, iodoacetic acid; MMP, matrix metalloproteinase; NAC, *N*-acetylcysteine; •NO, nitric oxide; NOX1, NADPH oxidase 1; PrEC, normal prostate epithelial cell; PrEGM, normal prostate epithelial growth medium; ROS, reactive oxygen species; RNS, reactive nitrogen species; siRNA, silencing RNA; SNAP, *S*-nitroso-*N*-acetyl-1,1-penicillamine; Trx1, thioredoxin 1.

* Corresponding author. Fax: +1 608 280 7087.
E-mail address: toberley@wisc.edu (T.D. Oberley).

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acting as an oxidant–reductant redox switch [6]. A reducing extracellular redox state modulated by Cys/CySS has been demonstrated to increase cell proliferation through a growth factor-signaling pathway in colon carcinoma cells [7]. A study in lung fibroblasts showed that an oxidizing extracellular redox state modulated by Cys/CySS stimulated cell proliferation and extracellular matrix expression [8]. These combined results suggest that extracellular Cys/CySS redox-dependent proliferation may be cell-type specific.

Nevertheless, the relationship between extracellular redox state and prostate cancer progression has not been fully established. In this study, we modulated Cys/CySS levels in the culture media of DU145 and PC3 prostate cancer cell lines and normal PrEC prostate epithelial cells. We were able to demonstrate that a reducing microenvironment with calculated redox potential of Cys/CySS (E_hCySS) of −180 mV favored prostate cancer invasive ability, at least partially through enhancing matrix metalloproteinase 9 (MMP9) activity and NADPH-mediated extracellular H_2O_2 production. In contrast, a reducing intracellular redox state induced by *N*-acetylcysteine (NAC) resulted in a significant decrease in cell invasion and MMP activity in PC3 cells. Media in which E_hCySS was altered (toward either oxidation or reduction) did not affect the in vitro invasive ability of PrECs. Thus, the invasive abilities of prostate cancer cells were relatively more susceptible to changes in microenvironmental or intracellular redox state than normal prostate epithelial cells. Our results indicate the possibility of innovative chemotherapy targeting both extracellular and intracellular redox states.

Materials and methods

Chemicals and reagents

All chemicals and reagents were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA), unless otherwise specified. Tissue culture supplies were from Falcon Becton–Dickinson Labware (Franklin Lakes, NJ, USA). All tissue culture reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA) except prostate epithelial growth medium (PrEGM), which was obtained from Lonza Walkersville (Walkersville, MD, USA); Cys/CySS-free RPMI 1640 medium, which was obtained from Sigma–Aldrich Co.; and fetal bovine serum (FBS), which was obtained from Tissue Culture Biologicals (Tulare, CA, USA). Amplex Red H_2O_2/Peroxidase Assay kit and Orange Green 488 maleimide were obtained from Invitrogen Life Technologies. Polycarbonated (PCF) inserts and Amicon filter units were purchased from Millipore Co. (Billerica, MA, USA). Pro-MMP2, pro-MMP9, and calcein-AM fluorescence dye were purchased from EMD Chemical Co. (Gibbstown, NJ, USA). Microspin G-25 columns were obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). All silencing RNAs (siRNAs) and reagents were purchased from Dharmacon (Lafayette, CO, USA). Fluorescein isothiocyanate (FITC)-conjugated gelatin was obtained from Elastin Products Co. (Owensville, MO, USA). All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), except Trx1 antibody, which was purchased from AbFrontier Co. Ltd. (Geumcheon-gu, Seoul, Korea), and IRDye 800CW goat anti-rabbit IgG, which was purchased from Li-Cor Biosciences (Lincoln, NE, USA).

Preparation of Cys/CySS-supplemented media

The Cys/CySS-supplemented media were prepared fresh daily. Media with varying E_hCySS were prepared by adding various concentrations of Cys/CySS (4 μM/300 μM or 450 μM/6 μM) to Cys/CySS-free RPMI 1640 medium containing 0.015 g/L methionine and 100 mg/L kanamycin sulfate (final pH 7.4). Stock solutions of Cys and CySS (10 mM, pH 7.4) were prepared fresh for every experiment and filtered through a 0.2-μm syringe filter before addition to Cys/CySS-free medium. Before and after incubation with various cells, Cys and CySS concentrations were determined by HPLC with fluorescence detection.

Cell culture and treatment

PC3 and DU145 cells were obtained from the American Type Culture Collection, whereas normal PrECs were obtained from Lonza Walkersville. PC3 and DU145 cells were tested and confirmed for authenticity using short-tandem-repeat DNA typing by Biosynthesis Cell (Lewisville, TX, USA). PC3 and DU145 cells were cultured in RPMI 1640 supplemented with 5% FBS, whereas PrECs were cultured in PrEGM for routine maintenance. PrEGM components are listed in Supplementary Table **1**. Cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Trypsin (0.05%)/0.53 mM EDTA and soybean trypsin inhibitor were used for routine subculture. To determine extracellular redox potential during steady state, PC3 cells were cultured in regular RPMI 1640 medium and PrECs were cultured in PrEGM for 0 and 24 h. To modulate extra- or intracellular redox states, DU145, PC3, and PrEC cells were cultured in RPMI 1640 serum-free medium for 24 h and washed with PBS, pH 7.4, three times before addition of Cys/CySS-free medium, Cys/CySS-supplemented medium, or the low-molecular-weight redox modulating compound NAC.

Analysis of Cys, CySS, GSH, GSSG, and CyS–GSH disulfide (CySGSH)

Concentrations of Cys, CySS, GSH, GSSG, and CySGSH in cell culture media were determined by HPLC with fluorescence detection as described elsewhere [9]. Briefly, media were added (1:1) to ice-cold 10% perchloric acid solution containing 0.2 M boric acid. Samples were derivatized with iodoacetic acid (IAA) and dansyl chloride. Derivatized samples were centrifuged and the aqueous layer was applied to a Supelcosil LC-NH_2 column. Thiols and disulfides were separated and quantified by integration of their HPLC peaks relative to the internal standard (γ-glutamylglutamate). Extracellular redox potentials of the redox couples Cys/CySS (E_hCySS) and GSH/GSSG (E_hGSSG) were calculated using the Nernst equation, $E_h\text{CySS} = -250 + 30 \log [(\text{CySS})/(\text{Cys})^2]$ and $E_h\text{GSSG} = -264 + 30 \log [(\text{GSSG})/(\text{GSH})^2]$, respectively.

In vitro invasion assay

Cells (5×10^5) were cultured in Cys/CySS-free medium or Cys/CySS-supplemented medium for 3 h before being seeded in the upper chamber of an in vitro cell invasion assay. Conditioned medium and cells were placed together in the upper chambers, whereas RPMI 1640 with 5% FBS was placed in the lower chambers. Invasion assays were performed as previously described [9].

Measurement of extracellular H_2O_2

Extracellular H_2O_2 levels were detected using the Amplex Red H_2O_2/Peroxidase Assay Kit based on the reaction of Amplex red reagent and H_2O_2 to produce resorufin [1].

Matrix metalloproteinase activity and zymography assays

Conditioned media were concentrated using an Amicon Filter unit. Samples of concentrated conditioned medium were analyzed using the MMP activity assay kit from Invitrogen Life Technologies or by electrophoresis (12% SDS–PAGE copolymerized with 1% gelatin as substrate) as previously described [9]; gelatinolytic activities were detected as white bands against a blue background.

siRNA transfection

Two different siRNAs for each protein were designed and synthesized by Dharmacon as follows: for NOX1 (Cat. No. D-010193), GCACACCUGUUUAACUUUG (I) and UGAGAAGGCCGACAAAUAC (III); for MMP9 (Cat. No. D-005970), GGAACCAGCUGUAUUUGUU (I) and GAAUACCUGUACCGCUAUG (III). Nonspecific siRNA, which does not have specificity