Extracellular Redox State Regulates Features Associated with Prostate Cancer Cell Invasion

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Abstract

We have examined the possible role of extracellular reduction-oxidation (redox) state in regulation of biological/biochemical features associated with prostate cancer cell invasion. DU145, PC-3, and RWPE1-derived human prostate cancer (WPE1-NB26) cell lines were used for the present *in vitro* analysis. Increasing levels of nitric oxide using *S*-nitroso-*N*-acetylpenicillamine resulted in a decrease in cell invasion ability, whereas increasing levels of extracellular superoxide radical ($O_2^{\bullet-}$) using xanthine/xanthine oxidase resulted in an increase in cell invasion ability in these three cell lines. WPE1-NB26 cells exhibited an increased glutathione/glutathione disulfide ratio in the medium in comparison with RWPE1 cells (immortalized but nonmalignant prostate epithelial cells), suggesting an alteration of extracellular redox state of WPE1-NB26 cells. We hypothesized that $O_2^{\bullet-}$ production at or near the plasma membrane or in the adjacent extracellular matrix at least partially regulated prostate cancer cell invasion. Using adenovirus-mediated extracellular superoxide dismutase (EC-SOD) gene transduction to enzymatically decrease $O_2^{\bullet-}$ levels, we showed that in the presence of heparin, adenovirus EC-SOD gene transduction resulted in an increase in the expression of EC-SOD outside the cells with resultant inhibition of cell invasion ability. This inhibition correlated with reduced metalloproteinase [matrix metalloproteinase (MMP) 2/membrane type 1-MMP] activities and increased levels of extracellular nitrite. Our results suggest a prominent role of extracellular redox status in regulation of cell invasion, which may provide opportunities for therapeutic interventions. [Cancer Res 2008;68(14):5820–6]

Introduction

Intracellular and extracellular redox states result from the net balance of reducing and oxidizing equivalents inside and outside the cell, respectively. Accumulating evidence has implicated intracellular redox state in the regulation of cancer cell behavior, including growth, invasiveness, and metastasis (1), but less is known about the biological effects of extracellular redox state on cancer cell properties.

Under physiologic conditions, the extracellular space is known to have a relatively more oxidized redox state than the interior of the cell (2). Extracellular redox state is determined largely by several known variables: (*a*) redox-modulating proteins that are located on the plasma membrane or outside of cells [including NADPH oxidase, extracellular superoxide dismutase (EC-SOD), thioredoxin 1 (TRX1), peroxiredoxin-IV, and extracellular glutathione peroxidase 3 (GPx3); refs. 3–5], (*b*) extracellular thiol/disulfide couples [including glutathione (GSH)/glutathione disulfide (GSSG), reduced thioredoxin (TRX-SH2)/oxidized thioredoxin (TRX-SS), and cysteine (Cys)/cystine (CySS); ref. 6], and (*c*) reactive oxygen species/reactive nitrogen species that are diffusible and thus capable of traveling across plasma membranes into the extracellular space [including hydrogen peroxide (H_2O_2) and nitric oxide ($NO^{\bullet}$)]. Initial studies have suggested that the redox status of the tumor extracellular microenvironment has an effect on cancer behavior. Extracellular Cys/CySS has been shown to regulate cell proliferation through a growth factor signaling pathway in colon carcinoma cells (7), whereas TRX1 levels in plasma or serum are markers for oxidative stress associated with hepatocellular carcinoma (8) and pancreatic cancer (9). In addition, $O_2^{\bullet-}$ released by tumor cells has been shown to play an important role in basement membrane degradation (10, 11). Overexpression of extracellular-located GPx3 resulted in inhibition of prostate cancer growth and metastasis both *in vitro* and *in vivo* (5). Despite these initial studies, the role(s) of extracellular redox state in regulating cancer behavior needs to be further analyzed. We hypothesized that (*a*) extracellular redox state of human prostate cancer cells is altered in comparison with nonmalignant prostate epithelial cells and (*b*) modulation of extracellular redox state regulates prostate cancer cell invasion.

Prostate cancer is the most common malignancy diagnosed in men and is the second leading cause of cancer deaths in males in the United States; a common cause of death is metastases to the bones (12). In the present study, we analyzed whether extracellular redox state plays a role in prostate cancer cell invasion, a biological process associated with metastasis. We analyzed the extracellular redox state of an aggressive prostate cancer cell line in comparison with its parental nonmalignant prostate epithelial cell line. Subsequently, we used EC-SOD overexpression as a tool to regulate redox state in the extracellular space of human prostate cancer cells. EC-SOD is the only isoform of SOD that is mainly expressed in the extracellular space via binding with heparan sulfate proteoglycans. EC-SOD is an antioxidant enzyme (AE) that converts $O_2^{\bullet-}$ to H_2O_2. The ultimate effects of SODs on redox state depend on cell milieu, environmental factors, reducing equivalents, and/or the balance of other AEs inside/outside of the cells (13).

Herein, we are the first to show that extracellular redox status was altered in an aggressive prostate carcinoma cell line compared with its parental immortalized but nonmalignant prostate epithelial cell line. More importantly, we show that redox state modulation in a highly aggressive prostate cancer cell line via

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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overexpression of extracellular EC-SOD negatively regulated prostate cancer cell invasion and matrix metalloproteinase (MMP), an enzyme that has the ability to degrade extracellular matrix (ECM). Our study is the first to show that extracellular redox state regulates prostate cancer cell invasion, a finding that may provide opportunities for therapeutic interventions.

Materials and Methods

Chemicals and reagents. All chemicals and reagents were purchased from Sigma Chemical Co., unless otherwise specified. Tissue culture supplies were from Falcon (BD Biosciences). All tissue culture reagents were obtained from Life Technologies-Invitrogen Cell Culture except for fetal bovine serum (FBS), which was purchased from Tissue Culture Biologicals. Griess reagent system was purchased from Promega Co. Polycarbonated (PCF) inserts were purchased from Millipore Co. Pro-MMP2 and calcein-AM fluorescence dye were purchased from Merck Chemicals Ltd. Matrigel was purchased from BD Biosciences. All antibodies were purchased from Santa Cruz Biotechnology, Inc., unless otherwise specified.

Cell culture and treatment. DU145, PC-3, RWPE1 (immortalized nonmalignant prostate epithelial cells), and WPE1-NB26 cells (aggressive prostate cancer cells) were obtained from the American Type Culture Collection. RWPE1 cells were derived from adult human prostate epithelial cells that were immortalized with human papillomavirus-18. WPE1-NB26 cells were derived from RWPE1 cells that were exposed to *N*-methyl-*N*-nitrosourea and then injected into nude mice (14). RWPE1 cells do not grow in agar and are not tumorigenic in nude mice, whereas WPE1-NB26 cells form large and poorly differentiated invasive tumors in nude mice. In addition, WPE1-NB26 cells form metastases in the lungs of male athymic nude mice after i.v. injection (15). DU145 and PC-3 cells were cultured in RPMI 1640 supplemented with 5% FBS and 100 mg/L kanamycin sulfate, whereas RWPE1-derived cell lines were cultured in keratinocyte serum-free medium (KSFM) supplemented with 50 mg/L bovine pituitary extract (BPE), 5 μg/L recombinant epidermal growth factor (rEGF), and 100 mg/L kanamycin sulfate for routine maintenance and subsequent experiments. All cell lines were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Various concentrations of *S*-nitroso-*N*-acetylpenicillamine (SNAP; a $NO^{\bullet}$ donor), apocynin (NADPH oxidase inhibitor), or xanthine/xanthine oxidase ($O_2^{\bullet-}$ generating system) were added to the medium for 6 h (DU145 and PC-3 cells) or 24 h (WPE1-NB26 cells, except for xanthine/xanthine oxidase, which were added for 6 h), and then cells and/or conditioned medium were collected for further analysis. For each cell line, preliminary time course and dose-response studies of these compounds were performed, with only the optimal conditions reported in the present study.

Adenovirus gene transduction. An adenoviral vector containing human EC-SOD cDNA (Adh*sod3*) was obtained from Dr. L.W. Oberley's or Dr. J.J. Cullen's laboratories at the University of Iowa. Control adenovirus with no gene inserted (AdEmpty) was purchased from ViraQuest, Inc. Adenovirus containing green fluorescent protein (Ad*gfp*) cDNA (ViraQuest) was used to determine transduction efficiency. Transduction efficiency of Ad*gfp* was >85% in all cell lines as measured by fluorescence intensity of GFP in flow cytometry analysis. The adenovirus constructs used were described previously (16).

The procedure of transduction of adenoviral vectors is illustrated in Supplementary Fig. S1. Cells were seeded in medium at 5×10^5 in 100-mm dishes or 2.5×10^4 in 24-well plates and allowed to attach for 48 h. Cells were then washed thrice with Dulbecco's PBS (pH 7.0; RWPE1-derived cell lines) or PBS (pH 7.4; DU145 and PC-3 cells) and transduced with either Adh*sod3* or AdEmpty at a specified multiplicity of infection (MOI) in 4 mL (for 100-mm dishes) or 200 μL (for 24-well plates) of medium (antibiotic-free with no BPE/rEGF or FBS added). At the end of the 24-h transduction period, virus-containing media were removed and complete media with or without heparin (500 μg/mL) were added to modulate the release of EC-SOD into media (as shown in Results). Cells were cultured for an additional 48, 72, 96, and 120 h after transduction, and then cells or conditioned media were harvested for further analysis. Every 48 h, complete medium with or without heparin was replaced. DU145 and PC-3 cells were transduced with 200 MOI, whereas WPE1-NB26 cells were transduced with 100 or 300 MOI, thus resulting in greater immunoreactive EC-SOD protein levels in the latter cells at the higher MOI.

Determination of protein levels. Protein concentrations of cell lysates and conditioned media were determined by the Bradford assay according to the manufacturer's instructions (Bio-Rad Laboratories).

***In vitro* invasion assay.** DU145 (2.5×10^5/mL), PC-3 (2.5×10^5/mL), or WPE1-NB26 (7.5×10^5/mL) cells were cultured in serum-free RPMI 1640 or KSFM with no BPE/rEGF added before seeding in the upper chamber (optimal cell numbers of each cell line were selected appropriate to their invasion ability). Serum-free RPMI 1640 or KSFM with no BPE/rEGF and complete medium (plus FBS or plus BPE/rEGF) were placed in the upper and lower chambers, respectively. After incubation for 6 h (DU145 or PC-3 cells ± redox-modulating compounds) or 24 h (WPE1-NB26 cells ± redox-modulating compounds or cells transduced with adenovirus) at 37°C in 5% CO_2/95% air, cells that had invaded through the coated PCF membranes were detached using the cell dissociation buffer and detected by the calcein-AM fluorescence dye. Collagen type I–coated (1 mg/mL) PCF membranes were used for WPE1-NB26 cells, whereas Matrigel-coated (basement membrane matrix, 100 μg/mL) PCF membranes were used for DU145 and PC-3 cells because the latter cells had greater cell invasion ability *in vitro*.

Extracellular nitrite measurements. External nitrite levels (which are a stable and nonvolatile breakdown product of $NO^{\bullet}$) were analyzed following the manufacturer's instructions based on the Griess reagent system as previously described (17). Media decanted from growing cells were analyzed in these experiments.

EC-SOD activity gel. Conditioned media were concentrated using an Amicon Ultra-15 filter (Millipore). Fifteen micrograms of protein obtained from concentrated conditioned medium were subjected to electrophoresis on 8% PAGE native gels. After electrophoresis, gels were stained with nitroblue tetrazolium (18). The presence of one or multiple achromatic bands on the purple background indicated EC-SOD activity.

Glutathione assay. Conditioned media were collected for extracellular glutathione assays (total GSH, reduced GSH, and GSSG levels). All the samples were protected from light and exposed to air for the minimal but the same amount of time. Sample preparations and assay protocol based on 5,5′-dithiobis-(2-nitrobenzoic acid)-GSSG reductase recycling were previously described (17). GSH/GSSG ratios were calculated as indicators of redox balance in the extracellular space.

MMP zymography. Conditioned media were concentrated using an Amicon Ultra-15 filter. Thirty micrograms of protein obtained from concentrated conditioned medium were subjected to electrophoresis (12% SDS-PAGE copolymerized with 1% gelatin as substrate). Gels were then washed with 2.5% Triton X-100 and incubated at 37°C overnight with Tris-HCl buffer (pH 7.6). Gels were stained with 0.25% Coomassie blue G-250 (Bio-Rad) for 1 h and destained with 10% acetic acid and 40% methanol until gelatinolytic activities were detected as clear bands against a blue background. The ability of membrane type 1-MMP (MT1-MMP) to catalyze pro-MMP2 to active MMP2 was analyzed by adding 20 ng of exogenous pro-MMP2 to medium for 24 h. Conditioned media were collected, concentrated, and subjected to electrophoresis as described above.

NADPH oxidase assay. The assay was performed following the protocol described by Cui and Douglas (19). Briefly, cells were washed and scraped in ice-cold Dulbecco's PBS followed by centrifugation at $750 \times g$ for 10 min. The pellets were resuspended and homogenized in lysis buffer (containing KH_2PO_4, EGTA, aprotinin, leupeptin, and phenylmethylsulfonyl fluoride). For 96-well plates, 90 μL of assay buffer (containing EGTA, sucrose, lucigenin, and NADPH in phosphate buffer, pH 7.0) were added to 10 μL of homogenate. Photoemission was measured by a Tropix luminometer and normalized by protein concentration.

Western blotting. The protocols of Western blot analysis were detailed elsewhere (17). Crude supernatants or concentrated conditioned media were placed in each well. Anti-EC-SOD (2 ng/mL; Assay Designs, Inc.) or anti-β-actin (1:1,000) antibodies were used. The data images were analyzed using ImageQuant software as previously described (17).

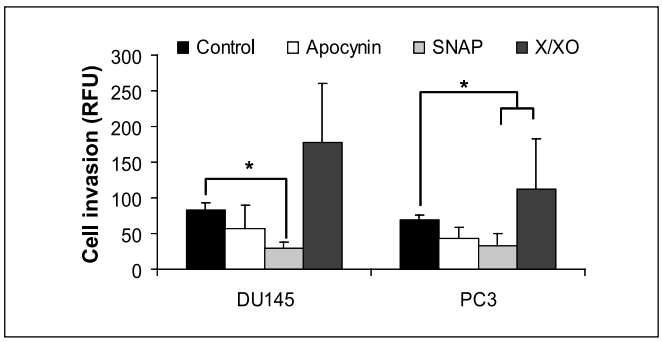

Figure 1. Effect of redox modulation on invasion ability of DU145 and PC-3 cells. Cells were cultured in serum-free RPMI 1640 for 24 h before seeding in the upper chamber. Apocynin (100 μmol/L), SNAP (100 μmol/L), or 4.5 μg/mL xanthine (*X*) plus 0.6 units/mL xanthine oxidase (*XO*) were added in the upper chamber for 6 h. Cells that had invaded through the Matrigel-coated membrane were detached and analyzed using calcein-AM. Data are results from three separate experiments. *RFU,* relative fluorescence units. *, $P < 0.05$.

Statistics. Statistical analysis was performed with Student's *t* test using SPSS 10 software (SPSS Inc.). Mean differences were considered significant at $P < 0.05$ unless otherwise specified. All data are presented as mean ± SE.

Results

Regulation of aggressive prostate cancer cell invasion through modulation of extracellular redox state. To determine whether redox state regulates prostate cancer cell invasion, redox-modulating compounds were added to the upper chamber of a cell invasion assay system. As shown in Fig. 1, treatment with 100 μmol/L apocynin (NADPH oxidase inhibitor) decreased invasive ability of DU145 and PC-3 cells (~31% and 35% decreases, respectively), whereas increase of $O_2^{\bullet-}$ levels using the xanthine (4.5 μg/mL)/xanthine oxidase (0.6 units/mL) system significantly increased invasion ability of DU145 and PC-3 cells (~2.2- and 1.6-fold increases, respectively). In addition, treatment of DU145 and PC-3 cells with a $NO^{\bullet}$ donor (100 μmol/L SNAP) resulted in reduction of invasion ability (~65% and 53% decreases, respectively). These results suggest a positive role of $O_2^{\bullet-}$ and negative role of $NO^{\bullet}$ in prostate cancer cell invasion.

Overexpression of EC-SOD immunoreactive protein in DU145 and PC-3 cells by adenovirus *sod3* gene transduction. DU145 and PC-3 cells were transduced with AdEmpty or Adh*sod3* at 200 MOI. At 96 and 120 h after Adh*sod3* transduction, PC-3 cells showed more EC-SOD protein in cell lysates than observed in DU145 cells; in addition, at 120 h, EC-SOD protein was significantly reduced in cell lysates of DU145 cells in comparison with PC-3 cells.

At 96 h after Adh*sod3* transduction, EC-SOD protein was detected outside the cell (in the medium) in the heparin-treated group but barely detected in the control group (nonheparin treatment) in both cell lines (Fig. 2*A*). At 120 h after Adh*sod3* transduction, EC-SOD protein in the medium of heparin-treated group was higher compared with the control group in both cell lines. Thus, cells that were transduced with Adh*sod3* without heparin treatment at 96 h are referred to as overexpressing intracellular EC-SOD to reflect the fact that the majority of the EC-SOD immunoreactive protein is within the cells (primarily modulation of intracellular redox state), whereas cells that were treated under the same conditions with heparin are referred to as overexpressing extracellular EC-SOD to reflect the fact that EC-SOD immunoreactive protein is detectable in the medium (thus modulating extracellular redox state).

Reduction of DU145 and PC-3 cell invasion abilities by overexpression of extracellular EC-SOD. In light of the above results, we further probed the possible role of extracellular redox state in cancer cell invasion. We used a Matrigel-coated membrane invasion assay system as described in Materials and Methods. Overexpression of extracellular EC-SOD at 96 h after Adh*sod3* transduction in DU145 or PC-3 cells resulted in a decrease in cell invasion ability (~49% decrease in both cell lines), whereas overexpression of intracellular EC-SOD in PC-3 cells showed a

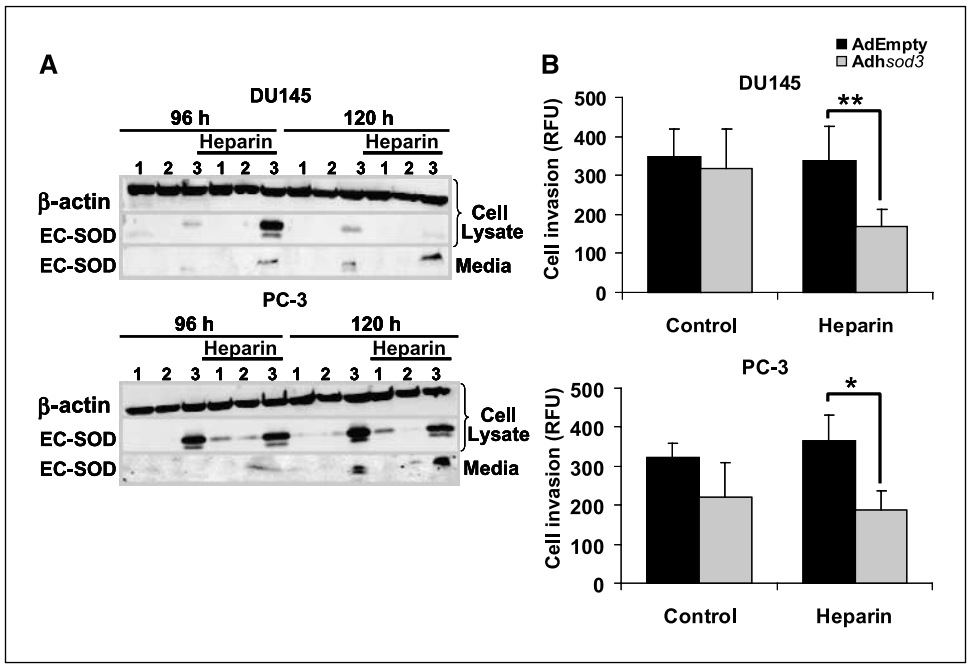

Figure 2. Effect of overexpression of intracellular or extracellular EC-SOD on DU145 and PC-3 cell invasion. Cells were transduced with AdEmpty or Adh*sod3* at 200 MOI with or without 500 μg/mL heparin. Cells and conditioned media were collected at 96 and 120 h after transduction. *A,* Western blot analysis of intracellular and extracellular EC-SOD protein expression. Thirty micrograms of protein from cell lysates or concentrated conditioned media were placed in each well. *Lane 1,* parental cells; *lane 2,* AdEmpty 200 MOI; *lane 3,* Adh*sod3* 200 MOI. *B, in vitro* invasion assay. Cells at 96 h after transduction were collected and seeded in the upper chamber for 24 h. Cells that had invaded through the Matrigel-coated membrane were detached and analyzed using calcein-AM. Data are results or representative of results (Western blot analysis) from three separate experiments. *, $P < 0.05$; **, $P < 0.1$.

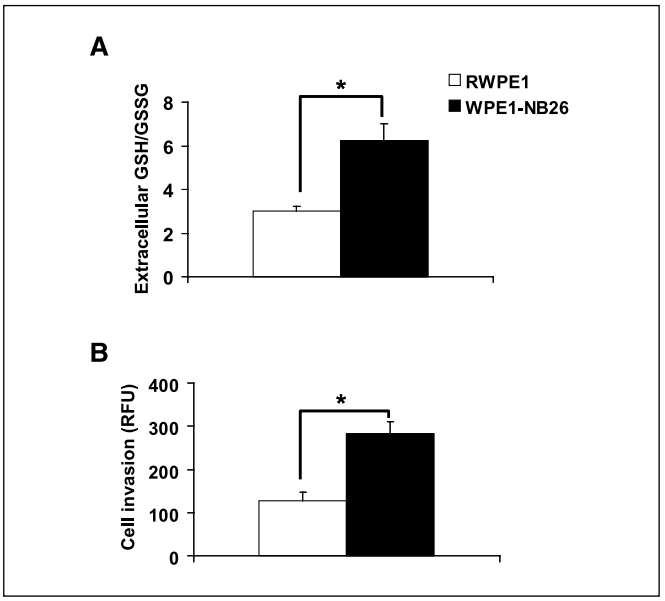

Figure 3. Extracellular redox state and invasion ability of RWPE1-derived cell lines. *A,* extracellular GSH/GSSG ratios. Conditioned media were used for the analysis. *B, in vitro* invasion assay. Cells were cultured for 24 h in KSFM with no BPE/rEGF added before seeding in the upper chamber. After incubation in the upper chamber for 24 h, cells that had invaded through the collagen type I–coated membrane were detached and analyzed using calcein-AM. Data are results from three separate experiments. *, $P < 0.05$.

small but not statistically significant decrease in cell invasion ability when compared with AdEmpty (Fig. 2*B*).

Extracellular redox states and invasion ability of RWPE1-derived cell lines. We analyzed the role of extracellular redox state in more detail in the WPE1-NB26 cell line because this allowed biochemical comparisons to the parental isogeneic nonmalignant RWPE1 cell line. We first analyzed extracellular redox state of RWPE1 versus WPE1-NB26 cells and found that highly aggressive WPE1-NB26 cells had a higher extracellular GSH/GSSG ratio than RWPE1 cells (~2-fold increase; Fig. 3*A*). Extracellular total and reduced GSH levels were higher, whereas extracellular GSSG levels were lower in WPE1-NB26 cells than RWPE1 cells (Supplementary Table S1). These changes in extracellular redox state are correlated with their cell invasion ability; WPE1-NB26 cells had a 2.2-fold increase in cell invasion in comparison with parental RWRE1 cells (Fig. 3*B*).

Regulation of WPE1-NB26 cell invasion through modulation of extracellular redox state. Redox-modulating compounds were added to the upper chamber of the cell invasion assay system to confirm the role of redox state on WPE1-NB26 cell invasion ability. Our results showed that reduction of NADPH oxidase activity (Supplementary Fig. S2*A*) by treatment with 500 μmol/L apocynin correlated with decreased invasive ability of WPE1-NB26 cells (Fig. 4*A*), whereas increase of $O_2^{\bullet-}$ levels using the xanthine (4.5 μg/mL)/xanthine oxidase (0.6 units/mL) system significantly increased invasion ability of WPE1-NB26 cells (1.4-fold increase; Fig. 4*B*). The observed increase in cell invasion ability induced by xanthine/xanthine oxidase correlated with induction of MMP2 and MT1-MMP activities (the conversion of pro-MMP2 to MMP2 was used as an indicator of MT1-MMP activity as described in Materials and Methods; Fig. 4*C*). Addition of SOD (2,000 units/mL) to medium inhibited xanthine/xanthine oxidase–induced cell invasion (~45% decrease) and MMP2 activity (~50% decrease), whereas catalase or GPX has no effect (data not shown). In addition, we showed that treatment of WPE1-NB26 cells with 100 μmol/L SNAP resulted in reduction of invasion ability (~29% decrease; Fig. 4*D*), with a concomitant increase in extracellular nitrite levels (Supplementary Fig. S2*B*). These results suggest a negative role of $NO^{\bullet}$ in prostate cancer cell invasion. The above results are consistent with those obtained from DU145 and PC-3 cells, although effects observed by extracellular redox state modulation seem greater in the more invasive DU145 and PC-3 cells than the less invasive WPE1-NB26 cells.

Elevation of EC-SOD immunoreactive protein and activity in WPE1-NB26 cells by adenovirus *sod3* gene transduction. WPE1-NB26 cells were transduced with AdEmpty or Adh*sod3* at

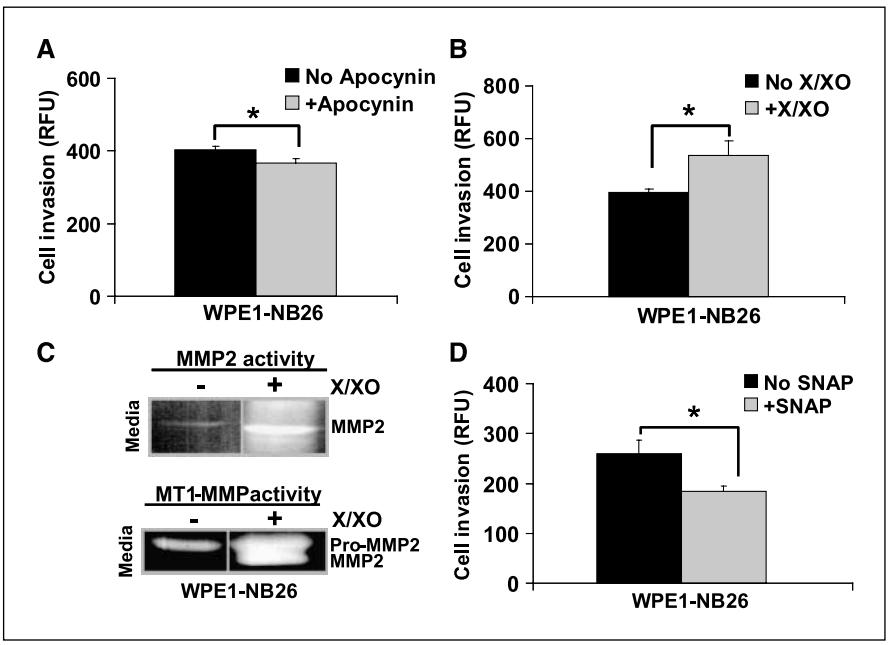

Figure 4. Effect of redox modulation on invasion ability of WPE1-NB26 cells. All compounds were added 24 h before analysis (except for xanthine/xanthine oxidase, which were added for 6 h). *A,* inhibition of invasion ability by apocynin (500 μmol/L). *B,* induction of invasion ability by xanthine/xanthine oxidase (4.5 μg/mL xanthine and 0.6 units/mL xanthine oxidase were added for 6 h). *C,* activation of MMP2 (*top*) and MT1-MMP (*bottom*) activities by xanthine/xanthine oxidase. Concentrated conditioned media (30 μg) from each sample were used for zymography gel assay. The ability of MT1-MMP to catalyze pro-MMP2 to MMP2 was analyzed by adding 20 ng of pro-MMP2 in the medium. MMP bands appear as smeared bands in the xanthine/xanthine oxidase treatment group. *D,* inhibition of invasion ability by SNAP (100 μmol/L). All data are results or representative of results (zymography analysis) from three separate experiments. *, $P < 0.05$.

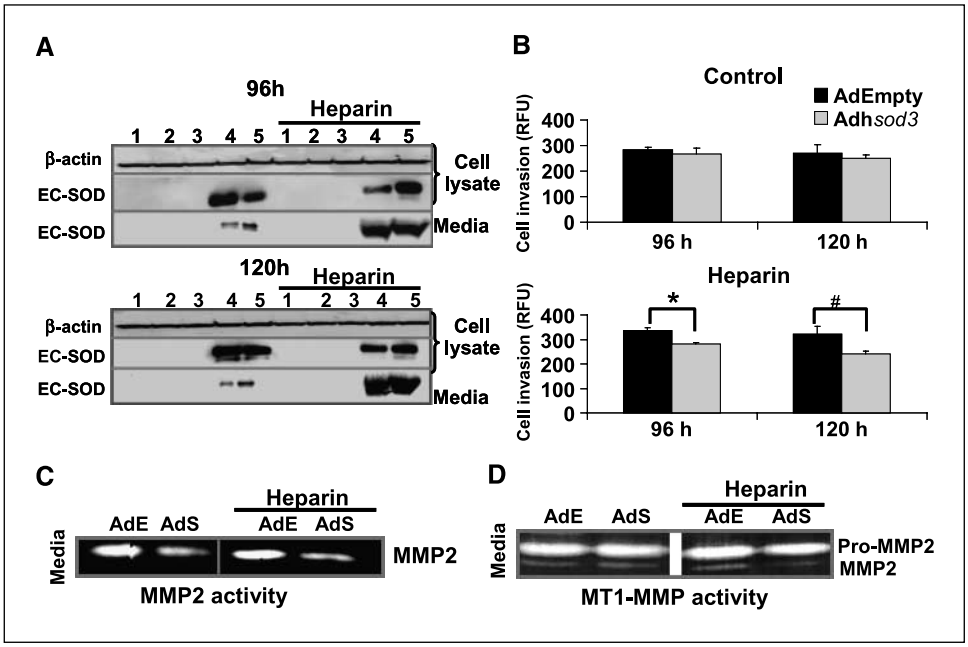

Figure 5. Effect of overexpression of extracellular EC-SOD on WPE1-NB26 cell invasion. WPE1-NB26 cells were transduced with AdEmpty or Adh*sod3* for 96 and 120 h with or without 500 μg/mL heparin. *A,* Western blot analysis of intracellular and extracellular EC-SOD protein expression. Twenty micrograms of protein from cell lysates or concentrated conditioned media were placed in each well. *Lane 1,* WPE1-NB26 cells; *lane 2,* AdEmpty 100 MOI; *lane 3,* AdEmpty 300 MOI; *lane 4,* Adh*sod3* 100 MOI; *lane 5,* Adh*sod3* 300 MOI. *B, in vitro* invasion assay. Cells were cultured in KSFM with no BPE/rEGF added before seeding in the upper chamber. After incubation in the upper chamber for 24 h, cells that had invaded through the collagen type I–coated membrane were detached and analyzed using calcein-AM. *C,* zymography gels showing MMP2 activity at 120 h after transduction. Thirty micrograms of concentrated conditioned media were placed in each well. *D,* zymography gels showing MT1-MMP activity at 120 h after transduction. The ability of MT1-MMP to catalyze pro-MMP2 to MMP2 was analyzed by adding 20 ng of pro-MMP2 in the medium. Data are results or representative of results (Western blot analysis) from three separate experiments. *AdE,* AdEmpty; *AdS,* Adh*sod3*. *, $P < 0.05$; #, $P = 0.08$.

100 or 300 MOI. As shown in Fig. 5*A*, at both 96 and 120 h after Adh*sod3* transduction at 300 MOI, more EC-SOD protein (~60% of total immunoreactive protein) in the heparin-treated group was secreted outside the cell compared with the control group (~30% of total immunoreactive protein). Western blot results correlated with EC-SOD activity gels (Supplementary Fig. S3*A*), confirming that conditioned medium collected from heparin-treated cells contained higher levels of enzymatically active EC-SOD. Consequently, WPE1-NB26 cells that were transduced with Adh*sod3* at 96 and 120 h without heparin treatment are referred to as overexpressing intracellular EC-SOD, whereas cells that were treated under the same conditions with heparin are referred to as overexpressing extracellular EC-SOD.

Reduction of MMP activity and cell invasion ability in WPE1-NB26 cells by overexpression of extracellular EC-SOD. To investigate the mechanism of extracellular EC-SOD on the invasion ability of WPE1-NB26 cells, we used a collagen type I–coated membrane invasion assay as described in Materials and Methods. Overexpression of extracellular EC-SOD in WPE1-NB26 cells resulted in a statistically significantly decrease in cell invasion ability at 96 and 120 h after Adh*sod3* transduction ($P < 0.05$ and $P = 0.08$, respectively), whereas overexpression of intracellular EC-SOD showed only a slight decrease in cell invasion ability when compared with AdEmpty at the same time points (Fig. 5*B*). These results are consistent with the results from DU145 and PC-3 cells. Notably, treatment of parental nontransduced WPE1-NB26 cells with heparin alone did not affect cell invasion ability (Supplementary Fig. S3*B*).

Further, we investigated the effects of extracellular redox state on biochemical properties associated with cell invasion. Zymography gels were used to determine whether overexpressing extracellular EC-SOD inhibited MMP2 or MT1-MMP activities. As shown in Fig. 5*C* and *D*, extracellular MMP2 and MT1-MMP activities were decreased in WPE1-NB26 cells overexpressing extracellular EC-SOD; extracellular MMP2 was only slightly decreased in WPE1-NB26 cells overexpressing intracellular EC-SOD.

Alteration of extracellular nitrite levels by overexpression of extracellular EC-SOD. If extracellular EC-SOD inhibits cell invasion by scavenging $O_2^{\bullet-}$, then extracellular nitrite levels may be modulated. WPE1-NB26 cells overexpressing extracellular EC-SOD showed a significant increase in extracellular nitrite levels at 96 and 120 h after transduction when compared with AdEmpty (~3- and 2.6-fold increases, respectively; Fig. 6*B*), whereas there was only a slight increase in extracellular nitrite levels in WPE1-NB26 cells overexpressing intracellular EC-SOD at the same time points (Fig. 6*A*).

Discussion

Prostate cell invasion and metastasis is a complex, multistep cascade that leads to secondary site deposits in distant tissues. Several *in vitro* studies have previously shown a relationship between intracellular redox state and cancer cell invasion. However, interpretation of these results from cell lysates is presently uncertain because the cell is compartmentalized into many organelles and redox state in each component is at present not precisely defined. We hypothesize that (*a*) extracellular redox state in prostate cancer cells is altered in comparison with nonmalignant prostate epithelial cells and (*b*) modulation

of extracellular redox state (toward either more reduced or oxidized states) regulates prostate cancer cell invasion. To test the first hypothesis, we analyzed the extracellular redox state of aggressive WPE1-NB26 prostate cancer cells in comparison with immortalized nonmalignant prostate epithelial cells and showed higher extracellular GSH/GSSG ratios in the cancer cells. We chose to analyze these two cell lines because they are syngeneic and can be grown in serum-free conditions, thus allowing rigorous control of cell culture conditions for subsequent analysis of biochemical data. Our results agree with the studies of Rubartelli and Lotze (20), which showed higher levels of free thiols using mercury orange staining in the intercellular space of colon carcinoma tissues compared with nonmalignant colon tissues.

To test whether extracellular redox state regulates cancer cell invasion, we added apocynin, SNAP, xanthine/xanthine oxidase, or overexpressed EC-SOD in the medium. Apocynin, SNAP, and overexpressed EC-SOD decreased cell invasion, whereas xanthine/xanthine oxidase increased cell invasion in three separate prostate cancer cell lines. Interestingly, responses to each of these redox-modulating compounds/proteins were greater in DU145 and PC-3 cells than in WPE1-NB26 cells. The results raise the intriguing possibility that redox state becomes progressively more abnormal as cancer cells become more aberrant. However, future studies are necessary to confirm this hypothesis.

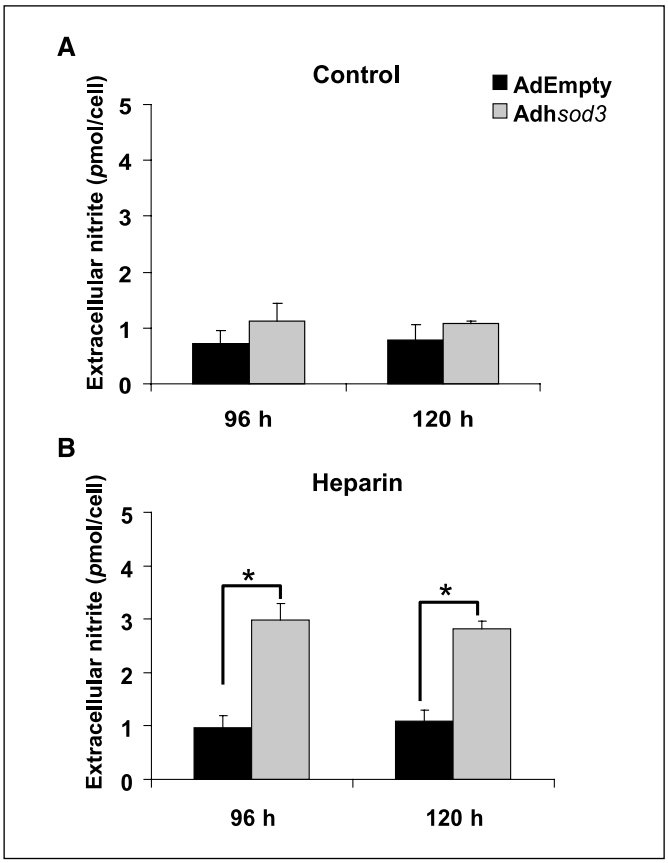

Figure 6. Effect of overexpression of extracellular EC-SOD on extracellular nitrite levels. WPE1-NB26 cells were transduced with AdEmpty or Adh*sod3* for 96 and 120 h at 300 MOI without (*A*) or with (*B*) 500 μg/mL heparin. Conditioned media (50 μL) were collected to measure nitrite levels based on the Griess reagent system. Data are results from three separate experiments. *, $P < 0.05$.

It has been shown that increased NADPH oxidase activity correlates with increasing prostate cancer cell tumorigenicity (21). We established that WPE1-NB26 cells had higher levels of NADPH oxidase protein than immortalized nonmalignant prostate epithelial cells (data not shown). Reduction of NADPH oxidase activity by treatment with apocynin and induction of $NO^{\bullet}$ levels by treatment with SNAP resulted in reduction of prostate cancer cell invasion ability, whereas increase in $O_2^{\bullet-}$ levels (xanthine/xanthine oxidase generated in the medium) resulted in enhancement of MMP activity and cancer cell invasion ability. Thus, modulation of plasma membrane/extracellular redox state resulted in alterations in prostate cancer cell behavior. Although our results showed that apocynin clearly reduced NADPH oxidase activity in WPE1-NB26 cells (Supplementary Fig. S2*A*), we cannot rule out the possibility that apocynin inhibited cell invasion by other unknown effects in cells.

In the present study, we used EC-SOD as a molecular tool to establish the effects of extracellular redox state on the behavior of aggressive prostate cancer cells. SOD is an AE that converts $O_2^{\bullet-}$ to H_2O_2 and O_2. SODs include manganese SOD (MnSOD), copper-zinc SOD (CuZnSOD), and EC-SOD. CuZnSOD is located in the cytoplasm, mitochondrial intermembrane space, and nucleus, whereas MnSOD is located exclusively in the mitochondrial matrix. Here, we focus on EC-SOD due to its primary location in the ECM. However, in the adenoviral transduction system described in the present study, EC-SOD was present both within cells and in the medium, and so its effect on redox state presumably depends largely on where it is located.

We used adenoviral vectors to transfer functional human EC-SOD cDNA into prostate cancer cells. Western blot analysis showed successful overexpression of EC-SOD immunoreactive protein, with the predicted two bands identified at ~31 and ~27 kDa, with the smaller protein shown by another study to be a result of COOH-terminal truncation (22). To modulate extracellular redox state, we added heparin to stimulate the release of EC-SOD outside the cells. Western blot analysis and activity gel assay of EC-SOD in the medium showed successful overexpression of EC-SOD extracellularly [Supplementary Fig. S2*A*, multiple bands observed in EC-SOD activity gels agree with EC-SOD multimer formation reported by Due and colleagues (23)].

For biochemical analyses, we studied the role of extracellular redox state in the WPE1-NB26 cell line because this allowed comparison with the parental isogeneic nonmalignant RWPE1 cell line. Our experiments showed that overexpression of EC-SOD primarily in the extracellular space (medium) significantly decreased WPE1-NB26 cell invasion ability, whereas overexpression of EC-SOD primarily intracellularly only slightly decreased cell invasion. The decrease in cell invasion was not due to increased cell killing by EC-SOD because cells in the upper chamber did not show a change in cell viability.

MMP proteins, including MMP2 and MT1-MMP, are overexpressed in many human malignant tumors and play an important role in tumor invasion (24). MMP2 (gelatinase A) is secreted as inactive zymogen forms (pro-MMP2) and catalyzed to an active form by many proteinases (25). One of the physiologic activators of pro-MMP2 is MT1-MMP (26). Several ECM components are degraded by MT1-MMP (26). The inhibition of cell invasion ability by overexpression of extracellular EC-SOD was correlated with the reduction of immunoreactive protein levels of MMP2 (Supplementary Fig. S4*A*) and MMP2/MT1-MMP activities. MMP activity is regulated at multiple levels; the activation of

proenzymes (secreted form) and the inhibition of MMPs by tissue inhibitors of metalloproteinases are important regulatory processes. Thus, we propose that overexpression of extracellular EC-SOD may regulate MMPs either at transcriptional, translational, posttranslational, secretion, or conformational levels. Alternatively, the changes of extracellular redox state in aggressive prostate cancer cells may regulate the expression of adhesion molecules or ligand binding activation in plasma membranes. Thus, modulation of extracellular redox state of prostate cancer cells by overexpression of extracellular EC-SOD may regulate proteins at the cell surface involved in the metastatic process, such as MT1-MMP.

In addition, we showed that overexpression of EC-SOD primarily in the extracellular space resulted in elevation of nitrite levels; however, there was no change in intracellular levels of inducible $NO^{\bullet}$ synthase protein expression (Supplementary Fig. S4*B*). $NO^{\bullet}$ reacts extremely rapidly with $O_2^{\bullet-}$ to produce peroxynitrite, a potential mediator of cell injury. Because overexpression of EC-SOD would result in lowering of $O_2^{\bullet-}$ levels, increase in $NO^{\bullet}$ levels may be due to the reduced reaction of $O_2^{\bullet-}$ with $NO^{\bullet}$. Our present data indicate that elevated levels of nitrite due to overexpression of extracellular EC-SOD correlated with reduction of MMP activities and cell invasion ability. In addition, increased levels of $NO^{\bullet}$ mediated by SNAP resulted in reduction of cell invasion ability. Because $NO^{\bullet}$ is freely diffusible, it may regulate cell invasion either from intracellular or extracellular locations. It has previously been shown that $NO^{\bullet}$ regulates MMP mRNA and protein expression levels (27). The exact mechanism(s) of how $NO^{\bullet}$ regulates MMP or cell invasion needs to be further analyzed.

To our knowledge, the present study is the first to provide evidence that extracellular redox state regulates prostate cancer cell invasion. Specific molecules used as mediators need to be further analyzed, although our current studies suggest that $O_2^{\bullet-}$ may play as a positive mediator, whereas $NO^{\bullet}$ may act as a negative mediator in regulation of prostate cell invasion. It is also possible that molecular species derived from these two molecules may play a role in prostate cancer cell invasion. Although the extracellular redox system is complicated, modulation of its various components singly and/or together as a network will provide valuable information that may potentially be applied to therapeutic interventions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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