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Original article

Extracellular redox state shift: A novel approach to target prostate cancer invasion

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ABSTRACT

Aim: Extracellular superoxide dismutase (ECSOD) and the cysteine/glutamate transporter (Cys)/(xCT) are tumor microenvironment (TME) redox state homeostasis regulators. Altered expression of ECSOD and xCT can lead to imbalance of the TME redox state and likely have a profound effect on cancer invasion. In the present study, we investigated whether ECSOD and xCT could be therapeutic targets for prostate cancer (PCa) invasion.
Results: Immunohistochemistry of tumor microarray PCa tissues (N = 165) with high Gleason scores indicated that xCT protein expression is significantly increased while ECSOD protein expression is significantly decreased. Metastatic PCa indicated ECSOD protein expression is significantly decreased in epithelial area whereas xCT protein expression is significantly increased in stromal area. Furthermore, inhibition of extracellular $O_2^{\cdot-}$ by overexpression of ECSOD or alteration of the extracellular Cys/CySS ratio by knockdown of xCT protein inhibited PCa cell invasion. Simultaneous overexpression of ECSOD and knockdown xCT inhibited PCa cell invasion more than overexpression of ECSOD or knockdown of xCT alone. In the co-culturing system, simultaneous overexpression of ECSOD and knockdown of xCT in prostate stromal WPMY-1 cells inhibited PCa cell invasiveness more than overexpression of ECSOD alone. The decrease in PCa invasion correlated with increased of extracellular H_2O_2 levels. Notably, overexpression of catalase in TME reversed the inhibitory effect of ECSOD on cancer cell invasion.
Conclusion: Impaired ECSOD activity and an upregulated of xCT protein expression may be clinical features of an aggressive PCa, particularly metastatic cancers and/or those with a high Gleason score. Therefore, shifting the extracellular redox state toward an oxidizing status by targeted modulation of ECSOD and xCT, in both cancer and stromal cells, may provide a greater strategy for potential therapeutic interventions of aggressive PCa.

1. Introduction

Redox state (reduction/oxidation) is the balance of reducing and oxidizing equivalents and plays important roles in several physiologic and pathologic processes. Therapies that target redox imbalances have been added to cancer regimens to target redox–related proteins that influence carcinogenesis and metastasis. Redox imbalance results from altered levels of reactive oxygen species/reactive nitrogen species (ROS/RNS) and/or antioxidant proteins. The major species of ROS/RNS include hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), superoxide radical ($O_2^{\cdot-}$), nitric oxide ($NO^{\cdot}$), and peroxynitrite [1]. Studies to date focused on the intracellular redox state; little is known about the redox

Abbreviations: H_2DCFDA, 2′,7′-dichlorofluorescein diacetate; 5-Aza-dC, 5-aza-2-deoxycytidine; CDCFDA, 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate; AdhSOD3, adenoviral vector containing human ECSOD cDNA; GFP, adenovirus containing green fluorescent protein; AdEmpty, adenovirus with no gene inserted; BPH, benign prostatic hyperplasia; CAT, catalase; Cys, cysteine; xCT, cysteine/glutamate transporter; CySS, cysteine; ECSOD, extracellular superoxide dismutase; FBS, fetal bovine serum; GSH, glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; ·OH, hydroxyl radical; IHC, immunohistochemistry; MOI, multiplicity of infection; $NO^{\cdot}$, nitric oxide; PrEC, normal human prostate epithelial cells; Nrf2, nuclear factor (erythroid-derived 2)-Like 2; PCF, polycarbonated; PCa, prostate cancer; RNS, reactive nitrogen species; ROS, reactive oxygen species; SASP, sulfasalazine; SOD, superoxide dismutase; $O_2^{\cdot-}$, superoxide radical; TMA, tissue microarray; TME, tumor microenvironment

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state in the extracellular spaces (extracellular matrix and stromal cells). We previously demonstrated that an imbalance of cysteine/cystine (Cys/CySS) pools in extracellular spaces promoted an alteration of the intracellular redox state that could be monitored by the redox status of thioredoxin 1 [2–4]. The extracellular redox state acts in concert with the intracellular redox state to control influx and efflux of ROS/RNS [1,2,5]. Both ROS/RNS and Cys/CySS are associated with invasion, hypoxia, angiogenesis, and acidosis in the extracellular spaces of cancerous tissues [1,6,7].

Among redox state regulators, the cysteine/glutamate transporter (xCT) and extracellular superoxide dismutase (ECSOD) are the primarily tumor microenvironment (TME)-redox state homeostasis regulators; the former controls the redox coupling ratio (Cys/CySS) and the latter controls the prooxidant levels including $O_2^{\bullet-}$ and H_2O_2 [1]. ECSOD (*SOD3*) is the isoform of superoxide dismutase (SOD) that contains a signaling peptide, which directs ECSOD to the extracellular spaces and facilitates its binding to heparan sulfate proteoglycans [1,8]. ECSOD serves as a modulator of redox homeostasis in extracellular spaces by removing plasma membranes/extracellular $O_2^{\bullet-}$ and producing H_2O_2 [1,8,9]. ECSOD has a longer half-life in the circulation relative to the other two SOD isoforms, copper zinc SOD and manganese SOD [10]. ECSOD expression is influenced by multiple stimuli, including angiotensin II, $NO^{\bullet}$, exercise training, and certain pathological states [8]. Several studies indicate that ECSOD has an anti-tumor effect [11], thus it is reduced in several human cancers including colon, lung, breast, thyroid, mammary, and pancreatic ductal adenocarcinoma [12–15].

On the other hand, xCT (*SLC7A11*) is a plasma membrane transporter of CySS from outside the cell to inside. While inside the cell, CySS is rapidly reduced to Cys by glutathione (GSH) or thioredoxin reductase 1, and is then either synthesized into GSH or exported to the extracellular spaces [16]. The constant flow of Cys from inside the cells provides a reducing extracellular microenvironment and consequently, Cys levels are low inside the cells [17]. Commonly, xCT is not universally expressed by cells; overexpression of xCT is reported for various cancers including aggressive prostate cancer (PCa) [3], colon [18], glioma [19], head and neck [20], lung [21,22], breast [23,24], ovarian [25,26], liver [27], and in cancer stem-like cells [28]. Due to its ability to export glutamate in exchange for CySS, down-regulation of xCT expression markedly decreases cancer cell viability and subsequently restricts the cancer cell's ability to switch between glucose and glutamine [16,22].

PCa incidence is increasing significantly worldwide [29]. Metastatic PCa is the most lethal form of this disease and a better understanding of its development may lead to effective targeted therapies. The redox state in PCa cells, stromal cells, and adjacent benign tissue has not been comprehensively studied. Herein, we analyzed a large number of prostate tissue samples to determine the expression levels of the ECSOD and xCT proteins in human benign tissues and in PCa tissues of varying Gleason scores and clinical parameters. In addition, we modulated the levels of ECSOD and xCT in both PCa epithelial and stromal prostate cells to investigate the extracellular redox state upon PCa invasion. The present study demonstrates that modulation of various TME components, for both PCa and stromal prostate cells, provides an effective anti-invasive effect that may have application as therapeutic interventions.

2. Materials and methods

2.1. Tumor microarray construction and immunohistochemistry staining

Paraffin-embedded tissue blocks were selectively cored from 165 PCa patients and 34 benign control patients, who underwent radical prostatectomy at the University of Kentucky, to construct a tissue microarray (TMA) through the Markey Biospecimen and Tissue Procurement Shared Resource, University of Kentucky-Lexington. Approval for use of human prostate tissue was obtained from the University of Kentucky Institutional Review Board. Tissue cores (2 mm) containing cancerous tissues, adjacent benign epithelial tissues, or human benign prostatic hyperplasia (BPH) were used for the construction of tissue microarrays with duplicate cores from each patient. There were 4 disease groups: Gleason scores $\geq$ 8, 7, $\leq$ 6, and BPH. PCa-TMA slides were deparaffinized in a 60 °C oven for 1 h and then rinsed in 3 changes of xylene for 10 min each. To block endogenous peroxidases, slides were immersed in 0.3% methanol/hydrogen peroxide for 20 min and then rehydrated for 1 min in 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) for 20 min at 110 °C in DAKO high pH EDTA or low pH citrate antigen retrieval buffer, as indicated. Endogenous peroxidase activity was quenched using reagent from Envision+ Kits (DAKO, Glostrup Municipality, Denmark), followed by incubation with primary antibodies ECSOD (high pH, 1:100) or xCT (low pH, 1:50) overnight at 4 °C in a humidified chamber. After washing, slides were incubated with Envision+ polymer-bound secondary antibody (DAKO), then visualized with 3,3′-Diaminobenzidine and lightly counterstained with hematoxylin. Slides were then dehydrated, cleared, and mounted with coverslips; followed by microscopic analysis. Negative control slides had the primary antibody replaced with rabbit or goat serum for quality control and validation of the staining. The information about PCa-TMA samples is displayed in Table 1. The assessments of ECSOD and xCT immunoreactivity were quantified with the Aperio imaging analysis system - Version 1 (Aperio Technologies, Inc., Vista, CA, USA), which can accurately measure protein expression patterns and morphometric characteristics in distinct tissue regions of interest (epithelial vs. stromal areas). The cytoplasmic algorithm was set to analyze the intensity (positive pixel count) of ECSOD and xCT in order to obtain an objective evaluation and avoid subjective interpretation (Supplementary Fig. 1). The threshold intensity for positive areas (weak, medium, and strong positive) ranged from 0 to 220. The threshold for strong positive intensity ranged from 0 to 100. The average intensities of ECSOD and xCT in the epithelial area were obtained from the strong intensity areas in each core, while the average intensities of ECSOD and xCT in the stromal area were obtained from all positive areas in each core (Fig. 1).

Table 1
Characteristics and clinical parameters of participants.

PCa-TMA description	Clinical parameters	# of Cases
Tissues	BPH	34
	PCa	165
PCa	Gleason ≤ 6	33
	Gleason 7	100
	Gleason ≥ 8	32
Clinical Stage	Stage I	3
	Stage II	86
	Stage III	47
	Stage IV	18
	Unknown	11
Metastatic PCa	Organ Confined	141
	Metastatic PCa	13
	Unknown	11
Smoking	Tobacco/cigar/others	52
	Non-smoking	56
	Unknown	57
Recurrence	Local/Distal Recurrence	31
	No Recurrence	82
	Unknown	52
PSA	< 10 ng/ml	3
	10–20 ng/ml	4
	> 20 ng/ml	66
Age range (years)	BPH	57–81
	PCa	41–75