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Oxidative Stress Promotes Benign Prostatic Hyperplasia

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Abstract

BACKGROUND—Benign prostatic hyperplasia (BPH) is characterized by increased tissue mass in the transition zone of the prostate, which leads to obstruction of urine outflow and significant morbidity in the majority of older men. Plasma markers of oxidative stress are increased in men with BPH but it is unclear whether oxidative stress and/or oxidative DNA damage are causal in the pathogenesis of BPH.

METHODS—Levels of 8-OH deoxyguanosine (8-OH dG), a marker of oxidative stress, were measured in prostate tissues from normal transition zone and BPH by ELISA. 8-OH dG was also detected in tissues by immunohistochemistry and staining quantitated by image analysis. Nox4 promotes the formation of reactive oxygen species. We therefore created and characterized transgenic mice with prostate specific expression of Nox4 under the control of the prostate specific ARR2PB promoter.

RESULTS—Human BPH tissues contained significantly higher levels of 8-OH dG than control transition zone tissues and the levels of 8-OH dG were correlated with prostate weight. Cells with 8-OH dG staining were predominantly in the epithelium and were present in a patchy distribution. The total fraction of epithelial staining with 8-OH dG was significantly increased in BPH tissues by image analysis. The ARR2PB-Nox4 mice had increased oxidative DNA damage in the prostate, increased prostate weight, increased epithelial proliferation, and histological changes including epithelial proliferation, stromal thickening, and fibrosis when compared to wild type controls.

CONCLUSIONS—Oxidative stress and oxidative DNA damage are important in the pathogenesis of BPH.

Keywords

oxidative stress; benign prostatic hyperplasia; Nox4; oxidative DNA damage; transgenic

INTRODUCTION

Benign prostatic hyperplasia (BPH) is one of the most common diseases of older men. Up to 80 percent of men have anatomic evidence of BPH by the 8th decade of life [1]. This benign

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growth of the prostate leads to urinary tract obstruction and thus, considerable morbidity in older men. Complications of BPH such as acute urinary retention and urinary tract infection can lead to death in some cases. Almost one third of men will require treatment for this condition in their lifetime and annually more than one billion dollars is spent on the medical and surgical treatment of this disease [2]. Thus BPH is a disease which affects the majority of older men and is of considerable medical importance.

The transition zone, which is located around the prostatic urethra, commonly increases more than 30-fold in size during the development of BPH and both epithelial and stromal elements contribute to this growth [3]. While other factors that can play a role in the symptom complex characteristic of lower urinary tract obstruction, the increased size of the transition zone tissue around the prostatic urethra is clearly of importance in the pathogenesis of this disease.

A number of studies of plasma [4–6] or urine [7] have shown increased levels of oxidative stress in patients with BPH but it has been unclear whether oxidative stress actually causes BPH. In this report we show that oxidative DNA damage in prostatic transition zone tissue is correlated with the presence and severity of BPH. In addition, a transgenic mouse model in which Nox4 is expressed in prostatic epithelium results in increased oxidative DNA damage as well as increased prostate weight, epithelial proliferation, and stromal alterations. These studies support the hypothesis that oxidative stress plays a causal role in BPH.

MATERIALS AND METHODS

Tissue Acquisition

Samples of prostate from normal transition zone (TZ) or hyperplastic transition zone (BPH) were taken from radical prostatectomies and snap frozen in liquid nitrogen. Paraffin embedded tissues from radical prostatectomy specimens were used for tissue microarray construction as described previously [8]. All tissues were collected with informed consent with the approval of the Baylor College of Medicine Institutional Review Board.

Enzyme-Linked Immune-Absorption Assay (ELISA) for Nox4

Tissue levels of Nox4 were determined using an ELISA (USCN Life Science Inc., Houston, TX: SEB924Mu/924Hu). Protein extracts were prepared and ELISA performed following the manufacturer's protocol. Absorbance was measured at 450 nm using a VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA). All determinations were performed in triplicate.

ELISA Determination of 8-Hydroxy-Deoxyguanosine (8-OH dG) Levels

Tissue levels of 8-hydroxy-deoxyguanine (8-OH dG) were determined using an ELISA (JaICA, Fukuroi, Japan; NNS-KOG-200SE-EX). DNA was extracted and ELISA performed following the manufacturer's protocol. Absorbance was measured at 450 nm using a VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA). All determinations were performed in triplicate.

Immunohistochemistry of Human and Mouse Tissues

Immunohistochemistry of the human prostate tissue microarray and mouse prostate tissues were conducted using paraffin-embedded sections. After deparaffinizing and rehydrating of the tissue section, antigen retrieval was performed for 20 min in a rice steamer in Tris-EDTA Buffer, pH 8.0 (Sigma, St. Louis, MO). The anti-Ki-67 antibody (RM-9106; Thermo Fisher Scientific, Waltham, MA) was used at 1:40 dilution. Sections were incubated with the primary antibody for 2 hr at room temperature (RT) and developed using the avidin-biotin peroxidase complex procedure (Vector Laboratories, Burlingame, CA). The detection of the antibody was performed using HRP visualization with Stable DAB Plus (Diagnostic Biosystems, Pleasanton, CA) for 2 min at RT.

For detection of 8-hydroxy deoxyguanosine (8-OH dG), samples were pretreated with RNase (100 µg/ ml) for 60 min at 37°C, then with proteinase K (10 µg/ ml) for 30 min at 37°C and finally with 4N HCL at RT for 7 min. Slides were then incubated with the primary antibody (Santa Cruz, SC-66036) at 1:500 dilution overnight at 4°C followed by blue AP visualization using Alkaline Phosphatase Substrate Kit III (Vector Laboratories).

Image Analysis of 8-OH dG Immunohistochemistry

All the images were taken using the Vectra™ Automated Multispectral Imaging System (Perkin Elmer, Waltham, MA) using Inform™ image analysis software as described previously [8] to separately analyze epithelium and stroma to quantify the number of positive nuclei in these two cell types.

Transgenic Mice

Mouse Nox4 cDNA was PCR amplified from Image consortium clone, 100066254, accession number BC165954 (Open BioSystems, Pittsburgh, PA) and subcloned into pCR2.1-TOPO (Life Technologies, Grand Island, NY). Forward and reverse primers were tagged with MfeI consensus sequences. The plasmid insert was sequenced in both directions using T7 and M13 reverse primers to ensure that no mutations occurred during amplification. The Nox4 coding sequence was excised with MfeI and ligated into the multiple cloning site on the ARR2PB-KBPA vector containing the ARR₂PB (probasin) promoter, CKR intron, and bGHpA [9]. Colonies were screened by PCR amplification and sequenced to identify clones in the appropriate orientation and to verify sequence. Restriction digested, gel-purified plasmid DNA was used by the Baylor College of Medicine Genetically Engineered Mouse Core for microinjection into FVB mouse egg pronuclei. All experiments and animal work involving transgenic mice and wild type littermates followed the Baylor College of Medicine IACUC approved animal protocol. Following microinjection, 22 pups were born from 6 litters. DNA was prepared from tail snips and screened for the presence of the transgene using primers Forward (Nox4): 5'-ATGAGGCTGCAGTTGAGGTT-3' and Reverse (ARRPB2): 5'-CTGGTCATCATCCTGCCTTT-3'. Sixteen week old transgenic and wild type (WT) littermates were sacrificed to detect levels of Nox4 gene transgene expression in the prostates of F1 progeny by quantitative real time RT-PCR using SYBR green [10] on iQ thermal cycler (Bio-Rad, Hercules, CA). Nox4 was amplified using primer pair 5'-ATCTTTGCCTCGAGGGTTT-3' and 5'-TGA-CAGGTTTGTGCTCCTG-3'. The

primer pair is specific to the transgene and does not amplify endogenous Nox4. The line of a founder with the highest levels of gene expression in prostate tissue was used for subsequent studies.

After germline transmission of Nox4 was determined, prostates were harvested from transgenic mice (Nox4) and wild-type (WT) littermate controls between 2 and 18 months of age. Prostates and seminal vesicles were harvested separately. Prostates were weighed and portions snap frozen and or fixed in neutral buffered formalin and paraffin embedded for histology and immunohistochemistry.

Quantitation of Ki-67 Immunohistochemistry

After staining prostate tissues from 2 month old WT and Nox4 mice, staining was quantitated by visual inspection. Five mice from each group were chosen at random and Ki-67 positive nuclei in the epithelial and stromal cells counted in five random 200 × microscopic fields from the dorsolateral and ventral prostate. The mean number of cells in each compartment was then calculated.

Quantitation of Stromal Thickening

Hematoxylin and eosin stained section from randomly chosen 15–18 month old WT and Nox4 mice were evaluated for stromal thickening. Five mice of each genotype were analyzed. Only ventral prostate was evaluated since the subepithelial stroma is normally thin in this lobe. Stromal thickening was quantitated in five random 200 × microscopic fields as follows. The percentage of glandular circumference in which the subepithelial stroma was thicker than three nuclei of the luminal epithelial cells was estimated in 5 percent increments. Foci with obvious tangential sectioning were excluded from analysis. The mean percentage of the glandular circumference with thickened stroma was then calculated.

RESULTS

Increased Oxidative DNA Damage in BPH Epithelium

Oxidative DNA damage is a marker of oxidative stress that is relatively stable [11]. To determine whether increased oxidative DNA damage is present in BPH, we analyzed the content of 8-OH deoxyguanosine (8-OH dG), a marker of oxidative DNA damage, in normal transition zone and BPH tissues. DNAs extracted from six normal transition zone and 32 BPH tissues were analyzed quantitatively for 8-OH dG content using an ELISA. The 8-OH dG content was significantly increased in BPH tissues compared to normal transition zone (Fig. 1A). Furthermore, the content of 8-OH dG was significantly correlated with prostate weight ($r^2 = .349$, $P < 0.04$, Pearson Product Moment; Fig. 1B).

To determine the cellular location of the 8-OH dG in the prostatic tissues, we carried out immunohistochemistry using an anti-8-OH dG antibody. Examples of 8-OH dG immunohistochemistry are shown in Figure 2A. The 8-OH dG immunoreactivity was more extensive within epithelial cells and was variable within the tissues, with some acini having strong staining while others had weaker or no staining. Staining of stromal cells was also observed. To quantify this variable expression, we carried out image analysis. There was a

significant increase in 8-OH dG staining in BPH epithelium compared to normal transition zone (Fig. 2B). The 8-OG dG level was somewhat higher in BPH stroma but this difference was not statistically significant.

Prostate Epithelial Expression of Nox4 Results in Increased Prostate Size in a Transgenic Mouse Model

The NADPH oxidases (Nox4) gene family [12] encodes a family of proteins which generate reactive oxygen species. These proteins differ in the subcellular localization and Nox4 has a primarily nuclear localization [13,14]. Nox4 has been reported to be expressed in rat prostate [15] and in human prostate cancer cells [16]. To evaluate the potential role of oxidative stress and oxidative DNA damage in promoting prostatic growth, we constructed transgenic mice in which Nox4 was expressed under the control of the a ARR2PB (probasin) prostate specific promoter. This promoter directs prostate epithelial specific expression of the transgene at the onset of sexual maturity in the transgenic male mice [17]. Transgenic mice (at least 4 months of age) were analyzed for expression of Nox4 mRNA (Fig. 3A) using a transgene specific primer set to confirm transgene expression, which showed some variability between different mice. Increased Nox4 protein expression was confirmed by Nox4 ELISA (Fig. 3B). Of note, there were substantial levels of Nox4 in the wild type mouse prostates. We then evaluated mice at 2 months of age for increased oxidative DNA damage using an 8-OH dG ELISA to confirm that Nox4 can induce oxidative DNA damage. We chose this early time point to minimize the impact of any secondary changes on this parameter. As shown in Figure 3C, 8-OH dG was significantly increased shortly after the onset of sexual maturity in the transgenic mice. Thus the Nox4 transgenic mice express the Nox4 transgene and shown increased levels of oxidative DNA damage.

These mice were followed and analyzed for gross and microscopic prostatic pathology at intervals over the course of more than 18 months. A significant increase in prostate weight was noted in mice compared to age matched littermate controls, both before the age of 12 months and subsequently (Fig. 4A). Immunohistochemistry for Ki-67, a proliferation marker, showed a significant increase in proliferating epithelial cells of the dorsolateral and ventral prostates of Nox4 mice compared the WT littermate controls (Fig. 4B) but no significant change in stroma.

Histological analysis showed variable histology with focally increased numbers of epithelial cells with piling up and crowding of cells in ventral and/or dorsolateral prostate (Fig. 5). This was most easily appreciated in ventral prostate, which has very thin stroma and mainly flat epithelium. Changes are illustrated in Figure 5A–D. Normal glands adjacent to the focal abnormal areas are indicated by arrows. This was accompanied in most (but not all) foci by stromal thickening and fibrosis with increased spindled cells that appeared to be fibroblasts. Trichrome stains accentuate the areas of fibrosis with collagen deposition which surrounded the somewhat hypertrophied smooth muscle cells (Fig. 5E–F). Similar changes were seen focally in the dorsolateral prostate as well (Fig. 5G–I). In addition, in agreement with others [18], we saw similar but less marked changes focally in older WT mice. We quantitated the extent of stromal thickening in the ventral prostates of WT and Nox4 mice and results are shown in Figure 5J. We confirmed the presence of focal stromal thickening in WT and that it

is significantly more common in the Nox4 mice although it only involves a small percentage of the total circumference of the acini even in the Nox4 mice. We noted some chronic inflammatory cells in the prostate of the Nox4 mice but this was not noticeably more than in the WT mice.

DISCUSSION

Oxidative stress can have direct and indirect growth promoting activities. Our studies of human tissues show that 8-OH dG, a marker of oxidative stress, is increased in BPH and significantly correlated with prostate weight. The 8-OG dG is predominantly in the epithelium but is also present in stromal cells as well. These studies confirm and extend previous observations by other groups. Olinski et al. [19] have shown that BPH tissues have higher levels of DNA base lesions typical of oxidative DNA damage in DNAs from BPH tissue (relative to matched normal tissue). Consistent with this finding, Malins et al. [20] have shown by infrared spectroscopy that there were higher levels of structural alterations in DNA from BPH tissue consistent with oxidative damage when compared to normal tissue. Our finding that 8-OH dG levels correlate with prostate weight argues that oxidative stress and/or oxidative DNA damage are functionally important in BPH. Our ARRR2PB-Nox4 transgenic mice also show increased 8-OH dG and over a period of 18 months, showed increased prostate weight which was associated with epithelial proliferation and stromal alterations including fibrosis. It should be noted that human BPH characterized not only by net cell growth but also the development of fibrosis [21–24]. Our studies strongly support the concept that oxidative stress and/or oxidative DNA damage plays a significant role in the pathogenesis of BPH.

A major question is why there is increased oxidative stress and/or oxidative DNA damage in BPH. Olinski et al. [19] have shown that there is decreased activity of proteins that decrease oxidative stress and oxidative DNA damage such superoxide dismutase and/or catalase in the BPH tissues. Consistent with these human observations, superoxide dismutase gene expression is decreased in the prostate of older rats [25]. Of note, expression microarray studies of aging rat prostate have revealed upregulation of multiple genes that are increased during oxidative stress [25]. Whether there is increased expression of proteins that can increase oxidative stress in BPH tissues is unclear. We have confirmed expression of Nox4 in both normal transition zone and BPH tissue by ELISA but there was no significant difference in protein levels (data not shown). Although Nox4 is not increased in BPH, it is present in normal transition zone and BPH epithelium and may generate oxidative DNA damage in this context. However, other reactive oxygen generating proteins have not been systematically examined. It should be noted that alterations in androgen and/or estrogens can increase oxidative stress in the prostate and alteration of both hormones have been described in patients with BPH. Finally, it is possible that inflammatory cells that are almost always present in BPH may contribute to oxidative stress/oxidative DNA damage by production of reactive oxygen species.

Several groups have shown that patients with BPH have increased plasma levels malondialdehyde [5,6,26], which is a product of oxidative damage. Some of the increased malondialdehyde is from the hyperplastic prostate tissue since plasma malondialdehyde

levels decrease after surgical removal of BPH tissue. However, patients with BPH appear to have a systemic increase in oxidative stress due to dietary, environmental, and/or genetic factors since malondialdehyde levels remain elevated even after surgery [5]. Such systemic factors would exacerbate local factors in the prostate transition zone leading to oxidative stress. The nature of the factors that might lead to systemic oxidative stress is unclear but deserves further study since dietary or environmental factors can be modified to potentially prevent disease.

The underlying mechanism by which oxidative stress/oxidative DNA damage results in increased prostate growth and other cellular changes is not known. We favor the hypothesis that this is related to two distinct processes. First, it is known that oxidative stress can directly increase activity of growth, promoting signaling pathways such as MAP kinase and PI-3K/AKT [27,28] and in this manner induce cellular proliferation. Second, oxidative DNA damage can lead to results cellular senescence in a subset of prostatic epithelial cells and secondary secretion of multiple cytokines due to the senescence associated secretory response. We have shown in prior studies [8,29–32], based on multiple markers, that senescent epithelial cells are significantly increased in BPH tissues, as are cytokines that are up regulated as part of the senescence associated secretory response such as IL-1 α and IL-8. The senescent epithelial cells are irregularly distributed within the epithelial acini, similar to the pattern seen in 8-OHdG immunohistochemistry. We have also observed smaller numbers of senescent stromal cells (compared to epithelium) and in this study, we also observed stromal cells with oxidative DNA damage. Of note, Nox4 induces cellular senescence when expressed in mouse NIH3T3 fibroblasts in vitro [33]. The senescent epithelial and stromal cells secrete cytokines such as IL-17 that may attract inflammatory cells into the prostate that further increases oxidative stress. However, we currently do not have evidence evaluating the relative importance of these two potential pathways in humans or in our mouse model and further studies are needed.

CONCLUSIONS

Our studies reveal that increased levels of oxidative DNA damage are present in BPH and play a causal role in the increased tissue proliferation and other changes seen in this disorder.

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Fig. 1.

Increased 8-OH deoxyguanosine (8-OH dG) is present in BPH and correlates with prostate weight. **(A)** 8-OH dG levels, as determined by ELISA of DNA extracts from BPH and normal transition zone tissues. Mean $\pm$ SEM is shown. ** $P < .01$; t -test **(B)** Correlation of 8-OH dG levels and prostate weight ($r^2 = .349$, $P = .03$; Pearson Product Moment).

Fig. 2.

Immunohistochemical localization of 8-OH dG in normal transition zone and BPH tissues.

(A) Immunohistochemistry using anti-8-OH dG antibody of normal transition zone and BPH tissues (100 ×). Note variable, primarily epithelial staining which is greater in BPH tissues.

(B) Image analysis of 8-OH dG staining in normal transition zone and BPH tissues. Data is expressed as percent nuclear area staining with 8-OH dG in either the epithelial or stromal compartments. Mean $\pm$ SEM is shown. ** $P < .01$; t -test.

Fig. 3.

ARR2PB-Nox4 transgenic mice express Nox4 and have increased oxidative DNA damage in the prostate. **(A)** Q-RT-PCR of RNAs from ARR2PB-Nox4 transgenic mice or wild type littermate controls using a transgene specific promoter. Some variability in expression of the transgene is noted. **(B)** Nox4 protein levels in prostate protein extracts from ARR2PB-Nox4 mice (Nox4) and wild type (WT) littermate controls as measured by ELISA. Mean $\pm$ SEM is shown. *** $P < .001$; t -test. **(C)** 8-OHdG levels in DNA extracts from ARR2PB-Nox4 mice (Nox4) and wild type (WT) littermate controls as measured by ELISA. Mean $\pm$ SEM is shown. *** $P < .001$; t -test.

Fig. 4. Increased prostate size and epithelial proliferation in ARR2PB-Nox4 transgenic mice. (A) Prostate weight of mice less than 12 months or greater than 12 months in ARR2PB-Nox4 mice (Nox4) and wild type (WT) littermate controls. Mean $\pm$ SEM is shown. * $P < .05$; t -test. (B) Ki-67 positive nuclei per 200 $\times$ field in 2 month old ARR2PB-Nox4 mice (Nox4) and wild type (WT) littermate controls in either the epithelial or stromal compartments. Mean $\pm$ SEM is shown. ** $P < .01$; t -test. (C) Examples of Ki-67 immunohistochemistry in ventral prostate of WT and Nox4 mice (200 $\times$).

Fig. 5.

Focal histopathological alterations of the prostate in ARR2PB-Nox4 transgenic mice. Histological appearance of ventral prostates (VP) and dorsolateral (DLP) and in ARR2PB-Nox4 transgenic mice and littermate controls at 15–18 months of age. (A) Normal ventral prostate in WT mouse. (B–D) Ventral prostate from Nox4 mice. Arrows show examples of normal acini while double arrows show abnormal acini with stromal thickening and epithelial piling up. Figure B shows scattered chronic inflammatory cells in stroma but similar areas were also seen in some WT prostates. (E, F) Trichrome stain of ventral prostate of WT (E) and Nox4 (F) mice. Note increased fibrous tissue (blue staining) in Nox4 mouse.

(G) Normal dorsolateral prostate in WT mouse. (H, I) Dorsolateral prostate from Nox4 mice. Note piling up of epithelial cells and stromal thickening (double arrows). All photomicrographs at $200\times$ original magnification. (J) Quantitative evaluation of stromal thickening in the ventral prostates of WT and Nox4 mice was performed as described in Materials and Methods. The mean percentage of the circumference of the acini is shown ($\pm$ SEM). ** $P < .01$, Mann Whitney.