Tumorigenesis and Neoplastic Progression

Sex Hormones Induce Direct Epithelial and Inflammation-Mediated Oxidative/Nitrosative Stress That Favors Prostatic Carcinogenesis in the Noble Rat

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Oxidative and nitrosative stress have been implicated in prostate carcinogenesis, but the cause(s) of redox imbalance in the gland remains poorly defined. We and others have reported that administration of testosterone plus 17β-estradiol to Noble rats for 16 weeks induces dysplasia and stromal inflammation of the lateral prostate (LP) but not the ventral prostate. Here, using laser capture microdissected specimens, we found that the combined hormone regimen increased the expression of mRNA of specific members of NAD(P)H oxidase (NOX-1, NOX-2, and NOX4), nitric-oxide synthase [NOS; inducible NOS and endothelial NOS], and cyclooxygenase (COX-2) in the LP epithelium and/or its adjacent inflammatory stroma. Accompanying these changes was the accumulation of 8-hydroxy-2'-deoxyguanosine, 4-hydroxyxenonenal protein adducts, and nitrotyrosine, primarily in the LP epithelium, suggesting that NOX, NOS, and COX may mediate hormone-induced oxidative/nitrosative stress in epithelium. We concluded that the oxidative/nitrosative damage resulting from the testosterone-plus-17β-estradiol treatment is not solely derived from stromal inflammatory lesions but likely also originates from the epithelium per se. In this context, the up-regulation of COX-2 from epithelium represents a potential mechanism by which the hormone-initiated epithelium might induce inflammatory responses. Thus, we link alterations in the hormonal milieu with oxidative/nitrosative/inflammatory damage to the prostate epithelium that promotes carcinogenesis. (Am J Pathol 2007, 171:1334–1341; DOI: 10.2353/ajpath.2007.070199)

Oxidative stress (OS) and nitrosative stress (NS) refer to a state of redox imbalance leading to excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), respectively, that overwhelms the antioxidant defenses of a cell. Such imbalances have recently been implicated in the genesis of prostate cancer (PCa). Thus, biomarkers of OS and NS have been reported in human prostatic intraepithelial neoplasia, also termed dysplasia purported PCa precursor, and in cancer of the prostate.1,2

Reactive oxygen species are normally generated as byproducts of aerobic respiration in the mitochondria, as well as a variety of inflammatory cells, producing large quantities of ROS and RNS.3 In addition to these two sources, superoxide-generating homologues of phagocytic NAD(P)H oxidase catalytic subunit gp91phox (NOX-1 and NOX-3, NOX-4, and NOX-5) and nitric-oxide synthase (NOS) family members, including inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS), are primary sources of superoxide (O2) and nitric oxide (NO), respectively, in nonphagocytic cells, as well as in various epithelial cell types.4 With regard to carcinogenesis, NOX-1 and NOX-5 have been reported to exert major effects on prostate tumor growth and angiogenesis.5,6-NO is also an important signaling molecule for inducing inflammation, including those of the prostate.7 Moreover, the conversion of arachidonic acid to prostaglandins via cyclooxygenases (COX-1 and COX-2) are potent inducers of inflammation.8 In addition to their proinflammatory actions, COXs also produce peroxyl radicals as a byproduct of prostaglandin synthesis.9,10 Apropos to carcinogenesis of the prostate, consistent overexpression of COX-2 was found in proliferative inflammatory atrophy (PIA)11 and prostatic intraepithelial

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neoplasia lesions, but its expression level in PCa remains controversial. Damage to cellular DNA and protein, produced directly in tissues or indirectly by ROS and RNS, as a consequence of inflammation, has been implicated as causative factors for a variety of cancers. In this regard, PIA, an inflammation-associated proliferative lesion in the human prostate, has been proposed as a precursor of prostatic intraepithelial neoplasia and PCa. It has been postulated that the genesis of PIA results from ROS/RNS produced by inflammatory cells that induce epithelial atrophy and subsequent regenerative proliferation, which has been considered to represent early changes in the carcinogenic process.

Although androgens and estrogens have major influences on the regulation of cell growth and differentiation, as well as on malignant transformation of the prostate, information is limited concerning the role that these hormones may play in the regulation of redox balance in this organ. We previously demonstrated that androgens and estrogens can influence redox status and antioxidant enzyme activity in the prostates of Noble (NBL) rats. Exposure of NBL rats to treatment with a combination of testosterone (T) and 17β-estradiol (E2) for 16 weeks induces dysplasia accompanied by inflammation selectively in the lateral prostates (LPs), and longer exposure of these steroids induces a high incidence of adenocarcinomas in the LP. However, neither of these lesions are induced in the ventral prostates (VPs) of all treated animals. Using the NBL experimental model, our current results provide evidence that this steroid treatment selectively causes major disruptive effects in the OS/NS of the LP that affects damage to DNA, protein, and lipids. Thus, for the first time, we provide a causative link to sex hormone-mediated alterations in redox imbalance and cellular damage in the prostate, which strongly implicates this mechanism in the development of cancer in the LP of the gland.

Materials and Methods

Animals and Hormone Treatments

Protocols of animal usage were approved by the University of Massachusetts Medical School Animal Care and Usage Committee. NBL rats were purchased from Charles River Laboratories (Kingston, NY), kept under standard conditions, and treated as previously reported. In brief, control rats (n = 8) were implanted with empty capsules. Hormone-treated rats (n = 8) were implanted with 2-cm lengths of Silastic tubing (1.0-mm inner diameter × 2.2-mm outer diameter; Dow Corning, Midland, MI) that were tightly packed with 14.4 ± 2.1 mg of T (Sigma, St. Louis, MO), and 1-cm lengths of the same tubing were packed with 14.8 ± 2.6 mg of E2 (Sigma). At the end of a 16-week treatment period, animals were sacrificed with an overdose of isoflurane, and VPs and LPs were excised. One half of each lobe was processed for histological examination, and the other half was snap frozen for RNA extraction or laser capture microdissection (LCM).

Histopathology and LCM

Formalin-fixed samples were processed for light microscopy. Twelve step-sections sampled serially through an entire half-LP/ventral prostate (VP), in a single-blinded manner, were subjected to histological examination for inflammatory, dysplastic, and malignant lesions by I.L. The other half of the LP was embedded in Tissue-Tek O.C.T. compound (Sakura Finetek USA, Torrance, CA) and then snap frozen in liquid nitrogen. Serial frozen sections were cryocut from each specimen. The first section of a replicate series was fixed, stained with hematoxylin, and used as a guide for the LCM (Arcturus, Mountain View, CA) conducted on the next serial sections, as previously described.

Radioimmunoassay (RIA) of Serum T and E2 Levels

Serum total T and E2 levels from at least five animals in the control and in the T+E2-treated groups were measured by RIA, which was conducted in the ILAT Steroid RIA Laboratory, University of Massachusetts Medical School (Worcester, MA). The RIA kits for T and E2 assays were provided by Diagnostic Products (Los Angeles, CA) and Diagnostic Systems Laboratories (Webster, TX), respectively. The sensitivities of the T and E2 assays are 0.04 ng/ml and 2.2 pg/ml, respectively.

RNA Isolation, cDNA Synthesis, and Real-Time Quantitative Polymerase Chain Reaction

Total RNA was isolated and reverse-transcribed into cDNA as previously described. Real-time quantitative polymerase chain reaction (PCR) was performed with the iCycler IQ Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA) as reported. Intron-spanning, gene-specific primers for nNOS (sense: 5'-CCCTGGCAATGTAAGGTT-3', antisense: 5'-TCTTCTCCCTCCTACAGATCC-3'), iNOS (sense: 5'-CAGTTTGGAGTCACCATG-3', antisense: 5'-ACCACGTTAATGATGAGGAGG-3'), eNOS (sense: 5'-TCAGGGAGGGCACCAGATC-3', antisense: 5'-CACGTTGTTCACGAGCAC-3'), COX-1 (sense: 5'-GGCTGAGTTAATGTC-3', antisense: 5'-ACGTGGTGTTTCATG-3'), and COX-2 (sense: 5'-GGCTTCAACACACCTGGA-3', antisense: 5'-CTGACTGATTCCACCCAC-3') were designed with PRIMER 3 software, purchased from MWG Biotech (High Point, NC), and optimized for real-time PCR. Primers and PCR conditions for NOX-1, NOX-2, NOX-4, superoxide dismutase 1 (SOD-1), superoxide dismutase 2 (SOD-2), and ribosomal protein L19 (RPL19) have been documented previously.

PCR products generated by each primer pair were column-purified by the Wizard SV gel and PCR clean-up system (Promega, Madison, WI) and quantified spectrophotometrically at 260 nm. The molecular mass of target cDNA was calculated on the following basis (Real-time PCR Instruction Manual; Applied Biosystems, Foster City, CA): mass of double-stranded DNA = number of bp × 1.096 × 10−21 g. Stock solutions of 300,000 copies of...
each standard cDNA/5 μl were prepared and serially diluted (1:10) with nuclease-free water. The target-gene transcript copy numbers in tissues/LCM samples were determined with a standard curve generated by plotting cycles at threshold against the logarithmic values of the standard copy number. The loading control was normalized by using the RPL19 level.

**Immunohistochemical Analysis of OS/NS Biomarkers**

Immunohistochemical staining for 8-hydroxy-2′-deoxyguanosine (8-OHdG), 4-hydroxynonenal (4-HNE), and nitrosyrosine was conducted on paraffin slides as previously described.\(^2\) For controls, the primary antibodies were preincubated with either excess antigens [1.5 mg/ml 8-OHdG] (Sigma) or 10 mmol/L nitrosyrosine (Calbiochem, San Diego, CA) or were replaced with the corresponding normal isotype serum (Zymed, South San Francisco, CA).

**Statistical Analyses**

The statistical significance of the difference in expression levels between treatment groups was determined with Systat software (Student version 6.0.1) (SPSS, Chicago, IL) for one-way analysis of variance, followed by Tukey’s post hoc analyses or Student’s t-test. A P value of ≤0.05 was taken as a statistically significant difference between two groups.

**Results**

**T+E2 Regimen Maintained Physiological T Levels and Elevated Levels of E2**

RIA analyses of serum T and E2 levels (Figure 1, A and B, respectively) revealed that the number and the size of Silastic capsules used maintained the serum T at physiological levels (untreated control rats 8.0 ± 0.49 ng/ml, P < 0.05) and increased E2 levels four- to fivefold (39.6 ± 3.5 pg/ml, P < 0.05) when compared with the untreated control rats (8.0 ± 1.14 pg/ml). The elevated E2 levels are within the physiological range of cyclic fluctuation of E2 (from 17 ± 2 to 88 ± 2 pg/ml) in female rats during their 4-day estrous cycle.\(^2\)

**T+E2 Co-Treatment Selectively Induced Inflammation and Premalignant Dysplasia in the LPS, but Not in the VPs, of Treated Rats**

The T+E2-induced histopathological changes in the NBL rat model have been reported previously.\(^2\) In this article, we give only the morphological alterations directly relevant to the present study. The LPSs and VPs of untreated rats were histologically normal (Figure 1, C and F, respectively). Dysplasia and inflammation developed specifically in the LPSs (Figure 1D), but not in the VPs (Figure 1G), of all treated rats. A spectrum of focal dysplastic epithelial lesions associated with variable degrees of chronic inflammation in the stroma was observed in the LPSs of treated rats, as has been reported previously.\(^2\) We found, however, that focal dysplastic lesions were sometimes evident in the LP region in the absence of inflammation (Figure 1, E and inset).

**T+E2 Co-Treatment Selectively Increased the Expression of NOXs, NOSs, and COXs in the LPSs, but Not in the VPs, of Treated Animals**

In the VP, only the expression of eNOS mRNA was significantly increased following T+E2 treatment (Figure 2B). In contrast, in the LP, T+E2 treatment significantly increased mRNA levels of all three NOX isoforms [NOX-1 (threefold, P < 0.005), NOX-2 (2.5-fold, P < 0.005), and NOX-4 (fourfold, P < 0.001)] (Figure 2A, left: bulk tissue), two NOS isoforms [iNOS (28-fold, P < 0.01) and eNOS (5.5-fold, P < 0.001)] (Figure 2B, left: bulk tissue), and two COX isoforms [COX-1 (twofold, P < 0.005) and COX-2 (sixfold, P < 0.005)] (Figure 2C, left: bulk tissue) compared with those of untreated controls. However, the mRNA levels of nNOS (Figure 2C, left: bulk tissue), SOD-1, and SOD-2 (data not shown) remained unchanged in the LPSs and VPs following T+E2 exposure.

**T+E2 Co-Treatment Increased Expression of Specific NOXs, NOSs, and COXs in the Stromal and Epithelial Compartments of the LPSs of Treated Animals**

Because most of the changes in ROS/RNS-generating enzymes induced by the combined hormone treatment occurred in the LP, we next used LCM to determine which tissue compartment, epithelium or stroma, was the major source of these alterations (Figure 2, A to C, right: LCM-derived cells). After the T+E2 treatment, marked increases in NOX-1 (no mRNA was detected in the epithelium of control LPSs), NOX-2 (threefold, P < 0.05), NOX-4 (no mRNA was detected in the epithelium of control LPSs), iNOS (30-fold, P < 0.05), eNOS (threelfold, P < 0.01), COX-1 (fivefold, P > 0.05), and COX-2 (110-fold, P < 0.005) mRNA were observed in the dysplastic epithelium. Comparably increased levels of expression of these genes also were found in the non-dysplastic epithelium of treated rats (data not shown). The combined hormone treatment also elicited pronounced up-regulation of NOX-1 mRNA expression (no mRNA was detected in the stroma of control LPSs), NOX-2 (twofold, P < 0.05), iNOS (16-fold, P < 0.05), and eNOS (fivefold, P < 0.01) in the stroma associated with inflammatory cells.
T+E2 Co-Treatment Induces Lobe-Specific Elevation of 8-OHdG, 4-HNE Protein Adducts, and Nitrotyrosine in the LPs, but Not in the VPs, of Treated Rats

For oxidative DNA and protein damage, the best recognized biomarkers are 8-OHdG bases in DNA and 4-HNE protein adducts, respectively. Regarding the nitrosative protein damage, nitrotyrosine, the stable modification of tyrosine residues, is formed. Extensive oxidative and nitrosative damage, as reflected by an accumulation of these surrogate markers, may lead to the genomic and proteomic alterations considered critical for neoplastic transformation.

In LPs and VPs of untreated control animals, modest levels of heterogeneous immunostaining for 8-OHdG and nitrotyrosine were seen frequently in the glandular epithelium and occasionally in the adjacent stroma. Nuclear staining of 8-OHdG was always relatively stronger in the ducts of both LP and VP than in their acini. However, the immunostaining of nitrotyrosine was more intense in the LP ducts than in the acini, whereas staining...
of nitrotyrosine in the ducts of VP (Figure 5H) was more conspicuous than that in the acinar region.

After T+E2 exposure, the LPs showed a widespread increase in 8-OHdG (Figure 3, C–F), 4-HNE (Figure 4, C–F), and nitrotyrosine (Figure 5, C–F) staining in ducts, dysplastic epithelia, and nondysplastic (apparently morphologically normal) glands. This dramatic increase in staining occurred whether inflammatory foci were immediately adjacent or distant to the involved ducts and glands. In most instances, vascular endothelium and inflammatory cells in the stroma also showed consistently high levels of nitrotyrosine immunostaining (data not shown). In marked contrast, glands and ducts in the VPs of the T+E2-treated animals exhibited no changes in 8-OHdG (Figure 3, I and J) and 4-HNE protein (Figure 4, I and J) immunostaining compared with that of untreated controls; however, a slight increase in nitrotyrosine accumulation (Figure 5, I and J) was evident in the epithelium. Immunopositivity was eliminated by preabsorption with counterpart antigens or replacement with isotype normal serum (data not shown).

Discussion

In the current study, we demonstrated that androgen-supported estrogen action induced oxidative/nitrosative damage to DNA (8-hydroxy-2-deoxyguanosine bases) and proteins (4-hydroxynonenal adducts and nitrotyrosine),
primarily in prostatic epithelia and, to a lesser extent, in the stroma of the cancer-susceptible LP of the NBL rat compared with values in untreated controls. These changes were associated with the elevated expression of mRNA of specific ROS/RNS-generating enzymes (NOX, NOS, and COX) in LCM-captured epithelial or stromal cells. In contrast, however, the administration of T and E2 together did not induce OS/NS-related changes in the VP, where this treatment does not cause dysplasia/carcinoma. Our findings suggest that hormone-induced OS/NS is closely correlated with the lobe-specific development for prostate cancer in this animal model.

Our data indicate that the sex hormone-induced OS/NS can arise both directly from prostatic epithelium and indirectly from inflammatory cells. This may occur through the dramatic elevation of steady-state levels of mRNA of ROS/RNS-generating enzymes (NOX-1, NOX-2, iNOS, eNOS, and COX-2) in either/both tissue components of LP following hormonal treatment. In contrast to LPs, inflammation, redox disruption, and elevation of the pro-oxidant/-pro-nitrosant gene expression was virtually absent in VPs of treated rats. In this context, it has been proposed that OS/NS of an inflammatory source, in particular, contribute to prostate carcinogenesis. As observed

Figure 4. Immunolocalization of 4-HNE protein adducts in the LP and VP after T+E2 co-treatment. A and B: Untreated control LP. Positive immunostaining is observed in the acinar (A) and ductal epithelium (B). C: T+E2-treated LP. A noticeable increase in immunoreactivity of 4-HNE protein adducts in a dysplastic gland closely associated with stromal inflammatory infiltration is shown. D: T+E2-treated LP. A representative high-grade dysplastic gland, which lacks the induction of stromal inflammatory response, exhibits intense immunostaining in the epithelium. E: T+E2-treated LP. A morphologically normal gland shows intense staining in the epithelium. Note the sparse staining in the adjacent stroma with no association with stromal inflammation. F: T+E2-treated LP. A modest increase in immunostaining is seen in the ductal epithelium. G and H: Untreated control VP. Weak and sporadic immunostaining is detected in acini (G), whereas relatively strong staining is found in ducts (H). I and J: T+E2-treated VP. No apparent increase of the staining in the glandular (I) and ductal (J) regions is seen. Scale bars = 20 μm.

Figure 5. Immunolocalization of nitrotyrosine in the LP and the VP after T+E2 co-treatment. A and B: Untreated control LP. Positive immunostaining is localized predominantly to the glandular (A) and ductal (B) epithelium. C: T+E2-treated LP. A representative dysplastic acinar region shows a remarkable increase in nitrotyrosine staining in the epithelium and the interstitial inflammatory stroma. D: T+E2-treated LP. High-grade dysplastic epithelium with minimal association with stromal inflammation shows a strong and uniform nitrotyrosine staining. Note the generally weak staining in the interstitial stroma. E: T+E2-treated LP. Intense nitrotyrosine immunoreactivity in a nondysplastic gland is shown. Note the sparse staining in the adjacent stroma without inflammatory infiltration. F: T+E2-treated LP. There is a modest increase of nitrotyrosine staining in the ductal epithelium. G and H: Untreated control VP. Immunostaining for nitrotyrosine in the acinar epithelium (G) is generally weak or negative. A representative duct (H) shows conspicuous staining in the epithelium. I and J: T+E2-treated VP. There is only a mild elevation of nitrotyrosine staining in acini (I) and ducts (J). Scale bars = 20 μm.
exhibit an increase in the ratio of estrogen to androgen in elderly men. In this regard, elderly men frequently obscure, chronic inflammatory lesions and PCa are com-factors or agents causing COX-2 elevation in PIA remain obscure. Chronic inflammatory lesions and PCa are com-factors or agents causing COX-2 elevation in PIA remain unclear. The "inflammatory stroma" comprises primarily epithelial cells infiltrates, fibroblasts, smooth muscle cells, and endothelium. PG, prostaglandins.

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in the current study, however, the weak topographic association between epithelial OS/NS and inflammatory cell infiltrates corroborates the concept that altered hormonal milieu can directly induce redox disruption in epithelium per se, which is independent from the indirect effects of stromal inflammatory cells.

COX-2 up-regulation in epithelium represents a potential mechanism by which the hormone-initiated epithelium might induce and dictate inflammatory responses in under-lying stroma. The T+E2-treated epithelium, which exhibits high levels of COX-2, may then release prostaglandins, which promote an inflammatory cell infiltration into the adjacent stroma that can augment the epithelial OS/NS-related injury in a reciprocal manner. Thus, we propose that these multifaceted, hormone-driven cellular events may create a vicious double-feed forward cycles of dysregulated ROS/RNS metabolism and inflammatory activation between the initiated epithelium and the inflammatory stroma that could ultimately lead to a pro-cancer microenviron-ment in the tissue. The hormonal actions on OS/NS are discussed in detail in the Discussion. The "inflammatory stroma" comprises primarily epithelial cells infiltrates, fibroblasts, smooth muscle cells, and endothelium. PG, prostaglandins.

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Therefore, the hormone-initiated epithelium may also provoke a local inflammatory response through the up-regulation of COX2. Thus, we propose that these multifaceted, hormone-driven cellular events may create vicious "double-feed forward cycles" of dysregulated ROS/RNS metabolism and inflammatory activation between the initiated epithelial cells and the reactive inflammatory stroma (Figure 6) that could ultimately create a pro-oxidant/pro-nitrosant microenvironment that favors the initiation of prostate cancer.47

In summary, for the first time we show evidence that the circulating hormonal milieu is a critical determinant of NOX, NOS, and COX expression in the prostate epithelium and/or infiltrating inflammatory cells. The resulting oxidative and nitrosative DNA and protein damage, as a direct conse-quence of ROS/RNS overproduction by the epithelium and/or mediated indirectly by stromal inflammation, may subsequently give rise to dysplasia and carcinoma. Notably, the hormone-initiated epithelium may also provoke a local inflammatory response through the up-regulation of COX2. Thus, we propose that these multifaceted, hormone-driven cellular events may create vicious “double-feed forward cycles” of dysregulated ROS/RNS metabolism and inflammatory activation between the initiated epithelial cells and the reactive inflammatory stroma (Figure 6) that could ultimately create a pro-oxidant/pro-nitrosant microenvironment that favors the initiation of prostate cancer.47

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