

Antioxidant Enzyme Expression and Reactive Oxygen Species Damage in Prostatic Intraepithelial Neoplasia and Cancer

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BACKGROUND. Oxidative stress results in damage to cellular structures and has been linked to many diseases, including cancer. The authors sought to determine whether the expression of three major antioxidant enzymes, copper-zinc superoxide dismutase (SOD1), manganese superoxide dismutase (SOD2), and catalase, was altered in human prostate carcinoma and its likely precursor, high grade prostatic intraepithelial neoplasia (PIN). The level of reactive oxygen species damage was evaluated by measuring the expression of the DNA adduct 8-hydroxydeoxyguanosine.

METHODS. The authors evaluated the tissue expression of the antioxidant enzymes in prostate carcinoma by immunohistochemistry, immunogold electron microscopy, and enzymatic assay. The polymerase chain reaction was used to amplify and screen tissue specimens for the genes of SOD1, SOD2, and extracellular SOD (SOD3). Matched paraffin embedded tissue sections were evaluated by RNA in situ hybridization for expression of SOD1 and immunohistochemically for the DNA adduct 8-hydroxydeoxyguanosine.

RESULTS. All prostatic tissues, including cancer, displayed immunoreactivity for the three antioxidant enzymes in epithelial cells, with no staining of the stroma, inflammatory cells, or endothelial cells. The number of immunoreactive cells was greater in benign epithelium than in PIN and cancer for each enzyme. The mean percentage and intensity of immunoreactive cells was greatest for SOD2, intermediate for SOD1, and lower for catalase. Staining in cancer was heterogeneous. Immunogold ultrasound studies revealed strong mitochondrial labeling for SOD2, which was greater in benign epithelium than in cancer; SOD1 labeling was invariably weaker, with nuclear labeling in benign epithelium and cytoplasmic labeling in cancer cells. There was no difference in enzyme activity for the three antioxidant enzymes between benign epithelium and cancer. No mutations were found in the 5 exons of SOD1, 5 exons of SOD2, and 3 exons of SOD3, except for 3 of 20 cases with polymorphisms for exon 3 of SOD1. Intense nuclear immunoreactivity for 8-hydroxydeoxyguanosine was present in fewer than 3% of epithelial cells, with no apparent differences among benign epithelium, PIN, and cancer.

CONCLUSIONS. SOD1, SOD2, and catalase had lower expression in PIN and prostate carcinoma than in benign epithelium. The number of immunoreactive cells in PIN was similar to cancer, indicating that these are closely related. Enzyme activities were variable, with no difference between benign epithelial cells and cancer, although this lack of change in enzyme activity could have been due to the presence of contaminating benign cells within the cancer specimens. The results of reactive oxygen species damage were found only in the epithelium and not in the stroma. Expression of the DNA adduct 8-hydroxydeoxyguanosine was present in fewer than 3% of cells, with no apparent differences among benign epithelium, PIN, and cancer. These findings suggest that oxidative stress is an early event in carcinogenesis. *Cancer* 2000;89:123-34. © 2000 American Cancer Society.

KEYWORDS: prostatic intraepithelial neoplasia, superoxide dismutase, catalase, reactive oxygen species, antioxidant enzymes, prostatic neoplasms, prostate carcinoma.

In 1999, about 179,300 American men were diagnosed with prostate carcinoma, most between the ages of 50 and 75 years.¹ The risk of prostate carcinoma increases dramatically after age 40 years, such that by age 80 years, about 80% of men have prostate carcinoma.^{2,3} What does prostate carcinoma have in common with aging other than declining androgen levels? Aging is closely linked with a shift in the pro-oxidant-antioxidant balance of many tissues toward an oxidative state with reactive oxygen species damage. We questioned whether alterations in the expression and activity of antioxidant enzymes and oxidative stress could be causally involved in the development of prostate carcinoma.

Antioxidant enzymes are endogenous proteins that work in combination to protect cells from reactive oxygen species (ROS) damage. ROS are physiologic by-products capable of directly injuring cells, presumably resulting in part from an imbalance of antioxidant enzymes and free radicals. Oxidative damage is diminished by antioxidant vitamins, non-provitamin A carotenoids, and trace elements such as selenium. In addition to aging, oxidative stress is linked to trisomy 21 (Down syndrome), familial amyotrophic lateral sclerosis, Alzheimer disease, ischemic-reperfusion injury, pulmonary oxygen toxicity, radiation effects, chemotherapy effects, mutagenesis, atherosclerosis, and select cancers such as mesothelioma.

Data are limited regarding the pro-oxidant-antioxidant balance in human prostate carcinoma and its likely precursor, prostatic intraepithelial neoplasia (PIN). Previous reports showed a decrease in immunoreactivity in PIN and cancer for some of the antioxidant enzymes when compared with benign epithelium.⁴ The activity of superoxide dismutase in prostatic stroma is higher than in the epithelium, and both decline with patient age.⁵ Benign prostatic hyperplasia has lower levels of antioxidant enzyme activity and DNA base modifications than normal tissue,⁶ although the source of the normal tissue in one study may have been contaminated by hyperplastic tissue.⁴ To our knowledge, there are no genetic studies of antioxidant enzymes in prostate carcinoma.

We undertook a comparative study of 27 radical prostatectomy specimens removed for clinically localized prostate carcinoma to determine the level of oxidative stress in benign tissue, PIN, and prostate carcinoma. Immunohistochemical methods were used to determine the pattern and extent of expression of three major antioxidant enzymes, including copper-

zinc-superoxide dismutase (SOD1), manganese-superoxide dismutase (SOD2), and catalase, as well as a marker of oxidative DNA damage, the DNA adduct 8-hydroxydeoxyguanosine. We also used immunogold electron microscopy to determine the precise intracellular location of SOD1, SOD2, catalase, and the DNA adduct 8-hydroxydeoxyguanosine in prostate carcinoma. In tissue homogenates, we measured the enzyme activity of each enzyme. Mutations in the genes for SOD1, SOD2, and extracellular SOD (SOD3) were examined by polymerase chain reaction. RNA in situ hybridization was employed to determine expression of mRNA for SOD1.

MATERIALS AND METHODS

Patient Specimens

We undertook immunohistochemical studies and enzymes assays of samples from 27 radical prostatectomy specimens removed at Mayo Clinic for clinically localized prostate carcinoma between March and June 1995. The mean patient age was 60.8 years (range, 48–73). The mean preoperative serum prostate specific antigen concentration was 12.6 ng/mL (range, 2.2–39.7); 11 patients had a concentration less than 10 ng/mL. The prostates were handled routinely by partial sampling, as previously described.⁷ Briefly, each was weighed, measured in 3 dimensions, and inked, and the apex and base were amputated at a thickness of 4 mm. The majority of the prostate was serially sectioned by knife at 4 to 5-mm intervals perpendicular to the long axis of the gland from the apex of the prostate to the tip of the seminal vesicles. Select frozen slices from transverse sections were prepared and stained with methylene blue for diagnosis; all results were confirmed subsequently on formalin fixed, paraffin embedded permanent sections. Pathologic cancer stage (TNM, 1997 revision) was T2N0M0 in 12 patients (44.4%), T3N0M0 in 12 (44.4%), and T3bN+M0 in 3 (11.1%). Gleason scores were 5 in 5 patients (18.5%), 6 in 7 (25.9%), 7 in 12 (44.4%), and 9 in 3 (11.1%). Twenty-one cancers (77.8%) were diploid by flow cytometry, 5 (18.5%) were tetraploid, and 1 (3.7%) was aneuploid.

For each case, approximately 1 cc of cancer and an equal portion of benign tissue, confirmed microscopically by frozen section, was snap frozen in liquid nitrogen within 15 minutes of surgical removal of the prostate. These specimens remained frozen at -70°C until the time of study. The amount of cancer in each study specimen was enriched to at least 80% (estimat-

ed visually by one of the authors [D.G.B.] by microdissection of the frozen tissue block.

Polymerase chain reaction (PCR), DNA sequencing, and RNA in situ hybridization studies were performed on frozen samples from 20 other patients. These 20 cases were selected from the surgical pathology files at Mayo Clinic from patients who had undergone radical retropubic prostatectomy and bilateral pelvic lymphadenectomy between 1991 and 1994. All patients had clinically localized prostate carcinoma and none had received preoperative androgen deprivation or radiation therapy. Fresh tissue blocks of primary prostate carcinoma and paired benign tissue were harvested, frozen, and microdissected for DNA extraction as previously described.⁸ Prostate specimens adjacent to those that had been frozen were also formalin fixed and paraffin embedded for routine surgical pathology. Two serial 5- μ m sections were prepared from a representative tissue block for RNA in situ hybridization analysis. The Gleason scores of carcinoma foci were 4–6 (6 foci), 7 (7 foci), and 8–10 (7 foci). Pathologic tumor stages were T2N0M0 (7 cases), T3N0M0 (10 cases), and T3N1M0 (3 cases). Based on previous loss-of-heterozygosity studies, all 20 cancers showed loss of heterozygosity on chromosome 21 (Cunningham), the chromosome which contains the SOD1 gene.

Antibodies

Rabbit polyclonal antibodies were produced and characterized by one of us (L.W.O.) against copper-zinc superoxide dismutase (SOD1) (anti-recombinant human placenta), manganese superoxide dismutase (SOD2) (anti-human kidney), and catalase (anti-bovine liver). Murine monoclonal antibodies against 8-hydroxydeoxyguanosine were produced and characterized by one of us (R.M.S.).⁹

Immunohistochemical Staining

For SOD1, SOD2, and catalase, immunohistochemical staining was performed by one of us (T.D.O.). Deparaffinized sections were rehydrated and incubated with 0.3% hydrogen peroxide–distilled water to block endogenous peroxidases. Tris/urea buffer was heated in a microwave oven to 90 °C, and slides were placed in the solution for 5 minutes. Slides were removed, the solution reheated, and slides immersed for an additional 30 minutes. Then the slides were exposed to a universal blocking reagent (Biogenex HK035-SK), which was diluted 1:10 for up to 10 minutes. After rinsing with Tris-buffered saline (TBS), slides were drained and placed in avidin solution (Vector Laboratories SP-2001, Burlingame, CA) for 20 minutes, and again rinsed in 3 changes of TBS. After 20 minutes of

incubation in biotin (Vector Laboratories), slides were incubated with primary antibody overnight at 4 °C. The following day, slides were warmed, rinsed in 3 changes of TBS at 37 °C, and developed by the labeled streptavidin biotin method (LSAB kit, DAKO Corp K0690, Carpinteria, CA). The chromogenic reaction was developed with Immunopure metal-enhanced diaminobenzidine substrate (Pierce Chemicals 34065, Rockford, IL) for up to 10 minutes. Slides were then rinsed with distilled water, lightly counterstained with hematoxylin, dehydrated, and mounted in Permount (Stephens Scientific; Kalamazoo, MI). Positive and negative controls were run in parallel with each batch and gave appropriate results.

For 8-hydroxydeoxyguanosine, immunohistochemical staining was performed by one of us (D.G.B.) using the avidin-biotin-peroxidase complex method. Briefly, deparaffinized sections were heated in a microwave oven for 15 minutes in 0.1 M citric acid buffer, boiled in the same solution in the microwave oven for another 15 minutes, and washed twice in tap water for 5 minutes. After two additional 5-minute washes in phosphate-buffered saline (PBS), slides were incubated for 1 hour at 37 °C in a mixture of 100 μ g/mL RNase in Tris buffer, pH 7.5. After two 5-minute washes in PBS, DNA was denatured by treatment with 4N HCl for 7 minutes at room temperature. Then 50 mM Tris base was added for neutralization for 5 minutes, and slides were washed 3 times in PBS for 5 minutes each. Blocking serum (10% normal horse serum) was applied for 1 hour at 37 °C. Anti-8-hydroxydeoxyguanosine antibody 1F7 was applied at 1:10 dilution in blocking serum overnight at 4 °C. The following day, slides were rinsed in 0.1% Triton X-100 solution for 30 seconds and washed in 3 changes of PBS for 5 minutes each. Biotinylated secondary antibody (ABC kit, Vector Laboratories) was applied for 30 minutes at 37 °C. Slides were rinsed in Triton X-100 followed by PBS. Endogenous peroxidases were quenched by immersion of slides for 30 minutes at room temperature in 0.3% hydrogen peroxide–methanol solution. After washing in PBS, the ABC reagent was applied for 30 minutes at 37 °C. Subsequently, slides were rinsed in PBS, followed by Triton X-100, and again in PBS. The chromogen solution, nickel-enhanced 3,3'-diaminobenzidine, was applied for 3–10 minutes, and slides were rinsed in tap water, lightly counterstained in ethyl green, and mounted in Permount. Positive and negative controls were run in parallel with each batch and gave appropriate results.

The percentage of stained cells was estimated by two of us (D.G.B., E.E.A.) in 10% increments from 0–100% for benign epithelium, high grade PIN, and adenocarcinoma. Intensity of staining was evaluated

independently on a scale from 0 to 3 (0 = negative staining; 1 = weak but unequivocal staining; 2 = moderate staining; 3 = intense staining).

Immunogold Labeling of Copper-Zinc Superoxide Dismutase (SOD1) and Manganese Superoxide Dismutase (SOD2)

Ultrastructural examination was performed by one of us (T.O.) on five other cases of prostate carcinoma to determine the cellular location of SOD1 and SOD2. We examined five sets of matched benign prostate and cancer tissue obtained at radical prostatectomy from patients at Mayo Clinic with previously untreated clinically localized cancer. All diagnoses were confirmed microscopically at the Middleton Veterans Hospital, Madison, Wisconsin. Samples were minced into 1-mm cubes, immersed in Carson-Millonig fixative, and washed in 0.1 M phosphate buffer for 30 minutes. After dehydration in graded alcohols to 90% ethanol, the tissue was immersed in a 2:1 mixture of LR white and 90% ethanol, then undiluted in LR white overnight. Polymerization was completed by incubation at 56 °C for 3 days. Sections were blocked with 4% bovine serum albumin and 0.5% Tween 20 in Tris-buffered saline for 10 minutes and then treated with primary antibody (1:200 for SOD1 and 1:200 for SOD2) overnight at 4 °C. Control sections were treated with pre-immune serum in place of the primary antibody. Sections were then washed, treated with gold-conjugated goat antirabbit immunoglobulin G diluted 1:50 in phosphate buffer for 90 minutes, washed, fixed in 2.5% glutaraldehyde for 10 minutes and stained with uranyl acetate for up to 20 minutes. Labeled sections were evaluated with a Hitachi 600 electron microscope.

Enzyme Activity Assays

Enzyme activities were determined separately for benign epithelium and cancer by one of us (L.W.O.). Enzyme activity for total superoxide dismutase was measured using the modified nitroblue tetrazolium method.¹⁰ One unit of superoxide dismutase activity was defined as the amount of protein that caused a 50% reduction in the background rate of the nitroblue tetrazolium reduction. NaCN was added to assay SOD2 activity. SOD2 activity was subtracted from the total activity to determine SOD1 + SOD3 activity. Results were expressed as units of activity per mg of protein. Protein content was measured by the method of Lowry et al.¹¹

Catalase activity was measured according to the decoloration of hydrogen peroxide spectrophotometrically at 240 nm.¹²

DNA Sequencing of SOD1, SOD2, and SOD3

The Sanger terminal termination sequencing method was applied on 20 samples using the Promega sequencing kit. Cold polymerase chain reaction (PCR) was performed to amplify each exon of these 3 genes using specific primers. The PCR product of each tumor was sequenced using a specific sequencing primer for each exon. Table 1 shows the sequences of primers used in this study. The sequencing primer was labeled with ³²P-dATP. Six percent polyacrylamide gel with 7M urea was used to separate the sequence products.

RNA In Situ Hybridization for SOD1

Sense and antisense oligonucleotides for exon 1 of SOD1 were synthesized in the Molecular Biology Core Facility at Mayo Clinic. The oligonucleotides were labeled with digoxigenin 11-dUTP at the 3' end in the mix which contained 1× TdT buffer, dATP, 5 μL digoxigenin 11-dUTP, 50 ng oligo probe, and 40 units terminal transferase (Promega). The reaction mix was incubated at 37 °C for 30 minutes. The labeled oligo probe was precipitated with 3 M Na-acetate, 10 mg/mL glycogen, and 100% ethanol, and dissolved in 62 μL diethylpyrocarbonate water.

Paraffin-embedded 5-micron-thick tissue sections from 20 cancers were deparaffinized in xylene, dehydrated in serial alcohol, treated with microwave irradiation for 15 minutes in 10 mM citrate buffer (pH 6.0), digested in proteinase K solution (25 mg/mL) for 10 minutes at 50 °C, rinsed in 0.1 M triethanolamine and acetic anhydride buffer for 15 minutes, and pre-hybridized at room temperature for 60 minutes. Ten μL of oligonucleotide probe was applied to these sections. Hybridization was performed in a humid chamber at 50 °C overnight. Slides were washed in 2× SSC, 1× SSC, and 0.5× SSC at 42 °C, incubated with anti-digoxigenin-AP at 37 °C for 2 hours, and probe binding sites were visualized after incubation in the solution of nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and levamisole. At medium magnification (×100), the pattern and extent of staining was graded from 0 (negative) to 3+ (intense staining).

RESULTS

Immunohistochemical Studies

Twenty-five of 27 patients had sufficient tissue available for immunohistochemistry for all markers, including SOD1, SOD2, catalase, and 8-hydroxydeoxyguanosine.

Antioxidant enzyme immunoreactivity

Copper-zinc superoxide dismutase (SOD1) immunoreactivity was both nuclear and cytoplasmic (Table 2)

TABLE 1
Primer Sequences for SOD Exons

Primer name	Primer sequence
SOD1 EXON1 A	5' GTC TGG CCT ATA AAG TAG TCG 3'
SOD1 EXON1 B	5' AGC GGC CTC GCA ACA CAA 3'
SOD1 EXON1 S	5' GCT GGT TTG CGT CGT AGT CTC CTG 3'
SOD1 EXON2 A	5' GGG ATT TCG GAC ACA GAT TT 3'
SOD1 EXON2 B	5' ACC CAC CTG CTG TAT TAT CT 3'
SOD1 EXON2 S	5' CAC TGT GAG GGG TAA AGG TAA ATC AG 3'
SOD1 EXON3 A	5' CAT AAT TTA GCT TTT TTT TCT TCT T 3'
SOD1 EXON3 B	5' TGG GGG AAA CAC GGA ATT AT 3'
SOD1 EXON3 3AS	5' GGG AAA CAC GGA ATT ATC TTA GGC AC 3'
SOD1 EXON4 A	5' CAT ATA GGC ATG TTG GAG AC 3'
SOD1 EXON4 B	5' ATT CGC GAC TAA CAA TCA AAG 3'
SOD1 EXON4 AS	5' TTC GCG ACT AAC AAT CAA AGT GAA AGA T 3'
SOD1 EXON5 A	5' ATT GTT GGG AGG AGG TAG TG 3'
SOD1 EXON5 B	5' ACA GCT AGC AGG ATA ACA GAT 3'
SOD1 EXON5 AS	5' GAG TTA AGG GGC CTC AGA CTA CAT C 3'
SOD2 EXON1 A	5' TTC GGC AGC GGC TTC AG 3'
SOD2 EXON1 B	5' CGG CCA CTG TCG CCA TT 3'
SOD2 EXON1 AS	5' TTC GCC CTT GGG CGC GTG ACC 3'
SOD2 EXON2 A	5' TGA CCG GGC TGT GCT TTC 3'
SOD2 EXON2 B	5' GTA CAA ATA CGA AGC GAG TT 3'
SOD2 EXON2 AS	5' TAC AAA TAC GAA GCG AGT TCT CCT C 3'
SOD2 EXON3 A	5' ACA GTG GTT GAA AAA GTA GGA 3'
SOD2 EXON3 B	5' CAA CAA GAG CAA AAC TCT TGT 3'
SOD2 EXON3 AS	5' CAA CAA GAG CAA AAC TCT TGT CTC AA 3'
SOD2 EXON4 A	5' TCT TGG GCC CTA TGA CAA AA 3'
SOD2 EXON4 B	5' GTT GAA TGC TTT ACA GTA GAG 3'
SOD2 EXON4 AS	5' AGA GCA TCT CTC CCA AAT GAA ATC AC 3'
SOD2 EXON5 A	5' GTT GAA ATT GAG AAG ATG CAA T 3'
SOD2 EXON5 B	5' GCA GTA CTC TAT ACC ACT AC 3'
SOD2 EXON5 AS	5' GTA CTC TAT ACC ACT ACA AAA ACA GTC 3'
SOD3 EXON1 A	5' CAG CCA CTG TGT TGT CAC T 3'
SOD3 EXON1 B	5' GTC CCA GCC TTG TCA CTT C 3'
SOD3 EXON1 AS	5' TCC CAG CCT TGT CAC TTC CTA TCT G 3'
SOD3 EXON2 A	5' CCT GCT TTT CCT CCC TGA A 3'
SOD3 EXON2 B	5' AAC CCA GCT TCA GAC TCA AT 3'
SOD3 EXON2 AS	5' CCC AGC TTC AGA CTC AAT AAG CAC A 3'
SOD3 EXON 3-1A	5' GTG CCC GAC TCC AGC CAT 3'
SOD3 EXON 3-1B	5' TGC CGG AAG AGG ACG ACG 3'
SOD3 EXON 3-1S	5' GCC CGA CTC CAG CCA TGC TGG 3'
SOD3 EXON 3-2A	5' CGC CTG CCA GGT GCA G 3'
SOD3 EXON 3-2B	5' GCC CAC GAT GGA GTG CG 3'
SOD3 EXON 3-2S	5' CGC CTG CCA GGT GCA GCC GTC 3'
SOD3 EXON 3-3A	5' GCA GCC TCT GGA GGT AC 3'
SOD3 EXON 3-3B	5' GCG AAG GTG AGA CCT CAG 3'
SOD3 EXON 3-3AS	5' GCG AAG GTG AGA CCT CAG AGT GG 3'
SOD3 EXON 3-4A	5' TTC GCC TCT GCT GAA GTC T 3'
SOD3 EXON 3-4B	5' GGG AAG ATC GTC AGG TCA A 3'
SOD3 EXON 3-4S	5' CTC CCC GCA GCC CTC TCC ACC 3'
SOD3 EXON 3-5A	5' GCC TTT GAC CTG ACG ATC T 3'
SOD3 EXON 3-5B	5' GAA TTG CTG AAT TTT TAA TCT GT 3'
SOD3 EXON 3-5S	5' GCC TTT GAC CTG ACG ATC TTC CC 3'

and present only in epithelium, with no staining of the stroma, inflammatory cells, or endothelial cells. The mean percentage of nuclei that stained was greater in benign epithelium (29.2% of cells) than in PIN and cancer (8.8% and 20.2%, respectively) (Fig. 1). Simi-

larly, the mean percentage of cells with cytoplasmic staining was greatest in benign epithelium (33.2% of cells), but this difference was only significant when compared with cancer and not with PIN. Cancer staining was heterogeneous in both nuclei and cytoplasm. The intensity of nuclear and cytoplasmic SOD1 staining was greatest in benign epithelium and weakest in high grade PIN.

Manganese superoxide dismutase (SOD2) immunoreactivity was invariably cytoplasmic (Table 2) and present only in epithelium, with no staining of the stroma, inflammatory cells, or endothelial cells. Staining was heterogeneous in cancer cells. There was intense staining at the leading edge of the cancer adjacent to benign epithelium, a finding not observed with the other antibodies in this study (Fig. 1). The mean percentage of cells staining was greater in benign epithelium (46.4% of cells) than in PIN and cancer (26.5% and 22.8%, respectively); there was no difference between PIN and cancer (Fig. 1). The intensity of staining was greater in benign epithelium than in PIN and cancer.

Catalase immunoreactivity was invariably cytoplasmic (Table 2) and present only in epithelium, with no staining of the stroma, inflammatory cells, or endothelial cells. Staining was heterogeneous in cancer cells (Fig. 1). The mean percentage of cells staining was greater in benign epithelium (14.4% of cells), with progressive decrease in PIN and cancer (10.5% and 8.6%, respectively). The intensity of staining was greater in benign epithelium than in PIN and cancer.

DNA adduct immunoreactivity

There was intense nuclear immunoreactivity for 8-hydroxydeoxyguanosine in the prostatic epithelium, with no staining in the stroma or endothelial cells. Background staining was observed in neutrophils, and these cells were excluded from analysis. No apparent difference was observed in the percentage of cells staining in benign epithelium, PIN, and cancer, although only a small percentage of cells stained, invariably fewer than 3%. There was intense staining of mitotic figures, and a predilection for staining in high Gleason grades of cancer (primary patterns 4 and 5). Occasionally, there was clustering of staining, but this was inconsistent. Cells with apoptotic bodies displayed heavy granular staining. In one case with an area of inflammation surrounding PIN, there was particularly intense staining of PIN. There was no apparent difference in staining at the leading edge of cancer when compared with the center.

TABLE 2
Immunohistochemical Expression of Antioxidant Enzymes in Benign Prostatic Tissue, PIN, and Prostate Carcinoma

Protein	Immunoreactive cells, mean % (SD)			Comparisons		
	Benign	PIN	Cancer	Benign vs. PIN	Benign vs. cancer	PIN vs. cancer
Nuclear CuZnSOD	29.2 (20.4)	8.9 (9.6)	20.2 (16.6)	0.0006	0.05	0.002
Cytoplasmic CuZnSOD	33.2 (19.5)	28.3 (23.1)	21.2 (17.2)	NS	0.02	NS
MnSOD	46.4 (27.1)	26.5 (26.8)	22.8 (27.0)	0.0001	0.0001	NS
Catalase	14.4 (12.3)	10.5 (20.6)	8.6 (16.2)	NS	NS	NS

Protein	Intensity of staining, mean (SD)			Comparisons		
	Benign	PIN	Cancer	Benign vs. PIN	Benign vs. cancer	PIN vs. cancer
Nuclear SOD1	1.84 (0.62)	0.67 (0.69)	1.48 (0.82)	0.0001	0.07	0.004
Cytoplasmic SOD1	1.48 (0.65)	1.11 (0.58)	1.12 (0.53)	0.01	0.03	NS
SOD2	2.12 (0.72)	1.2 (1.0)	1.4 (1.2)	0.0001	0.003	NS
Catalase	1.1 (0.7)	0.25 (0.44)	0.36 (0.49)	0.0004	0.0001	NS

PIN: high grade prostatic intraepithelial neoplasia; SD: standard deviation; NS: not significant.

Antioxidant Enzyme Activity Assays

Twenty-five cases had sufficient tissue for enzyme activity analysis of benign prostatic tissue and matched cancer (Table 3). Mean activity was greatest for catalase and lowest for SOD2. There was no significant difference between benign epithelium and cancer for any enzyme, although there was a trend toward lower activity of SOD1 in cancer.

Immunogold Labeling of SOD1 and SOD2

Labeling with SOD1 was extremely light in both benign and malignant epithelium. Label tended to be nuclear in benign epithelium and cytoplasmic in cancer cells (Fig. 2).

Benign epithelium contained mitochondria of varying sizes and shapes with heavy labeling for SOD2 (Fig. 3). Cancer cells from the same case contained small mitochondria with light labeling (Fig. 3). Benign tissue incubated with normal rabbit serum as control did not show labeling.

DNA Sequencing of SOD1, SOD2, and SOD3

We sequenced a total of 5 exons of SOD1, 5 exons of SOD2, and 3 exons of SOD3 in 20 cancers. No apparent mutations was detected for any exon, although 3 cases showed polymorphisms for exon 3 of SOD1. Studies were performed in duplicate and gave identical results.

RNA In Situ Hybridization for SOD1

Basal cells in benign prostatic epithelium displayed intense diffuse cytoplasmic staining for SOD1, whereas secretory cells showed light and patchy stain-

ing (Fig. 4). PIN displayed moderate patchy heterogeneous staining of the basal and secretory cells. Staining was predominately supranuclear. The staining in cancer cells varied greatly, with prominent intratumoral heterogeneity; nonetheless, staining was often uniform and diffuse, with accentuation of cell margins (Fig. 4). The extent and intensity of staining was invariably less than in the basal cells in benign epithelium. In 13 cancers, the majority of cells showed stronger cytoplasmic staining than benign secretory epithelial cells. In seven cancers, the majority of cells showed similar intensity of staining as benign secretory cells. Some stromal cells also showed equivocal or weak staining.

DISCUSSION

We found that there was lower antioxidant enzyme expression in PIN and prostate carcinoma than in basal cells of the benign prostatic epithelium. These findings were confirmed by down-regulation of mRNA for SOD1 according to in situ hybridization assays. Our findings confirm the immunohistochemical results of Baker et al., who used the same antibodies.⁴ Such results suggest that there is a shift in the prooxidant-antioxidant balance in prostate carcinoma, similar to that observed following exposure of the human prostate carcinoma cell line LNCaP to androgens.¹³ Antioxidant enzyme imbalance and oxidative stress alter cellular redox, arresting cell proliferation and growth by cell cycle arrest due to an activated p53 protein,¹⁴ inducing apoptosis;¹⁵ activating signal transduction pathways,¹⁶ activating transcription fac-

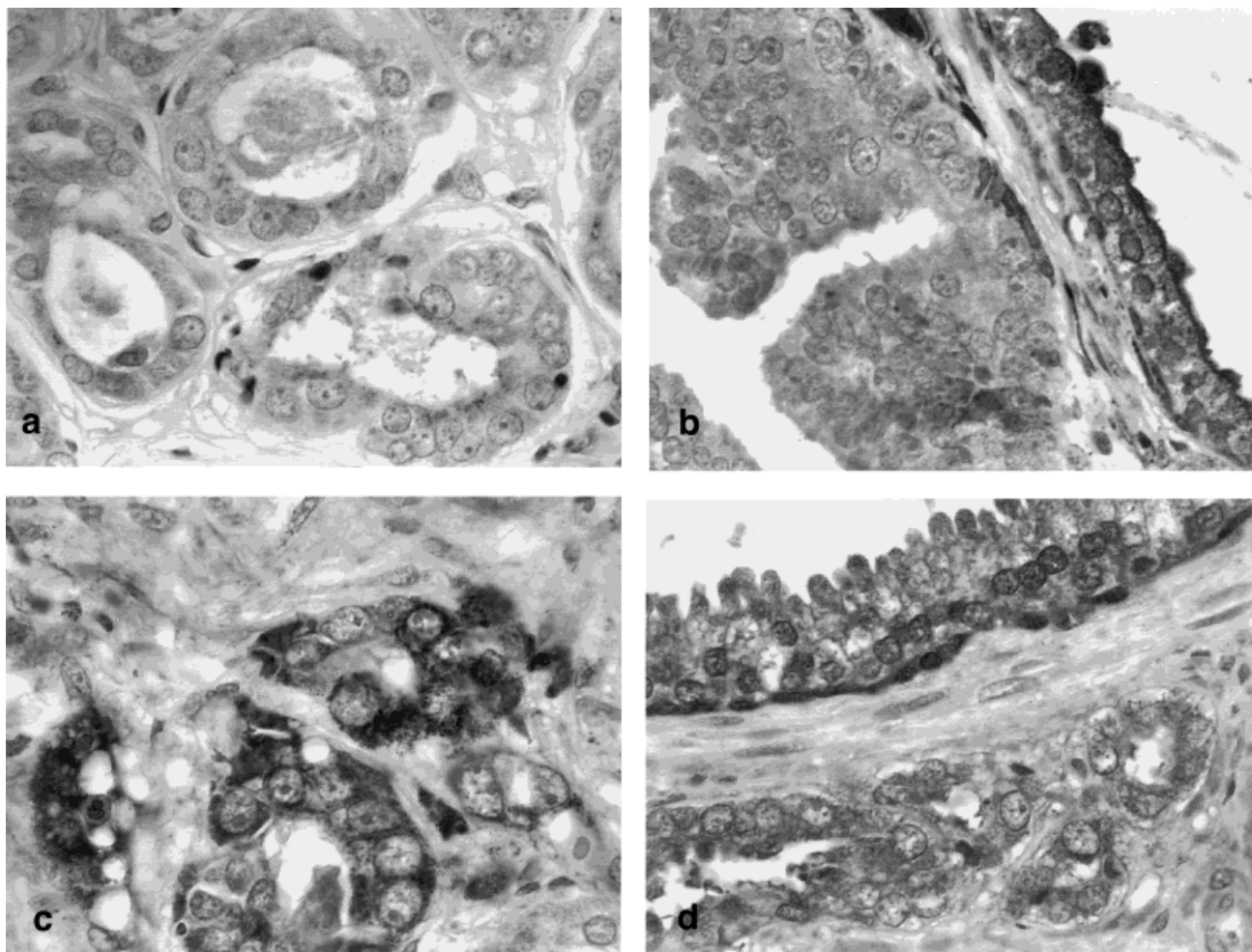


FIGURE 1. Antioxidant enzyme immunistochemical findings. (a) Catalase cytoplasmic immunoreactivity was minimal in adenocarcinoma, with weak staining at the luminal surface of some of the cancer cells. (b) Manganese superoxide dismutase (SOD2) cytoplasmic immunoreactivity in benign epithelium was intense in secretory luminal cells (right) and weak in high grade prostatic intraepithelial neoplasia. (c) Manganese superoxide dismutase (SOD2) cytoplasmic immunoreactivity at the leading edge of adenocarcinoma was intense in this case, but most cancer cells were much less immunoreactive or negative. (d) Copper-zinc superoxide dismutase (SOD1) cytoplasmic immunoreactivity was more intense in benign secretory and basal cells (top) than in adenocarcinoma (bottom).

tors such as Fos, Jun, and nuclear factor KB;^{15,17} and increasing mitochondrial activity.^{13,18}

Antioxidant enzyme activities were similar in benign prostatic tissue and cancer, with the greatest activity for catalase. The discrepancy between protein expression of antioxidant enzymes and their levels of activity (decreased vs. unchanged, respectively) may result from several different causes. The most likely cause is normal cell contamination. The enzyme activities were measured in cellular homogenates that contain both benign and malignant cells. Even though the immunohistochemical results did not show staining for these enzymes in benign prostatic cells, our previous work has shown that all mammalian cells have SOD activity or protein and most have catalase activity or protein.¹⁹ Thus, we believe it is likely that

prostate carcinoma cells also have diminished antioxidant enzyme activity similar in magnitude to the lowered protein levels measured by immunohistochemistry. Another possible explanation for our results is that prostate cells have substantial amounts of extracellular SOD (SOD3). Our enzymatic assays could not distinguish between intracellular CuZnSOD and extracellular SOD, so the results would be in error if extracellular SOD was present in large quantities. Prominent heterogeneity was observed in all cases, a characteristic of PIN and cancer phenotypically,²⁰ immunophenotypically,²⁰ and genotypically.^{8,20-23}

No genetic mutations were found in SOD1, SOD2, or SOD3 other than rare polymorphisms in one of the exons of SOD1. It is possible that mutations were present that could not be detected, but the identifica-

TABLE 3
Activity of Antioxidant Enzymes in Benign Prostatic Tissue and Prostate Carcinoma

Protein	Mean activity per mg protein (SD)		P Value
	Benign	Cancer	
SOD1	155 (99)	113 (47)	0.071
SOD2	53 (22)	64 (35)	0.085
Total SOD	207 (112)	176 (70)	0.204
Catalase	503 (196)	473 (175)	0.508

SOD: superoxide dismutase; SD: standard deviation.

tion of polymorphisms verified by direct sequencing validated our methods and suggested that there is a reasonable likelihood that we would have detected mutations within the exons of each SOD gene. Spontaneous mutations would have been expected in at least one SOD gene according to the mean spontaneous mutation frequency of $4.8-8.4 \times 10^{-7}$ per locus per cell division in human cells.²⁴ These findings suggest that SOD is required for cell survival or that SOD mutations are selected against in prostatic carcinogenesis. This refutes the recent suggestion that oxidative stress itself may damage genes coding for SOD1.²⁵ Mutations may be present in regulatory regions that would affect SOD expression but not coding. Alternatively, SOD mutations may not be selectively mutated in prostate carcinoma, and changes in oxidative stress may result from cancer rather than being causative. Further, ROS damage may be important in the development of prostate carcinoma through regulation of apoptosis or other signal transduction pathways rather than classic activation or suppression by mutations.

Previous analysis of these exact tissues in another study revealed substantial mutation rates in other genes of the corresponding chromosomes for each SOD, including chromosome 21 (SOD1), chromosome 6 (SOD2), and chromosome 4 (SOD3).²³ The absence of mutations in otherwise genetically unstable prostate carcinoma DNA suggests that intact oxidative pathways are important for cell viability and prostate carcinoma development.

We also measured nuclear 8-hydroxydeoxyguanosine, a DNA base lesion that is specific to oxidative damage whose expression can be determined immunohistochemically in archival tissue with a well-characterized monoclonal antibody. We questioned whether this factor would provide a measure of the cumulative effect of oxidative stress in relatively long-lived prostatic cells, unlike the protein or mRNA determinations of the antioxidant enzymes that might

reflect findings at only a single moment in time. There was a low (3% of cells) but significant level of 8-hydroxydeoxyguanosine immunoreactivity in epithelial cells, with no apparent difference between benign epithelium, PIN, and cancer, perhaps due to the inability of our methods to identify differences at low levels of expression. There may also be differential sensitivity of epithelial cells to oxidative stress, resulting in malignant transformation in only a subset of cells. These findings suggest that there is equivalent cumulative oxidative stress in benign and malignant epithelial cells that is not present in the prostatic stroma.

If oxidative stress is a contributing or risk factor for prostate carcinoma, then it should account for numerous unique attributes of this cancer. First, PIN and prostate carcinoma are exquisitely age-related, with very few autopsy cases prior to 30 years of age and few clinically detected cases prior to age 40-50 years, but a dramatic increase in both thereafter.^{2,3} Age-related changes result from an imbalance in cellular pro-oxidant-antioxidant status,²⁶⁻²⁸ with progressive accumulation of DNA strand breaks,²⁹ DNA adducts,³⁰ ring-saturation thymine derivatives, apurinic and apyrimidic rings, and oxidative modification of enzymes of DNA repair.³⁰⁻³² Antioxidant defense mechanisms, including ROS detoxification enzyme activity, decline with age.^{27,33} Overexpression of these enzymes prevents aging and extends the life span of fruit flies.²⁷ ROS-related DNA base modifications occur at the rate of about 100,000 per cell per day in the rat,³⁴ so it seems reasonable that this constant and lengthy bombardment of the antioxidant defenses could induce an increased amount of DNA damage with advancing age. Damage results from lipid peroxidation and other factors of oxidation created by highly reactive oxygen species, including superoxide radicals, hydroperoxyl radicals, hydroxyl radicals, and hydrogen peroxide, creating an optimal environment for mutagenesis and carcinogenesis.

The second unique attribute of prostate carcinoma is its virtually complete penetrance in men such that the prevalence is about 80% by age 80 years, the highest risk of any noncutaneous cancer in the body.^{2,3} The autopsy prevalence of prostate carcinoma is similar in populations around the world despite substantial differences in incidence and clinical detection. Oxidative stress fulfills the requirements for an universal age-related factor which is operative in all men regardless of genetic history, race, culture, lifestyle, and diet.

The third unique attribute of prostate carcinoma that a risk factor such as oxidative stress should account for is the remarkable multifocality of PIN and cancer.³⁵⁻³⁷ Our results and others⁴ indicate that oxi-

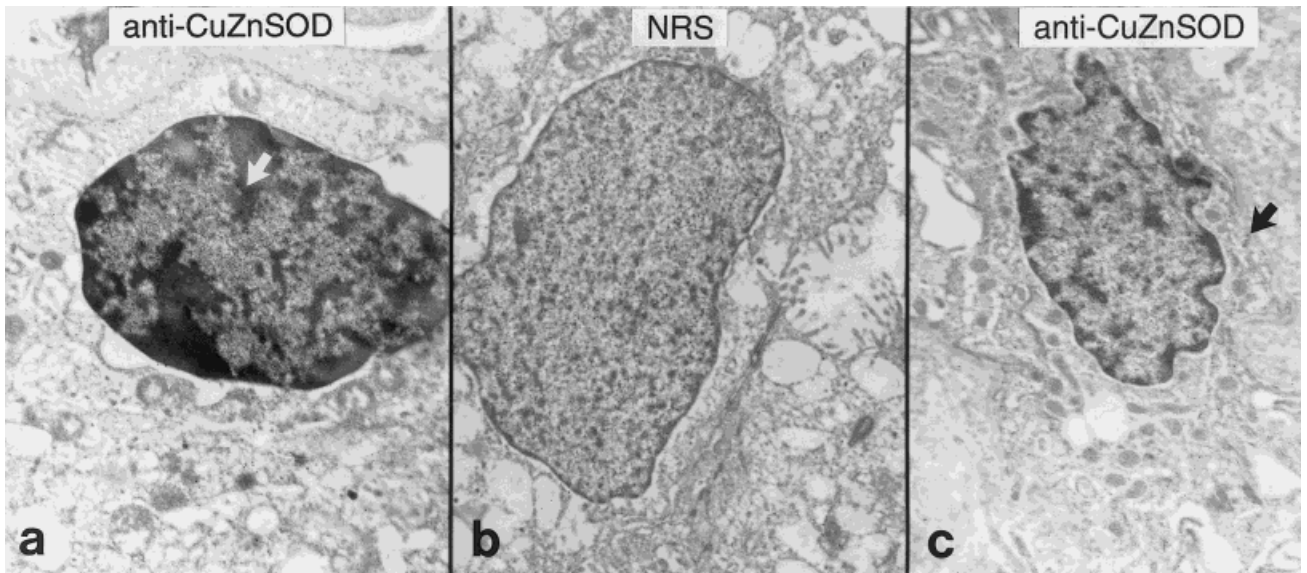


FIGURE 2. Immunogold ultrastructural findings with copper-zinc superoxide dismutase (CuZnSOD) (SOD1). (a) Benign epithelium displayed rare nuclear staining (arrow). (b) Normal rabbit serum (NRS) displayed complete absence of labeling. (c) Cancer cell revealed minimal cytoplasmic staining (arrow).



FIGURE 3. Immunogold ultrastructural findings with manganese superoxide dismutase (MnSOD) (SOD2). (a) Benign epithelium contained abundant labeling of mitochondria (arrow). (b) Normal rabbit serum (NRS) displayed complete absence of labeling. (c) Cancer cells contained light staining of mitochondria (arrow).

ductive stress and DNA adduct accumulation are present throughout the prostate in basal cells, the putative stem cell compartment, and could reasonably account for the field effect of prostatic carcinogenesis.^{8,38}

Another unique attribute of PIN and prostate carcinoma is its lack of a characteristic genotype. We previously observed marked heterogeneity of genetic alterations in both PIN and prostatic carcinoma.⁸ In 44% of cases of PIN with multiple informative loci of PIN, allelic imbalance was observed in one or more

foci of PIN, but not in other PIN foci, whereas allelic imbalance was observed for different markers in 80% of cases with multiple foci of carcinoma, similar to the findings of Sakr et al.²¹ Such genetic heterogeneity is expected to occur in the process of tumor progression and may be an underlying molecular mechanism for the diverse clinical and morphologic manifestations of prostate carcinoma. Other studies utilizing complementary techniques such as PCR, in situ hybridization, and DNA ploidy have also demonstrated intraglandular heterogeneity.^{21,22,39–41} Furthermore, small can-

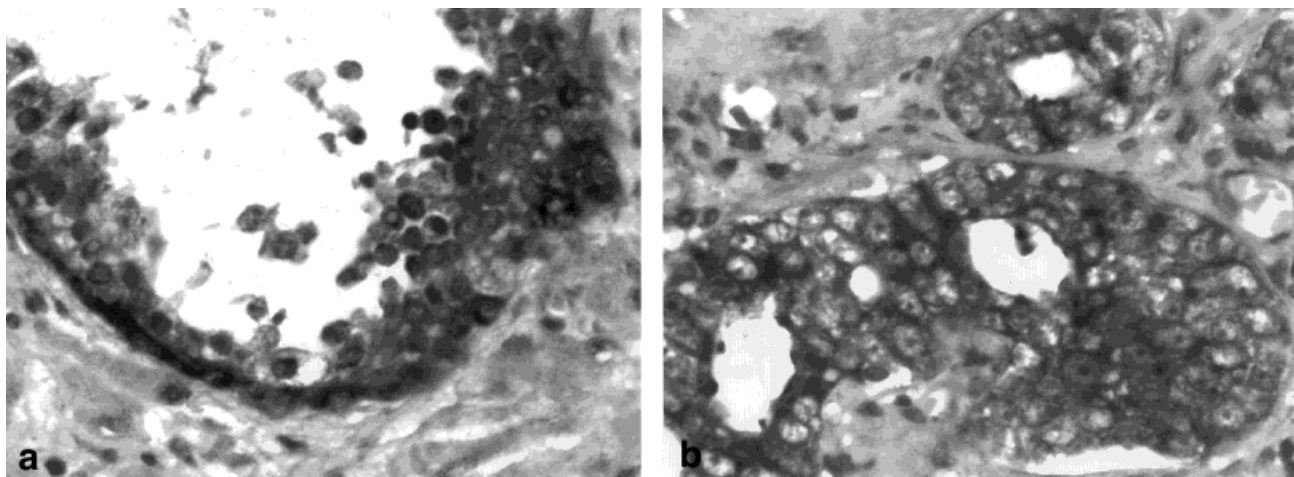


FIGURE 4. RNA in situ hybridization results for copper-zinc superoxide dismutase (SOD1). (a) Benign prostatic epithelium reveals strong staining of the basal cell layer and weaker staining of the secretory luminal cell layer. (b) Adenocarcinoma shows intense staining of virtually all cells in this focus.

cers can have significant genetic alterations. Therefore, the size of a cancer focus and its degree of histologic differentiation may not reflect the extent of its genetic alterations.^{36,39,41} Virtually the entire genome displays foci of allelic loss.²³ Select genes have a consistently higher prevalence of allelic loss than other genes, including 7q and 8p, but changes are present on many chromosomes in each case of prostate carcinoma. This diversity, heterogeneity, and relative lack of specificity of genetic abnormalities suggests that there are multiple pathways for development of prostate carcinoma, many of which could hypothetically be triggered or influenced by an epigenetic event, such as oxidative damage. A recent study showed abundant genomic alterations in colon carcinoma and colonic polyps, suggesting genomic destabilization as a cause rather than an effect of malignancy.⁴² Genomic destabilization may be caused by stressors, such as exposure to environmental agents or hazardous chemicals over a long period of time.⁴² These findings support our working hypothesis in prostate carcinoma.

Our study is limited by the inability to study systematically every antioxidant enzyme with all techniques due to the lack of available probes, reagents, and tissues. The methods of measurement may not be sufficiently sensitive to detect subtle differences between groups, particularly for 8-hydroxydeoxyguanosine expression. Finally, the observed differences in measures of pro-oxidant-antioxidant balance may be epiphenomena rather than causative.

Additional findings implicate ROS in prostatic carcinogenesis. Decreased levels of the detoxifying antioxidant enzyme GST- π activity may facilitate cancer progression due to impairment of cellular processing

of mutagens, allowing reactive oxygen species to damage DNA. Most prostate carcinomas fail to express GST- π ,⁴³⁻⁴⁸ and the regulatory sequence near the GST gene is commonly inactivated by hypermethylation during the early stages of prostatic carcinogenesis.⁴⁷ The extensive methylation of deoxycytidine nucleotides distributed throughout the 5' "CG island" region of GST- π is not detected in benign prostatic epithelium and may represent a potential cancer-specific molecular biomarker.⁴⁸ Patients with the highest percentage of GST- π immunoreactive cells in benign epithelium had better systemic progression free, cancer specific and overall survival.^{45,46} The antioxidant activity of selenium and vitamin E⁴⁹ may account for their apparent ability to prevent prostate carcinoma, according to results from recent epidemiologic studies.⁵⁰⁻⁵³

In summary, our results indicate that there is decreased expression and down-regulation of antioxidants in PIN and prostate carcinoma compared with benign epithelium which cannot be attributed to mutational changes in the corresponding genes. Enzyme activity was unchanged but widely variable from case to case, and there was a low but significant level of cumulative oxidative DNA damage limited to the epithelium. ROS damage is an attractive putative risk factor for PIN and prostate carcinoma accounting for many unique attributes, including close association with age, high prevalence, typical multifocality and heterogeneity, lack of a characteristic genotype, and androgen dependence. Further studies should be directed at confirming the role of ROS damage and the clinical potential for preventing or delaying the onset of prostate carcinoma.

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