GSTA1 Expression in Normal, Preneoplastic, and Neoplastic Human Prostate Tissue

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BACKGROUND. Glutathione S-transferases (GSTs), inducible enzymes that catalyze the detoxification of reactive electrophiles and oxidants, protect against neoplastic transformation. Prostatic adenocarcinoma and high-grade prostatic intraepithelial neoplasia (HGPIN) fail to express GSTP1, a major class of GST. This failure of expression is associated with methlyation of the *GSTP1* promoter, a somatic alteration proposed to be a critical step in prostatic carcinogenesis. However, simple atrophy and post-atrophic hyperplasia—proliferative lesions associated with chronic inflammation, which we have termed ''proliferative inflammatory atrophy'' (PIA)—express elevated levels of GSTP1. We postulated that this increase in GSTP1 expression in PIA occurs in response to increased oxidative stress. We examined the expression of another major class of GST, GSTA1, in the human prostate.

METHODS. We performed immunohistochemistry against GSTA1 on formalin-fixed radical prostatectomies (n = 45). A stereological grid point counting method was used to estimate the percent of cells staining positive for GSTA1 in normal prostate, PIA, HGPIN, and adenocarcinoma.

RESULTS. In contrast to GSTP1, normal peripheral zone epithelium was virtually devoid of GSTA1. Strikingly, though, epithelial cells in PIA demonstrated strong staining for GSTA1 (median of percent of cells staining positive = 44) as compared to those in normal peripheral zone (median = 3.0, $P < .00001$), HGPIN (median = 2.9, $P < .00001$), and adenocarcinoma (median = 3.8, $P < .00001$). Variations in GSTA1 were also detected between normal anatomic zones: the central zone showed an increase in the percentage of cells staining positive (median = 20.9) as compared to the transition (median = 0.47, $P < .0002$) and the peripheral ($P < .0001$) zones.

CONCLUSIONS. Expression of GSTA1 is increased in PIA, supporting the concept that cells within these lesions are subject to localized increases in oxidative stress. Low levels of GSTA1 and GSTP1 in HGPIN and adenocarcinoma suggest a broad lack of detoxification activity in these cells, which may be associated with carcinogenesis in the prostate. *Prostate 49: 30–37, 2001.* © 2001 Wiley-Liss, Inc.

KEY WORDS: **prostate cancer; oxidative stress; glutathione *S* transferase-alpha**

Grant sponsor: Public Health Services ; Grant number: K08 CA78588-0; Grant sponsor: NIH/NCI Specialized Program in Research Excellence (SPORE) in Prostate Cancer ; Grant number: P50CA58236; Grant sponsor: Bernard L. Schwartz.

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Received 9 March 2001; Accepted 11 June 2001

INTRODUCTION

The glutathione S-transferases (GST) are an important class of enzymes that play a prominent role in the intracellular detoxification of reactive electrophiles and products of oxidative stress by catalyzing the conjugation of these compounds to glutathione. Since conjugation to glutathione renders these potential carcinogens chemically inactive, and hence incapable of forming DNA adducts, it has been hypothesized that GSTs protect against neoplastic transformation [1]. There are five major families of cytosolic GST isoenzymes, designated as alpha, mu, pi, sigma, and theta. The most extensively studied of these in the human prostate is the pi-form of GST (GSTP1). Most basal cells in normal prostate epithelium express GSTP1 in large amounts [2–4]. The vast majority of prostate cancer cells, however, fail to express GSTP1 due to hypermethylation of the *GSTP1* gene promoter [2].The fact that *GSTP1* promoter hypermethylation also occurs in at least 70% of high-grade prostatic intraepithelial neoplasia (HGPIN) lesions [5]—the presumptive precursor lesions to adenocarcinoma—suggests that GSTP1 may serve as a ''caretaker'' gene [6], the decreased expression of which might render prostate cells vulnerable to malignant progression.

Although GSTP1 is highly expressed in the basal cells of normal epithelium, it is usually not expressed in normal secretory luminal cells. In contrast, prostate lesions consisting of both simple atrophy and postatrophic hyperplasia contain luminal epithelial cells with elevated levels of GSTP1 protein [3]. Since these focal lesions are hyperproliferative, and are often associated with chronic inflammation, the term proliferative inflammatory atrophy (PIA) was recently introduced in order to simplify terminology [3]. Chronic inflammation has been implicated in the development of tumors in a wide range of organ systems, including urinary bladder, liver, stomach, and large bowel. The mechanism of carcinogenesis in inflammatory tumor models is complex, but appears to depend upon repeated cycles of tissue damage and regeneration in the presence of phagocyte-derived reactive oxygen and nitrogen species. These cyclical periods of oxidative stress, it is postulated, lead to accumulated DNA damage and somatic mutations [7,8].

Several groups have invoked the inflammation-carcinoma sequence as a potential model for carcinogenesis in the prostate [3,9–13]. It is possible that increased expression of GSTP1 in PIA may result from the presence of an ongoing oxidative insult to this tissue, with silencing of GSTP1 function related to the development of cancer. If induction of GSTP1 is indicative of increased oxidative stress in PIA, then other GSTs might be induced in PIA as well.

Little is known, however, about the relative expression of other GSTs in normal prostate, PIA, HGPIN, and prostate adenocarcinoma. Though physiologically it appears to serve the same broad purpose as GSTP1, the GST alpha isoenzyme (GSTA1-5) follows a different pattern of tissue distribution and inducibility of expression in both rats and humans [1]. GSTA expression has been associated with various types of cancer, including renal, bladder, colon, and breast [14]. GSTA has also been identified within both normal prostate and prostate cancer [15,16]. Preliminary results from one study suggest that GSTA expression in the normal prostate is low [17]. Yet, it is unclear how GSTA is distributed within the different anatomic and cellular compartments of the prostate. Moreover, no quantitative data exists on the comparative levels of GSTA in normal prostate, PIA, PIN, and prostate adenocarcinoma, respectively.

Further elucidation of GSTA expression in both normal and diseased human prostate, therefore, may yield important clues for understanding the pathogenesis of prostate adenocarcinoma, and its potential relationship to chronic inflammation. In this study, we have quantitatively examined the expression of GSTA1 in normal prostate, PIA, HGPIN, and prostate adenocarcinoma.

MATERIALS AND METHODS

Surgical Specimens

Formalin-fixed, paraffin-embedded tissues were obtained from 45 randomly selected radical retropubic prostatectomies performed for prostate adenocarcinoma at the Johns Hopkins Hospital from 1997 to 2000. Patient ages varied between 44 and 67 years. Final Gleason sums and pathologic stages of the adenocarcinomas ranged from 6 to 8 and T2NoMx to T3bNoMx, respectively. All specimens consisted of portions of tissue that were dissected fresh immediately after surgical removal and immersed in 10% neutral buffered formalin prior to paraffin processing.

Immunohistochemistry

Polyclonal anti-GSTA1 antibody (rabbit 1:5000) was obtained from Calbiochem (San Diego, California). Manual immunohistochemistry was performed using the Vectastain Elite Kit (Vector, Burlingame, CA). After paraffin removal and hydration, slides were immersed in 10 mM citrate buffer pH 6.0 and steamed for 14 min to induce epitope retrieval. They were incubated with primary antibodies overnight at 4°C, then with secondary biotin-labeled antibodies for

30 min at room temperature (''universal'' anti-mouse/rabbit, diluted 1:1,000 in PBST with normal goat serum). To accomplish localization, Avidin-Biotin Complex–Horse Radish Peroxidase (ABC–HRP) was applied for 30 min, followed by peroxide/diaminobenzidine (DAB), substrate/chromagen. Slides were then counterstained with hematoxylin.

Evaluation of Immunohistochemical Staining

We employed a grid point counting method, based on the principles of stereology, in order to quantify the degree of immunohistochemical staining. This approach utilizes an eyepiece graticule (Weibel type 4) in order to generate an area fraction estimate of staining based upon evaluation of individual cells on which the tip of the gridline lands [18–21]. Counting only epithelial cells, we obtained a labeling index by determining the ratio of cells staining positive to the total number of cells counted. Both intra and inter-observer reproducibility for this process are quite high (MJ Putzi and AM De Marzo, written communication). Based on previous observations, we utilized epididymis and interstitial cells of the testis as negative and positive controls, respectively [14].

Areas of normal epithelium, PIA, HGPIN, and adenocarcinoma were individually identified and counted using the grid point counting method. On each slide, an initial region was selected at random and counted; the remainder of the slide was then systematically inspected and counted. Twenty different slides were assessed per lesion or prostate zone, representing 18 different patients for normal peripheral zone, 20 for PIA, 18 for HGPIN, and 20 for adenocarcinoma. For each separate region examined, 30–100 cells were assessed, which required examination of a minimum of 10 medium power fields (200× magnification using an Olympus BX-40 microscope) per slide. The percent number of cells that stained positive for all slides was recorded for normal peripheral zone, PIA, HGPIN, and adenocarcinoma. Additionally, 10 different slides were examined for normal transition zone (seven patients) and normal central zone (nine patients), and the number of positive cells for each of these areas was determined and compared to peripheral zone. Statistical calculations were performed utilizing the Kruskal–Wallis nonparametric test, followed by further analysis with the Wilcoxon-rank test. All calculations were performed using Stata (version 6.0) statistical computer software (Stata Corporation, College Station, Texas).

RESULTS

In comparing the amount of GSTA1 expressed in abnormal prostate epithelium—PIA, HGPIN, and adenocarcinoma—to that in normal epithelium, we initially utilized cells from the peripheral zone, since most of these lesions are known to arise from that region.

In contrast to GSTP1, which is expressed in the majority of normal basal epithelial cells, GSTA1 was nearly absent in normal peripheral zone epithelium (Fig. 1A): the median percent number of cells that stained positive in peripheral zone was only 3.8 (range 0–14.6) (Fig. 2). In addition, expression in peripheral zone occurred predominantly in luminal secretory cells. The median percent number of cells that stained positive in PIA lesions, however, was 43.9 (range = 26.5 to 62.1) (Fig. 2). This represented a marked increase in the expression of GSTA1 in PIA relative to that in normal peripheral zone ($P < .00004$). Interestingly, as with peripheral zone, expression in PIA was confined almost exclusively to luminal epithelial cells (Fig. 1C).

GSTP1 expression is virtually absent in HGPIN and adenocarcinoma as a result of *GSTP1* promoter hypermethylation [2,5]. We have, therefore, hypothesized that this loss of detoxification function might be compensated for by concomitant increased expression of other GST isoforms. In regards to GSTA1, though, this did not appear to be the case, since the overwhelming majority of HGPIN and adenocarcinoma cells were negative for GSTA1 (Fig. 1D–F). The median percent number of cells that stained positive in HGPIN was 2.9 (range = 0–26), and for adenocarcinoma it was 4.9 (range = 0–12.7). When compared to the percent number of positive cells in PIA, the difference was significant for both HGPIN ($P < .00001$) and adenocarcinoma ($P < .00001$). Levels of GSTA1 expression in peripheral zone, HGPIN, and adenocarcinoma were not significantly different from each other ($X^2 = .776$ with two degrees of freedom, $P < .6785$) (Fig. 2).

Although prior studies have looked at global expression of GSTA in the normal prostate, to our knowledge ours is the first to examine potential variations in GSTA expression between different anatomic zones. We detected low overall expression of GSTA1 throughout most of the normal epithelium, which correlates with previous observations [17]. However, when the percent number of cells staining positive was compared between the central, transition, and peripheral zones, expression in central (median = 20.9, range = 4.2–39.3) was significantly greater than in either transition (median = 0.47, range = 0–5.7, $P < .0002$) or peripheral ($P < .0001$) zones (Fig. 1B). GSTA1 expression was also slightly greater in the peripheral relative to the transition zone ($P < .0273$) (Fig. 3).

Since GSTA1 levels in the normal epithelium of central zone were highly elevated relative to the other

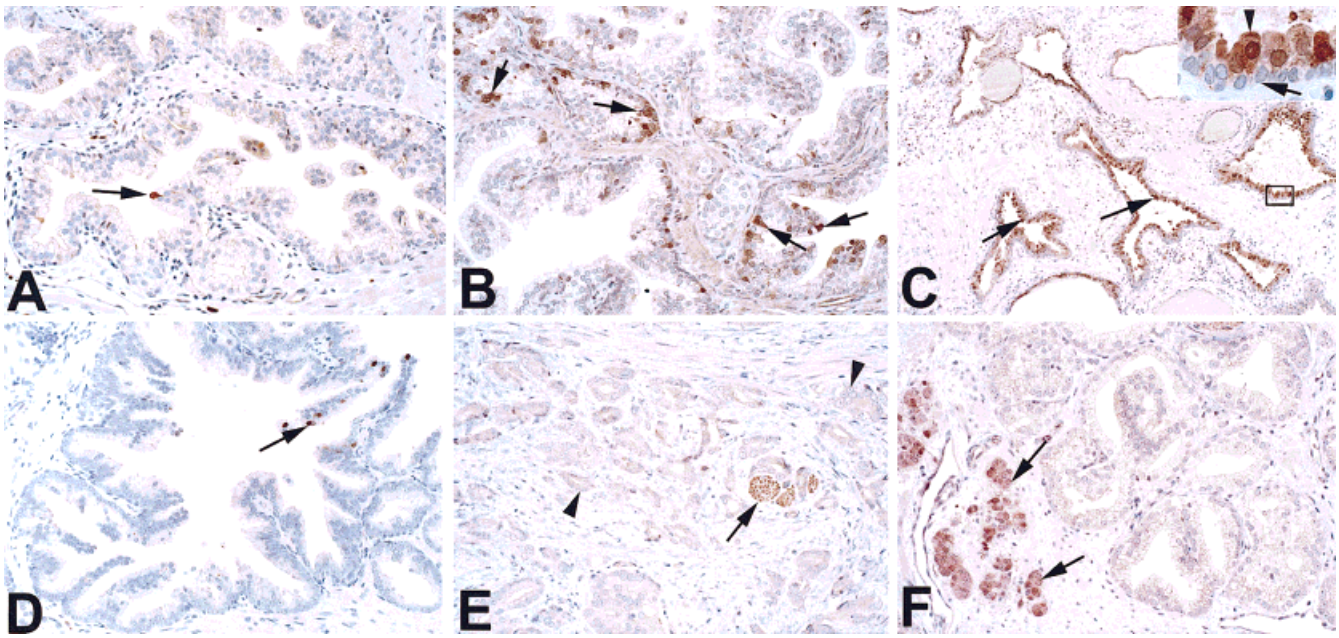

Fig. 1. Human prostate stained with polyclonal antibody to GSTAI (Immunoperoxidase). **A:** Normal peripheral zone demonstrating lack of significant staining. Arrow indicates a single positive luminal epithelial cell (×200). **B:** Normal central zone epithelium demonstrating a greater amount of staining. Arrows indicate positive luminal epithelial cells. Note lack of significant staining in the basal layer (×200). **C:** Focus of PIA with many cells staining positive (×100). Arrows indicate positive luminal epithelial cells. Inset: higher power of boxed area, demonstrating specificity of staining for luminal epithelial cells. Arrow indicates basal cell. Arrowhead indicates luminal cell (×600). **D:** Focus of HGPIN demonstrating infrequent staining. Arrow indicates a positive luminal epithelial cell (×200). **E:** Focus of adenocarcinoma demonstrating negative staining (Arrowheads), with a single nerve staining positive (Arrow) (×200). **F:** Heterogeneous area of adenocarcinoma, primarily staining negative but with some cells staining positive (Arrows) (×200).

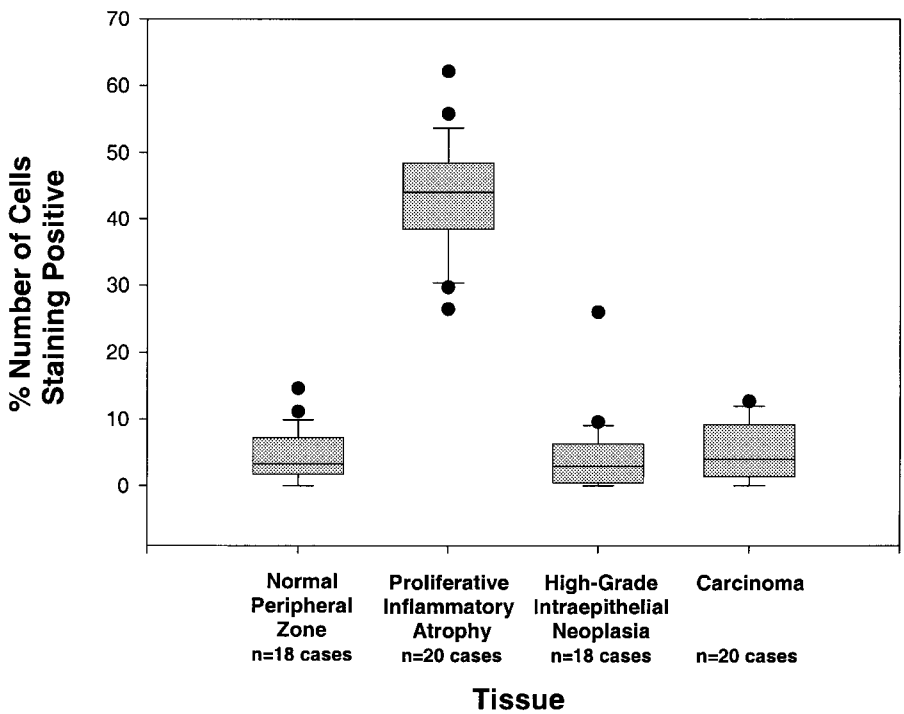

Fig. 2. Quantification of GSTAI staining in normal peripheral zone, PIA, high-grade prostatic intraepithelial neoplasia, and adenocarcinoma.

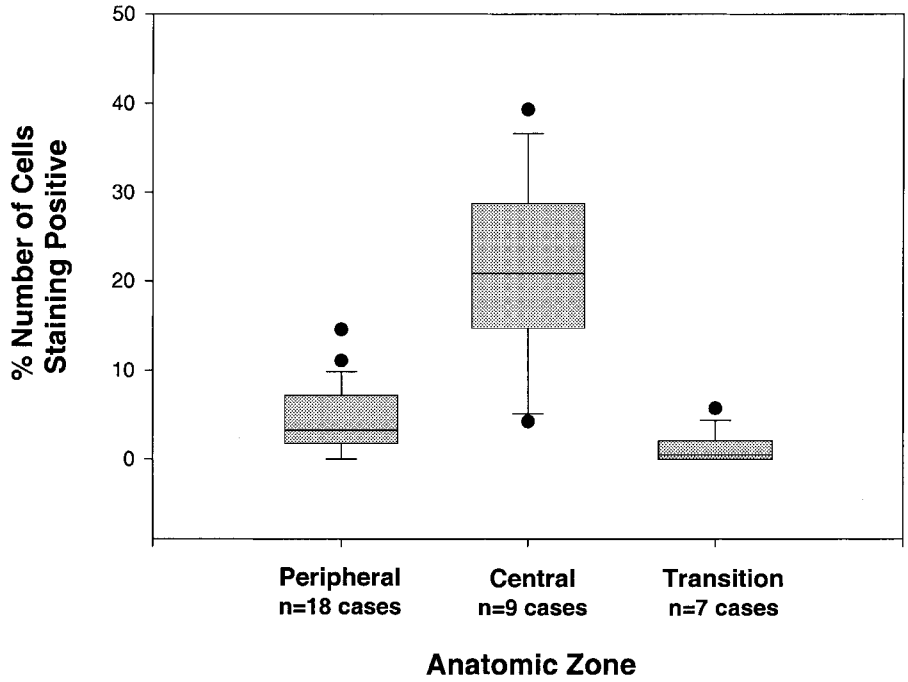

Fig. 3. Quantification of GSTAI staining in normal peripheral zone, normal central zone, and normal transition zone.

two zones, we compared levels in the central zone directly to those in PIA, and found that the PIA levels were significantly higher ($P < .0001$). Thus, GSTA1 expression in PIA was significantly greater than in all three zones of normal epithelium.

DISCUSSION

We have recently proposed that the type of atrophic prostatic epithelial cell found in PIA may serve as a precursor to both HGPIN and prostate adenocarcinoma [3,22]. We have based this hypothesis upon the following characteristics of these cells: (i) increased proliferation; (ii) decreased expression of $p27^{Kip1}$; (iii) increased expression of bcl-2; (iv) cytokeratin expression profiles consistent with intermediate cell differentiation (Van Leenders and De Marzo, written communication)—significant since intermediate cells are a proposed target for neoplastic transformation in the prostate [3,23]; (v) the tendency of atrophic cells to occur in association with HGPIN lesions; and (vi) shared morphological transitions between atrophic cells and HGPIN cells [3,22]. Additionally, recent data from FISH analysis indicate gains in signal for chromosome 8 centromere in PIA [21]. Moreover, while GSTP1 expression is decreased in adenocarcinoma cells, it is increased in many of the luminal cells in PIA—raising the possibility that these atrophic cells are producing GSTP1 in response to local oxidative stress.

We investigated this possibility further by measuring the expression of GSTA1, another major enzyme that is typically induced by oxidative stress. Our findings support the concept that atrophic prostatic epithelial cells are responding to stress by demonstrating that the expression of GSTA1, like GSTP1, is markedly increased in PIA relative to normal epithelium. While the vast majority of normal prostate epithelial cells did not stain for GSTA1, nearly 50% of luminal secretory cells in PIA did. This pattern clearly represents an abnormal process, and reinforces the concept that the epithelium in PIA is reacting to oxidative stress—perhaps from phagocytes or irritant chemicals located within the lumen of the duct. Since GSTP1 is abundant in normal basal cells, it is possible that GSTP1 elevation in luminal cells in PIA reflects the fact that these cells have adopted a basal phenotype. Yet, the absence of GSTA1 in normal basal cells, and its elevation in PIA luminal cells, undermine this argument. Interestingly, the overall number of cells staining positive for GSTA1 in normal epithelium in the present study was quite low when measured against the overall number of cells that stained positive for GSTP1 in previous studies. Indeed, in contrast

to the relative paucity of GSTA1 seen in normal prostate, GSTP1 is expressed in greater than 90% of normal-appearing basal cells. The vast majority of normal secretory luminal cells in the prostate do not, however, express GSTP1 [2].

Though previous studies have examined the presence of GSTA1 in prostate cancer, ours represents the first comprehensive effort to quantitatively analyze its expression in normal and diseased cells of the human prostate. Our data allowed us to compare the expression of GSTA not only between PIA, PIN, and adenocarcinoma lesions, but between different anatomic zones of the normal prostate as well. In spite of the overall low amount of GSTA1 activity detected in the normal prostate, we were able to discern distinct variations in its levels of expression between zones. Central zone epithelium demonstrated a statistically significant higher percentage of cells staining positive than epithelium from either the peripheral or transition zones, supporting McNeal's concept of a central zone cellular phenotype that is distinct from that of other zones [25]. Moreover, since carcinoma does not tend to develop in the central zone, this pattern suggests a potential link between constitutive expression of GSTA1 and the proclivity for particular anatomic zones of the prostate to develop cancer.

Expression of GSTA1 was also low in most HGPIN and adenocarcinoma lesions, with no statistically significant difference detected between positive staining in these cells and those in normal prostate peripheral zone. Murray et al have previously observed low amounts of GSTA1 in prostate adenocarcinoma [16]. However, our results differ somewhat in that while Murray's group reported 27% of cases to be positive (positive defined as at least one cell staining positive in the tumor), 85% of our lesions (17 of 20) contained at least one cell that stained positive. We used a different commercial source of rabbit polyclonal antibodies, which may account for this discrepancy. Elucidating the cause for low levels of expression of GSTA1 in adenocarcinoma cells represents an intriguing problem.

As noted above, we detected GSTA1 expression almost exclusively within secretory epithelial cells. This remained true for all normal, PIA, HGPIN, and adenocarcinoma cells. This observation contrasts with earlier studies documenting consistently strong immunoreactivity for GSTA in both layers of the normal epithelium [16]. Since GSTP1 is expressed exclusively in the basilar layer, our findings suggest that the expression of GSTP1 is under a different regulatory mechanism than GSTA1—a concept further supported by the lack of staining of GSTA1 in the seminal vesicle and ejaculatory duct epithelium, structures that normally stain strongly for GSTP1 [14].

Thus, in conjunction with earlier studies, our work has shown that the majority of HGPIN and prostate adenocarcinoma cells fail to express significant amounts of either GSTA1 or GSTP1. This denotes a rather broad lack of detoxification and anti-oxidant enzymatic activity in neoplastic cells in the prostate. Furthermore, it demonstrates that potential deficiencies in cellular detoxification created by the reduced expression of GSTP1 are not being compensated for by a concomitant increase in GSTA1 expression. Low levels of GSTA1 and GSTP1 in adenocarcinoma also imply a substantial relationship between the diminished expression of these cellular detoxification enzymes and prostate carcinogenesis. In the rat liver, for example, induced expression of GSTA and other detoxification enzymes enhances hepatocyte resistance to neoplastic transformation caused by aflatoxin B_1 [26–28]. Recently, in a large clinical trial, Wang et al demonstrated the significance of this chemoprotective mechanism in humans by showing that oltipraz, a potent GST-inducing agent, decreases urinary concentrations of aflatoxin-related toxic metabolites [29]. Since 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (PhIP) is a potent prostate carcinogen in the rat, it, therefore, would appear promising to investigate whether induction of GSTA1 and GSTP1 in prostate epithelial cells will protect against PhIP-mediated carcinogenesis.

Whether the low levels of GSTA1 and GSTP1 expression in prostate cancer are important to the process of neoplastic transformation, though, remains unclear. GSTP1 has been shown to be inactivated as a result of promoter hypermethylation in the majority of prostate cancers [2], liver cancers [30], and in many breast cancers [31]. There is mounting evidence that GSTP1 may possess some tumor suppressor features, since mice with inactivated GSTP1 alleles develop increased numbers of skin tumors in response to treatment with carcinogens [32]. Given the high level of expression of GSTA1 in PIA, one would expect that GST isoenzymes protect most PIA cells from the DNA-damaging reactive electrophiles generated by an inflammatory response. The absence or loss of such enzymes, under conditions of chronic oxidative stress, could lead to the accumulation of DNA damage, genetic alterations, and subsequent neoplastic transformation. In this sense, our study provides additional support for an inflammation-induced model for prostate cancer.

CONCLUSIONS

In the human prostate, expression of GSTA1 occurs primarily in luminal secretory cells. Although there is some variability in expression between different

anatomic zones, overall levels of GSTA1 in normal epithelium are low. In regions of PIA, however, GSTA1 expression is markedly increased. This pattern of expression supports the theory that cells in PIA lesions are responding to localized increases in oxidative stress. Low levels of GSTA1 and GSTP1 in HGPIN and prostate adenocarcinoma suggest a broad lack of detoxification activity in these cells, which may be associated with carcinogenesis in the prostate.

ACKNOWLEDGMENTS

The writers thank Elizaboth A. Platz for help with statistical analysis and Helen Fedor for preparing prostate tissues.

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