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Abstract

BACKGROUND—Epigenetic silencing of glutathione S-transferase π (GSTP1) is a hallmark of transformation from normal prostatic epithelium to adenocarcinoma of the prostate. The functional significance of this loss is incompletely understood. The present study explores the effects of restored GSTP1 expression on glutathione levels, accumulation of oxidative DNA damage, and prostate cancer cell survival following oxidative stress induced by protracted, low dose rate ionizing radiation (LDR).

METHODS—GSTP1 protein expression was stably restored in LNCaP prostate cancer cells. The effect of GSTP1 restoration on protracted LDR-induced oxidative DNA damage was measured by GC-MS quantitation of modified bases. Reduced and oxidized glutathione levels were measured in control and GSTP1 expressing populations. Clonogenic survival studies of GSTP1-transfected LNCaP cells after exposure to protracted LDR were performed. Global gene expression profiling and pathway analysis were performed.

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SUPPORTING INFORMATION
Additional supporting information may be found in the online version of this article at the publisher’s web-site.

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RESULTS—GSTP1 expressing cells accumulated less oxidized DNA base damage and exhibited decreased survival compared to control LNCaP-Neo cells following oxidative injury induced by protracted LDR. Restoration of GSTP1 expression resulted in changes in modified glutathione levels that correlated with GSTP1 protein levels in response to protracted LDR-induced oxidative stress. Survival differences were not attributable to depletion of cellular glutathione stores. Gene expression profiling and pathway analysis following GSTP1 restoration suggests this protein plays a key role in regulating prostate cancer cell survival.

CONCLUSIONS—The ubiquitous epigenetic silencing of GSTP1 in prostate cancer results in enhanced survival and accumulation of potentially promutagenic DNA adducts following exposure of cells to protracted oxidative injury suggesting a protective, anti-neoplastic function of GSTP1. The present work provides mechanistic backing to the tumor suppressor function of GSTP1 and its role in prostate carcinogenesis.

Keywords
prostate; GSTP1; oxidative stress; cancer

INTRODUCTION

Prostate cancer is the second most frequently diagnosed cancer in men and the second leading cause of cancer deaths in men in the United States [1,2]. The biochemical and genetic origins of prostate cancer, however, remain incompletely understood. In order to identify rational targeting strategies aimed at decreasing morbidity and mortality, an improved understanding of prostate cancer development and progression is needed. In recent years, epidemiologic and molecular studies have drawn increasing attention to the significance of the ubiquitous silencing of \( \pi \)-class glutathione s-transferase (GSTP1) expression in prostate carcinogenesis [3].

Glutathione S-transferases (GSTs) are enzymes known to inactivate reactive electrophiles through conjugation to reduced glutathione (GSH) yielding soluble byproducts that can be more effectively eliminated. This function for GSTs has led to the understanding that GSTP1 and other phase II enzymes in this class play a protective “caretaker” role early in prostate carcinogenesis [4]. In addition to their protective function during periods of oxidative stress, GSTs have also been implicated in antineo-plastic drug resistance as well as apoptotic and proliferative signaling pathways [5,6]. Within the family of cytosolic GSTs, \( \pi \)-class GST (GSTP1) has been shown to be overexpressed in various cancers [7,8], yet GSTP1 expression is reduced or absent in almost every case of human prostate cancer [3,9,10]. GSTP1 inactivation is mediated by somatic promoter CpG-island hypermethylation, an event that likely occurs early in malignant transformation [3,10–12].

Consistent with its protective role in mitigating oxidative stress, inactivation of GSTP1 is thought to play a role in the accumulation of genetic damage in prostate cancer following chronic exposure to oxidants or electrophiles from dietary sources [13]. However, the potential mechanistic roles of GSTP1 Loss in prostate cancer initiation and progression are not fully understood.
Given the role of GSTP1 in GSH-conjugation as well as GSH-redox reactions with organic peroxides [14,15], we hypothesized that prostate cancer cell exposure to protracted oxidative stress would lead to accumulation of oxidative DNA damage that might, in part, drive neoplastic transformation and, in the setting of GSTP1 expression, would result in depletion of cellular GSH stores. Bankruptcy of GSH stores in GSTP1-expressing cells has been shown to drive increased sensitivity to oxidative injury and ultimately cell death [8]. Here, we show that reconstitution of GSTP1 expression in LNCaP prostate cancer cells harboring DNA methylation mediated epigenetic silencing of GSTP1 leads to an increased sensitivity to prolonged low-dose rate oxidative stress, and reduced accumulation of potentially promutagenic oxidative damage to DNA. However, this was not accompanied by depletion of cellular GSH levels, indicating that GSH bankruptcy was not the mechanism by which the GSTP1 expression prostate cancer cells were sensitized to the protracted oxidative injury. Rather, we show that GSTP1 expression in LNCaP cells leads to a down-regulation of multiple cell survival pathways at the gene expression level.

**MATERIALS AND METHODS**

**Generation of Stable GSTP1 Clones**

LNCaP cells were grown in RPMI 1640 medium according to ATCC guidelines. LNCaP cells were transfected (Lipofectamine, Invitrogen) with a vector containing the full length GSTP1 coding sequence driven by a CMV promoter; a Neomycin resistance cassette was coexpressed from the same vector (pCMV-GSTP1). A vector-only control was transfected in LNCaP cells in parallel (pCMV-NEO). After selection in the presence of G418, the cells were plated at clonogenic density and single clones were selected. Stable expression of full length GSTP1 protein was verified by Western blot. GSTP1 enzymatic activity was measured in all clones as previously described [16].

**Measurement of GSH and GSSG Levels**

GSH and GSSG levels were assessed as described previously [17]. Briefly, cells were resuspended in 10 mM HCl and transferred to 37°C water bath followed by freezing in dry ice/ethanol bath. The freeze/thaw cycle were repeated three times. Protein was precipitated by adding sulfosalicylic acid to a final concentration of 6.5%, followed by incubation on ice for 10 min and centrifugation at 2,000 g for 15 min. Total intracellular glutathione was measured by 412 nm absorbance using the glutathione reductase-5,5′-dithiobis(2-nitrobenzoic acid) recycling assay. Total intracellular glutathione levels were determined by quantifying the intracellular glutathione levels and normalizing by the DNA concentration. The data were expressed as μM glutathione per μg DNA.

**In Vitro Measurements of Oxidized Bases**

Genomic DNA was isolated and purified from the various cell line cultures subjected to protracted, LDR (or no LDR) for 72 hr in a temperature controlled low-dose rate cesium irradiator. Gas chromatography/mass spectroscopy (GC-MS) with single ion monitoring analyses for the presence of oxidized guanine and adenine bases in the DNA samples were performed as described previously [18]. Briefly, samples were first hydrolyzed in 60% formic acid to obtain intact and modified bases and then treated with a solution of 99%
Bis(trimethylsilyl) trifluoroacetamide, 1% trichloromethylsilane dissolved in acetonitrile to convert the bases into volatile derivatives. To monitor the efficiency of base derivatization, samples were spiked with known quantities of the modified bases 8-azaguanine, 8-azadenine and 6-azathymine before acid hydrolysis. The base derivatives were analyzed by GC using a Hewlett-Packard 5890 gas chromatograph with a Hewlett-Packard 5970 mass selective detector (Hewlett-Packard, Charlotte, North Carolina).

Clonogenic Survival Studies

LNCaP cell sublines (differing in expression of GSTP1 polypeptides and enzyme activity) were exposed to protracted LDR for 24, 48, or 72 hr, a good model of protracted oxidant stress [19]. Clonogenic survival was assessed as previously described [19]. Briefly LNCaP cells obtained from ATCC (Manassas, VA, USA) and derivatized as described above were plated in triplicate at clonogenic density (100–1000 cells/10cm dish), treated and incubated in RPMI per ATCC guidelines for 3 weeks. Plates were fixed and stained in a 50% methanol 0.1% Crystal Violet solution and colonies >50 cells were counted.

Gene Expression and Pathway Analysis

Cy3 labeled cDNA was prepared from Trizol purified RNA derived from parental, vector control, and 3 stable GSTP1 expressing LNCaP cell sublines [LNCaP, LNCaP-Neo, GSTP1-1, GSTP1-3, GSTP1-5]. All lines were either sham irradiated or treated with protracted LDR (0.25 Gy/Hr) for 24 hr. The 10 labeled samples were resuspended in hybridization buffer and an overnight competitive hybridization against Cy5 labeled parental LNCaP cDNA was performed on spotted cDNA arrays (16,193 IMAGE clones, 13,815 unique genes/ESTs) similar to what has been described previously [20]. Raw data from the scanned arrays was filtered for spot quality (Q) >1 across arrays (yielding n = 7609 spots for analysis). Quality filtered features were subjected to Loess normalization and ANOVA analysis using “treatment” (LDR or sham), “construct” ([GSTP1-1, -3, -5] versus [LNCaP or LNCaP-Neo]) and the interaction term (treatment * construct) as factors (Partek®, Copyright, Partek Inc, St. Louis, MO). Mean Red/Green intensity of the Loess normalized data was used as gene expression measures. Pathway, gene network, and upstream regulator analysis were performed using Ingenuity Pathway Analysis tools (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity), as described previously [21].

RESULTS

Generation and Validation of GSTP1-Transfected LNCaP Cells

LNCaP prostate cancer cells are GSTP1-deficient due to promoter hypermethylation dependent epigenetic silencing [2,8]. In order to study the role of GSTP1 in these cells, we generated three LNCaP cell lines stably expressing GSTP1 (GSTP1 clones 1, 3, 5) and one expressing an empty control vector (LNCaP-Neo). SDS-PAGE analysis of protein extracts revealed very low GSTP1 expression in parental LNCaP and LNCaP-Neo control cells (Fig. 1A). The transfected cells expressed a range of significant levels of GSTP1 (in order of increasing qualitative expression levels: clone 3, clone 1, and clone 5). Functional validation of the expression was demonstrated by testing GSTP1 enzyme activity. As expected,
enzymatic activity in LNCaP and LNCaP-Neo cells was low and significantly increased activity was noted in the three GSTP1-clones (Fig. 1B).

**Oxidized Base Damage and Cell Survival Following Protracted Oxidative Injury**

To examine the role of GSTP1 in modulating the effects of oxidative injury, cells were treated with 72 hr of protracted LDR. Using GC-MS, we assessed DNA damage by measuring levels of the promutagenic oxidized bases 8-Hydroxyguanine (8OHdGua), 8-hydroxyadenine (8OHAde), and thymine glycol (TG). 5-methylcytosine (5-meCyt) was measured as a control. Evaluation of DNA from the GSTP1 expressing clones showed no significant increases in the accumulation of potentially promutagenic oxidized bases following protracted LDR exposure. In contrast, the GSTP1 deficient LNCaP-Neo cells exhibited over 17-fold increase of 8OHdGua, 14-fold higher levels of 8OHAde, and 3-fold elevation of TG with no corresponding increase (0.5-fold) in levels of 5-MeCyt (a non-oxidized, ubiquitously present DNA base used as control) after protracted LDR exposure (Fig. 1C). Clonogenic cell survival studies were conducted following varying durations of protracted LDR (6, 12, 18 Gy). In addition to the increased accumulation of oxidized bases as described above, clonogenic assays demonstrated on average greater than one log increased survival of LNCaP and LNCaP-Neo cell lines relative to GSTP1-expression subclones ($P < 0.01$ at 12 and 18 Gy, Fig. 1D).

**Glutathione Levels Before and After Protracted Oxidative Injury**

To study the role of GSH bankruptcy in mediating GSTP1 driven reduction in survival following exposure to oxidative injury, we assessed glutathione levels before and 6 hr after exposure to protracted, LDR. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured, and the oxidized: reduced glutathione ratio (GSSG:GSH) was determined. Comparing levels before and after protracted LDR, LNCaP-Neo cells showed a significant increase in GSH levels while the GSTP1 clones exhibited no significant change in GSH stores in response to LDR radiation (Fig. 2A), yielding no evidence of GSH bankruptcy in any of the GSTP1 clones 6 hr after oxidative injury. The GSSG:GSH ratio, conventionally used to report overall cellular oxidative stress, significantly increased in GSTP1 expressing clones, particularly in the GSTP1 clone with the highest qualitative protein expression as measured by western blot (GST-5, $P < 0.05$). By contrast, LNCaP-Neo clones showed no tendency towards increase in the GSSG:GSH ratio (Fig. 2B). Taken together, these findings suggest that the reduction in survival of GSTP1 reconstituted sub-clones exposed to protracted oxidative injury from LDR is not due to a bankruptcy of GSH.

**Global Gene Expression and Pathway Analysis**

To understand other mechanisms by which GSTP1 overexpression might influence cell survival, we undertook gene expression profiling before and after protracted LDR to identify systems level changes in the presence and absence of GSTP1. Spotted cDNA microarrays comprised of 16,193 IMAGE clones representing 13,815 unique genes/ESTs were used to query expression in radiated and untreated GSTP1 expressing clones as well as corresponding LNCaP and LNCaP-neo controls. A total of 7609 unique probes met spot quality criteria across all samples and were used for subsequent analysis. Differentially
expressed genes were defined as having a $|\text{Fold Change}| > 1.5$ and $P < 0.05$ by ANOVA analysis. There were 45 upregulated and 36 downregulated genes in GSTP1 reconstituted vs. control LNCaP cells (Fig. 3A, Supplemental Table SI). Under similar parameters, GSTP1 reconstitution in LDR treated LNCaP cells led to upregulation of 75 genes and downregulation of 85 genes compared to control cells.

Ingenuity Pathway Analysis (IPA) was used to identify key biological pathways among significantly differentially expressed genes between GSTP1 reconstituted and control cells ($P < 0.05$ and $|\text{Fold-change}| > 1.5$) to understand whether coordinated gene expression changes were underlying the observed DNA damage and survival phenotypes. Such analysis can be used to infer pathway activation state independent of the individual gene level transcription, which may be confounded by reactive positive or negative feedback mechanisms. Consistent with decreased survival following oxidative injury induced by protracted LDR, differentially expressed genes in GSTP1 expressing clones were most likely to represent key regulators of cell death and survival ($P = 4.11 \times 10^{-5}$, Figure 3B, Supplemental Table SII). Other biological functions invoked by coordinately regulated canonical pathways in GSTP1 expressing clones included cell-to-cell signaling, cell cycle arrest, and cellular growth and proliferation ($P < 0.01$, Fig. 3B). Gene network analysis, accounting for magnitude and direction of gene expression changes as well as downstream targets, predicted global activation of cell death ($P = 0.0053$, activation z-score = 1.977) and inhibition of cell viability ($P = 0.0345$, inhibition z-score = −2.185) in GSTP1 expressing clones, consistent with the observed phenotype of restored GSTP1 expression (Fig. 3C, Supplemental Table SII).

**DISCUSSION**

Studies describing the role of GSTP1 in modulating the cellular response in prostate cancer cells after oxidative DNA damage first emerged in the 1990s. GSTP1 is known to play a protective role via enzymatic GSH-conjugation (S-glutathionylation) of reactive electrophiles, which irreversibly consumes GSH, and via reversible GSH-redox reactions to reduce organic peroxides by oxidizing GSH to GSSG [8]. Initial experiments in prostate cancer cell lines performed by our group showed that GSTP1 inactivation resulted in a modest survival advantage following oxidative injury induced by protracted LDR [22]. It was subsequently observed that hepatic GSH levels decreased transiently in GSTP1-knockout mice after acetaminophen exposure followed by a rebound in GSH levels in GSTP null mice, in contrast to GSTP +/+ mice where levels remained low for prolonged periods [15]. The rebound in GSH stores observed in the absence of GSTP1 was hypothesized to play a role in mediating resistance to oxidative injury observed in the GSTP null state.

In the present study, GSTP1 restoration in LNCaP prostate cancer cells initially lacking GSTP1 expression at baseline resulted in a decrease in accumulation of potentially promutagenic oxidative DNA base damage and led to an increase in cell death to oxidative DNA injury induced by protracted LDR. Conversely, exposure of LNCAP-neo cells that lack GSTP1 protein expression to protracted LDR resulted in accumulation of substantially greater levels of promutagenic oxidative DNA damage and were more likely to survive the oxidant injury. The result is a cell that may be more prone to mutation, genetic instability
and further neoplastic transformation. This observation is consistent with prior in vitro and in vivo data demonstrating relative insensitivity to oxidative injury in the GSTP1 null state [15].

However, in contrast to previous studies, no depletion of cellular GSH stores was observed at any timepoint following protracted LDR in either GSTP1-transfected or GSTP1-deficient prostate cancer cells, suggesting glutathione bankruptcy alone is unlikely to be the principal mechanism underlying cytotoxicity following this particular form of oxidative injury in prostate cancer. Interestingly, gene expression patterns in GSTP1 positive and negative clones suggested GSTP1 Likely plays a broad and pleotropic regulatory role beyond its well-described anti-oxidant functions. GSTP1 appears to modulate a spectrum prostate cancer cell survival pathways, likely through direct and indirect perturbation of cell survival signal transduction and/or modulated androgen receptor signaling.

Analogous to the phenotype observed in null mice following acetaminophen treatment [15], we noted that loss of GSTP1 in prostate cancer cells resulted in a paradoxical resistance to oxidative cellular injury following protracted LDR. This resistant phenotype, manifesting as increased clonogenic survival, persisted in spite of the fact that GSTP1-deficient LNCaP-Neo clones accumulated substantially more oxidative DNA base damage. LDR functions in a manner analogous to chronic oxidative stress with damage accumulated in the form of sublethal oxidized base modifications. The ultimate mechanism of cell death and indeed the precise underlying cause of decreased clonogenic survival in GSTP1-transfected cells remain to be determined. However, given the gene expression and pathway analysis described in the present report, we speculate that GSTP1 can have roles in transcriptional control of cell growth and survival pathways. Various prior reports support such a function for GSTP1. For example, under oxidative stress GSTP1 is known to form oligomers that disrupt its sequestering interaction with downstream regulators, e.g., JUN N-terminal kinase (JNK). Free JNK subsequently phosphorylates its targets, thereby activating downstream effectors of transcriptional programs [6,23,24]. Such putative signaling functions, taken together with the observation that GSTP1 somatic hypermethylation and gene silencing is an early event in prostate carcinogenesis [3,9,25], may explain in part the rescued sensitivity observed in GSTP-expressing clones derived from parental null cell lines (as described here) which have evolved to thrive in the GSTP null state [26,27].

In our hands, GSTP1 expressing LNCaP clones exhibited no appreciable decline in GSH levels following protracted oxidative injury; the GSSG:GSH ratio, however, did rise following oxidative stress induced by protracted LDR, suggesting a relative excess of cellular GSH compared to reduced GSSG and presumably GSH conjugated byproducts. In contrast, LNCaP-Neo controls exhibited a rise in GSH and no change in GSSG:GSH after exposure to protracted LDR. Taken together, these observations have several implications. Prostate cancer cells likely have redundant oxidative stress responses capable of increasing GSH production at a level sufficient to compensate for oxidative stress induced GSH consumption, thereby maintaining GSH homeostasis. This would account for the stable GSH levels despite a rising GSSG:GSH ratio, as seen in the GSTP1-transfected clones after protracted LDR-induced oxidative injury. Coordinated increases in GSH production would also account for the rise in GSH levels in LNCaP-Neo cells following protracted LDR.
exposure, in this case without a corresponding rise in GSSG:GSH. In addition, though GSTP1 clearly mitigates oxidative damage (evidenced here by decreased oxidized base accumulation in GSTP1 clones), maintenance of cellular GSH levels is also consistent with a conserved role for GSTP1 in enzymatic and non-enzymatic downstream signaling interactions, which both likely depend on adequate levels of GSTP1 substrates [23,24].

Our findings suggest that epigenetic silencing of GSTP1 in prostate cancer results in accumulation of potentially promutagenic oxidative DNA damage and increased survival following oxidative injury. Enhanced survival with accumulation of promutagenic DNA bases can have pro-carcinogenic effects as it may lead to further mutation, genetic instability and enhanced neoplastic transformation. These data support an anti-carcinogenic role for GSTP1. Importantly, restoration of GSTP1 expression in LNCaP cells results in both reduced accumulation of oxidative base damage and decreased clonogenic survival following protracted LDR-induced oxidative injury, both of which would serve to reduce the risk of carcinogenic progression. GSH bankruptcy alone does not appear to be a primary mediator of increased cell death following such oxidative stress in GSTP1 positive prostate cancer cells. The protective effects of GSTP1 restoration likely involve both the enzymatic and non-enzymatic anti-neoplastic functions of GSTP1. Global gene expression profiling and pathway analysis demonstrate widespread biological changes in response to GSTP1 expression in prostate cancer cells with preferential activation of cell death and inhibition of survival pathways. These findings may have implications for prostate cancer prevention, for example through strategies aimed at induction of protective phase II enzymes such as GSTP1 [28]. Further studies are needed to validate key downstream regulators, elucidate the precise mechanisms underlying GSTP1’s protective effects, and to determine whether modulation of GSTP1 expression (e.g., epigenetic therapy) will confer potent protection against further accumulation of deleterious, promutagenic oxidative DNA injury that increases the risk of genetic instability and cancer progression.

Supplementary Material
Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
A: GSTP1 expression verified by Western blot (LNCaP-parental line, LNCaP-neo, vector control, LNCaP-GSTP-1, -3, -5, stable selected GSTP1 expressing clones). PCNA loading control. B: GSTP1 enzymatic activity as measured by glutathione conjugation assay (μMoles/min/mg). C: Relative Modified Base content: accumulation of oxidized bases: 8-Hydroxyguanine (8OHdGua), 8-hydroxyadenine (8OH Ade), thymine glycol (TG). 5-methylcytosine (5-meCyt) control. GC-MS values reported relative ratio of oxidized bases normalized to spike in aza-conjugated standard to unmodified endogenous base (dOH-N/Aza-N/N). D: Clonogenic Survival Assay. Log plot of surviving fraction vs LDR dose (0.25 Gy/hr, 6, 12, 18 Gy).
**Fig. 2.**

**A:** Total intracellular glutathione levels (GSH) measured by 412 nm absorbance, glutathione reductase 5,5'-dithiobis-2-nitrobenzoic acid recycling assay normalized to total DNA concentration (μM GSH per μg DNA), 0 and 18 Gy (0 and 72 hr LDR, 0.25cGy/hr).

**B:** GSSG:GSH ratio, GSSG measured as described above for GSH.
Fig. 3.
Significant gene expression changes following GSTP1 restoration in LNCaP clones (A) ($P < 0.05$ and $|\text{Fold-change}| > 1.5$). Expression reported as change relative to reference LNCaP parental line (blue = low, Red = high; median normalized across row). Control: LNCaP, LNCaP-Neo (2 Left columns). GSTP Transfected: LNCaP-GSTP-1, -3, -5. (3 right columns). B: GSTP1 modulated pathways and (C) Gene Network analysis (Ingenuity Pathway Analysis). Legend on the lower right depicts observed gene expression changes (Red = upregulated, Green = downregulated) as well as predicted downstream activation or repression status.