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Overexpression of Stably Transfected Human Glutathione *S*-Transferase P1–1 Protects against DNA Damage by Benzo[*a*]pyrene Diol-Epoxide in Human T47D Cells

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ABSTRACT

The (+)-anti enantiomer of benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) is a potent mutagenic and carcinogenic metabolite of benzo[*a*]pyrene (BP), and a major fraction is conjugated with glutathione *in vivo*. The chemopreventive role of glutathione *S*-transferases (GSTs) in protecting against covalent modification of DNA and other cellular macromolecules by BPDE was modeled in human T47D and MCF-7 cell lines previously stably transfected with human GSTπ1 (hGSTP1). Cells were exposed to [^{3}H]BPDE (30–600 nM). Dose-response experiments indicated that the high level of expression of hGSTP1–1 in the T47Dπ cell line (4411 $\pm$ 183 milliunits/mg of cytosolic protein, using 1-Cl-2,4-dinitrobenzene as substrate), resulted in 70–90% reduction in the covalent ^{3}H-adduct formation in DNA or RNA isolated from the GSTP1-transfected T47Dπ cell line. The lower level of hGSTP1–1 expression in the transfected MCF-7 cell line (91 milliunits/mg) provided only marginal protection against [^{3}H]BPDE adduct formation and did not affect sensitivity to BPDE-induced cytotoxicity. Protection against BPDE-induced cytotoxicity was observed only in the T47Dπ cell line, which had an IC_{50} value 5.8-fold greater than that of the T47D*neo* control cell line. Measurement of glutathione conjugates of BPDE indicated that the total conjugation was 5-fold higher in the GSTπ-transfected T47D line, most of which was exported into the culture medium over the 20-min exposure period. These results indicate that hGSTP1–1 protects effectively against DNA and RNA modification by BPDE, but moderate to high level expression may be required for strong protection against BPDE-induced genotoxicity and cytotoxicity.

Carcinogenic electrophiles, such as PAHs, are typically metabolized via a biphasic sequential mechanism, catalyzed by phase I activating and phase II detoxifying enzymes (Jakoby, 1980). GSTs constitute a multigene family of phase II conjugating enzymes that is broadly distributed phylogenetically. Detoxification of electrophilic compounds by GSTs may occur via catalytic conjugation of electrophilic intermediates with GSH, by GSH-dependent reduction of organic peroxides, or by direct binding to lipophilic compounds (Mannervik and Danielson, 1988; Hayes and Pulford, 1995). The conjugation reaction renders the compounds less reactive, more water-soluble, and more readily excretable. In chemoprevention studies in rodents, GST isoenzymes have been shown to be induced by a range of agents (such as oltipraz, diallyl sulfide, and such antioxidants as ethoxyquin) (Kensler *et al.*, 1986; Sparnins *et al.*, 1988; Clapper *et al.*, 1994). Studies with these agents have documented a significant decrease in DNA adduct formation and tumor incidence in animals exposed to carcinogens including BP and aflatoxin B_1. Chemopreventive compounds such as these have been classified as blocking agents because they prevent chemically induced DNA damage and subsequent mutation and initiation of carcinogenesis (Wattenberg, 1992).

An important caveat that complicates interpretation of mechanistic studies in animal models is that these chemo-

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ABBREVIATIONS : PAH, polycyclic aromatic hydrocarbon; GSH, glutathione; GST, glutathione *S*-transferase; *x*GSTM1–1, glutathione *S*-transferase μ-1, where *x* represents h (human) or m (murine); hGSTP1–1, human glutathione *S*-transferase π; BP, benzo[*a*]pyrene; BPDE, (+)-*anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10 epoxide; BPT-SG, benzo[*a*]pyrene-7,8,9-triol,10-glutathionyl conjugate; PBS, phosphate-buffered saline; FBS, fetal bovine serum; NQO, 4-nitroquinoline-1-oxide; MRP, multidrug resistance protein; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

preventive agents are often pleiotropic inducers of multiple enzyme activities that may each have a bearing on cellular sensitivity to carcinogen damage (Wattenberg, 1992; Talalay *et al.*, 1995). Although a number of studies have strongly implicated GST and other phase II detoxifying enzymes in protecting against BPDE-induced damage, it is difficult to separate their chemopreventive contributions in complex metabolic systems. A carefully controlled experimental system is needed to test the chemopreventive efficacy of individual components of cellular defensive phenotypes. Therefore, stably transfected cell lines were used as a well defined model system with which to test the extent of protection against BPDE by selected GST isoenzymes.

BP is a promutagenic PAH formed as a result of incomplete combustion of organic compounds (Jernström and Graslund, 1994). It is present along with other PAHs in cigarette smoke, diesel exhaust, and charred foods, for example, and has been implicated as an etiologic agent in smoking-related lung cancer (Yang *et al.*, 1977). The metabolic fate of BP *in vivo* involves initial activation by the CYP1A1 mixed-function oxidase (Shimada *et al.*, 1989) which results in the formation of reactive electrophilic intermediates that are capable of inducing DNA damage and mutations (Jernström and Gräslund, 1994). BPDE has been identified as the most mutagenic and carcinogenic metabolite of BP (Sims *et al.*, 1974; Slaga *et al.*, 1979). The C10 position of the arene oxide of BPDE can react spontaneously with nucleophilic groups such as thiols in proteins or with the exocyclic nitrogen groups in guanine and adenine in nucleic acids (Jernström and Gräslund, 1994). Alternatively, the epoxide can be hydrolyzed to yield a tetrahydroxyl derivative or conjugated with GSH to yield a thioether conjugate, reactions which result in loss of reactivity toward nucleophilic sites in cellular DNA (Hesse *et al.*, 1980). Although nonenzymatic conjugation of BPDE with GSH can occur spontaneously, it is greatly enhanced when GST isoenzymes are present (Sundberg *et al.*, 1997; Jernström *et al.*, 1996). The most active GST isoenzymes for BPDE conjugation belong to the μ and π class in humans (Robertson *et al.*, 1986). Because conjugation with GSH is a major route of systemic metabolism of BP, the π and μ class GST isoenzymes may play an important role at the cellular level in protection against BPDE mutagenesis.

Previous studies in this laboratory have shown that low to moderate expression of hGSTP1–1 or mGSTM1–1 in MCF-7 cells provided selective protection against some but not all cellular damage end-points by the carcinogen NQO (Fields *et al.*, 1994). A key observation from the experiments described herein was that high-level expression of hGSTP1–1 (human π-class GST) was protective against both DNA damage and cytotoxicity induced by BPDE, but only slight protection against DNA damage was seen at low activity levels. This suggests that a threshold level of GST activity may be required to achieve adequate protection against cellular damage by BPDE.

Experimental Procedures

Materials. [^{3}H]BPDE (975 mCi/mmol) and unlabeled BPDE were purchased from Chemsyn Science (Lenexa, KS). BP and other chemicals were reagent grade and were purchased from Sigma Chemical Company (St Louis, MO), Aldrich Chemical Company (Milwaukee, WI), or Fisher Scientific (Raleigh, NC)

Cell lines and culture. Establishment of clonal human MCF-7 cell lines stably transfected with hGSTP1–1 has been described previously (Moscow *et al.*, 1989; Townsend *et al.*, 1992). Cell lines were passaged as a monolayer in a 1:1 mixture of RPMI 1640 and Ham's F12 media (GIBCO, Long Island, NY) containing 5% FBS (GIBCO) and 50 μg/ml gentamycin (GIBCO) at 37° in a humidified 95% air/5% CO_2 atmosphere. Human T47D cell lines were stably transfected with hGSTP1–1 and clonal lines expressing the isoenzyme were isolated by similar procedures as described previously (Morrow *et al.*, 1992). The T47D cell lines were grown in the same medium except with 10% FBS added, and maintained as described above. The GSTP1–1 expressing lines were used for experimental comparison with the isogenic empty vector-transfected and geneticin-selected control lines, which are phenotypically identical except for GSTP1–1 expression levels.

Characterization of transfected T47D Cells. Transfected cells were grown and harvested as described above. Activity of GST was determined by a standard spectrophotometric assay using 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM GSH as substrates (Habig and Jakoby, 1981), and activities are expressed as nanomoles of substrate consumed per minute per milligram of protein (milliunits per milligram). Stable constitutive expression of the hGSTP1–1 cDNA in MCF-7 cells has been described previously and characterized by Northern and Western blot analysis (Moscow *et al.*, 1989; Townsend *et al.*, 1992; Fields *et al.*, 1994). The MCF-7 GST-transfected cell line, hGSTπ (hGSTP1–1), expresses specific activity of 91 munits/mg.

Total cellular RNA for Northern blotting was isolated from cells via isopyknic cesium chloride gradient ultracentrifugation separation and fractionated on a 1% agarose gel in 20 mM MOPS buffer pH 7.0, 5 mM sodium acetate, 0.22 M formaldehyde, and 1 mM EDTA. The gel was stained with ethidium bromide (5 μg/ml), photographed to verify even loading of undegraded RNA, and blotted to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) by capillary blotting. The RNA was then cross-linked to the blot by baking at 80° in a vacuum oven, and hybridized to ^{32}P-labeled human GSTP1–1 cDNA overnight followed by a high stringency wash at 60° for 1 hr and exposure to autoradiography film.

The Western blot analysis was done using cytosolic protein (50 μg/lane) that was electrophoresed on a 14% SDS-polyacrylamide gel electrophoresis gel and transferred by semi-dry electroblotting at 150 mA for 1 hr onto a nitrocellulose membrane. The blot was blocked with 5% nonfat powdered milk in PBS and then probed with a 1:1000 dilution of affinity purified rabbit polyclonal antihuman GSTP1–1 in 5% nonfat powdered milk. The purified antibody was prepared by coupling 25 mg of purified human GSTP1–1 in 5 ml 0.1 M HEPES buffer, pH 7.0, to ethanol-washed Affigel-15 (Bio-Rad, Hercules, CA), followed by blocking of unreacted groups with 1 M ethanolamine. Rabbit antisera developed against hGSTP1–1 and containing 10 mM KPO_4, pH 7.4, 0.4 M NaCl, and 10 mM EDTA was passed over the column followed by washing with several column volumes of the same buffer containing 1 M NaCl. Purified polyclonal antisera monospecific for hGSTP1–1 was eluted with 0.1 M glycine, pH 2.8, 1 M NaCl and immediately neutralized and then dialyzed against 10 mM KPO_4, pH 7.4, and 50% glycerol. The probed blot was washed several times with PBS, then incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat antirabbit IgG (Cappel/ICN, Costa Mesa, CA) and developed in a solution of 10 mM Tris·HCl, pH 7.5, 15% methanol, 0.5 mg/ml 4-Cl-1-napthol, and 0.015% H_2O_2.

Analysis of GSH conjugates of BPDE. Cells (2×10^6) were plated into 25-cm^2 flasks and incubated at 37° for 16–24 hr in a humidified 5% CO_2 atmosphere before use. The medium was changed to serum-free Ham's F12 at 30 min before addition of labeled BPDE. Subconfluent cells were exposed to 1.0 μM [^{3}H]BPDE for 20 min. Radiolabeled carcinogen was added to the fresh serum-free Ham's F12 medium immediately before exposure to the cells. Cells were rinsed briefly with cold PBS containing 5 mM EDTA and scraped into 1 ml PBS/EDTA and pelleted at low speed ($500 \times g$). The

cell medium and the combined cell PBS/EDTA wash and supernatant were concentrated by solid-phase extraction using Sep-Pak Vac cartridges (C18, 3 cc, 200 mg; Waters/Millipore, Milford, MA) according to the manufacturer's instructions, using methanol to elute the BPT-SG conjugates. The methanol was evaporated by N_2 gas and the residue was dissolved in 100 μl of 25 mM ammonium acetate/acetic acid, pH 4.0 (solvent A). The samples were analyzed for GSH conjugates by high performance liquid chromatography using a Nova Pak 4-μm C18 (3.9 $\times$ 150 mm) analytical column (Waters/Millipore) and a solvent system composed of solvent A and acetonitrile (solvent B) delivered at a flow rate of 1 ml/min. The elution system was 15–20% B linear for 10 min and 15–30% B linear gradient for 20 min. The effluent was monitored by UV-detection at 350 nm. Quantification of GSH conjugates was performed by comparison with authentic standard conjugate prepared as follows. BPDE was incubated with a large excess of GSH in NA_2CO_3-saturated H_2O, pH 8.5, followed by removal of the nonconjugated compound by ethyl acetate extraction as described previously (Jernström *et al.*, 1996). The cell pellets were diluted in buffer A and lysed by pulsed sonication for 20 sec at 20% of maximal energy output using a MSE Soniprep 150 with micro-tip. The samples were analyzed for BPT-SG conjugates as described above after precipitation of the proteins by perchloric acid (final concentration 5%).

Analysis of nucleic acid adducts. Cell plating and exposure were as described above for conjugate analysis except that experiments were done in serum-free Ham's F12 medium (GIBCO) which does not contain added GSH. Medium was changed from the 1:1 RPMI 1640/Hams F12 and 10% FBS to serum-free Ham's F12 medium at the time of addition of labeled BPDE. Subconfluent cells were rinsed briefly in prewarmed serum-free Ham's F12 and exposed to 30, 60, 100, 300, or 600 nM [^{3}H]BPDE for 20 min. Labeled BPDE was added to flasks immediately after dilution into Ham's F12. After [^{3}H]BPDE exposure, the cells were harvested in trypsin/EDTA and resuspended in a 50 mM Tris/5 mM EDTA buffer in 1.5-ml microfuge tubes. Cells were digested with proteinase K (20 μg/ml) at 50° for 1 hr in the presence of SDS (0.5%). The samples were extracted with phenol/chloroform (1:1), centrifuged at 12,000 $\times$ *g*, and the aqueous phase was extracted a second time with chloroform. The nucleic acids were precipitated from the aqueous fraction by addition of 0.1 volumes of 3 M sodium acetate, pH 5.2, and 2.5 volumes of cold 100% ethanol at −20° for 30 min or overnight. The nucleic acids were pelleted by centrifugation at 12,000 $\times$ *g*, washed once with 70% ethanol, recentrifuged, and resuspended in 400 μl of 10 mM Tris/1 mM EDTA and analyzed by scintillation counting. Labeling was normalized to nucleic acid content as determined by absorbance at 260 nm. Cells were exposed to [^{3}H]BPDE in the same manner for measurement of DNA and RNA adducts. Then cells were harvested by scraping into PBS/EDTA, pelleted and stored at −20° until processing. The DNA and RNA were isolated from the cells via isopyknic cesium chloride gradient ultracentrifugation separation and precipitated as previously described (Townsend *et al.*, 1992).

Cytotoxicity assay. The clonogenic survival assay was performed as described previously (Townsend *et al.*, 1992). Cells (200/well) were plated in 6-well plates for 18–24 hr before experiments. The cells were continuously exposed to the indicated concentrations of BP in RPMI/Ham's F12 + 10% FBS standard medium for 8–12 days. The T47D line has been shown previously to be sensitive to BP cytotoxicity because of activation via CYP1A1 (Vickers *et al.*, 1989). After the incubation period, the colonies were stained with methylene blue (0.16% in methanol) and counted. Clonogenic survival was expressed as percent of the control (vehicle treated) wells.

Results

Expression of hGSTP1–1 in stably transfected T47D cells. The T47Dπ (hGSTP1–1) cell line transfected with the pHD1013/hGSTP1–1 vector expressed specific activity of 4411 $\pm$ 183 munits/mg, compared with 15.9 $\pm$ 0.2 munits/mg (endogenous μ class GST) in control T47D*neo* cells. The higher GSTP1–1 expression in this cell line than in the MCF-7/hGSTπ line apparently results primarily from the high number of expression vector DNA copies inserted into the genome, as determined by Southern blotting (Morrow *et al.*, 1992). Analysis of the T47Dπ transfectant line by Northern and Western blotting indicated that both hGSTP1–1 mRNA and protein were highly expressed, as shown in Fig. 1, *left* and *right*, respectively. The mRNA and protein each migrated with the proper expected size, indicating that the hGSTP1–1 cDNA insert was correctly expressed. There was no detectable expression of hGSTP1–1 in control T47D*neo* cells, consistent with our previous observation by Western blotting that the low GST expression in the parental cell line is a μ class GST isoenzyme (data not shown).

Conjugation of BPDE with GSH. We analyzed BPT-SG conjugates formed in the T47D*neo* and T47Dπ cell lines to compare the amounts of the conjugate formed in each cell line and to determine whether the conjugate accumulated in cells or was transported out of the cells. Because previous experience indicated that the GSH conjugate of BPDE effluxes rapidly from some cell types, we analyzed the GSH conjugate in both the cells and the medium and PBS wash from each experiment. Exposure to 1.0 μM [^{3}H]-BPDE for 20 min resulted in 12-fold higher accumulation of BPT-SG in the hGSTP1–1 expressing T47Dπ cells than the control T47D*neo* cells (Fig. 2, ■). The concentration of BPT-SG in the culture medium was nearly 5-fold higher in the T47Dπ cells (Fig. 2, ▨), which indicates that most of the conjugate formed exited the cell over the 20-min incubation period. Overall, the total amount of conjugate formed in T47Dπ cells was slightly more than 5-fold greater than the amount formed in control cells. This difference is consistent with the decrease in nucleic acid alkylation, as detailed in the following section.

Nucleic acid adduct formation. Total nucleic acid adduct formation by [^{3}H]BPDE was measured over a dose range of 30–600 nM in the control and GST-transfected MCF-7 cell lines (Fig. 3). The results indicated that the highest degree of covalent modification occurred in the control cell lines. Although a consistent trend toward protection against [^{3}H]BPDE alkylation was observed in the hGSTπ (hGSTP1–1) cell line, the difference from the control did not achieve statistical significance ($p > 0.10$) for the hGSTπ line at either 300 nM or 600 nM. Therefore, we investigated whether a higher level of GST expression in a transgenic system would affect BPDE-induced damage. The dose response relationship for alkylation of total nucleic acids by [^{3}H]BPDE was determined with the control and GSTP1-transfected T47D lines, in which the T47Dπ cells have high GST activity that is comparable with the level of activity in human placenta. As was found previously, a high degree of covalent labeling was observed in the T47D*neo* (control) cell line. However, the T47Dπ cell line exhibited a 70–90% reduction in [^{3}H]BPDE labeling of nucleic acids over the dose range ($p < 0.001$). Hence, these results suggest that protection against BPDE-induced damage is dependent upon the level of GST activity, with relatively high expression required for protection in these epithelial breast cancer cell lines.

Alkylation of DNA and RNA was also measured separately in the T47D lines after [^{3}H]BPDE treatment and CsCl gradient ultracentrifugation. Protection against DNA adduct

damage was conferred by hGSTP1–1 in the T47Dπ cell line, as shown by a 77–85% reduction in DNA modification compared with the T47D*neo* control line (Fig. 4). Carcinogen adduct formation in RNA was similarly reduced in the T47Dπ cell line, with 80–84% reduction in comparison to the control cell line (not shown).

Modulation of BP cytotoxicity. Clonogenic survival of the MCF-7 control and MCF-7 GST-transfected cells was determined after exposure to BP concentrations ranging from 3–30 nM. The profile of the survival curves was similar in the MCF-7 cell lines (Fig. 5A), with no difference in sensitivity to BP between the control and GSTP1–1 expressing cell lines (Fig. 5A). Thus the low level of GST expression in the MCF-7 GST-transfected cell line did not convincingly protect against either the genotoxic or the cytotoxic effects of BPDE or BP. However, the higher level of hGSTP1–1 expression in the T47Dπ line rendered these cells nearly 6-fold more resistant to BP-induced cytotoxicity than the T47D*neo* control line, as indicated by an IC_{50} value of 35 nM compared with an IC_{50} of 6 nM in the control cell line (Fig. 5B).

Discussion

The progression from normal growth to malignancy involves multiple genetic changes, many of which are thought to result from exposure to chemical agents that damage DNA (Harris, 1991). Mutations in critical genes that control growth and other critical processes such as DNA repair may accelerate progression toward malignant transformation by increasing the probability of subsequent genetic alterations that promote carcinogenic progression (Loeb, 1991; Tlsty *et al.*, 1995). Hence a strong rationale exists to search for intervention strategies to block or decrease chemical damage to DNA, to prevent or delay progression to malignancy. Chemoprevention as an approach to reduction of human cancer mortality has been a focus of increasing recent investigation. Although natural mechanisms have evolved to defend against the cytotoxic or genotoxic effects of many types of carcinogens, the specificity and capacity of these natural chemoprotective defenses remains incompletely understood. In particular, development of successful chemoprevention strategies aimed at detoxifying DNA-damaging carcinogens requires an accurate understanding of the cellular metabolic activation and detoxification.

Activation of PAHs, such as BP, by cytochrome P450 isoenzymes is a preparatory step that generates substrates with sufficient reactivity to facilitate detoxification by phase II enzymes such as glucuronyltransferases, epoxide hydrolases, and GSTs. Hence, to minimize toxicity, phase I activation activities should ideally be rate-limiting and phase II detoxification activities present in excess. However, other factors such as membrane permeability and intracellular accumulation of intermediates are likely to complicate modeling of cellular metabolism beyond the simple kinetics of enzymology. Indeed, this was demonstrated clearly by the contrasting results obtained regarding sensitivity of transfected MCF-7 cells expressing hGSTP1–1 to the cytotoxic effects of NQO, depending on the presence of the MRP, a GSH conjugate efflux pump (Fields *et al.*, 1994; Morrow *et al.*, 1998). In the absence of MRP, hGSTP1–1 protected against DNA adduct formation by NQO but not against cytotoxicity (Fields *et al.*, 1994). However, in the presence of MRP, hGSTP1–1 also conferred synergistic resistance to NQO cytotoxicity as well

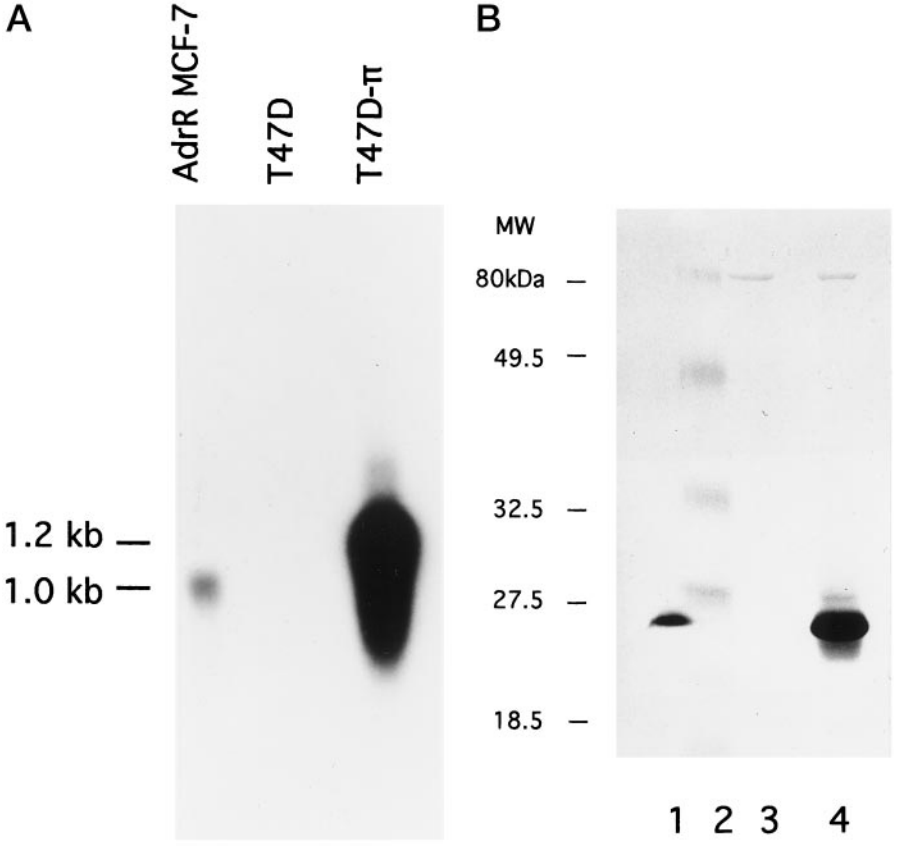

Fig. 1. Northern and western blot analysis of control and hGSTP1–1-transfected T47D lines. *Left*, Northern blot: Total cellular RNA (10 μg for MCF-7/ADR^R and T47D, or 1 μg for T47Dπ) was fractionated on a 1% agarose gel, transferred to nitrocellulose and probed with [^{32}P]–labeled hGSTP1 cDNA as described in Experimental Procedures. *Right*, Western blot: Cytosol (50 μg/lane) was fractionated on a 14% gel by SDS/polyacrylamide gel electrophoresis. Nitrocellulose was probed with affinity-purified rabbit polyclonal antibodies directed against human GSTP1–1 as described in Experimental Procedures. *Lane 1*, 50 ng of hGSTπ standard; *lane 2*, molecular mass ladder; *lane 3*, T47D*neo* (empty-vector control); *lane 4*, T47Dπ(hGSTπ).

as DNA adduct formation (Morrow *et al.*, 1998). The T47Dπ cell line used in this report was also found to be highly resistant to 4-NQO cytotoxicity (Townsend *et al.*, 1998), which suggested that the high level of expression of hGSTP1–1 in these cells might be sufficient to confer resistance to the toxic effects of other electrophilic GST substrates.

Previous studies have shown that addition of GSH and purified GST to C3H/10T1/2 cell lysates greatly reduced the concentration of free BPDE formed from labeled BP, whereas GSH alone had no effect (Ho and Fahl, 1984). Experiments with a mammalian co-culture mutagenesis assay system suggested that the presence of GSTP1–1 in the metabolic activation-competent H4IIE cell line conferred GSH-dependent protection against BP-7,8-dihydrodiol mutagenesis in the target V-79 mutagenesis tester cell line (Romert *et al.*, 1989). The experiments reported herein indicate that hGSTP1–1 expressed in the target cell also is highly protective against modification of cellular nucleic acids by BPDE added to extracellular medium. Chemoprotection conferred by hGSTP1–1 was more than 6-fold at the high level of hGSTP1–1 activity expressed in T47Dπ cells. The reductions in both nucleic acid adduct formation and cytotoxicity of BP were commensurate with the 5-fold higher total amount of GSH conjugate of BPDE formed by T47Dπ cells compared with T47D*neo* cells. In contrast, at the much lower GST expression levels in the transfected MCF-7 cells, protection was not statistically significant, although a trend toward reduction in nucleic acid adducts was noted.

Although the bulk of the BPT-SG conjugate was found in the medium of both T47D*neo* and T47Dπ cells, the ratio of intracellular to extracellular BPT-SG was higher in the T47Dπ cells (Fig. 2). This probably reflects a limitation or

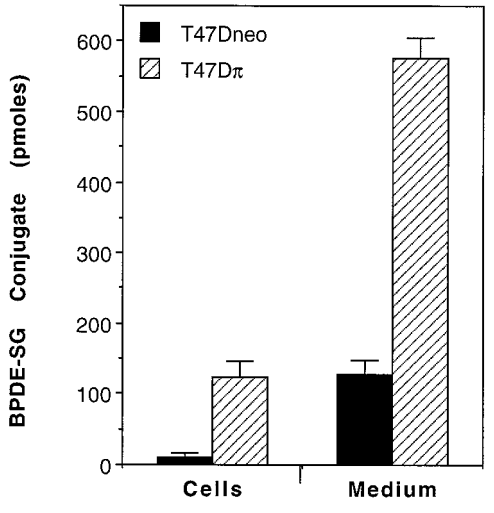

Fig. 2. Analysis of BPDE-SG conjugates in T47D cell lines. Cells (2×10^6) were plated into 100 mm dishes 16–20 hr before exposure to 1.0 μM [^{3}H]BPDE for 20 min. Cells were harvested and processed for analysis of GSH conjugates by reversed-phase high performance liquid chromatography as described in Experimental Procedures. Results are the mean ± standard deviation of three determinations.

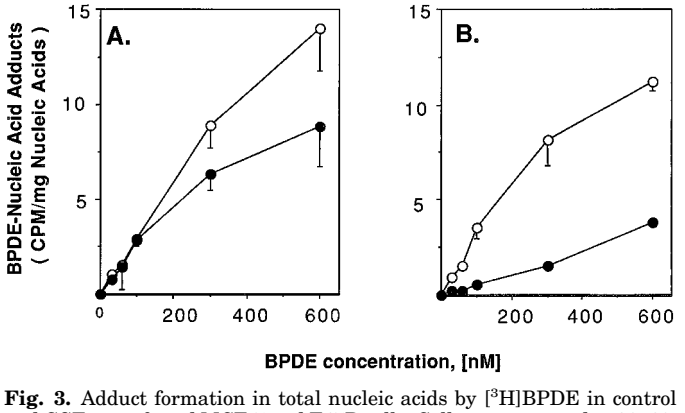

Fig. 3. Adduct formation in total nucleic acids by [^{3}H]BPDE in control and GST-transfected MCF-7 and T47D cells. Cells were exposed to 30, 60, 100, 300, or 600 nM [^{3}H]BPDE in GSH-free media for 20 min. Total nucleic acids were isolated from harvested cells and analyzed by scintillation counting to determine the degree of alkylation, expressed as cpm/mg. A, control (○) and hGSTP1–1 transfected (●) MCF-7 cells. B, control (○) or hGSTP1–1 transfected (●) T47D cells. Results are the mean ± standard error of 3 determinations.

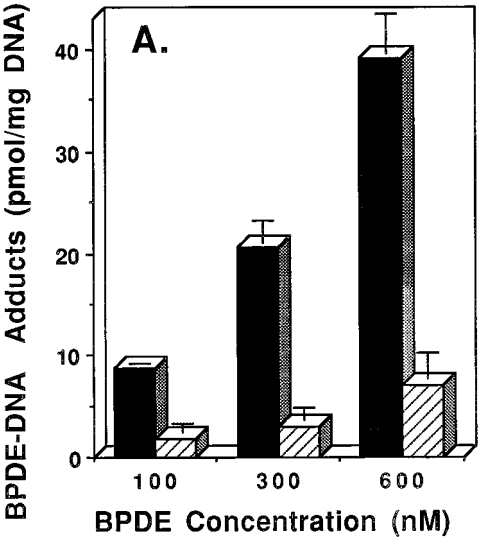

Fig. 4. Adduct formation in DNA by [^{3}H]BPDE in control and GST-transfected T47D cells. Cells were exposed to 100, 300, or 600 nM [^{3}H]BPDE in GSH-free media for 20 min. DNA was isolated from the cells via cesium chloride gradient ultracentrifugation separation, and analyzed by liquid scintillation counting to determine the degree of alkylation. Results are the mean ± standard error of three determinations.

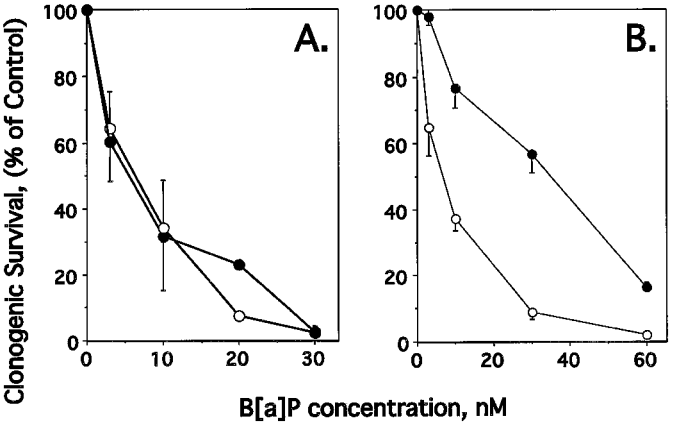

Fig. 5. Sensitivity of GSTπ transfected and control MCF-7 and T47D cell lines to BP cytotoxicity. Cells (200/well) were exposed 24 hr after plating to the indicated concentrations of BP, then grown for 2 weeks (continuous BP exposure), and stained. Surviving colonies were counted and survival expressed as percent of the control (untreated) plating efficiency. A, control (○) and hGSTP1–1 transfected (●) MCF-7 cells. B, control (○) or hGSTP1–1 transfected (●) T47D cells. Results are the mean ± standard deviation of three determinations.

saturation in the rate of efflux of the conjugate at the higher rate of its production in the T47Dπ cells. However, under normal conditions of exposure *in vivo,* the rate of production of the BPT-SG conjugate may be much slower because of limitations imposed by the rate of activation by CYP1A1. Neither the transporter responsible nor the nature of the transport mechanism for BPT-SG is presently known. Further studies will be required to understand the relative rates of metabolic flux from BP to BPDE and then to BPT-SG, the kinetics and significance of conjugate efflux capacity, and the impact of these on toxicity.

A recent and potentially important discovery was the finding that the GSTP1 gene is polymorphic in humans, with relatively low frequencies of two additional alleles that vary at amino acid positions 104 and 113 (Zimniak *et al.*, 1994; Ali-Osman *et al.*, 1997; Harries, 1997). The three variant allelotypes have functionally distinct enzymological characteristics. The recombinant variant homodimers have been expressed in *Escherichia coli*, purified, and used for enzymological comparison. The major hGSTP1a-1a isoenzyme employed in the present work (Ile104/Ala113) exhibited 3-fold higher catalytic efficiency (K_{cat}/K_m) than the other two variants toward the standard substrate 1-chloro-2,4-dinitrobenzene because of a lower K_m value, although the V_{max} values were similar (Ali-Osman, 1997). Another study compared the common hGSTP1a-1a and the purified hGSTP1b-1b (Val104/Ala113) variant homodimers for activity toward BPDE and showed a 3.4-fold higher V_{max} value for hGSTP1b-1b than for hGSTP1a-1a (Hu *et al.*, 1997b). However, the K_m values were also higher; hence, the two overall catalytic efficiencies were similar (Hu *et al.*, 1997b). In a more recent study with the hGSTP1a-1a and P1b-1b a significantly higher catalytic efficiency for BPDE conjugation was observed with the (Val104/Ala113) allelic variant (Sundberg *et al.*, 1998). In contrast to the results with the 1a and 1b homodimers, the hGST1c-1c variant (Val104/Val113) was at least 3-fold more efficient for conjugation of BPDE, apparently due to a higher affinity (lower K_m value) for the substrate as well as a slightly higher V_{max} value (Hu *et al.*, 1997c). This result suggests that the hGSTP1c allele might be more protective against BPDE than the common hGSTP1a allele. The protective efficacy of the hGSTP1b allele may be equal or lower, because the V_{max} and K_m for BPDE were both increased to a similar extent, yielding about the same catalytic efficiency but with a lower substrate affinity (Hu *et al.*, 1997b).

The significance of the enzymological differences between these hGSTP1 variant alleles remains to be established, but preliminary epidemiologic data suggests that the variant alleles are associated with increased risk of certain cancers (Ali-Osman *et al.*, 1997; Harries *et al.*, 1997). Intriguingly, genotyping results with a limited number of samples showed that the hGST1c allele occurred at 4-fold higher frequency in gliomas as compared with normal tissues (Ali-Osman *et al.*, 1997). Furthermore, separate studies found a higher frequency of the hGST1b allele in lung, bladder, and testicular cancer patients than healthy control populations (Harries *et al.*, 1997; Ryberg *et al.*, 1997). Finally, the level of hydrophobic DNA adducts was twice as high in smokers with the hGST1b allele (Ryberg *et al.*, 1997), which suggests weaker detoxification of BP metabolites in these individuals. The higher hGSTP1c allelic frequency in glioma seems to be inconsistent with its higher associated enzymatic activity for conjugation of BPDE as discussed above. However, BP exposure was not examined in this patient sample, and an epidemiologic study showed no association between smoking and risk of developing gliomas (Hurley *et al.*, 1996). Furthermore, gliomas may result from exposure to other carcinogens that are not effectively detoxified by the hGSTP1c-1c allele.

The cancer risk associated with the individual GSTP1 alleles may depend on several factors including the particular carcinogen involved, and the *in vivo* cellular carcinogen concentrations in relation to enzyme kinetic parameters (Hu *et al.*, 1997b), and the amount of each isoenzyme expressed (Hayes and Pulford, 1995). For example, catalytic efficiencies varied significantly with different activated PAHs; (±)-*anti*-chrysene diol-epoxide was conjugated more efficiently by the hGST1b-1b variant than by the hGSTP1a-1a isozyme (Hu *et al.*, 1997a), but efficiencies were comparable with BPDE. Thus protective functions of the three allelic variants should be compared directly, under well-controlled conditions. The stable transfection approach employed in this study enables parallel comparison of cellular functions of different isoenzymes and at different expression levels.

The π class GST is one of the most active, and the most stereoselective GST isoenzyme in humans for conjugation of BPDE (Robertson *et al.*, 1986). This is an important consideration since this enantiomer is substantially more mutagenic and carcinogenic than than the corresponding (−)-enantiomer (Slaga *et al.*, 1979; Wei *et al.*, 1994), both of which are more reactive with DNA than the (±)-*syn*-BPDE enantiomers. Although π class GST seems to play a key role in detoxification of (+)-*anti*–BPDE, recently reported kinetic analyses indicated that catalytic efficiency was even higher for conjugation by hGSTM1–1 (Sundberg *et al.*, 1997). This may have relevance to human risk of lung cancer, because the hGSTM1 gene is deleted in about half of some populations (Seidegård *et al.*, 1990). Molecular epidemiologic investigations have suggested that the risk of lung and other types of cancer may be higher in individuals with hGSTM1 deletion (Zhong *et al.*, 1993). Furthermore, significantly more DNA adducts were detected in smokers having both the GSTM1 deletion and the GSTP1b variant than either alone (Ryberg *et al.*, 1997). The frequency of the combined genotype with GSTM1 deletion and GSTP1b allele was also higher among lung cancer patients than either one alone in this study, indicating that the effect on cancer risk may be cumulative for GST polymorphisms.

The results of our studies with several GST-expressing transfectant cell lines, together with growing epidemiological evidence indicate an important role for GST expression in prevention of DNA damage and resultant mutagenesis and carcinogenesis. Still to be determined are the relationship between GST activity and protection at intermediate expression levels, and whether the altered enzymatic characteristics of the polymorphic variants of hGSTP1 affect protection against genotoxicity or cytotoxicity of (+)-*anti*-BPDE at the cellular level. Another area that is not well understood concerns the dynamic balance between activation and detoxification of PAHs and other procarcinogens metabolized sequentially *in situ* by phase I and phase II enzymes. The results presented herein suggest that protection against (+)-*anti*-BPDE DNA adduct formation may begin at low GST expression levels but clearly becomes much more effective at the high levels typically found in placenta and liver. The

cytotoxicity results also indicate that adequacy of the phase II detoxification activity is the key factor that determines the lethality of BP in T47D cells. These conclusions support the contention that induction of GST expression is an important component of the anticarcinogenic activity of some chemopreventive agents.

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