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Overexpression of GSTA2 Protects Against Cell Cycle Arrest and Apoptosis Induced by the DNA Inter-Strand Crosslinking Nitrogen Mustard, Mechlorethamine

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Abstract The effectiveness of bifunctional alkylating nitrogen mustard compounds in chemotherapy is related to their ability to form DNA inter-strand crosslinks. Patients exposed to DNA inter-strand crosslinking (ICL) agents subsequently experience an elevated incidence of myelodysplastic syndromes (MDS) and MDS related acute myeloid leukemia. Fanconi's anemia (FA) patients are deficient in the repair of crosslink DNA damage and they experience a high incidence of MDS. These observations indicate that hematopoietic cells are specific target for the transforming effects of DNA crosslinking damage. Changes in transcript levels were characterized in human hematopoietic cells occurring in response to the nitrogen mustard, mechlorethamine (HN2), but not in response to monofunctional analogs. Only modest changes in a few gene transcripts were detected in HL60 cells exposed to levels of HN2 tittered to maximal dose that caused growth suppression with minimal cell death and allowed eventual resumption of normal cell growth. Under conditions of transient growth suppression, a subset of glutathione-S-transferase (GST) isoenzyme genes was consistently upregulated three to fourfold by HN2, but not by monofunctional analogs. Subsequent efforts to confirm the changes detected by microarray analyses revealed an unexpected dependence on treatment conditions. The GST alpha class A2 subfamily member transcripts were upregulated 24 h after a 1 h exposure to HN2 that caused an extensive, but transient block in late S/G2 cell cycle phase, but were minimally altered with continuous exposure. The 1-h exposure to HN2 caused a transient late S/G2 cell cycle arrest in both the HL-60 cell line and the Colo 320HSR human colon cancer cell line. Overexpression of GSTA2 by transient transfection protected Colo 320HSR cells against both cycle arrest and apoptosis following exposure to HN2. Overexpression of GSTA2 in Colo 320HSR cells induced after exposure to HN2 did not alter cycle arrest or apoptosis. The results indicate that human GSTA2 facilitates the protection of cells from HN2 damage and not repair. Our results are consistent with the possibility that GSTA2 polymorphisms, variable isoenzyme expression, and variable induced expression may be factors in the pathogenesis of MDS. J. Cell. Biochem. 95: 339–351, 2005.

Key words: glutathione-S-transferase A2; hematopoietic cell line HL60; DNA inter-strand crosslinking; nitrogen mustard; MDS; cell cycle arrest

The myelodysplastic syndromes (MDS) are a heterogeneous set of clonal hematopoietic stem cell disorders with largely unknown etiology and pathogenesis [DeVita et al., 2001]. The biologic features of MDS suggest underlying genetic instability, with progressive genetic damage as a possible source of the disrupted differentiation of hematopoietic precursors and production of peripheral blood cells that characterizes MDS. Prior exposure to bifunctional alkylating chemotherapy agents that cause DNA inter-strand crosslinking (ICL) carries a high risk (10–20% at 5 years after treatment) of developing MDS and MDS-related acute myeloid leukemia (MDR-AML) [DeVita et al., 2001]. The possible role of ICL in the etiology of MDS is further strengthened by the high propensity of Fanconi's anemia (FA) patients to develop MDS or MDR-AML. FA is characterized by hypersensitivity to ICL. The frequency of MDS and MDR-AML increase exponentially with progressive age [Head, 1996; DeVita et al., 2001]; the elderly also display impaired capacity to repair ICL and exhibit a poor tolerance to

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chemotherapy [Rudd et al., 1995]. The genes involved in the complex cellular response to ICL damage and repair are not well defined [Venkitaraman, 2004].

Variation in glutathione levels in the bone marrow mononuclear cells of MDS patients correlates with the extent of oxidative DNA damage present [Peddie et al., 1997]. The results support the possibility that cellular defense mechanisms have a role in the pathogenesis of MDS [Peddie et al., 1997]. Glutathione-S-transferase (GST) isoenzymes are ubiquitous multifunctional enzymes that play a key role in cellular detoxification. Based on sequence homology, substrate specificity, and immunological crossreactivity, GST isoenzymes have been grouped into four cytosolic classes and one microsomal class [Boyland and Chasseaud, 1969; Jakoby, 1978; Morgenstern et al., 1979; Mannervik, 1985; Mannervik et al., 1985].

The GST isoenzymes protect cells against toxicants including alkylating drugs by conjugating them to glutathione, thereby neutralizing their electrophilic sites, and rendering the products more water-soluble [Boyland and Chasseaud, 1969]. Glutathione-electrophile conjugates are metabolized further to mercapturates and then excreted [Boyland and Chasseaud, 1969; Habig et al., 1974; Awasthi et al., 2003]. A significant number of individuals are null for isoenzymes GSTM1 and or GSTT1 due to gene polymorphisms. Individuals with null phenotypes have been found to exhibit a higher than normal incidence of certain cancers. Correlations between null GST phenotypes and higher incidence of MDS have been limited to certain ethnic groups, possibly related to variable frequencies of null genotypes and/or variable exposure to environmental factors [Tsabouri et al., 2000].

We postulated that variability in repair of ICL and/or levels of cellular defense mechanisms combined with naturally occurring or iatrogenic ICL DNA damage and unknown hematopoietic-cell-specific factors contribute to the pathogenesis of MDS. In order to identify candidate genes related to the pathogenesis of MDS, we evaluated gene expression changes limited to hematopoietic cells exposed to the DNA interstrand crosslinking nitrogen mustard, mechlorethamine (HN2), but lacking in response to monofunctional alkylating drugs that are not able to form ICL. Expression of a small number of genes was altered specifically by HN2 exposure and under certain treatment conditions, the GST family member GSTA2 was upregulated. DNA transfection was used to demonstrate that GSTA2 was able to protect cells from cell cycle block and apoptosis following exposure to HN2.

MATERIALS AND METHODS

Cell Culture

The human myeloid HL-60 cell line was obtained from the American Type Culture Collection and maintained in 15% fetal bovine serum (FBS) in RPMI 1640. Cultures were routinely cut to 0.2×10^6 per ml after they reached 1×10^6 per ml (every 3–4 days). The concentration of rapidly growing cells was adjusted to 0.4×10^6 per ml in normal growth medium in preparation for drug treatments. Cells were collected at 24 h by low speed centrifugation (400 RCF) and resuspended in serum-free medium at 0.4×10^6 per ml. Cells were then exposed to various drug concentrations for 1 h, collected by centrifugation, washed once and resuspended in complete growth medium. Cells exposed continuously were suspended in serum containing medium, drugs were added, and cultures were maintained for specified times. The bifunctional alkylating nitrogen mustard (mechlorethamine/HN2) and monofunctional alkylating mustard analogs 2-diethylaminoethyl chloride (DEE) and 2-dimethylaminoethyl chloride (DME) were obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared in medium without serum and added to cultures within 5 min of preparation to minimize inactivation in aqueous solutions (half-life of less than 1 h).

HL-60 cells were conditioned to HN2 by exposure to escalating levels of drug. HL-60 cells were initially exposed to 5 μM HN2 as described above and then cells were allowed to recover and grow in complete medium for 3 weeks. The treatment cycle was repeated using 7.5 μM HN2. The level of HN2 was increased by 2.5 μM each subsequent cycle until the cells were exposed to 20 μM HN2.

RNA

Total RNA was extracted from cultured cells with Trizol (Invitrogen, Carlsbad, CA) and then subjected to RNeasy Mini kit spin columns

combined with DNase treatment according to the manufacturer's instructions (Qiagen, Valencia, CA).

Microarray

Total RNA isolated from untreated cells or those exposed to HN2, DEE or DME for 1, 6, or 30 h was used as template for labeling with cy3 or cy5 and then hybridized to a human 11KcDNA microarray (Research Genetics) by the Vanderbilt Microarray Shared Resource (Vanderbilt University). GenePix Pro (Axon Instruments, Inc.) was used to scan microarrays and perform basic analysis of arrays and generating lists of differentially expressed genes. The microarray results and a description of the experiments in MIAME (minimum information about a microarray experiment) format are available at www.vmsr.net/supl/gsta2/.

Virtual Northern

Probes of candidate genes were hybridized to a blot containing cDNAs proportionally amplified from total cellular RNA. Full-length first strand cDNAs were generated from total RNAs of control and drug treated cells using SMART™ PCR cDNA synthesis kit (BD Biosciences, Clontech, Palo Alto, CA). Double stranded cDNA was then amplified by long distance (LD) PCR (Clontech), electrophoretically separated on a 1.2% agarose gel, denatured and transferred onto Hybond N+ Membrane (Amersham, Arlington Heights, IL). Clones of all cDNAs spotted on the Research Genetics' Microarray are available from The Vanderbilt Microarray Core. Clones corresponding to candidate genes selected from the Microarray were amplified and plasmids purified using the Promega wizard plasmid purification kit at mini-scale. Probes (cDNA inserts) were either excised by restriction enzyme digestion or PCR amplified from purified plasmids. The probes were labeled with fluorescein (ECL random primer labeling, Pharmacia) and hybridized with cDNA membranes in Quickhyb (Stratagene, La Jolla, CA) at 60°C for 2 h, and washed once with 1 × SSC, 0.1% SDS; 0.5 × SSC, 0.1%SDS; 0.1 × SSC, 0.1% SDS for 15 min each at 60°C. Detection was performed by the ECL luminescence detection method (Amersham). A probe for G3PDH was used as control to normalize cDNA loading.

Real-Time Quantitative PCR

Total RNA (500 ng) was used to prepare cDNA with the SuperScript™ cDNA synthesis kit and oligo dT (Invitrogen, Carlsbad, CA). The TaqMan® gene specific expression assays for GSTA1 and GSTA2 were obtained from Applied Biosystems (Foster City, CA) and reactions performed in triplicate using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). G3PDH was used as an internal reference standard and relative quantitation of transcript levels were analyzed using SDS Version 1.7 software (Applied Biosystems). The relative Ct (threshold cycle number) corrected for the internal reference standard with standard deviation and coefficient of variation of less than 3% in these experiments was calculated as described in ABI protocols (Applied Biosystems).

Plasmid Construction and Transfection

The full-length GST2A cDNA was cloned into the multiple cloning site of pTracer–EF bsd B (Invitrogen) with C-terminal GSTA2 in frame with a V5 epitope. This vector allows both GSTA2 and GFP gene expression under the control of separate regulatory sequences. Plasmid DNA was amplified in *E. coli* X1 Blue (Stratagene) and purified by a Qiagen plasmid purification column. Colo 320HSR cells (1×10^6) were seeded in a 60-mm dish and 24 h later transfected with 1 μg of purified plasmid DNA with 8 μl of enhancer and 25 μl Effectene reagent (Qiagen) using protocols recommended by the manufacturer. Control cells were transfected with empty vector (GFP alone), LacZ, or other substituted constructs. All cells expressing the GFP fluorescence marker also express cloned genes. Positive GSTA2 expressing cells were confirmed by immunoblotting with anti-V5 antibody in transfected cells.

Western Blot

Expression of GSTA2 and LacZ was measured in transfected cells. Control Colo 320HSR cells as well as those transfected with GSTA2 or LacZ were collected, washed with PBS and cytosol proteins solubilized in 100 μl PBS, 1 mM EDTA, 0.5% Triton X-100, 0.5 NP-40, and the cocktail of protease inhibitors for 15 min at room temperature. Insoluble material and nuclei were removed by centrifugation at 18,000 RCF for 10 min. Protein concentrations were estimated

by Bradford reagent. Cytosol proteins (20 μg) from each sample were separated on a 12% acrylamide gel and transferred onto nitrocellulose membranes. Membranes were blocked with 5% blotto in PBS/0.1% Tween-20. Membranes were probed with anti-V5 (peroxidase conjugated) (Invitrogen) and detected by ECL luminescence.

GST Assay

Total GST activity (cytosolic and microsomal) was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione. The conjugation is accompanied by an increase in absorbance at 340 nm [Habig and Jakoby, 1981].

Cell Cycle Analysis

The cells were prepared for cell cycle analysis by adjusting the concentration to 1×10^6 cells/ml with one wash in PBS + 2% FBS and then the pellet was fixed in 70% EtOH 1 h at room temperature in the dark. The cells were washed twice in the PBS + 2% FBS to remove excess EtOH. Equal volumes of RNase (Worthington Biochemical Lakewood, NJ) and the DNA intercalating nucleic acid dye, 7-aminoactinomycin D (Molecular Probes Eugene, OR), (500 μl) was added and then the cells were incubated in the dark at room temperature for 2 h. The cell cycle events were acquired (no more than 300 events per second) on a quality controlled Epics XL Cytometer (Beckman Coulter, Fullerton, CA). Analysis was performed using Modfit LT 3.0 (Verity Software Topsham, Maine) with DDE links to Microsoft Excel 5.0 (Microsoft Redmond, WA).

Apoptosis Assay

Treated cells were washed in RPMI 1640 using centrifugation at 400 RCF for 5 min. Supernatant was aspirated and cells dispersed by gentle vortexing. Annexin-V-PE (Caltag Laboratories, Burlingame, CA) was added in saturating amounts as determined by titration. Tubes were vortexed and incubated in the dark at room temperature for 1 h. Tubes were washed twice with RPMI, and cells were resuspended in 1 ml of RPMI. Cells were acquired on a quality controlled EPICS XL MCL cytometer (Beckman Coulter, Fullerton CA) collecting 50,000 events for each tube. An aliquot of cells was also collected by centrifugation, RPMI aspirated and cells fixed by drop-wise addition of 1 ml of ice-cold 70% EtOH for 1 h at room temperature in the dark. Cells were washed two times in Hanks + Balanced Salt Solution (Fisher Scientific, Houston, TX). Supernatant was aspirated and cells dispersed by gentle vortex. RNAse (500 μl) was added to each tube, vortexed, and then 500 μl of 7-AAD (Molecular Probes, Inc., Eugene, OR) working solution was added. Tubes were incubated at room temperature in the dark for 2 h. Tubes were then analyzed on the EPICS XL cytometer, gating on the FSC/SSC and singlet events as determined by the DNA peak versus linear signal. Fifty thousand events were collected from each tube. Analysis was performed using WinList 5.0 (Verity Software House, Topsham, MA) and Microsoft Excel 2000 (Microsoft, Redmond, WA).

RESULTS

GST Isoenzyme Transcripts Induced by HN2

The microarray experiments were designed to detect changes in levels of mRNA transcripts specific for exposure to bifunctional HN2, and not with the monofunctional alkylating analogs DEE and DME [Marathi et al., 1996]. The microarray analyses were completed with total RNA from cells treated with DEE and DME (samples combined) and cells treated with HN2 for 1, 6, and 30 h. A subset of GST isoenzyme transcripts were modestly upregulated specifically by HN2 (Table I). The virtual Northern method of evaluating transcript levels was used to confirm the microarray results and these analyses showed an unexpected dependence of some GST expression changes on treatment conditions. Although HN2 has a short half-life in aqueous solution, changes in certain GSTs depended on whether cells were exposed continuously (without washout) or limited to a 1 h

TABLE I. Microarray Results

	HN2/DEE+DME			HN2/NT[a]
GST	1 h	6 h	30 h	30 h
M3	2.7[b]	4.3	3.0	3.7
M5	1.6	2.7	1.7	2.0
T1	2.2	4.6	3.3	2.7
T2	3.0	2.8	2.4	2.5
A3	2.0	4.0	2.0	2.1
A2	1.4	1.6	1.4	1.6
P1	1.7	1.3	2.0	1.9

[a]Not treated.
[b]Fold increase in the level of the signals (ratio of means) in samples obtained from HN2 treated cells.

exposure (with washout at 1 h). The upregulation of GSTT1 observed in the microarray analyses of continuously exposed cells was not confirmed with 1-h washout and GSTA2 (relatively unaffected under the conditions of the microarray analyses) was upregulated over threefold in the virtual Northern analysis of RNAs isolated 24 h after a 1 h exposure to HN2 (Fig. 1). The monofunctional alkylating analogs, DME and DEE at 1 μM, did not influence GSTA2 expression (Fig. 1), but a low level of induced GSTA2 expression was observed with 20 μM DME and 100 μM DEE (data not shown). The virtual Northern analysis of RNA of some GST transcripts 24 h after a 1 h HN2 exposure were consistent with the microarray results and showed GSTM3 and GSTM5 (low level) upregulation and no change in GSTP1 (data not shown). A quantitative real-time RT-PCR analysis of GSTA2 and GSTA1 at 24 h after a 1 h exposure to HN2 showed a seven- and ninefold upregulation respectively over non-treated cells.

Total Cellular GST Activity

Total cellular GST activity was not elevated in HL60 cells following exposure to 1 μM HN2 (Fig. 2A). In addition, total GST activity measured over a 72 h time course following 1 μM HN2 showed no significant increase (Fig. 2B). The results were consistent with 1 μM HN2 inducing a specific subset of GST family members. Elevated total GST activity

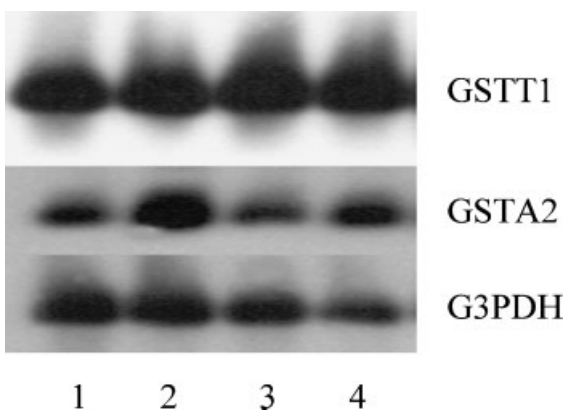

Fig. 1. Virtual Northern analysis of selected gene transcripts. Five micrograms of amplified full-length cDNA from untreated HL-60 cells (**lane 1**), HL-60 cells 24 h after exposure to 1 μM HN2 (**lane 2**), 1 μM DME (**lane 3**), or 1 μM DEE (**lane 4**) were separated on 1.2% agarose gel, alkaline denatured, and transferred onto nitrocellulose membranes. G3PDH, GSTA2, and GSTT1 cDNA probes were hybridized to replicate membranes.

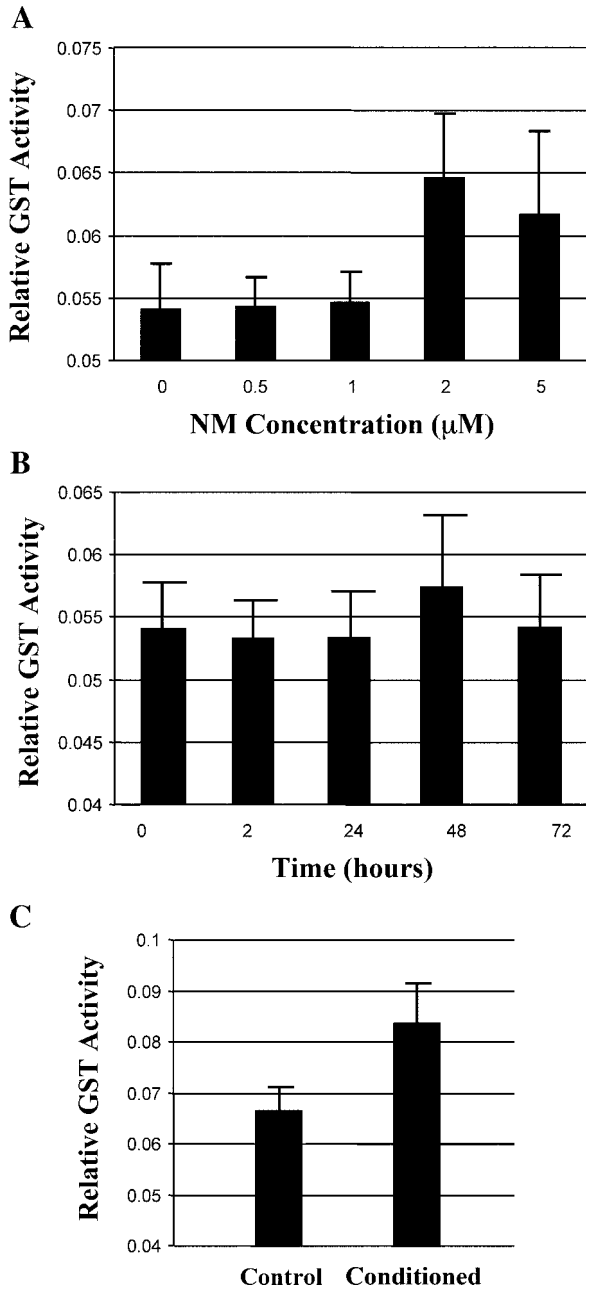

Fig. 2. Relative total GST activity in cells treated for variable times with variable levels of HN2. After 1 h exposure to HN2, HL-60 cells were washed with PBS and lysed after specified times in culture. Nuclei were removed by low speed centrifugation, and aliquots of supernatant were used to determine protein concentration and total GST activity. The relative total GST activity (normalized by equal protein concentration) after variable levels of HN2 treatment for 24 h (**A**); the relative total GST activity in HL-60 cells treated with 1μM HN2 and collected at different times (**B**); and the resting total cellular GST activity in control HL-60 and HL-60 cells conditioned with escalating doses of HN2 (see Materials and Methods) (**C**).

was detected at 2 μM HN2 (Fig. 2A). In addition, an increase in total GST activity was observed in HL-60 cells that had been exposed to escalating doses of HN2 over a prolonged period (Fig. 2C). These pretreated cells had a resting level of total GST activity 25% higher than in untreated HL60 cells (Fig. 2C). This significant increase in total GST activity protected cells from a single exposure to high levels of HN2 (2 and 5 μM), above the level required to block the cell cycle and to generate high numbers of cells with sub-G1 DNA contents consistent with extensive cell death (Fig. 3A).

HN2 Caused Cell Cycle Arrest and Apoptosis

A cell cycle DNA analysis of HL-60 cells 24 h after exposure to HN2 for 1 h showed that the number of cells accumulating in G2 progressively increased with higher levels (Fig. 3A). At the highest level of HN2, the cells appeared to accumulate with an apparent late S phase DNA content (Fig. 3A). The analysis also showed a progressive increase in the sub-G1 population of cells with higher levels of HN2 indicating that a greater fraction of cells were undergoing apoptosis (Fig. 3A). The percentages of cells in the cycle phases were calculated at each drug concentration (Fig. 3B). Cells with a sub-G1 DNA content were excluded from the calculation. The same accumulation of cells with G2 DNA content that occurred following HN2 treatment of HL60 cells was also observed in HN2 treated Colo 320HSR cells (Fig. 3C,D). The cell cycle arrest induced by HN2 was consistent with the formation of inter-strand DNA cross-links. The monofunctional alkylating analogs of HN2, DEE, and DME, did not cause the same cell cycle changes even at 50- to 100-fold higher concentrations (Fig. 3D).

GSTA2 Transfection and Protection

The functional significance of GSTA2 induced expression was examined using the transient DNA transfection approach. A full-length GSTA2 cDNA was subcloned into a eukaryotic expression vector and tagged with a V5 epitope at the c-terminal end of the open reading frame. To circumvent the inefficiency of transfecting HL-60 cells, a human colon cancer cell line was transfected with the GSTA2 expression vector. Over 20% transfection efficiency was consistently achieved with Qiagen's effectene reagent, based on fluorescence microscope and flow cytometric examination of co-expressed GFP. An immunoblot developed with antibody against the V5 epitope tag clearly showed GSTA2, and LacZ expression 24 h after Colo 320HSR transfection (Fig. 4).

The effects of GSTA2 overexpression on Colo 320HSR cells were analyzed by flow cytometry. Twenty-four hours after transfection, HN2 was added for 1 h and cultures were maintained for an additional 24 h. Transfected cells co-expressed GFP and made it possible to examine the cell cycle distribution simultaneously in both the transfected GSTA2 positive cells (GFP positive) and the non-transfected GFP negative cells in the same treated culture. As shown in Figure 5, cells that expressed GSTA2 did not arrest in G2 to the same extent following HN2 treatment as the cells transfected with GFP alone or the non-transfected cells (control) exposed to HN2 (Fig. 5). Similarly, use of constructs that expressed LacZ or MT-1B, had no effect on the number of cells shifting from the G1 (2N) to G2 (4N) DNA content (data not shown).

An experiment was completed to determine if overexpressed GSTA2 could facilitate repair of HN2 damage in addition to its suspected role in detoxifying drugs. Colo 320HSR cells were exposed to HN2 and after an hour of removing HN2, the cells were transfected with the GSTA2 expressing vector DNA. The cell cycle analysis was completed 24 h after transfection. The DNA histogram of cells with or without overexpressed GSTA2 (GFP negative) showed the same accumulation of late S/G2 cells after HN2 (data not shown). The results indicated that once HN2 damage occurred, GSTA2 was unable to prevent cell cycle arrest.

GSTA2 Protection From HN2 Induced Apoptosis

The possibility that overexpressed GSTA2 protected against HN2 induced apoptosis was evaluated using annexin V binding. Colo 320HSR cells were transfected to overexpress GSTA2 and then exposed to HN2. Annexin-V positive cells in the GSTA2 transfected cell population and non-transfected cells were quantified. As summarized in Table II, GSTA2 expression decreased the percentage of apoptotic cells.

Effects of Initial Exposure to HN2 on Cellular Response to Subsequent Exposures

An initial 1 h exposure of HL60 cells to 2.0 μM HN2 dramatically reduced the total number of

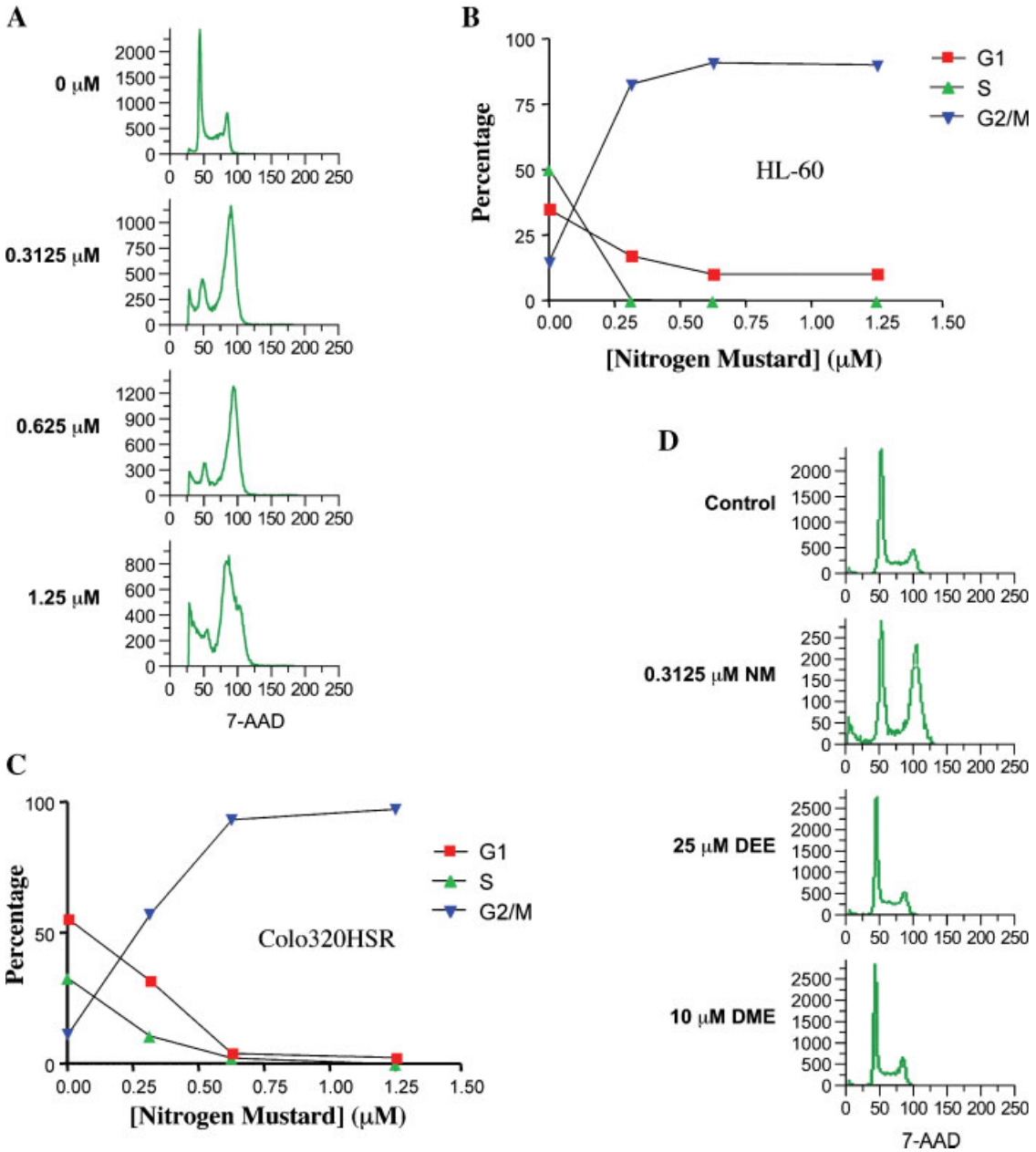

Fig. 3. Cell cycle analysis. HL-60 and Colo 320HSR cells were treated with HN2 in serum-free medium for 1 h at 37°C and then returned to complete medium for 24 h. DNA content was analyzed by flow cytometry using 7-AAD as described in the Materials and Methods. DNA histograms of HL-60 cells treated with a range of HN2 concentrations (**A**). The distributions of HL-60 cells in different cycle phases following HN2 treatment (**B**). Distribution of Colo 320HSR cells in cycle phases after HN2 treatment (**C**). DNA histogram of Colo 320HSR treated with selected concentrations of HN2 and two monofunctional alkylating analogs, DEE and DME (**D**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cells produced over the subsequent 7 days of culture compared to untreated cells (Table III). However, when HL60 cells were exposed to 1.0 μM HN2 and then rechallenged with 2.0 μM HN2 after 7 or 30 days, the cells resisted the growth suppressing effects of HN2 (Table III). The protection against rechallenge with HN2 was lost after 60 and 90 days.

DISCUSSION

The cytosolic GST isoenzymes are derived from a super family of four major classes of distantly related genes (α, μ, π, and θ). Each class comprises one or more related genes. Increased expression of GST isoenzymes in response to cytotoxic drug treatments or in

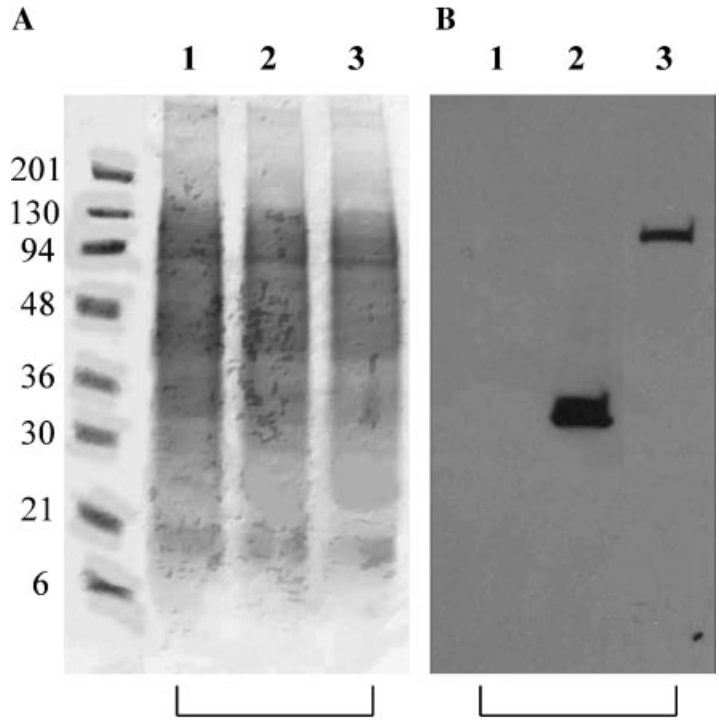

Fig. 4. GSTA2 expression 24 h after transfection of Colo 320HSR cells. One set of samples was stained with Coomassie blue to demonstrate equivalent protein loading (**A**). The second set of electrophoretically separated samples was used to detect the V5 epitope by immunoblotting (**B**). **Lane 1** contains the control (mock transfected) cell lysate; **lane 2** contains the Colo320HSR cells transfected with the GSTA2 expressing vector; and **lane 3** are cells transfected with the LacZ expressing vector. Molecular weight markers are on the left.

association with drug resistance is often cited as evidence for the role of GST isoenzymes in protecting cells from toxic agents [Tew, 1994; Hayes and Pulford, 1995]. Elevated expression of rodent alpha class GST isoenzymes has been repeatedly demonstrated in association with resistance to nitrogen mustard compounds [Robson et al., 1986, 1987; Buller et al., 1987; Lewis et al., 1988b; Schecter et al., 1991; Yang et al., 1992].

Attempts to extend the results from the rodent cells to nitrogen mustard treated human cells and tissues have not been as successful [Tew, 1994]. Human ovarian adenocarcinoma cell lines were established from a patient before and after the onset of resistance to combination chemotherapy [Lewis et al., 1988a]. The line established after the onset of drug resistance showed elevated levels of glutathione dependent enzymes and GST activity [Lewis et al., 1988a]. However, the GST alpha class isoenzymes measured were the same in the sensitive and chlorambucil resistant line [Lewis et al., 1988a]. Examination of chlorambucil resistance in human B-CLL also failed to document a role for GST alpha class isoenzymes [Schisselbauer et al., 1990; Tew, 1994; Panasci et al., 2001]. Further, the results were not consistent with glutathione or GST isoenzymes contributing to resistance. Recently, the findings with B-CLL were used to propose that DNA repair mechanisms account for resistance in B-CLL to nitrogen mustard compounds [Panasci et al., 2001]. An investigation of the mechanism responsible for melphalan resistance in multiple myeloma also showed that the rate of formation of DNA interstrand crosslinks in sensitive and resistant cells was the same indicating that protective or defense mechanisms were not involved [Spanswick et al., 2002]. The resistant cells exhibited greatly enhanced ability to repair ICL.

Escalating doses of the model GST substrate, CDNB, were used to elicit resistance in a human lung cancer cell line [Wareing et al., 1993]. The resistant cells expressed 20-fold higher levels of GSTA1 and GSTA2. The cells were not cross-resistant to chlorambucil, but were to cumene hydroperoxide. The ability of human alpha class GST isoenzymes to reduce organic hydroperoxides is well recognized [Hayes and Pulford,

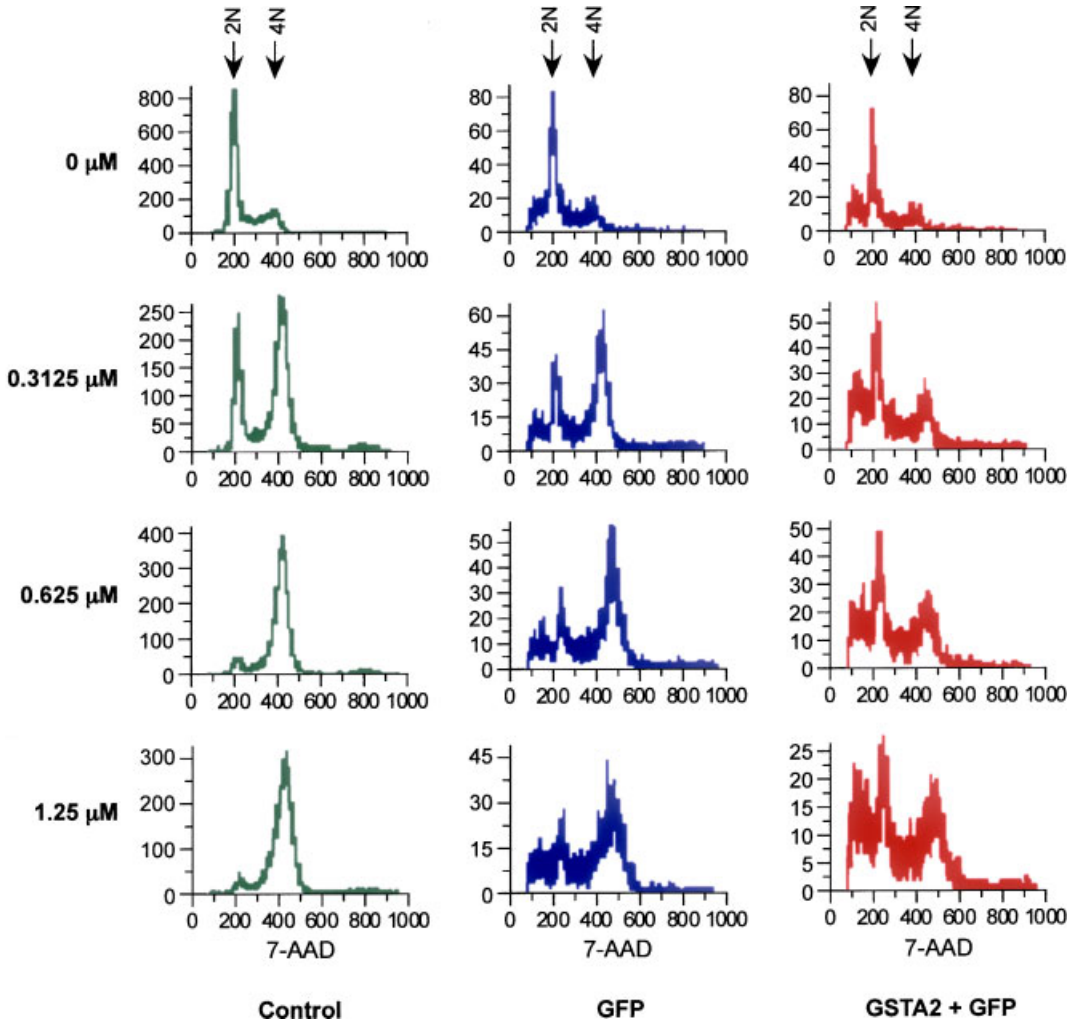

Fig. 5. Overexpression of GSTA2 protected Colo 320HSR from HN2 induced cell cycle arrest. Cultures were transfected with either a vector expressing GFP or GFP and GSTA2. After 24 h of growth in complete medium, the cultures were left untreated or treated with three levels of HN2 for 1 h. After washing, the cells were returned to complete medium for 24 h and then processed for flow cytometric analysis of GFP expression and DNA content. The first column of DNA histograms (Control) was derived from cells that were non-transfected (GFP negative following transfection with GFP + GSTA2). The second column of DNA histograms was derived from cells transfected with a vector expressing GFP alone (GFP). The third column of DNA histograms was obtained from cells transfected (selected for GFP expression) with a vector expressing both GFP and GSTA2 (GFP + GSTA2). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II. GSTA2 Protect Cells From Apoptosis Induced by HN2 Treatment

NH2 (μM)	Percentage of apoptotic cells in GSTA2 non-expressing population	Percentage of apoptotic cells in GSTA2 expressing (GFP positive) population
0	13.48 ± 1.22	11.45 ± 0.57
0.3125	16.25 ± 1.90	12.22 ± 0.95
0.625	21.21 ± 0.02	14.14 ± 0.13
1.25	22.71 ± 2.26	15.91 ± 0.46

Cultures of Colo 320HSR cells were transfected with vector DNA encoding GFP and GSTA2. Twenty-four hours after transfection, the cells were exposed to one of three levels of HN2 or untreated. Cells were then harvested for flow cytometric analysis of annexin V binding and GFP expression. The percent of annexin V positive cells was determined in both the GFP positive and negative cells.

TABLE III. Rechallenge

		Total cell production (% of non-treated)
Initial exposure		10
Rechallenge	7 days	78
	30 days	54
	60 days	15
	90 days	6

HL60 cells were exposed to 2.0 μM HN2 for 1 h (initial), and the total number of cells produced during the subsequent 7 days in culture was determined. HL60 cells exposed to 1.0 μM HN2 for 1 h were rechallenged with a 1-h exposure to 2.0 μM HN2 after 7, 30, 60, or 90 days. The total number of cells produced over 7 days following the retreatment was determined and expressed as % of untreated control cell production. Results from one of two experiments showing the same effects.

1995]. The absence of resistance to chlorambucil was attributed to the possible need for multiple factors [Wareing et al., 1993]. The possibility that resistance to nitrogen mustard compounds requires multiple factors has also been proposed to explain the difficulty in using DNA transfection of single GST genes to demonstrate responsibility for resistance to nitrogen mustards [Tew, 1994]. Transfection of GST expression vectors into the human MCF7 mammary carcinoma cells frequently did not show resistance to nitrogen mustard drugs and in a later report, both overexpression of multidrug resistance protein 1 and GSTA1 conferred resistance to chlorambucil [Morrow et al., 1998]. By contrast, the use of rodent cell lines in transfection assays has demonstrated a specific role for the GST alpha class isoenzymes in conferring resistance to nitrogen mustards, chlorambucil, and melphalan [Puchalski and Fahl, 1990]. That work also demonstrated the value of employing flow cytometry to obtain information specifically related to positively transfected cells in a mixture of positive and negative cells that result from using this approach [Puchalski and Fahl, 1990]. Our results showed that the human Colo 320HSR cells are a human target cell line suitable for demonstrating the cytoprotective effects of the single GST isoenzyme A2. Our results also demonstrate the usefulness of a flow cytometric analysis of changes in cell physiology associated with a subset of positive transfected cells in a culture where the non-transfected cells predominate.

The difficulty in relating rodent to human GST isoenzyme specific conjugating activity might be influenced by even small variations in amino acid sequences [Ciaccio et al., 1991; Gulick and Fahl, 1995; Dirven et al., 1996]. Differences in sequences between isoenzymes of the same class alter substrate specificity. Isolated human GST alpha class isoenzymes were found to catalyze the conjugation of chlorambucil to glutathione [Ciaccio et al., 1991; Meyer et al., 1992] and in one case, GSTA1 was considerably more effective than GSTA2 [Meyer et al., 1992]. In another report, GSTA1 was observed to be effective in catalyzing the conjugation of nitrogen mustard compounds, chlorambucil, melphalan, and phosphoramide mustard, while GSTA2 was not [Dirven et al., 1996]. Differences between human GSTA1 and GSTA2 isoenzymes in conjugating a number of different substrates have been reported [Bogaards et al., 1997; Coles et al., 2001a; Dreij et al., 2002].

Our results implicate both GSTA1 and GSTA2 in the detoxification of HN2 in a human hematopoietic cell line. The well-established correlation between exposures to nitrogen mustard drugs and the incidence of therapy related MDS and MDR-AML highlights the need for elucidating cytoprotective mechanisms in hematopoietic cells. Oxidative DNA damage and antioxidant defense (glutathione level) have been implicated in the pathogenesis of MDS [Peddie et al., 1997]. Evidence supporting the role of human alpha class isoenzymes in non-hematopoietic cellular responses to nitrogen mustard based chemotherapeutic drugs has favored the involvement of GSTA1. The role of GSTA2 was largely excluded by evidence from in vitro conjugating activity studies. Our results demonstrate that in a human myeloid leukemic cell line, GSTA2 appears to be involved in the cellular response to HN2. In addition, our array analysis showed that HN2 upregulated transcripts for the GSTM3 and GSTM5 genes. Neither of these human GST isoenzymes has been previously implicated in cellular responses to nitrogen mustard compounds. While 1 μM HN2 did not elevate the total GST activity in the HL-60 cells, the effects on specific GST isoenzymes and/or the effects on other uncharacterized cellular components enhanced the ability of the cell to maintain a normal rate of growth in response to a subsequent exposure to HN2 (Table III). The change in cellular phenotype was temporary, since the enhanced ability to respond to HN2 that was observed 30 days after the initial exposure to HN2 was not observed after 2 or 3 months.

Polymorphisms of the human GSTM1 and GSTT1 genes produce individuals with a null phenotype that correlates with increased incidence of certain cancers. Attempts to correlate the incidence of MDS with GSTM1 and GSTT1 null phenotypes have been inconsistent [Chen et al., 1996; Atoyebi et al., 1997; Basu et al., 1997; Sasai et al., 1999; Naoe et al., 2000; Tsabouri et al., 2000]. Variation of the frequency of the polymorphisms in different ethnic groups and variation in exposure to environmental factors in different locations may both contribute to the variable results of these analyses [Tsabouri et al., 2000]. The results of the present study indicate that GSTA2 might be an important GST to consider in the pathogenesis of MDS. While there are no reported polymorphisms of GSTA2 that produce null individuals, a wide interindividual variation in expression has been observed in human liver [Mulder et al., 1999; Coles et al., 2001b] and human pancreas [Coles et al., 2000]. In the human liver, the variable levels of GSTA2 were related to a polymorphism in the proximal promoter region of the GSTA1 gene that affected both GSTA1 and GSTA2 expression [Coles et al., 2001b]. It was also noted that because these two isoenzymes exhibit unique specificities in detoxifying carcinogens and chemotherapeutic drugs that this polymorphism should be of significance with regard to individual risk of cancer or response to chemotherapeutic agents. Although it is unknown if the same variation in GSTA2 expression occurs in hematopoietic cells, these cells are special targets in therapy related MDS and leukemia and our results indicate that this possibility should be investigated. An earlier examination of human liver primary hepatocyte cultures showed a wide variability in inducing GST alfa class transcripts with a number of agents that did not include nitrogen mustards [Morel et al., 1993]. This type of analysis should also be considered in hematopoietic cells including use of a number of nitrogen mustard compounds. The analysis of polymorphisms in the coding region of human GSTA2 was not consistent with variation in functional activity [Tetlow et al., 2001]. Transcription factors involved in antioxidant and xenobiotic responses have been implicated in the regulation of the mouse GST Ya gene [Pinkus et al., 1996]. Regulatory elements for these same factors have not been found in the human GSTA2 gene [Coles et al., 2001b].

In summary, our results implicate GSTA2 in the hematopoietic cellular response to the bifunctional alkylating agent HN2, demonstrate that cells exposed to this agent can develop transient resistance to this drug and demonstrate that ectopic overexpression of GSTA2 increases cellular resistance to this agent, but does not affect repair of damage caused by HN2. Variations in the expression of this gene should be considered as possible contributing factors to the development of MDS and MDR-AML in patients receiving bifunctional alkylating drugs.

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