Journal of Cellular Biochemistry 86:340–347 (2002)

Role of GST P1-1 in Mediating the Effect of Etoposide on Human Neuroblastoma Cell Line SH-SY5Y

S. Bernardini,[1,2]* L. Bellincampi,[1] S. Ballerini,[1] M. Ranalli,[3] A. Pastore,[2] C. Cortese,[1] and G. Federici[1,2]

[1]Department of Internal Medicine, University of Rome Tor Vergata, Rome, Italy
[2]Clinical Biochemistry Bambino-Gesù Children's Hospital-IRCCS, Rome, Italy
[3]Biochemistry Laboratory IDI-IRCCS, Department of Experimental Medicine, University of Tor Vergata, Rome, Italy

Abstract The oxidative stress could have a dual action on glutathione S-transferase (GST) P1-1 metabolism: transcriptional induction and/or polymerization. The former should represent a form of adaptation to oxidative stress and contribute to protect the cell, the latter one should activate apoptosis via c-Jun N-terminal kinase (JNK). We studied the effect of etoposide on human neuroblastoma cell line SH-SY5Y and on an etoposide-resistant clone to investigate whether a pleiotropic effect of etoposide on the redox status of the cell exists which is able to interfere with apoptosis through the GST P1-1 system. Etoposide treatment was able to induce GST P1-1 polymerization and activation of apoptosis. The data obtained from our etoposide-resistant clone and the possibility to reverse the sensitive phenotype to a resistant one by means of hexyl-glutathione preincubation, seem to suggest that cellular levels of glutathione have a key role in protecting GST P1-1 by oxidation and consequently the cell's decision between life and death. J. Cell. Biochem. 86: 340–347, 2002. © 2002 Wiley-Liss, Inc.

Key words: glutathione transferase P1-1; glutathione; etoposide; apoptosis; drug resistance

Glutathione S-transferases (GSTs, EC 2.5.1.18) belong to a large family of functionally different enzymes, which catalyzes the S-conjugation of glutathione (GSH) with a wide variety of electrophilic compounds including carcinogens, anticancer drugs, reactive oxygen species, and products of cellular metabolism. The soluble GSTs in human tissues are classified in eight classes: Alpha, Kappa, Mu, Pi, Sigma, Theta, Zeta, and Omega, some of which are present in multiple isoforms [Hayes and Strange, 2000]. GSTs are enzymes functionally active as homodimers or heterodimers formed between subunits of the same class. Even though the S-conjugation reaction protects the cell from cytotoxic and carcinogenic agents, many evidences show that some classes might be involved in anticancer drug-resistance and even be considered as tumor markers [Tew, 1994; Armstrong, 1997; Zhang et al., 1998]. In particular, elevated levels of GST P1-1 (GST P1-1) have been found in stomach, colon, bladder, testicular, prostate, breast, skin, acute myeloid and lymphoid leukemia, and lung tumors, compared with corresponding normal tissues [Helzlsover et al., 1988; Gilbert et al., 1993; Russo et al., 1994; Gaffey et al., 1995; Harries et al., 1997]. Furthermore, among individuals with similar levels of GST P1-1 expression in tumor, enzyme catalytic activity would be expected to vary according to the possible presence of variant *GSTP1* genotype. Indeed four allelic variants exist, which differ from each other by a single conservative amino acid substitution residing in the xenobiotic substrate-binding site (H site) at codons 105 and 114. The proteins encoded by the different *GSTP1* alleles show different ability to metabolize carcinogens and anticancer agents and several studies have indicated various degree of association between *GSTP1* polymorphism and

S. Bernardini and L. Bellincampi contributed equally to this work.

Grant sponsor: Ministero della Sanità; Grant numbers: RFS 1999, RF 2000.

*Correspondence to: S. Bernardini, MD, PhD, Department of Internal Medicine, University of Rome "Tor Vergata," Via di Tor Vergata 135, 00133 Rome, Italy.
E-mail: Bernardini@Med.UniRoma2.it

Received 27 February 2002; Accepted 8 April 2002
DOI 10.1002/jcb.10219

risk for a variety of cancers [Harries et al., 1997; Lee et al., 2000; Gsur et al., 2001; Maugard et al., 2001] as well as response to cancer treatment [Stanulla et al., 2000; Sweeney et al., 2000] or susceptibility to some diseases such as Parkinson's disease [Menegon et al., 1998], multiple sclerosis [Mann et al., 2000], and asthma [Spiteri et al., 2000]. Even if the results of many studies are often conflicting and the association between *GSTP1* polymorphism and disease susceptibility/outcome has not been definitively established, it is clear that it might be an ubiquitous "disease modifying" agent.

In this context, the role of GST P1-1 in modulating the stress kinases signaling pathways, and then apoptosis has to be considered. DNA damaging agents, such as UV irradiation and topoisomerase inhibitors (i.e., etoposide) seem to trigger the activation of the c-Jun N-terminal kinase (JNK) pathway, which is followed by CD95L expression and apoptosis [Kasibhatla et al., 1998]. Recently, it has been shown that monomeric GST P1-1 could form a complex with JNK and this association is inversely correlated with JNK activity. The inhibition of JNK activity by monomeric GST P1-1 is reversed by the oxidative stress, which causes an oligomerization of GST P1-1, and consequently, the dissociation of the GST P1-1-JNK complex [Adler et al., 1999] interfering with apoptosis [Bernardini et al., 2000]. On the other hand, the GST P1-1 inactivation is reversible by the binding of reduced glutathione to the enzyme [Shen et al., 1993]. In addition, several evidences suggest that aromatic compounds like etoposide, metabolized to generate phenols and reactive carbonyls could enter the "redox cycling," increase ROS production, and transcriptionally activate GST genes through the antioxidant reactive elements present in their promoter region (ARE) [Hayes and McLellan, 1999]. Therefore, the oxidative stress could have a dual action on GST P1-1 metabolism: transcriptional induction and/or polymerization. This might determine an opposite effect on cell fate, because transcriptional activation could represent a form of adaptation to oxidative stress contributing to protect the cell, while polymerization should activate apoptosis via JNK. Probably both mechanisms are present in the cell and the decision between life and death might be dependent from the functional moment of the cell as well as from the kind, level, and length of exposure to stressors.

The aim of our study was to investigate the modifications of GST P1-1 mRNA expression and protein polymerization after etoposide treatment in a human neuroblastoma cell line SH-SY5Y. In particular, we have investigated the effect of etoposide on the sensitive cell line and on an etoposide-resistant clone to better understand whether a pleiotropic effect of etoposide on the redox status of the cell exists, which might interfere with apoptosis by means of GST P1-1.

MATERIALS AND METHODS

Materials

The SH-SY5Y cell line was purchased from the American Type Tissue Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM) nutrient mix Ham's F12 (DMEM-F12), fetal bovine serum (FBS), *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), Ca^{2+}- and Mg^{2+}-free trypsin, L-glutamine and non-essential amino acids (NEAA) were purchased from Euroclone (Devon, UK). Tri-reagent, ribonuclease A, propidium iodide, etoposide, S-hexylglutathione, EDTA, Bicinchoninic acid solution and copper (II) sulfate assay, and all the reagents used for the measurement of intracellular glutathione levels were purchased from Sigma (St. Louis, MO). The first strand synthesis kit was from Roche Diagnostics (Mannheim, Germany). In addition, all the electrophoresis reagents, either on agarose or on acrylamide, were purchased from BioRad (Hercules, CA). The rabbit polyclonal antibody for the glutathione transferase P1-1 (NCL-GSTpi) was purchased from Novocastra (Newcastle, UK). The Supersignal West Pico Chemiluminescent substrate was by Pierce (Rockford, IL). All the amplifications primers used were from Invitrogen s.r.l. (Groningen, The Netherlands), while the Taq DNA polymerase and deoxynucleotides (dNTPs) were purchased from Amersham Pharmacia Biotech Europe (Freiburg, Germany). The pUC mix marker 8 was from M-medical-Genenco (Firenze, Italy). All tissue culture plastics were from Becton Dickinson labware (Bedford, MA).

Cell Cultures

The human SH-SY5Y neuroblastoma cell line was grown in DMEM nutrient mix Ham's F12 (DMEM-F12) supplemented with 10%

heat-inactivated FBS, 1% NEAA, 2 mM L-glutamine, and 15 mM HEPES, at 37°C with 5% CO_2 in a humidified atmosphere. Cells were splitted 1:5 twice weekly and routinely fed 24 h before each experiment. Cells were treated with 1 μM etoposide and/or 1 mM S-hexylglutathione at different time as reported in the result section. Each experiment was performed in triplicate. The SY5Y clone, resistant to 1 μM etoposide, was obtained after a 4 months' selection during which the cells were treated with increasing concentration of etoposide starting from 1 nM reaching 1 μM.

RT-PCR Analysis

Total RNA was extracted from 2×10^6 cells collected by centrifugation at 800*g* for 10 min using 1 ml of tri-reagent following the manufacturer's instructions. Total RNA was then reverse-transcribed with AMV reverse transcriptase according to the manufacturer's instructions. The cDNA was amplified by differential PCR using β2-microglobulin (β2-M) as internal standard. The PCR was performed with a Gene Amp PCR system 2400 (Perkin Elmer Applied Biosystems, Foster City, CA). PCR for GST P1-1/β2-M was carried out after a pre-heating at 94°C for 5 min through 25 cycles (denaturation at 94°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 1 min), and a final extension at 72°C for 7 min. The reaction mixture for a final volume of 50 μl was as follows: 8 μl of the RT reaction mixture, PCR buffer 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.25 U Taq Polymerase, and 0.5 μM of the listed primers: GST P1-1 sense: 5′-TCA CTC AAA GCC TCC TGC CTA T-3′ GST P1-1 antisense: 5′-CAG TGC CTT CAC ATA GTC ATC C-3′ β2-M sense: 5′-ACC CCC ACT GAA AAA GAT G-3′ β2-M antisense: 5′-ATC TTC AAA CCT CCA TGA TG-3′ β2-M primer sequence generated a 110-bp product; the GST P1-1 primers a 242-bp product, which includes exons 5 and 6. The RT-PCR product (15 μl) were separated on 3% agarose, 0.5 mg/ml ethidium bromide gels and visualized on Fluor-S-Max (BioRad). The ratio of target to control β2-M gene products was determined by bi-dimensional densitometry on Fluor-S-Max (BioRad).

Immunoblotting Analysis

The proteins were extracted from 5×10^6 cells collected by centrifugation at 800*g* for 10 min. The pellets, resuspended in 100 μl of 0.1 M potassium-phosphate buffer, pH 6.5, were sonicated by Sonics Vibra Cell (Danbury, CT) and then centrifuged at 12,000*g* for 5 min at 4°C to isolate the cytosolic proteins. Protein concentrations were measured by bicinchoninic acid solution and copper (II) sulfate assay (BCA-protein assay). Proteins were normalized to 50 μg/lane and applied in non-reducing conditions to a 12 % SDS–polyacrylamide gel for GST P1-1. The gel was blotted (3 h at 300 mA at 4°C) onto a nitrocellulose filter (0.2 μM). The filter was washed twice with TBS containing 0.05% Tween-20 (T-TBS) before blocking non-specific binding sites with a 2-h incubation at room temperature in 5% dry milk/T-TBS. The filter then was incubated overnight at room temperature with GST P1-1 polyclonal antibody (1:500 in 1% dry milk/T-TBS). After three washes in TBS and seven in T-TBS, the filter was incubated for 2 h with goat anti-rabbit horseradish peroxidase-conjugated IgG (1:5,000 in 1% dry milk/T-TBS) at room temperature. After three washes in TBS and seven in T-TBS, the filter was treated with the chemiluminescence detection system and detected on Fluor-S-Max (BioRad).

Determination of DNA Fragmentation

To estimate DNA fragmentation, 10^6 cells subjected to different treatments were collected at 800*g* for 10 min and fixed with 1:1 PBS and methanol–acetone (4:1 v/v) solution at −20°C. After a wash in PBS, the cells were incubated with 13 kU RNase for 20 min at 37°C and then with 40 μg/ml propidium iodide for 15 min at 37°C. The apoptotic bodies were evaluated by flow cytometry on a FACS-Calibur flow cytometer (Becton-Dickinson, San Jose, CA). Cells were excited at 488 nm using a 15 mW Argon laser, and the fluorescence was monitored at 578 nm at a rate of 150–300 events/s. Ten thousand events were evaluated using the Cell Quest Programme (ibid). Electronic gating FSC-a/vs/FSC-h was used, when appropriate, to eliminate cell aggregates. The experiments were performed in duplicate.

Measurement of Intracellular Glutathione Levels

The cells (5×10^6) differently treated were collected by centrifugation at 800*g* for 10 min. The cells were sonicated by Sonics Vibra Cell for three times of 2 s in 100 μl of 0.1 M potassium-phosphate buffer, pH 7.2 for GSSG determination, the buffer contains 5 mM

N-ethylmaleimide. After sonication, 50 μl of 12% sulfosalicylic acid was added; GSH (or GSSG) content on the acid-soluble fraction was determined; the protein pellet was dissolved in 150 μl of 0.1 N NaOH, and GS-Pro was determined. Protein concentrations were determined by BCA-protein assay.

The HPLC-system, with sample processor and solvent delivery system, was an Agilent 1100 Series, equipped with a fluorescence detector G1321A operating at an excitation wavelength of 390 nm and an emission wavelength of 478 nm; the data obtained were analyzed with the HP-chemstation program of Agilent Technologies (Hewlett-Packard Co., Amsterdam, The Netherlands). The derivatization procedure has been performed as follows: in a derivatization vial, containing 15 μl of sample and 15 μl of internal standard (50 μM cysteamine), were added 30 μl of 4 M $NaBH_4$ (dissolved in a solution of 333 ml/L of DMSO and 66 mN NaOH), 20 μl of a solution containing 2 mM EDTA and 2 mM DTT, 10 μl of 1-octanol and 20 μl of HCl 1.8 M. After a 3-min incubation at room temperature, 100 μl of *N*-ethylmorpholine buffer 1.5 M, pH 8.0, 400 μl of distilled water, and 20 μl of bromobimane 25 mM (in acetonitrile/H_2O 1:1) were added. After a 3-min incubation at room temperature, 40 μl of acetic acid was added and the autosampler injected 20 μl of this mixture into the column. The derivatized sample was injected into a 150 mm × 4.6 mm Hypersil-ODS column (ThermoQuest, Hypersil Division, Cheshire, UK) equilibrated with 30-mM ammonium nitrate and 40-M ammonium formate buffer, pH 3.6 (A). Glutathione was eluted from the column in 5 min with a gradient of acetonitrile (B) (0–4 min, 0–30% B; 4–5 min, 30–100% B) at a flow rate of 1.5 ml/min. Ambient temperature was used, and the retention time for the analyte were calculated using external standards at six different concentrations. The experiments were performed in duplicate.

RESULTS

Expression of GST P1-1 in Human Neuroblastoma Cell Line and Its Modulation by Etoposide

We have investigated the mRNA expression of GST P1-1 in the SH-SY5Y neuroblastoma cell line by means of differential RT-PCR using β2-microglobulin (β2-M) as housekeeping gene. The SH-SY5Y were treated with 1 μM etoposide for 6, 8, 10, 12, 18, and 24 h to investigate the possible presence of a modulatory effect of etoposide on the GST P1-1 expression.

As shown in Figure 1 (panel A), we did not detect any modulation of GST P1-1 during 24 h of incubation with etoposide. The percentage of variation between different times of treatment, expressed as GST P1-1/β2-M ratio was less than 20%. At 10 h of incubation with etoposide, there was a reduction in GST P1-1 mRNA levels, but there was also a comparable reduction in the housekeeping gene leading to an unchanged ratio value.

Immunoblotting of GST P1-1 in Human Neuroblastoma Cell Line and Its Modulation by Etoposide

We evaluated the GST P1-1 protein modulation due to etoposide at different times of treatment using an immunoblotting assay in non-reducing conditions.

As shown in Figure 1 (panel B), during 24 h of treatment, we did not detect any significant change in the monomeric GST P1-1 levels. Interestingly, we detected the appearance of an extraband (MW 46 kDa) in samples treated with etoposide for 8 and 10 h, indicating the presence of post translationally modified GST P1-1. Indeed, the extraband, about twice the molecular weight of monomeric GST P1-1, corresponds to that previously described as a dimer via intersubunit disulfide bonding between Cys-47 residues [Bernardini et al., 2000].

Evaluation of Oxidative Stress in Human Neuroblastoma Cell Line by Etoposide

We evaluated the oxidative stress as the percentage of GSSG + GS-Pro/GSH (GSSG, oxidated glutathione; GS-pro Glutathione bound to proteins; GSH, reduced glutathione).

As shown in Figure 1 (panel C), we observed a modification in the oxidative status of the cells after treatment with etoposide. In particular, the oxidative stress, reported as a ratio-fold over control, started to increase at 12 h of etoposide treatment and reached its highest value at 24 h of treatment.

Evaluation of Apoptosis Induced by Etoposide in Human Neuroblastoma Cell Line

The percentage of apoptotic bodies was measured by flow cytometry after propidium iodide staining, and expressed as fold over control.

As shown in Figure 1 (panel D), we observed a progressive increase in the apoptotic bodies

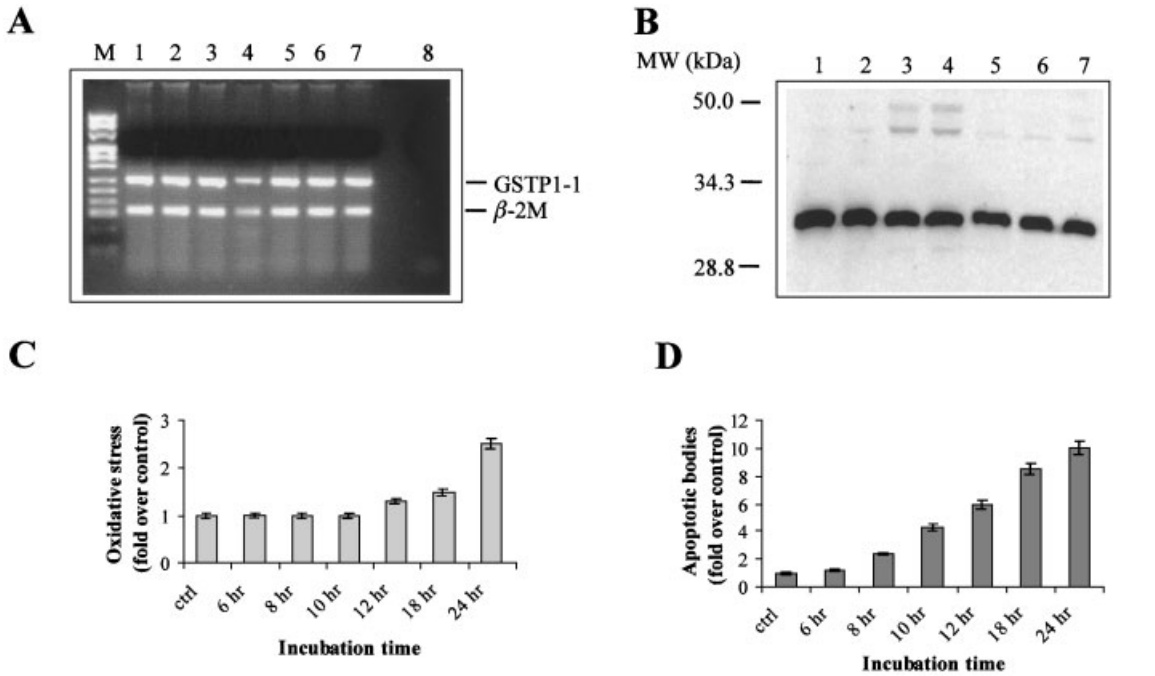

Fig. 1. Evaluation of GST P1-1 mRNA expression, protein polymerization, oxidative stress, and apoptosis in SH-SY5Y treated with 1 μM etoposide at different times. **Panel A**: Differential reverse transcription-polymerase chain reaction (RT-PCR) with co-amplification of GST P1-1 and β2-microglobulin (β2-M) in basal condition (ctrl, **lane 1**) and after treatment with 1 μM etoposide for 6 (**lane 2**), 8 (**lane 3**), 10 (**lane 4**), 12 (**lane 5**), 18 (**lane 6**), and 24 h (**lane 7**); RT-PCR negative control was loaded in **lane 8**. M: pUC mix marker 8. **Panel B**: Immunoblotting of GST P1-1 in non-reducing condition. Samples were analyzed in basal condition (ctrl, **lane1**) and after treatment with 1 μM etoposide for 6 (**lane 2**), 8 (**lane 3**), 10 (**lane 4**), 12 (**lane 5**), 18 h (**lane 6**), and 24 h (**lane 7**). **Panel C**: Oxidative stress reported as fold over control in basal condition (ctrl) and after treatment with 1 μM etoposide for 6, 8, 10, 12, 18, and 24 h. **Panel D**: The percentage of apoptotic bodies reported as fold over control in basal condition (ctrl) and after treatment with 1 μM etoposide for 6, 8, 10, 12, 18, and 24 h.

starting at 8 h, reaching the maximum level at 24 h of etoposide treatment.

Evaluation of GST P1-1 mRNA Expression, Protein Polymerization, and Oxidative Stress, in SH-SY5Y Etoposide-Resistant Clone

The results obtained for the etoposide resistant clone where compared with those obtained at 10-h treatment in the sensitive cell line. We chose this time of incubation because of a relevant value of apoptotic bodies and the presence of GST P1-1 polymerization. The SH-SY5Y etoposide-resistant clone presented an apoptotic bodies percentage comparable to the untreated sensitive cell line.

As shown in Figure 2 (panel A), we did not detect any significant variation in the GST P1-1 mRNA expression between the resistant clone and the sensitive cell line. On the contrary, we did not detect the extraband due to a lack of polymerization of GST P1-1 in the resistant cell clone (Fig. 2, panel B).

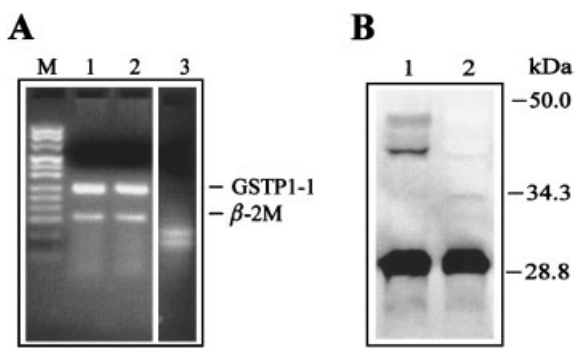

Fig. 2. Evaluation of GST P1-1 mRNA expression and protein polymerization in SH-SY5Y etoposide-resistant clone. **Panel A**: Differential RT-PCR with co-amplification of GST P1-1 and β2-microglobulin (β2-M) in SH-SY5Y etoposide sensitive cell line after treatment with 1 μM etoposide for 10 h (**lane 1**) and in SH-SY5Y etoposide resistant clone (**lane 2**). RT-PCR negative control was loaded in **lane 3**. M: pUC mix marker 8. **Panel B**: Immunoblotting of GST P1-1 in non-reducing condition. Samples were analyzed in SH-SY5Y etoposide-sensitive cell line after treatment with 1 μM etoposide for 10 h (**lane 1**) and in SH-SY5Y etoposide-resistant clone (**lane 2**).

The oxidative stress expressed as the percentage of GSSG + GS-Pro/GSH was about 2.5 times lower in the etoposide-resistant clone compared to the sensitive cell line. At the same time, the GSH levels was two times higher in the etoposide resistant clone (49.1 ± 2.4 μM/mg/ml total proteins) compared to the sensitive cell line (26.4 ± 1.6 μM/mg/ml total proteins).

Effect of S-Hexyl Glutathione on Human Neuroblastoma Cell Line

In order to investigate the role of the GSH on the ability of the cells to undergo apoptosis, we performed an experiment in which we pretreated the SH-SY5Y with 1 mM S-hexyl glutathione for 2 h, and then treated with 1 μM etoposide for 10 h. As shown in Figure 3 (panel A), the S-hexyl glutathione is able to prevent the GST P1-1 polymerization, and to determine a reduction in the apoptotic bodies formation (Fig. 3, panel B).

DISCUSSION

GSTs are a family of enzymes involved in the detoxification of electrophilic xenobiotics, including several anticancer drugs and environmental carcinogens. Therefore, GSH conjugation reaction catalyzed by GSTs might play a role in the inactivation of anticancer drugs in neoplastic cells. Many studies have confirmed their role in drug resistance, although the specific role of each isoenzyme has been difficult to assess; this is possibly due to the variability in the degree of resistance among different cell lines investigated to the different drugs used in relation to the isoenzymes transfected [Nakagawa et al., 1990; Leyland-Jones et al., 1991; Gaffey et al., 1995; Miyara et al., 1996]. Moreover, other GSH-related mechanisms involved in drug resistance, like GS-X and multidrug resistance associated protein (MDR) pumps, the activity of γ-glutamylcysteine synthetase, GSH peroxidase and GSH reductase, have to be considered [Zhang et al., 1998].

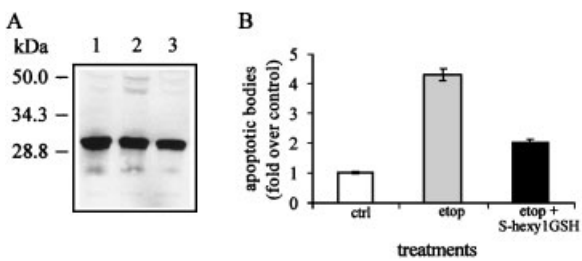

Fig. 3. Effect of S-hexyl glutathione on GST P1-1 protein polymerization and apoptosis in SH-SY5Y treated with 1 μM etoposide for 10 h. **Panel A**: Immunoblotting of GST P1-1 in non-reducing condition. SH-SY5Y etoposide-sensitive cell line were analyzed in basal condition (ctrl, **lane1**) and after treatment with 1 μM etoposide for 10 h without (**lane 2**) and with (**lane 3**) pre-treatment with 1 mM S-hexyl glutathione for 2 h. **Panel B**: The percentage of apoptotic bodies reported as fold over control in basal condition (ctrl) and after treatment with 1 μM etoposide for 10 h without (**lane 2**) and with (**lane 3**) pre-treatment with 1 mM S-hexyl glutathione for 2 h.

Much evidence suggests that the phenotype of drug resistance may result from reduction in the ability of neoplastic cells to start programmed cell death when exposed to antineoplastic drugs. This reduced ability could be intrinsic to the cell because of genetic changes in the apoptosis machinery or related to other factors that could interact with it.

It has been shown that monomeric GST P1-1 could form a complex with JNK. This association is inversely correlated with JNK activity. The inhibition of JNK activity by monomeric GST P1-1 is reversed by the oxidative stress, which causes an oligomerization of GST P1-1, and consequently, the dissociation of the GST P1-1-JNK complex. Thus, GST P1-1 hypothetically could interfere with the apoptosis machinery [Kasibhatla et al., 1998; Adler et al., 1999].

On the other hand, several evidences suggest that aromatic compounds like etoposide, metabolized to generate phenols and reactive carbonyls could enter the "redox cycling," increase ROS production and transcriptionally activate GST genes through the antioxidant reactive elements present in their promoter region (ARE) [Hayes and McLellan, 1999]. Therefore, the oxidative stress could have a dual action on GST P1-1 metabolism: transcriptional induction and/or polymerization. This might determine an opposite effect on cell fate, indeed transcriptional activation should represent a form of adaption to oxidative stress and contribute to protect the cell, on the contrary polymerization should activate apoptosis via JNK.

The aim of our study was to investigate the modifications of GST P1-1 mRNA expression and protein polymerization during etoposide treatment in a human neuroblastoma cell line SH-SY5Y. In particular, we have investigated the effect of etoposide on sensitive cell line and on an etoposide-resistant clone to better understand, if a pleotropic effect of etoposide on the redox status of the cell exists, which interfere with apoptosis by means of GST P1-1.

We exposed the SH-SY5Y human neuroblastoma cell line to etoposide for mainly three reasons: (1) etoposide is currently employed in the chemotherapeutic protocols for neuroblastoma; (2) etoposide is able to induce apoptosis both as DNA damaging agent, and acting through the JNK pathway, the activation of AP-1 (consensus activator protein 1), which in turn promotes CD95L expression to induce apoptosis [Shen et al., 1993]; (3) etoposide is an aromatic compound that on metabolization could generate phenols and reactive carbonyls, which enter in the "redox cycling" producing ROS.

Our experiments have been performed on SH-SY5Y human neuroblastoma cell line and on an etoposide-resistant clone of the same cell line.

The GST P1-1 mRNA expression and monomeric GST P1-1 protein levels did not change in a relevant manner after 6, 8, 10, 12, 18, and 24 h of incubation with etoposide 1 μM in the sensitive cell line. However, we detected the appearance of an extraband (MW 46 kDa) in samples treated with etoposide for 8 and 10 h, indicating the presence of post translationally modified GST P1-1. This polymerization represents a way to induce JNK activity removing the inhibition exerted by monomeric GST P1-1 [Kasibhatla et al., 1998].

The treatment with etoposide 1 μM induced apoptosis starting from 8 h, reaching its highest value at 24 h. In the same conditions, we also detected an increase of oxidative stress although it started only at 12 h. All these data suggest that the GST P1-1 polymerization plays an important role in the apoptotic mechanisms induced by etoposide and is not only an ephifenomen of the oxidative stress.

The etoposide-resistant clone, which presents a percentage of apoptotic bodies comparable to the untreated control of the sensitive cell line, did not show any difference in the GST P1-1 mRNA expression, but showed a lack of the extraband indicating non-polymerization of the GST P1-1. Furthermore, the etoposide-resistant clone showed a decrease in the oxidative stress and an increase of GSH levels when compared to the sensitive cell line treated with etoposide for 10 h.

This phenomenon prompted us to investigate the role of the GSH on the ability of the cells to undergo apoptosis. We pre-treated the SH-SY5Y with 1 mM S-hexyl glutathione for 2 h and then treated the cell line with 1 μM etoposide for 10 h. As anticipated, the S-hexyl glutathione was able to prevent the GST P1-1 polymerization and to determine a reduction in the apoptotic bodies formation.

Our data seem to suggest that etoposide treatment was unable, at least in our experimental conditions, to induce a transcriptional activation of GSTP1 in SH-SY5Y human neuroblastoma cell line. On the other hand, etoposide was able to induce GST P1-1 polymerization and activation of apoptosis via the JNK pathway. The data obtained from the etoposide-resistant clone and the possibility to reverse the sensitive phenotype to a resistant one by means of hexylglutathione preincubation, seem to suggest that cellular levels of GSH have a key role in protecting GST P1-1 from oxidation, and therefore, in the decision of the cell between life and death.

REFERENCES

Adler V, Yin Z, Fuchs SY, Benezra M, Rosario L, Tew KD, Pincus MR, Sardana M, Henderson CJ, Wolf RC, Davis RJ, Ronai Z. 1999. Regulation of JNK signaling by GSTp. EMBO J 18:1321–1334.

Armstrong RN. 1997. Structure, catalytic mechanism, and evolution of the glutathione transferases. Chem Res Toxicol 10:2–18.

Bernardini S, Bernassola F, Cortese C, Ballerini S, Melino G, Motti C, Bellincampi L, Iori R, Federici G. 2000. Modulation of GST P1-1 activity by polymerization during apoptosis. J Cell Biochem 77:645–653.

Gaffey MJ, Iezzoni JC, Meredith SD, Boyd JC, Stoler MH, Weiss LM. 1995. Cyclin D1 (PRAD1, CCND1) and glutathione S-transferase pi gene expression in head and neck squamous cell carcinoma. Hum Pathol 26: 1221–1226.

Gilbert L, Elwood LJ, Merino M, Masood S, Barnes R, Steinberg SM, Lazarous DF, Pierce L, d'Angelo T, Moscow JA. 1993. A pilot study of pi-class glutathione transferases expression and prognosis in node negative breast cancer. J Clin Oncol 11:49–58.

Gsur A, Haidinger G, Hinteregger S, Bernhofer G, Schatzl G, Madersbacher S, Marberger M, Vutuc C, Micksche M. 2001. Polymorphisms of glutathione S-transferase genes (GSTP1, GSTM1 and GSTT1) and prostate cancer risk. Int J Cancer 95:152–155.

Harries LW, Stubbins MJ, Forman D, Howard G, Wolf CR. 1997. Identification of genetic polymorphism at the glutathione S-transferases P1-1 locus and association with susceptibility to bladder, testicular and prostate cancer. Carcinogenesis 18:641–644.

Hayes JD, McLellan LI. 1999. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radiat Res 31:273–300.

Hayes JD, Strange RC. 2000. Glutathione S-transferase polymorphism and their biological consequences. Pharmacology 61:154–166.

Helzlsover KJ, Selmin O, Huang HY, Strickland PT, Hoffman S, Alberg AJ, Watson M, Comstock GW, Bell D. 1988. Association between glutathione S-transferases M1, P1, and T1 genetic polymorphism and development of breast cancer. J Natl Cancer Inst 90:512–518.

Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahoubi A, Green DR. 1998. DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-κB and AP-1. Mol Cell 1:543–551.

Lee JM, Lee YC, Yang SY, Shi WL, Lee CJ, Luh SP, Chen CJ, Hsieh CY, Wu MT. 2000. Genetic polymorphisms of *p53* and *GSTP1*, but not *NAT2*, are associated with susceptibility to squamous-cell carcinoma of the esophagus. Int J Cancer 89:458–464.

Leyland-Jones BR, Tawnsend AJ, Tu CPD, Cowan KH, Goldsmith ME. 1991. Anti neoplastic drug sensitivity of human MCF-7 breast cancer cells stably transfected human alpha class glutathione S-transferase gene. Cancer Res 51:587–594.

Mann CLA, Davies MB, Boggild MD, Alidersea J, Fryer AA, Jones PW, Ko C, Young C, Strange RC, Hawkins CP. 2000. Glutathione S-transferase polymorphisms in multiple sclerosis: Their relationship to disability. Neurology 54:552–557.

Maugard CM, Charrier J, Pitard A, Campion L, Akande O, Pleasants L, Ali-Osman F. 2001. Genetic polymorphism at the glutathione S-transferase P1 locus is a breast cancer risk modifier. Int J Cancer 91:334–339.

Menegon A, Board PG, Blackburn AC, Mellick GD, Le Couter DGL. 1998. Parkinson's disease, pesticides, and glutathione transferase polymorphisms. Lancet 352: 1344–1346.

Miyara H, Hida T, Nishida K, Takahashi T, Sugiura T, Ariyoshi Y, Morishita M, Takahashi T, Ueda R. 1996. Modification of chemo-radiosensitivity of a human lung cancer cell line by introduction of the glutathione S-transferase pi gene. Jpn J Clin Oncol 26:1–5.

Nakagawa K, Saijo N, Tsuchida S, Sakai M, Tsunokawa Y, Yokota J, Muramatsu M, Sato K, Tarada M, Tew KD. 1990. Glutathione S-transferase as a determinant of drug resistance in transfectant cell lines. J Biol Chem 265: 4296–4301.

Russo D, Marie JP, Zhou DC, Faussat AM, Delmer A, Maisonneuve L. 1994. Coexpression of anionic glutathione S-transferase (GST pi) and multidrug resistance (mdr 1) genes in acute myeloid and lymphoid leukemias. Leukemia 8:881–884.

Shen H, Tsuchida S, Tamai K, Sato K. 1993. Identification of cysteine residues involved in disulfide formation in the inactivation of glutathione transferase P-form by hydrogen peroxide. Arch Biochem Biophys 300:137–141.

Spiteri MA, Bianco A, Strange RC, Fryer AA. 2000. Polymorphisms at the glutathione S-transferase, GSTP1 locus: A novel mechanism for susceptibility and development of atopic airway inflammation. Allergy 55 (Suppl 61):15–20.

Stanulla M, Schrappe M, Brechlin AM, Zimmermann M, Welte K. 2000. Polymorphism within glutathione S-transferase genes (*GSTM1*, *GSTT1*, *GSTP1*) and risk of relapse in childhood B-cell precursor acute lymphoblastic leukemia: A case control study. Blood 95:1222–1228.

Sweeney C, McClure GY, Fares MY, Stone A, Coles BF, Thompson PA, Korourian S, Hutchins LF, Kadlubar FF, Ambrosone CB. 2000. Association between survival after treatment for breast cancer and glutathione S-transferase P1 Ile ^{105}Val polymorphism. Cancer Res 60:5621–5624.

Tew KD. 1994. Glutathione associated enzymes in anticancer drug resistance. Cancer Res 54:4313–4320.

Zhang K, Mack P, Wong K. 1998. Glutathione-related mechanisms in cellular resistance to anticancer drugs (review). Int J Oncol 12:871–882.