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Modulation of GST P1-1 Activity by Polymerization During Apoptosis

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Abstract Glutathione S-transferases (GSTs, EC 2.5.1.18) belong to a large family of functionally different enzymes that catalyze the S-conjugation of glutathione with a wide variety of electrophilic compounds including carcinogens and anticancer drugs. Drug resistance may result from reduction in apoptosis of neoplastic cells when exposed to antineoplastic drugs. The c-Jun N-terminal Kinase (JNK) belongs to the family of stress kinases and has been shown to be required for the maximal induction of apoptosis by DNA-damaging agents. Recently, an inhibition of JNK activity by GST P1-1, which was reversed by polymerization induced by oxidative stress, has been reported in 3T3-4A mouse fibroblast cell lines. The finding that GST P1-1 might inhibit JNK activity and that it is frequently highly expressed in tumor tissues suggests its possible implication in "apoptosis resistance" during antineoplastic therapy. We investigated the modulation of GST P1-1 during apoptosis in a neoplastic T-cell line (Jurkat) induced by hydrogen peroxide and etoposide. Apoptosis was paralleled by the appearance of a dimeric form of GST P1-1 on western blotting, associated with an increase in the Km^{GSH} and a reduction in GST P1-1 specific activity toward 1-chloro-2,4-dinitrobenzene, which reached statistical significance only in H_2O_2-treated cells. Our data seem to suggest that H_2O_2 and etoposide may partly act through a process of partial inactivation of the GST P1-1, possibly involving the "G" site in the process of dimerization, and thus favoring programmed cell death. J. Cell. Biochem. 77:645–653, 2000.

Key words: glutathione transferase P1-1; hydrogen peroxide; etoposide

Anticancer drugs are able to kill cancer cells by multiple mechanisms: intercalation into DNA, inhibition of DNA replication, cell membrane damage, or free radical generation. Cells respond to cytotoxic stress and DNA damage either by cell-cycle arrest and repair or by undergoing apoptotic cell death, depending on the cell type and the extent of damage. Understanding the mechanisms of anticancer drug-induced apoptosis is essential for developing effective strategies in tumor therapy. Although it is evident that different anticancer drugs act through a common downstream apoptotic pathway including caspases activation, the events occurring between the primary target of the drugs and the apoptotic cascade are not well understood at the molecular level. In particular, the role of the CD95/CD95L system is still an object of debate [Krammer, 1997; Tolomeo et al., 1988]. CD95 (APO-1/Fas) belongs to the family of death receptors, which is part of the superfamily of tumor necrosis factor receptors. After the interaction of the ligand (CD95L) with CD95, a death-inducing signaling complex, which includes the adapter protein FADD and the caspase-8/FLICE, is induced. Further downstream in the death pathway, caspase-8/FLICE triggers the proteolytic activation of other caspases that form an active tetrameric

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complex and cleave cellular substrates such as enzymes involved in genome function, regulators of cell cycle, and nuclear and cytoskeleton proteins, as well as the recently discovered inhibitor of a caspase-activated DNase [Enari et al., 1998; Krammer, 1999; Stroh and Schultze-Osthoff, 1998].

The transactivation of c-Jun, mediated by phosphorylation of the Ser/Thr residues in the activation domain by the c-Jun N-terminal Kinase (JNK), has been shown to be required for the maximal induction of apoptosis by DNA-damaging agents in Jurkat T cells, a T leukemia cell line [Kasibhatla et al., 1998]. DNA damaging agents, such as ultraviolet irradiation and topoisomerase inhibitors (i.e., etoposide), seem to trigger the activation of the JNK pathway, which is followed by CD95L expression. Indeed, a consensus AP-1-binding sequence has been identified on the CD95L promoter in Jurkat T cells and it is well established that AP-1 is induced via activation of the JNK pathway [Kasibhatla et al., 1998]. Moreover, ectopic expression of a constitutively active mitogen activated kinase-kinase, which acts upstream to JNK, leads to expression of CD95L and apoptosis in Jurkat T cells [Faris et al., 1998]. On the other hand, recent evidence suggests that etoposide-induced apoptosis does not require de novo synthesis of death ligands or CD95 interaction and that caspase-8/FLICE, the most proximal effector of the receptor complex, can be activated independently from a death receptor signaling [Wasselborg et al., 1999]. These contrasting results regarding the possible pathways involved in drug-induced apoptosis suggest that other mechanisms, possibly interconnected with those previously described, might exist.

Recently it has been shown that monomeric glutathione S-transferase P1-1 (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 that 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 [Adler et al., 1999]. Glutathione S-transferases (GSTs, EC 2.5.1.18) belong to a large family of functionally different enzymes that catalyze the S-conjugation of glutathione (GSH) with a wide variety of electrophilic compounds including carcinogens and anticancer drugs. The soluble GSTs in human tissues are classified in four main classes: alpha, mu, pi, and theta, some of which are present in multiple isoforms [Armstrong, 1997]. Although the reactions catalyzed by the GSTs should protect the cell from cytotoxic and carcinogenic agents, much evidence shows that GST P1-1 is represented in some tumors to the extent that it is considered a tumor marker and/or to be involved in anticancer drug-resistance [Zhang et al., 1998]. The genetic polymorphism described in *GSTP1*could be an important factor in cancer therapy, and three main isoforms have been described : *A** (Ile^{104}), *B** (Val^{104}), and *C** (Val^{104}; $Ala\text{-}Val^{113}$), each of which is characterized by point mutations within the active site of the GST P1-1 protein. This polymorphism could influence the GST P1-1 specific activity towards many substrates, including 1-chloro-2,4-dinitrobenzene (CDNB) [Ali-Osman et al., 1997]. A low basal JNK activity is believed to affect the half-life of JNK substrates including c-Jun and p53, since JNK targets the ubiquitination of its nonphosphorylated associated proteins [Fuchs et al., 1998]. The GST P1-1 inhibition of JNK is found primarily in normally growing, nonstressed cells, and is sustained by the monomeric form of the enzyme that is able to form a complex with JNK [Adler et al., 1999]. Ultraviolet irradiation as well as H_2O_2 incubation reduces GST P1-1/JNK association as a result of the formation of GST-GST dimers and multimers probably involving the cys-47 and cys-101 residues of the GST P1-1 [Shen et al., 1993]. Glutathione S-transferase dimers are unable to accommodate the Jun/JNK complex and, consequently, JNK can exert its kinase activity. Indeed the switch of GST P1-1 from the monomeric to the dimeric form might transmit the changes in the redox status of the cell to the JNK pathway, eventually influencing programmed cell death. The finding that GST P1-1 is frequently highly expressed in tumor tissues and that it is potentially able to inhibit JNK activity suggests its possible implication in a form of resistance to apoptosis during antineoplastic therapy. The aim of our study was to correlate the modulation of GST P1-1 expression, activity, and dimerization during incubation with H_2O_2 or etoposide with apoptosis in a neoplastic T-cell line (Jurkat).

MATERIALS AND METHODS

Reagents

Media, reagents, and plastics for cell culture, trypsin, EDTA, L-glutamine, sodium bicarbonate, phosphate-buffered saline (PBS), fetal bovine serum, and nonessential amino acid were from Flow Laboratories Ltd. (Herts, UK). Ham's F-12 and minimal essential medium (MEM) were from Gibco (Berlin, Germany) and fetal calf serum (FCS) from HyClone (Oud-Beijerland, Holland). N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES); ribonuclease A; propidium iodide (PI); purified reduced GSH; GSH reductase; 5,5′-dithio-bis (2-nitrobenzoic acid) (DNTB); trichloroacetic acid (TCA); and CDNB were from Sigma (St. Louis, MO). 2′,7′-Dichlorofluorescein diacetate was purchased from Molecular Probes (Eugene, OR). Electrophoresis reagents were from BioRad (Hercules, CA). Rabbit polyclonal antibodies NCL-GSTpi, NCL-GSTmuM2, and NCL-GSTα were from Novocastra (Newcastle, UK). The Taq DNA polymerase and dNTPs (deoxynucleotide triphosphates) were from Pharmacia-Biotech (Stockolm, Sweden); TRIZOL reagent and primers were from Life Technology (Milan, Italia). The 1st Strand cDNA Synthesis kit and DNA marker V were from Boehringer Mannheim (Indianapolis, IN).

Cell Cultures

The human leukemia Jurkat cell line (T cells) was grown in a 1:1 mixture of MEM and Ham's F-12 medium supplemented with 10% heat-inactivated FCS, 1.2 g/liter Na-bicarbonate, 1% nonessential amino acids, and 15 mM HEPES, at 37°C with 5% CO_2 in a humidified atmosphere. Cells were split 1:5 twice weekly and routinely fed 24 h before each experiment. The cells were incubated with *a*: H_2O_2 100 μM for 5 min, then the medium was removed and the cells were grown in H_2O_2-free medium for 3, 6, and 12 h; *b*: agonist antibody anti-CD95 250 ng/ml for 3, 6, and 12 h; *c*: etoposide 25 μM for 3, 6, and 12 h; *d*: control cells were harvested at the same times. Each experiment was performed in triplicate.

After removal of the culture medium, cells were centrifuged at 800*g* for 10 min. The pellets, suspended 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, 4°C. The resulting supernatants were assayed for GST activity. Pellets were immediately stored at −80°C to be subsequently utilized for immunoblot or RNA extraction.

Glutathione Transferase Activity and Kinetic Measurements

Glutathione transferase activity was determined using CDNB as cosubstrate, as previously described [Habig and Jacoby, 1981]. In a typical experiment, 20 μl of the cell supernatant (about 50 μg of protein) were added to 1 ml (final volume) of 0.1 M potassium phosphate buffer, 0.1 M EDTA (pH 6.5), containing 1 mM CDNB and 1 mM GSH. The reaction was monitored at 340 nm, where the product absorbs (ϵ = 9,600 $M^{-1}cm^{-1}$), by utilizing a double-beam Uvikon 940 spectrophotometer Kontron equipped with a thermostated cuvette holder at 25°C. The K_m^{CDNB} was calculated at fixed GSH concentration (5 mM) and variable CDNB concentration (from 0.1 to 2.0 mM) [Nuccetelli et al., 1998]. The K_m^{GSH} was determined at a fixed CDNB concentration (1 mM) and various GSH concentrations (from 0.02 to 5 mM). The K_m values were obtained from a Lineweaver-Burk double reciprocal plot. Total protein content was determined by bicinchoninic acid method. Statistical analysis was performed by Student's *t*-test.

Western Blot Analysis

Cytosol proteins were electrophoresed at 30 μg/lane in 12% sodium dodecyl sulfate (SDS) polyacrylamide gel, transfered onto nitrocellulose membrane (0.45 μm), and probed with rabbit polyclonal antibodies specific for GST P1-1, GSTmuM2, and GSTα. A goat antirabbit IgG-peroxidase conjugate was then added, and the GSTs visualized by a peroxidase substrate (4 chloro-1-naphtolo).

Determination of DNA Fragmentation

To estimate DNA fragmentation, 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. The cell cycle was evaluated by flow cytometry using PI staining (40 μg/ml) in the presence of 13 kU/ml ribonuclease A (20 min incubation at 37°C) on a FACS-Calibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). 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 (Becton-Dickinson). Electronic gating FSC-a/vs/FSC-h was used, when appropriate, to eliminate cell aggregates.

Measurement of Intracellular Glutathione Levels

Intracellular glutathione (GSH) concentration was estimated using a modification of Tietze's method. First, 10^7 cells were washed two times in PBS, pelleted, and dried. Then 200 μl 5% TCA, 0.1 N HCl, and 10 mM EDTA were added to the dry cell pellet and the tubes were vortexed vigorously for 1 min. Cellular debris was pelleted by a 1′-centrifugation and the supernatant was divided into two aliquots to detect reduced and total content of GSH. Following this, 240 μl 0.3 mM NADPH in stock buffer (125 mM sodium phosphate, 6.3 mM EDTA, pH 7.5) and 200 μl GSH reductase (1 U/ml in stock buffer) were added to 100 μl cellular supernatant to detect total GSH content; 40 μl of 6 mM DNTB in stock buffer was added to the cellular supernatant. After 30-min incubation in the dark, the production of 2-nitro-5-thiobenzoic acid from DNTB was followed spectrophotometrically at 412 nm. The concentrations of GSH were calculated from a titration curve established using known concentrations of purified reduced GSH.

Endonuclease Restriction Mapping

Total RNA was purified using a standard method and reverse transcribed with AMV reverse transcriptase, according to the manufacturer's protocol (Boehringer Mannheim). A 484 bp cDNA fragment spanning position +112 to +596 of the GST P1-1 cDNA was amplified by polymerase chain reaction (PCR) using primers and conditions previously described [Ali-Osman et al., 1997]. The cDNA product was purified and, after restriction with *MaeII* and *XcmI*, was electrophoresed in 2% agarose, 0.01% ethidium bromide.

RT-PCR Analysis

The cDNA was amplified by differential PCR using β2-microglobuline as internal standard. Kinetics analysis were performed to determine the condition in which data could be obtained before the amplification reaction reached the plateau phase (Fig. 1). Polymerase chain reaction was performed with a DNA Thermal cycler (Perkin Elmer, Norwalk, CT). Polymerase chain reaction for GST P1-1/β2-M was carried out after preheating 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.

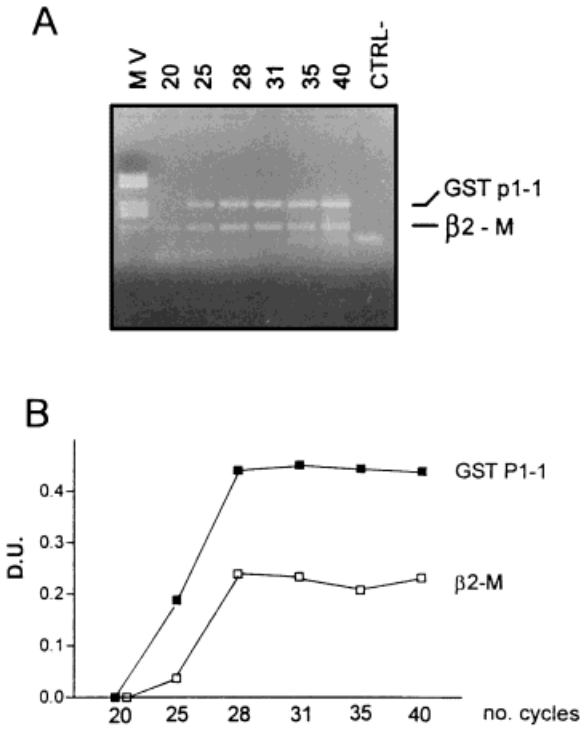

Fig. 1. **A:** Differential reverse transcription-polymerase chain reaction of glutathione S-transferases (GST) P1-1 and β2-M kinetic analysis. **B:** Densitometric analysis of same data.

The reaction mixture, with a final volume of 50 μl, was as follows: 10 μl of the reverse transcription (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 units 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′

The β2-M primer sequence generated a 110 bp product; and the GST P1-1 primers generated a 242 bp product, including exons 5 and 6 internal to the previously reported 484 bp

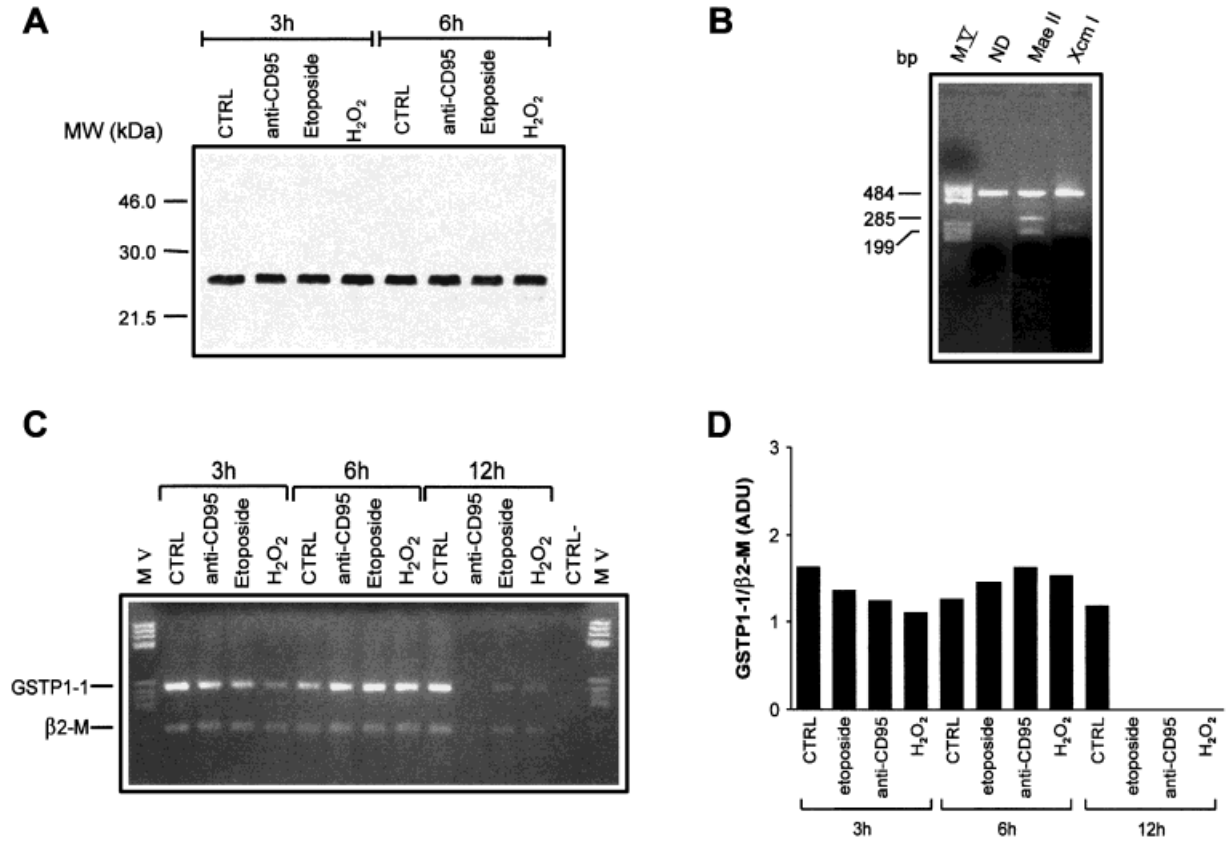

Fig. 2. **A:** Immunoblotting of glutathione S-transferase (GST) P1-1 in nonreducing conditions. Samples were analyzed in basal conditions (CTRL) and after incubation with 250 ng/ml of anti-CD95 antibody, 25μM etoposide, and 100μM H_2O_2 for 3 and 6 h (cells were incubated with H_2O_2 for 5 min, then the medium was removed and the cells were grown in H_2O_2-free medium for 3 and 6 h). **B:** *Mae II* and *XcmI* endonuclease restriction mapping of GST P1-1 cDNA, including exon 5 and 6 (amplification product of 484 bp) in Jurkat T cell line. MV, DNA molecular weight marker V (587–8 bp); ND, undigested sample. **C:** Differential reverse transcription-polymerase chain reaction (RT-PCR) with coamplification of GST P1-1 and β2-microglobulin (β2-M) in basal conditions (CTRL) and after incubation with 250 ng/ml of anti-CD95 ab, 25μM etoposide, and 100μM H_2O_2 for 3, 6, and 12 h (cells were incubated with H_2O_2 for 5 min, then the medium was removed and the cells were grown in H_2O_2-free medium for 3, 6, and 12 h). **D:** Densitometric analysis of data reported in C expressed as GST P1-1:β2-M ratio.

amplification product. The RT-PCR product (20 μl) was separated on 2% agarose, 0.01% ethidium bromide gels, and visualized on a transilluminator. The ratio of target to control β2-M gene products was determined by bidimensional densitometry on a GS-670 Densitometer (BioRad).

RESULTS

First, we evaluated the presence of GSTs in our cell-line model through both a Western blot and an enzyme activity study. The Western immunoblotting of the cytosol of the Jurkat T leukemia cell line, performed with polyclonal antibodies against alpha, mu and P1-1 GST classes, only showed the presence of GST P1-1 (Fig. 2A), while the alpha and mu classes were absent (data not shown). The K_m^{CDNB} and the K_m^{GSH}, obtained from a Lineweaver-Burk double reciprocal plot, were 1.0 mM and 0.12 mM, respectively.

By means of endonuclease restriction mapping of GST P1-1 cDNA, we showed the presence of a heterozygosity *A*/B** as indicated by the lack of digestion with *XcmI* (which recognizes a restriction site specific of variant *C**), the presence of one band digested with *MaeII* (specific for variant *B** and *C**), and the presence of one undigested upper band (specific for wild variant *A**; Fig. 2B).

The immunoblotting assay for GST P1-1 (following incubation with anti-CD95 antibody,

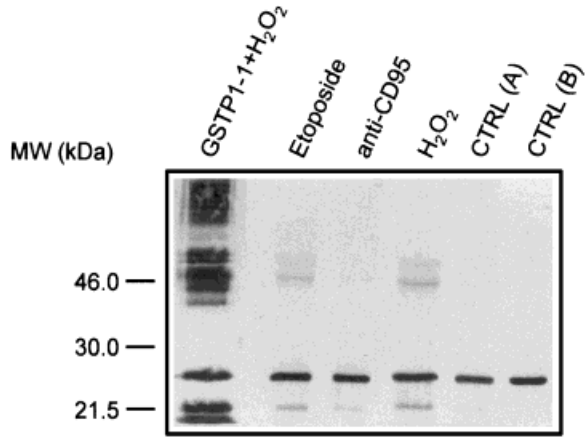

Fig. 3. Immunoblotting of glutathione S-transferase (GST) P1-1 in nonreducing conditions. Samples were analyzed in basal conditions [CTRL (A)] and after incubation with 250 ng/ml of anti-CD95 ab, 25μM etoposide, and 100μM H_2O_2 for 12 h (cells were incubated with H_2O_2 for 5 min, then the medium was removed and the cells were grown in H_2O_2-free medium for 12 hours). Control B [CTRL (B)] was analyzed in reducing conditions. This immunoblotting was performed simultaneously with the immunoblotting reported in Fig. 2A.

etoposide, and H_2O_2 in nonreducing conditions) did not show modifications of GST P1-1 steady state protein levels after 3 and 6 h of incubation (Fig. 2A). These data were confirmed by means of differential RT-PCR for GST P1-1 mRNA, using β2-microglobulin as standard (Fig. 2C and 2D). Our analysis was not able to detect appreciable amounts of GST P1-1 and β-2-M mRNA expression after 12 h of incubation, possibly as result of cell death. Indeed, the percentage of the apoptotic events increased significantly relative to control (6.1 ± 0.9) after 12 h of incubation with etoposide (25.4 ± 0.7) and H_2O_2 (25.2 ± 2.9), and to the same extent as the samples incubated with anti-CD95 Ab (27.3 ± 2.7).

After 12 h of incubation, the GST P1-1 levels still did not change significantly, but the appearance of the extra band F (46 kDa) and the increase of band B (21.5 kDa) in samples treated with etoposide and H_2O_2 indicated the presence of post-translationally modified GST P1-1 (Fig. 3). Indeed, band F, 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; band B corresponds to that formed by an intrasubunit disulfide bond between Cys-47 and Cys-101 residues [Shen et al., 1993]. The presence of a band F should involve a reduction in GST P1-1 specific activity towards CDNB and affinity for the cosubstrate GSH, because Cys-47 is located near the GSH site [Federici et al., 1996]. Indeed we found a reduction of GST P1-1 activity using CDNB as substrate after 12 h incubation (Table I) that reached statistical significance only after incubation with H_2O_2. This reduction was paralleled by an evident increase of the K_m^{GSH}.

After 12 h of incubation with anti-CD95 antibody, etoposide, and H_2O_2, all three samples showed an increase in the GSSG/GSH ratio relative to control (anti-CD95 = 280%, etoposide = 277%, H_2O_2 = 157%), indicating the presence of an oxidative stress during apoptosis induced by all three stimuli.

DISCUSSION

Glutathione S-transferases are a family of enzymes involved in the detoxification of electrophilic xenobiotics. The soluble GSTs in human tissues are classified in four main classes: alpha, mu, pi, and theta, some of which are present in multiple isoforms [Armstrong, 1997]. The GSH conjugation reaction catalyzed by GSTs might play a role in the inactivation of anticancer drugs in neoplastic cells. GST P1-1 activity has been correlated with drug resistance both in clinical and experimental models [Lewis et al., 1988; Mlyazaki et al., 1990; Tew, 1994]. On the other hand, GST P1-1 itself could be modulated by agents employed as adjuvants in anticancer therapy like retinoic acid [Bernardini et al., 1999]. Many studies, utilizing overexpression of GSTs in normal and neoplastic cell lines, 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 [Gaffey et al., 1995; Leyland-Jones et al., 1991; Miyara et al., 1996; Nakagawa et al., 1990]. Moreover, other GSH-related mechanisms involved in drug resistance such as GS-X, multidrug resistance associated protein (MDR) pumps, and the activity of γ-glutamylcysteine synthetase, GSH peroxidase, and GSH reductase have to be considered [Zhang et al., 1998].

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 antineo-

TABLE I. GST P1-1 Specific Activity[a]

	Control	anti-CD95 Ab	Etoposide	H_2O_2
S.A.	0.190 ± 0.017	0.191 ± 0.013	0.174 ± 0.023	0.147 ± 0.006*
K_m^{GSH}	0.12	0.17	0.25	0.53

[a]GST P1-1 specific activity expressed as μmol/min/mg of proteins (mean ± SD) and K_m^{GSH} expressed as mmol/liter after 12 h: incubation with anti-CD95 (250 ng/ml), etoposide (25 μM) and H_2O_2 (100 μM for 5 min). S.A., specific activity.
*$P < 0.01$.

plastic 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. Recently a new role, possibly correlated with drug resistance, has been proposed for GST P1-1. Indeed, in the 3T3-4A mouse fibroblast cell line, an inhibitory activity on JNK has been described for the glutathione transferase P1-1 [Adler et al., 1999]. This inhibition is apparently the result of complexes that monomeric GST P1-1 is able to form with JNK. Modulation of the cellular redox potential affects JNK inhibition by GST P1-1; the exposure to H_2O_2 decreases the amount of JNK complexed with monomeric GST P1-1 by increasing GST P1-1 polymerization.

Furthermore, it has been shown that GST P1-1 can be inactivated in vitro by H_2O_2 through the formation of an intrasubunit disulfide bond between cys-47 and cys-101 and a disulfide bond between the cys-47 of two subunits. After the formation of disulfide bonds, GST P1-1 exhibits altered mobilities in SDS-PAGE under nonreducing conditions, showing the presence of extra bands in addition to the native subunit band. The GST P1-1 inactivation is reversible by the use of antioxidants as well as prevented by the binding of GSH to the enzyme [Shen et al., 1993]. As a matter of fact, the formation of a disulfide bond between cys-47 and cys-101 decreases the affinity for GSH, as both cysteines are located near the G site [Federici et al., 1996].

Considering that apoptosis should be one of the end targets of JNK activity, the authors suggest that tumor cells overexpressing GST P1-1 [Gilbert et al., 1993; Harries et al., 1997; Helzlsover et al., 1988; Sclasselbauer et al., 1990] may escape programmed cell death, thus proposing a new model of drug resistance due to GST P1-1 [Adler et al., 1999]. We have studied the GST P1-1 mRNA expression, activity and, dimerization in Jurkat T cells after H_2O_2 exposure to verify if the model described for the 3T3-4A mouse fibroblast cell line is applicable to a neoplastic cell line and if the high levels of GST P1-1 present in Jurkat cells are capable of interfering with apoptosis. At the same time, we incubated Jurkat T cells with an agonist antibody anti-CD95, which should act downstream of the supposed interaction between JNK and GST P1-1, and that should not influence the behaviour of GST P1-1. We exposed the same cell line to etoposide, a DNA damaging agent that has been shown to act through the JNK pathway and the activation of AP-1, which in turn promotes CD95L expression [Kasibhatla et al., 1998] (Fig.4).

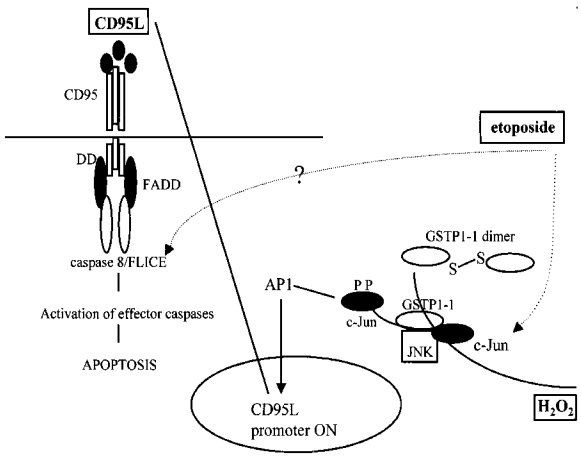

Fig. 4. Model of the interrelations between glutathione S-transferase (GST) P1-1, H_2O_2, etoposide, and apoptosis CD95/CD95L mediated.

The GST P1-1 mRNA expression, as well as GST P1-1 protein levels assessed by immunoblotting, did not change in a relevant manner after 3, 6, and 12 h of incubation with H_2O_2, anti-CD95, or etoposide. After 12 h of incubation with all three agents, the cells underwent apoptosis to a similar extent. In both H_2O_2- and etoposide-treated samples, the SDS-PAGE under nonreducing conditions showed the pres-

ence of an extra band of about 46.0 kDa, about twice the molecular mass of the native GST P1-1 subunit (23.5 kDa). As expected, this band was not apparent after ligation with anti-CD95.

Together with the appearance of the 46-kDa band, cells showed an increase in the K_m^{GSH} and a reduction in GST P1-1 specific activity toward CDNB, which reached statistical significance in H_2O_2-treated cells. These findings suggest a partial inactivation of GST P1-1 and the involvement of the G site in the process of dimerization.

By means of endonuclease restriction mapping of GST P1-1 cDNA, our cell line displayed an *A*/B** genotype, and even if the GST P1-1 polymorphism resides in the xenobiotic substrate binding site (H site), we cannot exclude the possibility that other genotypes may behave differently. It can be hypothesized that etoposide has a lower impact on GST P1-1 kinetic properties because it concomitantly might utilize other pathways to induce apoptosis. Although some authors suggest that DNA damaging agents trigger the activation of the JNK pathway, after which CD95L is expressed [Kasibhatla et al., 1998], other evidence in the same cell line suggests that caspase-8/FLICE, the most proximal effector of the receptor complex, can be activated in the absence of a death receptor signaling [Wasselborg et al., 1999]. In the latter case, etoposide could be able to bypass the GST P1-1/JNK pathway.

In conclusion, our data seem to suggest that the reported model [Adler et al., 1999] of interaction between H_2O_2 and GST P1-1/JNK complex described in 3T3-4A mouse fibroblasts is applicable to a human neoplastic cell line. Although only a small amount of GST P1-1 undergoes dimerization this phenomenon seems to be sufficient to favor apoptosis.

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