Glutathione transferase omega 1-1 (GSTO1-1) plays an anti-apoptotic role in cell resistance to cisplatin toxicity

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Several lines of evidence correlate the overexpression of glutathione S-transferase omega 1-1 (GSTO1-1) with the onset of drug resistance of cancer cells; however, no direct evidence is yet available. In order to investigate the mechanisms involved, stable transfection with GSTO1-1 complementary DNA was performed in HeLa cells, which spontaneous express very low levels of GSTO1-1. When transfected cells were seeded at low density, a sharp increase in GSTO1-1 expression was observed as compared with controls, along with an increased resistance against cisplatin cytotoxicity. When seeded at increasing densities, control untransfected cells also presented with an increase in GSTO1-1 expression, again accompanied by cisplatin resistance; the latter was significantly reduced after transfection with GSTO1-1 small interfering RNA. Cisplatin resistance of transfected cells was not accounted for by changes in the intracellular drug concentration nor in the amount of DNA cross-links or content of glutathione. Rather, transfected cells presented with a marked decrease of apoptosis as compared with controls, suggesting that GSTO1-1 overexpression may prevent cisplatin toxicity by interfering with the apoptotic process. Cisplatin treatment was in fact followed at early times (1–2 h) by activation of both Akt kinase and extracellular signal-regulated kinase (ERK)-1/2 in the transfected cells but not in controls. Conversely, in transfected cells, the strong activation of Jun N-terminal kinase (JNK)-1 induced by cisplatin at later times (10–20 h) was completely prevented. In conclusion, GSTO1-1 overexpression appears to be associated with activation of survival pathways (Akt and ERK1/2) and inhibition of apoptotic pathways (JNK1), as well as protection against cisplatin-induced apoptosis.

Introduction

Glutathione S-transferases (GSTs) are a superfamily of enzymes that mainly catalyze the conjugation of glutathione (GSH) to a wide range of exogenous and endogenous electrophilic substrates, including chemical carcinogens, therapeutic drugs and oxidative stress products. Seven cytosolic GST classes have been characterized in humans: alpha, mu, pi, sigma, theta, zeta and the most recently identified omega (glutathione transferase omega, GSTO). The latter was recognized through a bioinformatic analysis of the human Expressed Sequence Tag database (1). Members of the GSTO class have been subsequently identified in the pig (6), in Schistosoma mansoni (7) and Drosophila melanogaster (GenBank accession AAF50405).

Despite belonging to the GSTs superfamily, GSTOs lack any appreciable GST activity, while showing a series of unusual properties.

Abbreviations: ERK, extracellular signal-regulated kinase; GSH, glutathione; GST, glutathione S-transferase; GSTO, glutathione transferase omega; JNK, Jun N-terminal kinase; PI, propidium iodide; siRNA, small interfering RNA.

GSTOs have a cysteine residue in their active site, instead of the tyrosine or serine residues normally present in other mammalian GSTs, and an extra N-terminal tag of 19 amino acids (1). Human GSTs catalyze thioltransferase reactions, and a role in protein glutathionylation reactions has been envisaged (8). GSTOs also display dehydroascorbate reductase activity, which involves them in cellular antioxidant defense mechanisms (1–3,8–10). Further activities have been reported: GSTOs show mono- and dimethylarsonic reductase activity (10,11); GSTO1-1 plays a role in the activation of the inflammatory mediator interleukin-1β (12), as well as in the modulation of ryanodine receptor calcium release channels (13); the murine enzyme has been characterized as a GSH-binding stress protein, localizing into cell nucleus after heat shock treatment and overexpressed along with the acquisition of resistance against ionizing radiations (4). Two GSTO genes have been identified in humans: GSTO1-1, widely expressed in all tissues, and GSTO2-2, expressed at high levels in testis. Polymorphisms of either GSTO1-1 or GSTO2-2 could be a risk factor for incidence of different types of cancer. In particular, polymorphisms of the GSTO1-1 gene may be associated with the risk of hepatocellular carcinoma, cholangiocarcinoma and breast cancer (14), whereas polymorphisms in the GSTO2-2 seem to be associated with the risk of ovarian cancer (15). A recent proteomic analysis showed that GSTO1-1 is one of the proteins upregulated in human pancreatic cancer tissue, as compared with adjacent non-cancerous regions (16).

Analysis of human ovarian cancer cell lines has shown that GSTO1-1 is one of the proteins constantly upregulated in cisplatin-resistant sublines (17), thus suggesting a possible role of GSTO1-1 in cisplatin resistance. Cisplatin is one of the most potent and widely used anti-cancer agents for the treatment of solid tumors. Development of drug resistance to cisplatin and other agents is a major obstacle to anticancer treatments and can originate from various factors, including altered drug uptake or metabolism, inhibition of apoptotic cell death or increased repair of drug-induced DNA damage (18,19). The present study was aimed at investigating the role of GSTO1-1 in the acquisition of cell resistance to cisplatin. The experimental results, obtained with HeLa cells stably transfected with the GSTO1-1 complementary DNA, indicate that this protein can exert an anti-apoptotic role.

Materials and methods

Chemicals

Unless otherwise indicated, all reagents were from Sigma Chemical Co. (St Louis, MO).

Cell lines and culture conditions

Cells (HeLa, HeLa TetOff, CHO, Caco-2, PC12, leukemia U937 cells, human melanoma cell lines 665/2/21, 665/2/60, HK-2, INS-1E and Jurkat) were grown in the appropriate complete media. HeLa TetOff cells stably transfected with GSTO1-1 were grown in complete medium plus hygromycin.

For the cell density experiments, cells were seeded at low (4×103 cells/cm2) or high density (40×103 cells/cm2) and collected after 24 h or alternatively seeded at low density and allowed to grow up to different cell densities during 24, 48, 72 and 96 h of incubation.

At the end of the incubation times, cells were collected in phosphate-buffered saline and stored at –20°C until use.

Plasmid construction and stable transfection

GSTO1-1 complementary DNA was subcloned in the expression pTre vector (Clontech, Palo Alto, CA). The resulting pTreGSTO1 vector was used to transform Escherichia coli strain INVaF® (Invitrogen, Carlsbad, CA). The integrity of the constructs was confirmed by DNA sequencing using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

HeLa TetOff cells (Clontech) were transfected with lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Selection with
200 μg/ml of hygromycin was started after 48 h from the transfection. Cells were screened by immunoblotting with anti-GSTO1 antibody. A single clone with high level of GSTO1 expression (HeLa/GSTO1+) was thus obtained. The same transfection procedure was used for control cells (HeLa Cont), where an empty vector was employed.

Reverse transcription–polymerase chain reaction

Total RNA was extracted from cells by using the NucleoSpin RNA and Virus Purification kit (Clontech), following the manufacturer’s instructions. RNA yield and purity were checked by determining the A260 to A280 ratio. Semi-quantitative reverse transcription–polymerase chain reaction was performed using the TITANiUM One-Step RT-PCR kit (Clontech), using 1 μg of RNA, oligo dT primer and specific primers for amplification of GSTO1 and glyceraldehyde 3-phosphate dehydrogenase.

Cytotoxicity assay

Cytotoxicity was evaluated by tetrazolium colorimetric water-soluble tetrazolium-1 assay (Roche Diagnostics S.p.A, Milan, Italy) following the manufacturer’s instructions. HeLa/GSTO1+ and HeLa Cont clones were plated at low density in six-well plates and treated with different concentration of cisplatin for 60 min. At the end of treatments, cells were washed, plated in 96 multiwells and cultured for 24 h. Then 10 μl of cisplatin for 60 min. At the end of treatments, cells were washed, plated at low density in six-well plates and treated with different concentration of cisplatin for 60 min. Then 10 μl of cisplatin for 60 min. At the end of treatments, cells were washed, plated in 96 multiwells and cultured for 24 h. Then 10 μl of water-soluble tetrazolium-1 was added to each well and after an additional 4 h of incubation, the colored tetrazolium salt was spectrophotometrically read at 450 nm (Vic3® 1420 multilabel counter; Perkin-Elmer, Waltham, MA).

 Hoechst uptake

HeLa/GSTO1+ and HeLa Cont cells were plated in 96 multiwells (4000 cells/cm²). The following day, cells were treated with 20 μM cisplatin for 1 h, washed with fresh medium and after additional 20 h stained (10 min, 37°C) with 5 μg/ml of Hoechst 33342, with or without 2 μg/ml of propidium iodide (PI). At the end of the incubation time, both floating and adherent cells were collected and analyzed using a hemocytometer under a fluorescence microscope. Cells incorporating the Hoechst dye and showing typical morphologic apoptotic features, such as chromatin condensation, were considered apoptotic cells, according to Schmid et al. (20). The apoptosis index (ratio: apoptotic cells/total) × 100 was thus calculated. Hoechst- and PI-positive cells were considered in secondary necrosis.

Annexin V binding assay

HeLa/GSTO1+ and HeLa Cont cells were plated (3000 cells each) in Lab-tek II Chamber Slides (Nalge Nunc International, Rochester, NY). Twenty-four hours later, cells were treated with 20 μM cisplatin for 1 h and then washed with fresh medium. Further 24 h later, cells were stained using the Annexin V- FITC Fluorescence Microscopy Kit (BD Biosciences, Franklin Lakes, NJ) and 2 μg/ml of PI, following the manufacturer’s instructions, and observed under fluorescence microscope (Leica, Milan, Italy) equipped with an online image capture system (Leica DFC320).

Cisplatin treatment of cells for comet assay

Cells were plated at low density in 12-well tissue culture plates and allowed to grow for 20–24 h at 37°C before treatment. Cells were incubated with 20 μM cisplatin for 1 h at 37°C, washed with fresh medium and incubated for up to 24 h. Untreated controls were processed alongside the drug-treated samples.

Single-cell gel electrophoresis (comet assay)

Cross-link formation and repair were determined by the alkaline comet assay, as described by Olive et al. (21) with modifications. This version of the comet assay has been shown to allow for the detection of DNA cross-links from a variety of agents like ifosfamide (22), cisplatin and mitomycin C (23), oxaliplatin (24) and melphanal (25). Cisplatin-treated and control cells were exposed to 6.5 μM (final concentration) hydrogen peroxide for 20 min on ice, in the dark. At the end of such incubation, cells were centrifuged, suspended in 0.6% low-melting point agarose and dispensed onto glass microscope slides pre-coated with 1% normal melting point agarose. The slides were left overnight in ice-cold lysis buffer (100 mM disodium ethylenediaminetetraacetic acid, 2.5 M NaCl, 10 mM Trizma® base, pH 10.0, with 10 M NaOH and containing freshly added Triton X-100, 1% vol/vol) and then placed in a horizontal electrophoresis tank containing ice-cold alkaline electrophoresis buffer (300 mM NaOH and 1 mM disodium ethylenediaminetetraacetic acid) for 20 min, and electrophoresis was then conducted. Slides were neutralized with 0.4 M Tris–HCl, pH 7.5, for 20 min and washed with double-distilled water and then allowed to dry. All procedures were carried out under subdued light to minimize background DNA damage. For staining, slides were rehydrated in distilled water, incubated with a freshly made solution of 2.5 μg/ml PI for 20 min, washed again for 30 min and allowed to dry.

Comets were visualized with a fluorescence microscope (Leica) at ×200 magnification using a ×20 objective. Images were captured by an on-line Leica DFC320 camera and subsequently analyzed with the CometScore™ software (TriTek Corporation). A total of 100 cells were analyzed per sample, 50 per duplicate slide. The percentage of DNA in the tail of the comet (% tail DNA) was calculated for each of the analyzed comet. The tail length was expressed as the percentage decrease in % tail DNA compared with hydrogen-peroxide-only treated cells, calculated by the following formula:

\[
\text{Percentage of decrease in % tail DNA} = \left(1 - \frac{Tc}{Tco}\right) \times 100
\]

where \(Tc\) is the % tail DNA of cisplatin/hydrogen-peroxide-treated samples, \(Tco\) is the % tail DNA of untreated control samples and \(Th\) is the % tail DNA of hydrogen-peroxide-only treated samples.

Intracellular GSH determination

GSH determinations were performed according to Baker et al. (26). HeLa/GSTO1+ and HeLa Cont cells were plated at low density, treated with 20 μM cisplatin for 1 h and lysed in 5-sulfosalicylic acid (1% w/v) for 20 min at 4°C at the indicated times. Acid extracts were then collected and stored at 4°C, whereas cellular proteins were harvested in 0.1 M NaOH and stored at −20°C for protein determination (Bradford assay). Acid extracts and appropriate GSH standards were reacted with 0.2 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.3 mM NADPH and GSH reductase (1.0 U/ml) in sodium phosphate buffer; samples were then analyzed in a microtiter plate reader (Labsystems Multiskan® Plus) by using a single-wavelength (405 nm) kinetics for 3.0 min.

Cellular cisplatin content determination

Cisplatin content of cells was determined by electrothermal atomic absorption spectrometry (27). Stock standard solutions for platinum measurements were prepared by dilution from a 1000 μg/ml Pt standard for atomic absorption spectrometry (H₂PtCl₆ in 2 M HCl; Fluka, St Louis, MO). Cells were plated at low density, treated with 40 μM cisplatin for 1 h, washed at the end of the incubation time and collected at the indicated times. Measurements were carried out with a Perkin-Elmer atomic absorption spectrometer (Model 4100 ZL) equipped with a transverse heated graphite atomizer, a longitudinal Zeeman effect background corrector and an AS-71 autosampler. Transverse heated graphite atomizer graphite tubes with integrated platforms (Part no. B300-0653) were used as purchased.

RNA interference experiments

Small interfering RNAs (siRNAs): GSTO1siRNA-1 (HSC.RNALN004832.9-1-A1), GSTO1siRNA-2 (HSC.RNALN004832.9-2-A1) and GSTO1siRNA-3 (HSC.RNALN004832.9-3-A1) against GSTO1 were purchased from Integrated DNA Technologies (TEMA Italy). HeLa Cont cells were transfected using siGenome Non-Lipid Reagent (Bio-Rad Laboratories Srl, Milan, Italy). To optimize transfection conditions, fluorescent transfection efficiency controls (TYE563 DS Transfectin control; Integrated DNA Technologies) were used. As negative control, a scrambled oligonucleotide (DS Scrambled Negative; Integrated DNA Technologies) was used. GSTO1siRNA-1 resulted as the most active in silencing GSTO1-1, thus it was selected for the transfection experiments. HeLa Cont cells were transfected at 70% confluence using siLentFect according to the manufacturer’s recommendations. Forty-eight hours after transfection, cells were treated with increasing concentrations of cisplatin for 1 h and then incubated for 24 h in drug-free medium. Cell survival was determined by tetrazolium colorimetric water-soluble tetrazolium-1 assay, as described above.

Immunoblot analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed essentially as described by Laemmli (28), with 5% acrylamide for the stacking gel and 12% acrylamide for the separating gel. Proteins were transferred to nitrocellulose sheets (Bio-Rad) following the method of Towbin et al. (29).

For kinase detection, HeLa/GSTO1 and HeLa Cont cells were treated with 20 μM cisplatin for 1 h, washed, incubated in drug-free medium for additional times (1, 2, 10 and 20 h) and then collected with lysis buffer (25 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, pH 7.7, 0.5 M Triton X-100) and HeLa Cont cells were plated at low density, treated with 40 μM cisplatin for 1 h, washed, incubated in drug-free medium for additional times (1, 2, 10 and 20 h) and then collected with lysis buffer (25 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, pH 7.7, 0.5 M Triton X-100, 1.5 mM MgCl₂, 2 mM dithiothreitol, protease inhibitors—leupeptin 4 μg/ml, aprotinin 1 μg/ml and phenylmethylsulfonyl fluoride 1 mM—and phosphatase inhibitors—NaF 50 mM, Na3VO4 0.1 mM and β-glycerol phosphate 20 mM).

Immunodetection was performed employing antibodies against p-ERK1/2, p-Akt (ser473), p-Akt (thr308), Akt, p-p38 (Cell Signaling) and against extracellular signal-regulated kinase (ERK)-1/2, Jun N-terminal kinase (JNK) and p-JNK (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was obtained by peroxidase-labeled anti-rabbit IgG and BM Chemiluminiscence Blotting
Substrate (peroxidase) (Roche Diagnostics S.p.A, Milan, Italy). The acquisition and analysis of images were carried out with a Chemi-Doc apparatus (Bio-Rad).

Other determinations
Protein concentrations were determined using the bicinchoninic acid method, following the manufacturer’s instructions.

Results
Stable transfection and cell-density-dependent GSTO1-1 expression
By screening a series human cell lines, HeLa cells were found to express very low amounts of GSTO1-1 and were therefore chosen to perform stable transfection. In fact, expression of GSTO1-1 was present at various degrees in all other cell lines examined, including CHO, Caco-2, PC12, leukemia U937 cells, 665/2/21 and 665/2/60 human melanoma cells, HK-2, INS-1E and Jurkat cells (data not shown).

GSTO1-1 expression was verified by immunoblot. As shown in Figure 1A, in cultures seeded at low cell density (4 x 10^3 cells/cm²), intense immunoreactivity was detected in the stably transfected clone (HeLa/GSTO1⁺), as compared with a hardly detectable protein band in sham-transfected cells (HeLa Cont). On the other hand, endogenous GSTO1-1 expression was observed to increase along with cell proliferation and increasing cell density. Figure 1A reports the results obtained with cells seeded at high density (4 x 10^3 cells/cm²): in these conditions, the difference of expression between GSTO-transfected cells and controls becomes less evident.

Cell-density-dependent expression of GSTO1-1 was also apparent 24 h after seeding, in HeLa wild-type cells plated at low density (4 x 10^3 cells/cm²) and high density (4 x 10^3 cells/cm²) and high density (4 x 10^3 cells/cm²). Figure 1B shows the results of immunoblot; the densitometric analysis shows an amount of GSTO1-1 over three times higher in high-density cells as compared to low-density cells. Immunoblot data were matched by a similar increase of GSTO1-1 messenger RNA, as measured by reverse transcription–polymerase chain reaction (Figure 1C). In cells plated at low density and allowed to grow up to 96 h, a gradual increase of GSTO1-1 was observed (Figure 1D). Densitometric analysis showed a progressive increase of GSTO1-1, up to three times the basal levels at 96 h. Correspondingly, the amount of messenger RNA also progressively increased (Figure 1E).

Density-dependent upregulation of GSTO1-1 was observed also in other human cells, such as 665/2/21 and 665/2/60 melanoma cells, and even in cells growing in suspension, such as U937 and Jurkat cells (data not shown).

Cytotoxicity of cisplatin
Figure 2A shows the cytotoxic effects of increasing concentrations of cisplatin administered to HeLa/GSTO1⁺ and HeLa Cont cells seeded at low density, i.e. in conditions of highest difference in GSTO1-1 expression (Figure 1A). Twenty-four hours after cisplatin treatment, at all concentrations tested, the survival of HeLa/GSTO1⁺ cells was constantly much higher, with a maximum difference at the concentration of 20 µM cisplatin: 80% survival in HeLa/GSTO1⁺ as opposed to 20% in HeLa Cont cells. Comparable degrees of cisplatin resistance were observed in HeLa Cont cells seeded at high density, as compared with low-density conditions (Figure 2A). Also in this case, the maximum difference in survival was observed at the cisplatin concentration of 20 µM.

Fig. 1. Transfection-dependent and cell-density-dependent GSTO1-1 overexpression. (A) Immunoblot showing the expression level of GSTO1-1 in the stably transfected clone (HeLa/GSTO1⁺) and in the sham-transfected clone (HeLa Cont). Cells were seeded both at low density (LD, 4 x 10^3 cells/cm²) and high density (HD, 40 x 10^3 cells/cm²). Pur.E: recombinant purified GSTO1-1. (B) Immunoblot and densitometric analysis showing the amount of GSTO1-1 expressed in HeLa cells seeded both at low and at high density and collected after 24 h. Data are expressed as mean ± SD (n = 5). (C) Reverse transcription–polymerase chain reaction showing the corresponding increase of GSTO1-1 messenger RNA. (D) Immunoblot and densitometric analysis showing the amount of GSTO1-1 in HeLa cells seeded at low density and collected after 24, 48, 72 and 96 h. Data are expressed as mean ± SD (n = 4). (E) Reverse transcription–polymerase chain reaction showing the corresponding progressive increase of GSTO1-1 messenger RNA.
Knockdown of endogenous GSTO1-1 in high-density cells

To better evaluate the role of endogenous GSTO1-1 in cell protection against cisplatin, high-density HeLa cells were transfected with GSTO1-1 siRNA. Results are reported in Figure 2C, where a markedly decreased GSTO1 expression is apparent following transfection with three different sequences of siRNA as well as with their mixture. No such decrease was observed in cells transfected with mock RNA.

Figure 2B shows cell survival 24 h after treatment with increasing doses of cisplatin. Cell survival was reduced approximately to 40, 25 and 10% at 10, 20 and 40 μM cisplatin, respectively, in cells transfected with GSTO1 siRNA as compared with controls (transfected with a scrambled nucleotide). At the highest dose (80 μM), no difference was observed in % survival between the two samples. Maximal difference in cisplatin effects was observed in this case with a drug concentration of 10 μM, probably due to the fact that transient transfection with siRNA (specific or scrambled) can change drug sensitivity of cells.

Determination of cisplatin and GSH intracellular levels

Since resistance to cisplatin is often due to a decreased drug uptake in resistant cells compared with sensitive ones, the intracellular content of cisplatin was measured in transfected and control cells plated at low density. As shown in Figure 3A, intracellular concentrations of cisplatin were the same in both HeLa/GSTO1+ and HeLa Cont cells, 1 h after treatment with 40 μM cisplatin as well as at 15 h.

Fig. 2. Effects of GSTO1-1 expression on cisplatin cytotoxicity. HeLa/GSTO1+ and HeLa Cont cells were treated for 1 h with increasing doses of cisplatin and then incubated for 24 h in drug-free media. Cell survival was determined by tetrazolium colorimetric water-soluble tetrazolium-1 assay, as described in Materials and Methods. (A) % Survival of HeLa/GSTO1+ cells seeded at low density (4 × 10^3 cells/cm²) and HeLa Cont cells seeded both at low (LD) and high density (HD, 40 × 10^3 cells/cm²). (B) % Survival of high-density HeLa Cont cells transfected with GSTO1siRNA1 (see panel C) and of HeLa Cont cells transfected with a scramble negative siRNA. (C) Results of interfering experiments performed with three different GSTO1 siRNA (GSTO1siRNA-1, -2 and -3, see Materials and Methods) and with a mixture of the three (GSTO1siRNA1-2-3). Data shown in (A) and (B) are mean ± SD of at least five experiments performed in triplicate. Data were analyzed by two-way analysis of variance with Bonferroni multiple comparison test. *P < 0.001; **P < 0.01 for HeLa/GSTO1+ LD versus HeLa Cont LD; ***P < 0.05.

Fig. 3. Intracellular cisplatin and GSH content in HeLa/GSTO1+ and HeLa Cont cells. (A) Cells were treated with 40 μM cisplatin. Intracellular cisplatin was determined at the end of treatment (1 h) and at 15 h, by electrothermal atomic absorption spectrometry, as described in Materials and Methods. Data are expressed as mean ± SD (n = 4). (B) Cells were treated with 20 μM cisplatin. GSH was determined as described in Materials and Methods, before (0 time) and during the treatment (0.5 and 1 h), as well as at 6 and 15 h. Data are expressed as mean ± SD (n = 3).

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Conjugation with GSH and subsequent disposal of the drug is a major defense of the cell against cisplatin, and indeed, transfection of anti-apoptotic proteins can produce in some instances an increase of intracellular GSH (30,31). As shown in Figure 3B, however, GSH content in HeLa/GSTO1+ and in HeLa Cont cells (seeded at low density) was comparable, before, during or after cisplatin treatment, up to 15 h.

Single-cell DNA gel electrophoresis (comet assay)

DNA cross-links formation in response to cisplatin was investigated by using a modified alkaline comet assay (21–25). In this procedure, a fixed number of random DNA strand breaks are introduced by...
treatment of cells with H$_2$O$_2$ prior to analysis, which causes DNA to relax and become free upon electrophoresis. As cross-linking agents tend to retard the electrophoretic mobility of DNA, levels of cross-linking can be quantified from the decrease in the % tail DNA in cisplatin-treated cells as compared with untreated controls. As shown in Figure 4, DNA damage was detected neither in cisplatin-treated cells nor in controls when H$_2$O$_2$ treatment was not performed. On the contrary, H$_2$O$_2$ treatment revealed increasing levels of cisplatin-induced DNA cross-links in both clones, up to 14 h after treatment. No significant difference was observed, however, between HeLa/GSTO1$^+$ and HeLa Cont cells, suggesting that the higher resistance of HeLa/GSTO1$^+$ cells against cisplatin is not related to a decreased cisplatin binding to DNA. Twenty-four hours after cisplatin exposure, a marked decrease in DNA cross-links was observed in both clones; such a decrease was, however, more relevant in HeLa Cont cells as compared with HeLa/GSTO1$^+$.

**Activation of Akt and mitogen-activated protein kinases**

Mitogen-activated protein kinases and Akt represent the main kinases involved in apoptosis by either preventing or inducing the process. The activity of these kinases was evaluated in cells seeded at low density. Figure 6A shows the results with respect to ERK1/2: at both 1 and 2 h after cisplatin treatment, a clear decrease of the phosphorylated form of ERK1/2 was observed in HeLa Cont cells, whereas HeLa/GSTO1$^+$ cells maintained the initial degree of phosphorylation. No changes were observed in the total amount of ERK1/2.

Figure 6B shows the results with respect to Akt: the two phosphorylated forms of the protein (Ser-473 and Thr-308) progressively increased at 1 and 2 h after cisplatin treatment in HeLa/GSTO1$^+$, whereas no changes were observed in HeLa Cont cells. No changes were observed in the total amount of Akt.

No variations were observed in JNK1 activation at early times after cisplatin treatment (1 and 2 h; Figure 6C). However, at 10 h after treatment, a sharp increase in JNK1 activation was observed in HeLa Cont cells, whereas no such increase was apparent in HeLa/GSTO1$^+$ cells. This difference in JNK1 activation was still present at 20 h.

As far as p38, its activated form was almost undetectable in both HeLa/GSTO1$^+$ and HeLa Cont cells, and no variations were observed at 1 and 2 h after cisplatin treatment (data not shown).

**Discussion**

The development of cell populations displaying resistance to drug treatments is usually a major obstacle to efficient anticancer therapy, and this is also the case of cisplatin, one of the most widely used agents for the treatment of solid tumors. Recent data suggest that GSTO1-1 can play a primary role in such processes. Mouse GSTO was indeed identified because of its overexpression in a mouse lymphoma cell line presenting with resistance against a variety of chemotherapeutics (4). Overexpression of GSTO1-1 in transgenic *C.elegans*...
resulted in a significantly higher resistance to oxidative stress induced by several pro-oxidants, as well as by heat shock (32). More recently, GSTO1-1 has been identified as one of the proteins overexpressed in four platinum-resistant human ovarian cancer cell lines (17). Altogether these data suggest, but do not demonstrate, that GSTO1-1 could have a role in drug resistance.

The present study shows, for the first time, that GSTO1-1 overexpression can efficiently protect HeLa cells against cisplatin toxicity. Overexpression of GSTO1-1 was obtained both by transfection experiments as well as by culturing cells at increasing cell densities. In fact we found that GSTO1-1 expression was cell density dependent, i.e. it was very low when cells were seeded at low density but increased up with the increasing of cell density, as well as in cells directly seeded at high density. Such a behavior was also found in other cell lines (665/2/21 and 665/2/60 melanoma, U937 and Jurkat cells), allowing to hypothesize that cell-density-dependent GSTO1-1 overexpression may be a general phenomenon. Cell-density-dependent expression has also been reported for other proteins, such as phosphoenolpyruvate carboxykinase (33) and HSP27 (34). The latter is a well-known stress protein, itself involved in cell resistance to cisplatin and other anticancer drugs (35,36).

Cells overexpressing GSTO1-1 were efficiently protected against cisplatin toxicity. The results obtained in the two experimental models (GSTO1-1 transfection and high-density conditions) were very similar (Figure 2A), in particular, in both models the highest protection was apparent at the concentrations of 20 μM cisplatin, where a 80%
survival was observed in GSTO1-1 overexpressing cells as compared with only 20% in controls. The development of strong resistance against drug-induced cell death in cell cultures reaching confluence is a known phenomenon, at least in colon cancer cells. It was shown that the resistance phenotype may be strongly dependent on the overexpression of HSP27 (34). Our data suggest that a similar role can be played by GSTO1-1, at least with respect to resistance against cisplatin. Such an interpretation is supported by findings obtained with cells transfected with GSTO1-1 siRNA, in which a decreased expression of GSTO1-1 was matched by a strong decrease in cell survival 24 h after treatment with increasing doses of cisplatin (Figure 2B and C).

The potential role of GSTO1-1 in protection against cisplatin toxicity was recently questioned by Schmuck et al. (37), who investigated the cisplatin resistance of GSTO1-1-deficient T-47D breast cancer cells. When stable GSTO1-1 transfection was performed, T-47D cells acquired normal levels of GSTO1-1 S-(4-nitrophenyl)GSH reductase activity, but this was not accompanied by increased resistance against cisplatin or other anticancer agents. However, as discussed by the authors, such negative results could be the consequence of a major chromosomal rearrangement present in T-47D cells, probably resulting in the loss of components essential for the GSTO1-1-mediated resistance mechanism(s).

Development of cisplatin resistance has been shown to be frequently accompanied by decreased intracellular concentrations of the drug, probably as a consequence of decreased drug uptake or increased drug disposal (18). This was apparently not the case in our experimental model, where in fact comparable intracellular concentrations of cisplatin were detectable in both HeLa/GSTO1-1 and HeLa Cont cells, 1 and 15 h after treatment (Figure 3A). Another line of cell defense against cisplatin is given by intracellular GSH. In some instances, the stable transfection of cell lines with complementary DNA of proteins with protective function, like HSP27 (30) and Bcl2 (31), has been observed to produce a marked increase of intracellular GSH levels. No such increase was found, however, in our experiments, i.e. the transfection had no effect on GSH concentration either before or at the end of cisplatin treatment or 15 h later (Figure 3B).

A decreased amount of DNA cross-links has also been observed in cisplatin-resistant cells. No significant differences were, however, observed in the extent of DNA cross-links between HeLa/GSTO1-1 and HeLa Cont cells at any time up to 14 h (Figure 4), suggesting that GSTO1-1 expression does not interfere with cisplatin binding to DNA. Twenty-four hours after cisplatin exposure, a marked decrease in DNA cross-links was observed in both clones, a phenomenon explainable as the result of either activation of repair mechanism or apoptotic degradation of DNA. Actually, much higher levels of DNA cross-links were retained by GSTO1-1-transfected cells. Such observation can be explained by the higher rate of survival presented at 24 h by transfected cells as compared with controls (see Figure 2A). In other words, the sharp decrease in DNA cross-links detected in controls may simply be the consequence of a higher rate of DNA degradation during the apoptotic process.

The observations described above indicate that the protection against cisplatin toxicity observed in GSTO1-1-transfected cells cannot be ascribed to a decreased uptake of cisplatin, to an increased GSH-dependent disposal of the drug or to a decreased levels of DNA cross-linking. On the other hand, a strong difference between transfected and control cells in susceptibility to apoptosis was observed, as assessed with Hoechst method as well as by immunofluorescent determination of Annexin V (Figure 5A). Thus, altogether our data suggest that GSTO1-1 has a role in the resistance against cisplatin by interfering with the apoptotic process, as shown previously for other GSTs. glutathione S-transferase Mu1-1 can in fact bind to apoptosis-signal-regulating kinase-1, thus inhibiting apoptosis-signal-regulating kinase-1-dependent apoptotic cell death; heat shock causes the dissociation of the glutathione S-transferase Mu1-1—apoptosis-signal-regulating kinase-1 complex, thus removing the inhibition (38–40). Glutathione S-transferase P1-1 can bind and inhibit JNK (41); ultraviolet irradiation or H2O2 cause the activation of JNK by dissociating the Glutathione S-transferase P–JNK complex (42). We measured the activity of the main kinase systems involved in the apoptotic process, i.e. Akt kinases, which represent the main survival pathway, and the mitogen-activated protein kinases, involved either in proliferation–survival pathways or in pro-apoptotic mechanisms. Early after cisplatin treatment (1 and 2 h), both Akt phosphorylated forms (Thr-308 and Ser-473) were increased in HeLa/GSTO1-1 cells but not in controls. The Akt kinase family is regarded as the main survival pathway in a variety of cell types. Akt activation can block apoptosis induced by apoptotic stimuli such as growth factors withdrawal, ultraviolet irradiation, matrix detachment and DNA damage (43), by affecting several steps along the apoptotic machinery. It has been also reported that activation of Akt2 can inhibit the activation of JNK-p38 that is observed following cisplatin treatment (44). Activation of the JNK signaling cascade generally results in apoptosis (45). In our experimental model, a strong increase of the phosphorylated form of JNK was apparent in HeLa Cont cells at 10 and 20 h after cisplatin treatment, whereas such activation was completely prevented in HeLa/GSTO1-1 cells. Generally, activation of ERK-signaling pathway has a role in mediating cell division and survival (46); at 1 and 2 h after cisplatin treatment, a clear decrease of ERK1/2 activation was observed in HeLa Cont cells, which was not observed in HeLa/GSTO1-1, where ERK1/2 activation was maintained.

In conclusion, in our experimental model, the overexpression of GSTO1-1 appears to be associated—following cisplatin treatment—with the activation of two major pathways generally involved in survival mechanisms, i.e. Akt and ERK1/2, as well as with the (direct and/or indirect) inhibition of a pathway generally strongly associated with apoptosis, i.e. JNK activation.

These results can explain the protective effect afforded by GSTO1-1 overexpression against cisplatin-induced apoptosis. The mechanisms by which GSTO1-1 can interfere with the above mentioned signaling pathways are presently unknown and will hopefully be elucidated by studies currently in progress in our laboratory.

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References


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