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Enhanced resistance of HeLa cells to cisplatin by overexpression of γ-glutamyltransferase

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Abstract

γ-Glutamyltransferase (GGT), which is a key enzyme for the cellular glutathione (GSH) homeostasis, was shown to be overexpressed in human tumor cells selected for resistance to cisplatin and to influence the resistance of experimental tumors *in vivo*. We first established that cisplatin treatment of HeLa cells was accompanied by an early 3-fold induction of GGT synthesis, enhancing the possibility that this enzyme plays an important role in the cell defenses against this anticancer drug. This role was then studied using a GGT-transfected HeLa cell line (HeLa–GGT) exhibiting 10 times the activity of the parental HeLa cells (120–150 and 10–14 mU/mg protein, respectively). Both cell lines showed comparable intracellular GSH levels and cisplatin resistance when cultured in high (250 μM) or low (50 μM) cysteine-containing medium. When 50 μM of GSH were included in the low-cysteine culture medium only HeLa–GGT cells partially recovered their intracellular GSH and exhibited an increased resistance to cisplatin. Cisplatin treatment also inhibited GGT-dependent production of reactive oxygen species, a process depending on the availability of cysteinylglycine produced during GSH catabolism. Furthermore, we showed that cisplatin forms adducts with cysteinylglycine 10 times more rapidly than with GSH, and that these adducts were formed only in the extracellular medium of HeLa GGT cells. This extracellular mechanism could at least partially account for the increased resistance of GGT-rich cells to cisplatin.

Keywords: Cisplatin resistance; γ-Glutamyltransferase; GSH catabolism

1. Introduction

The tripeptide γ-Glu–Cys–Gly (GSH) is the main non-protein intracellular thiol. It plays a crucial role in the maintenance of the intracellular redox state and thus protects the cell against oxidative injuries. GSH reacts also with many toxic agents to form conjugates which can be easily excreted by means of specific transporters [1]. The elevation of intracellular GSH has been widely demonstrated in cells resistant to platinum agents, especially cisplatin (CDDP) [2] and the depletion of GSH sensitizes cells to these compounds [3]. However, it is not clear how GSH acts exactly, e.g. by complexing and exporting the drugs *via* the GSH-X pump [4], or by protecting cells against reactive oxygen species (ROS) [5], or also by increasing DNA repair [6]. The increase of intracellular GSH level in resistant cells could depend on induction of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in GSH synthesis, but several experimental evidences showed that under physiological conditions, cysteine availability, and not γ-GCS activity, is the key factor determining intracellular GSH synthesis [7]. In view of this, an important role could be played by γ-glutamyltransferase (GGT); in fact, GGT is a cell surface enzyme that initiates the cleavage of extracellular GSH, thereby providing GGT-positive cells with a secondary source of cysteine capable of enhancing the intracellular synthesis of GSH [8]. GGT activity was shown to be induced in cells selected for their resistance to CDDP [2,9] and in samples from a patient resistant to CDDP-based chemotherapy

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Abbreviations: GSH, glutathione; GGT, gamma-glutamyltransferase; cysgly, cysteinylglycine; γ-GCS, gamma-glutamylcysteine synthetase; CDDP, *cis*-diamminedichloroplatinum(II); LC-DMEM, low-cysteine DMEM; HC-DMEM, high-cysteine DMEM; ROS, reactive oxygen species; DHR-123, dihydrorhodamine-123; Rh-123, rhodamine-123.

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[10]. CDDP is one of the most efficient anticancer agents against a number of solid tumors, but its use is limited by the frequent emergence of CDDP-resistant cell populations. Many studies showed a relationship between resistance and enzymes related to GSH metabolism in tumor samples or in established cell lines [11]. *In vitro* studies by the National Cancer Institute Drug Screening Program showed no relationship between GGT activity and resistance to alkylating agents [12], however these studies were performed in culture medium rich in cysteine, thus likely obscuring the role of GGT in providing an additional source for this amino acid.

The aim of this study was to investigate the role of GGT overexpression in cancer cell resistance to CDDP under physiological conditions. To do this, we compared under standard culture conditions (250 μM of cysteine in the culture medium) and under culture conditions closer to the physiological concentrations of cysteine (50 μM), the sensitivity to CDDP of an isogenic cell line overexpressing human GGT, HeLa–GGT, and the parental cell line, HeLa.

2. Material and methods

2.1. Cell lines, culture conditions and treatments

The HeLa–GGT cell line was established in our laboratory from HeLa-Tet-off (Clontech) cells, after stable transfection by the vector pTRE-GGT containing the full length cDNA of human GGT [13]. The full length sequences of the human GGT were cloned in the Hind III/XbaI sites of the pTRE2 vector and transfected among with the pTK-Hyg selection plasmid to HeLa cells using lipofectamine. Twenty-four hours after transfection, cells were seeded in 90 μM Petri dishes at 4×10^5 cells/dish. The cells were allowed to attach, and hygromycine was added to the culture medium at 100 μg/mL. Hygromycine-resistant colonies, which appeared after 3 weeks were individually picked up using cloning cylinders (Sigma), and expanded. When sufficient material was available, the GGT activity of each clone was measured. One clone named HeLa–GGT was found to express 125–150 mU of GGT/mg protein, and retained for this studies. The parental cell line expresses 10–15 times lower GGT activity (10–14 mU/mg protein).

Cells were grown in DMEM (Sigma, this medium contains 250 μM cysteine and methionine, and will be referred as high-cysteine DMEM, HC-DMEM) supplemented with 10% (v/v) heat inactivated fetal calf serum (Boehringer), 1% (v/v) antibiotic–antimycotic solution (Sigma). The DMEM without cysteine and methionine (Sigma), was supplemented with 50 μM cysteine and 50 μM methionine (Gibco) (this medium will be referred as low-cysteine DMEM, LC-DMEM) and used for experiments in medium with low levels of sulfur-containing amino acids. Cultures were maintained at 37° in a humidified atmosphere of 95% air and 5% CO_2.

An amount of 5 mM *cis*-diamminedichloroplatinum(II) (CDDP) (Sigma) stock solution was prepared in 150 mM NaCl and stored at +4°. The stock was renewed every 4 weeks.

2.2. Analysis of GGT mRNAs expression

After 24 hr of CDDP treatment, total mRNA was extracted using the GenElute RNA extraction kit (Sigma). Total RNA of 4 μg were used for reverse transcription with 200 U of M-MLV reverse transcriptase (Promega) and 0.5 μg oligo$(dT)_{16–18}$ in 50 μL containing 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 1 mM of each dNTP. GGT total mRNA was amplified using primers Amp1–Amp2 (sense primer: 5′-AATGGACGACTTCAGCTCTCC-3′ and antisense primer 5′-AGCCGAACCAGAGGTTGTAGA-3′). β_2-Microglobulin sequences were amplified with sense primer (5′-ACCCCCACTGAAAAAGATGA-3′) and antisens primer (5′-ATCTTCAAACCTCCATGATG-3′). Analysis of GGT subtype A, and C mRNAs expression was performed using subtype A primers (sense: 5′-CACAGGGGACATAC-AGTGAG-3′, antisense: 5′-GAAATAGCTGAAGCACG-CGC-3′), and subtype primers (sense: 5′-GCCCAGAA-GTGAGAGCAGTT-3′, antisense: 5′-TCCAGAAAGCAG-CTAGAGGG-5′) [14]. Results were normalized for the β_2-microglobulin signal.

2.3. Measurement of GGT activity

GGT activity was determined using L-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycine as donor and acceptor substrates as previously described [15], and was expressed as mU/mg protein (nmol of 5-nitrobenzoate/min/mg protein). Protein concentration was determined using BioRad reagent (Coomassie Blue) according to the manufacturer's recommendations and bovine serum albumin as standard.

2.4. Measurement of intracellular GSH and of extracellular GSH and cysteinylglycine (cysgly)

2.4.1. Sample preparation for intracellular GSH measurement

The cells were seeded in 6-well plates and cultured either in HC-DMEM or in LC-DMEM during 24 hr. Cells were then incubated for additional 24 hr in HC-DMEM or LC-DMEM in the presence or absence of 5 μM CDDP. In addition the effect of 50 μM GSH was tested in LC-DMEM in the presence or absence of 5 μM CDDP. Cells were washed with cold PBS, scrapped in 500 μL of 3.3% (w/v) perchloric acid, and centrifuged at 12,000 *g* for 5 min at +4°. The supernatant was used for thiol measurement and the pellet for protein determination. Both extracellular and intracellular samples were kept at −80° until HPLC analysis.

2.4.2. Sample preparation for extracellular thiol measurement

HeLa and HeLa–GGT cells seeded in 6-well plates 24 hr before experiment were incubated in DMEM containing 1 mM GSH at pH 7.4. Two hundred microliter aliquots of medium were sampled after selected culture periods and mixed with 200 μL 3.3% (w/v) perchloric acid solution. Prior to HPLC analysis, samples were diluted 10-fold in a 0.9% (w/v) NaCl containing 4 mM EDTA.

2.4.3. HPLC analysis

The thiol measurements were performed using a reverse phase HPLC method including a reduction step of disulfide bonds with tri-*n*-butylphosphine (TBP) and precolumn derivatization with a thiol-selective fluorogenic reagent, 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide (ABD-F) as previously described [16]. Briefly, to 100 μL of the above described samples, 50 μL of the internal standard (thioglycolic acid, 0.3 M), 20 μL 0.5 M NaOH (100 μL in the case of intracellular medium), 250 μL 0.2 M borate buffer pH 9.0, 50 μL 5% (v/v) TBP, and 50 μL 2.3 mM ABD-F were added. The resulting mixture was incubated at 50° for 20 min; then the derivatization reaction was stopped by adding 50 μL 1 M HCl, and HPLC analysis was performed.

2.5. Survival assays

Cytotoxicity was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenil tetrazolium bromide) assay. HeLa or HeLa–GGT cells were plated in 96-well plates and cultured for 24 hr in the appropriate medium. Then, cells were incubated in 200 μL of medium containing the indicated concentrations of CDDP. After additional 24 hr of culture, 20 μL MTT (5 mg/mL) were added to the cells, followed by incubation for 3 more hours. The resulting formazan product was solubilized in 200 μL DMSO and spectrophotometrically measured at 595 nm.

2.6. Monitoring of reactive oxygen species production

Quantification of ROS was performed using the dihydrorhodamine-123 (DHR-123) probe which is oxidized by various ROS to the fluorescent rhodamine-123 (Rh-123) [17]. A stock solution of 10 mM DHR-123 was prepared in dimethylformamide. Cells were seeded in 6-well plates in HC-DMEM 24 hr before the experiment. Then, the cells were gently washed with 2 mL of PBS and incubated at 37° for 1 hr in PBS containing 1 mM GSH, 25 μM DHR-123, 150 μM Fe^{3+} and 165 μM EDTA. Two hundred microliter aliquots of medium were withdrawn and fluorescence was measured using a microplate fluorimeter (Cytofluor 2350; Millipore; Bedford, MA, USA) at an excitation and emission wavelengths of 485 and 530 nm, respectively. Acellular experiment containing 1 mM GSH, 25 μM DHR-123 and 150 μM Fe^{3+} and 165 μM EDTA in PBS was used as control.

2.7. Analysis of GSH– and cysgly–CDDP adducts

The analysis of GSH–CDDP and cysgly–CDDP adducts was based on the protocols of Bernareggi *et al*. [18]. The HPLC system used to monitor the production of CDDP–thiol adducts included an isocratic pump, a valve-injector fitted with a 20 μL loop (model Rheodyne 7125), a reverse-phase silica-based column (LiChrospher RP 18 end capped (5 μm) 250 × 4 mM; Merck Darmstadt) eluted with 0.35 mM triethylamine phosphate buffer pH 2.5 at a flow rate of 0.8 mL min^{-1} and a UV spectrophotometric detector operated at 230 nm. Data were recorded using an integrator. Retention times of CDDP, cysgly, GSH–CDDP, CDDP–cysgly, and GSH were ca. 2.7, 2.8, 3.0, 3.3 and 5.5 min, respectively. Stock solutions of CDDP and each thiol were prepared at a concentration of 3.3 mM in a 10 mM phosphate buffer pH 7.4 added with 150 mM NaCl. CDDP and thiol solutions were mixed in a 1:10 ratio (v/v), and the mixtures were incubated at 37°. At different time intervals ranging from 0 to 25 min, aliquots were withdrawn and immediately injected into the HPLC system. No significant difference between the molar absorbance of each thiol at 230 nm was observed and CDDP absorbance was at least 10-fold less than thiols at this wavelength. Thus, in order to calculate the formation rate of the adducts (expressed in mM/min), it was considered that the adduct absorbance was mainly due to the thiol contribution.

In order to study the formation of CDDP–cysgly adducts in the culture medium of the two cell lines, a new HPLC system was developed. It consists in a Aquasil C18 (3 μm) 150 × 3 mM i.d. column (thermo Hypersil) eluted with a 10 mM phosphate buffer pH 2.7 containing 5 mM pentanesulfonic acid, at a flow rate of 0.5 mL min^{-1}. UV detection was operated at 215 nm. The addition of the counter-ion (pentanesulfonic acid) allowed the efficient separation of CDDP–cysgly adducts from CDDP–GSH adducts, CDDP, GSH or cysgly. Retention times of CDDP, CDDP–GSH, GSH, cysgly and CDDP–cysgly and were ca. 2.0, 3.3, 5.8, 6.0 and 7.8 min, respectively.

2.8. Statistical analysis

All assays were set up in triplicate, and the results were expressed as the mean and standard deviation (mean ± SD). Student's *t*-paired test was used for statistical analysis.

3. Results

3.1. Induction of GGT expression in HeLa cells after CDDP treatment

While cell lines made resistant to CDDP often show elevated intracellular GSH, γ-GCS and GGT activities [2], the effect of a single short-term CDDP treatment on GGT activity before the onset of resistance is not well

documented, especially in human cell lines. Thus, we first studied the modulation of GGT activity in HeLa cells after CDDP challenge. Sub-confluent HeLa cells were treated with various concentrations of CDDP (0–10 μM) for 24 and 48 hr, and GGT activity was measured in cell extracts.

As shown in Fig. 1A, GGT activity was rapidly induced in response to CDDP (1.4-fold after 24 hr treatment with 2.5 μM of CDDP). Maximum induction was observed after 48 hr for treatments with 2.5 and 5 μM of CDDP (2.8-fold). Treatment with higher CDDP concentrations (10 μM)

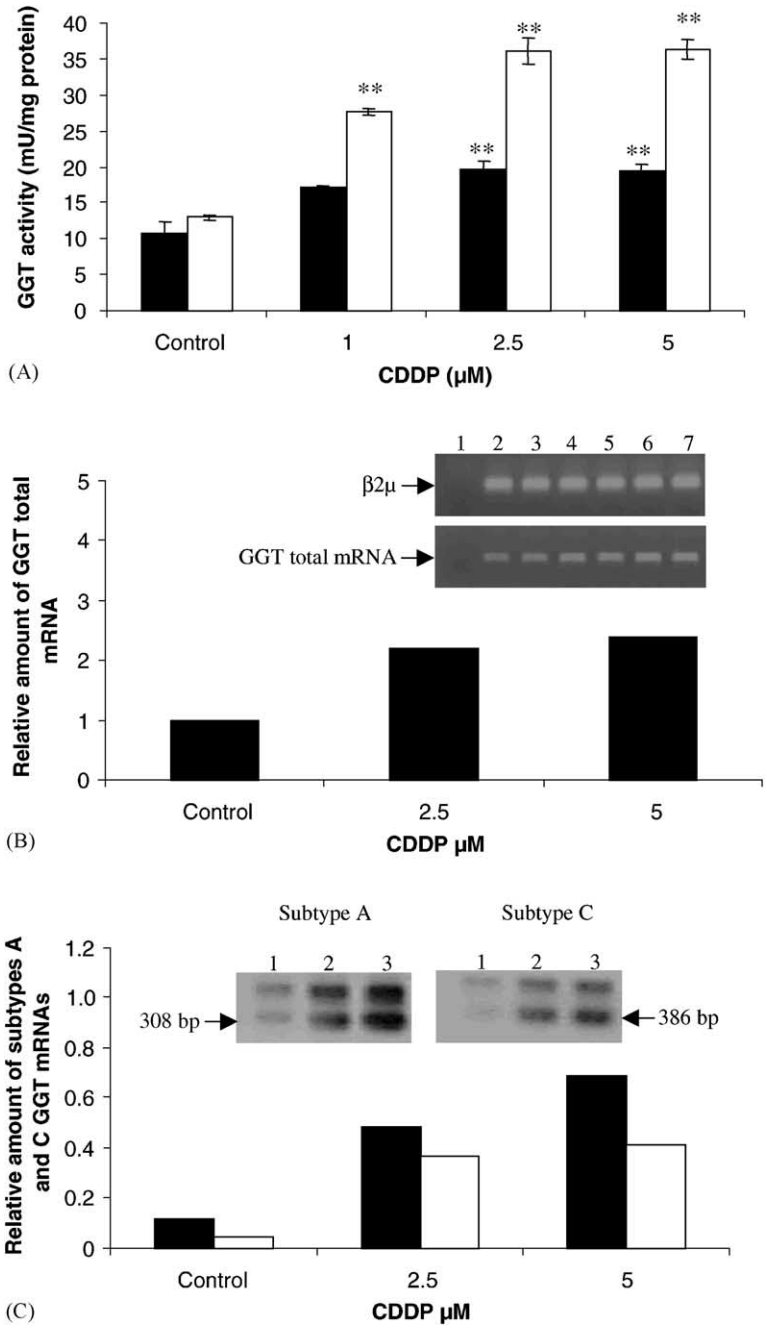

Fig. 1. CDDP treatment induces GGT synthesis in HeLa cells. (A) Induction of GGT activity: cells were treated with 0 (control), 1, 2.5, or 5 μM of CDDP for 24 hr (■) or 48 hr (□) and GGT activity was measured in whole cell extracts. Results shown are mean ± SD of three experiments. $^{**}P < 0.001$ vs. control cells. (B) Induction of GGT total mRNA level: cells were treated with 0 (control), 2.5 or 5 μM of CDDP 24 hr before total RNA extraction. RT-PCR was performed as described in Section 2. The relative amount of GGT total mRNA was obtained by the ratio GGT/β_2-microglobulin signals. Data are the mean of two independant experiments. Inserts: photographs of the corresponding agarose gels used for quantification of GGT and β_2-microglobulin signals; lane 1: negative control; lanes 2 and 3: control cells; lanes 4 and 5: cells treated with 2.5 μM CDDP; lanes 6 and 7: cells treated with 5 μM CDDP. (C) Induction of GGT mRNA subtypes: RT-PCR was performed using either subtype A (■) or subtype C (□) specific primers, respectively, and followed by Southern blot. The relative amount of each GGT mRNA subtype was obtained by the ratio GGT subtype/β_2-microglobulin signals. Inserts: photographs of the corresponding Southern blot used for quantification of each GGT mRNA subtype level. The arrows indicate the expected bands, and the numbers the corresponding sizes. Lane 1: control cells; lane 2: cells treated with 2.5 μM CDDP; lane 3: cells treated with 5 μM CDDP.

resulted in a lower induction of GGT activity, likely due to an observed massive cell death (data not shown).

We then examined whether the induction of the GGT activity was due to an increase in the enzyme synthesis. Total RNA was extracted from control and CDDP-treated HeLa cells, and the GGT total mRNA level was evaluated by RT-PCR using Amp1–Amp2 primers; β_2-microglobulin was used as an internal control. As shown in Fig. 1B, the GGT total mRNA level doubled after 24 hr treatment with 2.5 and 5 μM of CDDP, thus showing that the increase in GGT activity after CDDP treatment, depended on the increase of the corresponding mRNA.

GGT type I mRNAs show a polymorphism in their 5′ UTR due to alternative splicing events and probably to the use of different promoters [19]. We have previously shown that in HeLa cells, two subtypes (A and C) of GGT mRNA I were mainly expressed and that they were differently regulated after 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), TNFα or butyrate treatment [14]. As shown in Fig. 1C, 24 hr CDDP treatment induced both subtypes. Subtype A was induced in a dose-dependent manner (by 5- and 7-fold after 2.5 and 5 μM CDDP). Subtype C was induced by 4-fold by either treatments. Thus, the observed increase of GGT total mRNA I was due to the increase of at least these two particular subtypes.

Taken together, these results show that one of the early responses of HeLa cells to CDDP treatment is an increase in GGT synthesis and activity.

3.2. Establishment and characterization of HeLa–GGT cell line

We then asked whether the observed increase in GGT activity could provide an advantage to the cells exposed to CDDP. CDDP is mainly detoxified by conjugation with GSH and active transport outside the cell [4]. GGT can play a crucial role in CDDP detoxification by providing the precursors of GSH synthesis, especially when low concentrations of extracellular cysteine are present. In order to examine only the influence of an increased GGT activity, we transfected HeLa cells with the pTRE-GGT vector and we established a cell line expressing about 120–150 mU/mg of GGT. Both HeLa and HeLa–GGT cells had comparable γ-GCS activities (62 ± 2.5 and 58 ± 2 nmol γ-glucys formed/mg/min, respectively).

3.2.1. Intracellular GSH levels

We first studied the modifications in the intracellular GSH level of the parental HeLa and the transfected HeLa–GGT cell lines under different culture conditions, and in the presence or not of 5 μM CDDP. Results are summarized in Table 1. Under standard culture conditions (HC-DMEM, 250 μM cysteine), both cell lines presented comparable levels of intracellular GSH (39.5 ± 3.7 and 35.9 ± 3.5 nmol/mg for HeLa and HeLa–GGT cells, respectively). Decreasing the extracellular concentration of cysteine to 50 μM (LC-DMEM), which is the concentration found in human serum, resulted in a dramatic decrease of the intracellular level of GSH (8-fold) in both cell lines, which still exhibited comparable levels of intracellular GSH (5.3 ± 2.5 and 3.7 ± 0.6 nmol/mg for HeLa and HeLa–GGT cells, respectively). Despite the low intracellular GSH level, the cell proliferation was not affected (not shown). When 50 μM of GSH were added to the cells cultured in the LC-DMEM, we observed, only in HeLa–GGT cells, a 5-fold increase in the intracellular GSH level as compared either to the HeLa cells cultured in the same medium or to HeLa–GGT cells cultured in the LC-DMEM alone.

These results demonstrate that at physiologically low concentrations of cysteine, high GGT activity in the presence of extracellular GSH, can effectively enhance the intracellular GSH level.

Under every culture condition tested, in both HeLa–GGT and HeLa cell lines, treatment with 5 μM CDDP for 24 hr increased the intracellular GSH level (Table 1), suggesting that the CDDP-dependent increase of intracellular GSH concentration was independent from the composition of the culture medium and from GGT activity.

3.2.2. Cytotoxicity of CDDP

We then studied, under the same culture conditions, the cytotoxicity of CDDP on the two cell lines and the cell viability was evaluated using the MTT assay.

As shown in Fig. 2A and B, the cytotoxicity of CDDP, was substantially the same in both HeLa and HeLa–GGT cell lines in the presence of low- or high-cysteine concentrations in the medium (IC_{50} 7.5 μM in all conditions). These results indicate that, in these cellular models, variations of intracellular GSH, consequent to different cysteine availability, do not affect the CDDP toxicity. When 50 μM

Table 1
Influence of extracellular cyst(e)ine concentration and CDDP treatment on intracellular GSH level

	GSH (nmol/mg protein)					
	250 μM cys		50 μM cys		50 μM cys + 50 μM GSH	
	−CDDP	+5 μM CDDP	−CDDP	+5 μM CDDP	−CDDP	+5 μM CDDP
HeLa	39.5 ± 3.7	52.6 ± 6.4	5.3 ± 2.5	7.0 ± 0.8	4.0 ± 0.2	7.3 ± 2.8
HeLa–GGT	35.9 ± 3.5	43.6 ± 3.0	3.7 ± 0.6	9.3 ± 2.5	19.3 ± 4.7**	27.0 ± 2.6**

** $P < 0.001$ as compared HeLa cells cultured under the same conditions. Also significant to HeLa–GGT cells cultured in the presence of 50 μM cys.

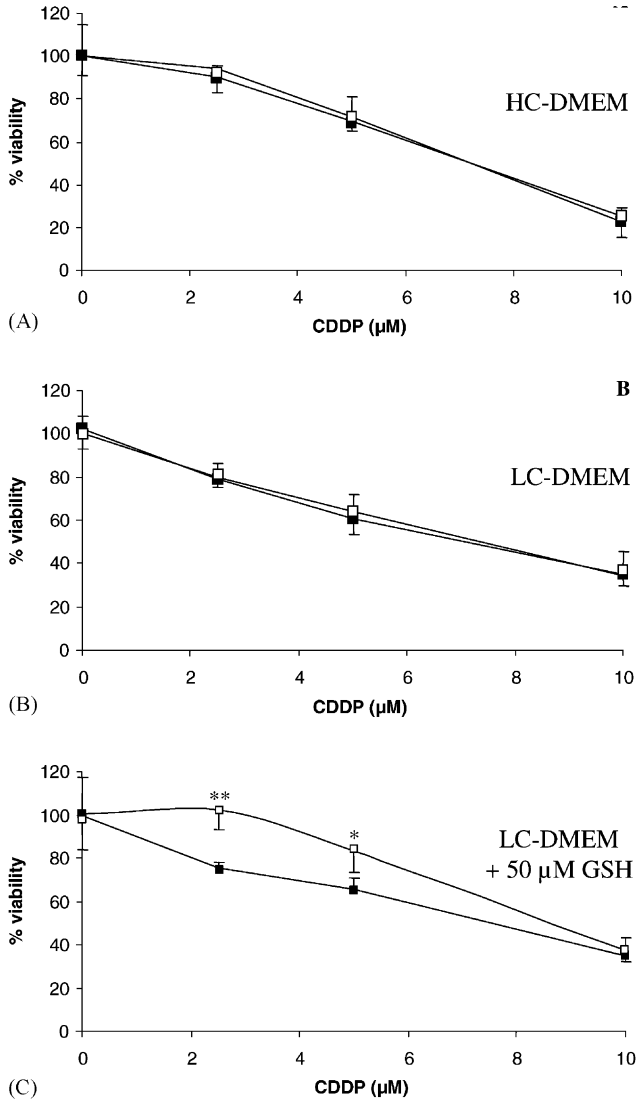

Fig. 2. CDDP cytotoxicity on HeLa and HeLa–GGT cells. HeLa (■) or HeLa–GGT (□) cells were seeded in 96-well plates and were incubated for 24 hr in the presence of indicated concentrations of CDDP. Viability was measured using MTT. (A) Cells were cultured in HC-DMEM (250 μM cys); (B) cells were cultured in LC-DMEM (50 μM cys); (C) cells were cultured in LC-DMEM supplemented with 50 μM GSH. Results expressed as % of untreated control cells are the mean ± SD of eight independent experiments. Statistical significance of differences in viability between HeLa and HeLa–GGT at the indicated CDDP concentrations: $^{*}P < 0.05$; $^{**}P < 0.001$.

GSH was included in the LC-DMEM medium, HeLa–GGT cells showed a significantly higher viability and IC_{50} when challenged with 2.5 or 5 μM of CDDP. In contrast there was no change of the observed toxicity of the drug on the HeLa cell line.

These results cannot be totally explained by the higher intracellular GSH content of HeLa–GGT cells (Table 1), because as we showed (Fig. 2A and B), the intracellular GSH concentration did not influence the CDDP toxicity. Therefore, we asked whether an extracellular event, depending on GGT and GSH, could be responsible for the observed higher resistance of HeLa–GGT cells.

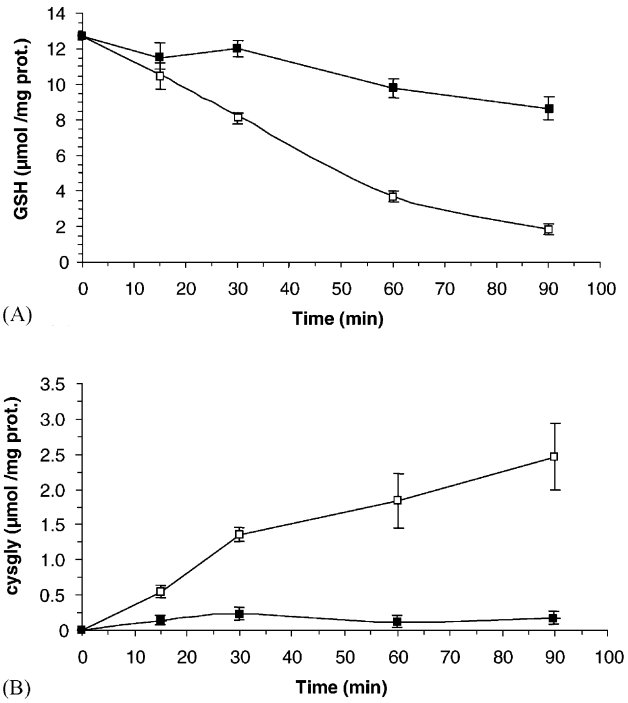

Fig. 3. Extracellular GSH catabolism and cysgly accumulation by HeLa and HeLa–GGT cells. HeLa (■) or HeLa–GGT (□) cells seeded in 6-well plates were incubated with 1 mM GSH in HC-DMEM. Samples of extracellular medium were withdrawn at various times, and GSH (A) and cysgly (B) concentrations were measured by HPLC. Values were adjusted for total cellular protein content. Results are mean ± SD of three distinct experiments.

3.2.3. GSH metabolism

HeLa and HeLa–GGT cells were incubated in the presence of 1 mM GSH, and the degradation of the tripeptide and the appearance of cysgly were measured through a 90 min period. As shown in Fig. 3A, after 90 min HeLa cells consumed only 20% of the GSH present in the medium whereas during the same time period, HeLa–GGT cells consumed 90% of the extracellular tripeptide.

The GGT-dependent GSH catabolism was accompanied in HeLa–GGT cells by an accumulation of cysgly in the medium (Fig. 3B). As expected, in HeLa cells the cysgly accumulation was by far lower. These experiments confirmed that HeLa–GGT cells, due to their GGT activity, presented a higher rate of extracellular GSH degradation and cysgly accumulation.

3.2.4. ROS production

It is now well documented that in the presence of transition metals, GSH catabolism leads to the production of ROS [20]. HeLa and HeLa–GGT cells were incubated in the presence of GSH and an iron source, and the ROS production was evaluated by the oxidation rate of DHR-123 to Rh-123. As shown in Fig. 4, both cell lines produced a significant amount of ROS. As expected, HeLa–GGT cells, due to their higher GGT activity produced 2.3 times more ROS than HeLa cells. When CDDP was included to the incubation medium, ROS production

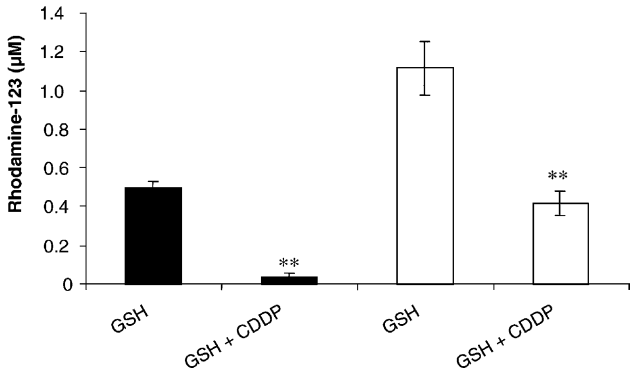

Fig. 4. Extracellular ROS production by HeLa and HeLa–GGT cells. HeLa (■) or HeLa–GGT (□) cells seeded in 6-well plates were incubated for 60 min with 0.5 mM GSH, 160 μM Fe^{3+}–EDTA and 25 μM DHR-123 in the absence or in the presence of 50 μM CDDP. ROS production was measured as DHR-123 oxidation. Results shown are mean ± SD of three distinct experiments. $^{**}P < 0.001$ vs. cells incubated in the absence of CDDP.

was inhibited by 92% for HeLa cells and by 62% for HeLa–GGT cells.

Production of ROS by the GGT-dependent catabolism is due to the high reactivity of the thiol group of cysgly. It is also well documented that CDDP can react with the thiol group of GSH to form different types of adducts [4,18]. An explanation for the inhibition of GGT-dependent ROS production by CDDP could be its reaction either with GSH or with cysgly thus blocking the reactive thiol group.

Consequently, we verified this hypothesis by measuring the rate of *in vitro* formation of adducts between GSH or cysgly and CDDP.

3.3. Formation of GSH– and cysgly–CDDP complexes

The production of each corresponding adduct with a 1:1 stoichiometric ratio (CDDP–GSH and CDDP–cysgly) was measured using HPLC after a short incubation period (within 15 min), and its production rate was considered in the present work to reflect the reactivity of each thiol with CDDP.

As shown in Fig. 5, cysgly–CDDP adduct formation reached very rapidly (within 5 min) a plateau while the production of GSH–CDDP adducts remained linear for up to 15 min. The rate of formation of cysgly–CDDP adducts (0.1126 mM/min), calculated on the linear part of the curve, was 10 times higher than the rate of formation of GSH–CDDP adducts (0.0102 mM/min).

The previous HPLC system, used to point out the difference between the reactivity of GSH and cysgly, was unable to fully separate GSH–CDDP adducts from cysgly–CDDP adducts. In order to characterize the presence of cysgly–CDDP adducts in the culture medium of the two tested cell lines, a new HPLC system using a counter ion in the mobile phase was developed. With this system cysgly–CDDP adduct is well separated from GSH and CDDP, while cysgly alone coelutes with GSH and GSH–CDDP adduct coelute with glygly (glycylglycine, an acceptor of γ-glutamyl group). HeLa and HeLa–GGT cells were incubated in PBS containing 1 mM GSH, 5 mM glygly and 100 μM CDDP. Samples were withdrawn at various time intervals and analyzed. As shown in Fig. 6, a peak corresponding to the cysgly–CDDP adduct was observed only for HeLa–GGT cells (compare Fig. 6B and C, incubation time 100 min), indicating that this mechanism is dependent on GGT catalytic activity.

Taken together, these results can explain why the HeLa–GGT cells show an increased resistance to CDDP only when GSH is present in the extracellular medium. GGT-dependent catabolism of GSH results in the production of highly reactive cysgly which in its turn forms extracellular adducts with CDDP, thus either lowering the availability or inactivate the drug. However, at higher concentrations of CDDP (10 μM), this mechanism seems to be unable to protect the cells.

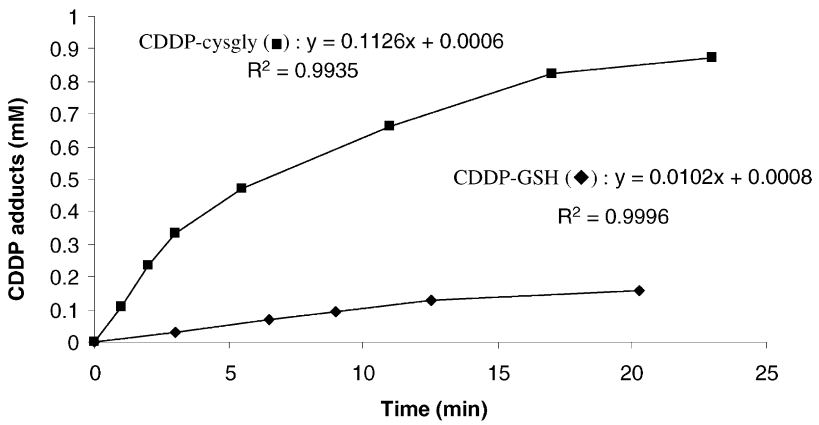

Fig. 5. Formation of CDDP–GSH and CDDP–cysgly adducts *in vitro*; 1 mM of CDDP was incubated either with 10 mM GSH (◆) or with 10 mM cysgly (■) in 10 mM phosphate buffer pH 7.4 at 37°. Samples were withdrawn at various times and the CDDP adducts were measured by HPLC. Values are from one out of three experiments which gave the same pattern. The equations were derived from the linear part of the graph (0–3 min for CDDP–cysgly adducts and 0–15 min for CDDP–GSH adducts, respectively).

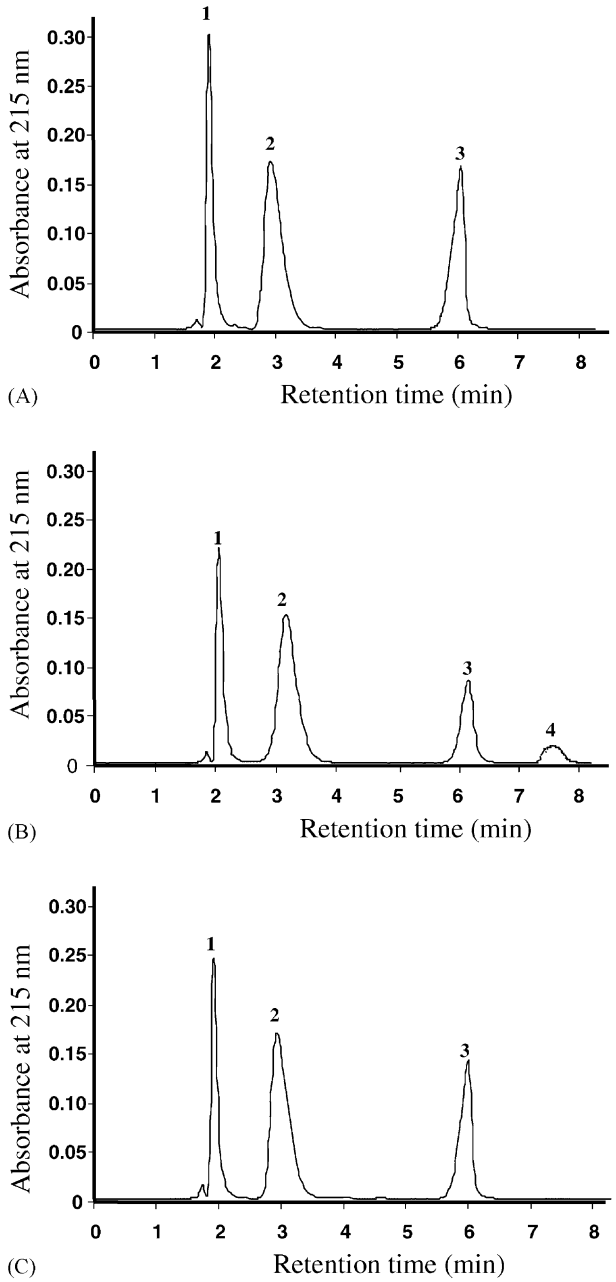

Fig. 6. Formation cysgly–CDDP adducts in extracellular medium. Cells were incubated with 1 mM GSH, 5 mM glygly and 100 μM CDDP in PBS at 37°. Extracellular medium was used for HPLC analysis at time = 0 (A) and 100 min for HeLa–GGT cells (B) or HeLa cells (C). Retention times are: 2, 3, 3.3, 5.8, 6 and 7.8 for, respectively, CDDP (1), glygly and GSH–CDDP adduct (2), GSH and cysgly (3) and CDDP–cysgly adduct (4).

4. Discussion

CDDP-resistant cell lines, isolated after prolonged exposure to low doses of this drug, have been valuable tools for the elucidation of the factors leading to CDDP resistance, which is often multifactorial. Resistance to alkylating agents and platinum derived drugs can involve a modification of the uptake of the drug [21], or an increased inactivation of the drug by sulfhydryl group containing molecules such as GSH [4] or an increased export [22]. It has been shown that in human leukemia cells, γ-GCS is increased after CDDP treatment coordinately with the MRP/GSH pump [22]. Such a response could allow the cells to increase their GSH synthesis and therefore to have a higher scavenging rate of the drug and to export more efficiently the GSH–CDDP complexes.

The resistance can also be the consequence of an enhanced DNA repair mechanism. CDDP challenge of human ovarian cancer cells resulted in the induction of ERCC-1 which is a critical factor in the nucleotide excision-repair pathway that performs CDDP-DNA adducts repair [23]. Furthermore, alterations in the expression of oncogenes (such as c-*fos*, c-*myc*, c-*jun* or c-*abl*) or tumor suppressor genes (such as *p53*) (for review see [24]) have also been implicated in the cellular resistance to CDDP.

GGT which is a key enzyme for the cellular GSH homeostasis has been shown to be overexpressed in different types of cancer such as ovarian [25], prostate [26] or lung [27]. Induction of GGT has been observed in human tumor cells selected for resistance to chemotherapeutic drugs [10].We first showed that an early response of HeLa cells to a short exposure to CDDP is an increase in GGT expression. The enzyme activity was induced 3-fold after 48 hr with 2.5 μM CDDP treatment and the inductions were correlated with an earlier accumulation of the GGT mRNA. Godwin *et al*. [2] have also shown a correlation between the degree of resistance of human ovarian cancer cell lines and the level of GGT mRNA. However, these cells were obtained after a long-term exposure to the drug. They also found that the GGT mRNA was induced earlier in the acquisition of the CDDP resistance than the γ-GCS mRNA. The mechanisms responsible for the increase of GGT synthesis cannot be well explained. CDDP treatment can increase c-*fos* and c-*jun* [24], and one possible mechanism could be through activation of AP-1. This could be true for the γ-GCS induction as the gene promoter sequences contain two AP-1 responsive elements [28]. For the human GGT gene only the promoter controlling the transcription of type B GGT mRNA I has been described and it contains AP-1-like responsive elements [19]. Although inducible by TPA [14], this type of mRNA is barely expressed in HeLa cells. In addition, type A and C GGT mRNA I are the major GGT mRNA forms and they are both induced by CDDP. Thus, the increase in the total GGT mRNA level can be attributed to the increase of these two subtypes. However, no information exists for the promoters controlling the expression of these two GGT mRNAs.

We then focused on the role of GGT in the response of HeLa cells to CDDP using two models: the HeLa-Tet-off cells expressing 10–14 mU/mg of GGT, and the same cell line stably transfected with the human GGT sequences and expressing a higher level of the enzyme (10–15-fold as compared to the parental cell line). This approach was chosen in order to avoid any interference from other pathways contributing to CDDP resistance. Indeed, to evaluate each parameter individually, studies with well-defined systems, such as the use of isogenic cell lines are needed.

Despite the difference in GGT activity, the two cell lines exhibited comparable γ-GCS activities and intracellular GSH concentrations either in low- or high-cysteine containing culture medium. Although culture in the low-cysteine medium resulted to a dramatic decrease (−80%) of the intracellular GSH, no differences in the cellular viability were observed. HeLa–GGT cells could recover about 50% of their intracellular GSH pool when GSH was added extracellularly. Thus, cells with high GGT activity can have the advantage for recovering intracellular GSH only when extracellular GSH is present and used as cysteine source. These data are in accordance with previous works on cellular and animal models which showed that a main function of GGT is to provide the cell with cysteine [29,30] and with the study of Saint Vincent *et al*. [7], who demonstrated that the limiting step in biosynthesis of GSH is not the step catalyzed by γ-GCS, but the availability of cysteine.

The CDDP cytotoxicity under both (low- and high-cysteine) culture conditions on the two cell lines remained comparable. These results suggest that, under these conditions, in HeLa cells, overexpression of GGT and intracellular GSH levels are not influencing the resistance to CDDP. In contrast to our observations, previous studies have shown that GSH depletion increased CDDP cytotoxicity [31,32]. In these studies the GSH depletion was achieved by pre-treatment of the cells with BSO, which is a potent inhibitor of γ-GCS, the key enzyme in GSH biosynthesis. This discrepancy can then be explained by the fact that BSO-treated cells have not only a low GSH content but that they are also unable to replenish their low GSH pool after CDDP treatment. Under our conditions, γ-GCS remains fully active and thus it can restore any GSH consumed by CDDP. In addition CDDP treatement of HeLa and HeLa–GGT cells led to an increase of the intracellular GSH under all conditions tested.

HeLa–GGT cells were found more resistant to CDDP treatment when a low (50 μM) amount of GSH were added to the culture medium. An hypothesis to explain this phenomenon can be that the HeLa–GGT cells can more efficiently catabolize the extracellular GSH, thus providing the cell with precursors for a higher turnover rate of the intracellular GSH. In this case cells could detoxify CDDP more efficiently. Earlier studies by Hanigan [33] also led to the same conclusion. By establishing experimental GGT-poor and GGT-rich tumors in animal models, these authors have shown that GGT-rich tumors grew twice faster than the GGT-poor counterparts. GGT-rich tumors were also more resistant to CDDP treatment, but they exhibited the same intracellular GSH content than the GGT-poor tumors. They concluded that the resistance of GGT-rich tumors may result from the ability of these cells to replenish intracellular GSH levels more rapidly when low amounts of cysteine (such as in blood) are available.

However, this explanation may not be satisfactory for our model. In fact, when HeLa and HeLa–GGT cells were cultured in a high- or low-cysteine containing medium, the IC_{50} for CDDP was the same. We can expect that in the high-cysteine medium the turnover of intracellular GSH is faster than in the low-cysteine medium and thus cells should exhibit an increased resistance, which is not the case in our model. We therefore looked for another GGT- and GSH-dependent mechanism to explain the increased resistance of HeLa–GGT cells when GSH was added extracellularly.

The extracellular degradation of GSH by GGT is accompanied by the appearance of extracellular cysgly, a strongly reactive thiol, which in the presence of transition metals can lead to the production of ROS [20]. We showed that HeLa and HeLa–GGT cells can produce ROS extracellularly, with the HeLa–GGT cells producing twice the amount of ROS as compared to their untransfected counterparts. This difference can be explained by the higher rate of GSH consumption by HeLa–GGT cells. Interestingly, when CDDP was included in the incubation medium, ROS production was partially inhibited. CDDP can react with the SH group of GSH or other SH containing molecules to form different types of adducts [18]. It is then logical to assume that CDDP can react with cysgly and even faster than with GSH as the SH goup of cysgly is by far more reactive. Accordingly to this and using an *in vitro* system, we showed that the rate of formation of cysgly–CDDP adducts is at least 10 times higher than the rate of formation of GSH–CDDP adducts. We also showed that significant amounts of cysgly–CDDP adducts accumulate only in the extracellular medium of HeLa–GGT cells demonstrating that extracellular production of cysgly by increased GGT activity allows the formation of these adducts.

It is also important to note that the CDDP concentrations used in this study were very close to the blood concentrations of the drug during cancer treatment. Indeed, Riva *et al*. [34] reported that the maximum concentration of CDDP in blood of patients during phase II trails reached 4 μM. Thus, in our experimental models the induction of GGT and the modulation of the resistance of the HeLa cells to CDDP toxicity due to an increased GGT activity are physiologically relevant.

In conclusion, we showed in this study that an early response of HeLa cells to CDDP treatment is an increase of GGT synthesis. Increased GGT activity can influence the intracellular GSH levels only under low-cysteine culture conditions and when GSH is present in the culture medium. Also, only under these particular conditions the cells exhibited an increased resistance to CDDP toxicity independently to the intracellular GSH content. We propose that the resistance to CDDP is due to a mechanism which in the first step occurs at the extracellular level. GGT cleavage of GSH results in the production of cysgly which in turn can rapidly form adducts with CDDP, thus detoxifying the drug. Further studies are in progress to ascertain whether this detoxification mechanism leads to a lower intracellular accumulation of the drug, or to a decreased formation of DNA-CDDP adducts.

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