Overexpression of the regulatory subunit of γ-glutamylcysteine synthetase in HeLa cells increases γ-glutamylcysteine synthetase activity and confers drug resistance

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γ-Glutamylcysteine synthetase (GCS) is reported to catalyse the rate-limiting step in glutathione biosynthesis, and is a heterodimer composed of a catalytic subunit [heavy subunit (GCS_h) of M_r 73000] and a regulatory subunit [light subunit (GCS_l) of M_r 31000]. In the present study, we have demonstrated for the first time a potential role for GCS_l in resistance towards doxorubicin and cadmium chloride. Addition of recombinant GCS_l to HeLa cell extracts *in vitro* was found to result in an increase in GCS activity of between 2- and 3-fold. Transient transfections of COS-1 cells with the GCS_l cDNA cause an increase in GCS activity of approx. 2-fold, and a small but significant ($P = 0.008$) increase in glutathione levels from 126.9 ± 4.2 nmol/mg protein to 178.8 ± 19.1 nmol/mg protein. We proceeded to make a HeLa cell line (LN73), which stably overexpresses GCS_l. These cells overexpress GCS_l approx. 20-fold above basal levels. LN73 was found to have a 2-fold increase in GCS activity (437.3 ± 85.2 pmol/min per mg) relative to the control cell line, HL9 (213.4 ± 71.8 pmol/min per mg). In contrast with the transient transfections in COS-1 cells, stable overexpression of GCS_l was found not to be associated with an increase in glutathione content. However, when the LN73 and HL9 cells were treated with the glutathione-depleting agent, diethylmaleate, the LN73 cells were found to have an enhanced ability to regenerate glutathione, compared with HL9 cells. The cell lines were treated with various anti-cancer drugs, and their cytotoxicity was examined. No obvious differences in toxicity were observed between the different cell lines following treatment with cisplatin and melphalan. The redox-cycling agent doxorubicin, however, was found to be more toxic (approx. 2-fold) to the HL9 cells than the LN73 cells. When the cells were treated with the carcinogenic transition-metal compound, cadmium chloride, LN73 cells were found to be approx. 3-fold more resistant than HL9 cells.

Key words: cadmium chloride, cisplatin, doxorubicin, glutathione, melphalan.

INTRODUCTION

Glutathione and glutathione-metabolizing enzymes have been implicated as key components in certain instances of resistance to cancer chemotherapy [1–6]. The significance of glutathione in resistance to different cytotoxic drugs is, however, poorly understood and the relative contribution of glutathione metabolism to anti-cancer drug resistance is unclear [1,7]. However, the fact that glutathione has the potential to detoxify several different anti-cancer drugs or the products of their metabolism, together with the finding that overexpression of certain glutathione S-transferases (GSTs) in cell lines can confer drug resistance [8–11], makes glutathione-associated detoxification mechanisms attractive candidates for mediating resistance to chemotherapeutic drugs.

Glutathione is synthesized in the cell cytosol by the sequential actions of γ-glutamylcysteine synthetase (GCS) (L-glutamate:L-cysteine γ-ligase, EC 6.3.2.2) and glutathione synthase (γ-L-glutamyl-L-cysteine:glycine ligase, EC 6.3.2.3) [12], but the mechanisms by which cells regulate glutathione homoeostasis are unclear. Evidence suggests that GCS catalyses the rate-limiting step in glutathione synthesis, since it can be regulated through feed-back inhibition by glutathione [13–16]. Increased GCS activity has been shown to be associated with the increased intracellular glutathione levels seen in many drug-resistant cancer cell lines [17–19].

GCS is a heterodimer, and is composed of a catalytic subunit [heavy subunit (GCS_h) of M_r 73000] and a regulatory subunit [light subunit (GCS_l) of M_r 31000] [14–16]. It has been shown previously [15] that GCS_h is responsible for all of the catalytic activity, and that GCS_l modulates the K_m for glutamate and sensitivity to feed-back inhibition by glutathione. Studies *in vitro* with recombinant rat GCS_l and GCS_h, and the purified holoenzyme, led Meister and co-workers to propose a mechanism whereby the subunit interactions regulate the activity of the enzyme *in vivo* [15,16]. This model, which is on the basis of evidence for both covalent and hydrophobic interactions between GCS_l and GCS_h, proposes that variations in intracellular glutathione concentrations can modulate disulphide-bridge formation between GCS_h and GCS_l, causing a conformational change in the enzyme. This affects the sensitivity of the enzyme to inhibition by glutathione, and results in regulation of GCS activity by redox status. When intracellular reduced glutathione concentrations are high, the disulphide bridge becomes reduced, and GCS_h is subject to competitive inhibition by glutathione with respect to glutamate. Moreover, the interaction between GCS_h

Abbreviations used: BSO, buthionine sulphoximine; DEM, diethylmaleate; GCS, γ-glutamylcysteine synthetase; GCS_l, γ-glutamylcysteine synthetase light subunit; GCS_h, γ-glutamylcysteine synthetase heavy subunit; GST, glutathione S-transferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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and GCS_l has been shown to decrease the K_m for glutamate; this also appears to be influenced by redox status, since treatment of the GCS holoenzyme with dithiothreitol results in an increase in the K_m for glutamate [15].

Historically, the majority of studies on the regulation of GCS in both adaptive-stress responses and drug resistance have focused on the catalytic subunit, GCS_h. It has been implicated as a determinant of glutathione levels, and has been shown to be up-regulated in tumour cell lines that have been made resistant to cisplatin or melphalan; increases in GCS_h levels have been found to coincide with increased glutathione levels [6,17,18]. By comparison, the expression of GCS_l has received very little attention, because a function was ascribed for it only within the last few years [15,16]. We have shown recently [20] that the regulatory subunit is co-ordinately up-regulated with GCS_h in response to oxidative stress in HepG2 cells. The role of the regulatory subunit in glutathione homoeostasis is, however, poorly defined, and it is unclear whether co-ordinate up-regulation of both GCS subunits is necessary to increase glutathione turnover in cancer cells *in vivo*. Increased expression of both GCS_h and GCS_l has been observed in cisplatin-resistant ovarian cancer cells [6], although this was later shown not to be accompanied by corresponding increases in the GCS_l mRNA [19].

GCS_h and GCS_l are encoded by separate genes [21–23] and, although we have shown that both subunits are induced by oxidative stress in human cell lines [20], it is unknown whether these are present in equimolar concentrations in the cytosol. Studies that have examined mRNA levels in human tissues have shown that the relative amounts of each of the subunits might be variable among different tissues [24], and this suggests a further level of complexity in the regulation of glutathione homoeostasis.

In order to understand further the role of GCS_l in glutathione homeostasis and drug resistance, we have made cell lines that stably overexpress GCS_l. These cell lines have increased GCS activity and enhanced capacity to synthesize glutathione, which results in resistance to doxorubicin and cadmium chloride.

EXPERIMENTAL

Chemicals

All chemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.) unless otherwise stated.

DNA and plasmids

Human GCS_l cDNA (EST clone no. 133938) was obtained from the I.M.A.G.E. consortium (St. Louis, MO, U.S.A.) [25] and has been described previously [20]. The *Escherichia coli* expression vector, pET15b, was purchased from Novagen (Abingdon, Oxon., U.K.). The mammalian expression vector, pCI-neo, was purchased from Promega (Southampton, U.K.). The GCS_l expression vector, pSTLN1, was constructed by ligating the cDNA encoding GCS_l into pCI-neo using *Mlu*I and *Sma*I restriction sites.

Expression and purification of recombinant GCS_l

The cDNA encoding human GCS_l was cloned into *Nde*I and *Xho*I restriction sites in pET15b, and the histidine-tagged recombinant protein was expressed in *E. coli* BL21(DE3) cells using 1 mM isopropyl β-D-thiogalactoside for 2.5 h at 30 °C. The GCS_l protein was purified on a HiTrap Chelating column (Pharmacia Biotech, Milton Keynes, U.K.) according to the manufacturer's instructions, and was eluted with 80 mM imidazole.

Cell culture and transfections

Transient transfections

COS 1 cells, maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Paisley, Renfrewshire, U.K.) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 50 units/ml penicillin and 50 μg/ml streptomycin, were seeded at a density of 10^6 cells/10-cm plate 24 h before transfection. Cultures were grown at 37 °C in a humidified air/CO_2 (19:1) atmosphere, and transfections were carried out by the method of calcium phosphate co-precipitation [26]. For each transfection, 10 μg of pSTLN1 or 10 μg of pSV-β-Galactosidase (Promega) was used. Cells were harvested by trypsin treatment 48 h after transfection and lysed by sonication in 10 mM sodium phosphate buffer, pH 7.5, containing EDTA (1 mM) and $MgCl_2$ (2 mM). Insoluble material was removed by centrifugation, and supernatants were retained for analysis.

Generation of stable cell lines

HeLa cells were maintained in minimal essential medium (MEM; Gibco BRL) containing 10% (v/v) fetal-calf serum, 1% (v/v) MEM non-essential amino acids (Gibco BRL), penicillin (50 units/ml) and streptomycin (50 μg/ml). Cultures were grown at 37 °C in a humidified air/CO_2 (19:1) atmosphere. Cells were seeded at a density of 10^6 cells/10-cm plate 24 h before transfection. The cells were transfected by the method of calcium phosphate co-precipitation with either 10 μg of pSTLN1 or 10 μg of pcDNA3.1HisLac (InVitrogen, Leek, The Netherlands) as a control. The media was replaced with fresh media containing 800 μg/ml G418 48 h after transfection, to select for stable incorporation of plasmid into the genome. Resistant cells were selected for 6–8 weeks, with replacement of the G418-containing media occurring every 3 days. Resistant colonies were picked and propagated for analysis of GCS_l protein by Western blotting.

Glutathione depletion by diethylmaleate (DEM) treatment

Cells were seeded at a density of 10^6 cells/10-cm plate 24 h before DEM treatment. DEM (150 μM) in fresh media was added to the cells and incubated at 37 °C for 2 h. The DEM-containing media was replaced with fresh media, and growth was allowed to continue at 37 °C for 3 h.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays

Cell metabolic activity was determined by using an MTT assay [27], following treatment with different drugs. Cells were seeded in 96-well plates at a density of 15000 cells per well 24 h before treatment. The cells were then treated with fresh media containing doxorubicin (10 nM, 100 nM, 200 nM, 400 nM, 500 nM, 750 nM or 1 μM), cadmium chloride (5 μM, 20 μM, 30 μM, 40 μM, 50 μM or 60 μM), cisplatin (5 μM, 10 μM, 50 μM, 100 μM or 250 μM) or melphalan (0.5 μM, 1 μM, 2.5 μM, 5 μM, 20 μM or 30 μM). The drug was removed 48 h after treatment, and 50 μl MTT (2 mg/ml) was added to each well. This was incubated for 4 h at room temperature, after which the MTT solution was removed and 50 μl of DMSO was added. A_{540} was determined. Cell metabolic activity for each treatment was expressed as a percentage of untreated controls.

Clonogenic survival assays

Cell survival following treatment with cadmium chloride or doxorubicin was determined by clonogenic assay [28]. Cells were

seeded at a density of 10^6 cells/10-cm plate 24 h before treatment with the drug. Cells were treated with either 100 nM or 250 nM doxorubicin for 1 h or 10 μM, 25 μM or 50 μM cadmium chloride for 4 h. After treatment, cells were washed three times with PBS, treated with trypsin and then reseeded in fresh media on 10-cm plates at a density of 2×10^4 cells per plate. Following incubation at 37 °C for 8 days, surviving colonies were stained with 1% (w/v) Crystal Violet and counted. Survival was expressed as a percentage of the colonies formed by cells treated with the drug solvent alone. Kruskal–Wallis non-parametric analysis-of-variance tests were performed to determine statistical significance.

Analytical methods

Protein concentrations and glutathione levels were determined by the methods of Bradford [29] and Tietze [30] respectively. These methods were modified for use on a Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, Herts., U.K.). GCS activity was determined by HPLC, by the method of Hamel et al. [31]. K_m and V_{max} values were determined by measuring initial reaction rates at glutamate concentrations of between 0.1 mM and 25 mM, and employing UltraFit curve-fitting software (Biosoft, Cambridge, U.K.) to fit the Michaelis–Menten equation using the Marquardt algorithm.

Western blot analysis

Western blotting was performed using antibodies raised against synthetic peptides corresponding to regions of GCS_l and GCS_h, as described previously [20].

RESULTS

Effect of recombinant GCS_l on GCS activity in HeLa cell extracts *in vitro*

Purified GCS has been shown to be a heterodimer comprising catalytic and regulatory subunits. However, the relative proportions of the GCS subunits *in vivo* are unknown. There is evidence to support the hypothesis that each of the subunits are subjected to different modes of regulation [24,32], and this suggests that differential regulation of the GCS_l and GCS_h subunits might provide an additional mechanism for regulation of GCS activity and glutathione homoeostasis. Studies of the specific activity of the GCS catalytic subunit have shown that without association with GCS_l, it has a low affinity for glutamate and is subject to competitive inhibition by glutathione [15]. If the GCS_l subunit were to be present at a relatively lower concentration than the GCS_h subunit in certain cells, increasing the concentration of GCS_l could potentially increase GCS activity. To test the hypothesis that GCS_l might be quantitatively rate-limiting for GCS activity, increasing amounts of recombinant GCS_l subunit were added to HeLa cell extracts and GCS activity was measured. Purified histidine-tagged recombinant GCS_l subunit (1 μg, 5 μg or 10 μg) was added to 100 μg of protein from HeLa cell extracts, and GCS activity was determined (Table 1). GCS activity of the HeLa cell extracts was found to be increased by 1.6-, 2.4- and 2.5-fold on addition of 1 μg, 5 μg and 10 μg of GCS_l respectively.

Table 1 Effect of recombinant GCS_l on GCS activity of HeLa cell extracts *in vitro*

Purified histidine-tagged recombinant GCS_l subunit (1 μg, 5 μg or 10 μg) was added to 100 μg of protein from HeLa cell extracts, and GCS activity was determined as described in the text.

Recombinant GCS light subunit (μg)	GCS activity of HeLa cell extracts (pmol/min per mg)
No addition (control)	235.9 ± 34.8
1	386.4 ± 86.0
5	554.6 ± 19.9
10	584.9 ± 2.5

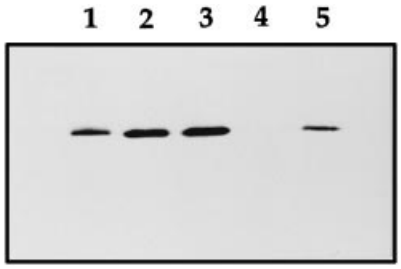

Figure 1 Overexpression of GCS_l in COS-1 cells

COS-1 cells were transfected with 10 μg of pSTLN1 or 10 μg of pSV-β-Galactosidase or 5 μg each of pSTLN1 and pSV-β-Galactosidase as described in the text. Cell extracts were prepared 48 h after transfection, and analysed by Western blotting using an antibody raised against a synthetic peptide from GCS_l [20]. The gel was loaded with 20 μg of protein from cell extracts as follows: lanes 2 and 3, cell extract from COS-1 cells transfected with pSTLN1; lane 4, cell extract from COS-1 cells transfected with pSV-β-Galactosidase; lane 5, cell extract from COS-1 cells transfected with pSV-β-Galactosidase together with pSTLN1. Lane 1 contains 10 μg of protein from rat kidney cytosol as a marker.

Transient transfection of COS-1 cells with the cDNA encoding GCS_l increases GCS activity

As we were able to show that recombinant GCS_l can increase GCS activity in HeLa cell extracts *in vitro*, it was necessary to determine whether excess GCS_l could increase GCS activity *in vivo*. It has been shown previously that transfection of COS-7 cells with GCS_l cDNA does not increase significantly GCS activity [33]. However, whereas these experiments demonstrated that an increase in GCS_l mRNA did not increase GCS activity, the levels of the GCS_l polypeptide were not determined, and it is unknown whether there was a substantial increase in GCS_l protein. In the present study, the GCS_l cDNA was cloned into pcDNA3 (Invitrogen) and used to transiently transfect COS-1 cells, as described for pSTLN1 in the Materials and methods section. Extracts from the transfected cells were subjected to Western blotting using an antibody raised against a synthetic peptide corresponding to a region of GCS_l [20]. No increase in GCS_l protein was observed (results not shown). To increase levels of expression of GCS_l, the GCS_l cDNA was cloned into pCI-neo (Promega) to create pSTLN1. The pCI-neo expression vector contains a chimaeric intron to increase expression levels of cDNAs. COS-1 cells were transfected with pSTLN1, using 10 μg of DNA/10-cm plate. Cells were transfected with the plasmid pSV-β-Galactosidase as a control, and were harvested 48 h after transfection; GCS_l levels were then determined by Western blotting. Figure 1 shows that COS-1 cells transfected with pSTLN1 have an increase in GCS_l levels of approx. 20-fold compared with cells transfected with pSV-β-Galactosidase. GCS activity of the transfected cell extracts was determined and it was found that the specific activity in COS-1 cells transfected with GCS_l is 1762.9 ± 502.9 pmol/min per mg compared with

Table 2 Effect of overexpression of GCS_l on glutathione levels and GCS activity in COS-1 cells

COS-1 cells were transfected with pSTLN1 or pSV-β-Galactosidase, using 10 μg of DNA/10-cm plate. Cells were harvested 48 h after transfection, and GCS activity and glutathione concentrations in the transfected cell extracts were determined.

Transfection plasmid	γ-Glutamylcysteine synthetase activity (pmol/min per mg)*	Glutathione concentration (nmol/mg protein)*†
pSV-β-Galactosidase	921.8 $\pm$ 4.1	126.9 $\pm$ 4.2
pSTLN1	1762.9 $\pm$ 502.9	178.8 $\pm$ 19.1

* Values are the means $\pm$ S.D. for determinations from five replica plates.
† The statistical significance value of $P = 0.008$ was determined by the Mann–Whitney Test.

Table 3 GCS activity of LN73 and HL9 HeLa cell lines

Values for specific activity are expressed as pmol/min per mg protein and are the means $\pm$ S.D. for three separate experiments, with assays each performed in duplicate.The V_{max} and K_m values for glutamate were determined by employing the UltraFit curve-fitting software (Biosoft) to fit the Michaelis–Menten equation using the Marquardt algorithm.

	Cell line	
Kinetic parameter	HL9	LN73
Specific activity	213.4 $\pm$ 71.8	437.3 $\pm$ 85.2
V_{max}	290.0 $\pm$ 71.1	478.0 $\pm$ 48.3
K_m for glutamate (mM)	2.6	1.1

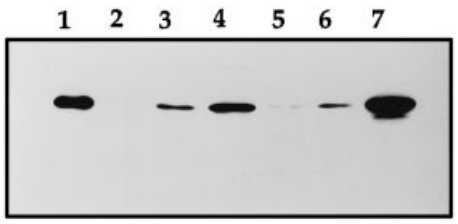

Figure 2 Identification of HeLa cell lines with stable overexpression of GCS_l

HeLa cells were seeded at a density of 10^6 cells/10-cm plate and transfected with 10 μg of pSTLN1, as described in the Experimental section. Following selection with G418, resistant colonies were propagated for analysis of GCS_l expression. Protein (20 μg) from HeLa cell sub-line extracts was analysed by Western blotting using an antibody raised against a synthetic peptide from GCS_l. Gels were loaded as follows: lane 2, extract from HeLa cells transfected with control vector; lane 3, cell extract from HeLa sub-line, LN53; lane 4, cell extract from HeLa sub-line LN54; lane 5, cell extract from HeLa sub-line LN68; lane 6, cell extract from HeLa sub-line LN72; lane 7, cell extract from HeLa sub-line, LN73. Lane 1 contains 10 μg of protein from rat kidney cytosol as a marker.

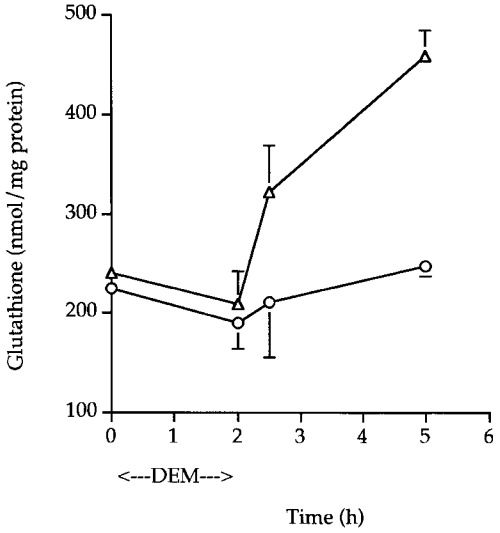

Figure 3 Glutathione concentrations in HeLa cells that overexpress GCS_l following treatment with DEM

Cells overexpressing GCS_l (LN73, $\triangle$) or the control cell line HL9 ($\bigcirc$) were seeded at 10^6 cells/10-cm plate 24 h before treatment. DEM (150 μM) was added to the cells in fresh media and incubated for 2 h at 37 °C. DEM-containing media was replaced with fresh media, and the cells were allowed to grow for a further 3 h. Samples were taken for analysis of glutathione content at $t = 0$, 2 h, 2.5 h and 5 h.

921.8 $\pm$ 4.1 pmol/min per mg in control cells (Table 2). In order to establish whether this increase in GCS activity is associated with an increase in glutathione levels, intracellular glutathione concentrations were determined 48 h after transfection of COS-1 cells with pSTLN1 or pSV-β-Galactosidase. Glutathione concentrations were found to be increased from 126.9 $\pm$ 4.2 nmol/mg protein to 178.8 $\pm$ 19.1 nmol/mg protein, a small but statistically significant ($P = 0.008$) increase of approx. 30 % (Table 2). The possibility that GCS_h had been up-regulated in these experiments, and had thus contributed to the increase in GCS activity and glutathione concentrations, was examined by Western blotting of COS-1 cell extracts from the transfected cells. Antibodies raised against a synthetic peptide corresponding to a specific region of GCS_h were used for Western blot analysis [20], and no increase in the GCS_h polypeptide was observed (results not shown).

Effect of stable integration of the cDNA encoding GCS_l into the genome of HeLa cells on GCS activity

During transient transfections of COS-1 cells, only a proportion of the cells (approx. 20–30 %) take up and express the cDNA. To examine the effect of GCS_l on GCS activity, glutathione homoeostasis and resistance to chemotherapeutic drugs, it was necessary to create cell lines that stably express the GCS_l cDNA at high levels. HeLa cells were transfected with pSTLN1, and following selection with G418, approx. 100 resistant colonies were isolated, propagated and analysed for GCS_l expression by Western blotting. Figure 2 shows the levels of the GCS_l polypeptide in five of the stably transfected HeLa cell lines. Of these cell lines, LN73 contains approx. 20-fold higher levels of GCS_l than the control cell line, HL9, and was selected for further analysis. Northern blotting showed that the increased levels of GCS_l protein in LN73 cells are accompanied by a corresponding increase in GCS_l mRNA levels (results not shown). GCS activity was measured in both LN73 and HL9 cell lines, and was found to be approx. 2-fold higher in LN73 cells (437.3 $\pm$ 85.2 pmol/min per mg) than HL9 cells (213.4 $\pm$ 71.8 pmol/min/mg) (Table 3). The K_m for glutamate was found to be 1.1 mM in LN73 cells and 2.6 mM in HL9 cells, showing a small increase in the affinity for glutamate in the HeLa cells transfected with GCS_l. LN73 cells do not, however, have increased glutathione levels compared with the HL9 control cell line.

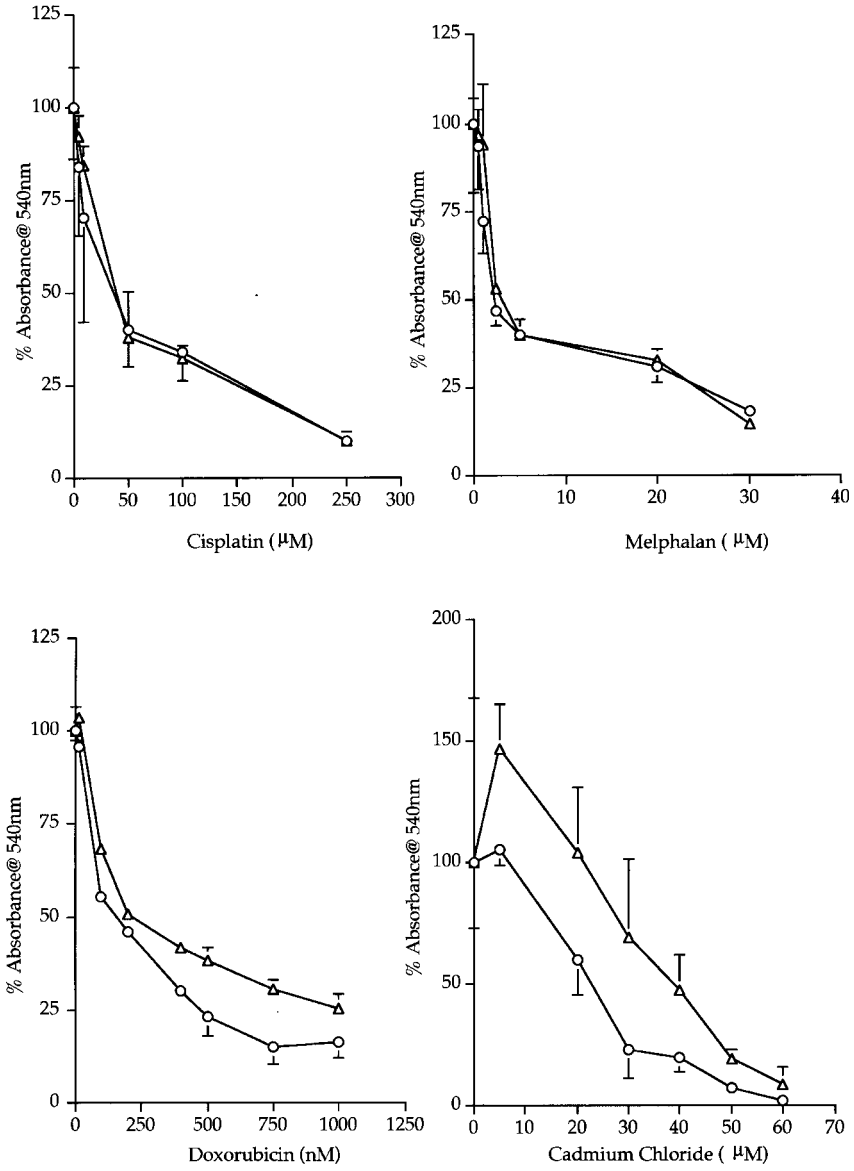

Figure 4 Cytotoxicity of cisplatin, melphalan, doxorubicin or cadmium chloride towards LN73 and HL9 cells

HeLa cells which overexpress GCS_l (LN73, △) or control HeLa cells (HL9, ○) were treated with cisplatin, melphalan, doxorubicin or cadmium chloride and subjected to MTT assay, as described in the Experimental section. A_{540} was determined, and values are expressed as a percentage of the absorbance obtained from cells treated with the drug solvent alone. Experiments were performed in quadruplicate and repeated on three separate occasions, with similar results being obtained on each occasion. The results shown are average values from a representative experiment.

The effect of DEM on glutathione levels in HL9 and LN73 cell lines

Whereas LN73 cells do not contain higher glutathione concentrations than HL9 cells, it is probable that they have an enhanced ability to synthesize glutathione, because of their increase in GCS activity. To test this hypothesis, LN73 or HL9 cells were treated with the glutathione-depleting agent DEM, and glutathione concentrations were determined at different time points after treatment. Cells were treated with 150 μM DEM in fresh media for 2 h, after which time the media was replaced with drug-free media. Glutathione concentrations were determined before treatment (at zero time), after 2 h (when the DEM was removed), 2.5 h and 5 h. Figure 3 shows that after 2 h, glutathione levels are decreased by approx. 15% in both cell lines. After replacement of the media containing DEM with fresh media, there is an overshoot in glutathione levels in the LN73 cells to approx. 2-fold higher than the starting concentration 5 h after DEM treatment. In contrast, 5 h after treatment HL9 cells recover glutathione concentrations to levels similar to the starting concentration.

Drug resistance of HeLa cells that overexpress GCS_l

Tumour resistance to several chemotherapeutic drugs is often associated with increased glutathione levels and glutathione metabolism [1–6]. As LN73 cells show an enhanced ability to synthesize glutathione, it was important to examine whether this cell line would show increased resistance to challenge with

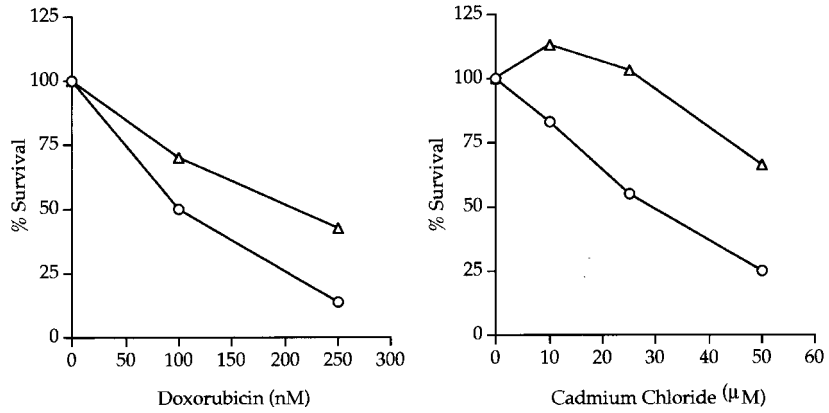

Figure 5 Clonogenic survival of LN73 cells following treatment with doxorubicin or cadmium chloride

HeLa cells which overexpress GCS_l (LN73, △) or control HeLa cells (HL9, ○) were treated with doxorubicin or cadmium chloride and cytotoxicity determined by clonogenic survival assay, as described in the text. Values are expressed as a percentage of colonies obtained from cells treated with the drug solvent alone. Experiments were performed in quadruplicate on two separate occasions, and similar results were obtained on each occasion. The results shown are average values from a representative experiment. Kruskal–Wallis non-parametric analysis-of-variance tests were performed to establish that there was a statistically significant difference in the response of HL9 and LN73 to doxorubicin ($P < 0.005$) and cadmium chloride ($P < 0.0005$).

certain chemotherapeutic drugs. The cytotoxicity of the anti-cancer drugs melphalan, cisplatin or doxorubicin was examined. In addition, the toxicity of the carcinogenic metal compound, cadmium chloride, was investigated, since glutathione has been proposed to have a direct role in its detoxification [34,35]. Cytotoxicity was examined by MTT assay, and Figure 4 shows that there was neither a difference in the toxicity of melphalan towards LN73 or HL9 cells, nor any difference in toxicity of cisplatin between the two cell lines. In contrast, HL9 cells were found to be more sensitive than LN73 to either doxorubicin or cadmium chloride. Treatment with cadmium chloride resulted in the greatest difference in cytotoxicity between the two cell lines. Cadmium chloride also appeared to stimulate growth of LN73 cells at the lowest concentration (5 μM) used. The reason for this is unknown, but comparison of the growth rates of untreated LN73 and HL9 cells revealed no significant differences between the two cell lines (results not shown).

The results from the MTT assays showing increased resistance of LN73 cells to doxorubicin and cadmium chloride were supported by clonogenic survival assays, which were performed with both HL9 and LN73 cell lines using 100 or 250 nM doxorubicin, or 10, 25 or 50 μM cadmium chloride. The cells were treated with doxorubicin for 1 h or cadmium chloride for 4.5 h, and reseeded at 2×10^4 cells/10-cm plate. Figure 5 shows that LN73 cells are more resistant than HL9 cells to doxorubicin [approx. 2-fold; IC_{25} (drug concentration that inhibits 25% of the colony formation) = 50 nM doxorubicin for HL9 cells and 90 nM doxorubicin for LN73 cells] and cadmium chloride (approx. 3-fold; IC_{25} = 14 μM cadmium chloride for HL9 cells and 44 μM cadmium chloride for LN73 cells), indicating that an enhanced capacity to synthesize glutathione might be sufficient to confer resistance to doxorubicin or cadmium chloride.

DISCUSSION

Enhanced detoxification of anti-cancer drugs by conjugation with glutathione is likely to make a significant contribution to the mechanisms by which cancer cells are resistant to chemotherapy. Several drug-resistant tumour-cell lines have been shown previously to contain higher levels of glutathione-metabolizing enzymes than the parental drug-sensitive cell lines [1,5,6,17–19]. Furthermore, treatment of cells with an inhibitor of GCS, buthionine sulphoximine (BSO), to deplete intracellular glutathione has been shown to potentiate cell killing by certain anti-cancer drugs and radiation [36–39]. However, to date there has been little direct evidence that increased glutathione turnover alone is sufficient to generate resistance to anti-cancer drugs in human cancer cells. The present study demonstrates that an increased ability to synthesize glutathione by overexpressing GCS_l in HeLa cells is sufficient to confer resistance to doxorubicin or cadmium chloride, but not cisplatin or melphalan.

Mulcahy et al. [33] demonstrated that transient expression of the GCS subunits in COS-7 cells resulted in increased glutathione concentrations and resistance to melphalan. Their experiments showed that transfection of GCS_l into COS cells caused a marginal (although not shown to be statistically significant) increase in glutathione levels. Transient transfection of GCS_h, or GCS_h and GCS_l together, stimulated significant increases in glutathione concentrations of 1.6- and 2.3-fold respectively. The increase in glutathione concentration was correlated with resistance to melphalan. In the present study, we were unable to demonstrate any difference in melphalan toxicity between the control cell line, HL9, and LN73 cells, which overexpresses GCS_l and have increased GCS activity. Nevertheless, we have clearly demonstrated that the LN73 cells have an increased capacity to synthesize glutathione, which is associated with resistance to doxorubicin or cadmium chloride, despite the absolute glutathione concentrations in LN73 cells being similar to the control cell line.

It is unclear why increased GCS activity in transiently transfected COS cells confers melphalan resistance, while increased GCS activity in stably transfected HeLa cells generates resistance to doxorubicin and cadmium chloride. Each of the four compounds examined in the present study has the potential to be detoxified by glutathione-dependent mechanisms, but it seems likely that the relative importance of glutathione-dependent detoxification will vary among different cell types. Furthermore, expression of GST isoenzymes will be different in HeLa and COS cells, since the former is derived from a human cervix carcinoma and the latter is a simian-virus-40-transformed monkey kidney cell line. Expression of GST isoforms appears to be of probable importance in determining the sensitivity of cells to anti-cancer

drugs [40]. In particular, evidence exists to suggest that Alpha class GST can confer resistance to alkylating agents, such as melphalan [2,9–11]. The human Alpha class GST A1-1 has been shown to be present at very low levels in HeLa cells, relative to Mu and Pi class GSTs [41].

The LN73 HeLa cell line has enhanced resistance to doxorubicin relative to the control cell line, HL9. Doxorubicin is one of the most extensively used anti-cancer drugs, but the mechanisms for its cytotoxicity remain unclear. Anthracyclines, such as doxorubicin, can damage DNA by intercalation, inhibition of topoisomerase II activity and alteration of the fluidity of the cell membrane [42]. Generation of free radicals by redox cycling of the quinone moiety also seems likely to contribute to toxicity. Although no single factor has been associated exclusively with doxorubicin resistance, evidence suggests that the drug-efflux pump, P-glycoprotein, and glutathione-associated mechanisms might each have a role in conferring resistance [36,37]. It has been shown previously that the doxorubicin resistance of a multidrug-resistant human breast carcinoma cell can be partially reversed by treatment with the p-glycoprotein inhibitor, verapamil, or the glutathione-depleting agent, BSO, and completely reversed by treatment with both agents [36]. Increased glutathione peroxidase activity has been observed in different doxorubicin-resistant cell lines [36,43]. Pi class GST has also been implicated in conferring low levels of resistance to doxorubicin [8], and it has been shown previously [44] that anthracyclines can form glutathione conjugates *in vitro*. Furthermore, the co-ordinated overexpression of genes encoding multidrug-resistance-associated protein and GCS have been shown to be correlated with doxorubicin resistance in human malignant mesothelioma cell lines [45]. Collectively, these data suggest that glutathione metabolism might have a role in protecting against doxorubicin toxicity, although its relative importance in different cells is unknown. It seems likely that glutathione exerts its protective effects at the level of scavenging free radicals, detoxifying peroxides or conjugating with products of lipid peroxidation.

The data presented here show that an increased ability to synthesize glutathione can provide a modest protection against doxorubicin toxicity, thus supporting the circumstantial evidence from previous studies that glutathione might be an important determinant of sensitivity to anthracyclines. It is notable, however, that the extent of protection against doxorubicin or cadmium toxicity is not greater than 3-fold. Previous studies have shown that overexpression of different GSTs in cell lines is also associated with relatively low levels of resistance to anti-cancer drugs [1,7–11]. However, owing to the complex nature of glutathione metabolism, it seems likely that more than one enzyme involved in glutathione-associated detoxification might be required to make a major impact on drug resistance. For example, an increase in GST or glutathione peroxidase expression might also require an enhanced supply of glutathione to confer substantial drug resistance.

In the present study, resistance to doxorubicin in LN73 cells is not as marked as that seen for cadmium chloride. Glutathione has been shown previously [35] to be the first line of defence against cadmium toxicity, and glutathione depletion has a pronounced effect on potentiating the toxicity of cadmium chloride in rat L6 myoblasts. Glutathione appears to be capable of protecting against lipid peroxidation generated by cadmium, as well as being able to bind cadmium directly [34,46].

Overexpression of the GCS_l subunit in HeLa cells does not appear to confer resistance to cisplatin. There are several reports in the literature to suggest that increased GCS activity is associated with resistance to platinum compounds. For example, it has been shown that cisplatin-resistant ovarian and bladder cancer cell lines have increased levels of the GCS_h mRNA [6,47]. Also, when the GCS_h subunit was overexpressed in a human small cell-lung-cancer cell line, the cells became resistant to cisplatin [48]. The involvement of glutathione metabolism in cisplatin resistance might, however, be dependent on the cell type. Two independent reports suggest glutathione might not represent an important mechanism for resistance to platinum compounds in HeLa cells: a cisplatin-resistant HeLa cell line was shown to have no increase in glutathione or GST levels compared with the parental cell line [49], and depletion of glutathione in a HeLa cell line made resistant to cisplatin by irradiation with γ-rays had no effect on resistance [50].

An important observation in the present study is that whereas LN73 cells have increased GCS activity and an increased ability to resynthesize glutathione after its depletion, the absolute or apparent steady-state levels of glutathione are the same as in the control cell line. This is in contrast with the transient transfections, where a 1.4-fold increase in glutathione concentrations was observed (Table 2). Although the reasons for this are unknown, it is possible that the transfection procedure itself introduces a transient stress that stimulates GCS activity; those cells containing increased levels of the GCS_l subunit will have the capacity to synthesize more glutathione than the pSV-β-Galactosidase-transfected cells. Glutathione homoeostasis is probably tightly regulated, and the data obtained in the present study support the hypothesis that the capacity to synthesize glutathione might have equal or more importance than the apparent steady-state level of glutathione in resistance to stress [51].

The enhanced ability to resynthesize glutathione following DEM treatment in LN73 cells was evident by an overshoot in glutathione concentrations by 2-fold over the starting levels 5 h after treatment. This overproduction of glutathione following its depletion was substantially less marked in HL9 cells, but it is of interest that previous studies have demonstrated increased glutathione levels following its depletion both *in vivo*, following cyclophosphamide treatment of mice [52], and in cell lines [53]. In menadione-resistant Chinese hamster ovary cells an increase in glutathione levels to approx. 2-fold higher than the starting level occurs 24 h after depletion by menadione treatment [53]. The control cell line showed a more marked glutathione depletion than the menadione-resistant cell line, and its glutathione levels did not exceed the starting concentrations during recovery.

The mechanism for the DEM-stimulated overproduction of glutathione is unknown, but it is likely that this could be on the basis of activation of GCS activity, which results in a transient increase in steady-state glutathione levels. The results obtained in the present study suggest that enhanced glutathione production following its depletion is on the basis of increased synthesis *de novo* mediated by GCS, and provide evidence that GCS_l probably has a critical role in regulating this adaptive response. It has been proposed that GCS_l can regulate the activity of GCS_h by reversible disulphide-bond formation between the two subunits, which is sensitive to the intracellular redox state [15]. The disulphide bridge is proposed to induce an enzyme conformational change, such that GCS has an increased affinity for glutamate and is therefore more active. Reduction of the disulphide bond results in a 2-fold increase in the K_m for glutamate and causes GCS to be subject to competitive inhibition by glutathione.

Evidence also exists to suggest the existence of hydrophobic interactions between the GCS_h and GCS_l subunits, although the extent of these associations *in vivo* is not clear [15]. The hydrophobic interactions also appear to be of importance in modulating enzyme activity, because the reduced holoenzyme has a substantially lower K_m for glutamate (2.8 mM) than the

recombinant or isolated GCS_h subunit (18.2 mM) [15]. In the present study, we have shown that addition of recombinant GCS_l into HeLa cell extracts results in an increase in GCS activity *in vitro*. Overexpression of GCS_l in COS-1 or HeLa cells also results in increased GCS activity, supporting the hypothesis that GCS_l is important in regulating GCS activity. The results suggest that either the GCS_h and GCS_l subunits do not exist in these cell lines in equimolar amounts, or that the nature of their association is such that an increase in the quantity of one subunit influences the dynamics of the on–off rate of subunit interaction, so that the probability of the enzyme existing as a heterodimer is higher. Both mechanisms might be pertinent. The addition of 1 μg of GCS_l to 100 μg of protein from HeLa cell extracts results in an increase in GCS activity of 1.6-fold. An addition of either 5 or 10 μg of GCS_l results in a 2.4- or 2.5-fold increase in GCS activity respectively, suggesting that the availability of GCS_h is both limiting and saturable. However, it is notable that relatively large amounts of GCS_l are required to increase GCS activity, as the added GCS_l will account for approx. 1 %, 5 % or 10 % of the total protein in the HeLa cell extracts. Rat kidney cytosol contains substantially higher levels of each of the GCS subunits than HeLa cells (between 20 and 30-fold; results not shown), and the purified GCS heterodimer appears to comprise less than 1 % of the total protein in rat kidney cytosol [54]. Thus it is likely that, in our *in vitro* measurements of GCS activity in HeLa cell extracts, the added GCS_l is present in considerable excess to GCS_h on a molar basis, even at the lowest concentration of GCS_l used.

Little is known about the expression of GCS_l in human cancer or drug-resistant cancer cell lines, since the majority of studies have focused on regulation of the GCS_h subunit. It has, however, been shown that a cisplatin-resistant ovarian cancer cell line, C200, contains elevated levels of both the GCS_h and GCS_l polypeptides [6]. The present study shows that GCS_l has the potential to impact on drug resistance and glutathione homoeostasis, but the implications of this on human cancer and drug resistance *in vivo* are, as yet, unknown.

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