Glyoxalase I in detoxification: studies using a glyoxalase I transfectant cell line

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The glyoxalase system (glyoxalase I, glyoxalase II and GSH as cofactor) is involved in the detoxification of methylglyoxal (a by-product of the glycolytic pathway) and other α-oxoaldehydes. We have transfected a 622 bp cDNA encoding human glyoxalase I into murine NIH3T3 cells. The recipient cells were shown to express elevated transcript and protein levels and a 10-fold increase in glyoxalase I enzyme activity. This was accompanied by an increased tolerance for exogenous methylglyoxal and enhanced resistance to the cytotoxic effects of two glyoxalase I inhibitors (*s*-*p*-bromobenzylglutathione diethyl ester and *s*-*p*-bromobenzylglutathione dicyclopentyl ester), a glutathione analogue [γ-glutamyl-(*S*)-(benzyl)cysteinyl-(*R*)-(−)-phenylglycine diethyl ester] and the anti-cancer drugs mitomycin C and adriamycin. Steady-state levels of GSH were significantly lower in the transfected cells, perhaps reflecting increased flux as a consequence of elevated glyoxalase activity. This decrease did not alter the sensitivity to the alkylating agent chlorambucil. Although transfection did not affect the growth or doubling time of the NIH3T3 cells, analysis of glyoxalase I activity showed a consistent increase in tumour tissue when compared with pair-matched controls. Thus increased glyoxalase I is associated with the malignant phenotype and may also contribute to protection against the cytotoxicity of certain anti-cancer drugs.

INTRODUCTION

The glyoxalase system is involved in the cellular metabolism of methylglyoxal and other α-oxoaldehydes [1]. Even though the precise biological significance of the glyoxalase system is not certain, the ubiquitous distribution of these enzymes suggests an important cellular function. Glyoxalase I catalyses the formation of *S*-D-lactoylglutathione from methylglyoxal and GSH [2]. Glyoxalase II in turn metabolizes *S*-D-lactoylglutathione to D-lactic acid and regenerates GSH. Methylglyoxal arises as a product of glycolysis after conversion of glyceraldehyde 3-phosphate into dihydroxyacetone phosphate. In prokaryotes, methylglyoxal synthetase catalyses the synthesis of methylglyoxal from dihydroxyacetone phosphate [3]. Glyoxalase I from bacteria [4] has 51% nucleotide homology with the cDNA of the human enzyme [5]. Thus this pathway appears to be evolutionarily well conserved, again indicating its biological importance.

Abnormal expression of the glyoxalase system has been demonstrated in a number of cellular disorders, including diabetes mellitus and cancer. Increased concentrations of methylglyoxal, *S*-D-lactoylglutathione and D-lactate were found in the blood samples of insulin-dependent and non-insulin-dependent diabetic patients when compared with non-diabetic individuals [6,7]. Studies by Ayoub et al. [8] showed increased expression of glyoxalase I and decreased glyoxalase II in several tumour cell lines of urological origin when compared with non-malignant cells. Chemically induced differentiation of HL60 cells resulted in a decrease in glyoxalase I and corresponding increase in glyoxalase II activities [9]. Previous studies from our laboratory have shown enhanced levels of glyoxalase I protein in human colon carcinomas when compared with corresponding normal tissue [10]. In an attempt to understand the significance of this overexpression, we cloned the human glyoxalase I cDNA and determined its sequence [5]. In the present study, we transfected the glyoxalase I cDNA into NIH3T3 cells and developed stable cell lines overexpressing the enzyme. Using one of these clonal cell lines, we investigated the effect of glyoxalase I overexpression on aspects of drug metabolism. Results indicate a possible role for glyoxalase I in drug detoxification and GSH homoeostasis.

MATERIALS AND METHODS

Development of glyoxalase I transfectants

Glyoxalase I cDNA containing the entire coding region [5] was subcloned into cytomegalovirus vector in both sense and anti-sense orientations. Reverse orientation (glyoxalase I rev.) served as a transfection control. NIH3T3 cells were transfected with 10–20 μg of DNA by the calcium phosphate method [11]. Several clones were selected in the presence of G418 and analysed. For the following experiments, one clone in each orientation was selected and used.

Preparation of cytosolic fractions and enzyme assays

Transfectant and control cell pellets were sonicated for 30 s on ice in 10 mM Tris/HCl, pH 7.8, containing protease inhibitors. Cytosolic fractions were isolated by sequential centrifugation at 10000 *g* for 20 min and 100000 *g* for 1 h. Proteins from normal and tumour biopsy specimens were isolated similarly. Protein concentrations were estimated by the method of Bradford [12].

Assay of glyoxalase I

Cytosolic fractions were used to measure the glyoxalase I enzyme activity by the method of Oray and Norton [13], as described previously [10].

Assay of GSH reductase

This was carried out by monitoring the oxidization of NADPH at 340 nm [14] using cytosolic extracts isolated as described above.

Abbreviations used: Ter.199, γ-glutamyl-(*S*)-(benzyl)cysteinyl-(*R*)-(−)-phenylglycine diethyl ester; Glyoxalase I rev., reverse orientation.

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Measurement of GSH levels

Proteins were precipitated with a final concentration of 3% sulphosalicylic acid. Intracellular GSH levels were determined by the method of Griffith [15].

Western-blot analysis

Cytosolic protein from transfectant cell lines and NIH3T3 cells (100 μg) was separated on SDS/12% polyacrylamide gels and transferred to poly(vinylidene difluoride) membrane. Membranes were immunostained with polyclonal glyoxalase I antibody [10].

Northern-blot analysis

RNA from NIH3T3 cells and glyoxalase I transfectants was isolated, electrophoresed and transferred to a nylon membrane. Glyoxalase I cDNA was labelled with [^{32}P]dCTP, hybridized with the membrane, washed and exposed to film as described [5]. Similarly, RNA samples from cells were analysed by using a labelled γ-glutamylcysteinyl synthetase probe [16].

Cytotoxicity assay

NIH3T3 and transfectant cells were plated into 96-well plates at 2000–5000 cells/well. These were treated with various concentrations of methylglyoxal, adriamycin, mitomycin C, chlorambucil (Sigma Chemical Co., St. Louis, MO, U.S.A.), glutathione analogue γ-glutamyl-(*S*)-(benzyl)cysteinyl-(*R*)-(−)-phenylglycine diethyl ester (Ter.199) and two glyoxalase I inhibitors, *s*-*p*-bromobenzylglutathione diethyl ester (BrBzGSHEt$_2$) and *s*-*p*-bromobenzylglutathione dicyclopentyl ester (BrBzGSH diCp) [17]. The last two compounds were gifts from Dr. P. J. Thornalley, University of Essex, Colchester, Essex, U.K. The GSH analogue was kindly provided by Terrapin Technologies, San Francisco, CA, U.S.A. [18]. Cytotoxicities were determined by staining with Sulphorhodamine B and monitoring the A_{560}, as described by Skehan et al. [19].

RESULTS

Figure 1 shows the overexpression of glyoxalase I in transfectants both by Northern- (Figure 1a) and Western- (Figure 1b) blot analysis. There was also an approximately 10-fold increase in glyoxalase I enzyme activity over that in control cell lines (Table 1). Neither the cDNA probe nor the antibodies for human glyoxalase I gave a detectable signal with the NIH3T3 murine glyoxalase I message or protein. This may reflect the lack of cross-species homology or the low levels of the intrinsic murine glyoxalase I. The latter explanation is supported by the relatively low specific activity (69 nmol/min per mg; Table 1) in both the wild-type and reverse-orientation transfectant cells.

When the cell lines were treated with methylglyoxal, a substrate for glyoxalase I, the transfectant cell line exhibited an approximately 2-fold resistance to the cytotoxic effects of methylglyoxal when compared with the control cell lines (IC_{50} 0.4 mM compared with 0.2 mM; Figure 2). The cytotoxicity profiles of a number of glutathione-based derivatives are shown in Figure 3. Figures 3(a) and 3(b) show that the transfectants maintain an approximately 2-fold resistance against glyoxalase inhibitors [17] and Figure 3(c) shows a similarly enhanced resistance to Ter.199, a previously characterized inhibitor of glutathione S-transferases [18]. As each of these three inhibitors was designed to allow passage across cell membranes and interfere

Table 1 Cellular properties of glyoxalase-I-transfected cells

Cytosolic fractions were isolated and enzyme assays were performed as described in the Materials and methods section. Results are means ± S.D. for three to five experiments.

Cell line	Cell doubling time (h)	Glyoxalase I activity (nmol/min per mg)	GSH concn. (nmol/mg of protein)	GSH reductase activity (nmol/min per mg)
NIH3T3	17 ± 1.4	69 ± 1.7 (1)	3.86 ± 0.84	28 ± 2.5
Glyoxalase I rev.	20.2 ± 0.71	69 ± 9.2 (1)	4.45 ± 0.89	54 ± 2.0
Glyoxalase I	23.7 ± 4.2	632 ± 25.6 (9.2)*	2.05 ± 1.4	62 ± 6.8

* Significantly different from control (NIH3T3 cell) group.

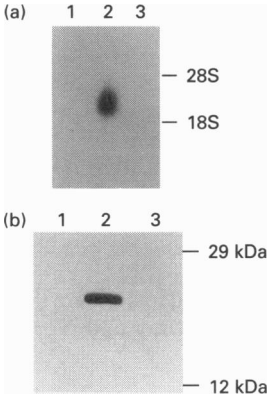

Figure 1 Analysis of (a) RNA and (b) protein from glyoxalase-I-transfected and control NIH3T3 cells

(**a**) Total RNA was isolated, electrophoresed, transferred to a membrane and probed with glyoxalase I cDNA as described in the Materials and methods section. Lane 1, NIH3T3 cells; lane 2, glyoxalase I; lane 3, glyoxalase I rev. (**b**) Cytosolic proteins were isolated, electrophoresed, transferred to a membrane and immunostained with glyoxalase I antibodies (1:500) as described in the Materials and methods section. Lanes are as in (**a**).

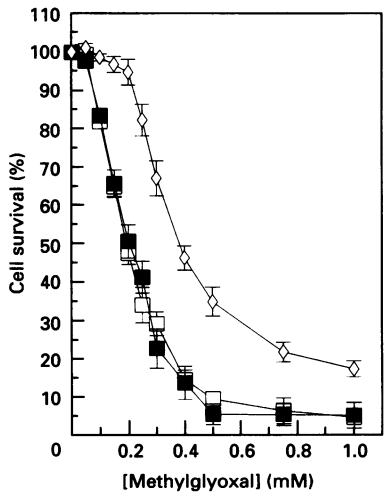

Figure 2 Methylglyoxal tolerance of glyoxalase-I-transfected NIH3T3 cells

Cells were treated with various concentrations of methylglyoxal, and cytotoxicity was determined as described in the Materials and methods section. □, NIH3T3; ■, glyoxalase I rev.; ◇, glyoxalase I transfectant. Results are means ± S.D. for 3 experiments.

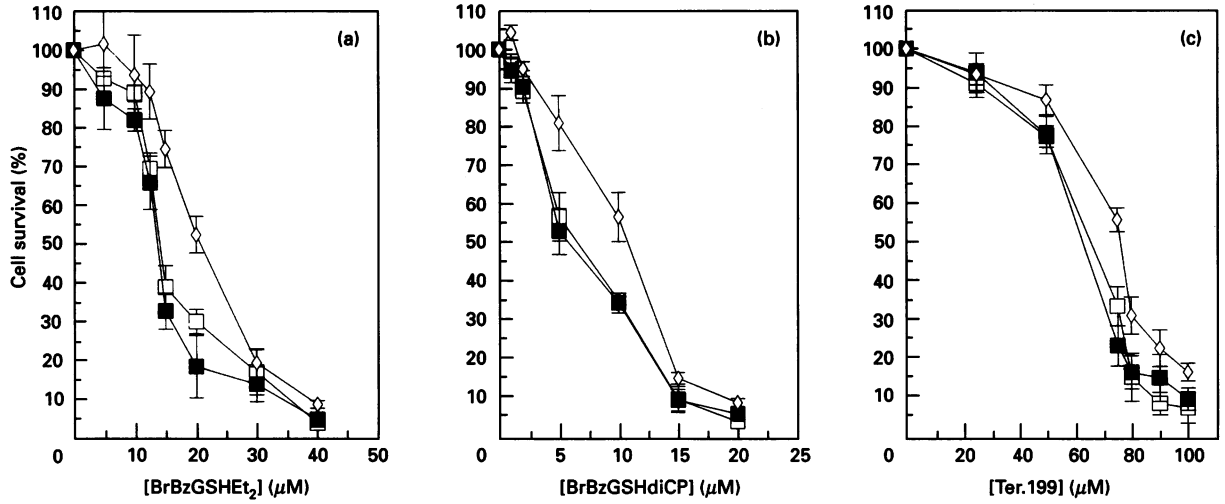

Figure 3 Effect of overexpression of glyoxalase I on the cytotoxicity of glyoxalase I inhibitors

□, NIH3T3; ■, glyoxalase I rev.; ◇, glyoxalase I transfectant. (**a**) $BrBzGSHEt_2$; (**b**) BrBzGSH diCp; (**c**) Ter.199. Results are means ± S.D. for 3 experiments.

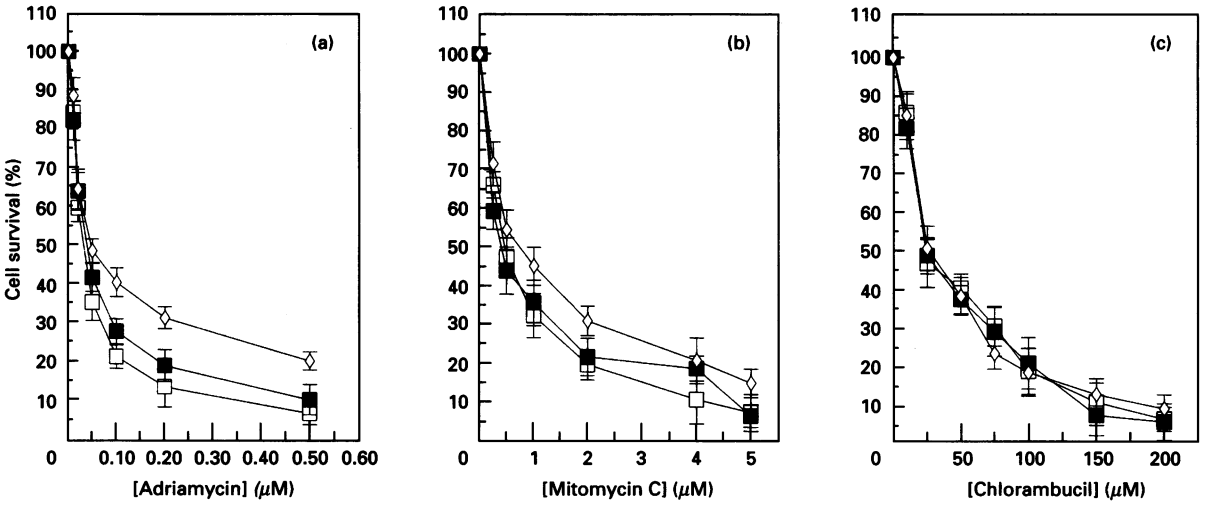

Figure 4 Effect of overexpression of glyoxalase I on the cytotoxicity of anti-tumour drugs

Cells were dosed and the cytotoxicity was determined as described in the Materials and methods section. □, NIH3T3; ■, glyoxalase I rev.; ◇, glyoxalase I transfectant. (**a**) Adriamycin; (**b**) mitomycin C; (**c**) chlorambucil. Results are means ± S.D. for 3 experiments.

with the GSH-binding site of target enzymes, it appears logical that the increased target/drug ratios of the transfectants would confer such a degree of resistance.

s-p-Bromobenzylglutathione is the non-esterified form of these inhibitors, therefore it does not readily traverse cell membranes. Accordingly, this analogue was non-toxic in both wild-type and transfected cell lines (results not shown).

In order to see the effect of increased glyoxalase I levels on the cytotoxicity of anti-tumour drugs, cells were treated with adriamycin, mitomycin C or chlorambucil (Figure 4). The results show that the glyoxalase I transfectant cells are resistant to adriamycin and slightly resistant to mitomycin C only at higher concentrations. No difference was observed among the cell lines treated with chlorambucil.

Because glyoxalase I is an important enzyme in the salvage pathways of GSH homoeostasis, intracellular levels of this compound were measured (Table 1). GSH concentrations were lower in the transfected line. This was not caused by the transfection procedure, as the reverse-oriented cDNA control had GSH levels similar to those of the NIH3T3 recipient cells. γ-Glutamylcysteine synthetase, the rate-limiting step in *de novo* GSH biosynthesis, was unaffected by glyoxalase I transfection when checked by Northern-blot analysis (results not shown). However, glutathione reductase activity was increased in both the forward and reverse-oriented transfectants, suggesting a non-specific effect. Glyoxalase II enzyme levels were undetectable, even with 200 μg of cytosolic protein from these cell lines.

In order to compare glyoxalase I activities in the transfectant

Table 2 Glyoxalase I activity of various normal and tumour samples

Cytosolic fractions were prepared as described in the Materials and methods section. Cytosolic protein from tissue samples (100 μg) was used to measure the enzyme activity, with methylglyoxal as substrate. Activities are the means ± S.D. of three to five individuals.

Sample	Glyoxalase I activity (μmol/min per mg)
Breast	
Normal	0.32 ± 0.11
Tumour	0.75 ± 0.15
Colon	
Normal	0.47 ± 0.07
Tumour	1.52 ± 0.08
Kidney	
Normal	0.50 ± 0.18
Tumour	0.70 ± 0.18
Lung	
Normal	0.32 ± 0.09
Tumour	0.44 ± 0.13
Prostate	
Normal	1.88 ± 0.14
Tumour	2.46 ± 0.19

cells with those in human tissues, enzyme activities were assessed in the cytosol of a number of human samples (Table 2). Glyoxalase I activities ranged from 0.32 to 2.46 μmol/min per mg, with the highest levels in prostate and colon. In each tissue, pair-matched tumour samples consistently showed an elevated enzyme activity compared with normal.

DISCUSSION

Abnormalities in the glyoxalase system have been linked with a number of human disease states including cancer, diabetes, malaria and muscular dystrophy [1,6,20,21]. Because methylglyoxal is a by-product of the glycolytic pathway in both prokaryotic and eukaryotic cells, the glyoxalase system is presumably critical in the detoxification of this endogenous metabolite. Free methylglyoxal is toxic to cells by a number of mechanisms which involve covalent modification of proteins and nucleic acids [22]. However, the full extent of the biological importance of this enzyme system may yet to be realized. For example, the high glyoxalase I levels in proliferative tissues such as embryonic cells [23], regenerating liver [24] and tumours of various origins (Table 2 and ref. [5]) indicate the co-ordinated up-regulation of the enzyme in cells with high glycolytic activity. Although transfection of glyoxalase I enhanced the capacity of the cell line to withstand exogenous methylglyoxal challenge, there was no indication of an increased growth rate as evidenced by altered doubling times. Similarly, the transfected cells did not display an altered capacity to grow in media containing different amounts of carbohydrate energy sources (results not shown).

At present, the importance of potentially limiting levels of glyoxalase II in converting *S*-D-lactoyl glutathione into D-lactate is not known. Conversion of methylglyoxal into *S*-D-lactoylglutathione is the essential detoxification step. Glyoxalase II serves primarily to regenerate GSH with the ultimate release of D-lactate. This factor may explain the significantly lower levels of intracellular GSH in the transfectants, as a higher proportion may be in the *S*-D-lactoyl conjugated form. NIH3T3 cells have a glyoxalase II activity below the level of detectability. This may predict a lower rate of conversion of *S*-D-lactoylglutathione into D-lactate. Both γ-glutamylcysteine synthetase and glutathione reductase also contribute significantly to GSH homoeostasis. However, there was no indication that altered expression of these enzymes could account for the observed differences. It is also relevant to note the unaltered sensitivity of the transfectant cells to the alkylating agent chlorambucil. Sensitivity to this class of agent is frequently influenced by depletion of intracellular GSH. Presumably, this reflects the fact that GSH has not been reduced to limiting levels for drugs exposure. Conversely, electrophilic anti-cancer drugs, such as chlorambucil, could be influenced to some extent by the presence of additional nucleophilic target sites provided by the transfected DNA. Once again, this did not appear to be the case, as the sensitivity of the wild-type, transfected and reverse-orientation transfected lines were the same.

Enhanced resistance was found with esterified GSH analogues and with adriamycin and mitomycin C. The cellular metabolism of both anti-cancer drugs is complex, but quinone electrophiles are generally considered to be important to the cytotoxic process. Although there is no obvious way that either drug can form an α-oxoaldehyde intermediate, the possibility that the parent drugs or their metabolites may be a substrate for glyoxalase I cannot be ruled out. Resistance is primarily expressed at higher drug concentrations for the anti-cancer drugs, suggesting that this may not be a critical clinical factor in determining sensitivity. For the GSH analogue, resistance appears to occur at all concentrations tested. The discrepancy between the 10-fold increase in glyoxalase I activity and 2-fold resistance may be explained by pharmacokinetic or pharmacodynamic aspects of the inhibitors. In addition, although these analogues were designed to be inhibitors of glyoxalase and glutathione S-transferases [17,18], it is possible that other enzymes that use GSH as a cofactor may be affected. This may serve to disrupt the stoicheiometry of the enzyme–inhibitor and alter any quantitative cytotoxic relationship.

In confirmation of earlier reports [5,10], tumour tissues expressed higher glyoxalase I activity than normal tissue from the same patient. These enzyme activities were roughly equivalent to the specific activity of the transfected cell line, but approximately 10-fold higher than the wild-type NIH3T3 cells. In general, fresh human tissues express higher levels of glyoxalase I than established cell lines. However, the ubiquitous nature of the enzyme is demonstrated by the fact that each of the 60 tumour cell lines that constitute the National Cancer Institute tumour cell line screening panel (brain, breast, colon, leukaemia, lung, melanoma, ovarian, prostate and renal) express significant levels of glyoxalase I transcript (S. Ranganathan, E. S. Walsh and K. D. Tew, unpublished work).

In conclusion, a stably expressing glyoxalase I transfectant cell line has been established. The enhanced enzyme levels contribute either directly or indirectly to the expression of resistance to GSH analogues and some anti-cancer drugs. Alterations in GSH pools are apparent in the transfectants, possibly the consequence of an enhanced sequestration of GSH as *S*-D-lactoylglutathione. The elevated levels of glyoxalase I in tumour tissues may increase the value of the transfected cells as a model system for studying endogenous and xenobiotic metabolism.

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