Extracellular Glutathione Is a Source of Cysteine for Cells That Express γ-Glutamyl Transpeptidase†

Marie H. Hanigan* and William A. Ricketts

Department of Anatomy and Cell Biology, Box 439, School of Medicine, University of Virginia, Charlottesville, Virginia 22908

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ABSTRACT: We show that γ-glutamyl transpeptidase (GGT) is a glutathionase that enables cells to use extracellular glutathione as a source of cysteine. We transfected NIH/3T3 mouse fibroblasts with a plasmid containing cDNA for human GGT, and obtained stably transformed cell lines that expressed GGT in its proper orientation on the outer surface of the cell. NIH/3T3 fibroblasts require cysteine for growth and are unable to use extracellular glutathione as a source of cysteine. We demonstrate GGT-positive fibroblasts are able to grow in cysteine-free medium supplemented with glutathione. Cysteine derived from the cleavage of extracellular glutathione can be used to maintain intracellular levels of glutathione. GGT-positive NIH/3T3 cells were able to replenish intracellular glutathione when incubated in cysteine-free medium containing glutathione. GGT-negative cells could not. Therefore, GGT is a glutathionase that provides the cell with access to a secondary source of cysteine.

γ-Glutamyl transpeptidase (GGT)[1] is a cell-surface enzyme that can either hydrolyze the γ-glutamyl bond of glutathione or transfer the γ-glutamyl moiety of glutathione to an acceptor molecule (Hanigan & Pitot, 1985). Aberrant expression of GGT has been noted in subpopulations of cells in human tumors from the liver, lung, breast, and skin (Gerber & Thung, 1980; Dempo et al., 1981; Bard et al., 1986; Levine et al., 1983; Groves et al., 1991). When tumors are treated with chemotherapeutic drugs such as cisplatin and L-phenylalanine mustard, drug-resistant variants arise that are GGT-positive and have elevated levels of intracellular glutathione (Godwin et al., 1992; Ahmad et al., 1987; Lewis et al., 1988). We are interested in determining what role the expression of GGT has on the development of tumors and their subsequent response to chemotherapy.

Expression of GGT in tumors has been interpreted as an indicator of cell dedifferentiation with no functional significance (Dempo et al., 1981; Groves et al., 1991; Uriel, 1979). We challenged this interpretation by proposing that GGT expression is part of a much broader system of detoxification within the cell (Hanigan & Pitot, 1985). Cleavage of the γ-glutamyl bond of glutathione by GGT liberates the cysteinylglycine dipeptide from glutathione. Our hypothesis states that by initiating the cleavage of glutathione in the serum, GGT increases the supply of cysteine that is accessible to the cell. Cysteine is an essential amino acid for most cells; only the liver, pancreas, and kidney can convert methionine to cysteine (Mudd et al., 1965). Cysteine is the rate-limiting component of glutathione synthesis and thus a critical element in the glutathione detoxification pathways within the cell (Reed et al., 1983).

To test the proposition that GGT can provide cells with access to the cysteine in extracellular glutathione, we constructed two lines of NIH/3T3 mouse fibroblasts that differed only in their expression of GGT. We exploited the fact that NIH/3T3 fibroblasts cannot grow in the absence of externally supplied cysteine, and asked whether expression of GGT enabled cells to use extracellular glutathione as a source of cysteine. We offer the first direct evidence showing that GGT expression enables cells to use extracellular glutathione as a source of cysteine. Further, we demonstrate that cysteine derived from extracellular glutathione can be used to increase intracellular levels of glutathione.

EXPERIMENTAL PROCEDURES

Cell Lines and Medium. NIH/3T3 mouse fibroblasts (ATCC CRL 1658) were obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in F12/DMEM medium with 25 μg/mL gentamicin (BRL/GIBCO Laboratories, Grand Island, NY) and 5% calf serum (HyClone Laboratories, Logan, UT). Cysteine-free medium was made in our laboratory according to the formulation for a 1:1 mixture of F12/DMEM as described in the GIBCO Laboratories catalogue. Fetal bovine serum and calf serum contain only 0.01 mM cysteine (HyClone technical representative, personal communication). Addition of 5% serum to cysteine-free medium results in a final concentration of 0.5 μM cysteine.

Extracellular Cysteinylglycine and Glutathione as Sources of Cysteine. We plated 5×10^4 NIH/3T3 cells per 60-mm dish. Twenty-four hours after plating, the medium was changed to cysteine-free F12/DMEM with 5% fetal bovine serum and gentamicin. The medium was supplemented with 350 μM cysteine, cysteinylglycine (Bachem Bioscience Inc., Philadelphia, PA), or glutathione. The number of cells per dish was determined 4 days after the medium change.

Construction of an Expression Vector Containing GGT. A full-length cDNA clone for human GGT was provided to us by Dr. Henry Pitot and David Goodspeed (Goodspeed et al., 1989). We inserted the human GGT cDNA, which is contained in a 2.32-kb *Eco*RI fragment, into the *Eco*RI restriction site of the polylinker region of the expression vector pLEN-PT. We obtained pLEN-PT from Dr. Paul Johnson and Dr. Richard Hynes (MIT, Boston, MA). The pLEN-PT vector was derived from the pLEN vector (Dr. Tyler White, California Biotechnology Inc., Mountain View, CA). The pLEN-PT vector is a pUC8-based vector with an SV40 origin

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* Author to whom correspondence should be addressed.

[1] Abbreviations: GGT, γ-glutamyl transpeptidase; FITC, fluorescein isothiocyanate.

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of replication, SV40 enhancer sequences, a human metallothionein II promoter, the polylinker region from the pECE vector (Ellis et al., 1986), and SV40 poly(A) addition signal, and a poly(A) tract. This clone was named GGT/pLEN-PT.

Transfection of NIH/3T3 Cells with GGT-pLEN/PT. GGT/pLEN-PT plasmid was transfected into NIH/3T3 cells using the calcium phosphate eukaryotic transfection kit from Stratagene (La Jolla, CA). Ten micrograms of GGT/pLEN-PT plus 2 μg of pWLneo (a plasmid provided by Stratagene that contains a G418 resistance marker) was precipitated and added to 5×10^5 cells. Control dishes were transfected with pWLneo alone. Stable transformants were selected by the addition of 0.8 mg/mL G418 to the culture medium. Individual colonies were isolated and maintained in medium containing 0.5 mg/mL G418. Periodic screening showed all cell lines to be free of mycoplasma contamination.

Determination of GGT and Aminopeptidase N Activity. Expression of GGT activity was determined biochemically (Tateishi et al., 1976) and histochemically (Rutenburg et al., 1969). The activity of aminopeptidase N (EC 3.4.11.2), previously referred to as aminopeptidase M, was measured as described by Hughey and co-workers (Hughey et al., 1978). Protein was determined with the BCA protein assay (Pierce, Rockford, IL).

Fluorescent Staining of GGT on the Cell Surface. GGT was purified from rat kidneys, and antibody directed against GGT was prepared in rabbits as previously described (Hanigan & Pitot, 1982). Cells were trypsinized from cultures and labeled with a rabbit antibody to rat kidney GGT or with preimmune serum as previously described (Hanigan & Pitot, 1982). The fluorescent secondary antibody, FITC-labeled goat anti-rabbit antibody (Jackson Immunoresearch, West Grove, PA), was diluted 1:240 in phosphate-buffered saline. All solutions contained 0.1% sodium azide to inhibit capping. The cells were visualized with a fluorescence microscope.

Uptake of Cysteine from Extracellular Glutathione. F12/DMEM medium supplemented with 20 μM glutathione and 0.5 μCi of [^{35}S]glutathione was added to confluent cultures of GGT-positive and GGT-negative NIH/3T3 cells. At 10 min, 2 h, 24 h, and 48 h after the addition of the radiolabeled glutathione, the medium was removed. The dishes were rinsed with phosphate-buffered saline, trypsinized off the dish, and resuspended in phosphate-buffered saline. The amount of ^{35}S was determined by adding an aliquot of cells to Ready-Safe scintillation fluid (Beckman Instruments, Fullerman, CA) and analyzing the samples with a Beckman LS 8000 scintillation counter. The BCA protein assay (Pierce, Rockford, IL) was used to determine the protein concentration in the sample. One $\times 10^6$ cells continued 0.25 mg of protein.

Growth of Cells in Cysteine-Free Medium. Five $\times 10^4$ cells were plated per 60-mm tissue culture dish. After 24 h, the medium was removed and replaced with one of the following: F12/DMEM medium, cysteine-free F12/DMEM medium, or cysteine-free F12/DMEM medium containing 100 μM glutathione. All of the media contained 5% fetal bovine serum and gentamicin. Three dishes were harvested at the time of the media change (t_0) and every 24 h thereafter.

Replenishment of Intracellular Glutathione. Confluent cultures of GGT-positive and GGT-negative NIH/3T3 cells were incubated in cysteine-free F12/DMEM for 24 h to deplete intracellular glutathione. The medium was then changed to cysteine-free F12/DMEM supplemented with 100 μM cysteine or 100 μM glutathione. After a 6-h incubation, the intracellular glutathione concentration was measured as described by Tietze (1969).

Table I: Growth of NIH/3T3 Cells in Various Sources of Cysteine[a]

medium	no. of cells per dish ($\times 10^4$)
F12/DMEM (contains 350 μM cysteine)	58.2 ± 3.3
cysteine-free F12/DMEM	0.73 ± 0.25
cysteine-free F12/DMEM plus 350 μM glutathione	1.07 ± 0.23
cysteine-free F12/DMEM plus 350 μM cysteinylglycine	25.1 ± 0.53

[a] Cysteine-free F12/DMEM medium supplemented with 350 μM cysteine, cysteinylglycine, or glutathione plus 5% fetal bovine serum and 25 μg/mL gentamicin was added to logarithmically growing cultures of GGT-negative NIH/3T3 cells. The number of cells per dish was determined 4 days after the medium change. Values represent the average of three dishes ± the standard deviation.

RESULTS

NIH/3T3 fibroblasts do not express GGT activity as determined by the biochemical and histochemical assays. NIH/3T3 cells were unable to grow in cysteine-free medium (Table I). They could not synthesize cysteine from the methionine supplied in the medium. When the tripeptide glutathione was added to cysteine-free medium, the NIH/3T3 cells were unable to utilize extracellular glutathione as a source of cysteine (Table I). The cells were able to use cysteinylglycine, a product of the GGT reaction with glutathione, as a source of cysteine (Table I).

The cells grow more slowly in cysteinyl glycine than in cysteine (Table I). There are two cell-surface enzymes that cleave the cysteinylglycine dipeptide: aminopeptidase *N* and cysteinylglycine dipeptidase (Rankin et al., 1980; McIntyre & Curthoys, 1982). The aminopeptidase *N* activity in NIH/3T3 cells is 3.3×10^{-3} ($\pm 1.6 \times 10^{-3}$) units of activity per milligram of protein. In our experiments, cysteinylglycine dipeptidase is not active in these cells. We have grown NIH/3T3 cells in cysteine-free medium containing 50–400 μM cysteinylglycine. Several investigators have reported that cysteinylglycine dipeptidase is inhibited by cysteinylglycine concentrations over 50 μM (Kozak & Tate, 1982; McIntyre & Curthoys, 1982). In our experiments, cysteinylglycine dipeptidase would be inhibited as we raised the cysteinylglycine concentrations. This inhibition would have blocked cell growth, since the only source of cysteine for the cells is from the cleavage of cysteinylglycine. Rather than inhibiting cell growth, we found that the rate of cell growth increased as we increased the cysteinylglycine concentration. Therefore, aminopeptidase *N* is the major dipeptidase that is active in these cells.

The GGT-positive line was constructed by cotransfecting NIH/3T3 cells with both GGT/pLEN-PT, an expression vector containing a full-length cDNA clone for GGT, and pWLneo, a plasmid containing a G418 resistance marker. Forty-eight hours after the transfection of NIH/3T3 cells with GGT/pLEN-PT and pWLneo, 1% of the cells were positive histochemically for the expression of GGT. After 2 weeks of selection in G418, 74% of the G418-resistant colonies in the cultures transfected with GGT/pLEN-PT and pWLneo were GGT-positive. None of the G418-resistant colonies on the dishes transfected with pWLneo alone were GGT-positive. Colonies were picked from the dishes and grown into cell lines.

A GGT-positive NIH/3T3 line that expressed 0.64 ± 0.04 unit of GGT activity/mg of protein was chosen for further experiments. A GGT-negative line was selected from NIH/3T3 cells transfected with pWLneo alone. The growth rate

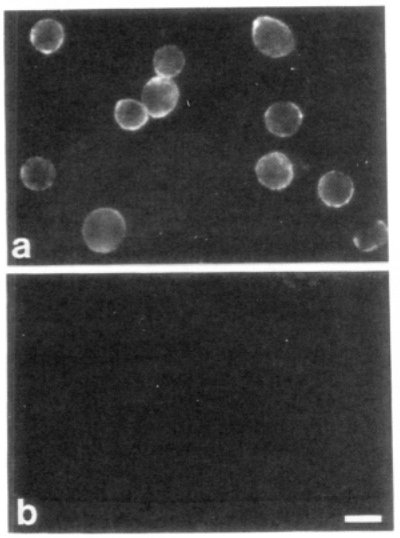

FIGURE 1: Localization of GGT on the surface of NIH/3T3 fibroblasts transfected with GGT/pLEN-PT. GGT-positive NIH/3T3 cells (a) or GGT-negative NIH/3T3 cells (b) were suspended in phosphate-buffered saline containing 0.01% sodium azide. They were incubated with a rabbit antibody to rat kidney GGT. FITC-labeled goat anti-rabbit antiserum was used as a secondary antibody. The bright fluorescent ring surrounding the GGT-positive cells is characteristic of antibodies bound to a cell-surface protein. No fluorescent staining was seen on cells incubated with preimmune serum. Bar indicates 20 μm.

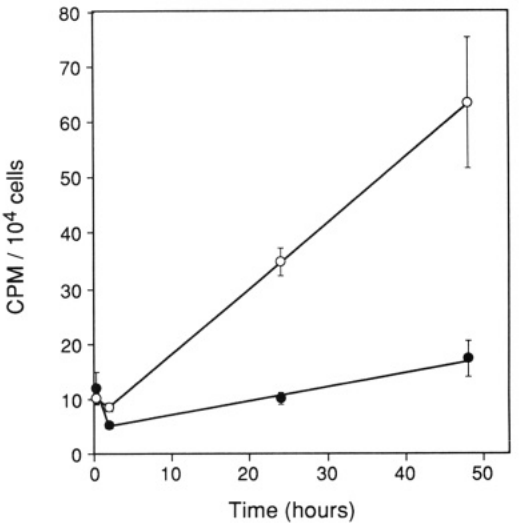

FIGURE 2: Uptake of cysteine from extracellular [^{35}S]glutathione by GGT-positive NIH/3T3 cells. F12/DMEM medium supplemented with 20 μM glutathione and 0.5 μCi of [^{35}S]glutathione was added to confluent cultures of GGT-positive NIH/3T3 cells (open circles) and GGT-negative NIH/3T3 cells (closed circles). The number of cell-associated counts was determined 10 min, 2 h, 24 h, and 48 h after the addition of radiolabeled glutathione. Values represent the average of three dishes ± the standard deviation.

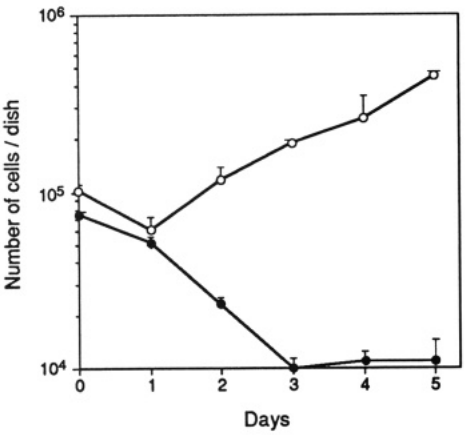

FIGURE 3: Growth of GGT-positive NIH/3T3 cells and GGT-negative NIH/3T3 cells in cysteine-free medium containing 100 μM glutathione. Cysteine-free F12/DMEM medium containing 100 μM glutathione, 5% serum, and gentamicin was added to logarithmically growing cultures of GGT-positive NIH/3T3 cells (open circles) or GGT-negative NIH/3T3 cells (closed circles). Values represent the average of three dishes ± the standard deviation.

of these two lines, when maintained in complete medium, was equivalent. There was no detectable GGT activity in NIH/3T3 cells or in the NIH/3T3 cells transfected with the pWL/neo plasmid alone.

GGT is located on the outer surface of the cell membrane. The GGT-positive transfected cells exhibited prominent staining on the outer surface when labeled with an antibody to GGT (Figure 1). This evidence confirms that the enzyme was processed and inserted into the outer surface of the membrane of transfected cells (Hanigan & Pitot, 1982). No fluorescent staining was observed on GGT-negative NIH-3T3 cells or on GGT-positive NIH/3T3 cells incubated with preimmune serum.

We asked whether the expression of GGT enabled cells to take up the cysteine from extracellular glutathione. ^{35}S-labeled glutathione was added to the medium at a concentration of 20 μM, which is equivalent to the concentration in serum (Kretzschmar et al., 1991). GGT-positive cells took up the ^{35}S-label, which is part of the cysteine moiety, in a linear and time-dependent fashion. No label was present in the GGT-negative cells (Figure 2).

NIH/3T3 fibroblasts require cysteine for growth as shown in Table I. GGT-positive cells grew logarithmically in cysteine-free medium containing 100 μM glutathione (Figure 3). In contrast, the GGT-negative cells were not able to access the cysteine contained within the glutathione and died within 3 days. These results demonstrate that GGT expression enables cells to use extracellular glutathione as a source of cysteine.

The GGT-positive cells did not grow during the first 24 h in cysteine-free medium containing glutathione. When GGT cleaves glutathione, it releases the cysteinylglycine dipeptide. Aminopeptidase *N*, which cleaves the dipeptide, is expressed at 3.3×10^{-3} unit of activity/mg of protein. Cell growth is limited by the rate at which the cysteinylglycine dipeptide is cleaved (see Table I). GGT activity in the GGT-positive cells is 0.64 unit of activity/mg of protein, 200-fold higher than a dipeptidase activity. Therefore, in the cultures of the GGT-positive NIH/3T3 cells, cleavage of the cysteinylglycine dipeptide is the rate-limiting step in the release of cysteine from extracellular glutathione. However, the initial cleavage of glutathione by GGT is essential for the cells to utilize extracellular glutathione as a source of cysteine (Figure 3).

Fibroblasts synthesize glutathione (Bannai & Kitamura, 1980). We asked whether GGT-positive cells could use cysteine derived from extracellular glutathione to synthesize intracellular glutathione. We depleted intracellular glutathione by incubating cells in cysteine-free medium for 24 h. After 24 h in cysteine-free medium, the glutathione level in the GGT-positive NIH/3T3 cells was 0.072 ± 0.031 nmol/10^6 cells, and in the GGT-negative cells, the level was 0.081 ± 0.024 nmol/10^6 cells. The medium was then changed to cysteine-free medium containing cysteine or glutathione. After a 6-h incubation in medium containing cysteine, both the GGT-positive and GGT-negative cells replenished their intracellular glutathione (Table II). When incubated in medium containing glutathione as the only source of cysteine, the GGT-positive

Table II: Ability of GGT-Positive and GGT-Negative NIH/3T3 Cells To Use Extracellular Glutathione as a Source of Cysteine To Replenish Intracellular Glutathione[a]

cells	supplements to cysteine-free F12/DMEM medium	intracellular glutathione (nmol of glutathione/ 10^6 cells)
GGT-positive	none	0.075 ± 0.021
NIH/3T3 cells	100 μM glutathione	3.1 ± 0.19
	100 μM cysteine	5.3 ± 0.53
GGT-negative	none	0.054 ● 0.024
NIH/3T3 cells	100 μM glutathione	0.32 ● 0.045
	100 μM cysteine	5.3 ± 0.22

[a] Confluent cultures of GGT-positive and GGT-negative NIH/3T3 cells were incubated in cysteine-free F12/DMEM for 24 h to deplete intracellular glutathione. The medium was then changed to cysteine-free F12/DMEM supplemented with cysteine or glutathione. The intracellular glutathione concentration was measured 6 h after the medium change. Values represent the average of three dishes ● the standard deviation.

cells replenished their intracellular glutathione whereas the GGT-negative cells did not (Table II). Our data show that cysteine can be derived from extracellular glutathione to support synthesis of intracellular glutathione in GGT-positive cells.

DISCUSSION

We have demonstrated that expression of GGT enables cells to use glutathione as a source of cysteine. Glutathione is a tripeptide consisting of glutamic acid, cysteine, and glycine. The glutamic acid is linked to cysteine via the carboxyl group on the side chain of the glutamic acid. This γ-glutamyl bond, which is resistant to cleavage by proteases, can be cleaved by GGT. NIH/3T3 fibroblasts do not express GGT. When transfected with a plasmid containing cDNA for human GGT, the NIH/3T3 cells synthesized the enzyme and inserted it into its proper location on the outer surface of the cell membrane. We have shown that cysteine is an essential amino acid for NIH/3T3 cells. An NIH/3T3 cell line that expressed GGT was able to grow in cysteine-free medium supplemented with glutathione while a control line that did not express GGT died.

Investigation into the role that GGT may play in the development of drug-resistant tumors has been hampered by an extensive debate as to whether the enzyme functions as a transferase or a glutathionase. GGT is normally present in the kidney and other cells that have excretory or absorptive function (Hanigan & Pitot, 1985). Meister has proposed that GGT transfers the γ-glutamyl group of glutathione to an amino acid acceptor and the resulting γ-glutamyl dipeptide is part of an amino acid transport system (Meister, 1973; Anderson & Meister, 1983). Others have used kinetic data to argue that the hydrolysis reaction predominates *in vivo* (Curthoys & Hughey, 1979). Amid the dispute over the fate of the glutamate moiety, little attention has been paid to the fact that both the transferase and hydrolysis reactions release the cysteinylglycine dipeptide from glutathione. This dipeptide can be cleaved by cell-surface aminopeptidases to generate free cysteine and glycine (McIntyre & Curthoys, 1982; Kozak & Tate, 1982). Studies with inhibitors of GGT suggest that in the kidney, GGT initiates the cleavage of glutathione present in the glomerular filtrate, allowing its constituent amino acids to be reabsorbed rather than being excreted (Griffith & Meister, 1979).

Increased expression of GGT has been documented in cell lines as a consequence of selection for chemotherapy resistance. Godwin and co-workers (1992) reported that human ovarian cancer cell lines selected *in vitro* for resistance to cisplatin showed an increase of 15–40-fold in the steady-state mRNA levels of GGT. The increase in GGT mRNA correlated with an increased level of intracellular glutathione and of enzymes involved in the synthesis of glutathione. Maintaining intracellular glutathione levels is a crucial element in the resistant phenotype. Resistance to the toxic effects of cisplatin can be overcome by depleting intracellular glutathione (Hamilton et al., 1985). Our studies demonstrate that GGT works in concert with enzymes that synthesize intracellular glutathione by providing the cell with access to this alternative source of cysteine. The sequence coding for GGT may be turned on as part of a coordinately regulated set of genes. The promoter region of GGT contains a putative antioxidant response element. Rushmore and co-workers (1991) have reported an antioxidant response element, of 11 base pairs, in the 5′-flanking region of the rat glutathione *S*-transferase Ya subunit gene and the NAD(P)H:quinone reductase gene. This same sequence is present in the promoter region of the rat GGT gene (Kurauchi et. al., 1991).

Rajpert-De Meyts and co-workers (1992) have also recently transfected GGT into NIH/3T3 cells and obtained stable lines expressing GGT. In their studies, they deplete the cells of glutathione by treatment with diethyl maleate, an inhibitor of glutathione synthesis. After removal of the diethyl maleate block, both the GGT-positive and GGT-negative cells are able to resynthesize glutathione. They show that the addition of glutathione to complete medium accelerates the resynthesis of glutathione in the GGT-positive cells. We interpret their results as further evidence that glutathione provides an additional source of cysteine to GGT-positive cells replenishing intracellular glutathione. However, all of their experiments were done with medium that contained cysteine; therefore, the critical role of GGT in providing cells with cysteine is not apparent in their studies.

We have demonstrated the role of GGT in providing cells with a secondary source of cysteine. The ability of the cell to use extracellular glutathione as a source of cysteine is important when the supply of cysteine becomes limiting for the synthesis of intracellular glutathione. These results indicate that expression of GGT may play a critical role in the resistance of tumors to chemotherapeutic drugs that are detoxified by glutathione. Our data would suggest that GGT is not simply a dedifferentiation antigen. When cysteine levels are stressed by the administration of chemotherapeutic drugs, GGT-positive cells may be more resistant to the toxic effects of these drugs than GGT-negative cells. Further studies are in progress with epithelial cells transfected with GGT to test their resistance to chemotherapeutic agents.

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