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Modulating Effect of Cystamine and Unsaturated Fatty Acids on Gamma-Glutamyl Transpeptidase: Relation to Cellular Oxidative Status

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Summary. Cell surface gamma-glutamyl transpeptidase activity in cultured neoplastic astrocytes was significantly increased upon treatment of the cells with the hepatoprotective disulfide, cystamine. The cystamine effect was sensitive to cycloheximide and could be significantly depressed by exogenous glutathione. Surface gamma-glutamyl transpeptidase activity was also modulated by the presence in the culture medium of the unsaturated fatty acids, linoleic acid and arachidonic acid. Metabolism of the fatty acids via the cyclooxygenase pathway was not a prerequisite for their modulation of the glycoprotein ectoenzyme. Lipoxygenase, however, was found to potentiate the unsaturated fatty acid effect in neoplastic astrocytes. Lipoxygenase is reported to catalyze the conversion of unsaturated fatty acids to their corresponding peroxides. The data indicate an oxidative influence on the control of gamma-glutamyl transpeptidase activity.

Key Words gamma-glutamyl transpeptidase · cystamine · glutathione status · linoleic acid · arachidonic acid · neoplastic astrocytes

Introduction

It has been known for some time that exposure of cells to a variety of carcinogens and xenobiotics [5, 20, 22, 23] results in an increased expression of cell surface gamma-glutamyl transpeptidase (GGTP). Conceivably, the shared capacity of these compounds to stimulate GGTP might be related to their dependence on the cellular detoxification system for their ultimate biotransformation and elimination from cells [10].

One of the consequences of oxidative detoxification is an increase in the oxidative status of cells [17]; therefore, the apparent modulation of GGTP by carcinogens and xenobiotics may be a function of the extent to which cells became oxidized during the microsome-directed conversion of these substances into excretable or potentially excretable forms.

In this report, we investigate the potential role of the cellular oxidative status in the control of GGTP expression in neoplastic astrocytes in culture. The dramatic elevations in GGTP which can be achieved in the neoplastic astrocytes make them ideal for studying effector-mediated modulations of the glycoprotein ectoenzyme.

Two approaches were taken. Cultured neoplastic astrocytes were treated with either the hepatoprotective disulfide, cystamine, [3, 4] or with unsaturated fatty acids, and the impact of these compounds on cell surface GGTP activity and the cellular oxidative status was monitored. The cellular oxidative status was defined in terms of the extent to which two types of activities were present in cells: (1) activities reported to generate initiators of membrane peroxidation and (2) those reported to protect against lipid peroxidation in cellular membranes. NADPH oxidizing activity and the accumulation and subsequent release of GSSG from cells were used as indices of activity types (1) and (2), respectively. The data are consistent with the thesis of a link between the oxidative status of cells and GGTP control.

Materials and Methods

Cell Cultures and Experimental Protocol

In this study we used a rat cell line of neoplastic astrocytes, the C_6, originally obtained from the American Tissue Culture collection, Rockville, Maryland. The cultures were maintained in Medium-199 supplemented with 10% bovine serum (BS), 5% fetal bovine serum (FBS), 100 μU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. This formulation will be referred to as the native medium.

Experiments were initiated by harvesting confluent stock cultures and transferring the cells at a ratio of 1 : 4 (surface area/surface area) into 12-well plates (3.94 cm^2/well) containing native medium. On the following day, the monolayers were treated with Medium-199 plus 2% FBS, plus or minus the test compound(s).

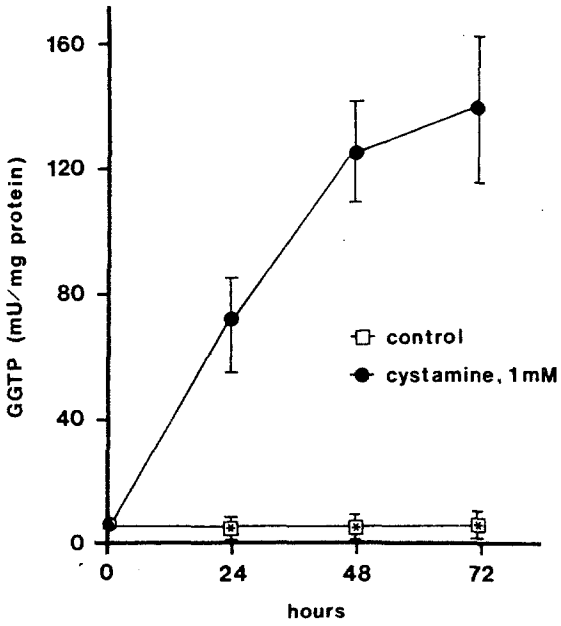

Fig. 1. Cystamine modulates GGTP activity in glial cells. Cystamine-treated cells exhibit a continued increase in GGTP over a period of 3 days. $n = 12$

In experiments testing the effects of unsaturated fatty acids on GGTP, serum was omitted from the test medium. For all experiments, the control and experimental groups were placed on a single 12-well plate and cell-surface GGTP was assayed on washed monolayers after 24 hr.

GGTP Activity

Cell surface GGTP activity was assayed in quadruplicate at 37°C in a reaction mixture which consisted of 100 mM Tris-HCl, 100 mM glycylglycine and 5 mM gamma-glutamyl-*p*-nitroanalide, all at pH 8 and mixed in a ratio of 3 : 1 : 1 [21].

Aliquots of the mixture (0.5 ml) were added to washed monolayers on 12-well plates and after an appropriate time, 0.4 ml of the reaction mixture was removed from individual wells and added to tubes containing 1 ml of 30% acetic acid. The latter step prevents the further hydrolysis of substrate by cells inadvertently removed from the well with the reaction mixture. The absorbance of the reaction product, *p*-nitroanaline, was measured at 405 nm, and GGTP activity was expressed as mU/mg protein. One milliunit is equivalent to the production of one nanomole of *p*-nitroanaline per minute. Proteins were determined according to the method of Bradford [1].

Cellular Glutathione Status

Intracellular concentrations of oxidized (GSSG) and reduced (GSH) glutathione were monitored to determine the impact of various treatment regimens on glutathione metabolism in the cultured neoplastic astrocytes.

The assay for oxidized glutathione was, in principle, the same as that reported by Oshino and Chance [16]. A 5% trichloroacetic acid (TCA) solution was directly added to washed monolayers, and after 10 min the TCA solution was removed from the culture dishes and centrifuged at 3000 × *g* for 10 min to remove precipitated proteins.

Table 1. Cystamine-associated increases in GGTP are concentration dependent[a]

Cystamine added (mM)	GGTP (mU/mg protein)
None	5 ± 1.4
0.5	38 ± 3.0
1.0	71 ± 15.0
1.5	83 ± 1.8

[a] Glial cells were treated with the indicated concentrations of cystamine over a period of 24 hr. GGTP is expressed as mU/mg protein. $n = 8$.

The assay mixture in a final volume of 0.9 ml contained 0.2 M potassium phosphate buffer, pH 7.4, 1 mM EDTA, 0.1 mM 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), 0.15 mM NADPH, 0.1 mg/ml bovine serum albumin and 0.04 ml of the TCA supernatant. The reaction was started by adding 0.1 ml of glutathione reductase (48 μg/ml). The rate of absorbance change at 412 nm was monitored for 2 min on a Gilford Response. The glutathione reductase solution was prepared daily from an ammonium sulfate suspension of glutathione peroxidase (Sigma) by diluting with 0.1 mg/ml bovine serum albumin/0.2 M potassium phosphate buffer, pH 7.4. GSH was determined according to the method of Ellman and Lysko [6]. GSSG was expressed as μm GSH equivalents/mg protein and GSH expressed as μm GSH/mg protein.

In some experiments, we assessed the cellular oxidative status by monitoring medium GSSG. Since GSSG is produced by GSH peroxidase, an enzyme which protects against lipid peroxidation in cellular membranes [18], the accumulation and subsequent spillage of GSSG into the culture medium is indicative of the extent to which cells are involved in protecting themselves against peroxidative influences.

To access medium GSSG concentrations, the spent medium (0.6 ml) was first added to 0.4 ml of a 5% TCA solution and after removal of precipitated proteins, the GSSG content was determined as described above.

NADPH Oxidation

NADPH oxidase activity was monitored as a means of assessing the impact of cystamine on the cellular oxidative status. Measurements of NADPH oxidation by control and cystamine-treated cells were carried out according to the method of Gillette, Brodie and LaDu [9].

Titration of SH Groups on Cell Monolayers and on Purified GGTP

Washed neoplastic astrocytes were titrated for surface SH groups by exposing cell monolayers to a DTNB assay mixture for 5 min at 25°C. The mixture was then removed and the absorbance at 412 was determined. The assay mixture consisted of 0.2 M potassium phosphate, pH 7.4 plus 0.1 mM DTNB. The titration of SH groups on purified GGTP was determined essentially as above except that the assay mixture contained in a final volume

Table 2. Effect of cycloheximide on cystamine-associated increases in GGTP[a]

	Cycloheximide (0.5 μg/ml)	GGTP (mU/mg protein)
Control	–	8.0 ± 1.5
	+	5.0 ± 0.0
Cystamine, 1 mM	–	95.0 ± 6.2
	+	12.0 ± 0.6

[a] $n = 4$.

of 1 ml, 0.8 ml 0.2 M potassium phosphate buffer, pH 7.4; 0.1 mM DTNB plus 0.1 ml purified GGTP. After approximately 25 hr at 25°C the absorbance at 412 was determined.

Results

Cystamine Stimulates GGTP Expression

Treatment of cultured neoplastic astrocytes with cystamine results in a time-dependent (Fig. 1) and concentration-dependent (Table 1) stimulation of GGTP. The cystamine-associated increases in GGTP activity were found to be sensitive to the protein synthesis inhibitor, cycloheximide (Table 2). Additionally, the simultaneous addition of GSH with the disulfide resulted in a significant depression of the cystamine effect on surface GGTP activity (Fig. 2). GSSG was found to be equally depressive (*data not shown*). Lastly, the glutathione synthesis inhibitor, buthionine sulfoximide (BSD) was found to prevent the depression by GSH of cystamine-associated increases in GGTP (Table 3).

The source of the depressive effect of exogenous GSH (or GSSG) remains to be elucidated; however, the observation that BSD inhibits this effect is indicative of a requirement for the exogenous GSH to first gain entry into cells prior to exerting its depressive effect. By inhibiting glutathione synthetase BSD effectively prevents the entry of exogenous GSH. To gain entry into cells, exogenous GSH must first undergo catabolism in medium and then the constituent amino acids transported and resynthesized into intracellular GSH.

The apparent inhibition of the cystamine effect by BSD is presumably due to a BSD-associated depletion of intracellular GSH followed by severe cystamine-mediated peroxidative damage to cells. The fact that BSD deprives the neoplastic astrocytes of their protection against oxidative stress most probably facilitates oxidative damage to GGTP and other enzymes as well [15].

Data which implicate cystamine as a potential source of cellular oxidative damage are illustrated in Fig. 3. Neoplastic astrocytes treated with cystamine exhibited a marked increase in their oxidative status as determined by their capacity to oxidize NADPH. NADPH oxidizing enzymes are reported to generate initiators of membrane lipid peroxidation [2, 7, 8, 22].

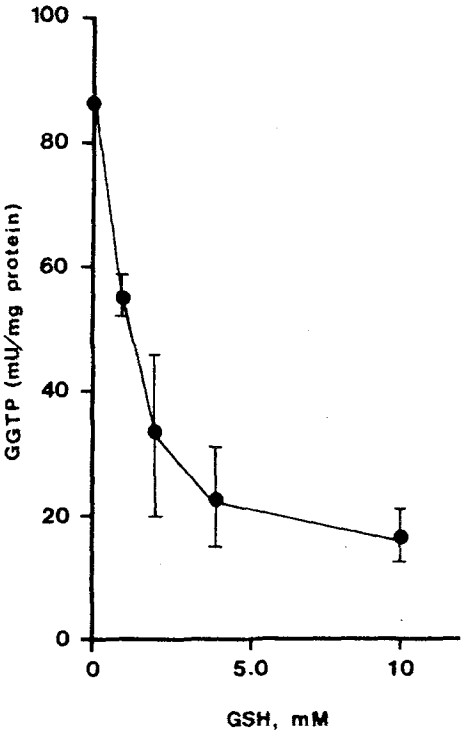

Fig. 2. Glutathione depresses the stimulatory effect of cystamine on GGTP. Glial cell monolayers were treated with 1 mM cystamine plus increasing concentrations of reduced glutathione. The depressive effect of glutathione was only effective if the sulfhydryl tripeptide was added simultaneously with cystamine

Table 3. Buthionine sulfoximide inhibits the GSH-related depression of cystamine-stimulated GGTP[a]

Additions	GGTP (mU/mg protein)	
	without cystamine	with cystamine, 1.5 mM
None	5.6 ± 1	93.0 ± 8
Glutathione, 2 mM	8.0 ± 1	49.0 ± 3
Glutathione, 4 mM	8.0 ± 1	31.0 ± 2
BSD, 0.5 mM + glutathione, 2.0 mM		56.0 ± 9
BSD, 1.0 mM + glutathione, 2.0 mM		82.0 ± 7
BSD, 0.5 mM	9.0 ± 1	10.6 ± 7
BSD, 1.0 mM	9.3 ± 1	4.0 ± 2

[a] $n = 4$.

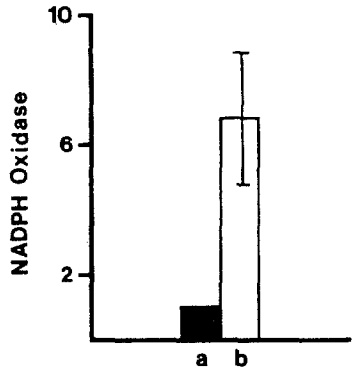

Fig. 3. NADPH oxidase activity in control (*a*) and cystamine-treated (*b*) glial cells. Glial cell-monolayers were treated with cystamine over a 24-hr period, then NADPH oxidase activity was assayed using methylene blue the electron acceptor [15]. NADPH oxidase activity is normalized to the control. $n = 3$

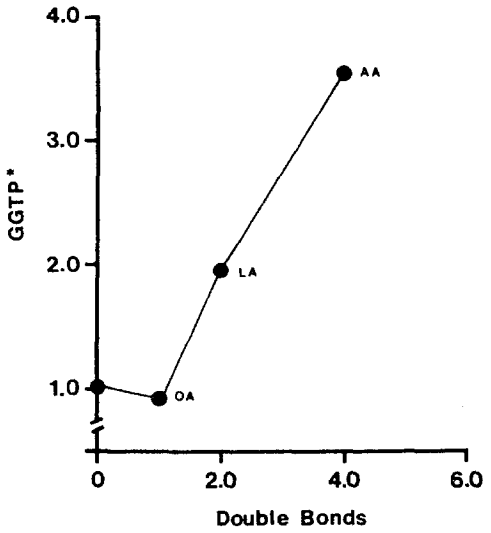

Fig. 4. Effect of unsaturated fatty acids on GGTP. Glial cell monolayers treated with oleic acid (OA, 18 : 1), linoleic acid (LA, 18 : 2) and arachidonic acid (AA, 20 : 4), respectively, were examined for GGTP activity after 24 hr. Each fatty acid was present in the serum-free growth medium at a concentration of 100 μM. GGTP is normalized to the control

Effects of Medium Fatty Acids of GGTP

Modulations in GGTP were also linked to the nature of the fatty acids present in the culture medium. Over a period of 24 hr, the presence of linoleic and arachidonic acid but not oleic acid (100 μM each) in the culture medium resulted in a cycloheximide-sensitive stimulation of GGTP bioactivity (Fig. 4, Table 4). The further treatment of cells with the combination of arachidonic acid plus inhibitors of cyclooxygenase failed to alleviate the impact of the unsaturated fatty acid on GGTP (Table 5). The data

Table 4. Cycloheximide inhibits unsaturated fatty acid-dependent increases in GGTP[a]

Additions	Cycloheximide (0.5 μg/ml)	GGTP (mU/mg protein)
None	–	26.0 ± 0.8
	+	33.0 ± 2.0
Linoleic acid, 100 μM	–	45.0 ± 3.0
	+	30.0 ± 2.0
Arachidonic acid, 100 μM	–	68.0 ± 0.0
	+	22.0 ± 5.0

[a] $n = 4$.

were interpreted to indicate that the modulatory effect of arachidonic acid was independent of the capacity of the cells to metabolize the effector at least by the cyclooxygenase pathway.

In other experiments, neoplastic astrocytes were treated with unsaturated fatty acids plus or minus lipoxygenase (Sigma), an enzyme which catalyzes the oxidation of unsaturated fatty acids [11, 14]. Lipoxygenase was found to potentiate the effect of the linoleic acid and arachidonic acid on GGTP (Figs. 5*A* and *B*), when the fatty acids were present at low concentrations. The apparent depression of GGTP by lipoxygenase at the higher fatty acid concentrations was conceivably due to the presence in the medium and/or uptake and incorporation of excessive amounts of fatty acid peroxides [13].

Intracellular Glutathione in Neoplastic Astrocytes

Using GSSG release as an index of the cellular oxidative status, it was observed that cystamine and unsaturated fatty acid-treated cells released more GSSG into the culture medium than did the corresponding controls (Table 6). The data were interpreted to indicate an increased involvement of both groups of treated cells in protective measures against oxidative stress [18]. For example, when super oxide radicals produced by NADPH oxidizing reactions (i.e. cystamine-treated cells) are acted upon by superoxide dismutase, H_2O_2 and eventually GSSG is reported to be produced. GSSG is also a product of the reaction wherein unsaturated fatty acid hydroperoxides are converted by glutathione peroxidase into their corresponding hydroxy fatty acids [11, 18].

Finally, the assessment of total glutathione in arachidonic acid and cystamine-treated cells revealed an increase in intracellular glutathione, reduced and oxidized (Table 7). Paradoxically, how-

Table 5. Effect of cyclooxygenase inhibitors on arachidonic acid-mediated modulations in GGTP[a]

	GGTP mμ/mg protein	
	no additions	arachidonic acid 0.1 mM
Control	14 ± 4	39 ± 6
ETOH, 0.21%[b]	13 ± 5	—
Ibuprofen, 0.25 mM	11 ± 4	34 ± 5
Acetylsalicyclic acid 0.25 mM	12 ± 3	38 ± 5

[a] Ibuprofen and acetylsalicyclic acid, respectively, are reversible and irreversible inhibitors of cyclooxygenase.
[b] Ethyl alcohol was present at a concentration of 0.21 percent in the cultures containing arachidonic acid plus cyclooxygenase inhibitors. $n = 8$.

Table 6. Release of GSSG by neoplastic astrocytes treated with cystamine and arachidonic acid[a]

Condition	GGTP inhibitor added[b]	Medium GSSG μM GSH Eq/mg protein
Control	−	0.54 ± 0.60
	+	0.98 ± 0.50
Cystamine, 0.5 mM	−	0.35 ± 0.36
	+	4.86 ± 1.40
Arachidonic acid 0.1 mM	−	1.18 ± 1.13
	+	1.70 ± 0.90

[a] $n = 8$ for the control and arachidonic acid groups; $n = 4$ for the cystamine group.
[b] Serine plus borate were added at concentrations of 20 mM each to prevent metabolism of GSSG released into the medium.

ever, the correlation between GGTP control and the cellular oxidative status appeared to break down when the ratio of oxidized-to-reduced glutathione was used as an index of the oxidative status of cells.

Discussion

The treatment of neoplastic astrocytes with cystamine resulted in increases in the cellular oxidative status (Fig. 3) and in GGTP enzyme activity (Fig. 1). The activity of the cell surface enzyme was also increased upon exposure of the cells to either unsaturated fatty acids or to their corresponding peroxides (Fig. 5*A* and *B*). The failure of these effectors to upmodulate GGTP in the presence of cycloheximide indicated that the synthesis of new protein was a prerequisite for the effect. It remains to be determined if this requirement is specific for GGTP

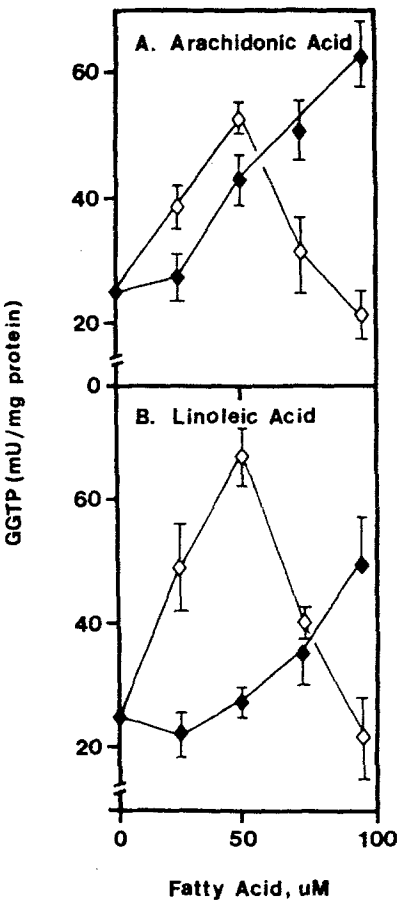

Fig. 5. Effect of lipoxygenase on fatty acid-dependent modulations in GGTP. Glial cell monolayers were treated with increasing concentrations of either (*A*) arachidonic acid or with (*B*) linoleic acid with (◇) and without (◆) 15 μg/ml lipoxygenase (Sigma)

Table 7. Intracellular glutathione status in neoplastic astrocytes treated with cystamine and arachidonic acid[a]

Condition	GGTP mU/mg protein	Glutathione		
		GSH μM/mg Protein	GSSG μM GSH equiv/mg Protein	GSSG/GSH ratio
Control	12 ± 2	45 ± 7	19 ± 8	0.42
Control + GSH, 4 mM	9 ± 2	36 ± 5	8 ± 1	0.22
Cystamine, 0.5 mM	58 ± 8	154 ± 27	63 ± 9	0.41
Cystamine + GSH, 4 mM	21 ± 4	82 ± 7	39 ± 1	0.48
Arachidonic acid, 0.1 mM	26 ± 1	60 ± 9	25 ± 1	0.42
Arachidonic acid + GSH, 4 mM	19 ± 3	8 ± 1	4 ± 1	0.50

[a] $n = 4$.

Table 8. DTNB titration of SH groups[a]

		Cell monolayers Ab_{412}/mg protein C	Purified GGTP[b] Ab_{412}/mg protein
Part I	Control	1.0	1.28 ± 0.27
	Cystamine, 0.5 mM	3.9	1.62 ± 0.10
Part II	Control	1.0	ND
	Arachidonic acid, 0.1 mM	3.6	ND

[a] All data are normalized to the control; $n = 4$ and $n = 8$ for the cell monolayers and purified GGTP, respectively.
[b] Neoplastic astrocyte monolayers were washed and removed from flasks by scraping, and the cells treated with papain (15 mg papain/1 mg cell protein) to solubilize the surface proteins. The solubilized surface proteins were chromatographed on a sephadex G-150.

itself or for some other protein which may be involved in the regulation of GGTP in the treated neoplastic astrocytes.

The unsaturated fatty acid effect on GGTP is consistent with either the uptake and incorporation of unsaturated fatty acids (or their hydroperoxides) into cellular membranes [19], or with their metabolism by the lipoxygenase pathways [24]. Our data do not distinguish between the two possible mechanisms. The net result of either mechanism would be an increase in the cellular oxidative status particularly if the assessment is based on the extent of involvement of cells in processes which protect against lipid peroxidation. The increased release of GSSG from arachidonic acid-treated cells (Table 6) is indicative of the involvement of the neoplastic astrocytes in reactions which protect against peroxidative damage presumably mediated by the presence of unsaturated fatty acids in cellular membranes or being metabolized in the cytoplasm [12].

With regard to the cystamine effect on GGTP there exists an additional mechanism which has not been considered. The possibility exists that cystamine might act in the neoplastic astrocytes by lowering intracellular GSH via a thiol-disulfide exchange between GSH and cystamine [25]. This potential effect, if it occurs to any extent at all, most probably represents only a minor component of the cystamine effect on GGTP. This assessment is based on unpublished data which show that cysteamine, the reduced form of cystamine, also modulates GGTP in neoplastic astrocytes. In contrast to cystamine, the interaction of cysteamine with GSSG via the same thiol-disulfide exchange reaction would tend to raise and not lower intracellular GSH.

The precise relationship between GGTP control and glutathione appears to be complex and not thoroughly understood at this point. Even with the apparent relationship between GGTP control and the oxidative status of cells with respect to one criteria (i.e. level of NADPH oxidizing activity and GSSG spillage into the culture medium), GGTP expression in treated neoplastic astrocytes bears no apparent relationship to the GSSG/GSH ratios in these cells. The assessment of the ratio of GSSG to GSH is an alternative means of evaluating the cellular oxidative status. Nevertheless, taking into consideration, the data in Table 8, a role for glutathione in GGTP control becomes perceivable. The data specifically indicated that treated cells and more importantly their purified GGTP (i.e. cystamine-treated cells specifically) contained more titratable SH groups than did the corresponding untreated cells and their purified GGTP, respectively. The data can be interpreted to indicate that the rise in intracellular GSH in cystamine and arachidonic acid-treated cells (Table 7) may be a key element in the upmodulation of GGTP activity in the treated neoplastic astrocytes. The rise in GSSG, one of the indicators of the involvement of cells in oxidation-related protective measures, may only serve as a signal for the "turn on" of GSH synthesis and the subsequent reduction and activation of GGTP.

Support for this thesis can be found in the BSD experiments (Table 3). Cells presumably prevented from synthesizing GSH were unable to respond to cystamine treatments by increasing the level of GGTP expressed. Further, in other experiments where the intracellular GSH response was reduced as a result of adding exogenous GSH, the upmodulation of GGTP was also reduced. Additional studies are required to determine the precise mechanism by which exogenous GSH impacts the modulation of intracellular glutathione in treated neoplastic astrocytes.

In further related studies, GGTP from control and cystamine-treated cells will be evaluated for specific activity (i.e. bioactivity/amount of GGTP antigen), for its immunoelectrophoretic properties, pH activity profiles and kinetic properties. These experiments will provide information on the extent to which the cellular oxidative status may ultimately influence the amount and character of GGTP protein expressed in cultured neoplastic astrocytes.

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