Gamma-Glutamyl Transferase Expression in Higher-grade Astrocytic Glioma

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Increased expression of γ-glutamyltransferase (GGT) has been detected in a range of human malignancies and is thought to be involved in neoplastic proliferation and treatment resistance. Since GGT expression and its role in malignant glioma biology remain largely unknown, we investigated this phenomenon by immunostaining 26 higher-grade human astrocytic gliomas (WHO grades III and IV) with a monoclonal anti-GGT-antibody (138H11). Further, human pancreatic *GGT* cDNA was used for liposome-mediated transfection of 9L gliosarcoma cells. GGT-expressing and control 9L cells were cultured in media containing different amounts of essential amino acids and/or cytotoxic agents. Cell viability was evaluated by microplate MTT assay. Immunohistochemical staining of tumor specimens demonstrated that GGT expression is a frequent feature of higher-grade human astrocytic gliomas, but not of normal brain tissue. Human tumors were strongly GGT-positive in 6 of 7 cases of grade III astrocytoma, and in 12 of 19 grade IV astrocytoma (*glioblastoma multiforme*, GBM) cases. In the cell culture model, 9L-GGT cells had a growth advantage over control cells in cysteine-deficient medium, but not in standard or glutamine-free medium. No significant difference in numbers of viable cells of either clone was found in media containing the alkylating drug BCNU (5–200 μg/ml). In conclusion, GGT is expressed in a high percentage of human WHO grade III astrocytomas and GBM, but not in normal brain tissue. This molecule seems to give neoplastic cells a moderate growth advantage under in vivo conditions.

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Glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH) is an essential cellular tripeptide with antioxidative and detoxifying functions (1–3). Intracellular GSH maintains the antioxidant state of the cell by catalyzing the reduction of H_2O_2 and other peroxides, and also plays an important role in protection of intracellular protein thiol groups (4). The membrane-bound ectoenzyme γ-glutamyl-transferase (GGT, E.C.2.3.2.2) is capable of cleaving the γ -glutamyl bond of GSH. After degradation of extracellular GSH and conjugates, the constituent amino acids (AA) and dipeptides are transferred back to the cell and are used in metabolic pathways for intracellular de novo synthesis of GSH (4, 5). Since malignant cells are highly dependent on cysteine, using extracellular GSH as a source of cysteine may confer a considerable growth advantage to a tumor. Orlowski & Meister (6) proposed the so-called γ-glutamyl cycle for participation of GGT in the cellular AA uptake. In this cycle, the γ-glutamyl group of GSH is transferred to other AA by means of a transpeptidation reaction, thereby allowing easy uptake of the resulting γ-glutamyl-AA (4, 7–9).

High GGT expression is physiologically present in kidney and pancreas (10–12), and has been demonstrated in many human malignancies, especially in epithelial cancers (10, 11, 13). In some studies, high GGT expression of the respective cells was found to correlate with multidrug resistance (14). Malignant glioma, however, has not been investigated previously, although it is known that normal brain astrocytes and neurons do not express significant amounts of GGT (13). Higher-grade astrocytomas (WHO grades III and IV) are primary brain tumors with an invasive and infiltrating growth, and are largely resistant to adjuvant treatments such as chemo- and radiotherapy (15–17). These tumors are known for their

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invariably fatal outcome. Despite extensive multimodal treatment regimens, the median survival time of patients with WHO grade IV tumors (*glioblastoma multiforme*, GBM) still does not exceed one year (17, 18).

The primary aim of the present study was to investigate GGT expression in human gliomas of different malignancy grades. Furthermore, the physiologic role of GGT in glioma was to be elucidated in a cell culture model with orthotopic expression of human GGT cDNA.

MATERIAL AND METHODS

Patients

This study involved 7 anaplastic astrocytoma WHO grade III (AA) patients (4 males and 3 females) and 19 GBM patients (9 males and 10 females) (see Table). All of these patients underwent surgery for removal of primary tumor, and most of them received postoperative fractionated radiotherapy (2 Gy per fraction, 56–60 Gy mean total tumor dose). Tumor specimens were paraffin-embedded and stained with hematoxylin and eosin (Sigma, Deisenhofen, Germany) for light-microscopic evaluation according to the WHO histological classification (18).

Immunohistochemistry

Tumor specimens were frozen and stored at −80°C until further processing. Cryostat sections (7 μm) were cut, air-dried and fixed in ice-cold acetone. Sections were blocked with 5% BSA (Sigma) for 10 min and then incubated for 1 h with the monoclonal mouse anti-human-GGT antibody (Ab) 138H11 (10, 11, 19) in a concentration of 1 : 100. The secondary Ab was a biotinylated rabbit anti-mouse IgG (DAKO, Hamburg, Germany) used in a concentration of 1:100 and incubated with the sections for 30 min. Avidin-linked peroxidase (Vectastain Elite ABC Peroxidase Kit, Vector Labs, Burlingame, CA) was used according to the manufacturer's instructions. Staining was done with AEC substrate (Sigma), and sections were counterstained with hematoxylin. For analysis, each section was evaluated independently by at least two observers. The used scoring system was semiquantitative.

Cell culture

Rat 9L gliosarcoma cells (gift from Dr Xandra O. Breakefield, MGH Boston, MA) were grown as monolayer cultures in DMEM (Sigma) supplemented with 10% fetal calf serum (FCS, Sigma) and 1% penicillin-streptomycin (this medium composition is referred to as standard medium) and maintained in an atmosphere of 5% CO_2 at 37°C.

Cell transfection

The recombinant cDNA encoding human pancreatic γ-glutamyl-transferase (20) was placed under the transcriptional control of the strong constitutive murine phosphoglycerate kinase promoter in a eucaryotic expression plasmid (pPGK, gift from Dr M. Sena-Esteves, MGH Boston, MA). 9L rat gliosarcoma cells were stably transfected with the resulting construct (pPGK-hGGT) using cationic liposomes (DOTAP, Boehringer Mannheim, Mannheim, Germany), and were selected by 1 mg/ml G418 (Sigma). The resulting G418-resistant clones were lysed, cell debris was centrifuged, and GGT activity of the lysate was determined by a commercially available GGT assay (Sigma) using L-γ-glutamyl-p-nitroanilide as substrate. We defined one unit of GGT as the amount of activity that will liberate 1 μmol of p-nitroaniline/min at 37°C. The 9L cell clone with the highest GGT activity was used for further experiments.

Immunocytochemistry

GGT-expressing cell clones were cultured in standard medium on chamber slides (Nunc Inc., Naperville, IL) until reaching approximately 70% confluence. They were then fixed with ice-cold acetone for 5 min and air-dried. Staining was performed analogous to the immunohistochemical technique described above. For immunofluorescence staining, a secondary anti-mouse Cy3-labeled Ab (Dianova, Hamburg, Germany) was used in a concentration of 1 : 200, and incubated for 1 h after standard incubation with primary Ab. The bright red immunofluorescence was visualized with a UV-equipped inverted microscope (Zeiss Axiovert 135).

Immunoelectron microscopy

GGT-expressing and control (mock-transfected) cells were harvested by trypsination. After centrifugation and washing in PBS, cells were fixed in 2% formaldehyde and 0.02% glutaraldehyde in PBS for 20 min at room temperature, carefully washed in PBS, and subsequently embedded in 7% gelatin (Sigma) in PBS. Small gelatin pieces were postfixed in 2% formaldehyde and 0.02% glutaraldehyde in PBS for 5 min at 4°C. After washing in PBS, all fixed samples were incubated with 1.15 M sucrose and 10% polyvinylpyrrolidone (PVP K15, Fluka, Buchs, Switzerland) in PBS for 2 days at 5°C. Ultrathin cryosectioning was performed with a Reichert-Jung FC 4E cryoultramicrotome. Cryosections were transferred to formvar-coated nickel grids (Bio Cell Plano, Marburg, Germany), washed in PBS, afterwards in PBS containing 50 mM glycine, and finally in PBS containing 0.5% albumin and 0.2% gelatin. Sections were incubated overnight at 5°C in a moist chamber with the 138H11 Ab diluted 1:200 in PBS. The reaction with gold-labeled secondary antibody (goat-anti-mouse IgG, GAM; Aurion Immuno Gold, Wageningen, The Netherlands) was carried out for 1 h at room temperature. After washing in PBS and tridistilled water, the sections were embedded in 1.1% tylose (Fluka) containing 0.5% uranyl acetate.

Cell viability assays

Cells in the logarithmic phase of growth were trypsinized, counted by trypan-blue exclusion in a hemocytometer, and plated at a density of 3×10^3 cells per well in 96-well microtiter plates. After an incubation period of 24 h, the medium was replaced by either 100 μl/well cysteine-deficient medium supplemented with GSH, or glutamine-deficient medium supplemented with GSH. On each of the following 5 days, the medium of one microtiter plate was replaced by 0.5 mg/ml MTT (Sigma) in DMEM. After 3 h incubation at 37°C, MTT was removed, and the intracellular formazan crystals were solubilized in 100 μl isopropanol/0.1 N HCl. Absorbance was measured at 570 nm and 630 nm on a microplate reader (EL $\times$ 800, Biotek Instruments, Heidelberg, Germany). Fractional absorbance was expressed as a percentage of the absorbance displayed by cells grown in standard medium. In experiments assessing cytotoxicity of 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU; Carmubris, Bristol-Myers Squibb GmbH, Munich, Germany), an alkylating agent widely used for chemotherapy of higher-grade glioma (16), various concentrations of BCNU were added after an initial 24 h incubation period in standard medium. After another 24 h incubation, BCNU-containing medium was replaced by standard medium, and the number of viable cells was assayed, as described above.

Measurement of GSH content

9L-GGT and 9L-control cells were grown in standard medium until reaching 70% confluence, and were then incubated for 24 h with standard medium or with cysteine-free medium supplemented with 100 μM GSH or with standard medium with 100 μM L-buthionine-sulfoximine (L-BSO, Sigma), an irreversible inhibitor of γ-glutamylcysteine-synthetase. Cells were than washed twice in cold PBS and afterwards scraped in ice-cold 3.3% $HClO_4$. After 5 min centrifugation at 14000 rpm, the supernatant was removed and stored at -70°C until assayed for GSH as previously described (21). The protein pellet was suspended in 300 μl 1 M NaOH and used for determination of total protein by the Lowry assay (Sigma).

RESULTS

Immunohistochemistry in human glioma

From a total of 26 fresh-frozen astrocytoma specimens, 18 (69%) stained positive for GGT, and 8 (31%) were GGT negative (see Table). Six out of 7 grade III astrocytomas (86%) were GGT positive (Table 1 and Fig. 1A). Twelve (63%) out of 19 GBM stained positive for GGT, and 7 were GGT negative (Table and Fig. 1B). As previously known (13), normal brain tissue was GGT negative, except for endothelial cells in brain capillaries, which stained strongly positive for GGT (data not shown). The subcellular distribution of GGT positivity in tumor cells was slightly variable and mainly membrane-bound with occasional cytoplasmic staining.

Cell culture model

The 9L gliosarcoma cell clone with the highest enzymatic activity had 120 mU GGT activity/mg of total protein, and was used for all further experiments. Immunocytochemistry with the 138H11 Ab confirmed high level GGT expression in the transfected and selected clones (Fig. 1C). Immunoelectron microscopy showed orthotopic membrane-bound expression of transgene GGT (Fig. 1D). Control 9L cells were mock transfected with the same plasmid without GGT cDNA (pPGK-4B1) and selected as described above. On immunocytochemistry and immunoelectron microscopy, they had no detectable GGT expression (data not shown).

No significant differences between viability of GGT expressing and control clones were noted in standard medium, in glutamine deficient medium (0.2 mM versus 2.0 mM in standard medium), as well as in glutamine-free medium supplemented with GSH (data not shown). However, 9L-GGT cells cultured in cysteine-free medium supplemented with GSH exhibited a statistically significant growth advantage over control cells ($p < 0.05$), and were able to compensate for lack of cysteine by GGT-mediated GSH metabolization (Fig. 2A). BCNU treatment of both cell clones in standard medium revealed no difference in viability at any BCNU concentration (Fig. 2B).

Control 9L cells and parental non-transfected 9L cells grown in standard medium contained slightly higher amounts of intracellular GSH than 9L-GGT cells (9L-control: 2.7 nmol GSH/mg protein; 9L-parental: 2.54 nmol GSH/mg protein, 9L-GGT: 2.15 nmol GSH/mg protein). These differences were not statistically significant. Cells grown for 24 h in cysteine-free medium supplemented with 100 μM GSH were almost totally depleted of intracellular GSH. Under these conditions, 0.04 nmol GSH/mg protein was measured in 9L-controls, which is twice the amount of GSH in 9L-GGT cells (0.02 nmol GSH/mg protein). Treatment of cells for 24 h with 100 μM L-BSO reduced the intracellular GSH content of both cell clones (0.08 nmol GSH/mg protein in 9L-control, and 0.05 nmol GSH/mg protein in 9L-GGT). The differences between intracellular GSH levels of cells cultured in standard medium vs. cysteine-free medium or L-BSO-containing medium are statistically significant ($p < 0.05$).

DISCUSSION

Our study describes immunohistochemically detected GGT expression in a high percentage of human WHO grade III and IV astrocytic gliomas. Sixty-nine percent of all tumors in our series stained positive for GGT. In a glioma cell culture model, GGT overexpression was shown to be of importance for cell proliferation in cysteine-deficient

medium, which is one of the limiting factors for tumor growth in vivo.

GGT expression in primary human glioma

Some GGT-overexpressing malignancies, such as liver (22) and lung (14) carcinomas, are derived from tissues with physiologically rather low GGT expression. Other GGT-overexpressing tumors, such as renal cell cancer (10, 11), arise from organs with high baseline GGT expression (5, 23). On the other hand, normal astrocytes and neurons usually do not express detectable amounts of GGT, as we and others (13) have shown. Therefore, our present findings that brain tumors express GGT suggest the possible use of this enzyme as a tumor marker for the CNS. This is underscored by the fact that the majority of cells in immunopositive tumors tend to express GGT, although the amount of expression in single tumor cells may vary considerably. This variation is probably due to the clonal heterogeneity of a given tumor, since cells with similar GGT immunopositivity were arranged in clusters (clones) which may represent the progeny of a single neoplastic cell (Fig. 1A and B). On the other hand, the subcellular distribution of GGT immunopositivity was only slightly variable. Mainly membrane-bound protein with occasional cytoplasmic staining was demonstrated immunohistochemically. These findings are consistent with the results of immunohistochemical staining with the mAb 138H11 in tumors of the kidney (10), and support the notion that functional GGT carrying the 138H11 epitope is mainly membrane-bound, while cytoplasmic staining may represent different precursor forms of the protein.

GGT expression in cell culture

Our experimental results with rat brain tumor cells in culture suggest a nutritional benefit of GGT-overexpressing cells rather than a direct advantage regarding detoxification and resistance to cytotoxic drugs. There are several opinions concerning the role of GGT in detoxification, and it seems that the function of this enzyme is highly dependent on cell type or the predominant metabolic pathways. Thus, Hanigan et al. (23) described a cytotoxic effect of GGT overexpression on kidney cells in the presence of cisplatin, whereas a human prostate tumor cell line transfected with GGT became resistant to cisplatin. Other

Table 1

Clinical history of patients and GGT immunostaining results of tumors

Pat. no.	Age (years)	Sex	Tumor type	Chemotherapy	Radiation	Recurrence-free survival (months)	Recurrence*	Total survival (months)	GGT immunostaining
1	56	M	GBM	– –	+	7	– –	11	+ +
2	61	M	GBM	– –	+	9	– –	14	+
3	78	F	GBM	– –	– –	1	– –	3	+
4	66	F	GBM	– –	+	10	– –	10	+ +
5	69	F	GBM	BCNU	+	10	– –	10	– –
6	68	M	GBM	– –	+	52	+	64	+ +
7	57	M	GBM	– –	–	5	+	10	– –
8	70	M	GBM	– –	+	6	+	13	+ +
9	62	M	GBM	TK/GCV	+	12	+	14	+ +
10	27	F	GBM	– –	+	4	+	26	+ +
11	76	F	GBM	– –	+	5	+	17	+
12	50	F	GBM	– –	+	3	+	8	– –
13	59	F	GBM	BCNU	+	3	+	6	– –
14	44	M	GBM	– –	+	6	+	16	– –
15	55	M	GBM	– –	+	7	+	7	+ +
16	67	F	GBM	–	–	1	– –	1	+
17	63	F	GBM	–	+	13	+	28	+
18	64	F	GBM	–	+	5	– –	5*	– –
19	59	M	GBM	BCNU	+	7	– –	7*	– –
20	35	F	AA	– –	+	9	– –	9*	+
21	33	M	AA	– –	+	52	+	57	+ +
22	64	F	AA	ACNU/A	+	27	+	38	+ +
23	62	F	AA	– –	+	60	– –	60*	+ +
24	45	M	AA	– –	+	82	– –	82*	+ +
25	72	M	AA	– –	+	19	– –	19*	–
26	36	M	AA	BCNU	+	7	+	9	+

Abbreviations: M = male; F = female; BCNU = chemotherapy with carmustine (BCNU); TK/GCV = gene therapy with thymidine kinase/ganciclovir; ACNU/C = chemotherapy with ACNU and Ara C; ACNU/V = chemotherapy with ACNU and VM26. *Presence of recurrent tumor on MRI or recurrence surgery as of study closure. Degree of GGT immunostaining: – – no GGT staining; + less than 50% GGT-positive tumor cells; + + more than 50% GGT-positive tumor cells.

studies focus on the role of increased intracellular concentration of GSH in resistance against alkylating drugs (24, 25). Our GSH assays revealed a decreased intracellular GSH level in transgene 9L-GGT cells, although GGT is thought to provide cells with abundant precursors of intracellular GSH for its de novo synthesis. Several mechanisms may account for this phenomenon. First, the intracellular amount of GSH is tightly regulated by a negative feedback on the level of cysteinyl-glycine-synthetase, the first enzyme in the intracellular GSH synthesis cycle (4). Because of this autoregulation mechanism, cells with high intracellular amounts of GSH precursor molecules need not necessarily produce more GSH than control cells. Of importance is the fact that cells cannot degrade intracellular GSH, since the presence of the C-terminal glycine protects the peptide against intracellular γ-glutamyl-cyclotransferase (26), and so GSH cleavage is possible only in cells expressing GGT (5, 7, 8). In fact, catabolism of the GSH pool has been postulated to be the main function of GGT in vivo (5, 27, 28). Intracellular GSH concentration is in the range of 0.5–10 mM, being 3 to 4 orders of magnitude lower in extracellular fluid (4, 8, 26). Thus, in a medium with higher extracellular GSH levels, GGT-mediated cleavage of external GSH may increase the intracellular levels of its constituent AA precursors, and therefore lead to an efflux of GSH out of the cell (29). This would provide GGT-expressing cells with a higher turnover but lower intracellular levels of GSH than control cells.

In our cell culture experiments we could not find evidence for a proliferation advantage created by increased GGT-mediated uptake of glutamine. Furthermore, GGT does not seem to enable tumor cells to compensate for a glutamine deficiency by metabolization of extracellular GSH, even when this peptide is present at high extracellular concentrations. Biochemically, there seem to be sound reasons for not cleaving GSH to replenish glutamine. Glutamine is the most abundant amino acid in the body (30, 31). In brain tissue, its concentration can be as high as 10 mmol/L. It serves as the major source of energy in rapidly dividing tumor cells and is utilized in protein and nucleotide synthesis (31, 32). On the other hand, GSH occurs in micromolar concentrations in extracellular fluid (4). Its intracellular synthesis is dependent on ATP-consuming enzymatic reactions and requires two moles of ATP per mole of GSH (4, 26). Therefore, it is unlikely that a physiologic mechanism in vivo would favor degradation of this valuable peptide for the purpose of glutamine substitution. Even if there were an organ or an area with glutamine deficiency, the low extracellular concentration of GSH would be insufficient for substitution.

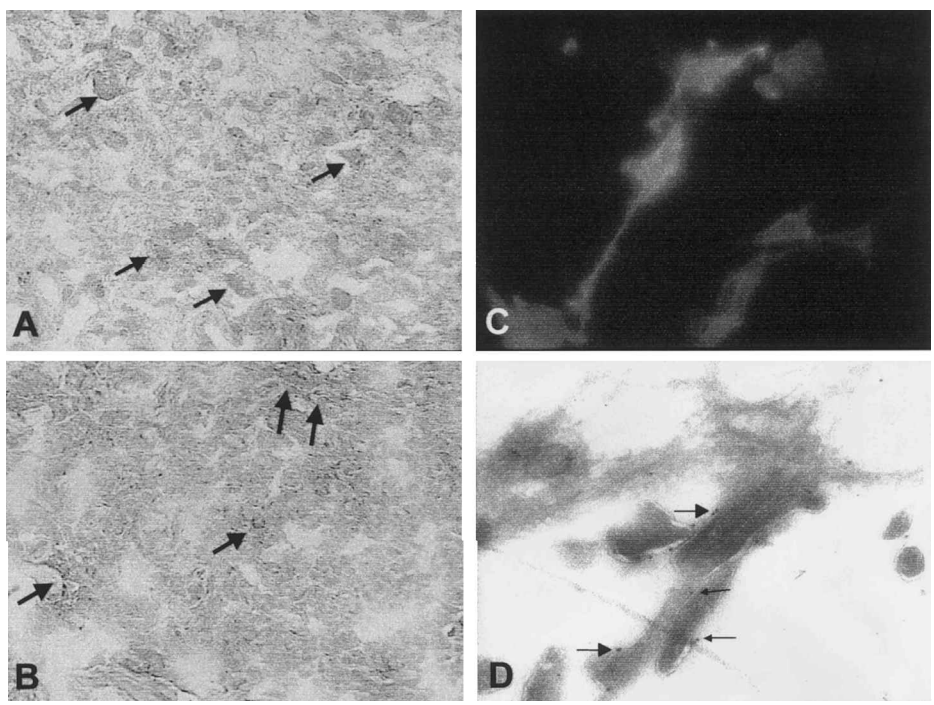

Fig. 1. Photomicrographs of tumor specimens and cultured glioma cells immunostained for GGT expression. A. Anaplastic astrocytoma (WHO G III) with high expression of GGT (arrows), patient #23. Original magnification 240 ×, section counterstained with hematoxylin and eosin. B. *Glioblastoma multiforme* (WHO G IV) displaying strong GGT immunopositivity (arrows), patient #1. Original magnification 240 ×, section counterstained with hematoxylin and eosin. C. Immunofluorescence microscopy of GGT-transfected 9L cells using a secondary Cy3-conjugated antibody demonstrates high expression of transgene GGT. Original magnification 400 ×. D. Immunoelectron microscopy in 9L-GGT cells shows orthotopic membrane-bound expression of transgene human GGT (arrows).

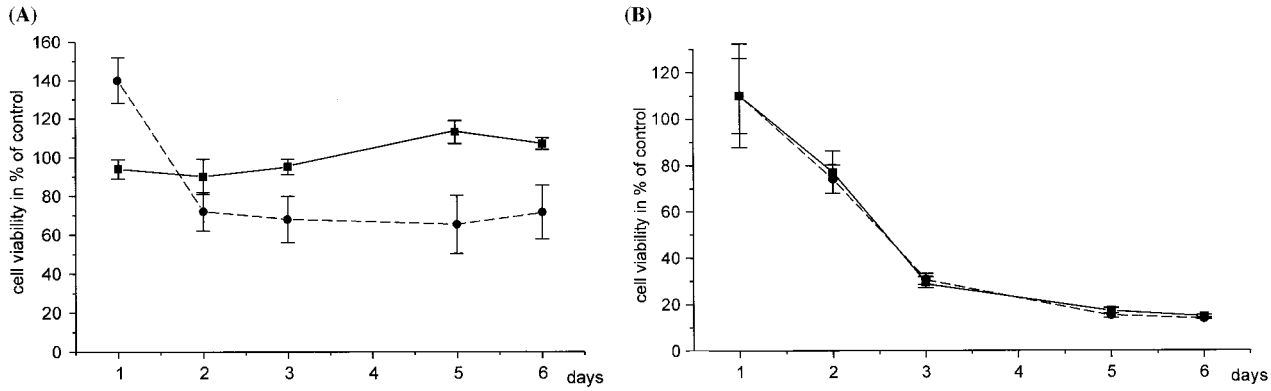

Fig. 2. MTT viability assay in cell culture. 9L-4B1 cells grown in standard medium were used as controls. Values expressed as mean ± standard error (SE). Data points represent averaged results of two independent experiments and four wells/experiments. A. Cell growth in cysteine-free medium supplemented with 100 μM GSH. Differences in numbers of viable cells between the clones are statistically significant ($p = < 0.05$). B. Cell growth in standard medium with 100 μg/ml BCNU. No significant differences were found in numbers of viable cells in either of the cell clones. Key: —■— 9L-GGT; —●— 9L-4B1.

L-cysteine is a non-essential AA in humans and is found in micromolar concentrations in plasma (4, 33). As malignant cells are highly dependent on cysteine, access to this AA may be limiting cell growth in rapidly dividing tumors in vivo (5, 33). Cysteine is cytotoxic in higher concentrations, as it auto-oxidizes rapidly to cystine and produces free radicals (34). Therefore, GSH may serve as a storage and transport form of the cysteine moiety (5). Glioma cells overexpressing GGT are apparently able to use GSH as a source of cysteine in cysteine-deficient medium, which gives them a growth advantage over non-expressing cells, as we (Fig. 2A) and others (5, 23, 35, 36) have shown. It is already known that exogenous cysteine-metabolizing enzymes (L-cysteine desulfhydrase, γ-cystathionase) may inhibit the growth of L1210 leukemia cells without being cytotoxic (33). Therefore, the growth pattern of 9L control cells in cysteine-free medium may reflect the fact that, after an initial crisis and death of tumor cells highly sensitive to low cysteine levels, the remaining less-sensitive tumor cells are not killed, but only blocked in their proliferative capacity (Fig. 2A). On the other hand, GGT-overexpressing 9L cells continue to survive and moderately proliferate in cysteine-free medium supplemented with GSH, as demonstrated by the statistically significant difference between the cell populations ($p < 0.05$, Fig. 2A).

In conclusion, our present findings may lead to the use of GGT as a tumor marker for human astrocytic tumors. GGT seems to endow tumor cells with a moderate growth advantage under in vivo conditions. Further prospective investigations of GGT expression and regulation in brain tumors and in CSF from tumor patients are currently under way.

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REFERENCES

1. Gomi A, Shinoda S, Masuzawa T, Ishikawa T, Kuo MT. Transient induction of the MRP/GS-X pump and γ-glutamylcysteine synthetase by 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea in human glioma cells. Cancer Res 1997; 57: 5292–9.
2. Lorico A, Rappa G, Finch RA, Yang D, Flavell RA, Sartorelli AC. Disruption of the murine MRP (multidrug resistance protein) gene leads to increased sensitivity to etoposide (VP-16) and increased levels of glutathione. Cancer Res 1997; 57: 5238–42.
3. Rappa G, Lorico A, Flavell RA, Sartorelli AC. Evidence that the multidrug resistance protein (MRP) functions as a co-transporter of glutathione and natural product toxins. Cancer Res 1997; 57: 5232–7.
4. Meister A, Anderson ME. Glutathione. Ann Rev Biochem 1983; 52: 711–60.
5. Hanigan MH. γ-glutamyltranspeptidase, a glutathionase: its expression and function in carcinogenesis. Chem Biol Interact 1998; 111–112: 333–42.
6. Orlowski M, Meister A. The gamma-glutamyl-cycle: a possible transport system for amino acids. Proc Natl Acad Sci USA 1970; 67: 1248–55.
7. Griffith O, Meister A. Translocation of intracellular glutathione to membrane-bound γ-glutamyl transpeptidase as a discrete step in the ?-glutamyl cycle. Glutathionuria after inhibition of transpeptidase. Proc Natl Acad Sci USA 1979; 76: 268–72.
8. Griffith OW, Bridges RJ, Meister A. Evidence that the γ-glutamyl cycle functions in vivo using intracellular glutathione. Effects of amino acids and selective inhibition of enzymes. Proc Natl Acad Sci USA 1978; 75: 5405–548.

9. Griffith OW, Novogrodsky A, Meister A. Translocation of glutathione from lymphoid cells that have markedly different γ-glutamyl transpeptidase activities. Proc Natl Acad Sci USA 1979; 76: 2249–52.
10. Fischer P, Scherberich JE, Schoeppe W. Comparative biochemical and immunological studies on gamma-glutamyltransferases from human kidney and renal cell carcinoma applying monoclonal antibodies. Clin Chim Acta 1990; 191: 185–200.
11. Fischer P, Störkel S, Haase W, Scherberich JE. Differential diagnosis of histogenetically distinct human epithelial renal tumours with a monoclonal antibody against γ-glutamyltransferase. Cancer Immunol Immunother 1991; 33: 382–8.
12. Rajpert-De Meyts E, Heisterkamp N, Groffen J. Cloning and nucleotide sequence of human γ-glutamyl transpeptidase. Proc Natl Acad Sci USA 1988; 85: 8840–4.
13. Hanigan MH, Frierson HJ. Immunohistochemical detection of γ-glutamyl-transpeptidase in normal human tissue. J Histochem Cytochem 1996; 44: 1101–8.
14. Hanigan MH, Frierson HF, Swanson PE, De Young BR. Altered expression of gamma-glutamyl transpeptidase in human tumors. Hum Pathol 1999; 30: 300–5.
15. Fine HA. The basis for current treatment recommendations for malignant gliomas. J Neurooncol 1994; 20: 111–20.
16. Shapiro WR, Grenn SB, Burger PC, et al. A randomized comparison of intra-arterial versus intravenous BCNU, with or without intravenous 5-fluorouracil, for newly diagnosed patients with malignant glioma. J Neurosurg 1992; 76: 772–81.
17. Shugg D, Allen BJ, Blizzard L, Dwyer T, Roder D. Brain cancer incidence, mortality and case survival. Observations from two Australian cancer registries. Int J Cancer 1994; 59: 765–70.
18. Kleihues P, Burger PC, Scheithauer BW. Histological typing of tumors of the central nervous system, Second ed. Berlin, Heidelberg: Springer, 1993.
19. Kaufmann O, Dietel M, Scherberich JE, Gaedicke G, Fischer P. Immunohistochemical differentiation of metastases of renal carcinomas versus other carcinomas with anti-γ GT monoclonal antibody 138H11. Histopathology 1997; 31: 31–7.
20. Courtay C, Oster T, Michelet F. GGT: nucleotide sequence of the human pancreatic cDNA. Biochem Pharmacol 1992; 43: 2527–33.
21. Leroy P, Nicolas A, Thioudellet C, Oster T, Wellman M, Siest G. Rapid liquid chromatographic assay of glutathione in cultured cells. Biomed Chromatogr 1993; 7: 86–9.
22. Cheng S, Nassar K, Levy D. γ-glutamyl transpeptidase activity in normal, regenerating and malignant hepatocytes. FEBS Lett 1978; 85: 310.
23. Hanigan MH, Gallagher BC, Townsend DM, Gabarra V. γ-glutamyl transpeptidase accelerates tumor growth and increases the resistance of tumors to cisplatin in vivo. Carcinogenesis 1999; 20: 553–9.
24. Blair S, Heerdt P, Sachar S, et al. Glutathione metabolism in patients with non-small cell lung cancers. Cancer Res 1997; 57: 152–5.
25. Ali-Osman F, Stein DE, Renwick A. Glutathione content and glutathione-S-transferase expression in 1,3–bis(2–chloroethyl)-1-nitrosourea-resistant human malignant astrocytoma cell lines. Cancer Res 1990; 50: 6976–80.
26. Wang W, Balatori N. Endogenous glutathione conjugates. Occurrence and biological functions. Pharmacol Rev 1998; 50: 335–55.
27. McIntyre TM, Curthoys NP. Comparison of the hydrolytic and transfer activities of rat renal γ-glutamyltranspeptidase. J Biol Chem 1979; 254: 6499–504.
28. Shaw LM, Newman DA. Hydrolysis of glutathione by human liver γ-glutamyltransferase. Clin Chem 1979; 25: 75–9.
29. Thioudellet C, Oster T, Wellman M, Siest G. Molecular and functional characterization of recombinant human gamma-glutamyltransferase. Coupling of its activity to glutathione levels in V79 cells. Eur J Biochem 1994; 222: 1009–16.
30. Souba WW. Glutamine and cancer. Ann Surg 1993; 218: 715–28.
31. Klimberg VS, McClellan JL. Glutamine, cancer, and its therapy. Am J Surg 1996; 172: 418–24.
32. Wasa M, Bode BP, Abcouver SF, Collins CL, Tanabe KK, Souba WW. Glutamine as a regulator of DNA and protein biosynthesis in human solid tumor cell lines. Ann Surg 1996; 224: 189–97.
33. Uren JR, Lazarus H. L-cyst(e)ine requirements of malignant cells and progress toward depletion therapy. Cancer Treat Rep 1979; 63: 1073–9.
34. Olney JW, Zorumski C, Price MT, Labruyere J. L-cysteine, a bicarbonate-sensitive endogenous excitotoxin. Science 1990; 248: 596–9.
35. Hanigan MH. Extracellular glutathione is a source of cysteine for cells that express γ-glutamyl transpeptidase. Biochemistry 1993; 32: 6302–6.
36. Hanigan MH. Expression of γ-glutamyl transpeptidase provides tumor cells with a selective growth advantage at physiologic concentrations of cysteine. Carcinogenesis 1995; 16: 181–5.