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Cisplatin induced gamma-glutamyltransferase up-regulation, hypertrophy and differentiation in astrocytic glioma cells in culture

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Summary. Gamma-glutamyltransferase (GGT) hydrolyses gamma-glutamylated peptides, including glutathione and transports amino acids into the cells. The enzyme is up-regulated in some tumors, especially those with a higher degree of malignancy and resistance to cytostatics. In this study we examined the effects of Cisplatin (1.6 x 10^{-5}M) on the activity of GGT in astrocytic C6 glioma cells in cultures monitored for growth, morphology and differentiation. Initially (24 h), the drug inhibited cell division and later (96 h), it caused apoptotic death of about half of the population. The more resistant and surviving cells became hypertrophic and more differentiated, as indicated by their larger size and higher protein content, including the maturation-specific GFAP. In addition, the activity of GGT was significantly elevated in these cells at 48 h and onwards. At 96 h, the biochemically determined enzyme activity was between 230% and 330% above the controls. Compared to the protein content, the GGT activity started to increase later (48 h) but it grew steeper towards 72-96 h. Similarly, histochemical analysis revealed a manifold increase in the number of GGT^+ cells in the population and higher intensity of staining per cell from at 48 h and onwards. The study showed that the transformed astrocytic cells can up-regulate GGT activity as part of an adaptation and/or, survival-enhancing reaction triggered by Cisplatin.

Key words: γ-glutamyltranspeptidase, C6 glioma cells, Cisplatin, cellular stress, astrocyte reactiveness

Introduction

Gamma-glutamyltransferase (GGT, EC 2.3.2.2.) catalyzes the hydrolysis of gamma-glutamylated peptides, including glutathione and transports amino acids into cells using the gamma glutamyl residues as an amino acid acceptor (Meister, 1982; Hanigan et al., 1998a). Under normal conditions, the activity of GGT is high in cells that are active in resorption and secretion (Lojda, 1981; Meister, 1982). Increased GGT activity has been reported in some types of tumors, especially those with a higher degree of malignancy or resistance to chemotherapeutics, including Cisplatin (Sulakhe, 1987; Paolicchi et al., 1996; Hanigan et al., 1998b, 1999; Huang et al., 2001). However, the actual role of GGT in the biology of tumor cells and their responsiveness to cytostatics is still a matter of dispute (Paolicchi et al., 1996; Hanigan et al., 1998a,b). By participating in the metabolism of glutathione, GGT can control the redox potential of cells and the other GSH-dependent functions, including cell division and drug metabolism (Meister, 1982; Chang et al., 1999; Murray, 2000; Schnelldorfer et al., 2000). GGT-rich tumor cells may, therefore, be more active in proliferation, inactivation of chemotherapeutics or the maintenance of the therapy-induced alterations of the oxido-reduction status of cells.

The sensitivity of brain tumors to chemotherapeutics, including Cisplatin varies, though it is low in most cases (Beppu et al., 2000; Ashby and Shapiro, 2001). The resistance has been attributed to an over expression of several molecular species, for instance, O6-alkyl-guanine-DNA alkyltransferase, the multidrug resistance-associated protein and the major vault protein (Cavenee et al., 2000; Mohri et al., 2000; Berger et al., 2001). Higher GGT expression has been recently reported in high-grade glial cell malignancies (Mares et al., 2000; Schafer et al., 2001) but information on the impact of GGT activity on tumor progression or its reactivity to cytostatics is not available though it may also be a factor controlling tumor growth and the results of some therapeutic procedures (see also Addendum).

GGT is well expressed in the transformed astrocyte-like C6 glioma cell line. Its activity decreases in culture at the stage of confluency accompanied by inhibition of

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growth (Morgenstern et al., 1992). Previously, we showed that the exposure of these cells to Cisplatin leads to decreased proliferation and cell death of a part of the population (Mareš et al., 1987; Krajčí et al., 2000). In the present study, we describe the Cisplatin induced up-regulation of GGT activity in C6 glioma cultures accompanied with a hyperplastic reaction, enhanced chemodifferentation and the better survival of cells.

Materials and methods

Cell cultures and Cisplatin treatment

C6 glioma cells (ATCC CCL 107, passage 50-80) were cultured for one to six days in Eagle´s MEM enriched by 10% bovine serum and gentamycin (LEK, Ljubljana, Slovenia) at 37 °C in a humidified air atmosphere with 5% CO_2. On day 3, the cells were exposed to 1.66×10^{-5} M Cisplatin (cis-dichlorodiamine-platinum II, Lachema, Brno, CR) for 90 min. After a brief washing, the cells were cultured in fresh medium for another 24 to 96 h. This treatment better defines the actual time of exposure of cells to Cisplatin and was best for inducing cytostatic effect and the differential survival of cells. Control and treated cells were cultured for the same time (age-matched controls).

Morphology and growth

The cells were followed-up intravitally by phase-contrast microscopy. Growth of the population was evaluated by counting cells in suspensions prepared by mild trypsinization (0.2% trypsin in PBS, Sigma, St. Louis).

Determination of glial acidic fibrillary protein (GFAP)

Light-microscope immunochemistry

The cells on cover slides were stained by monoclonal anti GFAP antibody (Exbio, Prague, C.R., 1:25) and goat anti-mouse IgG (Fab specific, 1:200) FITC conjugate or biotin conjugated goat anti-mouse IgG (Fab specific, 1:300) followed by Extravidin-Peroxidase (1:100, Sigma, St. Louis, USA for all secondary antibodies) and by the conventional diaminobenzidine reaction enhanced by 5% nickel chloride solution. The cells were examined by transmission light- or epi-fluorescence microscopy (Axioplan, Opton).

Flow cytofluorimetry

The cell suspensions prepared by trypsinization (see above) were washed in calcium-free phosphate-buffered saline (PBS) and fixed in 70% cold ethanol. The PBS-washed cells were incubated with monoclonal anti GFAP antibody (Exbio, Prague, C.R., 1:25) and rabbit anti-mouse IgG-FITC conjugate (Fab specific, 1:200, Sigma, St. Louis, USA). After washing, the cells were measured with a FACStar (Becton Dickinson, San José, USA) and the data were evaluated by Windi 2.8 software.

Determination of GGT activity and protein content

Histochemistry

The cells grown on glass coverslips in plastic Petri dishes were fixed in acetone and chloroform (2 min, 1:1, +4 °C). The samples were incubated in a solution of γ-glutamyl-4-methoxy-naphtylamide and Fast Blue B (2.0 mg and 2.5 mg in 10 ml PBS, respectively) (Bachem, Bubendorf, Switzerland and Fluka, Germany) and glycyl-glycin (20 mM, Sigma, St. Luis, USA, pH 7.4, +4 °C). Subsequently, the specimens were washed in 4% paraformaldehyde overnight at +4°C followed by a water bath and mounting in Apathy syrup. In staining controls, the GGT substrate or glycyl-glycin was omitted in the incubation medium (Lojda, 1981) or GGT was inhibited by serine borate (13 mM, Sigma, St. Louis, USA). The relative number of stained cells was determined under an occular grid (Axiophot, Opton, obj. x40). Quantitative evaluation of staining was performed in digitalized microscopic pictures (Olympus Provis) by the Advanced Image Data Analyzer Program (AIDA 2.11, Raytest Isotopengeraete GmbH, Germany) and the data were expressed as the average staining density per unit area.

Biochemistry

GGT activity was estimated at room temperature by the fluorogenic substrate γ-glutamyl-aminomethyl-coumarine (Glu-NHMec, Bachem, Bubbendorf, Switzerland) in the presence of glycyl-glycine (Gly-Gly-OH, Sigma-Aldrich, St. Louis, USA). We used a continuous rate assay in the modification for 96 well plates (Malík et al., 2001). Briefly, each well contained 180 μl 0.1M phosphate buffer, pH 8.0 with 50 mM Glu-NHMec, and 1 mM Gly-Gly-OH. The reaction was started by adding 20 μl aliquots of cell lysate (0.1% Triton X-100, Sigma, St. Louis). The NH2-Mec released from the substrate was monitored on an LS 50B fluorimeter (Perkin-Elmer, Ueberlingen, Germany) at excitation and emission wavelengths of 380 nm and 460 nm, respectively. The assays were standardized with NH2-Mec in the assay buffer. Enzyme activity was expressed as nkat/mg protein or as nkat/cell. Protein determination was carried out by a colorimetric method (Markwell et al., 1981) with bovine serum albumin as a standard. The cells were counted before each assay in suspensions prepared by 0.02% EDTA in Ca^{2+} free PBS (Sigma, St. Louis).

Statistics

The data are expressed as means ± S.E.M and were tested by Student´s t-test or the Newman-Keuls's one-

sample t-test against controls equal to 100% and within the group of treated cells by analysis of variance.

Results

Population growth, cell morphology and GFAP expression

Compared to age-matched controls, the number of cells in cultures treated with Cisplatin did not increase within the initial 24 h post-treatment (p-t.) interval. In agreement with this, mitoses were not found after this p-t. interval. At 48 h, the cell counts decreased below the values before administration of Cisplatin; at 96 h, only 48.5 % of the initial number of cells survived. The lethally damaged cells were retracting their processes, shrinking and breaking-down into apoptotic-like bodies. Finally, the damaged cells, or their fragments appeared either floating in the culture medium or remained loosely attached to the growth-support adherent living cells. The surviving cells progressively grew in size and formed long, thick and smooth processes free of the intermittent thickenings, gliosomatas, present in control cells (Fig. 2a,b) and their nucleoli were enlarged and of an activated appearance. As shown in our earlier EM study, these cells were often phagocytosing the cell debris formed at the earlier p-t. intervals (Krajčí et al., 2000). As was revealed by immunocytochemistry and flow-cytofluorometry (Figs. 1e,f, 2, 4), the GFAP content in the surviving populations was higher than in the control cultures at 48 h and onwards. The most brightly GFAP-fluorescing cells were often also the most hypertrophic and had the most pronounced cell processes (Fig. 1f).

GGT activity and protein content

Biochemical assay

Compared to age-matched cultures, the total activity of GGT per cell in Cisplatin- treated samples increased with post-treatment time, starting with +14.1% (n.s.) at 24 h and reaching +132.4%, +203.6% and +316.2% at 48, 72 and 96 h p-t. intervals, respectively (Fig. 3a, $p<0.001$). In treated cultures, there was also a substantial increase in the protein content per cell, starting with +63.9% at 24 h and reaching +186% towards 96 h (Fig. 3b, $p<0.001$). As a consequence, the activity of GGT per mg protein initially dropped (-31.2% at 24h, $p<0.001$), but later it started to rise and reached a 146% peak at the 72-96 h interval (Fig. 3 c, $p<0.001$).

Histochemical assay

Compared to age-matched samples, the frequency of GGT-positive cells (GGT^+) in Cisplatin-treated cultures dropped from 12.2% to 3.9% at the 24 h p-t. interval (Table 1). At 48 h, the number of GGT^+ cells exceeded controls by 102.7% ($p<0.05$) and at 72-96 h, by 180.7-186.0% ($p<0.001$, Table 1). As shown by microdensitometry, starting from the 48 h p-t. interval, the GGT^+ cells were also more densely stained (from +35 to +68%, $p<0.01$, Table 1, Fig. 1c,d). The maximum staining often appeared in the most hypertrophic cells.

Discussion

As revealed by the changes in C6 glioma cell numbers (Figs. 1, 2), Cisplatin inhibited their proliferation and induced apoptosis of a substantial part of the population. The surviving, and/or, the Cisplatin more resistant cells underwent hypertrophy and differentiation indicated by their enlargement, fiber growth, increased content of protein, including the astrocyte- specific GFAP. Importantly, GGT was up-regulated in most of these cells at 48 h and onwards. This was evidenced both biochemically and histochemically; the latter included an increased number of the GGT^+ cells in the population and the higher intensity of staining of the individual cells (Fig. 1c,d, Table 1). As follows from the growth curves (Fig. 2), the increased activity of GGT appeared at the time when the cytostatic effect was fully developed (Mareš et al., 1987; Krajčí et al., 2000). Moreover, the cells became clearly hypertrophic and more differentiated. Therefore, we

Table 1. Histochemistry of GGT in Cisplatin- treated C6 glioma cells.

GROUP AND TIME	CONTROLS		CISPLASTIN TREATMENT	
	Frequency	Staining density	Frecuency	Staining density
24 h	12.2±3.49%	0.059±0.0058	3.9±2.02% (-68.1%, n.s)*	0.057±0.005 (-3.4%, n.s)
48 h	25.6±6.91%	0.094±0.0045	51.9±6.46% (+102.7%, p<0.05)	0.158±0.011 (+68.1%, p<0.01)
72 h	19.2±2.94%	0.111±0.0091	53.9±4.38% (+180.7%, p<0.001)	0.150±0.006 (+35.1%, p<0.001)
96 h	25.0±6.26%	0.128±0.0068	71.5±6.72% (+186. 0, p<0.001)	0.180±0.007 (+40.6%, p<0.001)

*: Student's t-test; The "p" values refer to statistical significance of the differences between control and Cisplatin-treated samples.

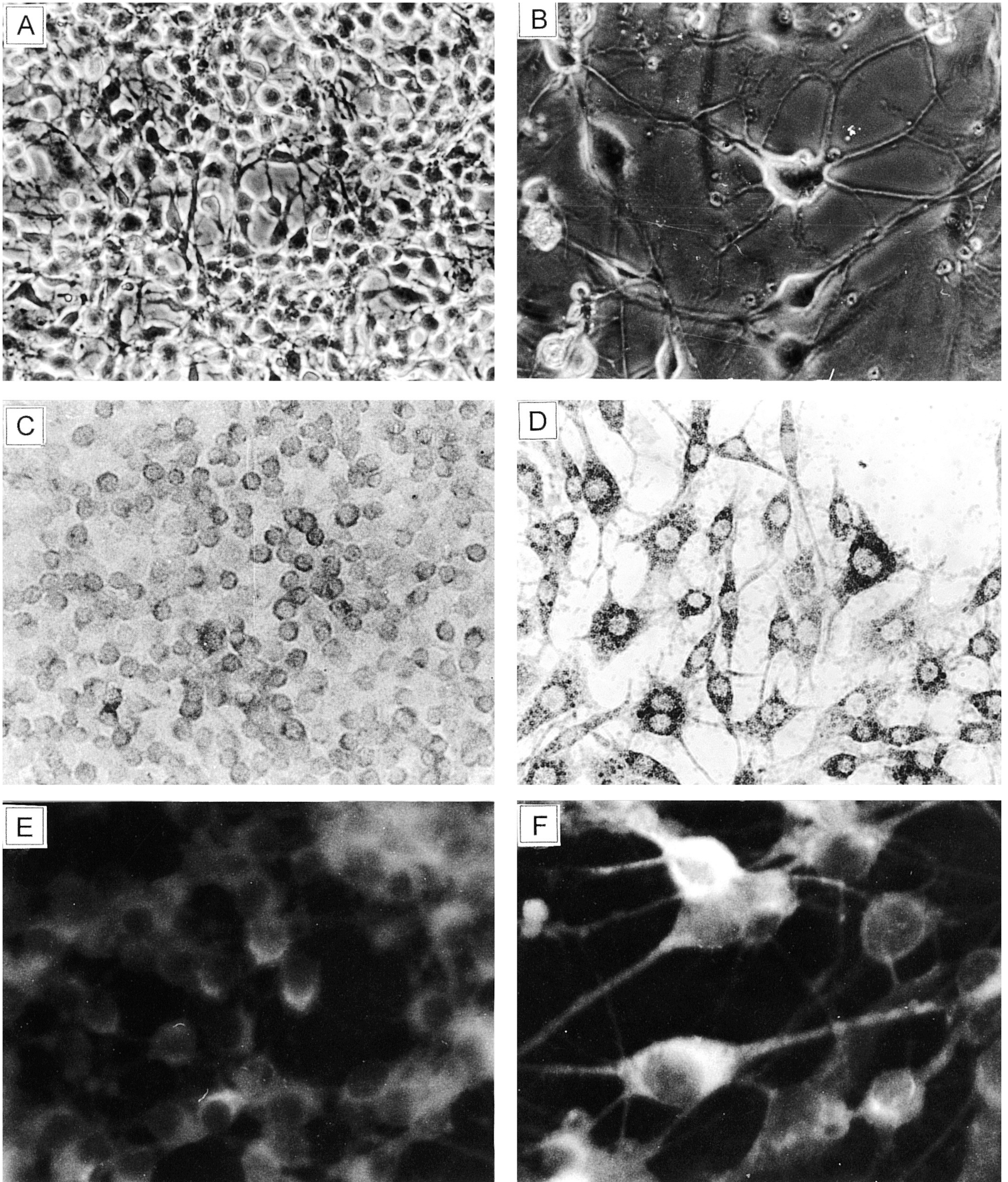

Fig. 1. Microscopical changes in morphology (**a, b,** phase contrast), activity of gamma glutamyltransferase (**c, d,** transmission microscopy) and Glial Fibrillary Acidic Protein (**e, f,** FITC-immunocytochemistry) in C6 glioma cells grown under basal conditions and after treatment with Cisplatin. Axiophot, Opton, obj. x 40.

assume that the up-regulation of GGT activity is a part of the adaptation, and/or, the defense reaction of cells to Cisplatin resulting in their better survival. This role of GGT may be anticipated both in the enhanced transport of amino acids into the cells undergoing hypertrophy or the increased turnover of GSH. GSH could be engaged in the attenuation of the Cisplatin effects via the formation of inactive GSH-Cisplatin conjugates or a more effective maintenance of the cellular redox potential, that was likely altered by the increase of oxygen- free radicals that results from the Cisplatin damage of mitochondria (Gordone and Gattone, 1986; Kruidering et al., 1997; Baliga et al., 1999; Krajčí et al., 2000; Murray, 2000). The importance of GSH in the cellular response to Cisplatin and some other cytostatics has been shown in several types of non-neuroectodermal tumors (Schnelldorfer et al. 2000; Hanigan et al., 1998a,b,1999; Zhang et al., 2001). The participation of GGT in GSH metabolism can, therefore, be one of the reasons for its up-regulation in some tumors after treatment with Cisplatin (Godwin et al., 1992; Borud et al., 2000).

Data on GGT activity in brain tumors, as well as its changes after chemotherapy, including by Cisplatin, are limited. A higher content of GGT-molecules was recently reported in higher-grade human astrocytic gliomas by immunocytochemistry (Schafer et al. 2001). A higher catalytic activity of GGT in some human glioblastoma biopsy samples has been shown also in our preliminary study (Mares et al., 2000).

The actual role of GGT in the response of cells to Cisplatin, especially as to their survival, requires further study, including the examination of other neuroectodermal cell lines and pathophysiological conditions, including other cytostatics. As shown in normal kidneys of human and animals, a high endogenous activity of GGT can also potentiate the Cisplatin effects due to a more intense formation of toxic metabolites from the Cisplatin-GSH conjugates (Townsed and Hanigan, 2002). In some studies, no significant correlation between GGT activity and the sensitivity of cells to Cisplatin, or tumor characteristics and their progression were found in ovarial tumors in human patients (Paolicchi et al., 1996, Hanigan 1998b, Hanigan et al., 1999). Also in the present study, some cells that survived Cisplatin treatment had only a

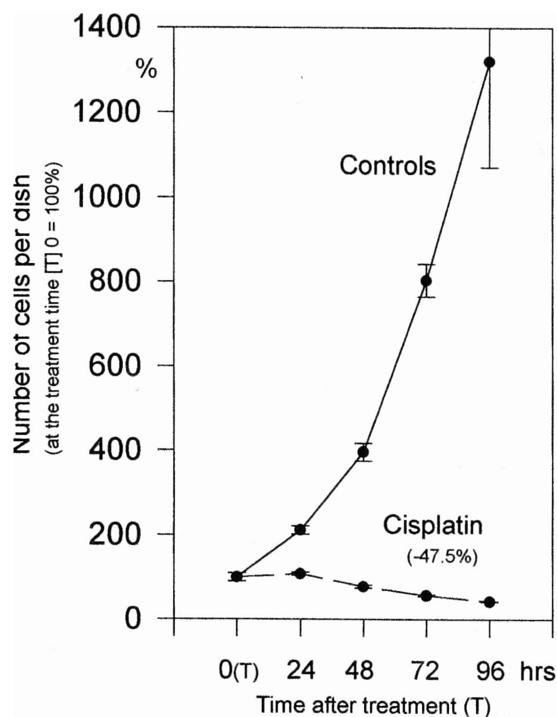

Fig. 2. Growth curves of C6 glioma cells in control- and Cisplatin-treated cultures.

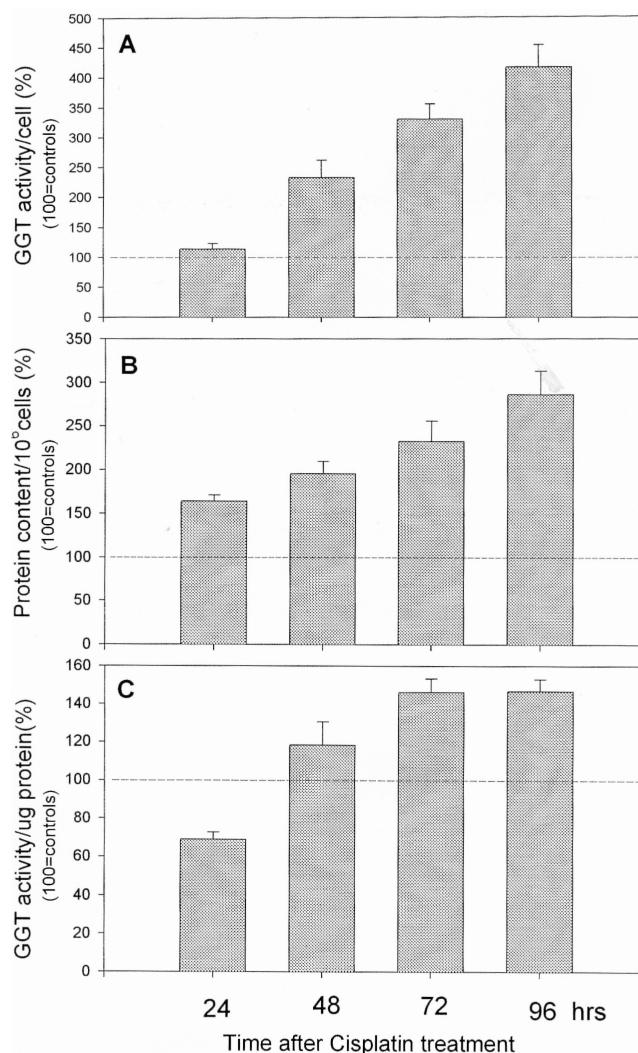

Fig. 3. Effects of Cisplatin on activity of gamma glutamyltransferase and protein content in C6 glioma cells determined biochemically.

threshold amount of GGT. In addition, the GGT up-regulation described in the present study should not be exclusively related to the cytostatic effects of Cisplatin. Because of the GGT involvement in GSH metabolism, its activity in astrocytic cells may increase also under other pathophysiological conditions accompanied by metabolic activation and oxidative stress. This is supported by our preliminary histochemical data on the increased activity of GGT in hypertrophic astrocytes in the human brain with post-irradiation gliosis (Mareš et al., 2000). Moreover, the up-regulation of GGT in astrocytes may also reflect alterations in GSH levels in neurons (Dringen et al., 1999). More information on GGT activity in tumor- and non-transformed astrocytes could, therefore, provide better insight into not only the growth and therapy of brain tumors but also the pathogenesis of some non-neoplastic glial cell hypertrophies that accompany, for instance, formation of glial scars, senile gliosis and neuronal degenerations. The high sensitivity of astrocytes to physiological and pathological agents has been demonstrated by changes in morphology, GFAP, glycogen and some growth factor receptors (Necchi et al., 1997; Castejon et al., 2001; Vinores et al., 2001). The present study extends the list of reactive molecules in astrocyte-like cells to GGT though its actual role and the molecular mechanisms of its up-regulation by Cisplatin remain to be determined.

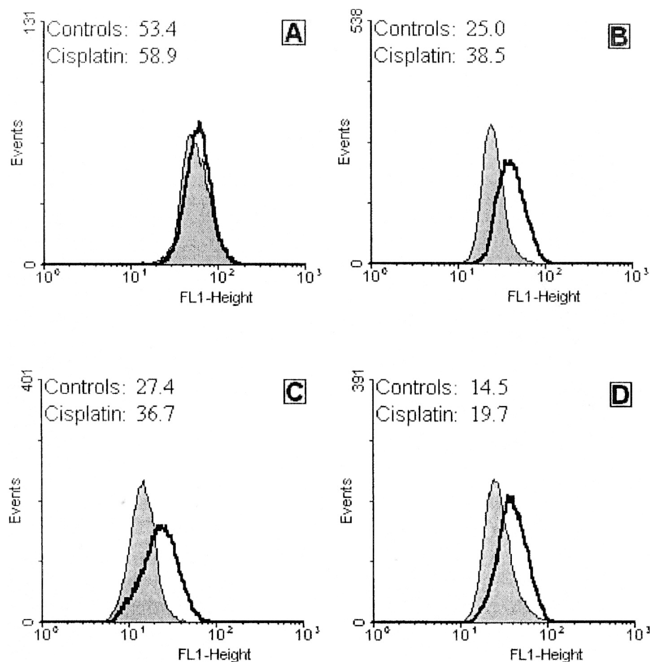

Fig. 4. The content of GFAP in C6 glioma cells treated with Cisplatin-measured by flow FITC-immunocytometry. Axes x and y: intensity of fluorescence per cell and number of cells, respectively. The inserted figures are median values of the fluorescence in the population. The filled and empty histograms indicate controls and treated cells. **A, B, C, D**: post-treatment intervals 24, 48, 72 and 96 h, respectively.

Addendum

During revision of the manuscript, the up-regulation of GGT, including the increase in the corresponding mRNAs has been reported in HeLa cells exposed to Cisplatin (Daubeuf et al. 2002, Biochemical Pharmacology 64, 207-216, 2002). This was more pronounced in the HeLa-GGT transfected cells which appeared also to be more resistant to Cisplatin due to a likely more effective inactivation of the drug by cysteinylglycin released from the extracellular GSH by the cell surface-bound GGT.

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