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Commentary

Expression of γ-glutamyltransferase in cancer cells and its significance in drug resistance

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ABSTRACT

The expression of γ-glutamyltransferase (GGT), a cell surface enzyme involved in cellular glutathione homeostasis, is often significantly increased in human tumors, and its role in tumor progression, invasion and drug resistance has been repeatedly suggested. As GGT participates in the metabolism of cellular glutathione, its activity has been mostly regarded as a factor in reconsitution of cellular antioxidant/antitoxic defences. On this basis, an involvement of GGT expression in resistance of cancer cells to cytotoxic drugs (in particular, cisplatin and other electrophilic agents) has been envisaged. Mechanistic aspects of GGT involvement in antitumor pharmacology deserve however further investigations. Recent evidence points to a more complex role of GGT in modulation of redox equilibria, with effects acting both intracellularly and in the extracellular microenvironment. Indications exist that the protective effects of GGT may be independent of intracellular glutathione, and derive rather from processes taking place at extracellular level and involving reactions of electrophilic drugs with thiol metabolites originating from GGT-mediated cleavage of extracellular glutathione. Although expression of GGT cannot be regarded as a general mechanism of resistance, the involvement of this enzyme in modulation of redox metabolism is expected to have impact in cellular response to several cytotoxic agents. The present commentary is a survey of data concerning the role of GGT in tumor cell biology and the mechanisms of its potential involvement in tumor drug resistance.

1. Introduction

γ-Glutamyltransferase activity (GGT; EC 2.3.2.2) is present in the plasma membrane of virtually all cells, but high enzyme levels are expressed in few districts of the body, i.e. kidney tubules, biliary epithelium and brain capillaries. On the other hand, GGT is normally found in serum where it is a marker of liver diseases, lower or trace levels are detectable in many more cell types, e.g. blood cells, endothelium, and notably secretory and absorptive cells [1]. GGT catalyzes the first step in the degradation of extracellular glutathione (GSH), i.e. the hydrolysis of the gamma-glutamyl bond between glutamate and cysteine (Fig. 1). In this process GGT releases cysteinyl-glycine, which is subsequently cleaved to cysteine and glycine by plasma membrane dipeptidase activities; thus, GGT-mediated metabolism of extracellular GSH appears to provide cells with a means for the recovery of cysteine, whose adequate supply is critical for protein synthesis especially in rapidly dividing neoplastic cells [2]. Therefore, this process could confer growth and survival advantages for tumor cells. Extensive research has been dedicated to the relationships between GGT activity and cellular glutathione levels, which

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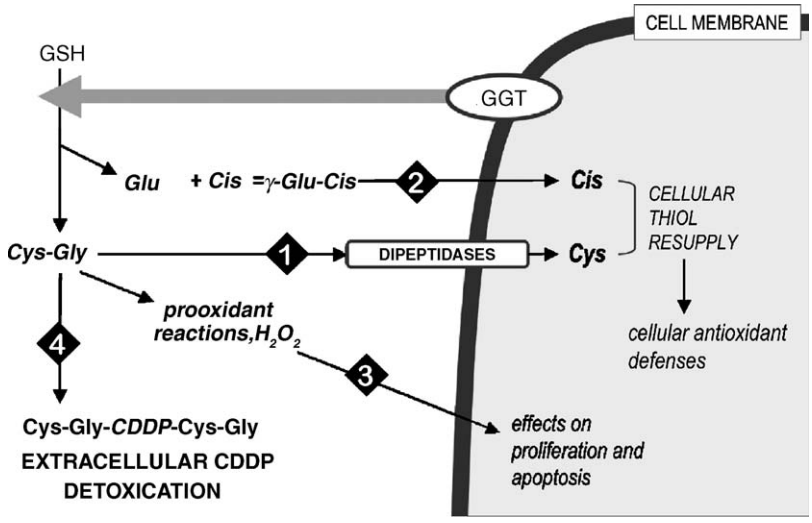

Fig. 1 – Distinct mechanisms through which γ-glutamyltransferase (GGT) activity can affect cell sensitivity to antitumor drugs. The cellular supply of thiols, critical for the maintainance of intracellular glutathione (GSH) levels, is favoured in two ways: (1) cysteinyl-glycine (Cys-Gly) originating from cleavage of extracellular GSH is hydrolyzed by membrane dipeptidases, and the resulting cysteine can be transported into the cell; (2) reaction of glutamic acid with cystine (Cis) leads to formation of γ-Glu-Cis, and in this form cystine can enter the cell. On the other hand, the prooxidant species produced extracellularly during GGT activity can independently affect the proliferative/apoptotic balance of the cell (3), and the prompt reactivity of cysteinyl-glycine with cisplatin (CDDP) leads to formation in the extracellular milieu of CDDP/thiol adducts whose cellular absorption is slower ("*extracellular* CDDP detoxication") (4).

are implicated in modulating the cytotoxic action of prooxidant and electrophilic agents. A number of studies have thus attempted to characterize GGT expression in tumors as a potential mechanism of cellular drug resistance; several aspects of the matter however require further elucidation.

2. GGT expression, GSH metabolism and cellular antioxidant defences

Cysteine resulting from GGT-mediated metabolism is essential for the intracellular de novo synthesis of glutathione. As the expression of GGT is often increased in tumors, it was proposed that GGT could play an important role in the utilization of GSH by tumors. Several lines of evidence support this interpretation. Rajpert-De Meyts et al. investigated the role of GGT in the cellular utilization of GSH by using transfected NIH-3T3 fibroblasts stably overexpressing GGT (≈200-fold). Following exposure of cells to GSH-depleting agents, GGT-transfected cells were able to utilize extracellular GSH much more efficiently than control cells, because lower concentrations of extracellular GSH were sufficient to sustain intracellular GSH levels, and the reconstitution of depleted GSH occurred more rapidly [3]. In 3-methylcholanthrene-induced rat sarcomas, Hochwald et al. observed a preferential utilization of circulating GSH as compared to host tissues; the phenomenon was dependent on GGT activity, as shown in experiments employing the irreversible GGT inhibitor acivicin [4].

Intracellular GSH is implicated in cellular defenses against prooxidant agents, although this is not to be considered the sole function of GSH within the cell [5]. A number of studies thus investigated the contribution of GGT-mediated utilization of extracellular GSH to protection of cells in conditions of oxidative stress. Shi et al. [6] used 3T3 fibroblasts stably overexpressing GGT activity, and observed that the exogenous administration of GSH to these cells could help maintain intracellular GSH and ATP levels, thus preventing injury caused by hydrogen peroxide; the protective effect was lost after GGT inhibition with acivicin. In agreement with these observations, a sublethal quinone-induced oxidative stress results in a rapid elevation of GGT-mRNA levels in rat lung epithelial cells, and this increase makes cells more resistant to a subsequent challenge with an otherwise lethal oxidative stress [7].

The metabolism of extracellular GSH can affect cellular functions also by modulating the availability of glutamic acid. The latter in fact, produced during GGT-mediated hydrolysis of extracellular GSH, can enter the cell and is direct precursor for the synthesis of glutamine by ATP-dependent glutamine synthase. Thus, GGT activity can cooperate in maintaining the intracellular supply of glutamine which, although not being an essential amino acid, is critical for the growth of normal as well as neoplastic cells. Kang et al. reported that the proliferative arrest observed when A549 lung carcinoma cells were cultured in glutamine-deficient medium was partially reversed by exogenous GSH, and the stimulatory effect was ascribed to the generation of glutamic acid via the GGT-mediated hydrolysis of GSH [8].

3. GGT expression in experimental carcinogenesis and human neoplasia

The connection between GGT and neoplastic transformation was highlighted in several experimental models of chemical

Table 1 – Patterns of GGT expression in human neoplasia

Site of cancer	Findings	References
Lung	Expression in bronchogenic carcinomas	[57]
	Expression in non-small cell cancer but not in normal/hyperplastic bronchial mucosa	[58]
Ovary	Extreme variability, but constantly lower levels in benign as compared to malignant lesions	[30]
	Expression in most common epithelian varieties, heterogeneous distribution	[59]
Breast	Diffuse cytoplasmic localization in malignant lesions, as compared to apical/luminal staining in normal epithelium and non-dysplastic lesions	[60]
	Correlation between GGT expression and unfavourable prognostic signs (lymph node metastasis, absence of oestradiol receptors)	[61]
	Increase of GGT-negative cells during progression from normal epithelium to atypical hyperplasias and infiltrating breast carcinomas	[62]
Prostate	No correlation between number of positive cells and parameters of tumor progression	[63]
Brain	GGT expression in astrocytic glioma associated with higher grade lesions	[64]
Skin	No expression in benign papillomas	[65]
	Intense positivity in squamous carcinomas, but not in basal cell carcinoma or most benign tumors	[66]
Melanoma	Correlation of expression with parameters of invasivity in metastatic melanoma	[67]
	Correlation of expression with increased metastatic potential of B16 melanoma cells	[68]
	Elevation of serum GGT in patients with metastatic melanoma, probably of tumoral origin	[69]
	Tumoral origin of serum GGT demonstrated in mouse melanoma model	[70]
Sarcoma	Expression present in Ewing's sarcoma	[71]
Leukemias	Higher percentage of GGT positive cells in chronic B-cell lymphocytic leukaemia and chronic myelogenous leukaemia	[72]
Liver	Hepatoma-specific GGT forms as sensitive marker for diagnosis of hepatocellular carcinoma	[73–75]
Colon	Increased expression in tumor tissue	[76]
	No increase of expression	[77]
Pancreas	Alterations in the protein glycosylation pattern in four of five GGT forms expressed by cancer cells	[78]

carcinogenesis in laboratory animals. A number of earlier, now classical, studies described the appearance of GGT activity in previously negative cells and areas of tissues (e.g. rat liver, mouse skin, hamster tracheal epithelium) exposed to the action of carcinogens. In the same areas, increased cell proliferation was also observed, often followed by the appearance of malignant tumors. GGT expression was thus interpreted as an early marker of neoplastic transformation.

The mechanisms underlying the increased GGT expression induced by carcinogenic treatments were however not at all clear. One early study focused the attention on the oncogene *ras*. In fact, while enabling cells to grow in nude mice, *ras*-transfection of hepatocytes also induced the expression of GGT; thus, a connection was established between activation of *ras*-dependent transduction pathways and regulation of cellular GGT [9]. This finding was recently confirmed in γ-radiated colon cancer cells. Indeed, Pankiv et al. have documented that the induction of GGT activity after low dose γ-radiation of CC531 colon carcinoma cells is an effect of radiation-induced activation of *ras* signalling [10].

Morevover, expression of GGT may have a role in tumor progression, as suggested by a number of observations. Transfection of a murine epidermal cell line with GGT cDNA resulted in a growth advantage of cells transplanted into nude mice [11]. Comparable results were obtained by Hanigan et al. with GGT-transfected PC3 prostate cancer cells [12]. Taken together, these and other studies of experimental carcinogenesis support the view that the expression of GGT in cancer cells may represent an important factor in the appearance of a more aggressive and resistant phenotype. As far as human malignancies, variations of GGT levels have been described in a number of human tumors and tumor-derived cell lines. A comprehensive analysis of GGT expression in 451 human malignant neoplasms showed that tumors derived from GGT-positive or negative cells generally maintained their phenotype, with the exception of most lung and ovarian carcinomas, expressing GGT although arising from GGT-negative cells [13]. A summary of the findings concerning GGT expression in a series of important human neplasms is reported in Table 1.

4. GGT expression and drug resistance

Since the first studies implicating GGT expression in carcinogenesis, the central role played by this activity in glutathione metabolism and cellular antioxidant systems has received high attention. GGT expression was considered as part of a '*resistance phenotype*' exhibited by pre-neoplastic transformed cells, i.e. a common pattern of biochemical changes suggesting that preneoplastic cells might be better equipped for defence against toxic xenobiotics. These changes included a decrease in several biotransformation activities (cytochrome P450, cytochrome *b*5, aminopyrine *N*-demethylase) along with increased glutathione, γ-glutamyltransferase and (cytosolic) glutathione *S*-transferase activities [14]. Cellular levels of reduced glutathione are a major defense factor against electrophilic agents like platinum compounds, including

cisplatin (cis-diamino-dichloro-platinum, CDDP) and oxaliplatin, and other alkylating agents (e.g. cyclophosphamide, melphalan) [15]. Thus, as GGT favours the reconstitution of intracellular GSH, it was proposed that GGT expression in tumor cells may participate in mechanisms of drug resistance [16].

Several studies have supported this interpretation. Exposure of ovarian cancer cells to cisplatin leads to the appearance of CDDP-resistant sublines, and the degree of resistance is closely correlated with increased expression of GGT mRNA and glutathione levels [15]. Ahmad et al. found that murine leukemia cells resistant to L-phenylalanine mustard expressed two- to three-fold GGT activities, and presented higher GSH contents as compared to drug-sensitive counterparts [17]. In ovarian carcinoma cells, the onset of resistance to CDDP, chlorambucil and 5-fluorouracil was associated with higher intracellular GSH and a marked increase (6.5-fold) of GGT activity [18]. Similarly, it was reported that CDDP-resistant astrocytic C6 glioma cells exhibited a significant elevation of GGT activity, histochemically related to a manifold increase in the number of GGT positive cells [19]. Transfection of PC3 prostate cancer cells with GGT cDNA was found to result in an increased resistance to cisplatin [12]. Consistently, Benlloch et al. reported that acivicin-induced inhibition of GGT activity resulted in sensitization of B16 melanoma cells towards endothelium-induced oxidative/nitrosative injury [20].

The favouring action of GGT expression on the reconstitution of intracellular glutathione can be explained first of all by the fact that GGT-mediated breakdown of extracellular GSH provides cells with a secondary source of cysteine [21]. In GGT-transfected HeLa cells cultured in cysteine-deficient media, it was shown that after GSH depletion only the expression of GGT allowed the recovery of intracellular GSH [22]. Hanigan compared the proliferation abilities of GGT-negative and GGT-transfected Hepa 1–6 mouse hepatoma cells; in standard culture medium both cell lines grew equally well, but when the cysteine concentration of the medium was reduced to physiologic levels similar to those existing in vivo, the GGT-positive cells exhibited a growth advantage [23]. Akin results had been reported previously, comparing the growth ability of a mouse epidermal cell line in vitro and in vivo after transplantation in nude mice [11]. In both studies, the interpretation was that GGT-positive tumor cells are able to recover cysteine from the cleavage of extracellular glutathione, thereby overcoming the growth restriction imposed by low cysteine availability.

It should be considered however that the facilitation effect offered by GGT on the cellular supply of thiols is mediated through an additional mechanism, i.e. the facilitation of cellular transport of cystine. Direct transport of cystine can occur through the X_c^- aminoacid system, and an up-regulation of this transporter has been recently observed in cisplatin-resistant A2780 ovarian carcinoma cells [24]. But cystine actually also is a good substrate for the transpeptidation reaction effected by GGT, and the γ-glutamyl-cystine formed in the reaction can be efficiently transported [25]. Such a mechanism was shown to participate in cellular resynthesis of GSH in HUVEC endothelial [26], PaTu8902 pancreatic duct [27] and rat liver oval cells [28].

The data summarized above support a potential contribution of GGT expression in drug resistance of cancer. However, several studies focused on this aspect have reported conflicting results, and controversy still exists concerning the mechanisms underlying such inconsistencies. In particular, no precise correlation was observed between GGT expression and resistance to cisplatin in a series of patients with germ cell tumors [29]. Ovarian cancers exhibit a large variability in GGT activity values among the different patients, and no induction was found following platinum-based therapy [30]. A specific point of controversy is represented by intracellular GSH levels. Glutathione content and related enzyme activities were assessed in a series of human tumor xenografts representative of platinum-responsive (i.e. small-cell lung cancer and ovarian carcinoma) and platinum-resistant tumor types (i.e. non-small-cell lung cancer and colorectal carcinoma). No significant correlations were found between glutathione levels and response to cisplatin treatment [31]. Lower levels of GGT were actually found in cisplatin-resistant A2780 ovarian cancer cells, and no correlation between GGT expression and intracellular GSH levels was found [32]. Conflicting results even exist concerning the favouring effect of GGT expression on intracellular GSH content. Warren et al. reported that GSH content in transplanted tumors derived from GGT-transfected cells was actually one-third of that detectable in control tumors [11]. Lower levels of intracellular GSH were observed in Ramos lymphoblastoid cells following GGT transfection [33], and the same result was found by us after inhibition of GGT activity in U937 histiocytoma cells [34]. These apparent inconsistencies can be explained in the light of recently described *prooxidant* effects of GGT activity. In fact, GGT-mediated metabolism of GSH is accompanied by metal-catalyzed redox reactions, during which prooxidant species are produced; as discussed below, an upregulation of GGT may therefore paradoxically impose an increased oxidative burden on the cell, which may in turn result in a decrease (rather than increase) of cellular GSH stores.

5. GGT and extracellular versus intracellular thiols

The available data do not consistently support a general role of GGT-mediated modulation of intracellular GSH in drug resistance. Recent observations suggest rather that critical processes may occur *extracellularly* and that the species primarily involved in the resistance of GGT-expressing tumors to electrophilic drugs can be instead cysteinyl-glycine, i.e. the reactive thiol metabolite originating from GGT activity. It had been shown by Kröning et al. that sulfur aminoacids (in particular, cysteine) can reduce CDDP cytotoxicity in cultured renal tubule cells, due to the formation of a CDDP/cysteine complex which is less toxic than free CDDP [35]. Our findings with HK-2 renal tubule cells confirmed that thiol adducts of CDDP (CDDP/GSH, CDDP/cysteinyl-glycine) are less toxic than the parental drug, and that pre-complexation of CDDP with both thiols results in a decreased intracellular accumulation [36]. Further investigation is needed to understand whether the reduced absorption of thiol–CDDP complexes is a peculiar feature of the HK-2 cells. On the other hand, CDDP/cysteinyl-

glycine complexes were also found in the supernatant of other cells, i.e. GGT-transfected CDDP-resistant HeLa cells [37]; this finding seems to indicate rather that a poor membrane permeability may be a specific property of thiol/CDDP conjugates. Finally, it should be considered that cysteinyl-glycine also reacts with CDDP more promptly, approx. five-fold faster than GSH [37], likely as the result of a lower pK_a of its SH group as compared to that of GSH [5]. Altogether, these observations consistently support the view that GGT activity, by converting the poorly reactive GSH into the highly reactive cysteinyl-glycine, is able to trigger the formation of CDDP/thiol complexes in the extracellular space [37], resulting in decreased cellular accumulation of CDDP, decreased DNA platination and decreased cytotoxicity [36].

This interesting mechanism of GGT-mediated "*extracellular detoxication*" of CDDP can help explain another important aspect related with CDDP treatments, i.e. the long envisaged connections between GGT activity of renal tubule cells and nephrotoxicity of cisplatin. Conflicting results have been reported about the precise role of tubular GGT in this toxic effect of CDDP in vivo. GGT knockout mice, as well as mice treated with the GGT inhibitor acivicin, do exhibit lower nephrotoxicity after treatment with CDDP [38,39]. The hypothesis was thus proposed that CDDP–GSH complexes reaching the tubular lumen with the glomerular filtrate may undergo a sequential extracellular hydrolysis by tubular GGT and membrane dipeptidase, resulting in cysteine–CDDP complexes; the hydrolysis of this complex by intracellular cysteine-conjugate β-lyase (CCBL) would cause then the formation of a reactive thiol, ultimately responsible for the renal toxicity of CDDP. According this hypothesis, tubular GGT would thus sensitize cells to drug-induced injury. In line with this hypothesis, Towsend and Hanigan have recently shown that the administration in mice of the CCBL inhibitor aminooxyacetic acid reduces mortality, blood urea nitrogen elevation, and histopathologically assessed renal tubular damage after administration of a subletal dose of CDDP [40]. At variance however, transfection of LLC-PK1 cells with the full-length cDNA coding for CCBL did not increase, but on the contrary decreased the antiproliferative effect of CDDP [41]. This finding does not support the proposed involvement of GGT in enhancing CDDP nephrotoxicity.

Based on the effects that GGT deletion or inhibition produce on *plasma* GSH levels, an alternative explanation of apparent protection provided by GGT can be proposed. It is in fact known that after GGT inhibition by acivicin [42], as well as in GGT knockout mice [43], plasma GSH concentrations are several-fold increased, and this in turn results in increased glomerular filtration of GSH. This behaviour, in association with water reabsorption and active GSH transport into tubular lumen, can increase the urinary concentration of GSH up to 5–30 mmol/L [21]. Such a concentration is expected to provide *direct* protection against CDDP cytotoxicity, irrespective of GGT activity [36]. It is known that i.v. administration of high doses of GSH can afford a substantial protection against CDDP nephrotoxicity [44], and that kidney function is anyway dependent on an adequate supply of GSH [45]. Thus, a direct complexation of CDDP in the tubular lumen can take place in conditions of GGT inhibition, and this phenomenon may provide an alternative explanation of the observations mentioned above [38,39].

6. Concluding remarks

The important roles played by GSH-centered mechanisms in detoxication processes and cellular response to cytotoxic injuries suggest that the manipulation of GSH system can be an exploitable approach to increase the sensitivity of cancer cells to cytotoxic drugs and/or to overcome drug resistance. Various strategies have been proposed to obtain such manipulation, aiming, e.g. at the GSH-*S*-transferase activities [46]. GSH is however also a major physiological regulator of the cell redox status, and to date all approaches targeting the GSH systems have raised problems related to undesired side effects being produced on normal tissues. Nevertheless, as far as GGT is concerned, a differential expression of this enzyme does exists in several tumor types, and it is conceivable that this fact might be exploited to increase the therapeutic efficacy of selected drugs. The involvement of GGT in CDDP detoxication will likely offer an additional target for the improvement of platinum-based therapies, but the heterogeneous expression of GGT among the different tumor types, and even different tumors of the same type, may become an important determinant in this perspective. On the other hand, it is now evident that cellular resistance to platinum compounds or alkylating agents is a multifactorial phenomenon involving not only defence mechanisms, but also cellular response to the genotoxic stress (e.g. DNA repair efficiency, DNA damage tolerance, stress response and susceptibility to DNA damage-induced apoptosis). Thus, it is likely that the relevance of GGT-mediated detoxication may be dependent on the specific biological context, including GGT expression level as well as the concomitant expression of other resistance mechanisms.

Finally, although expression of GGT may not be regarded as a general mechanism of resistance, the involvement of this enzyme in modulation of redox metabolism is expected to have impact in cellular response to several cytotoxic agents. It should be emphasized that the implication of GGT activity in tumor biology and its relevance as a therapeutic target may extend beyond the above discussed roles in cellular thiol supply and detoxication pathways. GGT has been shown to affect other cellular functions, which can have relevance in the behaviour of malignant cells. It has been in fact documented that GGT enzymatic activity can exert *prooxidant effects* at the membrane surface level and in the extracellular microenvironment [reviewed in 47]. Such endogenous "oxidative stress" might help explain some apparent contradictions in the literature, i.e. the lower (not higher) GSH levels occasionally reported after transfection of cells with GGT. Several studies from our and other laboratories have shown that an "autocrine" production of hydrogen peroxide occurs in GGT-expressing cells [48–50], resulting in redox effects on several cellular targets, e.g. modulation of SH groups in cell surface proteins and receptors [51,52]. The responsibility for these phenomena was again identified in cysteinyl-glycine produced during the GGT-mediated cleavage of extracellular GSH. Cysteinyl-glycine is far more reactive thiol as compared to parent tripeptide, and this reactivity can favour the occurrence of metal–catalyzed redox cycling reactions ultimately leading to generation of reactive oxygen species, ROS [48,53]. Indeed, GGT-dependent oxidative changes could be

demonstrated histochemically in GGT-expressing hyperplastic nodules induced in rat liver by chemical carcinogenesis [54,55]. ROS (hydrogen peroxide, in the first place) can have effects on a number of cellular functions, e.g. the cellular proliferation/apoptosis balance, and our previous studies in fact documented that GGT-generated ROS can mediate both antiproliferative and antiapoptotic effects, depending on the experimental conditions used [34,56].

A summary of the functions of GGT discussed in the present commentary is reported in Fig. 1. The biological events related to the expression of this enzyme activity in tumors represent a complex phenomenon, with potential implications on antitumor therapy. Further studies on the GGT-mediated functions may provide more precise information on the relevance of the implicated events.

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