# γ-Glutamyl Transpeptidase Overexpression Increases Metastatic Growth of B16 Melanoma Cells in the Mouse Liver

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**B16 melanoma (B16M) cells with high glutathione (GSH) content show rapid proliferation *in vitro* and high metastatic activity in the liver *in vivo*. γ-Glutamyl transpeptidase (GGT)-mediated extracellular GSH cleavage and intracellular GSH synthesis were studied *in vitro* in B16M cells with high (F10) and low (F1) metastatic potential. GGT activity was modified by transfection with the human GGT gene (B16MF1/Tet-GGT cells) or by acivicin-induced inhibition. B16MF1/Tet-GGT and B16MF10 cells exhibited higher GSH content (35 ± 6 and 40 ± 5 nmol/$10^6$ cells, respectively) and GGT activity (89 ± 9 and 37 ± 7 mU/$10^6$ cells, respectively) as compared ($P < .05$) with B16MF1 cells (10 ± 3 nmol GSH and 4 mU GGT/$10^6$ cells). Metastasis (number of foci/100 $mm^3$ of liver) increased in B16MF1 cells pretreated with GSH ester (~3-fold, $P < .01$), and decreased in B16MF1/Tet-GGT and B16MF10 cells pretreated with the GSH synthesis inhibitor L-buthionine (S,R)-sulphoximine (~5-fold and 2-fold, respectively, $P < .01$). Liver, kidney, brain, lung, and erythrocyte GSH content in B16MF1/Tet-GGT- or B16MF10-bearing mice decreased as compared with B16MF1- and non–tumor-bearing mice. Organic anion transporting polypeptide 1–independent sinusoidal GSH efflux from hepatocytes increased in B16MF1/Tet-GGT– or B16MF10-bearing mice (~2-fold, $P < .01$) as compared with non–tumor-bearing mice. Our results indicate that tumor GGT activity and an intertissue flow of GSH can regulate GSH content of melanoma cells and their metastatic growth in the liver. (HEPATOLOGY 2002;35: 74-81.)**

γ-Glutamyl-cysteinyl-glycine (GSH) is involved in many cellular functions, *e.g.*, bioreductive reactions, maintenance of enzyme activity, amino acid transport, protection against oxidative/nitrosative stress, and detoxification of xenobiotics.[1] Induction of GSH deficiency in tumors was shown to be potentially useful in cancer therapy.[2] Elevation of intracellular GSH levels is associated with mitogenic stimulation[3] and regulation of DNA synthesis.[4] We found that GSH controls the onset of tumor-cell proliferation by regulating protein kinase C activity and intracellular pH,[5] and that a direct increase of intracellular GSH levels promotes survival and metastatic growth of B16M cells in the liver.[6,7]

In rapidly growing tumors, cyst(e)ine, whose concentration in blood is low, may become limiting for GSH synthesis and cell growth.[8,9] Thus, malignant cells might require alternative pathways to ensure free cyst(e)ine availability. Recently, as it was shown in 3-methylcholantrene–induced sarcomas growing in rats, blood GSH can be used by solid tumors.[10] γ-glutamyl transpeptidase (GGT) is the only known enzyme that cleaves the γ-glutamyl-cysteine peptide bond in GSH and other γ-glutamyl compounds.[11] GGT contains a heavy subunit (41.6 kd) that anchors the enzyme to the plasma membrane, and a light subunit (19.7 kd) orientated towards the outer surface of the cell membrane, which contains the catalytic site.[12] GGT cleaves GSH releasing γ-glutamyl amino acids and cysteinylglycine, which is further cleaved by membrane-bound dipeptidases into cysteine and glycine.[13] Free γ-glutamyl-amino acids, cysteine, and glycine entering the cell serve as GSH precursors.[8] Hence GGT expression may provide tumor cells with a growth advantage at physiologic concentrations of cyst(e)ine.[9] Moreover, an interorgan flow of GSH was proposed as a distribution system of cysteine used by tissues with substantial GGT activity for protein and GSH synthesis.[8,14] Here we examined whether, at physiologic levels of extracellular GSH and cyst(e)ine, GGT activity regulates tumor GSH levels and metastatic growth of low and/or high GGT expressing B16 melanoma (B16M) cells in the liver.

*Abbreviations: GSH, glutathione; GGT, γ-glutamyl transpeptidase; B16M, B16 melanoma; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; KHBM, Krebs-Henseleit bicarbonate medium; DMSO, dimethylsulfoxide; CDNB, 1-chloro-2,4-dinitrobenzene; DNP-SG, S-(2,4-dinitrophenyl)-glutathione; MRP, multidrug resistance-associated protein; gsht, putative sinusoidal GSH transporter; GSSG, glutathione disulphide; γ-GCS, γ-glutamylcysteine synthetase; GSH-S, glutathione synthetase.*

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## Materials and Methods

***Culture of B16M Variant Cell Lines.*** Murine B16M tumor cell lines with low (B16MF1 from the American Type Culture Collection, Manassas, VA) or high (B16MF10, a gift from Dr. F. Vidal-Vanaclocha, University of the Basque Country, Spain) growth potential *in vivo*[15] were cultured[6] in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories., Grand Island, NY), pH 7.4, supplemented with 10% fetal calf serum (Gibco), 10 mmol/L HEPES, 40 mmol/L $NaHCO_3$, 100 U/mL penicillin and 100 $\mu$g/mL streptomycin. For perifusion and animal studies B16M cells were harvested by exposure to 0.02% EDTA (5 minutes at 37°C), washed twice in ice-cold fresh phosphate-buffered saline (PBS), and resuspended in DMEM. Intracellular and extracellular compartments were separated as described for Ehrlich ascites tumor cells.[16] Cell integrity was assessed by trypan blue exclusion and leakage of lactate dehydrogenase activity.[7]

***GGT Transfection and Assays.*** The Tet-off gene expression system (Clontech, Palo Alto, CA) was used to transfect the B16MF1 cells with the human GGT gene following the manufacturer's instructions. Tet-off B16MF1 cells stably expressed Tet-regulated pTRE-GGT giving up to an increased GGT gene expression (B16MF1/Tet-GGT cells). GGT expression was also analyzed by immunohistochemistry using GGT129, an antibody directed against a 20–amino acid sequence in the human GGT protein.[17] GGT activity was measured as previously described.[18] Protein was determined with the BCA protein assay (Pierce, Rockford, IL).

***Perifusion of B16M Cells.*** Isolated B16M cells, suspended in DMEM, were incubated in a perifusion system similar to that previously described for rat hepatocytes.[19] Briefly, a buffer gassed with 95% $O_2$ to 5% $CO_2$ was constantly pumped by an LKB multiperpex roller pump (type 2115) to a chamber containing a final volume of 10 mL and $3 \times 10^6$ B16M cells/mL. The filter (Amicon YM30, Bedford, MA) was placed at the top of the chamber. The tumor cell suspension was perifused at 37°C and maintained homogenous by using a magnetic stirrer placed at the bottom of the chamber. The perifusion buffer was Krebs-Henseleit bicarbonate medium (KHBM; pH 7.4) containing plasma concentrations (aortic blood) of L-amino acids found in non–tumor bearing C57BL/6J mice (395 ± 34 $\mu$mol/L Ala, 56 ± 8 $\mu$mol/L Asn, 24 ± 3 $\mu$mol/L Asp, 8 ± 1 $\mu$mol/L Cyst(e)ine, 72 ± 18 $\mu$mol/L Glu, 442 ± 37 $\mu$mol/L Gln, 229 ± 36 $\mu$mol/L Gly, 46 ± 11 $\mu$mol/L His, 62 ± 13 $\mu$mol/L Ile, 78 ± 16 $\mu$mol/L Leu, 265 ± 23 $\mu$mol/L Lys, 44 ± 6 $\mu$mol/L Met, 56 ± 9 $\mu$mol/L Phe, 107 ± 11 $\mu$mol/L Pro, 87 ± 24 $\mu$mol/L Ser, 117 ± 14 $\mu$mol/L Thr, 70 ± 15 $\mu$mol/L Trp, 83 ± 10 $\mu$mol/L Tyr, 115 ± 18 $\mu$mol/L Val, 129 ± 16 $\mu$mol/L Arg; n = 15), glucose (1 g/L), sodium pyruvate (10 mg/L), and supplemented with 10 U/mL penicillin and 10 $\mu$g/mL streptomycin. Perifusate flow (2 mL/min) was constant throughout the experiment. Effluent flow was monitored continuously for $O_2$ and pH with Philips electrodes. Tumor cell viability was always greater than 97% along the experimental time. To take samples (0.5 mL) of the cell suspension without interrupting the flow a syringe was introduced into the chamber through a rubber septum.

***Experimental Metastases.*** Male C57BL/6J mice (8 to 10 week old) from IFFA Credo (L'Arbreole, France) were fed *ad libitum* on a stock laboratory diet (Letica, Barcelona, Spain), and kept on a 12-hour light/dark cycle with the room temperature maintained at 22°C. All experiments were started between 10:00 and 12:00 h. Procedures involving animals were in compliance with the national and international laws and policies (EEC Directive 86/609, OJ L 358. 1, December 12, 1987; and NIH Guide for the Care and Use of Laboratory Animals, NIH Publ No 85-23, 1985).

Hepatic metastasis were produced by intrasplenic injection into anesthetized mice (Nembutal, 50 mg/kg intraperitoneally) of $2 \times 10^5$ viable B16 cells suspended in 0.1 mL DMEM. Mice were cervically dislocated 10 days after B16M inoculation. The livers were fixed with 10% formaldehyde in PBS (pH 7.4) for 24 hours at 22°C and then paraffin-embedded. Metastasis density (mean number of foci/100 $mm^3$ of liver detected in fifteen $10 \times 10$-$mm^2$ sections per liver) and metastasis volume (mean percent of liver volume occupied by metastasis) were determined as described earlier.[7]

***Isolation and Incubation of Hepatocytes.*** Isolation of hepatocytes from non–tumor-bearing and B16M-bearing C57BL/6J mice followed Berry and Friend's method.[20] Parenchymal liver cells were purified from the crude cell suspension by density gradient centrifugation in a vertical rotor.[21] The crude liver cell suspension (50 mg dry weight in 2 mL) was added to a medium (40 mL) containing 40% (vol/vol) of Percoll, 3% (wt/vol) of defatted bovine serum albumin, 10% DMEM, 10 mmol/L MOPS, 120 mmol/L NaCl, 6.7 mmol/L KCl, 1.2 mmol/L $CaCl_2$, and adjusted to pH 7.4 with 0.1 N NaOH. Centrifugation was carried out at 4°C in a Beckman-Coulter Optima XL-100K ($7 \times 10^4$ $g_{av}$ for 15 minutes). Metabolic viability and integrity of isolated hepatocytes removed from the gradient medium after centrifugation was assayed as previously described.[21] For incubations, hepatocytes (10-12 mg dry weight/mL) were suspended at 37°C in KHBM (pH 7.4) containing 1.3 mmol/L $CaCl_2$. The gas atmosphere was 95% $O_2$ to 5% $CO_2$.

***Culture of Hepatocytes.*** Isolated hepatocytes ($3 \times 10^6$) were seeded onto 60-mm culture dishes (Life Technologies, Prat de Llobregat, Spain) in 4 mL Williams' medium E (Life Technologies) supplemented with 5 mmol/L L-glutamine, 100 U/mL penicillin, 100 $\mu$g/mL streptomycin, 200 U/mL insulin, 1 $\mu$mol/L dexamethasone, and 10% fetal calf serum (Gibco). The dishes were incubated at 37°C in a humidified 5% $CO_2$ atmosphere for 6 hours before starting assays.

***Taurocholate Uptake Into Hepatocytes.*** Hepatocyte monolayers were washed 3 times with KHBM (pH 7.4, 37°C). Uptake studies were initiated by adding 4 mL KHBM supplemented with 2 $\mu$Ci of [$^3$H]-taurocholic acid (NEN, Boston, MA) and 20 $\mu$mol/L nonradiolabeled taurocholic acid (Calbiochem, Darmstadt, Germany). The oatp1 and/or oatp2 inhibitors, rifamycin SV (sodium salt) and rifampicin[22] (Sigma), were dissolved in dimethylsulfoxide (DMSO) and subsequently diluted to 1:100 in the incubation medium (1% DMSO had no effect on the uptake, not shown). The incubation period was 2 minutes, because preliminary experiments showed that uptakes were linear ($r > 0.99$) for at least 3 minutes. Uptake was finished by aspirating the transport buffer and rinsing the culture dishes twice with 4 mL of ice-cold

KHBM. This removes more than 99% of extracellular label without affecting intracellular radioactivity. Then 2 mL of Triton X-100 (0.5% wt/vol) were added to the dishes and the cells were solubilized at room temperature. One-milliliter aliquots of the lysates were mixed with 9 mL toluene (Merck, Darmstsdt, Germany), and the cell-associated radioactivity was measured in a TriCarb 2700TR liquid scintillation analyzer (Canberra Packard, Tecnasa, Madrid, Spain).

***S-dinitrophenyl Glutathione Efflux From Hepatocytes.*** Cultured hepatocytes were incubated for 15 minutes at 10°C with 1 mmol/L 1-chloro-2,4-dinitrobenzene (CDNB) and 5 $\mu$Ci of [$^{14}$C]CDNB (Amersham, Bucks, UK), dissolved in DMSO and diluted in KHBM (pH 7.4) containing 0.5% bovine serum albumin (DMSO was 0.05% of the total incubation volume). This allowed diffusion of CDNB into the cells and conjugation with GSH to produce S-(2,4-dinitrophenyl)-glutathione (DNP-SG). Subsequently, the cells were rapidly washed twice with ice-cold KHBM (this removes more than 99% of extracellular label), and culture medium (37°C) was added again. Then, 200-$\mu$L samples from the medium were taken at 1-minute intervals for 5 minutes, added to 10 mL toluene, and measured in the liquid scintillation counter. Under these conditions, secretion of DNP-SG was linear ($r > 0.99$).

To measure total DNP-SG, immediately after CDNB loading, 2 mL of Triton X-100 (0.5% wt/vol) were added to the dishes, and the cells were solubilized at room temperature. Quantitation was accomplished by measuring absorbance of the conjugate at 340 nm and by using a standard curve made up of known amounts of DNP-SG, synthesized and purified as previously described.[23] To prevent interferences with proteins, aliquots used for spectrophotometric analysis were pretreated with an equal amount of 10% trichloroacetic acid and centrifuged.

***Electroporation and Selection of Hepatocyte Multidrug Resistance Protein-2 Double Knockout Clone.*** Murine multidrug resistance protein (MRP) genomic sequences were isolated from a λ Charon 35 phage library constructed with DNA from mouse strain C57BL/6J. The preparation of the gene-targeting construct, electroporation into mouse hepatocytes (BioRad system to permeabilize cell membranes, Hercules, CA), and selection and screening of the clones, first by polymerase chain reaction analysis and then by DNA blot analysis, were carried out as previously described.[24] The multidrug resistance-associated protein-2 double knockout clone (MRP−/−2) was obtained by exposing a single knockout clone to high concentrations of G418 for 2 weeks.

***Blood Collection and Processing.*** Blood was collected into 1-mL syringes containing sodium heparin (0.05 mL of a 5% solution in 6.9% NaCl). Plasma and erythrocytes were separated as previously described.[25] Erythrocytes were resuspended in ice-cold KHBM (pH 7.4) to yield the original hematocrit.

***Amino Acid Analysis.*** Proteins were precipitated by treating 0.1 mL of intracellular compartment or plasma with 0.4 mL of 3.75% (wt/vol) ice-cold sulphosalicylic acid in 0.3 mol/L lithium citrate buffer (pH 2.8). After centrifugation, 0.25 mL of the supernatant were injected into an LKB 4151 amino-acid analyzer.[16]

***Cystine Uptake.*** B16M cells were plated in 35-mm culture dishes. At the required times, cells were rinsed 3 times with prewarmed transport medium (10 mmol/L PBS, pH 7.4, with 0.01% $CaCl_2$, 0.01% $MgCl_2$, and 0.1% glucose). Uptake measurement was initiated by addition of 1.0 mL transport medium containing 1 $\mu$Ci of L-[$^3$H]cystine (Amersham) and nonradioactive cystine (0.5 mmol/L). After incubation at 37°C, uptake was finished by rinsing several times with ice-cold PBS until less than 0.001% of the initial radioactivity was present in the supernatant. Cells were then dissolved with 0.5 mL of 0.5 N NaOH, and an aliquot was used for determining radioactivity and another for protein assay. To correct for trapping, transport at 4°C was studied in parallel.[26]

***Determination of GSH and Glutathione Disulfide.*** Organs, tissues, erythrocytes, plasma, or B16M cells were treated as previously described.[2,7,25] GSH was measured by the glutathione S-transferase reaction.[27] Glutathione disulfide (GSSG) was determined by high performance liquid chromatography.[28] Total glutathione (GSH + 2GSSG) was determined in plasma by a kinetic assay in which a catalytic amount of GSH or GSSG and glutathione reductase cause the continuous reduction of 5,5′dithiobis-2-nitrobenzoic acid (Sigma) by NADPH.[27]

***Preparation of GSH Ester.*** GSH monoisopropyl(glycyl) ester was prepared as previously described.[29]

***GSH Synthesis-Related Enzyme Activities.*** B16M cells were detached, washed twice at 4°C in KHBM (without $Ca^{2+}$ or $Mg^{2+}$) containing 0.5 mmol/L EGTA, pH 7.4, resuspended and homogenized in 0.1 mol/L phosphate buffer (pH 7.2) at 4°C.[7] γ-Glutamylcysteine synthetase (γ-GCS) and GSH synthetase (GSH-S) activities were measured as described elsewhere.[30]

***Expression of Results and Statistical Analyses.*** Data are presented as mean ± SD. Data were analyzed by one- or two-way analysis of variance (ANOVA) or unpaired *t* tests where appropriate (SPSS 9.0 software for Windows; SPSS Inc., Chicago, IL). The homogeneity of the variances was analyzed by the Levene test. The null hypothesis was accepted for all the values of the tests in which the F-value was nonsignificant at $P > .05$. The data for which the F-value was significant was examined by Tukey's test at $P < .05$.

## Results

***GGT-Dependent GSH Content of Melanoma Cells.*** Is GGT involved in regulating GSH homeostasis in growing tumor cells? To answer this question a perifusion chamber, containing a suspension of melanoma cells, was used as an experimental setup that mimics *in vivo* conditions by providing a constant supply of GSH and amino acids at physiologic plasma concentrations. Melanoma cell lines with different GGT activities and rates of extracellular GSH cleavage were used (Table 1). Diethylmaleate (an α,β-unsaturated carbonyl compound)-induced intracellular GSH depletion (see note to Table 1)[2] was used to study rates of intracellular GSH replenishment (net difference between GSH synthesis and efflux).[7] When GSH was present in the perifusion buffer, B16MF1/Tet-GGT cells showed a 3-fold increase in the rate of GSH replenishment ($P < .01$) and in their maximum intracellular GSH content ($P < .01$) as compared with control nontransfected B16MF1 cells (Table 1). Comparably, when the B16MF10 cell line (whose capacity to accumulate GSH is 4-fold higher than B16MF1 cells) was preincubated with acivicin (an irreversible GGT inhibitor[31]) both the rate of intracellular GSH replenishment ($P < .01$) and the maximum intracellular GSH content ($P < .05$) decreased (Table 1). These changes appear to be a direct

**Table 1. GGT Activity and Intracellular GSH Content in B16M Variant Cell Lines Perifused With Physiologic Plasma Concentrations of Amino Acids and GSH**

| Melanoma | GGT (mU/$10^6$ cells) | Rate of Extracellular GSH Cleavage (nmol/$10^6$ cells × h) | Rate of Intracellular GSH Replenishment (nmol/$10^6$ cells × h) | | Maximum Intracellular GSH Content (nmol/$10^6$ cells) | | γGCS (mU/$10^6$ cells) | GSH-S (mU/$10^6$ cells) |
|---|---|---|---|---|---|---|---|---|
| | | | −eGSH | +eGSH | −eGSH | +eGSH | | |
| B16MF1 | 4 ± 2[c] | 17 ± 4[c] | 0.9 ± 0.2[c] | 1.1 ± 0.3[c] | 12 ± 2[c] | 10 ± 3[c] | 106 ± 25[b] | 9 ± 1[a] |
| B16MF1/Tet-GGT | 89 ± 9[a] | 255 ± 26[a] | 0.8 ± 0.2[c] | 3.5 ± 0.4[a] | 15 ± 4[c] | 35 ± 6[a] | 112 ± 16[b] | 11 ± 2[a] |
| B16MF10 | 37 ± 7[b] | 131 ± 17[b] | 3.0 ± 0.5[ab] | 4.3 ± 0.5[a] | 29 ± 5[ab] | 40 ± 5[a] | 227 ± 34[a] | 10 ± 2[a] |
| B16MF10 + acivicin | ND | ND | 2.8 ± 0.4[ab] | 2.6 ± 0.3[b] | 25 ± 3[b] | 27 ± 3[b] | 209 ± 28[a] | 9 ± 2[a] |

NOTE. Cultured B16M tumor cell lines were isolated (24 hours after seeding) and perifused, and determinations were performed as described under Materials and Methods. Acivicin (10 μmol/L) was added to the B16MF10 culture medium 3 hours before harvesting. B16M cells were preincubated for 10 minutes with diethylmaleate (25 μmol/L for B16MF1 and B16MF1-Tet-GGT cells; and 100 μmol/L for B16MF10 cells) before incubation in the perifusion chamber. GSH content in all melanoma cell lines was similar after diethylmaleate-induced GSH depletion (*e.g.*, 5 ± 2 nmol/$10^6$ B16MF10 cells). Extracellular GSH (eGSH) concentration in the perifusion buffer was of 9.2 μmol/L (see under Discussion). In each case, data are means ± SD for 12 independent experiments. A one-way (two-ways for those parameters determined ± eGSH) analysis of variance (ANOVA) was used to make comparisons among melanoma groups. Different superscript letters within a column indicate differences, $P < .05$.

Abbreviation: ND, not detectable.

consequence of the difference in GGT activity among melanoma cell lines since γ-GCS and GSH-S, the enzyme activities involved in GSH synthesis, were found similar in B16MF1/Tet-GGT and B16MF1 cells or in B16MF10 cells ± acivicin (Table 1).

We also found that the intracellular concentration of amino acids was similar in the different B16M cell lines along the perifusion time. In particular, concentrations of free glutamate and glycine were, *e.g.*, in B16MF1 cells, of 2.1 ± 0.3 mmol/L and 1.3 ± 0.2 mmol/L, respectively (which ensures maximal rates of GSH synthesis[32]); whereas glutamine (a major fuel for cancer cells[33]) and cyst(e)ine (cysteine + cystine) (rapidly used for protein and GSH synthesis[7]) were undetectable. No significant differences were found in the rate of cystine uptake among B16MF1, B16MF1/Tet-GGT, and B16MF10 cells (0.28 ± 0.05, 0.28 ± 0.05, and 0.35 ± 0.06 nmol cystine/mg protein × min, respectively; $P > .05$, n = 7). Moreover rates of GSH replenishment and the maximum intracellular GSH content for B16MF1/Tet-GGT and B16MF10 cells perifused with amino acids and extracellular GSH (Table 1), were reproduced in the absence of extracellular GSH by increasing 2-fold (up to 16 μmol/L) the concentration of cysteine in the perifusion buffer (not shown). Hence, intracellular cysteine availability appears modulated by its GGT-dependent generation from extracellular GSH.

***GGT Activity Influences Metastatic Growth of Melanoma Cells.*** We showed that hepatic metastasis in mice intrasplenically injected with high-GSH content B16M cells increases.[6] Therefore, possibly, GGT-dependent changes in melanoma GSH content may be associated with changes in the metastatic potential. Indeed, metastatic growth in the liver of mice intrasplenically injected with B16MF1 cells increased ($P < .01$) by transfecting the GGT gene (B16MF1/Tet-GGT cells) or by preincubation with GSH ester (which enters the cell and delivers free GSH) ($P < .01$) (Table 2). Besides, the metastatic growth of the B16MF10 and the B16MF1/Tet-GGT cell lines was decreased ($P < .01$) by preincubation with BSO (L-buthionine (S,R)-sulphoximine), a specific inhibitor of γ-GCS.[13]

***Intertissue Flow of GSH as a Tumor Growth-Promoting Mechanism.*** GSH exported from cells enters the blood plasma thus creating an interorgan flow of GSH.[14] Under physiologic conditions, the liver is the major source of plasma GSH (over 90% of the total GSH inflow) perhaps with minor contributions from other organs.[8,14] We found that GSH levels decrease in the liver and kidney of Ehrlich ascites tumor-bearing mice.[5] Hence, we investigated if B16M cells growing in the liver induce in hepatocytes changes in the rate of GSH synthesis and/or efflux. Hepatocytes isolated from B16MF10 tumor-bearing mice synthesize GSH at a similar rate to hepatocytes from non–tumor-bearing mice (or hepatocytes from B16MF1 or B16MF1/Tet-GGT tumor-bearing mice, not shown) (Table 3). However, hepatocytes from B16MF10 tumor-bearing mice release glutathione (GSH + GSSG) (98 ± 1% as GSH) at a rate 2-fold higher than hepatocytes from non–tumor-bearing mice (or hepatocytes from B16MF1 tumor-bearing mice, not shown) ($P < .01$ in all cases). This tumor-induced increase in GSH release from hepatocytes facilitates additional GSH molecules to metastatic cells.

To investigate whether this effect is localized in the tissue affected by the growing metastasis, GSH levels were measured in different tissues. As compared with non–tumor-bearing mice or

**Table 2. Measurement of Metastatic Growth in the Liver of Mice Intrasplenically Injected With Different B16M Variant Cell Lines**

| Melanoma | Intracellular Tumor GSH Before Inoculation (nmol/$10^6$ cells) | Metastasis Density (No. of foci/100 $mm^3$) | Metastasis Volume (% liver volume) |
|---|---|---|---|
| B16MF1 | 11 ± 4[b] | 5 ± 2[a] | 2 ± 1[d] |
| B16MF1 + GSH ester | 33 ± 7[a] | 16 ± 4[a] | 14 ± 3[b] |
| B16MF1/Tet-GGT | 36 ± 5[a] | 20 ± 3[a] | 25 ± 5[a] |
| B16MF1/Tet-GGT + BSO | 9 ± 3[b] | 4 ± 1[c] | 7 ± 2[c] |
| B16MF10 | 38 ± 6[a] | 23 ± 4[a] | 27 ± 4[a] |
| B16MF10 + BSO | 12 ± 4[b] | 12 ± 2[b] | 9 ± 3[c] |

NOTE. Data represent average values ± SD of 40 mice per group. Where indicated GSH ester was added to the culture medium 20 hours after plating, whereas BSO (200 μmol/L) was added 4 hours after plating.[7] A one-way ANOVA was performed for comparison among melanoma groups. Different superscript letters within a column indicate differences, $P < .01$.

**Table 3. Rates of GSH Synthesis and Glutathione Efflux in Isolated Hepatocytes From Non–Tumor-Bearing Mice and B16MF10-Bearing Mice**

| | Rate (nmol/g × min) | | | |
|---|---|---|---|---|
| | GSH Synthesis | | Glutathione Efflux (GSH + 2GSSG) | |
| Additions | Non–Tumor-Bearing Mice | B16MF10 | Non–Tumor-Bearing Mice | B16MF10 |
| None | 3 ± 1 | 5 ± 2 | 3 ± 0.5 | 6 ± 2* |
| L-Amino acids | 28 ± 7† | 26 ± 6† | 12 ± 4† | 23 ± 5†‡ |

NOTE. The initial GSH concentration was of 4.0 ± 0.4 μmol/g in isolated hepatocytes from non–tumor-bearing mice and of 3.1 ± 0.5 μmol/g in hepatocytes from B16MF10-bearing mice. Hepatocytes were incubated in 10 mL-Erlenmeyers (final volume 2 mL) for 60 minutes (see under Materials and Methods) in the presence or in the absence of amino acids (5 mmol/L Gln, 2 mmol/L Gly, 1 mmol/L Ser, 1 mmol/L *N*-acetylcysteine).[7] Only L-amino acids were used. Glucose (5 mmol/L) and bovine serum albumin (2%) were present in all incubations. Rates of GSH synthesis were calculated from total GSH content in incubations at 0, 20, 40, and 60 minutes. Rates of glutathione efflux were calculated from contents of GSH and GSSG in extracellular medium at 0, 20, 40, and 60 minutes. The significance test (Student's unpaired *t* test) refers, for both groups, to the comparison of GSH concentration in the absence or in the presence of amino acids (†$P < .01$), and also to the difference between results for the B16MF10 group and the non–tumor-bearing group (*$P < .05$; ‡$P < .01$). All values are means ± SD for 12 observations. Data obtained using hepatocytes from B16MF1-bearing mice were not significantly different from those shown in the table for the non–tumor-bearing group. Data obtained using hepatocytes from B16MF1/Tet-GGT–bearing mice were not significantly different from those displayed in the table for the B16MF10 group (not shown) (n = 12 in both cases).

B16MF1-bearing mice, in mice inoculated with B16MF10 or B16MF1/Tet-GGT cells GSH decreases ($P < .05$) in liver, kidneys, brain, lungs, and erythrocytes (Table 4). This multiorgan GSH depletion is not the consequence of changes in the tissue pool of amino acids, GSH synthesis activity, glutathione redox status (GSH/GSSG) or the GGT activity in those tissues, as compared with non–tumor-bearing mice (not shown).

***Export of GSH in Hepatocytes From Melanoma-Bearing Mice.*** The liver is quantitatively the major site of GSH synthesis in the body[34]; thus, and taking into account our results (Table 3), we investigated the mechanisms involved in export of GSH from cultured hepatocytes isolated from non–tumor-bearing mice and from mice bearing melanoma cells with high metastatic potential and GGT activity. Hepatocellular export of GSH involves oatp1 (the sinusoidal organic anion transporter) and gsht (a putative GSH transporter on the sinusoidal side); whereas the GSH-activated MRP2 (the multidrug resistance-associated and ATP-dependent organic anion transporter) is located on the canalicular membrane.[35] As shown in Table 5, GSH efflux (2-fold higher than controls in hepatocytes from B16MF10-bearing mice) was not significantly inhibited by rifamycin SV or rifampicin (inhibitors of oatp1 and/or oatp2),[22] although both inhibited taurocholate uptake. Besides, sulfinpyrazone-induced inhibition[36] or abrogation (MRP−/−2)[24] of MRP2-dependent canalicular GSH efflux did not affect the rate of GSH release (Table 5). However, GSH efflux was significantly inhibited by methionine, an inhibitor of the gsht transporter.[35] This suggests the gsht-mediated sinusoidal GSH efflux as the main mechanism for the effect observed in hepatocytes of melanoma-bearing mice.

## Discussion

The intracellular GSH content of melanoma cells can be modulated by the tumor GGT-dependent cleavage of extracellular GSH (Table 1). This is important because multidrug and/or radiation resistance, which are characteristic features of malignant tumors, frequently associate with high GSH content in the cancer cells.[37] Indeed, exogenous GSH may protect cells from oxidative damage.[38-41] GGT activity generates cysteine,[42] the rate-limiting amino acid for GSH synthesis,[13] promoting an increase in tumor GSH (Table 1) and, thereby, facilitating metastatic growth (Table 2). Using an MCA sarcoma as a model, it was shown that tumors *in vivo* utilize host GSH via GGT.[10] Our results establish a relationship among GGT activity, intracellular GSH synthesis and content, and metastatic activity *in vivo* (Table 2). We also show that sinusoidal GSH export from hepatocytes appears facilitated in a tumor-bearing host (Tables 3 and 5).

Plasmatic (aortic blood) levels (μmol/L) of cyst(e)ine in non–tumor-bearing mice and in mice inoculated with B16MF1, B16MF1/Tet-GGT, or B16MF10 cells (10 days after inoculation) were of 9.2 ± 1.3, 8.8 ± 0.9, 7.5 ± 0.8, and 7.3 ± 1.0 ($P < .05$ in the last 2 values versus non–tumor-bearing mice), respectively. Usually plasma concentration of cystine is higher than that of cysteine, and cystine is readily converted to cysteine in cells.[43] Thus, we measured cystine uptake, which enters the cell through the $Na^+$-independent $x_c^-$ system in exchange for glutamate,[43] and found that it was similar in the 3 melanoma cell lines (see Results). However, if extracellular levels of glutamate are high, as may occur in patients with advanced cancer,[44] cystine uptake is competitively inhibited, decreasing intracellular cystine availability. Then, cysteine released from extracellular GSH by GGT may represent a fundamental difference. Cysteine is transported mainly by system ASC,[43] thus a defect in amino acid transport affecting this system

**Table 4. GSH Content in Tissues From Mice Intrasplenically Injected With Different B16M Variant Cell Lines**

| | | Tumor-Bearing Mice | | |
|---|---|---|---|---|
| Tissue | Non–Tumor-Bearing Mice | B16MF1 | B16MF1/Tet-GGT | B16MF10 |
| Brain | 1.7 ± 0.2[a] | 1.8 ± 0.3[a] | 1.1 ± 0.1[b] | 1.2 ± 0.3[b] |
| Lung | 1.6 ± 0.2[a] | 1.6 ± 0.2[a] | 0.9 ± 0.1[b] | 1.0 ± 0.2[b] |
| Heart | 0.7 ± 0.1[a] | 0.6 ± 0.2[a] | 0.7 ± 0.2[a] | 0.5 ± 0.1[a] |
| Liver | 6.7 ± 0.5[a] | 6.5 ± 0.3[a] | 4.0 ± 0.2[b] | 3.5 ± 0.4[b] |
| Glandular stomach | 5.0 ± 0.4[a] | 5.2 ± 0.5[a] | 4.5 ± 0.3[a] | 4.5 ± 0.3[a] |
| Kidney | 2.8 ± 0.3[a] | 2.7 ± 0.4[a] | 1.4 ± 0.2[b] | 1.7 ± 0.2[b] |
| Pancreas | 0.7 ± 0.1[a] | 0.7 ± 0.2[a] | 0.5 ± 0.1[a] | 0.5 ± 0.2[a] |
| Skeletal muscle (soleus) | 0.5 ± 0.1[a] | 0.5 ± 0.1[a] | 0.4 ± 0.05[a] | 0.5 ± 0.1[a] |
| Bone marrow | 0.4 ± 0.05[a] | 0.3 ± 0.1[a] | 0.3 ± 0.1[a] | 0.4 ± 0.1[a] |
| Testis | 3.0 ± 0.4[a] | 2.7 ± 0.5[a] | 2.5 ± 0.2[a] | 2.7 ± 0.3[a] |
| Erythrocytes | 7.5 ± 0.5[a] | 7.3 ± 0.3[a] | 5.0 ± 0.3[b] | 5.3 ± 0.2[b] |

NOTE. Animals were killed 10 days after inoculation of tumor cells. Organs or tissues were quickly dissected and removed, washed at 4°C in KHBM (pH 7.4) without $Ca^{2+}$ or $Mg^{2+}$ and containing 0.5 mmol/L EGTA, dried on tissue paper, frozen in liquid nitrogen, powdered in a mortar and treated as described under Materials and Methods. Data are means ± SD for 20 different mice. Values are μmol GSH per gram tissue, except in erythrocytes for which GSH is expressed as μmol/g hemoglobin. A one-way ANOVA was performed. Different superscript letters within a row indicate significant differences, $P < .05$.

**Table 5. GSH and GSH-Conjugate Efflux and Organic Anion Uptake in Cultured Hepatocytes From Non–Tumor- and Melanoma-Bearing Mice**

| Additions | GSH Efflux (nmol/g × min) | | DNP-SG Efflux (nmol/g × min) | | Taurocholate Uptake (nmol/g × min) | |
|---|---|---|---|---|---|---|
| | Non-tumor | B16MF10 | Non-tumor | B16MF10 | Non-tumor | B16MF10 |
| None | 10 ± 2 | 20 ± 3* | 374 ± 84 | 493 ± 124 | 276 ± 55 | 257 ± 43 |
| Rifamycin SV (0.1 mmol/L) | 11 ± 3 | 23 ± 3* | 415 ± 112 | 466 ± 106 | 64 ± 12† | 77 ± 15† |
| Rifampicin (0.1 mmol/L) | 13 ± 2 | 21 ± 2* | 396 ± 64 | 484 ± 78 | 159 ± 31‡ | 133 ± 26‡ |
| Sulfinpyrazone (2 mmol/L) | 10 ± 1 | 20 ± 4* | 79 ± 17† | 166 ± 39*† | 255 ± 46 | 268 ± 50 |
| $MRP^{-/-}2$ | 12 ± 4 | 19 ± 2§ | 31 ± 8† | 45 ± 10†§ | 250 ± 53 | 274 ± 62 |
| Methionine (1 mmol/L) | 5 ± 1† | 11 ± 3*† | 365 ± 77 | 510 ± 158§ | 298 ± 37 | 264 ± 67 |
| Methionine (1 mmol/L) + acivicin (0.1 mmol/L) | 4 ± 1† | 12 ± 2*§ | 387 ± 80 | 477 ± 111 | 309 ± 72 | 291 ± 59 |

NOTE. The initial GSH concentration was of 4.8 ± 0.3 μmol/g in cultured hepatocytes from non–tumor-bearing mice and of 4.0 ± 0.3 μmol/g in cultured hepatocytes from B16MF10-bearing mice (n = 7 in both cases). Results are means ± SD of 5 independent experiments where each sample was run in duplicate. Rates of GSH efflux were calculated from the contents of total glutathione (GSH + GSSG) and GSSG in the extracellular medium at 0, 30, 60, and 120 minutes of incubation. DNP-SG efflux and taurocholate uptake were determined as explained under Materials and Methods. The significance test (Student's unpaired *t* test) refers, for both groups, to the comparison in the absence or in the presence of additions (‡$P < .05$; †$P < .01$), and also to the difference between results for the B16MF10 group and the non–tumor-bearing group (§$P < .05$; *$P < .01$).

should be reflected by changes in the plasma concentration of other amino acids transported by the same system. This is not the case, because plasma levels of alanine and proline did not change significantly between controls and tumor-bearing mice (not shown). Therefore, although not affecting cysteine transport directly, tumors expressing high GGT levels efficiently degrade plasma GSH, providing abundant cysteine for uptake.[45] Hence GGT activity may be critical in regulating tumor GSH synthesis.

We recently reported that GSH protects B16MF10 cells against nitrosative and oxidative stress in the murine hepatic microvasculature.[6,46] Here, we show that metastatic growth can be implemented in B16MF1 cells by directly increasing their GSH content with GSH ester; whereas BSO-induced GSH depletion decreases the high metastatic activity displayed by B16MF1/Tet-GGT or B16MF10 cells (Table 2). In agreement with our findings, a recent report, in which the role of GSH in the growth of HepG2 cells was examined, shows that changes in cell growth and DNA synthesis paralleled changes in GSH levels suggesting a causal relationship between the two.[47] Nevertheless, the molecular mechanism(s) that link GSH and metastasis progression remain(s) unknown. Hypothetically, GSH could regulate DNA synthesis by providing reducing equivalents to glutaredoxin and/or thioredoxin, both required for ribonucleotide reductase. Moreover, the glutathione redox state could regulate nuclear binding activity of different redox-dependent transcription factors, *e.g.*, nuclear factor κB or apolipoprotein 1[48]; or function in regulating mechanisms of genomic surveillance, *e.g.*, cell cycle checkpoint systems.[49]

Cells that have membrane-bound GGT can utilize translocated GSH, whereas GSH exported from cells that have low or no detectable GGT activity enters the blood stream.[14] Translocation of GSH provides a means of delivering the thiol to a cell membrane, where it may function, *e.g.*, in transport, protection, or as a source of cysteine. To investigate if a growing tumor may indirecly affect the GGT activity in different tissues, we measured it in kidney, brain, and liver of melanoma (B16MF1, B16MF1/Tet-GGT, or B16MF10) and non–tumor-bearing mice (125 ± 17 U/g in kidney; 89 ± 21 mU/g in brain; and 26 ± 9 mU/g in liver; n = 10) and found no significant differences. Besides, plasma GSH levels (aortic blood) were found to be similar in B16MF1-bearing mice and non–tumor-bearing mice (9.2 ± 1.4 μmol/L; n = 10), but lower in B16MF1/Tet-GGT (5.7 ± 1.5 μmol/L; n = 10; $P < .05$) or B16MF10-bearing mice (6.6 ± 1.1 μmol/L; n = 10; $P < .05$) 10 days after inoculation. Therefore, although the liver and in minor proportion other tissues (Table 4) contribute to plasma GSH levels, melanoma cells with high GGT activity induce a decrease in circulating GSH. Moreover, melanoma cells growing in the liver may benefit from the increased sinusoidal GSH release from hepatocytes (Table 3), likely through the putative gsht system (Table 5).

The fact that B16MF10 cells do not cleave extracellular GSH in the presence of acivicin (Table 1) indicates that melanoma use of GSH is mediated by GGT. Indeed the GSH utilization ratio for the systemic and the tumor circulations, after acivicin administration to MCA sarcoma-bearing rats, were significantly lowered.[10] Nevertheless, although acivicin inhibited more than 95% of the MCA sarcoma GGT activity, the tumor GSH utilization ratio was still 45%.[10] Therefore, we cannot rule out the possibility that in some tumors a non–GGT-dependent pathway for GSH utilization may exist.

Extracellular GSH may exert a cell-specific cytotoxic effect on tumor cells, which appears mediated by GGT-dependent prooxidant reactions.[50] However, no evidence of cancer cell damage was found even by increasing 4-fold the physiologic GSH concentration (see note to Table 1) in the perifusion buffer (which can only be expected *in vivo* under conditions of severe/massive liver/tissue damage) (not shown).

In conclusion, GGT activity, by eliciting an increase in GSH content within melanoma cells, can promote metastatic growth in the liver. Intertissue flow of GSH, where the liver plays a key role, serves as a growth-promoting mechanism for GGT-positive tumors. Our findings point out the importance of GGT as a potential target in the therapy of melanoma and, possibly, of other tumors where an increased expression of this enzyme has been found (including human tumors of the liver, lung, breast, and ovary, see Hochwald et al.[10] and references therein).

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