Differential induction of γ-glutamyl transpeptidase in primary cultures of rat and mouse hepatocytes parallels induction during hepatocarcinogenesis

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Introduction

Studies of liver carcinogenesis in both rats and mice have been based on the analysis of the preneoplastic hepatocytes (4–7). Prenecplastic lesions are identified by an alteration in the level of one or more marker enzymes (8). In the rat, induction of γ-glutamyl transpeptidase (GGT*) is the most commonly used marker for the identification of preneoplastic hepatocytes. In the mouse, GGT is not induced in the preneoplastic hepatocytes or tumors induced by most hepatocarcinogenic regimens. Other marker enzymes, such as reduced levels of glucose-6-phosphatase and adenosine triphosphatase, are observed in both species. It is unclear why the induction of GGT differs between these two species.

GGT is a cell-surface enzyme that initiates the cleavage of extracellular glutathione into its constituent amino acids: cysteine, glycine and glutamic acid (9). Most cells maintain intracellular glutathione levels by de novo synthesis as they are unable to take up extracellular glutathione. By cleaving extracellular glutathione, GGT provides the cell with the amino acids necessary for intracellular synthesis (9). Hanigan and Pitot have suggested that in the rat, GGT-positive hepatocytes are better able to maintain intracellular glutathione levels and therefore have a selective advantage over GGT-negative hepatocytes when animals are treated with promoting agents that deplete intracellular glutathione (10).

GGT is induced in rat hepatocytes when they are isolated and maintained in vitro (11). We designed a series of experiments to compare the induction of GGT in isolated rat and mouse hepatocytes to gain a better understanding of the species differences in the regulation of this enzyme. In this study, the level of GGT activity was determined in primary cultures of rat and mouse hepatocytes over a 7-day time course. Intracellular glutathione levels were also measured. RNase protection assays were used to identify which GGT promoter was activated by the in vitro culture conditions.

Materials and methods

Animals

Female C57BL/6 mice (20–25 g) and female Sprague–Dawley rats (180–300 g) were obtained from Hilltop Lab Animals (Scottdale, PA). The animals were housed in plastic cages on hardwood chip bedding with a 12-h light–dark cycle. Rodent Chow 3000 (Agway; New York, NY) and tap water were available ad libitum.

Hepatocyte isolation

Rats and mice were anesthetized with 80 mg ketamine HCl (Fort Dodge Laboratories, Fort Dodge, IA) and 10 mg xylazine (Miles Inc., Shawnee Mission, KA) per kg body wt. The liver was perfused by the method of Krausing et al. (12). For both rats and mice, the liver was perfused for 4 min at 10 ml/min with calcium and magnesium-free Hanks’ Basic Salt Solution (GIBCO/BRL, Grand Island, NY) supplemented with 50 mM HEPES and 0.5 mM EGTA, pH 7.4 at 37°C. This was followed by a 10-min perfusion with Leibovitz modified L-15 medium (GIBCO/BRL), which contained 18 mM HEPES and 1 mg/ml collagenase D (Boehringer-Mannheim, Mannheim). The liver was excised, and the cells dispersed in L-15 medium and filtered through a 260-µm pore Nitex mesh (TETKO, Briarcliff Manor, NY). The cells were pelleted twice in L-15 medium supplemented with 0.2% bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO) and 18 mM HEPES at 30 g at 4°C for 5 min. The viability was determined by trypan blue exclusion.

A total of 5×10⁶ viable cells were plated per 60-mm dish in 3 ml of L-15 medium supplemented with 0.2% BSA, 18 mM HEPES, 8.3 mM glucose (Sigma), 0.5 µg/ml insulin (GIBCO/BRL), 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 50 units/ml penicillin G and 50 µg/ml streptomycin sulfate (GIBCO/BRL). The medium was changed after the first 4 h and every 24 h thereafter.

GGT assays

For histochemical staining, one dish for each time period was rinsed three times with saline and the cells stained for GGT activity according to the method of Rutgersburg et al. (13). The percentage of GGT-positive cells (stained red) was estimated by counting the number of stained and unstained cells in each of 10 microscope fields per dish. Totals of 300 to 1000 cells were counted on each dish. For biochemical determination of GGT activity, dishes were rinsed with saline then stored at −80°C. The dishes were thawed, the cells scraped off the dish in saline and assayed for GGT activity by the method of Tateishi et al. (14). One unit of GGT activity was defined as the amount of enzyme that released 1 µmol of d-p-nitroaniline per min at 25°C. Three dishes were assayed independently for each time point. Protein con-
centration was determined by the BCA protein assay (Pierce, Rockford, IL). The GGT assays were done for each of five rat and four mouse experiments.

**Glutathione assay**

At each time point, the media was removed from three dishes, the dishes were rinsed with saline and placed on ice. The cells were lysed with ice-cold 0.2 M 5-sulfosalicylic acid, scraped off the dish and centrifuged at 10 000 g for 5 min at 4°C in an Eppendorf microfuge. The supernatant was stored at −80°C. Glutathione levels in the supernatant were determined by the method of Tietze (15). The glutathione levels were determined in each of three rat and four mouse experiments.

**GGT mRNA analysis**

Template preparation and synthesis of GGT 5′UTR antisense cRNA probes specific for mRNA I, mRNA II, mRNA III and mRNA IV were carried out, as previously described, to yield cRNAs of 155, 160, 256 and 341 nt in size, respectively (16). These GGT 5′UTR-specific cDNA plasmid constructs were kindly provided by Dr Yannick Laperche (Hôpital Henri Mondor, INSERM U-99, Créteil, France). A cyclophilin antisense cRNA was used as an internal control for equal loading of RNA. The cyclophilin template was modified such that an antisense transcript of 107 nt was obtained (17). Antisense probes were synthesized and radio labelled with [α-32P]UTP (650 Ci/mmol; ICN Radiochemicals) in vitro, with the Gemini II transcription system (Promega Corp., Madison, WI), and purified on polyacrylamide-gels.

An aliquot of rat liver cells isolated by collagenase perfusion was centrifuged at 50 g, the supernatant removed and the pellet stored at −80°C. The remaining cells were plated and maintained in culture as described above. On day 7, the medium was removed and dishes were stored at −80°C. RNA protection assays were done as previously described (17). Briefly, total cellular RNA was isolated by the acid- guanidium thiocyanate–phenol–chloroform extraction procedure (18). Total cellular RNA was simultaneously hybridized to 5 × 10^6 c.p.m. of each of the four GGT cRNA probes, and the cyclophilin probe. A tRNA control sample was hybridized to all five probes to verify the specificity of the RNAse protection assays. Unhybridized RNAs were digested by adding RNAse digestion solution containing 47 U RNAse-A and 344 U RNAse-T1 (US Biochemical Corp., Cleveland, OH) for 30 min at 30°C. After digestion with proteinase-K for 15 min at 30°C, samples were extracted with phenol–chloroform, then ethanol precipitated and electrophoresed on 5% denaturing polyacrylamide gels. 32P-Labelled RNA size standards were generated by in vitro transcription of an RNA marker template set (Ambion, Austin, TX). Protected probe fragments were visualized by autoradiography with pre-flashed Kodak X-Omat AR5 film (Eastman Kodak) and intensifying screens at −80°C. Protected fragments were quantified by scintillation counting. GGT mRNA was analyzed independently for each of five experiments (five individual animals).

**Statistical analysis**

The data for the percentage of cells that expressed GGT and the GGT activity in the rat and mouse liver cells were analyzed for statistically significant differences over time by Kruskal–Wallis one-way ANOVA of ranks (19). For each species Dunnett’s method was used to isolate the group(s) that differed significantly from the control group (4-h time point). The data for the glutathione levels in the rat and mouse liver cells were normally distributed and of equal variance. They were analyzed by a two-way ANOVA (time and species). Tukey’s test was used to isolate the group(s) that differed significantly from the others.

**Results**

GGT activity in primary cultures of rat hepatocytes increased with time in culture. Histochemical staining for GGT activity revealed that 2.7% of the cells in the initial preparation of rat liver cells were GGT-positive. These strongly GGT-positive cells were smaller than the hepatocytes and were probably bile duct cells. After 3 days in culture, the hepatocytes, which were larger and often binucleate, began to stain positive for GGT activity. By day 5 there was a significant increase (P < 0.05) in the percentage of GGT-positive cells. On day 7, 54% of the hepatocytes were GGT-positive (Figure 1). Biochemical determination of GGT activity confirmed the histochemical observations (Figure 2). The activity increased 20-fold from 0.0011 ± 0.0008 units GGT activity/mg protein on day 1 to 0.022 ± 0.008 units GGT activity/mg protein on day 7. A statistically significant increase over the starting value was detected by day 3.

In contrast to the rat cells, GGT was not induced in mouse hepatocytes maintained in vitro. Results from the histochemical staining showed that the initial preparation of mouse liver cells did not contain any GGT-positive cells (Figure 1). None of the cells became GGT-positive over the course of the experiment. Biochemical assays of GGT activity also showed that GGT was not induced in mouse liver cells during the 7-day experiment (Figure 2). Protein assays revealed a 25% decrease in the number of cells per dish between days 1 and 7 in both the rat and mouse experiments.

Intracellular levels of glutathione were determined throughout the course of the experiment in both rat and mouse liver cell cultures (Figure 3). At 4 h after plating, the glutathione concentration in the rat liver cells was 27.5 ± 15.1 nmol/mg protein. In the mouse liver cells, the glutathione concentration was 45.8 ± 14.5 nmol/mg protein. During the first 24 h in culture, the glutathione level in both the rat and mouse liver cells rose to the same level. Over the next 2 days the glutathione concentration in the rat cells declined, while in the mouse cells, the glutathione level continued to rise and reached a plateau by 3 days. At each of the subsequent time points, days 3, 5 and 7, the glutathione concentration in the mouse cells was approximately two-fold higher than the concentration in

![Fig. 1. Induction of GGT in primary cultures of rat and mouse liver cells as determined by histochemical staining. Percentage of GGT-positive rat cells (C) and mouse (○) was determined over 7 days in culture. At each time point, one dish was stained in each of five rat and four mouse experiments. The results are the average values ± SD. Values that differ significantly (P < 0.05) from the starting value (4 h) are indicated by an asterisk.](image-url)
the rat cells. The glutathione concentrations in the mouse and rat liver cells did not differ significantly from one another during the first 24 h in culture, but did differ significantly \( (P < 0.05) \) at 3, 5 and 7 days. In one experiment 30 \( \mu \text{M} \) buthionine sulfoximine, an inhibitor of \( \gamma \)-glutamyl cysteine synthetase, was added to cultures of mouse hepatocytes to reduce intracellular glutathione levels. In this experiment, glutathione levels in the mouse liver cells were maintained below 50 nmol/mg protein, but there was no induction of GGT in the mouse hepatocytes.

RNase protection analysis was used to determine which of the rat GGT transcripts, rat GGT mRNAs I–IV, were induced in primary cultures of rat hepatocytes. No GGT mRNA could be detected in rat liver cells collected immediately following liver perfusion. After the cells were in culture for 7 days, only GGT mRNAIII was present (Figure 4). Bands were analyzed by scintillation counting and compared with cyclophilin mRNA, a loading control. mRNA pooled from two experiments showed that after 7 days in culture GGT mRNAIII was 18.0 \( \pm \) 4.6% of the level of cyclophilin mRNA.

Discussion
In this study we found that GGT is not induced in mouse hepatocytes when they are maintained in vitro under the same conditions that induce GGT activity in primary cultures of rat hepatocytes. Hepatocytes do not replicate under the culture conditions used for these experiments (11). Comparison of intracellular glutathione levels in rat and mouse liver cells showed that the glutathione level was higher in the mouse liver cells than the rat. Blocking glutathione synthesis with buthionine sulfoximine reduced the intracellular glutathione concentration in mouse liver cells, but did not trigger an induction of GGT. Analysis of the GGT mRNA in primary cultures of rat hepatocytes showed that only GGT mRNAIII is induced. This is the same GGT mRNA species that is present in GGT-positive, carcinogen-induced, preneoplastic, rat liver lesions and liver tumors (1–3).

GGT can be induced in mouse hepatocytes. In fetal mice, GGT is expressed in hepatocytes from the 17th day of gestation until birth. At birth, the level of GGT declines precipitously (14). A similar pattern of expression is seen in rat liver (20). Although induction of GGT is rare in preneoplastic liver lesions or hepatic tumors in mice, four hepatocarcinogens have been shown to induce GGT in these lesions (21–26). In the rat, GGT is observed in preneoplastic hepatocytes and

**Fig. 2.** GGT activity determined biochemically in primary cultures of rat (○) and mouse (●) liver cells. Three dishes were assayed per time point in each of five rat and four mouse experiments. The values shown are the average of the mean \( \pm \) SEM. Values that differ significantly \( (P < 0.05) \) from the starting value (4 h) are indicated by an asterisk.

**Fig. 3.** Glutathione levels in primary cultures of rat (○) and mouse (●) liver cells. Three dishes were assayed per time point in each of three rat and four mouse experiments. The values shown are the average of the mean \( \pm \) SEM. Glutathione levels in mouse liver cells that differ significantly \( (P < 0.05) \) from the starting level (4 h) are indicated by an asterisk. At the 3-, 5-, and 7-day time points the glutathione concentration in the mouse liver cells differed significantly \( (P < 0.05) \) from the concentration in the rat liver cells.
hepatocellular carcinomas generated by a wide array of structurally distinct hepatocarcinogens (27,28).

The level of GGT induction observed in the rat liver cells is similar to the level seen in preneoplastic liver lesions. Previous studies have shown that preneoplastic GGT-positive hepatocytes isolated from carcinogen treated rats had 0.037 units GGT activity/mg protein (29). In the present study, after 7 days in culture, the GGT activity in the rat hepatocytes was 0.022 units/mg protein.

Sirica and co-workers were the first to report that GGT is induced in rat hepatocytes when they are maintained in vitro (11). Other investigators have shown that the level of induction can be suppressed by modifying the isolation procedure, the substrate on which they are plated or the composition of the tissue culture medium (30–32). Meredith reported that supplementing the medium with compounds that maintain cystathionase activity, cysteine levels and intracellular glutathione levels, suppressed the induction of GGT in primary cultures of rat hepatocytes (33). In vivo hepatic glutathione concentrations are similar in well-fed rats and mice (34). In this study the intracellular glutathione concentrations were similar in the rat and mouse hepatocytes during the first 24 h in culture. After 3 days in culture, the glutathione concentration in the mouse liver cells was approximately twice the concentration in the rat hepatocytes. Inhibition of glutathione synthesis in mouse liver cells lowered the intracellular glutathione level but did not cause an induction of GGT activity.

In both the rat and the mouse, GGT is a single copy gene that can be transcribed from any of at least four promoters (35–40). Each promoter gives rise to one or more distinct GGT mRNAs that can be distinguished with probes to unique sequences upstream of the coding region (1). In both species, the hepatic GGT mRNA is transcribed from promoter III during fetal development (1,40). Data in this study revealed that GGT mRNAIII is the only GGT mRNA that is induced when rat hepatocytes are maintained in culture. This is the same GGT mRNA species that is induced in the rat during hepatocarcinogenesis (2,3). Therefore, promoter III is activated in rat hepatocytes both in vitro and during tumor formation in vivo.

Our examination of the published sequence for promoter III in the rat GGT gene shows sequence homology to the putative consensus sequence for NF-kB binding. NF-kB is a nuclear transcription factor that regulates a series of genes involved in stress responses (41). The sequence GGGGAGT-TCC, which is –82 to –73 relative to the translation start site, is in accord with the consensus sequence GGGRNN(YYC)C (41,42). This enhancer sequence is present in the GGT mRNAIII in the rat but is absent in GGT mRNAIII in the mouse (40). The activity of NF-kB is modulated by oxidation–reduction status and glutathione level of the cell (43,44). The reduced levels of intracellular glutathione in the rat hepatocytes in vitro may have triggered the induction of GGT mRNA via an NF-kB-mediated pathway. Further studies are necessary to test this hypothesis.

In this study we have demonstrated that GGT in vitro is induced in primary cultures of rat hepatocytes but not in mouse hepatocytes. The data show that in vitro, the level of GGT induction is similar to that seen in preneoplastic hepatocytes. In addition, the same promoter was activated in vitro and in vivo. Primary cultures of rat and mouse hepatocytes provide a model system with which to study interspecies differences in the regulation of this enzyme and to better understand the role of GGT in normal and neoplastic processes.

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References


