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Characterization of γ-Glutamyltransferase from Neoplastic and Non-Neoplastic Liver Tissues in Man and During Rat Liver Hepatocarcinogenesis

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Abstract—*The activity and the affinity for concanavalin A-Sepharose (Con A) of liver γ-glutamyltransferase (γ GT) were investigated in man, under various clinical conditions and in rats during experimental hepatocarcinogenesis. In man, γ GT activity was higher than normal in hepatomas and (except for 1 case of hemochromatosis) also higher in the surrounding cirrhotic liver. The proportion of γ GT which did not bind to Con A (Con A⁻ form) was also increased in the tumors and in the surrounding liver, yet (with the same exception as above) to a greater extent in the hepatomas.*

In rat, γ GT activity was higher in fetal liver (15-fold) and in hepatocarcinomas (10-fold) than in normal adult liver; total liver γ GT activity gradually increased during progression from foci of altered cells to neoplastic nodules and tumors.

The proportion of the Con A⁻ form of γ GT in the early or late stage of the carcinogenic process did not significantly differ from that in normal adult or regenerating rat liver, i.e. about 20% of the total activity. By contrast, nearly all the γ GT from fetal rat liver bound to Con A. This suggests that γ GT expression in rat liver carcinoma does not correspond to so-called retrodifferentiation process.

INTRODUCTION

γ-GLUTAMYLTRANSFERASE (γ GT) is a membrane-bound glycoprotein which catalyzes the transfer of the γ-glutamyl residue from glutathione and other γ-glutamyl compounds to amino acids or peptides [1].

Previous histochemical and biochemical studies have shown that, in rat and man, the γ GT activity was higher in fetal liver and in hepatomas than in liver of adults [2–9].

In adult liver, γ GT activity has been detected in Kupffer cells, endothelium of periportal vessels and bile duct epithelium, whereas virtually no activity could be observed in hepatocytes [10–12].

Based on these quantitative changes and the appearance of γ GT activity in the hepatocytes during the course of experimental hepatocarcinogenesis in the rat, it was proposed that γ GT could be used as an oncofetal marker for neoplastic transformation of livers [11–16].

Kinetic and immunological properties of γ GT were found to be similar in fetal liver, adult liver and hepatoma in rat and human [5, 8, 17–23], suggesting that the elevated level found in fetal liver and in hepatoma corresponds to a quantitative increase of an otherwise identical isoenzymic form of γ GT.

However, it was demonstrated that rat liver γ GT exists in 2 different types that may be distinguished by Con A-Sepharose affinity chromatography: a sialic acid-rich type found in the fetus, and a sialic acid-poor adult type, practically absent in the fetus [24]. The fetal type of the γ GT appeared also in the regenerating rat liver, 24 hr after partial hepatectomy [25].

Comparative studies on the γ GT isolated from hepatoma tissue and normal adult or fetal liver, in rat and human, suggested significant tissue differ-

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Abbreviations: Con A: concanavalin A. DENA: diethylnitrosamine. γ GT: γ glutamyltransferase (EC 2.3.2.2). Con A⁻ γ GT: the enzyme form that does not bind to Con A—Sepharose.

ences in the carbohydrate moieties of this enzyme [17, 19, 21, 23, 24, 27].

On the other hand, other authors did not find differences in the binding properties of the γ GT to Con A-Sepharose between fetal, normal adult and neoplastic liver in rat and man [22, 26]. The enzyme was also claimed to be similar in normal regenerating and preneoplastic rat liver [26].

In view of these controversial data, we investigated the activity and the lectin-binding properties of the γ GT from liver lesions induced during the course of experimental hepatocarcinogenesis in rat and from normal and tumor liver in man.

The aim of the study was to search for the appearance of a new or modified form of γ GT during the progression of the carcinogenic process, which might help in improving the differential diagnosis of various hepatobiliary diseases in human clinics.

MATERIALS AND METHODS

Chemicals

α-Methyl-D-mannopyranoside (grade III), concanavalin A-Sepharose 4B and neuraminidase from *Clostridium perfringens* (type V) were obtained from Sigma Chemical (St. Louis, MO, U.S.A.).

All other chemicals used were of the highest analytical quality available.

Enzyme assay

γ GT activity was determined at 25°C on an automated analyzer, with a kinetic method as developed by Szasz [28], using γ-glutamyl-*p*-nitroanilide as substrate and glycylglycine as acceptor. This activity was expressed in international milliunits (mUI) per mg of protein.

Protein determination

Protein concentration was determined by the method of Bradford [29] with bovine serum abumin as the standard.

Animals and human tissues

The Scherer–Emmelot hepatocarcinogenesis model [30] was used. Female Sprague–Dawley or Wistar rats weighing 180–220 g, were submitted to a 70% partial hepatectomy [31] and 24 hr later, were treated with diethylnitrosamine (DENA), at a total dose of 70 mg/kg, given either as a single i.p. injection or p.o., as fractionated doses over 10 consecutive days. Three, 11 and 15 months after DENA treatment, 2–3 animals from each group were sacrificed and liver samples were either immediately homogenized or frozen in liquid nitrogen and stored at −20° C until used.

Histological examination was performed using hematoxylin–eosin staining and γ GT staining [10]. γ GT-positive foci of altered hepatocytes were observed at 3 months; hepatic nodules and tumors were confirmed by macroscopical inspection 11 and 15 months, respectively, after the carcinogenic treatment. Hepatoma tissue was carefully dissected and isolated from the surrounding tissue.

Fetal rat livers were obtained on day 18 of gestation; pooled livers from 10 to 12 fetuses were used.

For studies on regenerating liver, 2 animals were partially hepatectomized [31] and killed 24 hr later.

Human liver tissues were obtained at the time of surgery, following partial or total hepatectomy. The situations investigated are summarized in Table 1.

Preparation of γ GT from rat and human liver tissues

Fresh or thawed tissues were minced and homogenized in 3 vol. medium (10 mM Tris–HCl, pH 7.5, containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$) at 4° C, using a Teflon–glass homogenizer according to Köttgen *et al.* [25].

A sample of the homogenate was solubilized in 4.0% (v/v) Triton X-100 during 40 min at 37° C. After centrifugation for 5 min at 10,000 ***g***, the supernatant was used to determine total γ GT activity.

Con A-Sepharose column chromatography

The supernatant was loaded on a 1 × 5 cm column on Con A-Sepharose 4B, previously washed with 10 mM Tris–HCl, pH 7.5, containing 0.5 M NaCl, Triton X-100 (5 g/l), 1 mM of each $CaCl_2$, $MgCl_2$, $MnCl_2$ and sodium azide (0.2 g/l).

One hour after application of the enzyme solution, the column was rinsed with equilibration buffer (10 mM Tris–HCl, pH 7.5, containing 0.15 M NaCl, Triton X-100 (5g/l), 1 mM of each $CaCl_2$, $MgCl_2$ and $MnCl_2$). Equilibration buffer containing successively 0.2 M and 0.5 M α-methyl-D-mannopyranoside was then applied onto the column; a temperature of 20–25°C and a flow rate of 30 ml/hr was maintained. Fractions of 380 μl were collected. (Note that these fractions may not be frozen because of Mn precipitate in these conditions.)

The total recovery of γ GT from the Con A-Sepharose affinity column was 80–100%. The proportion of γ GT not bound to Con A (Con A^- γ GT) was expressed as a percentage of the total activity recovered.

The capacity of absorption of the column corresponded, in our study, to a binding of 7 mg of porcine thyroglobulin per ml of Con A-Sepharose (close to the 8.5 mg value indicated in the manufacturer's notice). This capacity was never approached, even in terms of total proteins loaded on the column (about 1 mg/ml Con A for human liver tissues and for fetal rat liver and about 5 mg/ml Con A for the other various rat livers).

Table 1. Clinical and histological findings concerning human livers studied

Case	Age	Sex	Associated clinical condition	Histologic findings	Tumor size
1	16	M	Brain death; seric hepatic enzymes normal	Liver macroscopically normal	—
2	41	M	Alcoholism	Cirrhosis	—
3	35	F	Oral contraception	Normal liver* liver cell adenoma	16 × 14 × 6 cm
4	65	M	Chronic hepatitis	Post-hepatitic cirrhosis* differentiated and necrotic HCC	3.5 cm dia.
5	56	M	NANB hepatitis 4 years prior to diagnosis	Post-hepatic cirrhosis + dysplasia* multifocal differentiated HCC	5, 3, 1 cm dia.
6	62	M	Diabetes	Hemochromatosis* well-differentiated HCC	N.D.
7	55	M	Viral hepatitis 13 years prior to diagnosis	Normal liver* poorly-differentiated HCC	23 × 22 × 5 cm

*Histological findings in surrounding non-tumoral liver.
N.D.: not determined, HCC: hepatocellular carcinoma, NANB hepatitis: Non-A–Non-B hepatitis.

Periodically, a biological sample with near 100% of Con A-bound γ GT was rechromatographed, showing again near 100% binding; this excludes that eventual absence of binding in a run resulted from saturation of Con A-Sepharose due to cumulative improper regeneration of the column.

Neuraminidase treatment of γ GT preparations

After dialysis against 0.05 M sodium acetate buffer, containing 10 mM $CaCl_2$, pH 5.0, for 12 hr at 4° C, the liver extract was incubated for 48 hr at 20° C with neuraminidase (0.6 U/ml), before being applied to the column.

Incubation of the homogenate without neuraminidase then served as control. In these conditions, γ GT activity in the control dropped, at most, by 15%. The activity of neuraminidase was tested as described by Warren *et al.* [32].

Statistical methods

All data were expressed as means with standard deviation. Significance of the differences between groups was evaluated by Student's *t*-test. *P* values above 0.05 were considered not significant.

RESULTS

Human liver

The clinical and histological findings concerning human livers are summarized in Table 1.

In human hepatoma, γ GT activity was 1.8 to 4.9 times (3.2 on average) the normal liver value (Table 2).

This was not different in hepatoma tissue and in surrounding non-neoplastic liver tissue, when cirrhosis was present, with (case 5) or without (case 4) foci of dysplasia.

Total γ GT activity thus would not discriminate all hepatomas from cirrhotic liver. It might however be that, when hemochromatosis is present, a near normal level of γ GT activity would be found in cirrhotic liver, as suggested by case 6.

In the liver cell adenoma investigated (case 3), the γ GT activity was similar to that observed in normal liver and in its own histologically normal counterpart. This suggests a possible differential diagnosis between benign and malignant tumors on this basis.

Figure 1 shows a typical elution profile obtained when the homogenate from a human hepatoma tissue (case 5) was applied to a column of Con A-Sepharose. When the column was washed through with equilibration buffer, 60.7% of the γ GT activity was retained; the remainder of the enzymatic activity eluted with the void volume, with most of the other proteins.

Figure 1 further shows that elution with 0.2 M α-methyl-D-mannopyranoside removed all the enzyme from the column. The same protocol was applied for all liver homogenates.

In man (Table 2), the proportion of the Con A^- form of γ GT was about 6 times higher, on average,

Table 2. Total γ GT activity and percentage of the form without affinity for concanavalin A (Con A⁻ γ GT) in human liver

	Tissue	Specific activity (mUI/mg protein)	Range	Fraction of Con A⁻ γ GT activity (% total)	Range
a.	Normal adult liver				
	Case 1	80	(64–95)	1.6	(1.3–1.9)
	Case 3*	94	(91–97)	3.3	(1.0–5.7)
	Case 7*	54	(46–66)	7.7	(4.4–10.9)
		Mean: 76± 20		Mean: 4.2 ± 3.1	
b.	Cirrhotic liver				
	Case 2	180		13.8	
	Case 4*	283	(229–390)	9.5	(8.2–10.8)
	Case 5*	297	(278–329)	10.9	(8.5–13.3)
	Case 6*	77	(37–132)	15.2	(13.5–17)
		Mean: 209 ± 102†		Mean: 12.4 ± 2.6‖	
c.	Hepatoma				
	Case 4	378	(246–474)	31.4	(30.3–32.6)
	Case 5	243	(221–269)	36.9	(34.5–39.3)
	Case 6	217	(146–323)	12.9	(9.8–16.0)
	Case 7	138	(111–174)	20.8	(14.2–27.4)
		Mean: 244 ± 100‡§		Mean: 25.5 ± 10.7‡¶	
d.	Adenoma				
	Case 3	90	(88–92)	7.4	(6.3–8.4)

Con A-Sepharose column chromatography was performed as described in Materials and Methods.
*Adjacent to tumor, see corresponding cases in the adenoma or hepatoma groups.
†Significantly different from normal liver at P level < 0.05.
‡Significantly different from normal liver at P level < 0.025.
§Not significantly different from cirrhotic liver.
‖Significantly different from normal liver at P level < 0.01.
¶Significantly different from cirrhotic liver at P level < 0.05.

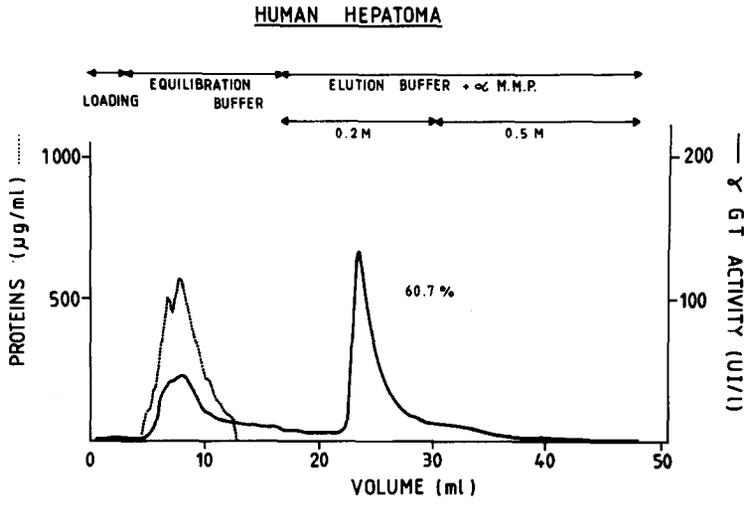

Fig. 1. Con A-Sepharose chromatography of one human hepatoma homogenate (case 5); γ GT activity and protein concentration were determined as described in Materials and Methods. One hour after loading the enzyme sample onto the column, equilibration buffer and then elution buffer containing α-methyl-D-mannopyranoside (α-M.M.P.) were passed through, at a flow rate of 30 ml/min.

in hepatomas than in normal liver; in 3 out of 4 cases, it was also higher than in cirrhotic liver.

There was, however, overlap between individual values from each of those groups.

In the 1 case of adenoma investigated, the proportion of Con A⁻ γ GT was within the range of values for normal liver.

Table 3. Total γ GT activity and percentage of the form without affinity for concanavalin A (Con A⁻ γ GT) in rat liver

Tissue*		Specific activity of γ GT (mUI/mg protein) (Mean ± S.D.)	Fraction of ConA⁻ γ GT activity (% total) (Mean ± S.D.)
a. Normal liver			
1. Adult normal liver (3)		3.5 ± 0.9	21 ± 4
2. Regenerating liver (2)†		6.05 ± 2.05	22 ± 2
3. Fetal liver (2)‡		51.6 ± 5.2	1.2 ± 0.8
b. Stages in hepatocarcinogenesis	(treatment§ duration)		
1. Foci of altered cells (2)	(3 months)	6.9 ± 4.5	21 ± 1
2. Nodular liver (3)	(10–11 months)	13.4 ± 5.3	37 ± 9
3. Liver carcinoma (2)	(15 months)		
Tumor		36.5 ± 18.2	16 ± 4
Surrounding liver		12.6 ± 6.9	21 ± 1

Con A-Sepharose column chromatography was performed as described in Materials and Methods. The fraction unbound to Con A was expressed as a percentage of the total activity recovered from the column.

*Number of independent experiments.

†24 hr after partial hepatectomy.

‡18th day of gestation.

§Refers to the time elapsed since application of the carcinogenic treatment (i.e partial hepatectomy + DENA).

Rat liver

Table 3 shows the specific γ GT activity in homogenates of normal adult and fetal rat liver, as well as in regenerating rat liver and in pre-neoplastic and neoplastic liver lesions induced following the protocol described by Scherer and Emmelot [30].

This activity was about 10-fold higher in liver carcinoma (range 5- to 20-fold) and about 15-fold higher in fetal liver, than in normal adult liver. Total liver γ GT activity gradually increased with the progression of the hepatocarcinogenic process.

A slight increase in γ GT activity was observed in the early (foci) stage of this process and in the regenerating liver but, in view of the high S.D. value, this may not be considered as significant and would require further investigation.

In the rat (Table 3), the results of Con A-Sepharose chromatography indicate similar proportions of the Con A⁻ form of γ GT in adult normal or regenerating liver, as well as in livers containing foci of altered cells, neoplastic nodules or liver carcinomas and in the tumors themselves.

By contrast, practically all of the enzyme in fetal liver bound to Con A.

DISCUSSION

As γ GT is associated with membranes, treatment with detergents or proteolytic enzyme is necessary to solubilize the enzyme.

When solubilized with detergent, γ GT is a hydrophobic protein, similar to the native enzyme and contains the domain that anchors the enzyme to the membrane [33, 34]. On the other hand, treatment with proteolytic enzyme removes this anchoring portion and affects some properties of the enzyme, e.g. behavior on Con A-Sepharose [35].

In order to avoid possible selection of particular isoforms, or production of artefacts during purification in these conditions, affinity chromatography was performed with γ GT solubilized by Triton X-100 from tissue homogenates.

Our results show that in human liver tissues, the γ GT activity was higher in all hepatomas than in adult normal liver (Table 2).

The γ GT was the least augmented in the poorly-differentiated hepatoma (case 7). A low γ GT activity has been reported in poorly-differentiated hepatoma cell lines, but this did not seem to constitute a general rule [6, 13]. No correlation was noted between tumor size and the γ GT activity in the hepatomas (Tables 1 and 2).

In this study, we also analyzed the activity and affinity for Con A-Sepharose of human cirrhotic liver γ GT.

As γ GT activity measured by biochemical methods represents the sum of the activities present in the bile duct elements and in the altered cells foci, it was not unexpected that it was increased in the cirrhotic liver tissue, adjacent to tumors. We know of no similar studies in man, but similar findings have been reported in experimental cirrhosis in rats [12, 36].

In liver cell adenoma and in the surrounding liver, no significant change in the enzyme activity was detected (case 3). This is in agreement with a previous histochemical study [7].

On the basis of their behavior in Con A-Sepharose chromatography, it was apparent that γ GT from

human hepatomas and from the surrounding non-neoplastic tissue differed in their heterosaccharide moieties and/or sugar accessibility in 3 out of 4 cases (Table 2).

The exception concerns a case (case 6) in which hemochromatosis was observed, with the hepatoma exhibiting a proportion of the Con A^- γ GT form close to the average value for the cirrhosis group.

The chromatographic behavior of γ GT after neuraminidase treatment (results not shown) indicates that the abnormal properties of the human hepatoma enzyme in this respect relates to other factors (glycosyl moieties and/or molecular structure) than the content in sialic acid residues alone. A similar finding was reported by Yamamoto *et al.* [19]. Similarly, α FP in newborn mouse serum exists as Con A^- and Con A^+ forms, yet no variation in the extent of sialylation was ever demonstrated, also suggesting that differences in the amount of sialic acid do not explain differences in reactivity with Con A in that case [37].

The presence of γ GT forms with glycosyl moieties different from those of the normal enzyme has been demonstrated in several human cancer tissues including malignant hepatoma [19, 23], breast cancer [38] and renal [39], colorectal [40] and pancreatic [41] carcinomas.

In rats, very low γ GT activity was detected in adult liver. This agrees with previous reports [4, 9, 13, 36]. Total γ GT activity did not increase significantly in regenerating rat liver 24 hr after partial hepatectomy, as also observed by others [4, 16, 25, 36].

The level of liver γ GT activity gradually increased with the progression of chemically-induced hepatocarcinogenesis (Table 3): it was higher than normal in the precancerous nodular stage, and further increased in the hepatocarcinomas. It was also very high in fetal liver.

Our results also indicate that the determination of the Con A^- form of γ GT cannot discriminate between the early or late stages of the carcinogenic process and normal adult or regenerating rat liver (Table 3).

The only other work on γ GT during progression of hepatocarcinogenesis in rat [26] concerns a different model, using continuous feeding of a choline-deficient diet supplemented with ethionine, during 23 weeks. Our study thus confirms, with a different model, that the fraction of Con A^- γ Gt is not an appropriate marker of neoplastic lesions in rat liver.

Under almost similar experimental conditions to those of Köttgen *et al.* [24, 25], we did not find the high proportion of Con A^- γ GT in fetal and regenerating rat liver (Table 3), reported by those authors.

Our results better agree in this, with those obtained by Ellison *et al.* [26], using the same methodology.

We cannot offer any explanation for this discrepancy. Among the possible basis for the differences in results, the temperature at which chromatography is performed and the ratio of lectin to γ GT protein might intervene.

Binding at 4°C (as used by Kottgen *et al.* [24, 25]) is lower than at 25°C, as used in our assays [42]. Artefactually low binding to Con A might also results from improper ratio of lectin to γ GT protein [42].

The difference in the proportion of Con A^- γ GT, between preneoplastic and neoplastic liver as compared to fetal rat liver suggests that increased γ GT in the former cases does not reflect a simple retro-differentiation process.

In conclusion, our experiments confirm that γ GT activity increases in malignant liver lesions over adult normal level in both human and rat species. They further show that the 2 models differ in that, whereas in man, the Con A^- fraction of γ GT was higher in malignant liver lesions (25%) than in benign lesions (7%, 12%) and in normal liver (4%), in rats, the proportion of Con A^- γ GT was the same in normal, regenerating, pre-neoplastic and neoplastic liver (23%).

Despite some overlap between normal, preneoplastic and malignant groups in this respect, our results indicate that an increase in the proportion of the Con A^- form of γ GT to about 5 times the normal value might represent a specific marker of liver malignancy in man (1/4 false-negative; 0/8 false-positive). The fact that in the false-negative case, hemochromatosis was present, suggests that search for seric signs of hemochromatosis might allow to define situations in which the γ GT–Con A binding assay would not be applicable for detecting liver malignancy.

If sensitivity is expected from the test, a total γ GT activity higher than twice the normal average value in a liver biopsy sample, appears as a potentially good marker, yet at the expense of specificity (0/4 false-negative; 3/8 false-positive).

In this respect, it is noteworthy to mention, that in the case of adenoma investigated, γ GT level and the proportion of the Con A^- form of the enzyme were close to normal.

Further investigation is therefore warranted in order to determine how γ GT activity and the proportion of the Con A^- γ GT form (alone or in combination) would have optimal value for differential diagnosis of liver lesions.

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