HISTOCHEMICAL DEMONSTRATION OF INCREASED ACTIVITY OF γ -GLUTAMYL TRANSPEPTIDASE IN RAT LIVER DURING HEPATOCARCINOGENESIS*

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Histochemical demonstration of γ -glutamyl transpeptidase (γ -GTP) during chemically induced hepatocarcinogenesis in the rats was studied. The carcinogens employed were N-OH-FAA (N-hydroxy derivative of N-2-fluorenylacetamide), and aflatoxin B₁.

In the control rats, activity of γ -GTP was demonstrated in the bile duct cells and occasionally along the bile canaliculi, but was not in the hepatocytes. Proliferating bile duct cells including so-called oval cells and cholangiocarcinoma cells, and hepatocytes of hyperplastic areas and nodules and hepatocellular carcinoma induced by N-OH-FAA with or without aflatoxin B₁ revealed positive activity of γ -GTP, histochemically. Only focal demonstration of γ -GTP activity in the hepatocyte was observed in association with a mild hyperplastic change in rats treated with aflatoxin B₁ alone.

The elevation of γ -GTP activity in serum and liver tissue with appearance of fetal type of its isoenzyme pattern was also demonstrated biochemically and correlated with the histochemical appearance of γ -GTP activity in the liver tissue from the early phase of hepatobiliary carcinogenesis.

These results seem to demonstrate carcino-embryonic nature of the induced γ -GTP in the development of cholangiocellular and hepatocellular carcinomas.

Since it was suggested by Hanes et al. (11) that the initial reaction of glutathion breakdown was carried out by transpeptidation reaction with γ -glutamyl transpeptidase (γ -GTP), there have been appeared many biochemical studies on this enzyme (6, 22). Szczeklik et al. (24) first reported on increased activity of γ -GTP in serum in most varieties of human liver diseases including hepatocellular carcinoma. Recently, biochemical studies on γ -GTP activity during experimental hepatocarcinogenesis have been carried out and suggested the activation of γ -GTP in relation to the carcinogenic process induced by chemical compounds (8, 9).

However, there is almost none of the systematic histochemical work on this enzyme involved in the experimental hepato-biliary carcinogenic process. The

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present study was carried out for histochemical demonstration of γ -GTP activity in the livers of rats treated with a strong hepatocarcinogen; N-OH-FAA, a carcinogenic mycotoxin; aflatoxin B₁ and their combination. Biochemical determination of γ -GTP activity in the sera and liver tissues was also included for evaluation of the histochemical findings.

MATERIAL AND METHODS

Animals

A total of 94 male albino rats of the Sprague-Dawley strain obtained as weanlings from CLEA Japan Inc. were used for these experiments. The animals were housed four or five per cage in an air-conditioned room.

Carcinogen dosage

Animals were fed CE-2 pellet (regular basal diet, Clea Japan Inc., Tokyo). At 6 weeks of age, rats divided into five groups were placed on the same basal diet with or without: N-hydroxy derivative of N-2-fluorenylacetamide (N-OH-FAA, mp 147°C, was kindly supplied by Professor Kei Sato of Hirosaki University School of Education, Hirosaki, Japan) at 0.01% for the first two weeks, 0.012% for the following 4 weeks and 0.015% for the last 13 weeks in groups 1 and 2; and at 0.032% for 16 weeks in group 4. Aflatoxin B₁ (Makor Chemicals, Jerusalem, Israel) was administered to rats by stomach tube at a dose of 10 μ g in 0.4 ml olive oil three times a week at two days intervals for the first two weeks, at dose of 25 μ g in 1.0 ml olive oil three times a week at two days intervals for the following 5 weeks and finally, after pause of 24 weeks, at a dose of 40 μ g in 1.2 ml olive oil twice a week at three days intervals for 5 weeks in groups 2 and 3. All animals were allowed food and water *ad libitum*.

Four animals in groups 1, 2, 3 and 5 were sacrificed after 16, 36, 58 and 78 weeks, respectively, and three or four animals in groups 4 and 5 were sacrificed after 1, 2, 4, 8, 12 and 16 weeks of experimental feeding, respectively. Total intake of carcinogen per rat in each group is shown in Table 1.

Histological and histochemical procedures

The liver specimens obtained under light anesthesia with ethylether were immediately fixed in cold 10% buffered formalin or Carnoy's solution. Usually processed tissue sections were stained by hematoxylin and eosin and periodic acid-Schiff reaction.

Histochemical demonstration of γ -GTP was performed using the modified method of Albert-Orlowska-Orlowski-Szewczuk (1). Frozen sections, 5–10 μ in thickness, were prepared by cryostat and incubated at room temperature in the following reaction mixture for 5–20 minutes (non-fixed specimen) or 15–30 minutes (fixed specimen with cold acetone): 6 ml 0.9% NaCl containing 0.02 M of γ -L-glutamyl- α -naphthylamide and 4 ml phosphate buffer 0.1 M, pH 6.7. Immediately before use, 7 mg of fast-garnet GBC were added to 10 ml of the incubation mixture. After being rinsed in saline three times for one minute each time, the sections were immersed for 2 minutes in 0.1 M CuSO₄ solution. The sections were then counterstained with hematoxylin for 1–2 minutes and mounted with glycerin. Good results were also obtained with paraffin-embedded sections following fixation in cold acetone, but not following fixation in cold formalin.

To establish the specificity of the histochemical reaction, some critical control experiments have been conducted. Incubation of specimens in medium, from which the substrate was omitted, did not produce reaction product. The enzymatic reaction did not occur at 0-time incubation. Diffusion of enzyme was protected within limit of incubation-time as described above. No effect of counterstain with hematoxylin on γ -GTP stain was observed. These results indicated that the reaction products were not due to non-enzymatic chemical reaction.

In addition to γ -GTP, histochemical demonstration of the following enzymes was also carried out by the respective method of staining on the frozen sections: Non-mitochondrial adenosine triphosphatase (ATPase) by Wachstein Meisel's method (5), alkaline phosphatase (Alk.Pase) by Anderson-Barka's method using Naphthol AS-MX phosphate and Fast Red LB (5) and β -glucuronidase by Hayashi-Fishman's method (13).

Biochemical determination of γ -GTP

Histochemical studies were paralleled by biochemical assays of γ -GTP activity in serum and liver homogenates. Freshly prepared liver tissue was immediately homogenized in three volumes of 0.25 M sucrose with Tefrone homogenizer on ice bath and separated into subcellular fractions according to the method of Hogeboom and Schneider (14). γ -GTP activities were determined by using the Sankyo kit (Sankyo Pharmaceutical Co. Ltd., Tokyo), which is a modification of the method of Szczeklik (24) and uses γ -glutamyl-p-nitroanilide as a substrate. Isozyme pattern of γ -GTP was obtained on 0.5% polyacrylamide disc electrophoresis (19).

RESULTS

Histology and histochemistry of the liver

In the control group, all the rats sacrificed at the various experimental weeks revealed non remarkable morphological change throughout liver tissue. The activity of γ -GTP was demonstrated in the epithelium of bile ducts and ductules at the portal area (Fig. 1). Occasionally, faintly positive reaction was observed along the bile canaliculi of the acinus-peripheral hepatocytes. ATPase was localized chiefly in the bile canaliculi and bile ducts and vascular endothelium of the portal area. Alk. Pase was demonstrated only in the portal area, especially in the bile ducts, vascular endothelium and in connective tissue surrounding the bile ducts. Almost all of the hepatocytes and Kupffer cells contained β -glucuronidase-positive granules in their cytoplasm.

Remarkable histochemical findings as the sequential change during hepatobiliary carcinogenesis was obtained in γ -GTP activity. The rats of group 4, which were treated with higher dose of N-OH-FAA than those of group 1 and 2, showed the earlier appearance of the proliferative changes of both the bile duct cells and hepatocytes. Bile duct proliferation associated with increased activity of γ -GTP at the portal area appeared in liver of the rats sacrificed after four-week's feeding of N-OH-FAA.

The rats of group 4 after 8 and 12 experimental weeks and those of group 1 and 2 after 16 experimental weeks showed the advanced changes in the livers, as described by previous workers using FAA (N-2-fluorenylacetylamide), (20, 15). Bile ductal and ductular proliferation was associated with irregularity of the limiting

FIG. 1. Liver of the control rat. $\times 400$

Activity of γ -GTP is confined in the epithelium of bile ducts at the portal area.

FIG. 2. Liver of the rat fed N-OH-FAA for 16 weeks. $\times 200$

Activity of γ -GTP is demonstrated in the hyperplastic hepatic cells, proliferating bile-duct cells and oval cells.

FIG. 3. Liver of the rat, 36 weeks after start of the feeding-experiment with N-OH-FAA and aflatoxin B₁. $\times 100$

Positive histochemical reaction of γ -GTP is seen in the hepatocellular tumor cells as well as the hyperplastic hepatic cells and bile-duct cells.

FIG. 4. Cholangiocellular carcinoma developed in the rat fed N-OH-FAA for 19 weeks and sacrificed at the 58th experimental week. $\times 300$

Activity of γ -GTP is demonstrated in these tumor cells.

FIG. 5. Liver of the rat treated with aflatoxin B₁ alone and sacrificed at the 36th experimental week. $\times 100$

Scattered foci of hyperplastic hepatic cells showing positive reaction of γ -GTP are seen mainly in the acinus-peripheral region.

FIG. 6. The higher magnification of Fig. 5. $\times 400$

plate, periportal fibrosis and occasional oval cells. Areas of hyperplasia and hyperplastic nodules showed a predilection for the portal zones. The activity of γ -GTP was demonstrated in these hyperplastic parenchymal cells, proliferating bile-duct cells and oval cells, taking form of a homogeneous red-brown mass (Fig. 2). The apical portion of epithelium and cell membrane along the bile canaliculi showed intense activity.

The rats showed more manifest changes after 16 experimental weeks in group 4 and after 36 and 58 weeks in groups 1 and 2 than the above described morphological alterations. In addition to the benign hepatocellular tumor—adenoma, differentiated type of hepatocellular carcinoma showing trabecular pattern was also observed at these stages. A positive histochemical reaction of γ -GTP was also detected in the cytoplasm of these hepatocellular tumor cells as a fine granular particle. However, the reactivity of these cells was demonstrated with slight irregularity among the cell to cell (Fig. 3). Bile duct showed focal proliferation with occasional cystic dilatation—cystadenoma. A few cases of cholangiocellular carcinoma were also found in group 1 and 2. Strong reactivity of γ -GTP was demonstrated in these bile duct cells (Fig. 4) (Table 1).

The liver of the rat treated with aflatoxin B₁ after 16 experimental weeks showed a few scattered foci of hepatocytes with multi-vacuoles in their cytoplasm as well as slight chromatin irregularity of nuclei. These foci were more prominent associated with focal proliferation of the atypical hepatocytes including megalocytes in the liver of rats sacrificed after 36 experimental weeks. The activity of γ -GTP was observed not only in the bile duct and ductules along the portal areas, but also in these hepatocytes showing the focal atypical changes (Figs. 5 and 6) (Table 1).

As for the histochemical findings other than those with γ -GTP, a correlation was observed between the experimental morphological changes and the gradual increase in lysosomal granules of the hepatocytes, positive β -glucuronidase staining, or decrease in both vascular and canalicular ATPase. Some of the hyperplastic nodules with γ -GTP activity showed increase in sinusoidal alkaline phosphatase and contained glycogen-rich hepatocytes. Histochemical findings, exclusive of γ -GTP, varied considerably amongst different rats as well as amongst different experimental stages.

Biochemical Analysis

The results of biochemical analysis on serum γ -GTP activity are shown in Table 1. The animals treated with N-OH-FAA alone and with combined administration of aflatoxin B₁ and N-OH-FAA showed higher activity of serum γ -GTP than those treated with aflatoxin B₁ alone. However, serum values of γ -GTP in aflatoxin B₁-treated rats after 36 and 78 experimental weeks were approximately twice and three times higher than that of control rats, respectively. Thus, relatively good correlation was observed between the histochemical demonstration and serum level of γ -GTP.

The biochemical results of tissue γ -GTP activity including their isozyme analysis were reported previously (18). The γ -GTP activity in livers of the rats treated with N-OH-FAA or aflatoxin B₁ alone is shown in Table 2. And γ -GTP activity in each fraction of the livers obtained from N-OH-FAA treated rats is shown in Fig. 7. The γ -GTP activity in each fraction of the livers of N-OH-FAA treated rats was

TABLE 1. Carcinogenic dose, morphological findings accompanied with increased histochemical activity of γ -GTP and serum level of γ -GTP

Group No	Carcinogenic Compound	Exper. Weeks	No. of Rats Sacrificed	Total Carcinogen Dose	Morphological Change of Liver*				γ -GTP Activity in Serum (mean $\pm$ S.E.)
					Bile duct proliferation	Bile duct carcinoma	Areas of hyperplasia	Nodular hyperplasia	Hepatocellular carcinoma
1.	N-OH-FAA	16	4	295mg	++**	—	+	—	63.0 $\pm$ 18.0
		36	4	352mg	++	—	+	+	126.5 $\pm$ 47.0
		58	4	352mg	++	— $\sim$ +	+	+	
2.	Aflatoxin B ₁ + N-OH-FAA	16	4	435 μ g + 295mg	+	—	+	—	58.0 $\pm$ 9.2
		36	4	870 μ g + 352mg	++	—	+	— $\sim$ +	108.0 $\pm$ 68.7
		58	4	870 μ g + 352mg	+	— $\sim$ +	+	+	
3.	Aflatoxin B ₁	16	4	435 μ g	— $\sim$ +	—	—	—	2.0 $\pm$ 1.0
		36	4	870 μ g	— $\sim$ +	—	+	—	11.0 $\pm$ 8.7
		78	4	870 μ g	—	—	+	—	7.2 $\pm$ 2.5
4.	N-OH-FAA	1	4	33.6mg	—	—	—	—	3.2 $\pm$ 5.2
		2	4	78.4mg	— $\sim$ +	—	—	—	1.3 $\pm$ 0.7
		4	4	168mg	+	—	—	—	4.0 $\pm$ 5.8
		8	4	347mg	++	—	+	+	17.9 $\pm$ 16.8
		12	3	526mg	++	—	+	+	37.4 $\pm$ 22.8
		16	3	706mg	++	—	+	+	37.5 $\pm$ 34.4
5.	None	1	4	—	—	—	—	—	— 3.5 $\pm$ 4.5
		2	4	—	—	—	—	—	
		4	4	—	—	—	—	—	
		8	4	—	—	—	—	—	
		12	4	—	—	—	—	—	
		16	4	—	—	—	—	—	
		36	4	—	—	—	—	—	
		78	4	—	—	—	—	—	

* All these changes were accompanied with increased histochemical reaction of γ -GTP.

** ++ shows marked proliferation of bile duct.

TABLE 2. γ -GTP Activity of the Liver Homogenates of the Rats Treated with N-OH-FAA or Aflatoxin B₁

Group No.	Carcinogenic compound	Exper. weeks	γ -GTP activity (mu/mg*)	
			Range Unitt	Mean Units $\pm$ S.D.
3	Aflatoxin B ₁	16	0.5—1.1	0.6 $\pm$ 0.3
		36	0.6—4.4	2.5 $\pm$ 1.9
		78	2.7—8.0	4.9 $\pm$ 1.9
4	N-OH-FAA	1	1.9—3.9	3.1 $\pm$ 0.8
		2	2.3—3.4	2.9 $\pm$ 0.4
		4	2.4—7.2	4.3 $\pm$ 1.8
		8	7.8—16.2	10.9 $\pm$ 3.4
		12	17.4—20.5	19.1 $\pm$ 1.3
		16	15.5—21.0	17.4 $\pm$ 2.6
5	None	—	0.6—5.0	1.6 $\pm$ 1.1

* mu/mg: Results are expressed in milliunits per miligram of liver protein; a unit of activity being the number of μ moles of substrate transformed per 30 minutes at 37°C.

FIG. 7. γ -GTP activity in each fraction of the livers obtained from N-OH-FAA treated rats after 16 and 36 weeks of experiments, respectively (18).

estimated approximately 7 to 12 times higher than that of control rat. The predominant enzymatic activity was found in the granular fractions—mitochondrial and microsomal fractions—of the liver tissue.

Electrophoretic analysis on the isozyme pattern of serum and liver γ -GTP revealed the predominant elevation of its fetal type enzyme, corresponding with α -globulin region (14th fr.) from microsome fraction of the liver homogenates, in all

FIG. 8. Zymogram of γ -GTP in the hepatoma tissue (hepatocellular carcinoma) of rat fed N-OH-FAA for 19 weeks. The first peak at 7th fraction (tube) shows elevated isozyme pattern corresponding to β -globulin region and the second peak at 14th fraction corresponds to the fetal type isozyme of γ -GTP (18).

of the rats showing hyperplastic or neoplastic changes of the hepato-biliary cells in their livers (Fig. 8).

DISCUSSION

Histochemical demonstration of γ -GTP activity in the tissues of mammals including humans was firstly reported by Albert et al. (2). Since then, there have appeared many histochemical works concerned with this enzyme in the liver (1-4, 10, 12, 21, 23, 26, 27). Although intensive activity of γ -GTP was exclusively demonstrated in the wall of bile ducts and ductules, hepatocytes normally showed no activity in the liver of mammals except for the acinus-peripheral hepatocytes of adult guinea pig and rabbit or the hepatocytes of the fetal or newborn rat and mouse (3, 4, 7). In the control rats used in our study, activity of γ -GTP was localized in the epithelium of bile ducts and ductles of the liver. The observation of positive reaction along the bile canaliculi, which was noted in the acinus-peripheral hepatocytes in some of these control rats, was also reported (1, 21). Although some workers (1, 23) have described positive activity in Kupffer cells, no apparent activity was observed in Kupffer cells or sinusoidal endothelium.

Increased activity of γ -GTP, not only in the proliferating bile ducts but also in periportal hepatocytes, was demonstrated following experimental biliary obstruction in rat (12, 21) and chronic active hepatitis and liver cirrhosis in man (26, 27). Later works also showed γ -GTP activity in human liver specimens on hepatic cell membrane and further in their cytoplasm following development of hepatocellular carcinoma. However, the significance of the positive reaction of γ -GTP of the hepatocytes in morphogenesis of any of the hepatic lesions including hepatobiliary carcinogenesis was not elucidated yet.

Fiala et al. (8, 9) and Taniguchi et al. (28) have recently emphasized the close correlation of increased γ -GTP activity of the liver with hepatocarcinogenesis based

on their biochemical studies on rats treated with chemical hepatocarcinogens. The present study has revealed the early appearance of γ -GTP activity in the hepatocytes of hyperplastic areas and nodules following bile duct proliferation including appearance of the oval cells in the periportal areas. This finding seems to reflect the development of a progressive process supervening upon mere repair or regeneration in these hyperplastic regions, because neither an increase of γ -GTP activity in the liver tissue nor positive reaction of γ -GTP in the hepatocytes was observed in rats after partial hepatectomy (7, 8). Cells of hepatocellular tumor as well as those of cholangiocellular tumor also presented considerable activity of γ -GTP in their cytoplasm. Results of biochemical analyses on activity of γ -GTP in the serum and liver tissue of rats corresponded well to the histochemical appearance from the early phase of the experiments. Thus, the elevation of γ -GTP activity in serum and liver tissue seems to be derived not only from proliferative change of bile duct cells but also from hepatocytes which may show altered functional activity during carcinogenic process. As reported previously (17), α -fetoprotein levels in the serum, measured at various intervals during the feeding of rats with N-OH-FAA in group 4, showed the similar increase just like the increase of γ -GTP in serum and liver in precancerous stage. However, α -fetoprotein appeared in the blood in the earlier stage, already from the first week after the start of N-OH-FAA feeding, than the appearance of γ -GTP activity.

Electrophoretic demonstration of predominance of fetal-type isozyme, showing a peak in α -globulin fraction in the elevated level of γ -GTP in the serum and liver tissue of rats treated with hepatocarcinogens in the present study may show induction of embryonic enzyme during the hepatocarcinogenic process, as Fiala and Fiala (8) suggested in their biochemical study on γ -GTP.

Although measurement of serum γ -GTP is a sensitive test for liver disease, elevation is not confined to a single disease entity (22). However, histochemical study on γ -GTP activity in the variety of experimental liver lesions in rats and mice (7) revealed the characteristic appearance of γ -GTP reaction again in the hepatobiliary cells showing hyperplastic changes in the liver of rats treated with carcinogenic N-monomethyl-p-aminoazobenzene and carbon tetrachloride and liver of mice treated with carcinogenic mycotoxin, luteoskyrin. Increased γ -GTP activity was only confined to the proliferating bile ducts in the rats treated with ethanol, lithocholic acid or after ligation of the common bile duct. Adult guinea pig hepatocytes, which normally contain γ -GTP activity, showed no change after feeding of non-carcinogen p-aminoazobenzene, but lost all activity after administration of the hepatocarcinogen diethylnitrosamine (7, 25). Okabe et al. found an isoenzyme pattern in the albumin region, consisting of the major activity of γ -GTP in the serum of normal adult guinea pig, by their electrophoretic analysis, giving a clue for interpretation to this paradoxical phenomenon (16).

Further study of re-appearance of embryonal components of this enzyme during neoplastic transformation of the cell is needed to elucidate its significance from the morphological as well as biochemical standpoints. For this purpose, ultrastructural demonstration of γ -GTP activity in the animal liver during hepatocarcinogenesis is now in progress.

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