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γ-Glutamyl Transpeptidase, Glutathione S-Transferase, Alkaline Phosphatase, and Glutathione Levels during Different Stages of Chemically Induced Hepatocarcinogenesis in the Rat

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Summary Chemically induced hepatocarcinogenesis in rats with partial hepatectomy was followed morphologically and enzymatically at 4, 6, 8, 12, and 16 weeks after injection of the inducer, diethylnitrosamine. The enzymes determined were plasma and liver γ-glutamyltranspeptidase (GGT), alkaline phosphatase (ALP), and glutathione S-transferase (GST). The livers of the treated rats killed after 8 weeks appeared to be rough, pale, and larger compared with the control ones. After 6 weeks, large nodules were observed on the treated liver. Staining of the liver sections histochemically and immunohistochemically revealed that the enzyme-positive foci increased with time ($r=0.93$, $p<0.05$, for the placental form of GST (PGST); not significantly for GGT). The number of enzyme-positive foci per tissue surface area did not correlate with time. GGT, ALP, and GST activities in the plasma and liver of the treated rats were higher than those in the controls. Blood glutathione levels were not affected during chemically induced hepatocarcinogenesis in the rat.

Key Words: rat, hepatocarcinogenesis, marker enzymes, histochemistry, glutathione

Chemical hepatocarcinogenesis is a multistage and complex process comprising the initiation, promotion, and progression stages, after which the affected

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tissue is transformed to a malignant phenotype [1]. Gross morphological changes, which are usually not observed during the early stages of hepatocarcinogenesis, become apparent after the tissue has been transformed.

Tumor marker enzymes such as γ-glutamyltranspeptidase (GGT), alkaline phosphatase (ALP), and the placental form of glutathione S-transferase (PGST) have been found to be useful in the detection and monitoring of the progression of carcinogenesis [2–8]. The change in the expression of these enzymes is usually a reflection of the change in the tissue, and the correlation of these enzyme activities with carcinogenesis is evident. Elevated activities of GGT have been strongly correlated with the extent of carcinogenesis [9]. The expression of variant ALP isoenzymes has been shown in various cancers [10], and the selective expression of PGST has been shown to occur during the preneoplastic state [7].

Since the activities of such tumor marker enzymes have been reported to increase in chemically induced carcinogenesis, the determination of these activities provides a useful method for monitoring the progress of hepatocarcinogenesis. Furthermore, the activities of the tumor marker enzymes are useful as indicators during the preneoplastic state, during which no morphological changes are observable. We report herein the determination of the above three tumor marker enzyme activities during the progress of chemically induced hepatocarcinogenesis in the rat.

MATERIALS AND METHODS

Chemicals. Diethylnitrosamine (DEN), 2-acetylaminofluorene (AAF), 5,5-dithio-bis-(2-nitrobenzoic acid), γ-glutamylcarboxynitroanilide, glycylglycine, and all other reagents used were of the highest grade commercially available (Sigma Chemical Co., St. Louis, MO). A basal diet was purchased from Gold Coin Co., Ltd. (Kuala Lumpur, Malaysia).

Treatment of animals. Chemical induction of hepatocarcinogenesis in the rat was carried out essentially following the method of Solt and Farber [11]. Sixty male *Rattus norwegicus*, 120–150 g, 6–8 week, were used. The rats were caged individually in a well-ventilated room at 27°C, maintained on a basal diet or a diet containing 0.02% AAF, and provided water *ad libitum*. The rats were divided into 10 groups of 6 rats in each. The rats in groups 1 to 5 were given a single intraperitoneal injection of DEN (200 mg/kg body weight) to induce hepatocarcinogenesis. After 2 weeks, the rats were fed a diet containing 0.02% 2-AAF for a further 2 weeks. The rats were then fed a basal diet until sacrifice by cervical dislocation at 4, 6, 8, 12, and 16 weeks after the DEN injection. All the rats were hepatectomized partially (2/3) 3 weeks after the DEN injection. The rats in groups 6 to 10 served as controls and were maintained on the basal diet for the duration of the experiment. They were also hepatectomized partially (2/3) in the third week of the experiment and sacrificed concomitantly at the times stated for the treated rats.

Immediately after death, blood was collected from the heart into heparinized tubes and then centrifuged. The resulting plasma was used for plasma enzyme assays. The liver was immediately excised, weighed, and stored frozen before use. The crude homogenate and cytosolic and microsomal fractions of the liver were prepared according to the method of Speier and Wattenberg [12].

Enzyme assays. γ-Glutamyltranspeptidase was assayed essentially by the method of Jacobs [13] but with slight modifications as described previously by Rahmat *et al.* [14]. Enzyme activity was expressed as IU/liter for plasma and as IU/g for the liver microsomal enzyme fraction.

Alkaline phosphatase activity in the plasma and crude liver homogenate was assayed by the method of Jahan and Butterworth [15]. The enzyme activity was expressed as IU/liter for plasma and as IU/g protein for the liver fraction.

Liver cytosolic glutathione S-transferase activity was measured by the method of Habig *et al.* [16]. Blood glutathione was determined by the method of Ellman [17]. Protein concentrations in the plasma and liver fractions were determined according to Bradford [18].

Immunohistochemical staining. The excised liver was sliced into 5-μm sections and used for the histochemical detection of GGT activity by the method of Rutenberg *et al.* [19]. The placental form of glutathione S-transferase (PGST) was detected immunohistochemically by the method of Hsu *et al.* [20]. The enzyme-positive lesions observed in the liver sections were quantified with a Kontron image analyzer (Zeiss, Germany) and expressed as the percentage of the total tissue surface area occupied by the enzyme-positive foci. The number of enzyme-positive foci per cm^2 tissue surface area was also determined. The primary antibody to PGST was a gift from Professor Kiyomi Sato, Hirosaki University, Japan.

Statistical analysis. The results obtained were analyzed by analysis of variance and Student's t-test. A value of $p<0.05$ was considered as significant.

RESULTS

Gross morphology of the liver

The livers of the treated rats killed after 8 weeks appeared to be rough and pale compared with the deep red and smooth appearance of the normal livers. The livers of the treated rats were generally larger than those of the controls and showed generalized nodule formation; and the nodules formed were quite obvious, especially in the livers of treated rats killed after 12 or 16 weeks.

Histochemical and immunohistochemical staining

Liver sections were stained histochemically for GGT activities and immunohistochemically for PGST activities. Control livers did not contain any enzyme-positive foci, whereas all of the liver sections of the treated rats did (Table 1). The size of the area occupied by PGST-positive foci appeared to increase with time, the

Table 1. Glutathione S-transferase (placental form)- and γ-glutamyltranspeptidase-positive foci in the liver of normal and treated rats over time.

Time (weeks)	Percentage area			Number of foci/tissue area (Number/cm²)		
		PGST	GGT		PGST	GGT
	Control	Treated	Treated	Control	Treated	Treated
4	0	6.91±2.26	6.17±1.23	0	47.50±7.99	40.45±5.28
6	0	8.69±2.05	6.04±1.36	0	40.30±8.48	29.92±3.75
8	0	8.56±2.98	10.09±5.61	0	16.32±1.98	17.68±2.24
12	0	10.81±2.99	10.39±6.10	0	24.22±4.52	24.27±5.94
16	0	16.82±2.57	10.77±2.29	0	30.46±3.64	19.85±4.56

Values shown are mean±SE of mean, $n=5$–8. PGST, glutathione S-transferase (placental form); GGT, γ-glutamyltranspeptidase. Glutathione S-transferase (placental form) was stained with primary antibody to the enzyme, whereas γ-glutamyltranspeptidase was stained for activity by immersion of the sections in substrate solution.

Table 2. Enzyme activities and total glutathione concentration in the plasma during chemically induced hepatocarcinogenesis.

Time	GGT (IU/liter)		ALP (IU/liter)		Total GSH (μmol/liter)	
	Control	Treated	Control	Treated	Control	Treated
4 weeks	4.71±1.32	28.37±16.36*	415.48±61.93	832.46±56.15*	6.43±1.19	7.71±0.91
6 weeks	5.82±0.58	11.42±6.22	461.58±73.39	553.89±123.96	6.27±0.84	6.92±1.20
8 weeks	5.01±0.87	19.73±18.82	473.89±66.06	582.49±169.14	7.67±0.33	8.74±1.37
12 weeks	5.36±1.41	11.11±2.32*	355.20±64.70	411.40±53.18	5.55±0.64	6.21±0.54
16 weeks	5.86±0.65	10.82±2.25*	310.23±81.52	484.50±54.44*	5.46±0.57	6.37±0.77*

Values shown are mean±SE of mean, $n=5$–8. *$p<0.05$ compared with corresponding control. GGT, γ-glutamyltranspeptidase; ALP, alkaline phosphatase; GSH, reduced glutathione.

correlation being strong ($r=0.93$, $p<0.05$). The GGT-positive foci similarly appeared to increase in area with time, although the correlation in this case was not significant ($r=0.85$, $p>0.05$). However, in terms of the number of enzyme-positive foci per tissue surface area, there seemed to be no correlation with time. Enzyme-positive foci less than 0.02-mm diameter in size were not quantified.

There were numerous enzyme-positive foci observed in the liver sections from treated rats after 4 weeks, as evident from the high number of foci per unit surface area. Initially, the foci were evenly distributed over the whole area of the liver sections. Later, the number of enzyme-positive foci decreased in number but grew in size per unit surface area. Distinct patches of enzyme-positive foci were obvious in liver sections of the rats killed after 8 weeks.

Plasma marker enzyme activities

Plasma GGT activities in the controls showed a general trend to increase with time (Table 2). In the treated rats, GGT activities were significantly higher than those in the corresponding controls, but did not show any time dependency.

However, values for the 6-, 8-, and 12-week samples were not significantly different from the controls because one of the rats in each of the different time groups had a very high GGT activity, resulting in very high values for standard error of the mean. In addition, the liver of these rats had extensive nodule formation on the liver, markedly more noticeable than that for the other members of the group.

In contrast to GGT activities, plasma ALP activities in the controls tended to decrease with time, indicating that ALP activity is affected by age. The trend was also evident in the treated rats. ALP activities in the treated rats were generally higher than those in the controls, with values for the 4- and 16-week samples being significantly higher than those of the corresponding controls.

The concentration of glutathione in the plasma remained fairly constant with time for both the controls and treated rats. Treatment with DEN/AAF and partial hepatectomy did not affect plasma glutathione concentrations.

Liver marker enzyme activities

Marker enzyme activities were determined in the crude homogenate and in microsomal and cytosolic fractions of the liver (Table 3). The mean value for microsomal GGT activity was generally higher in the treated rats than in the controls, and it was significantly so ($p<0.05$) for the 4- and 16-week groups. ALP values in the crude liver homogenate of the treated rats tended to be higher than those for the controls, with only the 6- and 16-week values being significantly ($p<0.05$) higher. Cytosolic GST activities were significantly higher ($p<0.05$) than the control ones with the exception of the 8-week value. There was no correlation with time for either microsomal GGT or crude homogenate ALP activity for the treated rats. However, cytosolic GST activities showed a decreasing trend with time, the correlation being significant ($r=-0.88$, $p<0.05$).

Overall, the activities of GGT and ALP in the plasma, microsomal GGT, and cytosolic GST were significantly higher than the control ones at 4 weeks after treatment. Certain members in each of the 6-, 8-, and 12-week groups of rats seemed to be in a very severe state of hepatocarcinogenesis compared with the other members in each group. The formation of nodules on the livers of these rats was

Table 3. Enzyme activities in the liver during chemically induced hepatocarcinogenesis.

Time	GGT (IU/g)[a]		ALP (IU/g)[b]		GST (U/mg)[c]	
	Control	Treated	Control	Treated	Control	Treated
4 weeks	2.63±0.51	6.31±2.11*	7.45±1.55	9.00±3.15	0.53±0.07	1.18±0.22*
6 weeks	3.41±0.14	6.02±3.07	7.79±1.31	8.28±1.86*	0.56±0.03	1.01±0.17*
8 weeks	5.88±0.56	9.24±6.00	9.73±1.57	14.52±7.04	0.56±0.04	0.90±0.40
12 weeks	5.35±0.32	7.67±4.13	6.04±1.22	7.76±1.66	0.45±0.02	0.80±0.12*
16 weeks	4.54±0.61	8.87±2.65*	6.47±0.86	10.43±1.24*	0.53±0.10	0.81±0.07*

Values shown are mean±SE of mean, $n=5$–8. *$p<0.05$ compared with corresponding control. GGT, γ-glutamyltranspeptidase; ALP, alkaline phosphatase; GST, glutathione S-transferase. [a] measured in microsomal fraction; [b] measured in crude homogenate; [c] measured in cytosolic fraction.

very extensive. The activities of the enzymes determined in these rats resulted in higher standard error of mean values, making the differences between these groups and their controls nonsignificant.

DISCUSSION

Several methods have been used to monitor carcinogenesis. These include the examination of gross morphology, ultrastructure, histology, and the determination of tumor marker enzyme activities in the plasma or subcellular fractions. GGT, ALP, and GST, including PGST, have been determined in chemically induced preneoplasia in the rat [2–8, 14, 21, 22]. In the present study, the activities of these enzymes were monitored sequentially during hepatocarcinogenesis in the rat at 4, 6, 8, 12, and 16 weeks after injection of DEN.

The appearance of the treated liver was different after 4 weeks compared with that of the control, indicating that carcinogenesis had been induced. However, the presence of nodules was obvious only after 8 weeks. PGST- and GGT-positive foci were detected after 4 weeks, and the percentage area of the enzyme-positive foci increased with time. The location of enzyme-positive foci appeared to be generalized throughout the liver sections stained. The number of enzyme-positive foci per unit surface area was highest at 4 weeks, i.e., immediately after administration of AAF, decreased to a minimum at 8 weeks, and then increased again. Numerous large patches of enzyme-positive foci were obvious after 8 weeks, the time coinciding with that for the minimum number of foci per unit surface area. By that time the appearance of the enzyme-positive foci had altered from a generalized pattern to a more localized one. In addition, the number of larger size foci had also increased.

The results obtained in the present study seem to be in agreement with those observed by Pitot and Sirica [23], who had reported that hepatocellular carcinomas appeared initially as small "enzyme-altered foci" that subsequently developed to become large and apparent tumors after initiation and promotion of carcinogenesis.

Plasma GGT activity was significantly increased at 4 weeks and again at 12 and 16 weeks, and the liver activity, at 4 and 16 weeks. GGT activity was not significant between 6 and 8 weeks in the plasma and 6 and 12 weeks in the liver. Thus, the treatment given to the rats was effective in inducing GGT activities almost immediately, as had been reported by Elhkim *et al.* [2]. The reduction in plasma GGT could be due to regression of a portion of the altered hepatic foci while the remainder progressed toward the neoplastic state [3]. The significant difference in activities during the later sample times could be attributed to the severity of carcinogenesis, as had been reported by Murray *et al.* based on studies involving humans [9].

Generally, plasma ALP activities were higher in the treated rats than in the controls. This is in accordance with the results of previous work [10, 24]. ALP

activities in the crude homogenate of the liver of the treated rats were also higher than those for the controls. However, we could not ascertain whether the increase in plasma ALP activities was due to this increased liver ALP activity. It has been proposed that the increase in plasma ALP activities could be due to an overflow from the initiated cells of the affected tissue [25, 26]. Age also seemed to affect ALP activities, as ALP activities decreased with advancing age. Thus, total ALP activity measurement would not give a reliable assessment of the disease. A more accurate measure would be to determine specific ALP isoenzyme activities in the affected tissue itself, preferably by electrophoretic methods [27].

Glutathione S-transferase activities in the liver of the treated rats were also higher than those for the controls, in accordance with the results of Jensson *et al.* [7]. There tended to be a decrease with time in the activities in the treated rats. However, GST activities remained constant with time for the controls. This suggests that GST is not affected by age, in contrast to ALP activity.

Altered expression of GST isoforms has been implicated in carcinogenesis. In the rat, different GST isoforms, including PGST, are preferentially induced by carcinogens such as 3′-methyldiaminoazobenzene and 2-acetylaminofluorene [6, 8, 27]. Since PGST is only expressed during carcinogenesis, it has been regarded as a very reliable marker for hepatocarcinogenesis in the rat.

Reduced glutathione is ubiquitous in blood, and it is a substrate for several glutathione-dependent enzymes, of which GST is a member. The concentration of GSH seemed to be unaffected during hepatocarcinogenesis.

Induction of carcinogenesis by chemicals followed by partial hepatectomy resulted in elevated enzyme activities. Determination of the appropriate tumor marker enzyme activities coupled with histochemical tests provides a reliable procedure for monitoring the severity of hepatocarcinogenesis, especially during the early stages when gross morphological change are not obvious.

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