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Differential Effects of Nongenotoxic and Genotoxic Carcinogens on the Preneoplastic Lesions in the Rat Liver

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Glutathione *S*-transferase placental form (GST-P) positive foci development and its expression in liver exposed by nongenotoxic carcinogens phenobarbital (PB) and clofibrate (CF), and genotoxic carcinogen 2-amino-3-methylimidazo[4,5-*f*] quinoline (IQ) were investigated as a measure of carcinogenic potential of these chemicals. Male F344 rats were initially given a single intraperitioneal injection of diethylnitrosamine (200 mg/kg), and 2 weeks later, animals were fed diets containing 0.03% IQ or 0.5% CF or 0.05% PB or basal diet as a control for 6 weeks. All rats were subjected to two-thirds partial hepatectomy (PH) at week 3. Sequential sacrifice of rats was performed at 8 weeks or 52 weeks, and liver tissues were examined for immunohistochemical staining of GST-P positive foci. The numbers (No./cm^2) and areas (mm^2/cm^2) of GST-P positive foci were increased by IQ or PB, but were decreased by CF compare to the control. Consistent with the development of GST-P positive foci, a time-related increase in the expression of GST-P mRNA was found in the rats treated with IQ, whereas CF decreased it. The incidence of hepatocellular carcinoma at 52 weeks was increased by all three chemicals. These results show that PB and IQ induced GST-P positive foci, but the peroxisome proliferator CF did not, which suggest that the prediction of carcinogenic potency based on the development of prenoplastic foci may cause false negative in a particular category compounds like peroxisome proliferators.

Key words : Genotoxic carcinogen, Nongenotoxic carcinogen, Glutathione *S*-transferase, Preneoplastic lesions

INTRODUCTION

A hepatic peroxisome proliferator, clofibrate (CF) and phenobarbital (PB) have been found to be nongenotoxic hepatocarcinogens in rodents (Reddy and Lalwani, 1983; Reddy and Quershi, 1979; Reddy *et al.*, 1979; 1980). 2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ), a potent genotoxic carcinogen isolated from pyrolysates of amino acids and protein, has also been found to be a hepatocarcinogen in rodents and non-human primates (Ohgaki *et al.*, 1984;Takayama *et al.*, 1984).

In the experimental hepatocarcinogenesis, neoplastic and putative preneoplastic lesions show alternative phenotypic properties. Various markers are available for detection of preneopastic changes in hepatocarcinogenesis. γ-glutamyltranspeptidase (γ-GT) and glutathione *S*-transferase placental form (GST-P) have been mostly used for identifying these lesions (Tatematsu *et al.*, 1985; Satoh *et al.*, 1985; Ito *et al.*, 1997; Shirai *et al.*, 1997). It has been found that the incidence of preneoplastic foci by medium-term exposure of hepatocarcinogens using the two stage hepatocarcinogenesis models is correlated to their ability to induce tumors after the long-term exposure (Ito *et al.*, 1988; Ogiso *et al.*, 1990). Previous studies also showed that the degree of dose-dependent induction of GST-P positive foci in the liver exposed by hepatocarcinogens for 6 weeks closely corresponded to dose-dependent induction of hepatocellular carcinoma (HCC) after long-term chronic administration (Ogiso *et al.*, 1985 and 1990).

However, the expression of these markers is dependent upon the type and nature of tumor promoters. For example, phenobarbital (PB) enhanced γ-GT in the neoplastic foci initiated by diethylnitrosamine (DEN), whereas peroxisome proliferator CF did not, even though both chemicals have tumor promoting activity (Tatematsu *et al.*, 1988; Rao *et al.*, 1986).

In the present study, we determined comparative ef-

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fects of non-genotoxic carcinogens PB and peroxisome proliferator CF, or of genotoxic carcinogen IQ on the expression of GST-P, development of GST-P positive foci in the preneoplastic lesions and incidence of HCC initiated by DEN.

MATERIALS AND METHODS

Chemicals

DEN and CF were obtained from Tokyo Chemical Industry Co (Tokyo, Japan). Synthetic IQ was obtained from Nard Inst. (Osaka, Japan). PB was obtained from Iwaki Pharmaceutical Co. (Tokyo, Japan).

Animals and treatments

A total of 170 male 5 week old F344 rats (Charles River Japan Inc., Japan), housed five per plastic cage on wood chips for bedding, were maintained under constant conditions (12h light/dark cycle, 50% humidity at 23±1°C) with Oriental MF diet (Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*. After 2 week of acclimation, the rats were given a single i.p. injection of DEN (200 mg/kg) dissolved in 0.9% NaCl. Two weeks later, rats were placed on diets containing 0.03% IQ, 0.5% CF, 0.05% PB, or basal diets for 6 weeks. All animals were subjected to the two-thirds partial hepatectomy (PH) at week 3 to induce proliferation of hepatocytes. Experimental protocol was shown in Fig. 1. At 8 weeks, rats were anesthesized under ether and killed. Each liver lobe was weighed separately, and then cut into 2-3 mm thick slices with a razor blade. The sections were fixed in ice-cold acetone for immunohistochemical staining of GST-P positive foci. After 8 weeks, the remaining rats were fed with the basal diet for further 44 weeks, and then animals were killed for detection of HCC incidence.

Immunohistochemical staining for GST-P positive foci

The avidin-biotin-peroxidase complex (ABC) method described by Hsu *et al.* (1981) was used to determine the location of GST-P binding in the liver. Affinity-purified biotin-labelled goat anti-rabbit IgG and ABC (Vectastain ABC kit, PK 4001) were obtained from Vector Laboratories Inc. (Burlingame, CA, USA). Paraffin sections were routinely passed through petroleum benzene and a graded alcohol and then treated sequentially with normal goat serum, rabbit anti-GST-P (1:5000 dilution), biotin-labelled goat anti-rabbit IgG (1:400 dilution) and ABC. The peroxidase binding sites were determined by the diaminobenzidine method. Sections were then counter-stained with hematoxylin for microscopic examination. As a negative control for the specificity of anti-GST-P antibody binding, preimmune rabbit serum was used instead of antiserum.

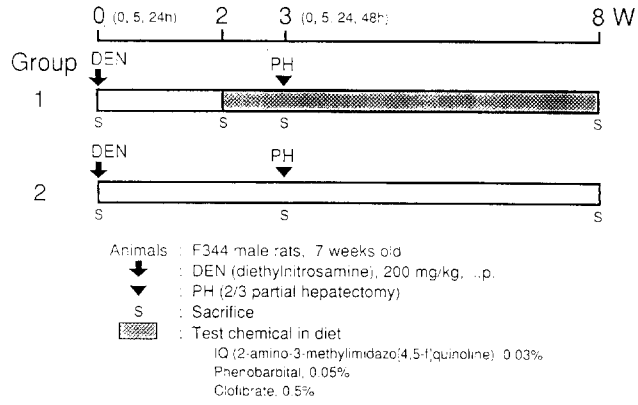

Fig. 1. Experimental protocol. See Materials and Methods section for details. Group 1, DEN + test chemical; Group 2, DEN alone. All rats were subjected to PH at the week 3.

Histopathological analysis

The numbers and areas of GST-P postitive foci, more than 0.1 mm in diameter, were measured using a color video image processor (VIP-21C, Olympus-Ikegami Tsushin Co., Tokyo) as described previously (Satho *et al.*, 1985; Tatematsu *et al.*, 1988).

For detection of HCC, rats exposed for 52 weeks were killed, and then the livers were examined under microscopy. All pathological lesions were recorded and processed for histological analysis or H&E staining to detect HCC.

RNA extraction and northern blotting

Total RNA was extracted by the method of Chomczynski and Sacchi (1987) using product specifications (Stratagene, La Jolla, CA). Liver tissues were washed with ice-cold PBS buffer, and then lysed with denaturating solution containing guanidine isothiocyanate and 2-mercaptoethanol followed by phenol-chloroform extraction.

For the northern blot, 10 μg of each RNA sample was dissolved in 10 μl denaturing buffer [5 μl formaldehyde, 2 μl formimide, 1 μl 10×MOPS buffer (MOPS; 0.1 M Sodium acetate, 10 μM EDTA, pH 7.0)] plus 1 μll ethidium bromide. The samples were electrophoresed through a 1.25% agarose gel. Fractionated RNA was transferred to a nylon membrane, and the membrane was dried and baked. A 0.6 kbp fragment of rat GST-P cDNA was used for hybridization. The ^{32}P-labeled probe for GST-P was generated with a random primed labeling kit (Ambion, Austin, TX, USA). The labeled GST-P probe (1×10^6 dpm/ml) was denatured, and then added directly to prehybridization buffer (QuickHyb®, Ambion, Austin, TX, USA). The membrane was hybridized for 30 min at 68°C and washed three times in 2×SSC containing 0.5% SDS

followed by one washing in 0.1×SSC with 0.1% SDS for 30 min. The final wash was done in 0.1×SSC with 0.1% SDS for 1 h at 56°C. The membrane was dried and then autoradiographed using Kodak MR film at -80°C overnight. Autoradiographs of northern blots were quantitated by scanning densitometry, using a laser densitometer. The mRNA level was expressed by the ratio of GST-P level/β-actin as an internal positive.

Statistics

Statistical comparisons of data concerning body and relative liver weights, numbers and areas of GST-P positive foci were performed using the one way analysis of variance followed by Scheffe's test as a post hoc test.

RESULTS

Sequential changes in mean body and relative liver weights of rats are shown in Fig. 2. Body weights of group treated with IQ or PB were not significantly different from those of control at week 8, but body weights of group treated with CF significantly decreased compared with those of control ($p<0.01$) at week 8 (Fig. 2A). However, relative liver weights of group treated with CF or PB or IQ were markedly increased compared with those of control ($p<0.01$) at week 8 (Fig. 2B). At 52 weeks, IQ significantly decreased body weight, but significantly increased absolute and relative liver weight (Table I).

Table I. Body weight, liver weight and Incidence of tumors in the rat treated for 52 weeks

Agents doses (%)	No. of rats	FBW (g)	ALW (g)	RLW (g%)	No. of tumors
Control	16	401±16	10.9±2.1	2.7±0.5	+
0.03% IQ	12	349±23**	18.0±8.2**	5.2±2.5**	+++
0.05% PB	14	397±22	10.8±2.9	2.7±0.5	++
0.5% Clofibrate	13	412±28	12.7±5.1	3.1±1.4	++

All animals were treated with a single i.p. injection of DEN 200 mg/kg, and then given partial hepatectomy at the third week, and treated for 8 weeks with each chemical. The animals were then maintained on the normal diet for further 44 weeks. The number of tumors per each lobe (tumor size was more than 0.1 mm in diameter) was counted (+; spontaneous incidence, less than 2 tumors/++;more than 2 but less than 10 tumors/each lobe, +++; more than 10 tumors/each lobe).
** : Significantly different from the value of control group at $p<0.01$.
IQ; 2-amino-3-methylimidazo(4,5-f)quinoline, PB; phenobarbital, FBW; final body weight, ALW; absolute liver weight, RLW; relative liver weight

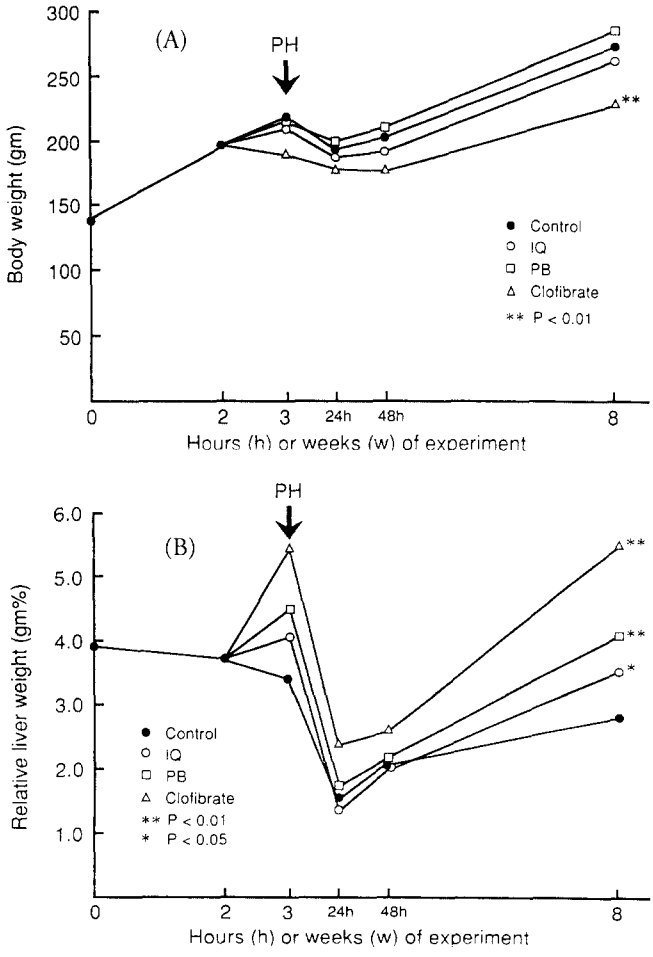

Fig. 2. Sequential changes in the rat mean body (A) and relative (B) weights. Results are expressed as means. * and **; Significantly different from control group values at $p<0.05$ or 0.01, respectively.

The development of GST-P positive foci determined in terms of numbers (No.) or areas (mm^2) per unit area of sections (cm^2) is shown in Fig. 3A and B. The numbers or areas of GST-P positive foci in the liver of rats treated IQ or PB were significantly increased compared with respective data of control group. In contrast, CF markedly lowered GST-P positive foci. The values for GST-P positive foci induced by IQ administration were approximately two times of the PB levels. In agreement with the development GST-P positive foci, the expression of GST-P mRNA in the DEN-initiated liver followed by IQ was increased, while, CF decreased the expression (Table II).

It was also found that a number of HCC were induced in the all part of lobes tested by treatments for 52 weeks, being most effective with IQ treatment (Table I).

DISCUSSION

In experimental hepatocarcinogenesis, enzyme-altered foci are thought to represent initiated cells, some of which develop into HCC (Farber, 1980; Schulte-

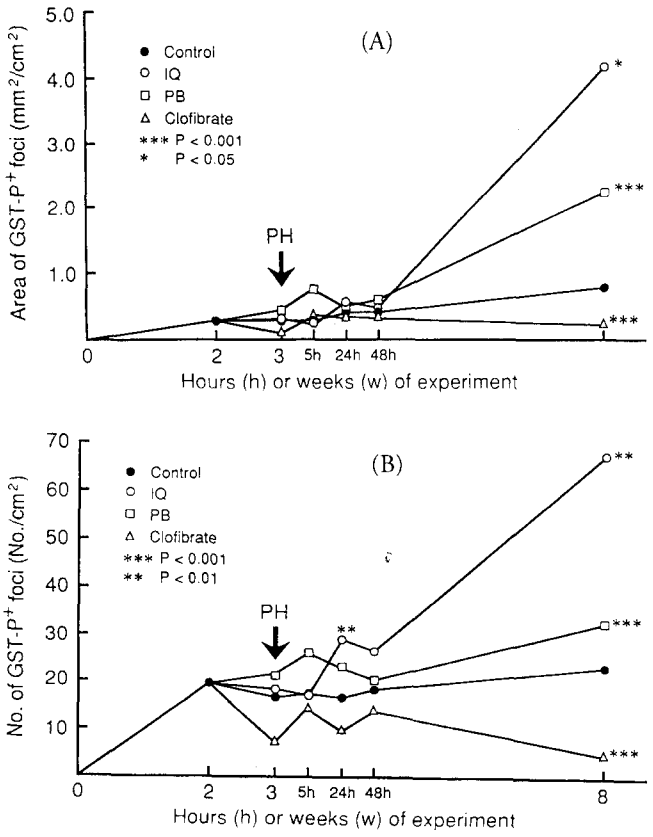

Fig. 3. Sequential changes in GST-P positive foci in rats. Results are expressed either by number of foci (No./cm^2, A) or area of foci (mm^2/cm^2, B). *, ** and ***; Significantly different from control group values at $p<0.05$, 0.01 or 0.001, respectively.

Table II. Level of GST-P mRNA and in the preneoplastic lesions of rat treated by IQ or CF

Time	IQ (GST-P/β-actin)	CF (GST-P/β-actin)
0	0.00±0.00	0.00±0.00
2 weeks	0.15±0.06	0.15±0.06
3 weeks	0.47±0.21	0.00±0.00
8 weeks	1.32±0.53	0.09±0.03

All animals were treated with a single i.p. injection of DEN 200 mg/kg, and then given partial hepatectomy at the 3rd week, and treated for 8 weeks with each chemicals.
Autoradiographs of northern blots were quantitated by scanning densitometry, using a laser densitometer. The mRNA level was expressed by the ratio of GST-P level/β-actin as internal positive.
Data were expressed as mean±SD (n=5 rats)
IQ; 2-amino-3-methylimidazo(4,5-*f*)quinoline

Hermann *et al.*, 1982). In the present study, we compared the effect of three hepatic carcinogens on the development of GST-P positive foci, expression of GST-P, and HCC incidence in the liver initiated DEN using a medium-term bioassay. All three chemicals have been found to have tumor promoting and carcinogenic activities (Reddy and Lalwani, 1983; Reddy *et al.*, 1980; Hosokawa *et al.*, 1989). Earlier data have shown that there is good correlation between GST-P positive foci development and cancer incidence after prolonged treatment of carcinogens (Ogiso *et al.*, 1985 and 1990).

In contrast, γ-GT, another commonly used marker for identifying of preneoplastic liver lesions, was not induced by the peroxisome proliferators including CF in two-step hepatocarcinogenesis (Staubli *et al.*, 1984; Numoto *et al.*, 1984). Furthermore, CF was found to even inhibit the development of the GST-P positive preneoplastic lesions initiated by DEN at the medium-term (8 weeks) treatment (Glauert *et al.*, 1986; Kluwe *et al.*, 1982). It was also reported that peroxisome proliferators enhanced the development of morphologically distinguishable GST-P negative preneoplastic lesions (Staubli *et al.*, 1984). The liver carcinogens as well as peroxisome proliferating agents such as di(2-ethyl-hexyl) phthalate and ciprofibrate, a derivative of clofibrate, were also found to induce phenotypically different preneoplastic foci (Reddy *et al.*, 1979; Numoto *et al.*, 1984; Kluwe *et al.*, 1982; Ward *et al.*, 1986).

In contrast to CF, the genotoxic carcinogen IQ and the non-genotoxic tumor promoter PB enhanced the development of the DEN-initiated GST-P positive preneoplastic lesions when administered after DEN treatment (Fig. 3A and B). The degree of development of GST-P positive foci induced by IQ was about 2 fold higher than that obtained with PB. We also examined the expression of GST-P mRNA in the liver initiated DEN followed by either IQ or CF to see whether the development of GST-P foci can be related to the expression of its mRNA. Similar to the other finding (Imai *et al.*, 1997), we also found that IQ increased the expression of GST-P whereas, CF decreased it. These results suggest that the expression of GST-P and the development of GST-P neoplastic foci may be coincidentally induced.

The mechanisms of this difference in the development of prenoplastic foci by the three chemicals which all have been demonstrated their carcinogenicity after long-term exposure in the rodents are not clear. It is noteworthy that the cell proliferation in the DEN-initiated liver is related to the development of GST-P positive foci. In fact, it was reported that the expression of GST-P mRNA in putative initiated hepatocytes was controlled by the transcription factor AP-1 which is the complex of c-*fos*/c-*jun* or c-*jun*/c-*jun* (Okuta *et al.*, 1989). Therefore, it is possible that IQ may provide initiated cells with favor environments (such as increase production of growth factor or comitogenic factor or inhibition of apoptosis) for growth, which may lead to development of preneoplastic foci. Moreover, cultured hepatocytes exposed to a peroxisome proliferator and PB *in vivo* responded to hepa-

tocyte growth factor differently (Lindroos and Michalopoulos, 1993). It is also interesting to know that the preneoplastic lesions induced by PB are eosinophilic whereas those induced by peroxisome proliferators are slightly basophilic (Marsman and Popp, 1994). However, it is not clear whether the phenotypic difference may be related to the development of neoplastic foci.

It has also been suggested that the difference of eicosanoids metabolism may be related to the tumor promoting activity between PB and CF since PB increases the hepatic eicosanoid concentrations, whereas peroxisome proliferators decrease (Hong *et al.*, 1995; Hong and Glauert, 1996). Furthermore, inhibitors of arachidonic acid metabolism inhibit the promotion of altered hepatic foci and HCC by PB (Hendrich *et al.*, 1991; Denda *et al.*, 1989; Tang *et al.*, 1993). Eicosanoids have been found to be implicated in the hepatocyte replication (Hong and Glauert, 1996).

We also found that the HCC incidences were increased after one year, being most effective in the IQ treatment for 8 weeks. There have also been several reports that the chemicals tested in this study have eventually induced hepatic tumor with long-term exposure. The incidences of HCC in this study were much lower than those observed in other studies (Reddy and Lalwani, 1983; Reddy *et al.*, 1980; Hosokawa *et al.*, 1989; Tagayama *et al.*, 1984). In this study, animals were treated for 8 weeks, and maintained on normal diet for further 44 weeks. Therefore, it might be possible that the tumor formation was reversible and reduced after withdrawal of chemicals. Although the genotoxic carcinogen IQ and nongenotoxic tumor promoter PB have proved positive relationship between neoplastic foci development and HCC incidence, peroxisome proliferator CF did not induced GST-P development.

Therefore, the prediction of the carcinogenicity of chemicals by detection of the development of prenoplastic foci may cause false negative in the some particular category compounds such as peroxisome proliferators, and alternative markers are thus required for such chemicals. In addition, Further studies understanding of the mechanisms in the different properties of chemicals in the development of neoplastic foci are required.

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