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Modifying Influence of Swine Serum-induced Liver Fibrosis on Development of Preneoplastic Lesions in Rat Liver

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Modifying effects of fibrosis or a cirrhotic state, caused by treatment with swine serum (SS), on the induction of preneoplastic focal lesions were assessed in a rat medium-term liver bioassay model for the detection of environmental carcinogens, in which the test compound is administered during the promotion phase after initiation with diethylnitrosamine. In experiment I, repeated intraperitoneal administration of SS concomitantly with the hepatopromoting agent deoxycholic acid (DCA) or phenobarbital (PB) resulted in a cirrhotic state and a significant increase in the number or size of preneoplastic glutathione S-transferase placental form (GST-P)-positive liver cell foci as compared to the corresponding DCA or PB alone groups. In experiment II, SS was given prior to commencement of the same medium-term bioassay system, in which a known hepatopromoting agent, DCA, 17-α-ethynylestradiol, or 2-acetylaminofluorene, was applied. In this case, the liver did not show obvious cirrhotic change and, rather than any enhancement, slight inhibition of promotion occurred. The results indicate that a coexisting, but not a pre-existing, cirrhotic condition acts to increase growth pressure on GST-P$^+$ preneoplastic foci, and suggest that concomitant administration of SS with the promoting agent could be applied to improve the sensitivity of the assay protocol.

Key words: Modification — Hepatocarcinogenesis — Liver fibrosis — Swine serum — Rat

Various experimental systems have been developed and put into practice for the detection of carcinogenic potential of environmental compounds.[1)] In our laboratory, we have concentrated on an *in vivo* medium-term bioassay model to screen potentially hazardous compounds using preneoplastic foci as endpoint marker lesions. We have found a good correlation between development of these lesions and known long-term carcinogenicity, irrespective of mutagenicity.[2)] However, the assay model is not fully satisfactory because negative or borderline results have been obtained with some weak carcinogens.

Repeated intraperitoneal administration of SS[5] has been established as an effective protocol for inducing fibrosis or cirrhotic lesions in the rat liver.[3,4)] This is of potential interest, given the well-documented association between liver cell cancer development and cirrhosis in man.[5)] However, one feature which distinguishes the heterologous serum model is the lack of obvious parenchymal cell damage and proliferation.[6–8)] The present study was conducted to assess the influence of SS-induced fibrotic or cirrhotic conditions on the sensitivity of our medium-term bioassay system for environmental agents. The relatively weak promoting agent DCA was compared with PB for this purpose. In addition, the influence of a pre-existing cirrhotic condition was investigated using the same model and DCA, EE or 2-AAF as a promoter.

MATERIALS AND METHODS

Animals Totals of 60 (experiment I) and 115 (experiment II) five week-old male F-344 rats (Charles River, Inc., Atsugi), were housed five to a plastic cage on soft wood chips for bedding in an air-conditioned room at a constant temperature of 24±2°C and relative humidity of 60±5% with a 12/12 h light/dark cycle. They were maintained on Oriental MF basal diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*.

Chemicals Sources of the compounds used were as follows: DEN, Tokyo Chemical Industry Co. Ltd., Tokyo; SS, Nippon Bio-Test Co., Tokyo; DCA and EE, Wako Pure Chemical Industry, Ltd., Tokyo; 2-AAF, Nacalai Tesque, Kyoto; and PB, Iwaki Pharmaceutical Co., Tokyo.

Experimental protocol In experiment I, rats were initiated with a single intraperitoneal injection of DEN

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[5] Abbreviations: SS, swine serum; DCA, deoxycholic acid; PB, phenobarbital; EE, 17-α-ethynylestradiol; 2-AAF, 2-acetylaminofluorene; DEN, diethylnitrosamine; PH, partial hepatectomy; GST-P, glutathione S-transferase placental form.

(200 mg/kg b.w.) and after 2 weeks on basal diet were given dietary supplementation with DCA (0.15%) or PB (0.05%) for 6 weeks with PH performed at week 3, and with SS (0.5 ml/animal undiluted) being applied intraperitoneally twice weekly over the same period (12 times). In week 3, SS was injected 2 to 3 h after PH. Rats were scheduled to be killed at week 8 for immunohistochemical analysis of GST-P-positive liver cell foci and histopathological assessment of cirrhotic lesion development (Fig. 1).

In experiment II, rats were initially treated with SS intraperitoneally twice a week for 12 weeks (24 times), and then maintained for 1 week without treatment, before receiving a single dose of DEN (200 mg/kg, intraperitoneally) dissolved in 0.9% NaCl solution for initiation of hepatocarcinogenesis. Starting two weeks later, they were given basal diet containing DCA (0.15%), EE (0.5 ppm) or 2-AAF (0.01%) for 6 weeks, with PH being performed 3 weeks after the DEN injection. All survivors were scheduled to be killed at week 21 under ether anesthesia for immunohistochemical analysis of preneoplastic GST-P-positive liver cell foci and histopathological examination (Fig. 2). In both experimental studies, diets were prepared once a month and stored in a cold, dark room (4°C) until use. Body weights and food consumption of animals were measured once every week.

Tissue processing Immediately upon killing, the livers were excised and slices about 2–3 mm in thickness were cut with a razor blade. Four slices, one each from the right posterior and caudate lobes and two from the right anterior lobe, were fixed in ice-cold acetone and processed for embedding in paraffin and subsequent immunohistochemical demonstration of GST-P expression and detection of fibrous tissue by Azan-Mallory staining. Additional slices were fixed in 10% phosphate-buffered formaldehyde solution for routine processing and staining with hematoxylin and eosin.

Immunohistochemical staining Anti-GST-P antibody, raised as described previously,[9] was the generous gift of the late Professor Kiyomi Sato, Hirosaki University, Japan. The avidin-biotin-peroxidase complex (ABC) method described by Hsu *et al.*[10] was used to determine the location of GST-P binding in the liver sections. ABC kits were obtained from Vector Laboratory Inc., Burlingame, CA. Sections were lightly counterstained with hematoxylin for microscopic examination. As a negative control for the specificity of antibody binding, pre-immune rabbit serum was used in its place.

Quantitative analysis The numbers and areas of GST-P-positive foci of more than 0.2 mm in diameter[11] were measured in both experiments I and II using a color video image processor (Olympus VIP-21C; Olympus Ikegami Tsushin Co., Tokyo) and expressed as number/cm^2 and area (mm^2/cm^2). The analysis was carried out without reference to the treatment group, and the statistical significance of inter-group differences was assessed by using Student's *t* test.

RESULTS

Experiment I Data for mean body weight and absolute as well as relative liver weight of rats at week 8 are summarized in Table I. Repeated injection of SS did not cause obvious anaphylactic reactions. No significant

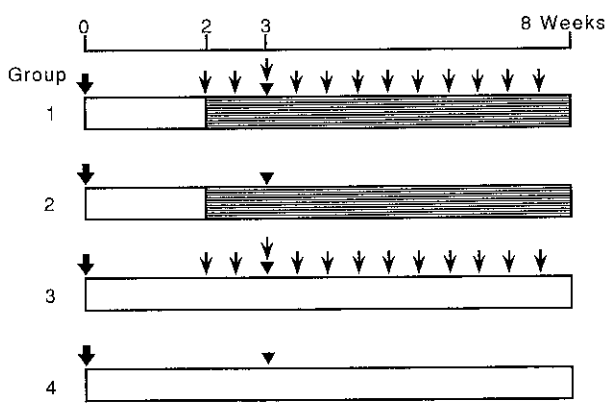

Fig. 1. Protocol for experiment I. A DEN-PH and test chemical (DCA or PB) model was used, in which rats were subjected to intraperitoneal injection of DEN (200 mg/kg) for initiation of hepatocarcinogenesis followed by PH and feeding of DCA (0.15%) or PB (0.05%) combined with intraperitoneal injections of SS (0.5 ml/rat, twice a week for 12 times) over 6 weeks. Group 2, control without SS; Group 3, control with SS alone; Group 4, control with test chemical alone. ↓, SS; ⬇, DEN; ▼, PH; ▤, DCA, EE or 2-AAF.

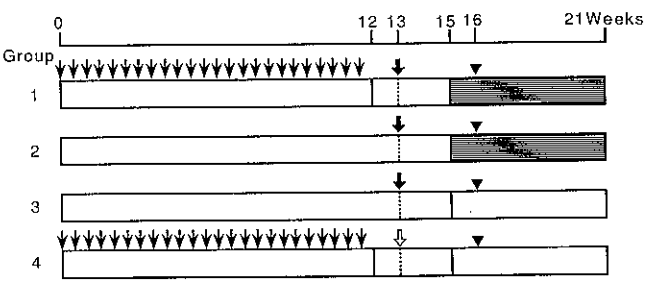

Fig. 2. Protocol for experiment II. Group 1 rats were subjected to intraperitoneal injections of SS, 0.5 ml/rat twice a week (24 times), over 12 weeks prior to application of DEN-PH and test chemical in the same way as in experiment I. Rats were fed DCA (0.15%), EE (0.5 ppm) or 2-AAF (0.01%) as a positive test compound for 6 weeks. Group 2, control without SS; Group 3, control without test chemical; Group 4, control with SS alone. ↓, SS; ⬇, DEN; ⇩, saline; ▼, PH; ▤, DCA, EE or 2-AAF.

Table I. Final Body and Liver Weights in Experiment I

Group	Treatment	No. of rats	Final body weight (g)	Liver weight (g)	Relative liver weight (% body weight)
1	DEN→DCA+SS	7	278.2±5.4	8.7±0.4***	3.13±0.13***
	DEN→PB+SS	9	256.6±14.1	9.2±1.0*	3.56±0.23**
2	DEN→DCA	9	279.9±19.0	10.6±0.8	3.79±0.11
	DEN→PB	10	261.1±9.1	10.1±0.7	3.88±0.15
3	DEN→SS	7	261.9±15.9	6.9±0.6	2.65±0.29
4	DEN	10	266.6±12.0	6.9±0.4	2.57±0.15

Data are mean±SD values.
Significantly different from the corresponding group 2 value at *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Table II. Numbers and Areas of GST-P$^+$ Liver Cell Foci in Experiment I

Group	Treatment	No. of rats	No./cm^2	Area (mm^2/cm^2)	Bridging fibrosis
1	DEN→DCA+SS	7	14.99±4.31	0.76±0.15***	+
	DEN→PB+SS	9	17.37±2.47*	0.80±0.17	+
2	DEN→DCA	9	12.41±2.97	0.41±0.11	−
	DEN→PB	10	14.02±2.61	0.72±0.16	−
3	DEN→SS	7	10.10±5.09	1.05±0.94	+
4	DEN	10	8.27±3.65	0.47±0.21	−

Data are mean±SD values.
Significantly different from the corresponding group 2 value without SS at *, $P<0.05$; ***, $P<0.001$.
−, not present; +, moderately present.

Table III. Final Body Liver Weights in Experiment II

Group	Treatment	No. of rats	Final body weight (g)	Liver weight (g)	Relative liver weight (% body weight)
1	SS→DEN→DCA	12	337.3±15.0	8.3±1.8	2.46±0.48
	SS→DEN→EE	11	262.3±17.8	7.0±0.9	2.67±0.31
	SS→DEN→2-AAF	8	324.9±21.1	9.7±2.4	2.96±0.60
2	DEN→DCA	12	344.3±17.6	8.9±1.7	2.57±0.40
	DEN→EE	15	263.3±17.3	6.9±0.8	2.62±0.33
	DEN→2-AAF	12	325.1±14.8	9.1±2.2	2.80±0.63
3	DEN	5	343.6±5.6	7.2±0.5	2.10±0.15
4	SS	18	359.2±16.4	10.3±1.1	2.86±0.28

Data are mean±SD values.

difference in body weight was observed between groups 1 and 2. However, SS was associated with decreased absolute and relative liver weights in rats receiving DCA or PB. No equivalent reduction was found for SS alone. In terms of histological appearance, the livers of rats treated with SS exhibited an increase in fibrotic tissues of variable thickness, with bridging formation from portal area to portal area (P-P bridging) and occasionally from portal areas to central regions of the lobules (P-C bridging), resulting in pseudolobule formation that resembled the cirrhotic conditions found in man. Although normal lobular structure was irregularly divided by fibrous tissue, no obvious regenerating nodule formation was observed. Furthermore, no inflammatory cell reaction was apparent (Fig. 3) and obviously necrotic cells or apoptotic bodies were absent.

Numbers of GST-P-positive liver cell foci in rats given PB+SS in group 1 were significantly increased ($P<$

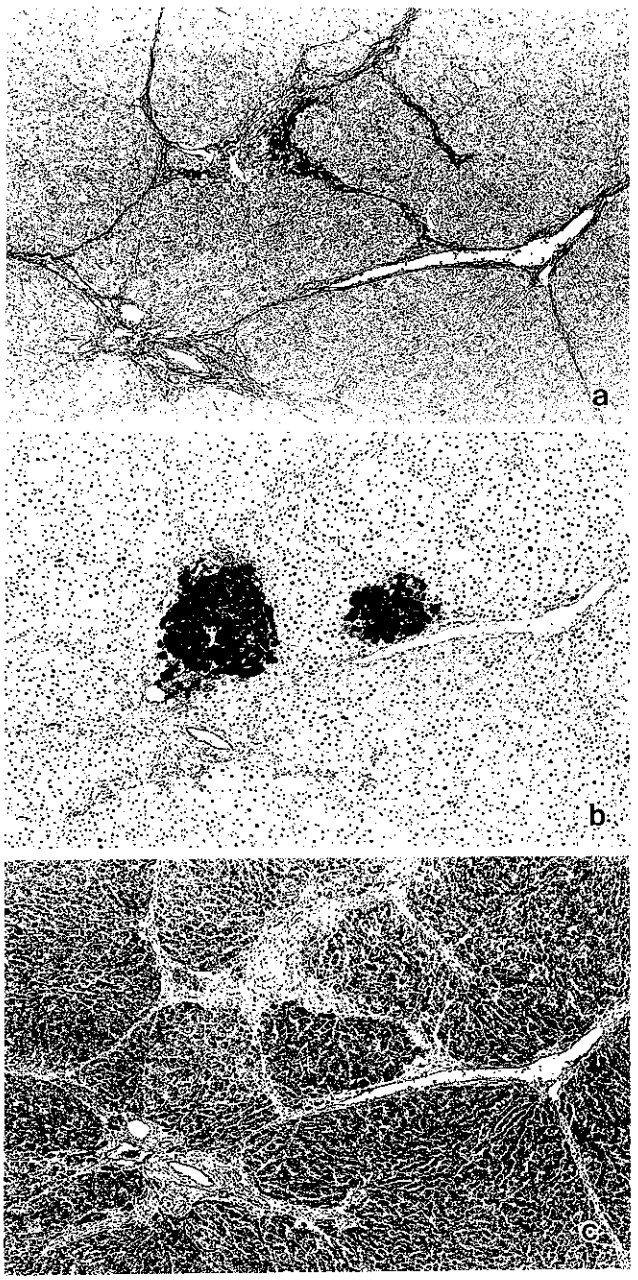

Fig. 3. Histological appearance of the liver in a rat of group 1 in experiment I, given DEN followed by DCA and SS combined with PH. a, Bridging fibrosis is revealed by Azan-Mallory staining; b, GST-P-positve foci; c, Hematoxylin and eosin. (×66, semi-serial section).

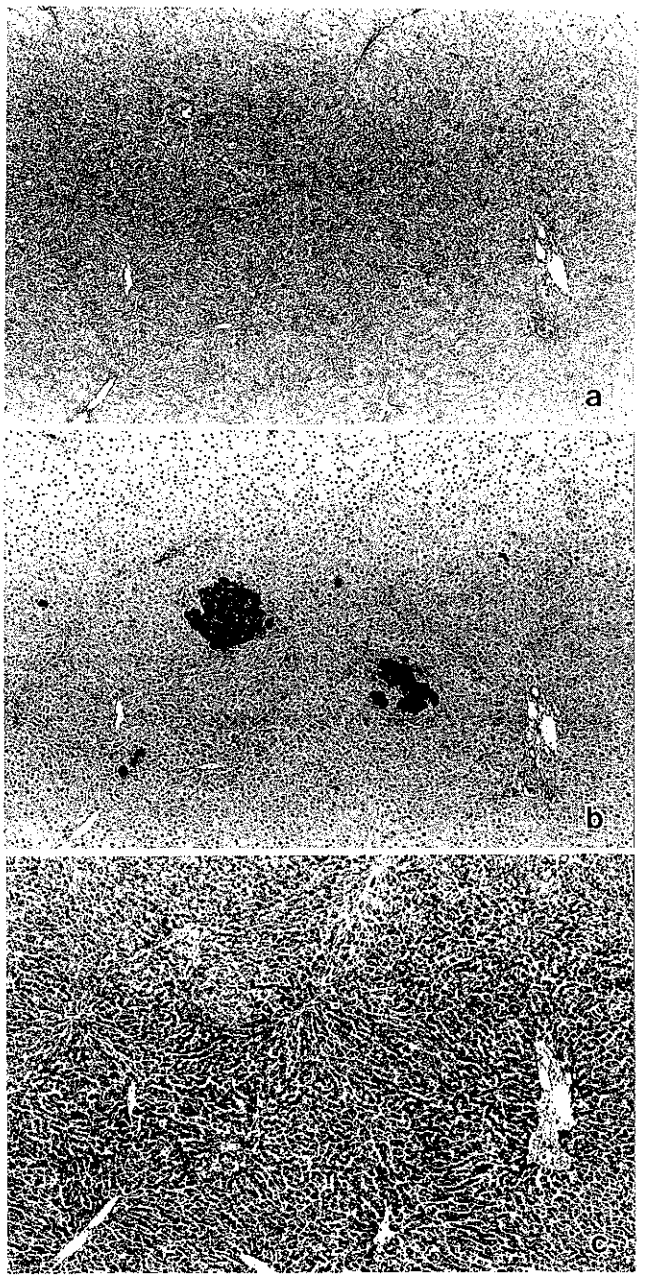

Fig. 4. Histological appearance of the liver in a rat of group 1 in experiment II, given SS prior to the DEN-PH-hepatopromoter regimen. a, Fibrosis is not as evident as in Fig. 3 (Azan-Mallory staining); b, GST-P-positive foci; c, Hematoxylin and eosin. (×66, semi-serial section).

0.05) and areas showed a slight tendency for enlargement (Table II). The values for rats given DCA+SS were also elevated, and in this case, the areas were also significantly affected. Administration of SS alone after DEN initiation was associated with tendencies for increase in both number and area of GST-P-positive liver cell foci, but without statistical significance.

Experiment II Data for mean body weight and absolute

Table IV. Numbers and Areas of GST-P$^+$ Liver Cell Foci in Experiment II

Group	Treatment	No. of rats	No./cm^2	Area (mm^2/cm^2)	Bridging fibrosis
1	SS→DEN→DCA	12	30.1±6.7*	1.6±0.3**	±
	SS→DEN→EE	11	26.6±5.7	1.4±0.3	±
	SS→DEN→2-AAF	8	81.1±28.5	6.5±2.8	±
2	DEN→DCA	12	37.0±7.2	2.2±0.5	−
	DEN→EE	15	25.1±8.8	1.2±0.5	−
	DEN→2-AAF	12	107.1±41.8	9.4±3.9	−
3	DEN	5	9.3±2.6	0.9±0.4	−
4	SS	18	0.0±0.0	0.0±0.0	±

Data are mean±SD values.
Significantly different from the corresponding group 2 value without SS at *, $P<0.05$; **, $P<0.01$.
−, not present; ±, negative to very weakly present.

as well as relative liver weight of rats at week 21 are summarized in Table III. No significant difference was observed for each subgroup among the four groups. Body and absolute liver weights were generally smaller in rats given EE in groups 1 and 2. The fibrosis development was, in most cases, less obvious than in experiment I (Fig. 4).

Quantitative values for GST-P-positive liver cell foci at week 21 are summarized in Table IV. Numbers and areas in group 1 animals given SS prior to DCA and 2-AAF exposure tended to be smaller than in group 2, this being significant in the DCA case. No induction of foci was observed after SS alone.

DISCUSSION

The results of the present study indicate that production of fibrosis by repeated administration of SS, given during administration of a hepatopromoting agent, is associated with increased preneoplastic development in our established medium-term assay for carcinogenic/modification potential. This was particularly evident in conjunction with the relatively weak promoter, DCA.

Chemically induced hepatic fibrosis in experimental animals has attracted a great deal of attention[12, 13] but exhibits obvious differences from the circumstances surrounding liver cirrhosis in man.[14, 15] Continuously occurring liver cell regeneration enhances the development of hepatocellular carcinoma. For example, in human cases, chronic liver cell necrosis and compensatory regeneration occurring during active viral hepatitis, which is always accompanied with progressive fibrosis, may be intimately involved in promoting hepatocellular carcinoma development.[16, 17] However, in the sixties, Paronetto and Popper[3] established a more comparable model in which the immune system plays a role, using a different approach that involves injecting heterologous serum into rats. Though the precise mechanisms remains unclear, septa connecting terminal hepatic venules appear without conspicuous hepatic parenchymal damage or increase in cell turnover.[6] In the present study, the lack of obvious inflammatory cell infiltration also suggests that parenchymal cell necrosis is not a major contributory factor for fibrosis in this model. This is in contrast to the LEC rat, in which liver cells undergo continuous damage and reactive cellular proliferation without obvious inflammatory cell reactions and fibrosis. LEC rats spontaneously develop hepatocellular carcinomas and are highly susceptible to carcinogens.[18, 19]

The significant changes in quantitative values for GST-P-positive focal lesions when SS was administered along with PB (in number) or DCA (in area) demonstrate a clear potential of SS to enhance the second promotion stage. A non-significant tendency for increase was also observed after SS alone after DEN initiation, in all cases associated with fibrotic changes. These were most pronounced in the DCA case where the total area of the lesions was almost doubled. Since DCA is a relatively weak promoter,[20, 21] as confirmed in the present study, the additional SS treatment was of clear benefit in allowing detection of its influence. The underlying mechanism remains to be established, but the question of whether this effect was indeed independent of any alteration in cell turnover is of obvious interest.

In contrast, no appreciable fibrotic condition was observed in rats given SS prior to initiation by DEN in experiment II, in contrast to the picture after 6 weeks of exposure in experiment I. This is possibly due to reversibility, given the longer interval after the last SS injection (9 weeks). The finding of a slight but significant reduction of GST-P-positive liver cell foci in the DCA case is interesting in this respect, but whether this was due to a memory effect acting on the promotion stage or an influence on DEN initiation could not be clarified.[22, 23] Further experiments are required to establish whether a cirrhotic condition can decrease the initiation potential

of DEN by altering drug-metabolizing enzymes or responsiveness to the necrogenic effect of DEN.[22,23]

Although cell death has been demonstrated at early stages with this experimental approach to induction of fibrosis, regenerative hyperplasia is not found.[7] Numbers of mesenchymal cells are increased,[4,24] and this has been suggested to play a causal role in the fibrogenesis. In view of its apparent independence from chronic regenerative processes, this type of fibrosis clearly warrants further investigation, especially in terms of altered collagen metabolism[25–28] and interstitial cell populations,[29–32] but for the purposes of the present study the positive influence on GST-P-positive lesion development was the major interest.

The assay model employed has undergone extensive investigation in our laboratory and has been established as a reliable approach to detection of carcinogenic or modification potential.[2] The test compounds and doses selected for the promotion stage in the present experiments were chosen on the basis of earlier findings (0.05% PB,[33] 0.01% 2-AAF,[34] 0.5 ppm EE,[20] 0.15% DCA[21]). In each case the results gained were essentially similar to those reported earlier.

In conclusion, the results are consistent with the hypothesis that fibrotic conditions act to increase growth pressure on preneoplastic foci, and suggest that SS could be applied to improve the sensitivity of our assay protocol.

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