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Hepatocyte Growth Factor Enhancement of Preneoplastic Hepatic Foci Development in Rats Treated with Diethylnitrosamine and N-Ethyl-N-hydroxyethylnitrosamine

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Effects of hepatocyte growth factor were investigated in a two-stage rat liver carcinogenesis protocol. Male F344 rats were first treated with diethylnitrosamine (200 mg/kg, i.p.) and then, starting two weeks later, with N-ethyl-N-hydroxyethylnitrosamine (EHEN) for 6 weeks at a dose of 0.01% in drinking water. Hepatocyte growth factor, which was injected i.v. at a dose of 200 μg/kg body weight one (at week 3) or two times (at weeks 3 and 4) during EHEN administration, significantly increased the development of preneoplastic glutathione S-transferase placental form-positive foci. Although the observed effects of hepatocyte growth factor were weaker than that of the two-thirds partial hepatectomy (PH) performed at week 3, the present results suggest that the enhancing effects of PH performed during the promotion stage may be largely mediated through induction of hepatocyte growth factor.

Key words: Hepatocyte growth factor — Liver carcinogenesis — GST-P-positive foci — Rat

We have developed a medium-term liver bioassay system of 8 weeks duration for rapid detection of carcinogenic agents using diethylnitrosamine (DEN) as an initiator, glutathione S-transferase placental form (GST-P)-positive hepatic foci as endpoint marker lesions and two-thirds partial hepatectomy (PH) as an accelerating factor.[1–4] The enhancing effects of PH on hepatocarcinogenesis have been repeatedly demonstrated[5,6] and its application during test chemical administration in the promoting stage of our rapid system has been well established.[7,8] Although similar enhancing effects have been observed with carbon tetrachloride[9,10] and other hepatonecrotic agents,[11,12] PH is considered to have advantages over such chemically induced regeneration since possible interactions with test chemicals can be avoided.[2]

Among the mechanisms underlying the enhancing potential of PH on hepatocarcinogenesis performed in the post-initiation stage, synchronized extensive induction of hepatocyte growth factor (HGF) may play the most important role. HGF was first identified in sera of partially hepatectomized rats[13–15] and was subsequently purified from rat platelets.[16,17] HGF has remarkable mitogenic activity in mature parenchymal hepatocytes and also stimulates DNA synthesis in other epithelial cells[13,18,19]; it may be a pleiotropic factor with biological activities required to construct normal tissues during organogenesis and regeneration.[20]

In the present experiment, we investigated the possible enhancing influence of HGF itself on hepatocarcinogenesis in comparison with PH using our medium-term liver bioassay system[1–4] and a strong hepatic and renal carcinogen, N-ethyl-N-hydroxyethylnitrosamine (EHEN), as a model compound.[21] Several types of hepatic altered lesions have been proposed as appropriate endpoints for prediction of liver carcinogenicity,[22,23] and GST-P-positive foci are considered to be the most appropriate.[24] The relationship between regeneration and carcinogenesis is also discussed.

MATERIALS AND METHODS

The experimental protocol is shown in Fig. 1. Male F344 rats (Charles River Japan, Inc., Atsugi), 6 weeks old at the commencement, were used. The rats were housed in an air-conditioned animal room at 23±2°C and 50±10% humidity and food and water were available *ad libitum*. Human recombinant HGF (lot no.: D21564, purity: more than 98%) was purified from the culture medium of CHO cells transfected with an expression vector containing HGF complementary DNA, as described previously.[25] The HGF solution, in an aqueous solvent (1.3 *M* NaCl, 0.01% Tween 20, and 10 m*M* Tris-HCl, pH 7.5), was diluted with saline immediately before use. Each injection was performed through the tail vein at a concentration of 200 μg/kg body weight (b.w.).

The animals were initially given a single intraperitoneal injection with DEN (Tokyo Chemical Co., Ltd.,

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Tokyo) at a dose of 200 mg/kg b.w. dissolved in saline to initiate hepatocarcinogenesis. After a 2 week recovery period, the rats received EHEN (Sakai Research Laboratory, Fukui) in the drinking water for 6 weeks at a concentration of 0.01% as a model promoter. The rats in group 1 were subjected to a single intravenous injection of HGF at week 3 and the rats in group 2 received two injections at weeks 3 and 4. The group 3 rats were intravenously injected with saline, 0.5 ml/kg b.w., as a vehicle control at weeks 3 and 4. The rats in group 4 were subjected to PH (two-thirds of the liver was removed) at week 3 under light ether anesthesia as a positive control and the rats in group 5 underwent a sham operation in place of PH.

Water consumption was recorded weekly (week 2 to 6) during the EHEN administration period. All animals were killed at week 8. The livers were removed and weighed, and slices from the left lateral and median lobes in groups 1, 2, 3 and 5, and from cranial and caudal parts of the right lateral lobe and the caudal part of the caudate lobe in group 4 were fixed in ice-cold acetone. The tissues were then routinely embedded in paraffin and sections were immunohistochemically stained for GST-P as previously reported.[24] The kidneys were also removed, weighed, and fixed in 10% phosphate-buffered formalin. One horizontal slice for the right kidney and one vertical slice for the left kidney from each animal were sampled, and hematoxylin and eosin-stained tissues were microscopically examined.

The numbers and the areas of GST-P-positive hepatic cell foci larger than 0.1 mm in diameter, the numbers of foci larger than 0.2 mm in diameter, and the total areas

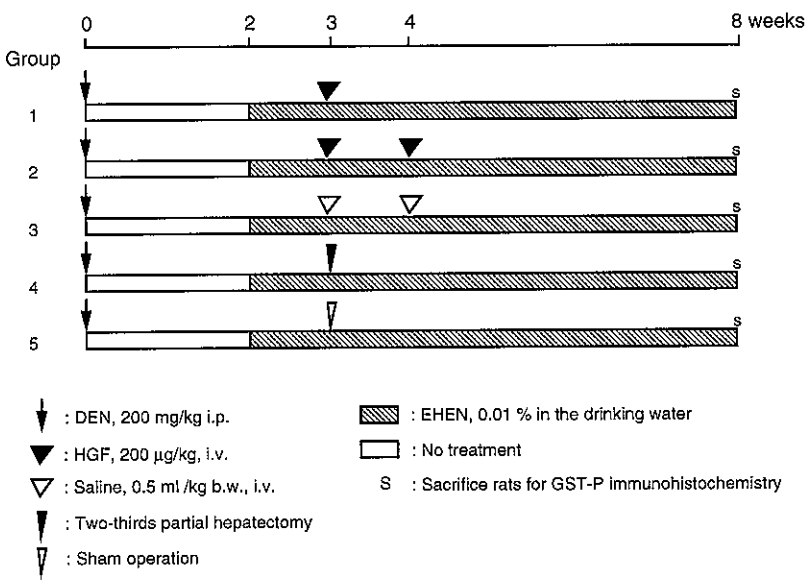

Fig. 1. Experimental protocol. Male F344 rats were subjected to a single DEN administration and then EHEN treatment along with intravenous HGF injection(s) or two-thirds partial hepatectomy as accelerating factors.

Table I. Final Body and Organ Weights of Rats

Group/ Treatment	No. of rats	Body weight (g)	Liver		Kidneys/Body (%)	Average intake of EHEN[a] (mg/day/rat)
			Absolute (g)	Liver/Body (%)		
1. HGF×1	10	288±10	9.66±0.35[b]	3.35±0.09[c]	0.63±0.03	1.96
2. HGF×2	10	287±8	9.68±0.27[b]	3.38±0.07[b]	0.62±0.02	2.02
3. Saline×2	11	289±12	9.70±0.51	3.36±0.09	0.61±0.03	2.06
4. PH	11	284±15	9.00±0.54[e]	3.17±0.19[d]	0.60±0.03	2.08
5. Sham operation	10	283±12	9.84±0.64	3.48±0.11	0.61±0.02	1.81

Data are mean±SD values.
a) Water consumption was recorded at weeks 2, 3, 4, 5, and 6 over 2 days at each time point.
Significantly different at *b*) $P<0.01$ and *c*) $P<0.05$ as compared to group 4, and at *d*) $P<0.001$ and *e*) $P<0.01$ from the respective control group values (vs. group 5).

of liver sections examined were measured using a video image processor, VIP 21C (Olympus-Ikegami Tsushin Co., Tokyo). Generally, we have analyzed foci larger than 0.2 mm in diameter for the purpose of rapid detection of hepatocarcinogenic agents using this protocol.[1-4] However, GST-P-positive foci larger than 0.1 mm in diameter (foci of more than about 12 hepatic cells) were mainly measured in this study since there is no reason to cut off smaller foci in this type of experiment.[26]

Statistical analysis of differences between means was carried out using Student's t test or Welch's t test after application of the preliminary F-test for equal variance for each appropriate pair.

RESULTS

Final numbers of animals and body and organ weights are summarized in Table I. No rats died during the experimental period. Final body weights were almost the same in groups 1–5. The treatment with HGF did not affect the body weight gain. Liver weights in group 4 were statistically significantly lower than in the other groups in terms of both absolute and relative to body values. The kidney weights were not influenced by the HGF treatment. As illustrated in Fig. 2, the water consumption was decreased in groups 4 and 5 after surgery and soon returned to the levels for groups which had received no surgical operation. The average intakes of EHEN calculated based on the 5 measurements of water consumption were similar in all groups.

Data for numbers and areas of GST-P-positive foci are summarized in Table II. There were no significant differences in foci development between the two controls, vehicle injections (group 3) and sham operation (group 5). On analysis of GST-P-positive foci larger than 0.1 mm in diameter, the numbers of foci were found to be almost the same between the groups subjected to a single HGF injection (group 1) and to PH (group 4). However, the average size of foci was significantly smaller in the HGF-injected groups (groups 1 and 2) than in group 4, resulting in lower total areas of foci in those two groups. Furthermore, a weak inverse relationship was observed between the number of HGF injections and the values of GST-P-positive foci. Numbers of foci larger than 0.2 mm in diameter were also significantly increased by HGF injection(s) (groups 1 and 2) and PH (group 4) as compared to the vehicle or sham operation groups. However, the values for the HGF-injected groups were significantly smaller than for the PH group, and no frequency-dependent enhancement of foci development was observed for HGF treatment. Areas of foci larger than 0.2 mm in diameter were not separately determined.

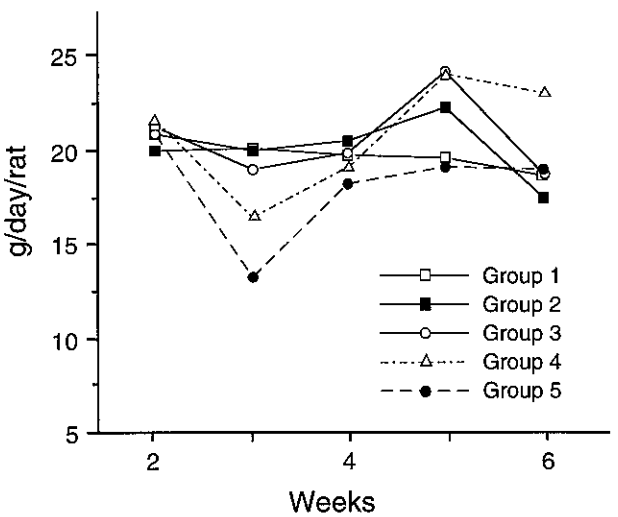

Fig. 2. Changes in water consumption during EHEN administration. Slight decrease in water intake was observed in rats after surgical operation. At each time point, water consumption was measured as values for two day-intake per cage (three cages per group).

Table II. Effects of HGF and PH on GST-P-positive Foci Development in the Rat Liver

Group/ Treatment	No. of rats	0.1 mm in diameter or larger			0.2 mm in diameter or larger
		Number (no./cm^2)	Area (mm^2/cm^2)	Average size (10^{-2} mm^2/focus)	Number (no./cm^2)
1. HGF×1	10	50.8±9.3[a)]	1.08±0.25[a, d)]	2.12±0.17[b, d)]	8.97±3.32[a, d)]
2. HGF×2	10	43.2±10.3[c, e)]	0.94±0.32[c, d)]	2.13±0.33[c, d)]	7.74±4.21[c, d)]
3. Saline×2	11	33.0±8.6	0.62±0.21	1.83±0.19	3.44±1.96
4. PH	11	53.7±9.4[a)]	1.73±0.29[a)]	3.24±0.37[a)]	16.62±2.72[a)]
5. Sham operation	10	30.3±12.2	0.57±0.27	1.85±0.18	3.62±2.73

Data are mean±SD values.
Significantly different at *a*) $P<0.001$, *b*) $P<0.01$, and *c*) $P<0.05$ in groups 1 and 2 vs. group 3, and group 4 vs. group 5, and at *d*) $P<0.001$ and *e*) $P<0.05$ in groups 1 and 2 vs. group 4.

Kidney weights were not affected by the HGF co-administration or PH. Microscopically, dysplastic tubules were sporadically observed in all EHEN-treated groups, and no effect of HGF administration or PH on their development was evident.

DISCUSSION

HGF is a potent mitogen that was originally isolated from serum on the basis of its ability to stimulate the *in vitro* growth of hepatocytes.[14, 15, 27] In rats, plasma HGF increases immediately after 70% PH, reaches a peak 2–3 h later, and remains about 10-fold higher than the control level for at least 72 h after resection.[28, 29] In our liver bioassay protocol, it has been repeatedly demonstrated that PH is requisite for enhancement of the promoting effects of chemicals and, therefore, for increasing the sensitivity of this bioassay for rapid detection of carcinogens.[1, 2, 6]

In the present experiment, effects of HGF injection(s) were compared with those of PH in our medium-term bioassay. The dose of HGF used in the present experiment was chosen based on the data from mouse experiments.[30] We found that intravenous injections of HGF performed once or twice during the hepatocarcinogen (EHEN) administration clearly accelerated liver carcinogenesis, as assessed using preneoplastic GST-P-positive hepatic foci as phenotypically altered endpoint indicators. However, the effects of HGF were less than that of PH in the present experiment and there were no apparent differences arising from treatment frequency (once or twice) of HGF. The average size of foci in the HGF-treated groups was smaller than in the PH group, meaning that the enhancing effect of one or two intravenous injections of 200 μg/kg HGF was weaker than that of 70% PH. Although the serum HGF levels were not compared between the two treatments in the present experiment and no data are available for direct comparison of serum HGF kinetics after intravenous injection(s) and PH in rats, it is possible that the difference might have been caused by different time-courses of serum HGF levels. As mentioned above, plasma HGF reaches a peak at 2–3 h and remains elevated for at least 72 h after resection.[28, 29] Since restoration of the liver volume generally takes 10–14 days after 70% PH in rats,[31] elevated plasma HGF levels may continue during this period. On the other hand, it is probable that plasma HGF levels immediately decrease after intravenous injection.[30] It was completely unexpected that the enhancing effect of HGF was almost the same, or even less, in the group subjected to two injections than in the group given a single injection. The reasons why there were no apparent differences between one and two injections of HGF in terms of foci development are unknown. One possibility is an immunological reaction which acted to decrease the HGF efficacy after the second injection.

In the present experiment, EHEN was chosen as a model compound since Ishiki *et al.*[30] had suggested that the mitogenic effect of HGF is much greater in damaged liver. HGF is also effective in renal regeneration,[20] and EHEN is carcinogenic in both liver and kidney.[21] It was reported that, when mice were intravenously injected with HGF (1 or 5 μg) after 30% PH or carbon tetrachloride administration, the labeling index of hepatocytes was markedly increased.[30] In the present experiment, liver weight in the PH group was significantly lower than in the sham operation control group. It is possible that EHEN toxicity appeared more clearly when this agent was combined with PH treatment.

It is very interesting in the context of the present results that HGF suppresses the growth of cultured hepatocellular carcinoma cells *in vitro*.[32] In addition to its well known hepatotropic effects, it has recently been demonstrated to exert a variety of biological influences, including acting as a mitogen in organs other than the liver,[18, 33] as a motogen[34, 35] and a morphogen.[36] Details of the mechanisms underlying the observed enhancing effect of increased plasma HGF on hepatocarcinogenesis or selective growth of preneoplastic lesions remain to be clarified, but it is likely that HGF-induced regeneration simply increases the possibility of preneoplastic initiated cells growing to become foci without any direct carcinogenic effect.[37] EHEN is a renal carcinogen[38] and tubular dysplasia was observed in the EHEN-treated rats after a 6-week exposure. Although HGF has been reported to be a renotropic agent,[20] no obvious effect of HGF on histopathological changes in this organ was observed in the present study.

In conclusion, the present results indicate that intravenously applied HGF could act as a coenhancing factor in rat liver carcinogenesis.

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