

Human Placental Extract Stimulates Liver Regeneration in Rats

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The effect of human placental extract (HPE) on liver regeneration in rats was investigated. After intravenous administration of HPE to α -naphthylisothiocyanate (ANIT)-intoxicated rats, the labeling index in hepatocytes was significantly increased to a level 16.5 times higher than that of the control. A 1/500 dilution of HPE directly stimulated DNA synthesis of the hepatocytes in primary culture. HPE heated at 121 °C did not stimulate the labeling index *in vivo* or hepatocyte DNA synthesis in primary culture, suggesting that HPE contains heat-unstable but potent mitogens for hepatocytes. HPE contains hepatocyte growth factor (HGF), but the mitogenic effect of HPE cannot be explained by the effect exerted by HGF alone, since both the labeling index *in vivo* and hepatocellular DNA synthesis *in vitro* stimulated by HPE were much higher than those stimulated by HGF alone when the applied doses of HGF were set to be almost the same level between each case. When HPE was fractionated on a heparin-sepharose column, the mitogenic effect of HPE was found to be located mainly in the heparin-bound fraction. Hepatocyte DNA synthesis induced by this fraction was enhanced cooperatively by the heparin-unbound fraction, suggesting that there are some modulators in the heparin-unbound fraction which enhance the proliferative activity of the heparin-bound fraction by a synergetic mechanism. Both HPE and heated HPE completely recovered the biochemical marker activity for liver function (glutamic-pyruvic transaminase, GPT; alkaline phosphatase, ALP; lactate dehydrogenase, LAP; γ -glutamyltransferase, γ -GTP activities and the bilirubin concentration) almost to the control level in the serum of ANIT-intoxicated rats, indicating that HPE also contains a heat-stable fraction which repairs liver function.

Key words human placental extract (HPE); liver regeneration; hepatocyte growth factor (HGF)

Human placental extract (HPE) has been used to treat a number of liver diseases including hepatitis and cirrhosis.^{1–3)} In recent years it has become evident that human placenta is both a source and a target of a large number of biologically active molecules.^{4–6)} Many of these agents are believed to act in an autocrine/paracrine fashion within the human placenta, regulating the production of other biologically active substances in a complex interplay of factors with a potential for treating various diseases. Recently, a number of growth factors^{4–10)} and their receptors^{11–13)} have been identified in human placenta, such as hepatocyte growth factor (HGF),^{4,7)} epidermal growth factor (EGF),¹⁰⁾ transforming growth factor- β (TGF- β)⁸⁾ and transforming growth factor- α (TGF- α).⁹⁾ Wolf *et al.*⁴⁾ have purified HGF, a heparin-binding glycoprotein which is a very potent mitogen for hepatocytes, from human placenta, and reported that this purified HGF markedly stimulates DNA synthesis in rat hepatocytes in primary culture.

Although many drugs have been used clinically to treat liver diseases, very few of these directly stimulate liver regeneration. Thus, it would be an important advance to develop hepatocyte mitogens as therapeutic agents to repair the damaged liver. We have already reported that Laennec, a hydrolyte of human placenta, stimulates liver regeneration both *in vivo* and *in vitro* in rats.³⁾ Laennec is prepared by treating HPE with heat and acid. Although we have found that Laennec does not contain HGF, probably because HGF is heat- and acid-labile, it still induces liver regeneration.³⁾ It is suggested that factors in HPE, other than HGF, are responsible for the effect of Laennec. It would be expected that HPE contains such heat- and acid-labile mitogens for hepatocytes. However, until now, no mitogens in HPE have been identified as being active in treating liver diseases. In the present study, to identify the HPE fraction responsible for liver regeneration and the improvement of liver function, we examined the ef-

fect of HPE on liver regeneration *in vivo* and *in vitro*. In addition, we also investigated the effect of heat-stable, -unstable, heparin-bound, -unbound fractions in HPE on liver regeneration.

MATERIALS AND METHODS

Animals and Materials Male Wistar rats weighing 250 g (for *in vivo* experiments) and 150 g (for *in vitro* experiments) (Nisseizai, Tokyo, Japan) were used. All animals were treated humanely. The studies reported in this manuscript have been carried out in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. ¹²⁵I-deoxyuridine was obtained from New England Nuclear (Boston, MA), 3,3'-diaminobenzidine from Sigma (St. Louis, MO) and the Predicta TGF- β 1 kit was purchased from Genzyme (U.S.A.).

Preparation of HPE Fresh normal mature placentas with an average weight of approximately 450 g were obtained after delivery. Each placenta was washed with PBS to remove any blood and homogenized in 450 ml 2 M sodium chloride solution. The homogenate was clarified by centrifugation at 15,000 *g* for 30 min at 4 °C, then the suspension was dialyzed against culture medium (William's E) using membrane dialysis (MW=1000, Spectra/Por, U.S.A.). The dialyzed suspension was used as the HPE for both *in vivo* and *in vitro* studies.

HPE Injection α -naphthylisothiocyanate (ANIT) (Sigma, St. Louis, MO) dissolved in olive oil was injected intraperitoneally at a dose of 50 mg/kg body wt. HPE, HPE diluted with saline at the designated times, and HPE heated at 121 °C for 20 min (2.0 ml/kg body weight, respectively) were administered through the penis vein or injected into a leg muscle 30 min before and at 8, 24, 32, 46 h after the ANIT

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intoxication. Rats were sacrificed 48 h after the ANIT intoxication.

Measurement of Labeling Index At 1 h before sacrificing the rats, 5-bromo-2'-deoxyuridine (100 mg/kg body weight) dissolved in normal saline was injected intraperitoneally. One hour after injection, the rats were exsanguinated *via* the abdominal artery under light ether anesthesia. The liver was then removed and fixed in 10% buffered formalin for 24 h. The fixed samples were embedded in paraffin and sections (4 μ m) were mounted on a glass slide. After deparaffinization of the liver sections, endogenous peroxidase was inactivated in 0.3% hydrogen peroxide in absolute methanol, and nuclei incorporating 5-bromo-2'-deoxyuridine were stained using a Cell Proliferation Kit from Amersham (Arlington Heights, IL). The labeling index of hepatocytes was determined by counting more than 500 nuclei in photographs of three randomly selected fields under light microscopy.

Determination of Bilirubin Concentration and the Activities of Biochemical Markers for Liver Function in Serum The total bilirubin concentration (BIL) and activity of liver-specific cytosolic enzymes, such as glutamic-pyruvic transaminase (GPT), lactate dehydrogenase (LAP), alkaline phosphatase (ALP) and γ -glutamyltransferase (γ -GTP), in rat serum obtained 48 h after the ANIT treatment, were determined using the appropriate assay kits (Wako Pure Chemical Industries, Osaka, Japan).

Assay of DNA Synthesis in Primary Cultured Rat Hepatocytes Parenchymal hepatocytes were plated at a density of 1.25×10^5 cells/1.88 cm² and cultured for 24 h as described previously.¹⁴ The non-attached cells were removed by washing and the culture medium containing HPE was applied to the monolayer. Then, 22 h after HPE addition, [¹²⁵I]-deoxyuridine was added and its incorporation for 6 h was assayed as described previously.¹⁴ Cellular protein was determined by the method described by Bradford, using the Bio-Rad protein assay kit with BSA (bovine serum albumin) as a

standard.¹⁵

Elution of Heparin-Bound and -Unbound Fractions Using Heparin-Sepharose Column Chromatography One ml HPE was added to a heparin column (1 ml bed volume, heparin-sepharose CL 6B, Pharmacia) at the rate of 0.3 ml/min and left for 30 min on ice. Then, 1.0 ml PBS was added to the column and the eluted fraction was collected as heparin-unbound fraction (F1). After that, 1.0 ml sodium chloride with a step-wise gradient was applied to the column at the same rate. The eluted fractions were obtained as designated for the heparin-bound fractions (F2—F11).

Determination of HGF and TGF- β 1 Concentrations The concentrations of HGF and TGF- β 1 in HPE were determined by enzyme-linked immunosorbent assay (ELISA) with IMMUNIS HGF EIA kits (Institute of Immunology) and PREDICTA TGF- β 1 ELISA kits (Genzyme).

Statistical Analysis Statistical analysis was performed by Student's *t*-test to identify significant differences between various treatment groups.

RESULTS

Effect of Human Placental Extract on Liver Regeneration in ANIT-Intoxicated Rats *in vivo* After the intravenous or intramuscular administration of HPE to ANIT-intoxicated rats, the hepatocyte labeling indices were 16.5 and 14.1 times that of the controls, respectively (Fig. 1). When 1/2, 1/8, and 1/20 dilutions of HPE were administered intravenously, the labeling indices were 5.78, 5.64, and 0.96 times that of the controls, showing an HPE dose-dependence (Fig. 1). The labeling index in the hepatocytes was almost the same as that of the controls when heated HPE was injected intravenously (Fig. 1). To examine whether the effect of HPE on liver regeneration was due to HGF contained in the HPE, we measured the HGF content of HPE and compared the labeling indices obtained by the administration of HPE and HGF alone¹⁹ (Fig. 1). In the case of HGF alone, the labeling

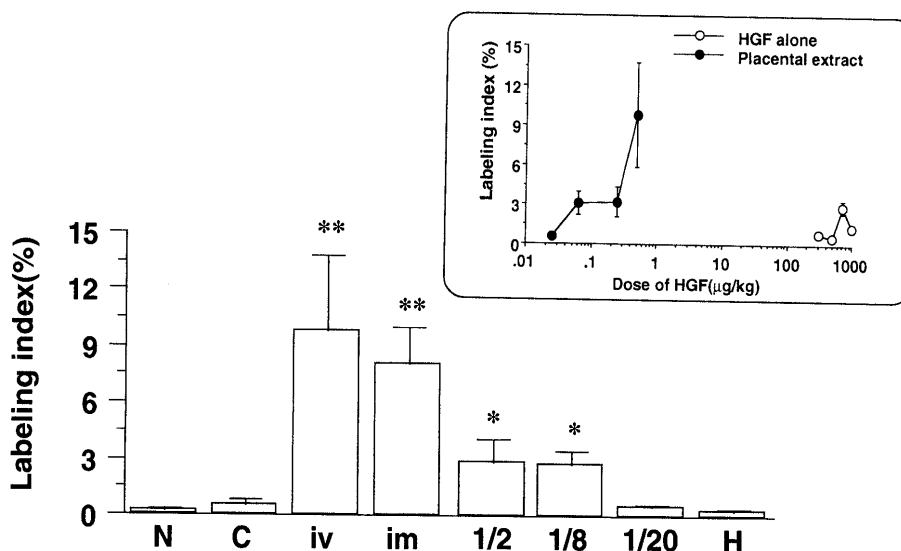


Fig. 1. Effect of HPE on Liver Regeneration in ANIT-Intoxicated Rats

ANIT-intoxicated rats were treated with HPE and the labeling index in hepatocytes was determined 48 h after ANIT intoxication. N: Normal rats without ANIT treatment; C: Control rats with ANIT treatment; i.v. and i.m.: Intravenous and intramuscular administration of HPE, respectively; 1/2, 1/8, 1/20: Intravenous administration of diluted HPE at the designated times; H: Intravenous administration of heated (121 °C 20 min) HPE. Inset: Comparison of the effects of HPE (●) and recombinant HGF (○) on liver regeneration. HGF content in HPE was determined by EIA. Labeling index was plotted against the HGF content in HPE. The effect of HGF alone was taken from Reference 20. Each value and vertical bar represents the mean \pm S.E. of 3—6 rats. * Significantly different from C ($p < 0.05$); ** ($p < 0.01$).

indices were only 0.80 ± 0.083 , 0.53 ± 0.104 , 2.90 ± 0.50 , and $1.33 \pm 0.17\%$ when the doses of HGF were 300, 500, 710, and $1000 \mu\text{g/kg}$, respectively (Fig. 1 inset). However, the labeling index was $9.80 \pm 3.93\%$ after the intravenous administration of undiluted HPE, the HGF content of which was only $0.5 \mu\text{g/kg}$.

Effect of HPE on the Activities of Biochemical Markers for Liver Function in ANIT-Intoxicated Rats To examine whether HPE promotes the repair of liver integrity in ANIT-intoxicated rats, we determined the change in BIL and the activities of biochemical markers for liver function such as GPT, LAP, ALP, and γ -GTP in serum from rats after the intravenous or intramuscular administration of HPE (Fig. 2). The increase in BIL and the activities of the biochemical markers for liver function in serum caused by ANIT-intoxica-

tion were significantly countered by the injection of HPE or HPE dilutions (Fig. 2). Heated HPE also suppressed the increase in BIL and maintained normal levels of activities of biochemical markers for liver function (Fig. 2).

Mitogenic Response of Primary-Cultured Rat Hepatocytes to HPE To examine the direct effect of HPE on hepatocytes, we examined the effect of HPE on DNA synthesis in primary cultured hepatocytes (Fig. 3A) and compared it with the effect of various concentrations of HGF on DNA synthesis in rat hepatocytes¹⁹⁾ (Fig. 3B). The concentrations of HGF and TGF- β 1 in the diluted HPE at the designated times were also determined by ELISA (Fig. 3A). When a 1/500 dilution of HPE was added to the cultured hepatocytes, DNA synthesis in the hepatocytes appeared to be maximum (Fig. 3A). This maximum effect was comparable with the effect exerted

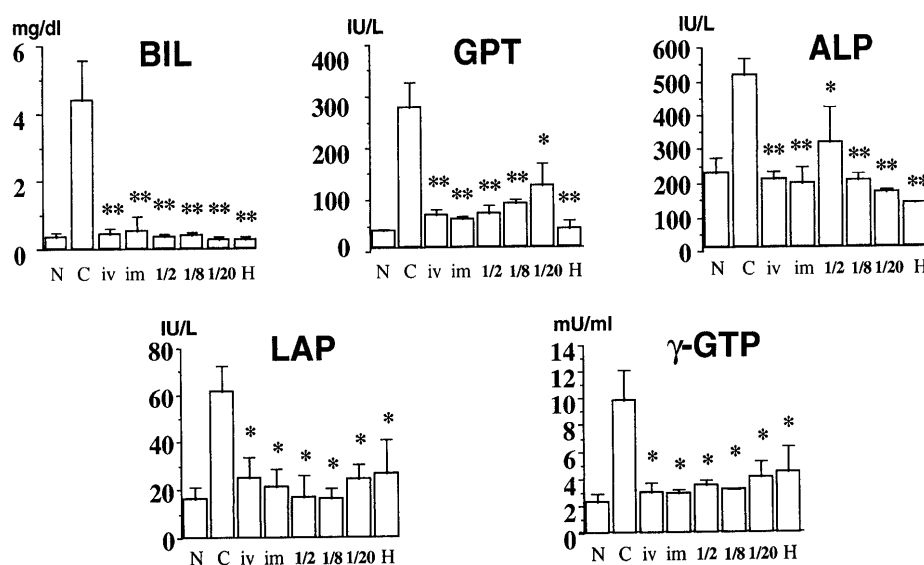


Fig. 2. Change in Bilirubin Concentration and the Activities of Biochemical Markers for Liver Function in Serum in ANIT-Intoxicated Rats Treated with HPE

ANIT-intoxicated rats were treated with HPE. Bilirubin concentration and the activities of biochemical markers for liver function in serum were determined 48 h after ANIT intoxication. N: Normal rats without ANIT treatment; C: Control rats with ANIT treatment; i.v. and i.m.: Intravenous and intramuscular administration of HPE, respectively; 1/2, 1/8, 1/20: Intravenous administration of diluted HPE at the designated times; H: Intravenous administration of heated (121°C 20 min) HPE. Each value and vertical bar represent the mean \pm S.E. of 3 rats. * Significantly different from C. ($p < 0.05$); ** ($p < 0.01$).

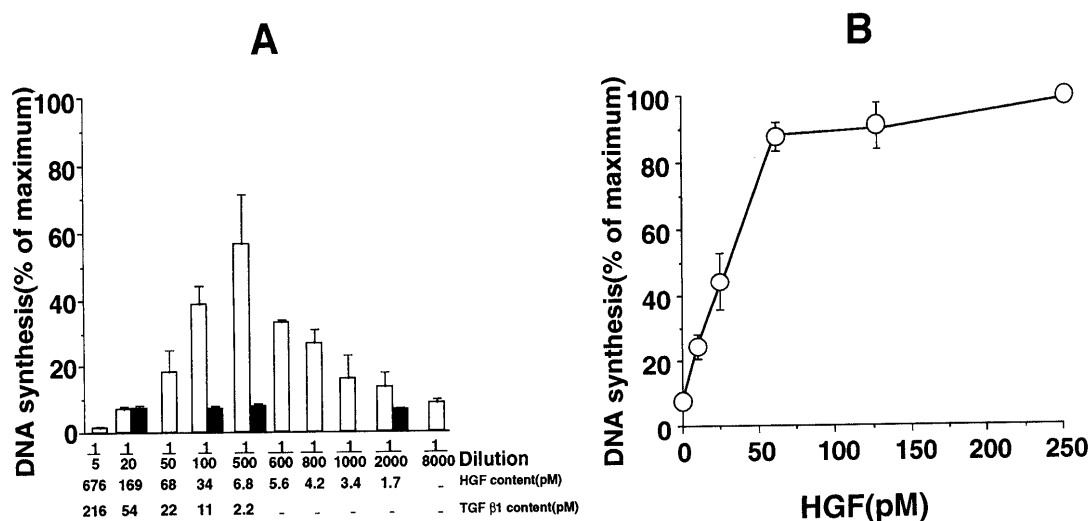


Fig. 3. Effect of HPE (A) and HGF (B) on DNA Synthesis in Rat Hepatocytes in Primary Culture

In primary cultured rat hepatocytes, heated (■) and unheated (□) HPE at the designated dilution (A) were applied to serum-free medium. 22 h after the addition, ^{125}I -deoxyuridine was added; its incorporation for 6 h was assayed as described in "Materials and Methods". Data for recombinant HGF was taken from Reference 20 and are shown in panel (B). Data are expressed as the values normalized with respect to the maximum value of mitogenic response in the presence of 250 pM recombinant HGF alone. The final concentration of HGF and TGF- β 1 in each dilution of unheated HPE is also shown. Each value and vertical bar represents the mean \pm S.E. of 3 rats.

by approximately 40 pM HGF (Fig. 3A and B). Under these conditions, the concentration of HGF in diluted HPE was only 6.8 pM (Fig. 3A) and the TGF- β 1 concentration was only 2.2 pM (Fig. 3A). The mitogenic effect of HPE appeared to occur in a bell shape: when the dilutions were less than 1/500, the mitogenic responses of hepatocytes appeared to increase as the dilutions increased, showing a dose-dependent reduction (Fig. 3A). The DNA synthesis in the hepatocytes fell as the dilutions exceeded 1/500 (Fig. 3A). Heated HPE at any designated dilution could not stimulate DNA syntheses in hepatocytes (Fig. 3A) and, in this case, the concentrations of HGF and TGF- β 1 in the original heated HPE were lower than the detection limit of the determination (<0.5 pM and <1.0 pM), respectively.

The Effect of the Heparin-Bound and -Unbound Fraction in HPE on DNA Synthesis in Rat Hepatocytes in Primary Culture The partial purification of HPE was performed with a heparin-sepharose column, and the effects of heparin-bound and -unbound fractions in HPE on DNA synthesis in rat hepatocytes were investigated (Fig. 4A). The through fraction (F1), termed the heparin-unbound fraction, did not increase DNA synthesis in rat hepatocytes (Fig. 4A). On the other hand, the 1/20 diluted heparin-bound fraction (F9), which contained 40.2 pM HGF, increased DNA synthesis in hepatocytes to 40% of the maximum (Fig. 4A). The effect of F9 on DNA synthesis in hepatocytes was similar to the effect of 40 pM HGF on DNA synthesis when the result was compared with the standard curve of recombinant HGF (Figs. 4A and 3B). To investigate whether the stimulative factor(s) for HGF mitogenic activity are present in the HPE, 10 pM HGF was added to each fraction at designated dilutions and the experiment was repeated (Fig. 4B). The DNA synthesis of hepatocytes was 20% of maximum in the presence of 10 pM HGF alone, but increased to approximately 40% of maximum when 10 pM HGF was applied to 1/20 diluted F1 (Fig. 4B). The DNA synthesis in the presence of F1 alone was almost comparable with the control level (Fig.

4A). These results suggest that some unknown stimulating factors for the HGF effect are present in F1.

The Synergetic Effect of the Heparin-Bound and -Unbound Fraction in HPE on DNA Synthesis in Rat Hepatocytes in Primary Culture To examine whether the heparin-bound fraction (F9) can enhance the effect of the heparin-unbound fraction (F1) on DNA synthesis in rat hepatocytes in primary culture, the synergetic effect of F1 and F9 was investigated (Fig. 5). F9 alone at a 1/80 dilution (Fig. 5A) contained 10 pM HGF and a 1/20 dilution (Fig. 5B) contained 40.2 pM HGF. These diluted F9 fractions stimulated DNA synthesis to 20% and 40% of maximum, respectively (Fig. 5A and B). These effects were almost the same as the effects induced by 10 pM and 40 pM recombinant HGF, respectively (Fig. 3A). The DNA synthesis stimulated by F1 alone at 1/20, 1/100, and 1/500 dilutions were 5.05 ± 0.54 , 8.9 ± 0.84 , and $10.71 \pm 0.85\%$ of maximum, respectively (Fig. 5A and B). When F9 was added to the 1/20, 1/100, and 1/500 dilutions of F1, the DNA synthesis increased to 41.3 ± 2.58 , 64.6 ± 2.24 , and $29.0 \pm 0.71\%$ of maximum for F9 at a 1/80 dilution (Fig. 5A) and 53.2 ± 5.9 , 102 ± 8.8 , $67.8 \pm 12.7\%$ of maximum for F9 at a 1/20 dilution (Fig. 5B).

DISCUSSION

We found that HPE markedly stimulates the labeling indices of hepatocytes in ANIT-intoxicated rats *in vivo* (Fig. 1), and this effect is dose-dependent (Fig. 1). However, when HPE was heated at 121 °C for 20 min, there was no increase in the labeling indices of hepatocytes (Fig. 1). This suggests that the active substances in HPE capable of stimulating liver regeneration are unstable when heated. HPE contains HGF, which is also unstable to heat but is a very potent mitogen for hepatocytes. Therefore, we suspected that HGF in HPE was responsible for the mitogenic effects of HPE. However, we found that the labeling indices in hepatocytes after the injection of recombinant HGF alone were much lower than that

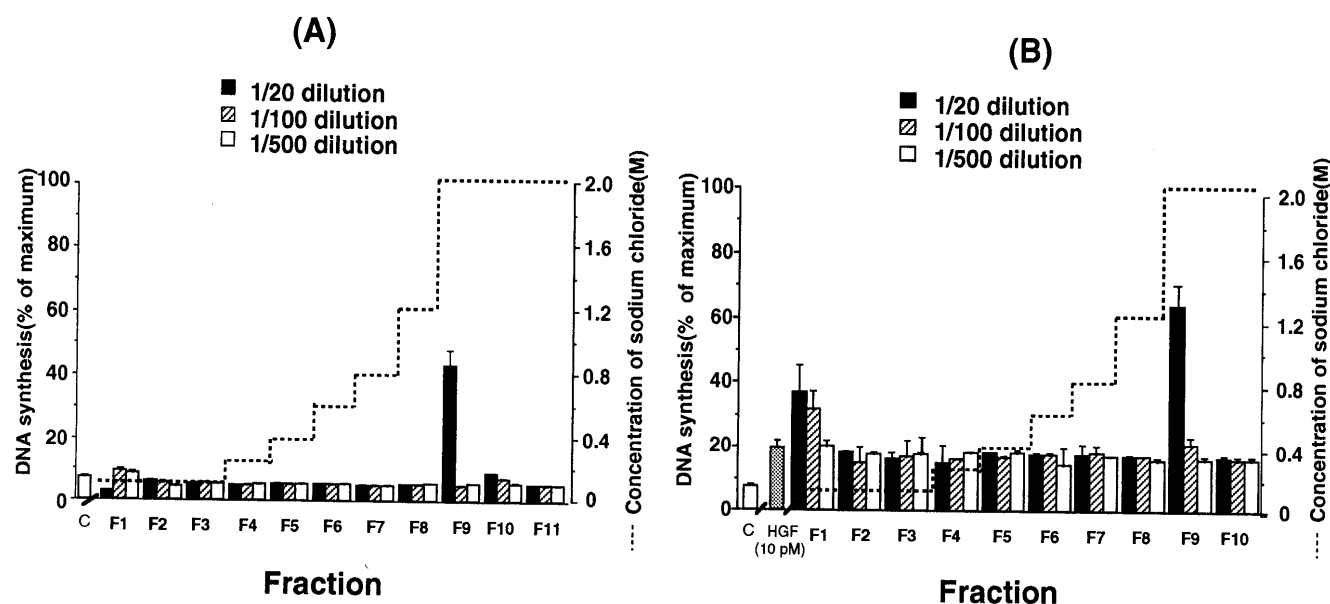


Fig. 4. Effect of HPE Fractionated Using Heparin-Sepharose Column Chromatography on DNA Synthesis in Rat Hepatocytes in Primary Culture

(A): Heparin-unbound fraction (F1) and -bound fraction (F2—F11) eluted by PBS and sodium chloride using gradient concentrations (-----) were diluted as shown and added to primary cultured hepatocytes. (B): 10 pM recombinant HGF was also added to each diluted fraction (F1—F10) in culture medium. DNA synthesis in hepatocytes was determined as described in "Materials and Methods". C is the control with medium alone without HGF. Each value and vertical bar represents the mean \pm S.E. of 3—5 rats. *: Significantly different from the mathematic additive levels of 1/20 diluted F1 and 10 pM HGF ($p < 0.05$).

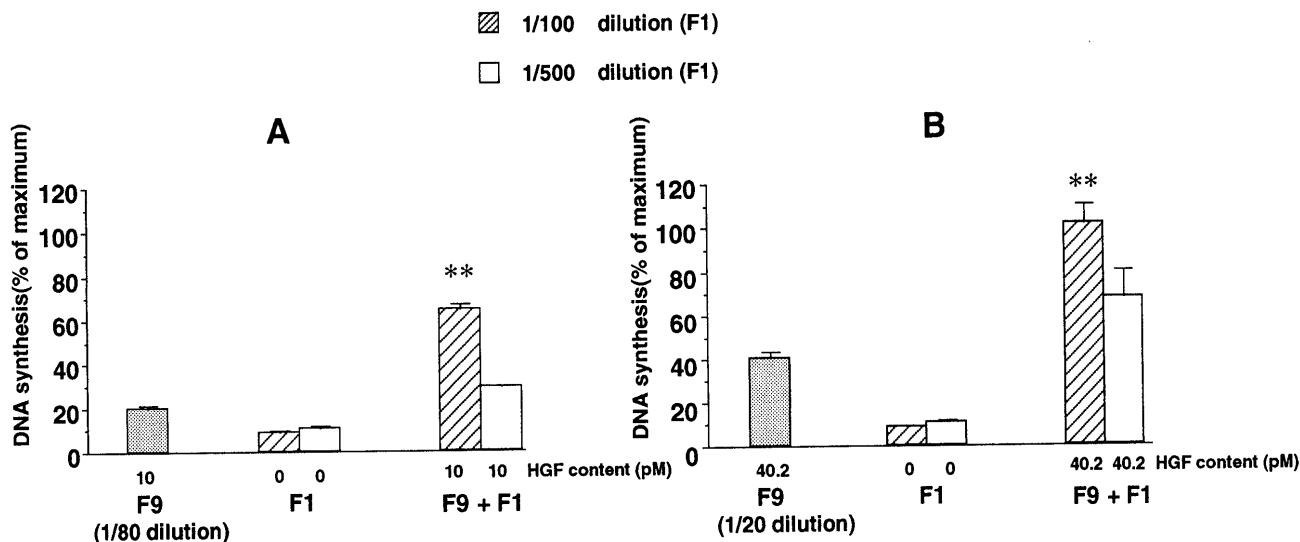


Fig. 5. The Synergetic Effect of Heparin-Bound and -Unbound Fractions in HPE on DNA Synthesis in Rat Hepatocytes

DNA synthesis in hepatocytes stimulated by 1/80 (A) and 1/20 (B) diluted heparin-bound fraction (F9) and -unbound fraction at the designated dilutions (F1) was assayed. F9+F1 is the DNA synthesis in hepatocytes with a mixture of F9 (1/80 (A) or a 1/20 (B) dilution in the final concentration) and F1 (1/100 or 1/500 dilutions in the final concentration); this was also determined. The final concentration of HGF in each fraction is also shown. Each value and vertical bar represents the mean \pm S.E. of 3 rats. **: Significantly different from the additive level of F9 alone and F1 alone at a 1/100 dilution ($p < 0.01$).

after undiluted HPE; even doses of HGF in an injection of HGF alone were 600–2000 times more than the HGF content in the HPE injection (Fig. 1). This indicates that the effect of HPE on liver regeneration cannot be explained simply by the effect exerted by the HGF in the HPE. In addition, in the *in vitro* study, we found that the DNA synthesis in hepatocytes reached a maximum in the presence of a 1/500 dilution of HPE (Fig. 3A), which contains only 6.8 pM HGF (Fig. 3A). This maximum effect also could not be explained by the 6.8 pM HGF in the 1/500 dilution of HPE when compared with the DNA synthesis induced by the recombinant HGF (Fig. 3A and B). It may be that there are other heat-unstable substances that stimulate liver regeneration in the HPE besides HGF, or that there is some factor in HPE which enhances the effect of mitogens.

The elevation of serum BIL and the activities of biochemical markers for liver function were significantly suppressed by either the intravenous or intramuscular administration of HPE in ANIT-intoxicated rats (Fig. 2). This result indicates that HPE repairs the integrity of the liver. However, this effect was not dose-dependent (Fig. 2). In addition, the active substance for repairing the integrity of the liver appears to be heat-stable (Fig. 2). It should be noted that when the heated HPE was injected into the ANIT-intoxicated rats, no mitogenic response was found in the hepatocytes (Fig. 1), although the elevation of serum BIL and the activities of biochemical markers for liver function were almost completely suppressed (Fig. 2). This phenomenon prompts us to consider that there are at least two active substances in the HPE; one is a heat-unstable material which stimulates liver regeneration, the other is a heat-stable material which repairs the liver integrity but cannot stimulate regeneration of the damaged liver. Because there are at least two such active substances present in HPE, HPE not only stimulates liver regeneration but also repairs the integrity of the liver under conditions of ANIT-intoxication (Figs. 1 and 2).

The pattern of DNA synthesis in hepatocytes in the presence of different dilutions of HPE appeared to be symmetri-

cal (Fig. 3A), indicating that HPE contains some inhibitor of hepatocyte proliferation as well as hepatocyte mitogens. This inhibitor in HPE was also diluted with the dilution of HPE so that the material could not exert its inhibitory effect on DNA synthesis in hepatocytes as the dilution of HPE increased. Nakamura *et al.*¹⁶⁾ reported that TGF- β 1, a very potent inhibitor of hepatocyte mitosis, completely inhibits DNA synthesis in rat hepatocytes induced by HGF at a TGF- β 1 concentration of 40 pM. Immunocytochemical studies have revealed that TGF- β 1 is present, throughout gestation, in the cytotrophoblast layer of chorionic villi¹⁷⁾ and in the extravillous cytotrophoblast of the cytotrophoblastic shell in human placenta.¹⁸⁾ We found that the 1/5 dilution of HPE contained 216 pM TGF- β 1 (Fig. 3A), and that DNA synthesis of hepatocytes was not stimulated when the 1/5 dilution of HPE was added to cultured hepatocytes (Fig. 3A). At the 1/500 dilution, the concentration of TGF- β 1 in human placenta was only 2.2 pM (Fig. 3A), and this low concentration of TGF- β 1 cannot inhibit DNA synthesis.¹⁶⁾ Thus, the TGF- β 1 in HPE might inhibit DNA synthesis in hepatocytes in a concentration-dependent manner.

The heparin-unbound fraction (F1) alone could not stimulate DNA synthesis in hepatocytes (Fig. 5), but it enhanced the stimulative effect of the heparin-bound fraction (F9) on DNA synthesis in rat hepatocytes (Fig. 5), suggesting that there are some enhancers in the heparin-unbound fraction of HPE which contribute to the mitogenic effect of the heparin-bound fraction, and the two fractions exert biological activity in liver regeneration by their synergetic effects (Fig. 5). Because the direct effect of the heparin-bound fraction on (F9) DNA synthesis can be explained by the effect of the HGF contained in the heparin-bound fraction (F9) (Fig. 5), we believe that the enhancer may be an enhancer for the HGF effect. In fact, this F1 fraction enhanced the mitogenic response to 10 pM HGF in hepatocytes (Fig. 4B). Thus, HPE contains a heparin-unbound factor which stimulates the mitogenic effect of HGF.

We conclude that (1) HPE stimulates liver regeneration

and liver repair in rats; (2) the mitogenic activity of HPE is governed by heat-unstable factors including HGF, while the improvement in liver integrity is related to a heat-stable substance in HPE; and (3) the synergetic effect of the heparin-bound and -unbound fractions may explain, at least in part, the mechanism of liver regeneration induced by HPE.

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