Purification and Partial Characterization of Hepatocyte Growth Factor from Plasma of a Patient with Fulminant Hepatic Failure

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Abstract

Human hepatocyte growth factor (hHGF) has been purified ~ 209,000-fold with 18% yield from plasma of a patient with fulminant hepatic failure. The purification involves heat treatment of plasma, ammonium sulfate precipitation, and chromatography on Affi-Gel Blue, heparin-Sepharose, and hydroxylapatite. Purified hHGF shows several bands with molecular weights between 76,000 and 92,000. Each band shows growth-stimulating activity on cultured hepatocytes which is proportional to the intensity of the band. After reduction of the sample with 2-mercaptoethanol, SDS-PAGE yields two chains with molecular weights of 31,500–34,500 and 54,000–65,000. The effect of hHGF on DNA synthesis by hepatocytes is half-maximal at 3.5 ng/ml. hHGF stimulates proliferation of cultured hepatocytes more effectively than human epidermal growth factor (hEGF) or insulin, and the effect of hHGF is additive or synergistic with the maximal effects of hEGF and insulin. These results suggest that hHGF is a new growth factor which is different from hEGF.

Introduction

Patients with fulminant hepatic failure show massive necrosis of liver cells or other sudden and severe impairment of hepatic function (1). Management of the disease is difficult, and mortality of the patients with severe (grade IV) hepatic encephalopathy is still ~ 80% (2, 3). Liver regeneration is vital to survival of the patients, but nothing is known about the factors controlling this process in the patients. In experimental animals, however, evidence for the presence of a humoral control system for liver regeneration has been reported (4–10), and factors have been isolated (11–17). Recently, Díaz-Gil et al. (18) have purified a factor from plasma of partially hepatectomized rats, and Nakamura et al. (19) have reported purification of a hepatocyte growth factor from rat platelets. These purified factors showed a single band with molecular weights of 64,000 (18) and 27,000 (19) on SDS-polyacrylamide gels, but no evidence was given which confirmed that either of these single bands was the factor itself rather than a contaminant. Hepatocyte growth stimulating factors were also isolated from weanling and regenerating rat liver (20–23) and regenerating dog liver (24, 25).

We found recently that serum and plasma from patients with fulminant hepatic failure with grade III to IV coma markedly stimulates growth of adult rat hepatocytes in primary culture (26, 27), and have partially purified a growth-stimulating factor, human hepatocyte growth factor (hHGF),[1] from a patient's plasma. It is a heat-labile protein with a molecular weight of ~ 85,000 (27). This paper reports the purification and partial characterization of this hHGF.

Methods

Materials. Collagenase (type I) and insulin were purchased from Sigma Chemical Co., St. Louis, MO. Fetal calf serum was obtained from Filtron, Altona, Australia. Affi-Gel Blue and hydroxylapatite were from Nippon Bio-Rad Laboratories, Tokyo, and heparin-Sepharose CL-6B and molecular weight standards for SDS-PAGE were from Pharmacia Japan Ltd., Tokyo. [Me-^{3}H]Thymidine (78.0 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Mouse epidermal growth factor (mEGF) was from Wako Pure Chemical Industries, Osaka, and human epidermal growth factor (hEGF) was from Wakunaga Pharmaceutical Co., Osaka. Plasma was obtained from a patient with fulminant hepatic failure due to hepatitis B (no. 12 described in the previous paper [27]) during plasma exchange therapy and was stored at −20°C. Plasma obtained from another patient, no. 13, with non-A, non-B hepatitis gave the same results.

Purification of hHGF from patient's plasma. The first three steps in the purification procedure were essentially the same as described previously (27). A sample of 930 ml of plasma from the patient was subjected to heat treatment and ammonium sulfate fractionation as described (27). The precipitate formed between 1.15 and 2.05 M ammonium sulfate was dissolved in 300 ml of Ca^{2+},Mg^{2+}-free phosphate-buffered saline [PBS(−)], dialyzed for 36 h against three changes of 4 liters of PBS(−), and applied to a column (3.9 × 11 cm) of Affi-Gel Blue, equilibrated with PBS(−), at a flow rate of 120 ml/h. The column was washed successively with 250 ml of PBS(−) and 250 ml of 1.4 M NaCl in PBS(−) (pH 7.4). The activity of hHGF was then eluted with 350 ml of 2 M guanidine-HCl (pH 7.4) at 300 ml/h, and the eluate was dialyzed for 72 h against at least five changes of 4 liters of PBS(−).

Concentrated Triton X-100 solution was added to the dialyzed preparation at a final concentration of 0.013%, and the mixture was applied to a heparin-Sepharose column (1.6 × 5 cm), equilibrated with 0.013% Triton X-100 in PBS(−), at a flow rate of 25 ml/h. The column was washed with 75 ml of 0.013% Triton X-100 in PBS(−) and then with PBS(−) containing 0.5 M NaCl and 0.013% Triton X-100 (pH 7.4), until the absorbance of the eluate at 280 nm reached 0.3. (This

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1. *Abbreviations used in this paper:* hEGF, human epidermal growth factor; HGF, hepatocyte growth factor; hHGF, human hepatocyte growth factor; mEGF, mouse epidermal growth factor; PBS(−), (Dulbecco's) Ca^{2+},Mg^{2+}-free phosphate-buffered saline.

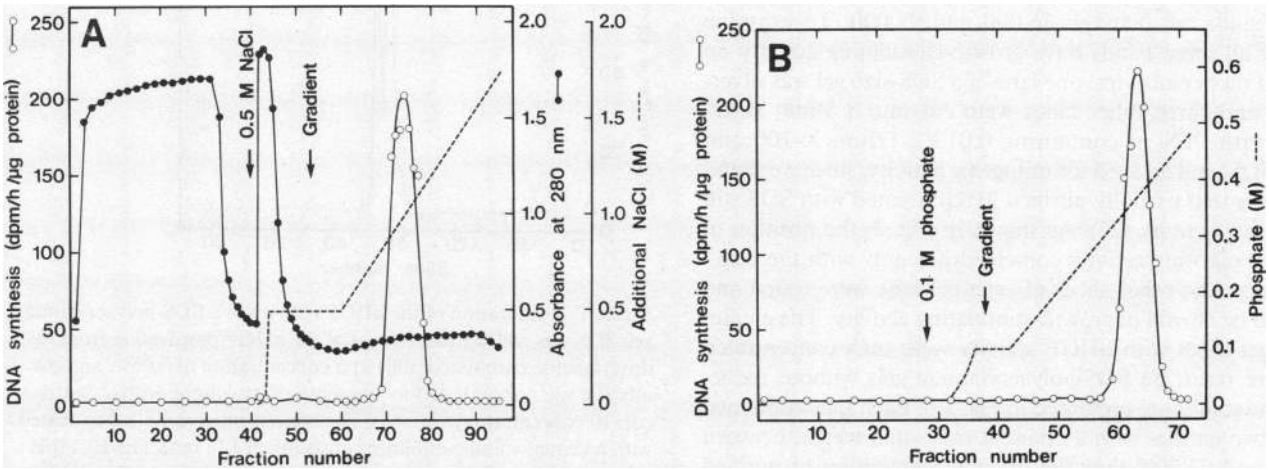
concentration of Triton X-100 in PBS(−) had an absorbance of 0.21 or greater depending on salt concentration.) The growth-promoting activity was then eluted with a linear salt gradient of 0.5–1.75 M NaCl in PBS(−) containing 0.013% Triton X-100 (pH 7.4, total gradient volume, 80 ml). A single peak of the growth-stimulating activity was eluted at ~ 1 M NaCl.

Peak fractions from the heparin-Sepharose column were pooled, diluted with an equal volume of 0.013% Triton X-100 in PBS(−), and applied to a hydroxylapatite column (1.6 × 5 cm), equilibrated with 0.013% Triton X-100 in PBS(−) at a flow rate of 20 ml/h. The column was washed with 20 ml of the same buffer and with 20 ml of 0.1 M sodium phosphate buffer (pH 7.1) containing 0.15 M NaCl and 0.013% Triton X-100. The hHGF activity was then eluted with a linear gradient of 0.1–0.5 M sodium phosphate buffer (pH 7.1) containing 0.15 M NaCl and 0.013% Triton X-100 (total gradient volume, 60 ml) at room temperature, and 2-ml fractions were collected. All hHGF activity bound to the column, and there was only one peak of activity eluting between 0.31 and 0.42 M sodium phosphate. Fractions with the highest activity were pooled, passed through a Millex-GV (Millipore Corp., Bedford, MA) filter and kept frozen at −20°C.

Primary culture of parenchymal cells from adult rat liver. Parenchymal liver cells were isolated from adult male Wistar rats, weighing ~ 200 g, by the method of Seglen (28). The cells were plated in Nunc 24-well plastic dishes, unless stated otherwise, at a density of 5×10^4 cells/0.2 ml per cm^2 and were cultured as monolayers at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, essentially as described by Tanaka et al. (29). The culture medium was Williams medium E, supplemented with 5% fetal calf serum, 1 μM dexamethasone, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (basal medium). The medium was changed after the first 4 h and then once a day. Samples were added to the basal medium at the first medium change (4 h after seeding) and thereafter at each medium change.

Determination of DNA synthesis and autoradiography. The rate of DNA synthesis was determined by pulse-labeling cultured cells with [^{3}H]thymidine (4 μCi/ml, 2 Ci/mmol) at 37°C for 2 h between 40 and 42 h after seeding in the presence and absence of 10 mM hydroxyurea as described previously (27). Activity of replicating DNA synthesis was calculated as the difference between the values with and without hydroxyurea. Protein was assayed by the method of Lowry et al. (30) with crystalline bovine serum albumin as standard. For autoradiography, hepatocytes cultured on Lux Thermanox (Miles Laboratories, Inc., Naperville, IL) coverslips were labeled with [^{3}H]thymidine (4 μCi/ml, 2 Ci/mmol) between 25 and 50 h after seeding. Autoradiograms of the hepatocytes were prepared as described previously (26), except that the cells were fixed in PBS(−)/methanol/glacial acetic acid (4:3:1 by vol) and then in methanol/glacial acetic acid (3:1 by vol) (31). At least 250 cells in randomly selected fields of a coverslip were examined to determine the labeling index, which was expressed as the percentage of the nuclei heavily labeled with [^{3}H]thymidine.

SDS-PAGE. SDS-PAGE was performed at room temperature by the method of Laemmli (32) using a 3% stacking gel and a 8% or 13.5% separating gel 1 mm thick. Samples were prepared by mixing with an equal volume of buffer consisting of 125 mM Tris-HCl (pH 6.8), 6% SDS, 20% glycerol (vol/vol), and 0.0025% bromphenol blue in the presence or absence of 1% 2-mercaptoethanol and boiled for 5 min before use, unless stated otherwise. In the case of reduced samples, they were further alkylated with iodoacetamide at a concentration three times greater than that of 2-mercaptoethanol to reduce artifact bands observed upon silver staining. After electrophoresis, gels were fixed in 10% trichloroacetic acid overnight (8% separating gel) or in methanol/glacial acetic acid/water (5:1:4 by vol) for at least 30 min followed by overnight washing in a solution consisting of 5% methanol and 7% acetic acid in water (13.5% separating gel) (33). The gels were silver-stained by the method of Wray et al. (34). Molecular weight standards used for SDS-PAGE were phosphorylase b (94,000 mol wt), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).



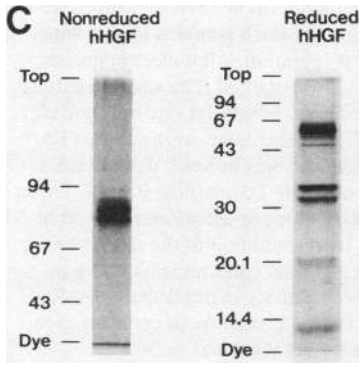


Figure 1. Purification of hHGF and SDS-PAGE of purified hHGF. (A) Elution profile of hHGF activity from a heparin-Sepharose column during the purification. Fractions of 10 ml were collected during sample loading and column washing with PBS(−) containing 0.013% Triton X-100, and fractions of 4 ml were collected during column washing with PBS(−) containing 0.5 M NaCl and 0.013% Triton X-100, and fractions of 2 ml were collected during gradient elution. (*B*) The elution profile of hHGF activity from a hydroxylapatite column during the purification. Fractions of 2 ml were collected during sample loading, column washing, and gradient elution. Absorbance at 280 nm is not given because of the UV absorbance of Triton X-100. For assay of activity, fractions were added to the medium to yield final concentrations of 0.42% by vol for *A* and 0.5% by vol for *B*. Each point represents the mean activity in duplicate wells. (*C*) SDS-PAGE of purified hHGF. The purified hHGF preparations nonreduced (79 ng of protein) and reduced (70 ng of protein) were subjected to SDS-PAGE using 8% and 13.5% separating gels, respectively. Molecular size standards (in kilodaltons) are also indicated.

Table I. Purification of hHGF

Step	Total protein*	C_{50}‡	Total activity§	Specific activity	Purification	Yield
	mg	*μg/ml*	*units × 10⁻⁴*	*units/mg*		%
Heated plasma	42,900	465	24.3	5.66	1	100
Ammonium sulfate	14,800	286	13.7	9.20	1.6	56.2
Affi-Gel Blue	910	30.0	7.98	87.7	16	32.8
Heparin-Sepharose	0.730	0.0398	4.83	66,100	11,700	19.9
Hydroxylapatite	0.0366	0.00223	4.32	1,180,000	209,000	17.8

* Protein was determined by a modification (35) of the method of Lowry et al. (30), using crystalline bovine serum albumin as a standard. ‡ The protein concentration of a hHGF preparation required for 50% of the maximal response to mEGF. § One unit of activity is defined as the quantity of hHGF required for 50% of the maximal response to mEGF in the assay system described.

Results

The purification procedure described here consists of five steps. The elution profiles of hHGF activity from columns of heparin-Sepharose and hydroxylapatite are shown in Fig. 1, *A* and *B*, respectively. All hHGF activity bound to both columns, and only one peak of activity was observed to elute in each case. Results of a typical purification are summarized in Table I. Starting with 930 ml of plasma from a patient, we obtained 37 μg of hHGF, respresenting a 209,000-fold purification and a 18% yield. The most effective step was heparin-Sepharose chromatography which produced a 750-fold increase in the specific activity.

The final preparation revealed several bands detectable with silver stain after SDS-PAGE under nonreducing conditions (Figs. 1 *C* and 2). The two major bands had molecular weights of 79,000 and 86,000 while the molecular weight of the minor bands fell between 76,000 and 92,000. To examine whether all these bands have growth-stimulating activity on cultured rat hepatocytes, one lane of a SDS-slab gel was silver-stained and three other lanes were cut into 1.5-mm slices, eluted with PBS(−) containing 0.013% Triton X-100 and 0.02% SDS, and assayed for mitogenic activity, since we found previosuly that partially purified hHGF treated with SDS still retained its activity (27). As shown in Fig. 2, the position of growth-promoting activity coincided precisely with the staining bands. The other slices of each gel lane were tested and found to be devoid of growth-stimulating activity. The eluates of the gel slices with hHGF activity were then concentrated and were rerun on SDS-polyacrylamide gels without reduction. The results are presented in Fig. 3 *A*. Each slice contained one or two entities with a different molecular weight between 76,000 and 92,000; thus the original preparation of purified hHGF consisted of at least seven different molecular weight entities. The potency of the growth-promoting activity of the eluates paralleled the darkness of stained bands. These results suggest that all bands are different molecular weight forms of hHGF rather than contaminants.

When purified hHGF was reduced with 2-mercaptoethanol and subjected to SDS-PAGE, one major group of bands with molecular weight between 54,000 and 65,000, two major bands with molecular weights of 31,500 and 34,500, and at least three minor bands with molecular weights of 48,000, 21,000, and 13,000 were detected on silver staining (Fig. 1 *C*). The eluted and concentrated fractions from the slices of nonreduced SDS-gels described above were rerun on SDS-gels after reduction. As presented in Fig. 3 *B*, the eluates of all slices (nos. 29–34) with hHGF activity showed two groups of bands with molecular weights of 54,000–65,000 and 31,500–34,500. Judging from both this result and the above finding that molecular weights of nonreduced hHGF were in the range of

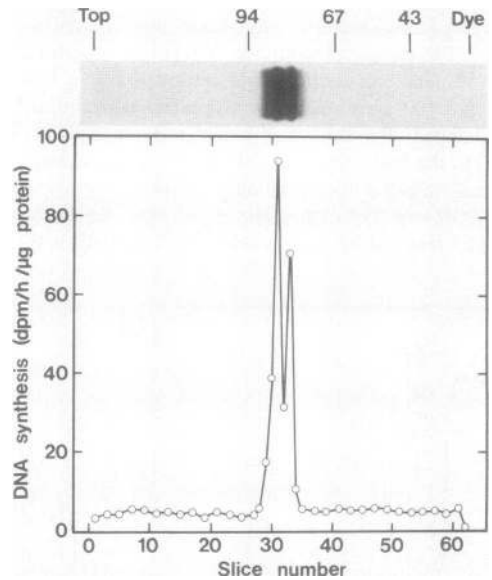


Figure 2. Localization of the hHGF activity in a SDS-polyacrylamide gel. SDS was added to an aliquot of the hHGF preparation from hydroxylapatite chromatography at a concentration of 0.05%, and the mixture was concentrated to one-tenth of its volume with a Centricon-10 concentrator (Amicon). The concentrated sample was treated with an equal volume of buffer consisting of 125 mM Tris-HCl (pH 6.8), 6% SDS, 20% glycerol (vol/vol) and 0.0025% bromphenol blue in the absence of 2-mercaptoethanol for 1 h at 25°C and subjected to SDS-PAGE using an 8% separating gel. Each lane was loaded with 20 μl of the concentrated hHGF preparation. After electrophoresis, one lane of the separating gel was silver-stained. The staining pattern is shown in the upper part of the figure. Molecular size standards (in kilodaltons) are also indicated. Three other lanes were cut into 1.5-mm slices with a razor blade. Each slice was minced, placed into a test tube, and incubated at 1 ml of PBS(−) containing 0.013% Triton X-100 and 0.02% SDS with shaking at room temperature for 20 h. hHGF activity was then assayed by the addition of the eluates to the medium of cultured hepatocytes at a final concentration of 5% by vol. Each point represents the mean activity in duplicate wells. Recovery of the hHGF activity during the procedure of concentration, electrophoresis and elution from the gel slices was ~ 30%.

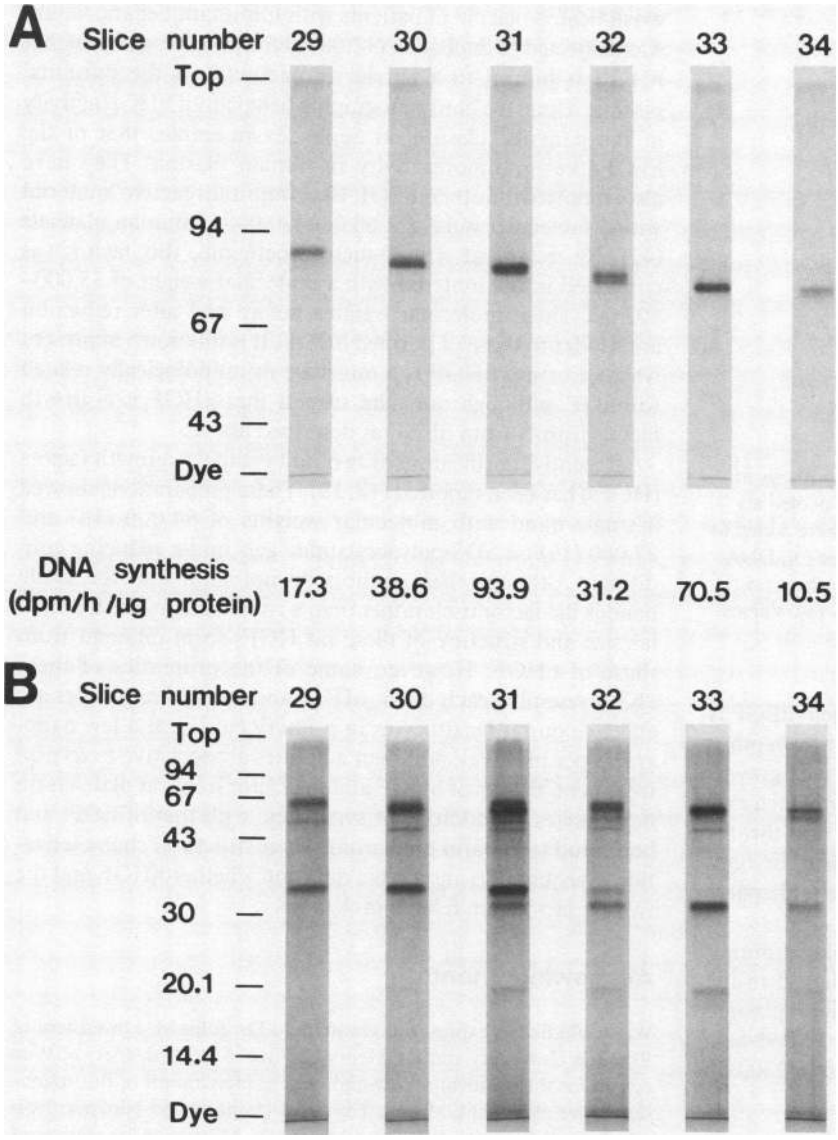


Figure 3. Electrophoretic analysis of hHGF bands eluted from the SDS-polyacrylamide gel described in Fig. 2. A 0.75 ml portion of each eluate from gel slices 29–34 described in Fig. 2 was concentrated with a Centricon-10. After addition of 2 ml of 20 mM Tris-HCl (pH 6.8) containing 0.15 M NaCl, 0.013% Triton X-100, and 0.05% SDS, the solutions were concentrated again to 0.2 ml. Aliquots (20 μl) of the concentrated eluates were used as loading samples. (*A*) Silver stain of nonreduced gels (8% separating gel). DNA synthesis in hepatocytes cultured with 5% by vol of unconcentrated eluates from gel slices is also shown. (*B*) Silver stain of reduced gels (13.5% separating gel). Molecular size standards (in kilodaltons) are indicated at left.

76,000–92,000, hHGF appears to consist of the two chains which are linked together by disulfide bonds. At least four different heavy chains appear to exist. In the case of the light chain, however, only two different sized chains were detected (Fig. 3 *B*). Since two other minor bands with molecular weights of 48,000 and 21,000 were also detected in some lanes, these components also seem to be part of the nonreduced entities with molecular weights between 76,000 and 92,000.

Fig. 4 shows the dose-response curve of DNA synthesis in rat hepatocytes as a function of purified hHGF. Half-maximal stimulation of DNA synthesis was observed at 3.5 ng/ml.

The growth-promoting activity of hHGF on hepatocytes was compared with that of other growth factors. Both DNA synthesis and labeling index were determined. As shown in Table II, hHGF stimulated proliferation of cultured hepatocytes more effectively than hEGF or insulin. Since molecular weights of hHGF and hEGF are 76,000–92,000 and 6,200 (36), respectively, on a molar basis hHGF stimulated DNA synthesis in hepatocytes at less than one-tenth the concentration of hEGF. Effects of combinations of optimal concentrations of hHGF, hEGF, and insulin on hepatocyte growth are also presented in Table II. The effect of hHGF was additive with the maximal effect of hEGF and synergistic with that of insulin. Labeling index of cultures with these three growth factors was 85%, showing that most hepatocytes were induced to proliferate. Hepatocytes whose DNA synthesis was stimulated by hHGF entered the mitotic phase and the number of nuclei increased significantly (data not shown).

Discussion

Results shown in Figs. 1 *C*, 2, and 3 strongly suggest that hHGF shows multiple forms with molecular weight between 76,000 and 92,000 and consists of the two chains with molecular weight of 54,000–65,000 and 31,500–34,500 which are linked together by disulfide bonds. Thus, molecular weights of hHGF and its components are much higher than that of hEGF. hHGF stimulated DNA synthesis in rat hepatocytes at less than one-tenth the concentration of hEGF on a molar basis (Fig. 4 and Table II). Moreover, the effect of hHGF was additive with the maximal effect of hEGF (Table II). Therefore, hHGF is probably a growth factor different from hEGF. This is supported by our recent finding that hHGF did not stimulate DNA synthesis in non-dividing confluent Swiss 3T3

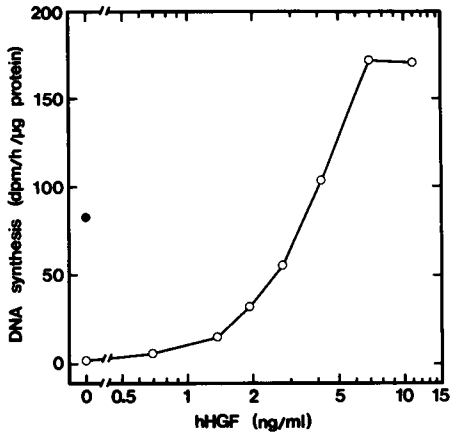


Figure 4. Dose-response curve for stimulation of DNA synthesis in cultured rat hepatocytes by hHGF. Various doses of the pooled active fractions of hHGF from a hydroxylapatite column were added to cultured hepatocytes, and DNA synthesis was determined (○). DNA synthesis in hepatocytes cultured in the basal medium with an optimal concentration of mEGF (25 ng/ml) are also shown (●). Values are means for duplicate wells.

cells (Gohda et al., unpublished observations), which mEGF is known to enhance (37). hHGF seems also different from other well-known growth factors, including platelet-derived growth factor, fibroblast growth factor, insulinlike growth factors I and II, transforming growth factor-β, transferrin, and thrombin, because these factors inhibit, do not stimulate, or only slightly stimulate DNA synthesis in cultured adult rat hepatocytes (12, 19, 38–40).

Kurobe et al. (41) have recently found hEGF-like immunoreactivity with high molecular weight, which emerged in the void volume of a Sephadex G-100 column, in human plasma. As we reported previously (27), hHGF activity eluted at a position corresponding to a molecular weight of ~ 230,000 when heated plasma of patients with fulminant hepatic failure was subjected to Sephadex G-200 gel filtration, suggesting that hHGF is bound to a carrier molecule(s) in the patients' plasma. Thus, the chromatographic behavior of hHGF activity in the patients' plasma on Sephadex resembles that of the hEGF-like immunoreactivity in human plasma. They have also reported another hEGF-like immunoreactive material with a molecular weight of 60,000–70,000 in human platelets (42). By treatment with 2-mercaptoethanol, this factor was converted into a material with a molecular weight of 35,000–40,000. These molecular weights before and after reduction are similar to those of purified hHGF. It is unknown at present whether or not hHGF is a molecule immunologically related to hEGF, although our data suggest that hHGF is a growth factor distinct from hEGF as described above.

Recently, purification of two rat hepatocyte growth factors (HGFs) has been reported (18, 19). These preparations showed a single band with molecular weights of 64,000 (18) and 27,000 (19) on SDS-polyacrylamide gels under reducing conditions. Although these authors do not show that the single band is the factor itself rather than a contaminant, the molecular size and structure of these rat HGFs seem different from those of hHGF. However, some of the properties of these HGFs resemble each other. hHGF and the two rat factors are effective on rat hepatocytes in primary culture at a few nanograms per milliliter, and their activities are sensitive to trypsin treatment. Effects of hHGF and the factor from rat platelets on hepatocytes are additive or synergistic with that of EGF, and both bind to heparin-Sepharose. Their structural characterization is required to answer the question whether hHGF and the two rat factors are related molecules.

Table II. Effects of Insulin, hHGF, and hEGF on DNA Synthesis and Labeling Index of Cultured Hepatocytes

Addition to the basal medium	Concentration	DNA synthesis	Labeling index*
	ng/ml	*dpm/h per μg protein*	%
None	—	1.7±0.4‡	<0.2‡
Insulin	600	7.8±0.8	4.1
hEGF	2.1	42.7±3.3	9.2
	6.3	175.4±3.3	24.7±7.7
	9.4	197.2±3.1	31.7±3.4
hHGF	8.3	297.9±19.4	50.1±1.2
hHGF + hEGF	8.3 + 6.3	578.9±47.2	66.8
	8.3 + 9.4	569.1±3.2	64.0
Insulin + hEGF	600 + 6.3	503.9±6.5	63.1±5.6
Insulin + hHGF	600 + 8.3	461.5±21.6	77.9±5.3
Insulin + hHGF + hEGF	600 + 8.3 + 6.3	684.0±41.0	85.0±4.0

* Linbro 12-well plastic dishes were used for culture of hepatocytes instead of Nunc dishes.
‡ Values are means±SD for triplicate wells or means for duplicate wells.

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