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Effect of Hepatocyte Growth Factor/Scatter Factor on Lipogenesis in Adult Rat Hepatocytes in Primary Culture[1]

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Hepatocyte growth factor (HGF)/scatter factor is known to be the most potent mitogen for hepatocytes. In this paper, we report that lipogenesis in primary cultured rat hepatocytes treated with 10 ng/ml of recombinant human HGF (rhHGF) for 24 h was stimulated, as measured by the incorporation of ^3H_2O into long-chain fatty acids, to more than twice as much as the control. Insulin (0.1 μM) was more effective than rhHGF but rhHGF did not show an additive or synergistic effect when added to insulin. We also showed that treatment with rhHGF increased the activities of glucose-6-phosphate dehydrogenase (G6PDH) and malic enzyme, key enzymes which supply NADPH for lipogenesis, and acetyl-CoA carboxylase, the rate-limiting enzyme of lipogenesis. The increase in G6PDH and acetyl-CoA carboxylase activities was accompanied by increases in the levels of mRNA for the enzymes. These results suggest that HGF is involved in liver regeneration not only by stimulation of cell proliferation but also by acceleration of differentiation of hepatocytes.

Key words: acetyl-CoA carboxylase, cell differentiation, hepatocyte, hepatocyte growth factor/scatter factor, lipogenesis.

Human hepatocyte growth factor (hHGF) was initially found in the plasma or sera of patients with fulminant hepatic failure (*1*) and was purified from the plasma of such patients as a hepatotrophic factor (*2*). Active hHGF is a heterodimer of about 90 kDa (*2*). Analysis of cDNA for hHGF which has been cloned from a human placental cDNA library revealed that the two peptide chains of active hHGF are derived from an inactive single-chain peptide precursor (pro-hHGF) encoded in a single mRNA (*3*). We have shown that recombinant hHGF (rhHGF) prepared from the culture supernatant of Chinese hamster ovary (CHO) cells transfected with hHGF cDNA (*4*) is a powerful mitogen for both rat and human hepatocytes *in vitro*, like native hHGF purified from plasma of patients with fulminant hepatic failure (*5*).

hHGF is now known to be the same molecule as scatter factor (*6*) and tumor cytotoxic factor (*7*), to be a broad-spectrum and multi-functional cytokine, and to be the specific ligand for the c-met receptor (for reviews, see book Ref. *8*). It is also thought to be involved in liver regeneration following liver injury such as hepatitis or liver resection based on the following observations (for review, see Ref. *9*). (1) Plasma or serum levels of hHGF in patients with fulminant hepatic failure were far higher than those in normal subjects. These levels showed a significant correlation with the clinical grade of hepatic encephalopathy (coma), a parameter of hepatic dysfunction (*10*). (2) The high plasma levels of hHGF in patients with fulminant hepatic failure rapidly declined to near-normal levels when the patients recovered from the disease (*10, 11*). (3) hHGF levels in plasma or sera in patients with liver diseases other than fulminant hepatic failure were also found to be significantly higher than those in normal subjects whose plasma hHGF concentration was as low as 0.2 ng/ml (*12*). (4) rhHGF also stimulates proliferation of human intrahepatic biliary epithelial cells (hBEC) *in vitro* (*13*). In addition, Ishii *et al.* (*14*) recently reported that administration of rhHGF to normal rats *in vivo* increased the labeling index of hepatocytes nearly 6-fold and that, when given to partially hepatectomized rats, rhHGF stimulated the regeneration rate of hepatocytes and liver weight. We have also recently shown that normal human serum contains an active hHGF-activator, serine protease(s), which catalyze(s) proteolytic processing of inactive pro-hHGF to form active hHGF heterodimer, and that plasma from patients with liver diseases such as fulminant hepatic failure contains significant amounts of pro-hHGF as well as active hHGF (*15*). In experimental animals, HGF activity in the liver and plasma was also shown to increase markedly before liver regeneration peaked, in rats subjected to

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Abbreviations: ACC, acetyl-CoA carboxylase; C/EBP, CCAAT/enhancer-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; hBEC, human intrahepatic biliary epithelial cells; HGF, hepatocyte growth factor; hHGF, human HGF; IRE, insulin-responsive element; NF-IL6, nuclear factor for IL-6 expression; PBS, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline; PCR, polymerase chain reaction; rhHGF, recombinant hHGF.

partial hepatectomy (*16*), and in the blood of mice with hepatic injury induced by administration of a hepatotoxin such as carbon tetrachloride (*17*).

Regeneration of tissues requires two steps: proliferation of the cells and differentiation of immature cells to express the phenotype of the cells. There are a number of reports regarding the effect of HGF in stimulating the proliferation of hepatocytes (for review, see Ref. *9*) but little is known about the effect of HGF on differentiation of the cells. In this regard, Takehara *et al.* (*18*) reported that HGF stimulates albumin synthesis by rat hepatocytes in primary culture, and Strain *et al.* (*19*) showed that hBEC cultured with rhHGF maintain expression of hBEC phenotypic markers for several passages. We have shown that the rates of synthesis and gene expression of rat fetuin, which is the rat counterpart of human α_2-HS glycoprotein and a negative acute reactant of hepatocytes, are stimulated by rhHGF *in vitro* and *in vivo* (*20*). In addition, Ishii *et al.* (*14*) recently reported that administration of rhHGF to normal and partially hepatectomized rats increases the levels of blood proteins such as albumin and blood coagulation factors. They also showed that rhHGF stimulates gene expression of albumin in normal rat liver *in vivo* (*14*). These observations suggest that HGF is not only a mitogen to hepatocytes, but also an activator of cell differentiation.

In this paper, we report that rhHGF stimulates lipogenesis, one of the most important functions of the liver, in rat hepatocytes in primary culture. We also describe the stimulation by rhHGF of gene expression of some key enzymes involved in lipogenesis.

MATERIALS AND METHODS

Materials—[α-^{32}P]dCTP (111 TBq/mmol), [^{3}H]H_2O (925 MBq/ml), and [^{14}C]sodium bicarbonate (0.21 GBq/mmol) were purchased from Du Pont-New England Nuclear, Boston, MA; bovine insulin, NADP and acetyl-CoA (sodium salt) were from Sigma Chemical, St. Louis, MO; dithiothreitol was from Nacalai Tesque, Kyoto; size markers of RNA (RNA ladder) were from Bethesda Research Laboratories Life Technologies, Gaithersburg, MD; Hybond-N$^+$ nylon hybridization membrane, multiprime DNA labeling system and rapid hybridization buffer were from Amersham-Japan, Tokyo; Taq DNA polymerase [EC 2.7.7.7] was from Boehringer Mannheim GmbH, Mannheim, Germany; X-ray film for autoradiography was from Konica, Tokyo. Recombinant hHGF (rhHGF) was prepared and purified as described previously (*4*). cDNAs for rat glucose-6-phosphate dehydrogenase (G6PDH) [EC 1.1.1.49] (*21*) and rat acetyl-CoA carboxylase [EC 6.4.1.2] (*22*) were generously supplied by Dr. Nobuko Iritani, Tezukayama Gakuin, Osaka. cDNA for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was kindly supplied by Dr. S. Sakiyama, Chiba Cancer Center Research Institute and Hospital, Chiba. Other materials used for preparation of adult rat hepatocytes, electrophoresis and Northern blot analysis were as described elsewhere (*2, 20, 23*).

Preparation of Primary Cultures of Parenchymal Cells from Adult Rat Liver—Male rats of the Wistar strain, weighing 150-200 g, were maintained with laboratory chow and water *ad libitum*, and hepatocytes in primary culture were prepared as described previously (*2*). Unless otherwise mentioned, the cells were suspended at a density of 7.5×10^5 cells/ml of Williams medium E containing 5% fetal calf serum, 1 μM dexamethasone, and 100 μg/ml of kanamycin sulfate (basal medium), and plated into 6-well plastic plates (Nunc A/S, Roskilde, Denmark) at 2.0 ml/well (1.5×10^5 cells/0.2 ml/cm^2). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air, and the medium was changed to basal medium containing 20 mM glucose 4 h after seeding because the rate of lipogenesis in rat liver is reported to reach a maximum at 15-20 mM glucose in perfusate (*24*) and, because the periportal blood level of glucose in rats was more than 10 mM 1 h after the end of the 3-hour feeding schedule (*24*). The cells were incubated for 24 h, and the medium was then changed to the same medium with or without rhHGF or insulin, or both, at the concentrations indicated and further incubated for the time indicated.

Determination of the Rate of Lipogenesis in Hepatocytes in Culture—The rate of *de novo* synthesis of fatty acids was determined by pulse-labeling cultured cells with ^3H_2O (18.5 MBq/ml) (*25-27*), and the cells were incubated for 2 h. The cells were washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) and kept in 1 ml of water at −20°C overnight. Total lipids were extracted by the method of Bligh and Dyer (*28*) with a mixture of chloroform-methanol (1 : 1, v/v), and the total lipids were saponified at 80°C for 2 h with 5 N NaOH (*26*). The solution was diluted with water and acidified with 10 N H_2SO_4. Long-chain fatty acids were then extracted with petroleum ether (b.p. 40-60°C), the petroleum ether layer was washed twice with 5 ml of water, and the radioactivity was measured by a liquid scintillation counter (model LSC-903S, Aloka, Tokyo). In order to determine the specific radioactivity of ^3H_2O in the medium, a sample of 1 μl of the medium was withdrawn 1 h after the addition of radioactive water.

Assays of Enzyme Activities—The cells were harvested with a rubber policeman in 1 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM dithiothreitol and 5 mM EDTA (*29*) for assay of G6PDH, or in 0.5 ml of 50 mM Tris-HCl (pH 7.4) containing 0.1 mM EDTA and 0.25 M sucrose (*30*) for malate dehydrogenase (oxaloacetate-decarboxylating) ($NADP^+$) [EC 1.1.1.40] (so-called "malic enzyme"). The cells were homogenized with a hand-held micro-homogenizer (Physcotron, model NS-310E, Niti-on Irika, Tokyo) and centrifuged at $30,000\times g$ for 30 min, and the resulting supernatant was used to determine the enzyme activity by the methods of Deutsch (*31*) and Hsu and Lardy (*32*), for G6PDH and malic enzyme, respectively. One unit of enzyme activity was defined as 1 μmol of NADPH formed/min at 25°C and expressed as unit/mg cellular protein.

For assay of acetyl-CoA carboxylase, hepatocytes were cultured as above, harvested, and washed with 5 ml of 20 mM triethanolamine-HCl buffer (pH 7.4) containing 0.2 M sucrose, 1 mM dithiothreitol, and 1 mM EDTA (sucrose solution) (*33*), then collected by centrifugation. The cell pellet was suspended in 0.25 ml of the sucrose solution and homogenized with a hand-held micro-homogenizer as above. The homogenate was centrifuged at $100,000\times g$ for 30 min and the supernatant was used as an enzyme source of acetyl-CoA carboxylase. The enzyme activity was determined by measuring $H^{14}CO_3^-$ incorporation into malonyl-

CoA in the presence of 50 mM sodium citrate as described by Shafrir and Bierman (*34*). Two blank series of reaction mixtures without acetyl-CoA and with the sucrose solution instead of the supernatant were always run. Both blank series gave similar values, and the value was subtracted from the assay series.

Northern Blot Analysis—Hepatocytes were cultured in 8.5 cm plastic dishes (Nunc "100×20," 56.7 cm^2) at the same cell density as above with or without rhHGF, the total cellular RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform method (*35*) from 1.5×10^7 cells, and poly(A)$^+$ RNA was prepared using Oligotex-dT30 (super) (Daiichi Pure Chemicals, Tokyo) as described previously (*36*).

To analyze the expression of G6PDH mRNA, total cellular RNA was electrophoresed in 1% agarose gel in 2.2 M formaldehyde and transferred to a Hybond-N$^+$ nylon membrane as described previously (*37*). The membrane was then hybridized in rapid hybridization buffer with the cDNA for rat G6PDH labeled with [α-^{32}P]dCTP by using a multi-prime labeling system. To analyze the expression of acetyl-CoA carboxylase mRNA, poly(A)$^+$ RNA (3 μg per lane) was electrophoresed in 0.8% agarose gel in 2.2 M formaldehyde and transferred to nylon membrane as described above. The membrane was hybridized with cDNA for rat acetyl-CoA carboxylase labeled with [α-^{32}P]dCTP by the polymerase chain reaction (PCR) method. Labeling of the cDNA fragment of acetyl-CoA carboxylase by PCR was carried out essentially as described by Schowalter and Sommer (*38*). Briefly, plasmid pUC18 which contains a fragment of acetyl-CoA carboxylase [nucleotide number 6685-7010, (*22*)] was used as a template DNA and subjected to PCR amplification with Taq DNA polymerase using the sense strand (5′-TGTAAAACGACGGCCAGT-3′) and antisense strand (5′-GAAACAGCTATGACCATG-3′) primers. The reaction mixture contained 1.0 μM of each primer, 0.32 mM each of dATP, dGTP, and dTTP, 0.66 μM of [α-^{32}P]dCTP (111 TBq/mmol) and 2 U of Taq DNA polymerase in 50 μl of 10 mM Tris-HCl (pH 8.3) containing 50 mM KCl and 2.5 mM $MgCl_2$. The reaction was carried out with 30 cycles of denaturation for 45 s at 88°C, annealing for 1 min at 60°C, and extension for 2 min at 72°C, using a Thermal Cycler (model PHC-3, Techne, Duxford, Cambridge, UK). Unincorporated nucleotides were removed using a Quick Spin column (Boehringer), and the PCR product was fractionated in 2% agarose gel. The predicted band size (330 bp) was excised, extracted and used as the probe for Northern blot hybridization. The radioactivity on the hybridized membrane was determined either by autoradiography or with a Bioimaging analyzer (model BAS-1000, Fujifilm, Tokyo). Equal loading of the lanes were checked by re-hybridizing the membrane with ^{32}P-labeled GAPDH cDNA as described by Sambrook *et al.* (*39*).

Other Methods—Protein was measured by the method of Lowry *et al.* (*40*). Statistical evaluation was done by using Student's *t* test for unpaired samples to compare the mean values of groups.

RESULTS

When rat hepatocytes were treated with 10 ng/ml of rhHGF for 24 h, the rate of lipogenesis was stimulated to about 2 times the control (Table I). Insulin, a positive control, was more effective than HGF, but the HGF did not show an additive or a synergistic effect when added to insulin under the conditions we used. We obtained similar results in four other separate experiments (data not shown). Since we used ^3H_2O as a labeled precursor of fatty acid, one can calculate the amount of acetyl group incorporated into fatty acids from the values of hydrogen from

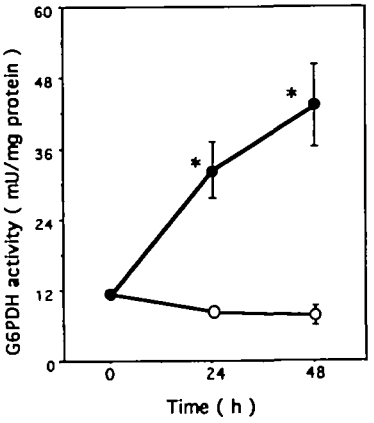

Fig. 1. **Changes in G6PDH activity after addition of rhHGF in rat hepatocytes in primary culture.** Cells (1.5×10^5/0.2 ml/cm^2) were incubated in 6-well plastic dishes with a basal medium containing 20 mM glucose for 24 h, then further incubated with (●) or without (○) 10 ng/ml rhHGF for the times indicated. After the end of the incubation, the cells were harvested and the G6PDH activity was determined as described in "MATERIALS AND METHODS." The points and bars show the means and SDs for triplicate wells. *$p<0.001$ *vs.* control (absence of rhHGF).

TABLE I. **Effects of rhHGF and insulin on the rate of lipogenesis and activity of G6PDH in rat hepatocytes in primary culture.** Hepatocytes cultured in a medium of 20 mM glucose for 24 h were further incubated with or without rhHGF or insulin, or with both at the concentrations indicated for 24 h. For measurement of the rate of lipogenesis, the cells were labeled with ^3H_2O for an additional 2 h as described in "MATERIALS AND METHODS." Amount of acetyl group incorporation into fatty acids was calculated from the specific radioactivity of the ^3H_2O of the medium and the radioactivity in fatty acids as described by Jungas (*25*). Activity of G6PDH at 0 time was 10.6±1.5 mU/mg protein ($n=6$). Data are expressed as mean±SD of 3 wells, and figures in parentheses are rate of stimulation (fold).

Addition (concentration)	Rate of lipogenesis		G6PDH activity
	^3H_2O incorporation (dpm/mg protein)	Acetyl group incorporation (nmol/mg protein/h)	(mU/mg protein)
None	182.4±39.2	4.87±0.84 (1.0)	9.8±1.6 (1.0)
rhHGF (10 ng/ml)	464.5±79.0	10.47±0.94[a] (2.15)	20.2±1.5[b] (2.1)
Insulin (0.1 μM)	1,047.0±193	27.18±3.27[b] (5.58)	29.8±2.6[b] (3.0)
rhHGF (10 ng/ml) + insulin (0.1 μM)	1,131.8±195	27.27±3.30[b] (5.60)	34.1±5.9[b] (3.5)

[a]$p<0.005$ and [b]$p<0.001$ *vs.* control (no addition).

^3H_2O incorporated into fatty acids using a formula originally reported by Jungas (*25*) (see also Ref. *26*) (Table I).

We next examined the effect of HGF on the activity of enzymes involved in lipogenesis. The activity of G6PDH, a key enzyme which supplies NADPH for lipogenesis, was found to increase to about twice the control level, when the cells were treated with 10 ng/ml of rhHGF for 24 h (Table I). Insulin was more effective in stimulating the activity than HGF. This stimulation of G6PDH activity by rhHGF was time-dependent and continuously increased up to 48 h (Fig. 1). The stimulation of G6PDH activity by rhHGF was also dose-dependent, and a significant increase was observed at 5 ng/ml (Fig. 2A). Because the activity of hepatic G6PDH is known to increase during liver regeneration in humans and experimental animals (*41-43*), we also measured the activity of malic enzyme, another key enzyme which supplies NADPH for lipogenesis, and found that it increased to about twice the control level, when the cells were treated with 10 ng/ml of rhHGF for 24 h (42.5 ± 3.4 and 21.3 ± 6.9 mU/mg protein with and without rhHGF, respectively, $p < 0.001$, $n = 3$). Insulin (0.1 μM) was more effective (58.7 ± 4.6 mU/mg protein) in stimulating malic enzyme activity than rhHGF, but the addition of rhHGF to insulin did not produce any additive or synergistic effect (data not shown). Because the results described above strongly suggested that HGF stimulated *de novo* synthesis of key enzymes of lipogenesis, we examined the effect of rhHGF on levels of mRNA for G6PDH in cells by Northern blot hybridization. As shown in Fig. 2B, levels of G6PDH mRNA (2.3 kb in size) (*21*) were increased dose-dependently by treatment with rhHGF. The intensities of bands measured by a densitometer and values normalized with respect to the intensity of the band of an internal control are plotted in Fig. 2A. The level of G6PDH mRNA increased virtually in proportion to the activity of the enzyme.

Finally, we examined the effect of rhHGF on the activity of acetyl-CoA carboxylase and on the levels of the mRNA for this enzyme, which is the rate-limiting enzyme in the biosynthesis of long-chain fatty acids. As shown in Fig. 3, hepatocytes treated with rhHGF at a concentration of 10 ng/ml stimulated the activity of acetyl-CoA carboxylase continuously up to 48 h of culture, and the level of stimula-

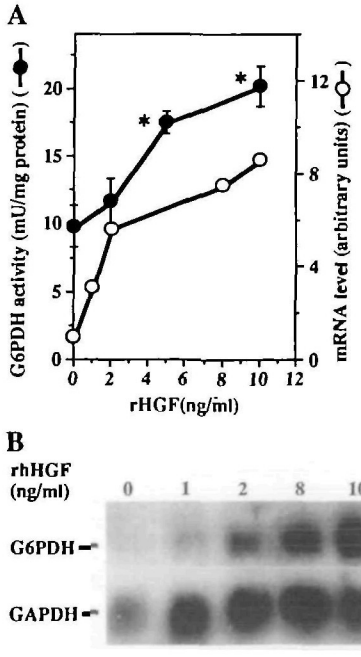

Fig 2 **Dose-response curves for stimulation of G6PDH activity (●) and G6PDH mRNA expression (○) by rhHGF in rat hepatocytes in primary culture.** Panel A: Cells were incubated with rhHGF at the concentrations indicated for 24 h. Other experimental conditions were the same as for Fig. 1. The points and bars show the means and SDs for triplicate wells. *$p < 0.001$ *vs.* control (without rhHGF). Panel B: The total cellular RNA (8 μg/lane) extracted from the cells incubated under the same conditions as in A, except in Nunc "100 × 20" (56.7 cm²) plastic dishes, was electrophoresed and blotted onto a nylon membrane, and the G6PDH mRNA (2.3 kb) was determined by Northern blotting with ^{32}P-labeled cDNA for rat G6PDH as a probe For quantitation of the mRNA level, the intensities of the bands of G6PDH mRNA were measured with a densitometer, and the relative values of G6PDH mRNA (2 3 kb) were normalized with respect to those for GAPDH mRNA (1 5 kb) and then plotted in A

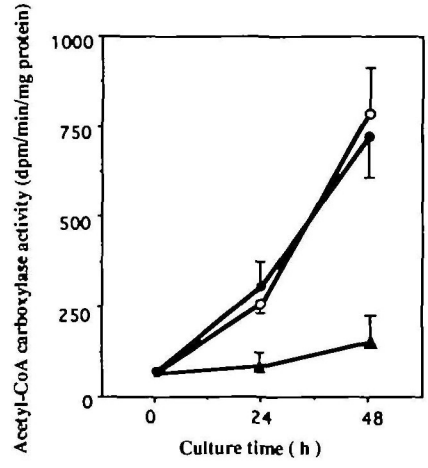

Fig. 3 **Effects of rhHGF and insulin on acetyl-CoA carboxylase activity in rat hepatocytes in primary culture.** Culture conditions were the same as in Fig. 1, except for the addition of 10 ng/ml rhHGF (●), 0 1 μM insulin (○), or none (▲) during the incubation period. The points and half bars show the means and SDs for triplicate wells

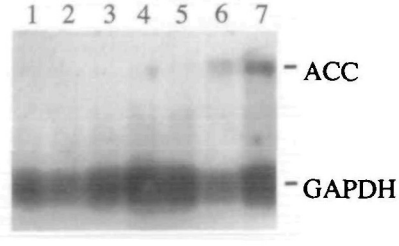

Fig. 4. **Effects of rhHGF and insulin on expression of mRNA for acetyl-CoA carboxylase in rat hepatocytes in primary culture.** Culture conditions were the same as in Fig. 2B, except for the incubation time and amounts added during the incubation period. Poly(A)$^+$RNA (3 μg/lane) extracted from cells without incubation (lane 1), or incubated for 24 h (lanes 2-4) or 48 h (lanes 5-7) were analyzed by Northern blot hybridization as described in "MATERIALS AND METHODS" Lanes 2 and 5: without addition; lanes 3 and 6: with 10 ng/ml rhHGF; lanes 4 and 7: with 0.1 μM insulin ACC: acetyl-CoA carboxylase mRNA (7.5 kb); GAPDH: glyceraldehyde-3-phosphate dehydrogenase mRNA (1.5 kb).

tion was about 2- and 5-fold that of the control (without rhHGF) at 24 and 48 h, respectively. Insulin, at a concentration of 0.1 μM showed almost the same effect as that of rhHGF (Fig. 3), but rhHGF did not show additive or synergistic effects when added to insulin (data not shown). The levels of mRNA for the enzyme (7.5 kb in size) (*22*) also increased time-dependently (Fig. 4). The intensities of the bands which had been normalized with respect to that of the internal control (GAPDH mRNA) increased virtually proportionally to the activity (5.4 and 7.9 fold with rhHGF and insulin for 48 h, respectively).

The results described above indicate that HGF stimulates lipogenesis in rat hepatocytes in primary culture by induction of key enzymes involved in lipogenesis and suggest that HGF is involved in liver regeneration not only by stimulation of cell proliferation, but also by stimulation of differentiation of hepatocytes.

DISCUSSION

The advantage of the use of labeled water (^2H_2O or ^3H_2O) to measure the rate of lipogenesis has already been discussed by Jungas (*25*) and others (*24, 26, 44*). In this study, we used tritium-labeled water as a precursor of fatty acids and found that the rate of lipogenesis in primary cultured rat hepatocytes was 4.87 nmol acetyl group incorporated into fatty acid/mg protein/h, corresponding to the value obtained in isolated rat liver cells by Nilsson *et al.* (*45*). The rate of lipogenesis in liver *in vivo*, however, is reported to be 69 μmol acetyl group incorporated into fatty acid/g liver/h (*44*) or 58 μmol/g dry wt of liver/h (*24*) in rats receiving a scheduled diet (3 h/day) consisting of laboratory chow. Assuming that the protein content of the liver is 23%, these values can be calculated to be 300 or 252 nmol acetyl group incorporated into fatty acid/mg protein/h, which is about 50 times higher than the value we obtained in this study. Although the feeding conditions for the rats we used and the *in vivo* studies described above were slightly different, the discrepancy between the values obtained *in vivo* and in cultured hepatocytes could be due to the difference in the environmental conditions of the cells, such as lower pO_2 than *in vivo* and hormonal status.

HGF is now known to be a multi-functional growth factor acting as a mitogen, motogen, and morphogen for various types of epithelial cells and is thought to be a physiological hepatotrophic factor for liver regeneration following liver injury in humans and experimental animals (*9*). In addition to the well known multiple biological activities of HGF, it has recently been shown that HGF stimulates differentiated functions of hepatocytes, such as synthesis of albumin, rat fetuin, and blood coagulation factors *in vivo* (*14, 18, 20*). In this study, we found that rhHGF induced key enzymes involved in lipogenesis, and stimulated the rate of lipogenesis, one of the most important differentiated functions of the liver, in rat hepatocytes in primary culture. As far as we know, this is the first report showing that HGF regulates a metabolic pathway consisting of several enzymes.

In contrast to HGF, epidermal growth factor (EGF), a potent hepatotrophic factor, suppressed insulin-stimulated lipogenesis, as measured by the incorporation of ^{14}C-acetate into triglyceride and phospholipid in rat hepatocytes in primary culture (*46*). Although the use of ^{14}C-acetate to measure the rate of lipogenesis is open to serious criticism as discussed by Jungas (*25*) and others (*24, 26, 44*), the above results may indicate that EGF stimulates only growth of hepatocytes, and may not be involved in differentiation of the cells. Yoshimoto *et al.* (*46*) also reported that G6PDH was induced dose-dependently by EGF with or without insulin in rat hepatocytes, but the induction of malic enzyme by insulin plus 3,3',5-triiodothyronine was strongly suppressed to near basal levels by the addition of EGF. Because hepatic G6PDH is known to increase in regenerative conditions in humans during hepatitis and cirrhosis (*41*) and in experimental animals after treatment with liver poisons such as carbon tetrachloride (*42*) and D-galactosamine (*42*), induction of G6PDH by EGF may participate in the synthesis of nucleic acid in growing cells. Therefore, the present and previous findings strongly suggest that HGF may participate in the process of liver regeneration, not only by stimulating the proliferation of hepatocytes but also by enhancing some impaired differentiated liver functions, such as the synthesis of blood proteins and lipogenesis.

Under the conditions we used, HGF (10 ng/ml) did not show an additive or a synergistic effect on stimulation of lipogenesis or induction of the key enzymes involved in lipogenesis when it was used with insulin (0.1 μM). However, this could be because 0.1 μM insulin stimulated the rate of lipogenesis and induction of the key enzymes to the maximum, in which case HGF could not show an additive effect. Therefore, it is possible that when the cells are treated with insulin at a lower concentration than 0.1 μM, HGF may show an additive or a synergistic effect, but further study is necessary to confirm this possibility.

Although the mechanism by which key enzymes involved in lipogenesis are induced by HGF is not clear at present, we previously showed that HGF stimulates the rate of synthesis and gene expression of rat fetuin, which is a negative acute phase reactant of hepatocytes *in vitro* and *in vivo* (*20*), and we suggested that HGF is likely to stimulate rat fetuin gene by inducing CCAAT/enhancer-binding protein-α (C/EBP-α) (*20*), which is postulated to stimulate the expression of a broad-spectrum of liver-specific genes of negative acute phase proteins (*47*) and is present in the promoter regions of rat fetuin (*48*) and other negative acute phase proteins such as albumin and transthyretin (*49*). Alternatively, HGF may suppress nuclear factor for IL-6 expression (NF-IL6), which is involved in the regulation of the gene expression of various positive and negative acute phase reactants in mouse liver (*49, 50*). In this regard, it is noteworthy that genes for G6PDH and acetyl-CoA carboxylase also contain the NF-IL6 binding sequence in their promoter regions (*51, 52*), whereas the C/EBP-α binding sequence is present only in that of G6PDH, but not in that of acetyl-CoA carboxylase. Preliminary results in our laboratory showed that C/EBP-α is not involved in the regulation of the gene expression of rat fetuin in hepatocytes in primary culture (Ohnishi *et al.*, unpublished observation). Therefore, HGF may stimulate the gene expression of these enzymes by only suppressing NF-IL6. On the other hand, there are many reports showing the presence of insulin-responsive element (IRE) sequences in some of the genes whose expression is regulated by insulin, such as acetyl-CoA carboxylase (*53*), phosphoenolpyruvate carboxykinase (*54*), and glyceraldehyde-

3-phosphate dehydrogenase (*55*), although the reported IRE sequences are somewhat variable and there is no consensus sequence. Therefore, it is possible that HGF stimulates the gene expression of G6PDH and acetyl-CoA carboxylase by regulating the activity of these IRE binding proteins, because the effects of HGF on such enzyme activity and the rate of lipogenesis do not increase when HGF is added to insulin, as we reported in this paper. The mechanism of the induction of gene expression of these enzymes by HGF is currently the subject of further investigation.

Liver regeneration requires two steps: (1) proliferation of hepatocytes and other surrounding cells, and (2) differentiation of proliferating cells into mature hepatocytes that express liver-specific functions. The mechanism of the overall regenerative process of liver is not fully understood at present. However, a wide variety of factors such as nutrients, hormones, neurotransmitters, and growth factors, are thought to regulate the regenerative process (*56, 57*). Although it is necessary to determine the effect of HGF on various liver-specific functions, such as glycolysis, gluconeogenesis, amino acid metabolism, and detoxication, the present and previous results suggest that HGF is a very important physiological hepatotrophic factor for liver regeneration because HGF stimulates proliferation and differentiation of hepatocytes *in vitro* and *in vivo*.

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REFERENCES

1. Nakayama, H., Tsubouchi, H., Gohda, E., Koura, M., Nagahama, J., Yoshida, H., Daikuhara, Y., and Hashimato, S. (1985) Stimulation of DNA synthesis in adult rat hepatocytes in primary culture by sera from patients with fulminant hepatic failure. *Biomed. Res.* **6**, 231-237
2. Gohda, E., Tsubouchi, H., Nakayama, H., Hirono, S., Sakiyama, O., Takahashi, K., Miyazaki, H., Hashimoto, S., and Daikuhara, Y. (1988) Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. *J. Clin. Invest.* **81**, 414-419
3. Miyazawa, K., Tsubouchi, H., Naka, D., Takahashi, K., Okigaki, M., Arakaki, N., Nakayama, H., Hirono, S., Sakiyama, O., Takahashi, K., Gohda, E., Daikuhara, Y., and Kitamura, N. (1989) Molecular cloning and sequence analysis of cDNA for human hepatocyte growth factor. *Biochem. Biophys. Res. Commun.* **163**, 967-973
4. Yoshiyama, Y., Arakaki, N., Naka, D., Takahashi, K., Hirono, S., Kondo, J., Nakayama, H., Gohda, E., Kitamura, N., Tsubouchi, H., Ishii, T., Hishida, T., and Daikuhara, Y. (1991) Identification of the N-terminal residue of the heavy chain of both native and recombinant human hepatocyte growth factor. *Biochem. Biophys. Res. Commun.* **175**, 660-667
5. Strain, A.J., Ismail, T., Tsubouchi, H., Arakaki, N., Hishida, T., Kitamura, N., Daikuhara, Y., and McMaster, P. (1991) Native and recombinant human hepatocyte growth factors are highly potent promoters of DNA synthesis in both human and rat hepatocytes. *J. Clin. Invest.* **87**, 1853-1857
6. Weidner, K.M., Arakaki, N., Hartmann, G., Vandekerckhove, J., Weingart, S., Rieder, H., Fonatsch, C., Tsubouchi, H., Hishida, T., Daikuhara, Y., and Birchmeier, W. (1991) Evidence for the identity of human scatter factor and human hepatocyte growth factor. *Proc. Natl. Acad. Sci. USA* **88**, 7001-7005
7. Shima, N., Nagao, O., Ogaki, F., Tsuda, E., Murakami, A., and Higashio, K. (1991) Tumor cytotoxic factor/hepatocyte growth factor from human fibroblasts: Cloning of its cDNA, purification and characterization of recombinant protein. *Biochem. Biophys. Res. Commun.* **180**, 1151-1158
8. Goldberg, I.D. and Rosen, E.M. (eds.) (1993) *Hepatocyte Growth Factor-Scatter Factor and the c-Met Receptor*, pp. 1-397, Birkhäuser Verlag, Basel
9. Tsubouchi, H., Gohda, E., Strain, A.J., and Daikuhara, Y. (1993) The role of HGF-SF in animal and human hepatic physiology and pathology in *Hepatocyte Growth Factor-Scatter Factor and the c-Met Receptor* (Goldberg, I.D. and Rosen, E.M., eds.) pp. 251-274, Birkhäuser Verlag, Basel
10. Tsubouchi, H., Hirono, S., Gohda, E., Nakayama, H., Takahashi, K., Sakiyama, O., Miyazaki, H., Sugihara, J., Tomita, E., Muto, Y., Daikuhara, Y., and Hashimoto, S. (1989) Clinical significance of human hepatocyte growth factor in blood from patients with fulminant hepatic failure. *Hepatology* **9**, 875-881
11. Tsubouchi, H., Hirono, S., Gohda, E., Nakayama, H., Takahashi, K., Sakiyama, O., Kimoto, M., Kawakami, S., Miyoshi, H., Kubozono, O., Kawarada, Y., Mizumoto, R., Arakaki, N., Daikuhara, Y., and Hashimoto, S. (1991) Human hepatocyte growth factor in blood of patients with fulminant hepatic failure. I. Clinical aspects. *Dig. Dis. Sci.* **36**, 780-784
12. Tsubouchi, H., Niitani, Y., Hirono, S., Nakayama, H., Gohda, E., Arakaki, N., Sakiyama, O., Takahashi, K., Kimoto, M., Kawakami, S., Setoguchi, M., Tachikawa, T., Shin, S., Arima, T., and Daikuhara, Y. (1991) Levels of the human hepatocyte growth factor in serum of patients with various liver diseases determined by an enzyme-linked immunosorbent assay. *Hepatology* **13**, 1-5
13. Joplin, R., Hishida, T., Tsubouchi, H., Daikuhara, Y., Ayres, R., Neuberger, J.M., and Strain, A.J. (1992) Human intrahepatic biliary epithelial cells proliferate in vitro in response to human hepatocyte growth factor. *J. Clin. Invest.* **90**, 1284-1289
14. Ishii, T., Sato, M., Sudo, K., Suzuki, M., Nakai, H., Hishida, T., Niwa, T., Umezu, K., and Yuasa, S. (1995) Hepatocyte growth factor stimulates liver regeneration and elevates blood protein level in normal and partially hepatectomized rats. *J. Biochem.* **117**, 1105-1112
15. Arakaki, N., Kawakami, S., Nakamura, O., Ohnishi, T., Miyazaki, H., Ishii, T., Tsubouchi, H., and Daikuhara, Y. (1995) Evidence for the presence of an inactive precursor of human hepatocyte growth factor in plasma and sera of patients with liver diseases. *Hepatology* **22**, 1728-1734
16. Kinoshita, T., Hirano, S., Matsumoto, K., and Nakamura, T. (1991) Possible endocrine control by hepatocyte growth factor of liver regeneration after partial hepatectomy. *Biochem. Biophys. Res. Commun.* **177**, 330-335
17. Gohda, E., Hayashi, Y., Kawaida, A., Tsubouchi, H., and Yamamoto, I. (1990) Hepatotrophic growth factor in blood of mice treated with carbon tetrachloride. *Life Sci.* **46**, 1801-1808
18. Takehara, T., Matsumoto, K., and Nakamura, T. (1992) Cell density-dependent regulation of albumin synthesis and DNA synthesis in rat hepatocytes by hepatocyte growth factor. *J. Biochem.* **112**, 330-334
19. Strain, A.J., Wallace, L., Joplin, R., Daikuhara, Y., Ishii, T., Kelly, D.A., and Neuberger, J.M. (1995) Characterization of biliary epithelial cells isolated from needle biopsies of human liver in the presence of hepatocyte growth factor. *Am. J. Pathol.* **146**, 537-545
20. Ohnishi, T., Nakamura, O., Arakaki, N., Miyazaki, H., and Daikuhara, Y. (1994) Effects of cytokines and growth factors on phosphorylated rat fetuin biosynthesis by adult rat hepatocytes in primary culture. *Biochem. Biophys. Res. Commun.* **200**, 598-605
21. Katsurada, A., Iritani, N., Fukuda, H., Matsumura, Y., Noguchi, T., and Tanaka, T. (1989) Effect of nutrients and insulin on transcriptional and post-transcriptional regulation of glucose-6-phosphate dehydrogenase synthesis in rat liver. *Biochim. Biophys. Acta* **1006**, 104-110
22. Katsurada, A., Iritani, N., Fukuda, H., Matsumura, Y., Nishimoto, N., Noguchi, T., and Tanaka, T. (1990) Effect of nutrients and hormones on transcriptional and post-transcriptional regulation of acetyl-CoA carboxylase in rat liver. *Eur. J. Biochem.* **190**, 435-441

23. Tamura, M., Arakaki, N., Tsubouchi, H., Takada, H., and Daikuhara, Y. (1993) Enhancement of human hepatocyte growth factor production by interleukin-1α and -1β and tumor necrosis factor-α by fibroblasts in culture. *J. Biol. Chem.* **268**, 8140-8145
24. Brunengraber, H., Boutry, M., and Lowenstein, J.M. (1973) Fatty acid and 3-β-hydroxysterol synthesis in the perfused rat liver. Including measurements on the production of lactate, pyruvate, β-hydroxybutyrate, and acetoacetate by the fed liver. *J. Biol. Chem.* **248**, 2656-2669
25. Jungas, R.L. (1968) Fatty acid synthesis in adipose tissue incubated in tritiated water. *Biochemistry* **7**, 3708-3717
26. Lowenstein, J.M., Brunengraber, H., and Wadke, M. (1975) Measurement of rates of lipogenesis with deuterated and tritiated water in *Methods in Enzymology* (Lowenstein, J.M., ed.) Vol. 35, pp. 279-287, Academic Press, New York
27. Bijleveld, C. and Geelen, M.J.H. (1987) Measurement of acetyl-CoA carboxylase activity in isolated hepatocytes. *Biochim. Biophys. Acta* **918**, 274-283
28. Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911-917
29. Nakamura, T., Yoshimoto, K., Aoyama, K., and Ichihara, A. (1982) Hormonal regulations of glucose-6-phosphate dehydrogenase and lipogenesis in primary culture of rat hepatocytes. *J. Biochem.* **91**, 681-693
30. Wise, E.M. and Ball, E.G. (1964) Malic enzyme and lipogenesis. *Proc. Natl. Acad. Sci. USA* **52**, 1255-1263
31. Deutsch, J. (1983) Glucose-6-phosphate dehydrogenase in *Methods of Enzymatic Analysis* (3rd ed.) (Bergmeyer, H.U., ed.), Vol. 3, pp. 190-197, Verlag Chemie GmBH, Weinheim
32. Hsu, R.Y. and Lardy, H.A. (1969) Malic enzyme in *Methods in Enzymology* (Colowick, S.P., Kaplan, N.O., and Lowenstein, J.M., eds.) Vol. 13, pp. 230-235, Academic Press, New York
33. Inoue, H. and Lowenstein, J.M. (1975) Acetyl-CoA carboxylase from rat liver in *Methods in Enzymology* (Lowenstein, J.M., ed.) Vol. 35, pp. 3-10, Academic Press, New York
34. Shafrir, E. and Bierman, E. (1981) Acetyl-CoA carboxylase activity in cultured human fibroblasts (induction by insulin in relation to cell growth and triacylglycerol metabolism). *Biochim. Biophys. Acta* **663**, 432-445
35. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
36. Ohnishi, T., Nakamura, O., Ozawa, M., Arakaki, N., Muramatsu, T., and Daikuhara, Y. (1993) Molecular cloning and sequence analysis of cDNA for a 59 kD bone sialoprotein of the rat: Demonstration that it is a counterpart of human α2-HS glycoprotein and bovine fetuin. *J. Bone Miner. Res.* **8**, 367-377
37. Kawakami, S., Tsubouchi, H., Nakagawa, S., Hirono, S., Arakaki, N., Nakamura, O., Daikuhara, Y., and Arima, T. (1994) Expression of hepatocyte growth factor in normal and carbon tetrachloride-treated monkeys. *Hepatology* **20**, 1255-1260
38. Schowalter, D.B. and Sommer, S.S. (1989) The generation of radiolabeled DNA and RNA probes with polymerase chain reaction. *Anal. Biochem.* **177**, 90-94
39. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual* (2nd ed.) pp. 7.37-7.52, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
40. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275
41. Schmidt, E. and Schmidt, F.W. (1970) Enzyme activities in human liver. *Enzymol. Biol. Clin.* **11**, 67-129
42. Watanabe, A. and Taketa, K. (1973) Actinomycin D-insensitive induction of rat liver glucose-6-phosphate dehydrogenase by carbon tetrachloride injury. *J. Biochem.* **73**, 771-779
43. Daikuhara, Y., Tamada, F., Takigawa, M., Takeda, Y., and Mori, Y. (1979) Changes in polyamine metabolism of rat liver after administration of D-galactosamine. Favorable effects of putrescine administration on galactosamine-induced hepatic injury. *Gastroenterology* **77**, 123-132
44. Lowenstein, J.M. (1971) Effect of (−)-hydroxycitrate on fatty acid synthesis by rat liver *in vivo*. *J. Biol. Chem.* **246**, 629-632
45. Nilsson, Å., Sundler, R., and Åkesson, B. (1973) Biosynthesis of fatty acids and cholesterol in isolated rat-liver parenchymal cells. Effect of albumin-bound fatty acids. *Eur. J. Biochem.* **39**, 613-620
46. Yoshimoto, K., Nakamura, T., and Ichihara, A. (1983) Reciprocal effects of epidermal growth factor on key lipogenic enzymes in primary cultures of adult rat hepatocytes. Induction of glucose-6-phosphate dehydrogenase and suppression of malic enzyme and lipogenesis. *J. Biol. Chem.* **258**, 12355-12360
47. Isshiki, H., Akira, S., Sugita, T., Nishio, Y., Hashimoto, S., Pawlowski, T., Suematsu, S., and Kishimoto, T. (1991) Reciprocal expression of NF-IL6 and C/EBP in hepatocytes: Possible involvement of NF-IL6 in acute phase protein gene expression. *New Biol.* **9**, 1897-1906
48. Falguerho, L., Patey, G., Paquereay, L., Rossi, V., Lahuna, O., Szpirer, J., Szpirer, C., Lavan, G., and Le Cam, A. (1991) Primary structure of the rat gene encoding an inhibitor of the insulin receptor tyrosine kinase. *Gene* **98**, 209-216
49. Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. (1990) A nuclear factor for IL-6 expression (NF-LI6) is a member of a C/EBP family. *EMBO J.* **9**, 1897-1906
50. Hirano, T., Akira, S., Taga, T., and Kishimoto, T. (1990) Biological and clinical aspects of interleukin 6. *Immunol. Today* **11**, 443-449
51. Rank, K.B., Harris, P.K., Ginsberg, L.C., and Stapleton, S.R. (1994) Isolation and sequence of a rat glucose-6-phosphate dehydrogenase promoter. *Biochim. Biophys. Acta* **1217**, 90-92
52. Luo, X.C. and Kim, K.H. (1990) An enhancer element in the house-keeping promoter for acetyl-CoA carboxylase gene. *Nucleic Acids Res.* **18**, 3249-3254
53. Park, K. and Kim, K.-H. (1991) Regulation of acetyl CoA carboxylase gene expression: Insulin induction of acetyl-CoA carboxylase and differentiation of 30A5 preadipocytes require prior cAMP action on the gene. *J. Biol. Chem.* **266**, 12249-12256
54. O'Brien, R.M., Lucas, P., Forest, C.D., Magnuson, M.A., and Granner, D.K. (1990) Identification of a sequence in the PEPCK gene that mediates a negative effect of insulin transcription. *Science* **249**, 533-537
55. Nasrin, N., Ercolani, L., Denaro, M., Kong, X.F., Kang, I., and Alexander, M. (1990) An insulin response element in the glyceraldehyde-3-phosphate dehydrogenase gene binds a nuclear protein induced by insulin in cultured cells and by nutritional manipulations in vivo. *Proc. Natl. Acad. Sci. USA* **87**, 5273-5277
56. Michalopoulos, G.K. (1990) Liver regeneration: Molecular mechanisms of growth control. *FASEB J.* **4**, 176-187
57. LeBrecque, D. (1994) Liver regeneration: A picture emerges from the puzzle. *Am. J. Gastroenterol.* **89**, S86-S96