The Mitogenic Activity of Hepatocyte Growth Factor on Rat Hepatocytes Is Dependent upon Endogenous Transforming Growth Factor- α

Tomoaki Tomiya,* Itsuro Ogata,* Miho Yamaoka,[†] Mikio Yanase,* Yukiko Inoue,* and Kenji Fujiwara[†]

From the First Department of Internal Medicine,* Faculty of Medicine, University of Tokyo, Tokyo; and the Third Department of Internal Medicine,[†] Saitama Medical School, Saitama, Japan

Both transforming growth factor- α (TGF- α) and hepatocyte growth factor (HGF) induce DNA synthesis in hepatocytes *in vitro* and *in vivo*. Hepatic and circulating levels of HGF have been reported to increase before an increase in TGF- α levels in several rat models of liver regeneration. In addition, serum TGF- α levels increase after an increase in serum HGF levels in patients with either partial hepatectomy or acute hepatitis. In this study, we investigate the significance of TGF- α in hepatocyte proliferation. TGF- α contents and DNA synthesis in cultured rat hepatocytes increased in response to HGF addition to the culture medium in a dose-related manner. These increases were suppressed by the addition of anti-sense TGF- α mRNA oligonucleotide. Furthermore, the addition of anti-TGF- α rabbit IgG suppressed the increase in DNA synthesis. When the anti-TGF- α antibody was administered to rats after partial hepatectomy, the number of mitotic hepatocytes was reduced in comparison to rats treated with normal rabbit IgG. These results were observed even though hepatic HGF levels were increased equally in rats given either anti-TGF- α antibody or normal rabbit IgG. Our results suggest that HGF stimulates TGF- α production in rat hepatocytes, and that the mitogenic activity of HGF depends on endogenous TGF- α activity. (*Am J Pathol* 2000, 157:1693–1701)

Several mitogens have been identified for hepatocytes in culture.^{1–3} Among them, transforming growth factor- α (TGF- α) and hepatocyte growth factor (HGF) have been reported to stimulate hepatocyte proliferation *in vivo*.^{4–9} Hepatocytes express both TGF- α mRNA and mRNA encoding the TGF- α /epidermal growth factor (EGF) receptor.^{1,10–16} In contrast, whereas hepatocytes express mRNA encoding the receptor for HGF, the *c-met* proto-oncogene gene product, only nonparenchymal cells of the liver produce HGF.^{17–24} Therefore, TGF- α and HGF

are thought to act in an autocrine and paracrine manner, respectively.^{1,3}

Previous reports have demonstrated that HGF mRNA and the circulating and hepatic levels of HGF are increased in rats either after partial hepatectomy or after acute liver injury induced by carbon tetrachloride intoxication. These increases occur before an increase in endogenous TGF- α and TGF- α mRNA.^{13,25–28} In humans, serum HGF levels have been shown to increase before an increase of serum TGF- α levels after partial hepatectomy or in patients with acute hepatitis.^{29–33} These observations raise questions as to whether TGF- α is required in addition to HGF for the proliferation of hepatocytes.

The present study investigates the significance of TGF- α in hepatocyte proliferation even in the presence of HGF. We examined the effect of the inhibition of the synthesis and activity of TGF- α , through the use of anti-sense TGF- α mRNA oligonucleotide and anti-TGF- α antibodies, respectively, on the DNA synthesis by rat hepatocytes cultured in the presence of HGF. Furthermore, we examined the effect of anti-TGF- α rabbit IgG administration on hepatocyte proliferation in rats after partial hepatectomy. Our results suggest that HGF stimulates TGF- α production in rat hepatocytes, and that the mitogenic activity of HGF depends on endogenous TGF- α activity.

Materials and Methods

Sources

Type I collagen-coated tissue culture dishes were purchased from Iwaki Glass (Corning, Tokyo, Japan); the 96-well assay plate was from A/S Nunc (Roskilde, Denmark); 20% neutral buffered formalin solution was from Muto Pure Chemicals Ltd. (Tokyo, Japan); phenylmethylsulfonyl fluoride was from Sigma Chemical Company (St. Louis, MO); Williams' medium E (WE) from ICN Biochemicals Inc. (Costa Mesa, CA); fetal calf serum (FCS) from Life Technologies Inc. (Grand Island, NY); insulin from Novo Industri A/S (Copenhagen, Denmark); dexametha-

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Address reprint requests to Tomoaki Tomiya, M.D., Ph.D., First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan. E-mail: tomiya-1m@h.u-tokyo.ac.jp.

sone from Takeda Medical Industries Ltd. (Osaka, Japan); blocking agents derived from skim milk, ie, Block Ace (BA) from Snow Brand Milk Products Co., Ltd. (Sapporo, Japan); peroxidase-conjugated anti-rabbit IgG solution from Otsuka Assay Laboratories (Tokushima, Japan); free and peroxidase-conjugated anti-rabbit IgG antibodies preabsorbed with rat serum from Chemicon International Inc. (Temecula, CA); normal rabbit IgG from InterCell Technologies Inc. (Hopewell, NJ); rat TGF- α from Bachem Feinchemikalien AG (Bubendorf, Switzerland); rat EGF from Otsuka Assay Laboratories (Tokushima, Japan); heparin-binding EGF-like growth factor (HB-EGF) from R&D Systems Inc. (Minneapolis, MN); 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate⁶] from Boehringer Mannheim (Mannheim, Germany); and dye reagent concentrate of Bio-Rad Protein Assay from Bio-Rad Laboratories (Hercules, CA). Recombinant HGF, a 5-amino acid-deleted form of HGF (dHGF), and anti-TGF- α rabbit IgG were generously provided from Snow Brand Milk Products Co., Ltd. and Kyowa Medex Co., Ltd., (Tokyo, Japan) respectively.³⁴ Rat HGF enzyme-linked immunosorbent assay (ELISA) kit, 5-bromo-2'-deoxy-uridine (BrdU) labeling and detection kits II and III, and rat interleukin-6 (IL-6) ELISA kits were obtained from the Institute of Immunology (Tokyo, Japan), Boehringer Mannheim, and Immuno-Biological Laboratories (Fujioka, Japan), respectively.³⁵ Other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animals

Male Sprague-Dawley rats, 5 to 6 weeks old, were obtained from Japan SLC (Shizuoka, Japan). They were housed in cages at 22°C under a 12-hour light-dark cycle and fed a commercial diet and water *ad libitum*. All animal study protocols conformed to the guideline of Faculty of Medicine, University of Tokyo for humane care.

Preparation of Oligonucleotides

Oligonucleotides were synthesized with an automated DNA synthesizer (Model 391 PCR-Mate DNA Synthesizer; Applied Biosystems, Foster City, CA) using the phosphoramidite protocol provided by the manufacturer.^{36,37} The pentadecadeoxynucleotide (5'-GGCCGCG-GGGACCAT-3'), complementary to the initiation codon and the next four codons of rat TGF- α mRNA,³⁸ was used as an anti-sense TGF- α mRNA oligonucleotide. A non-sense pentadecadeoxynucleotide, 5'-CGCGAGGCTAG-GCGC-3', was prepared containing the same numbers of G, C, A, and T as the anti-sense oligonucleotide.

Determination of BrdU Incorporation, TGF- α Content, Mitochondria Function, and Total Protein Content of Cultured Hepatocytes

Fixation of hepatocytes and BrdU detection were performed according to the manufacturer's instructions for the BrdU labeling and detection kits. Incorporated BrdU

was determined by ELISA using BrdU labeling and detection kit III. In addition, immunohistological detection of BrdU was performed using BrdU labeling and detection kit II, and the number of labeled nuclei were counted in >1,000 hepatocytes in each well for calculations of BrdU nuclear labeling index. The TGF- α content in fixed hepatocytes was determined using an immunoblotting method as previously described^{39,40} with some modifications. Briefly, 200 μ L of methanol containing 0.5% H₂O₂ was added to each culture well, incubated for 30 minutes at 4°C, and removed by aspiration. After five washes with 350 μ L of 10 mmol/L phosphate-buffered saline (PBS), pH 7.2, the wells were filled with 350 μ L of BA, incubated for 5 hours at 4°C, and washed three times with 350 μ L of 10% BA in 10 mmol/L PBS. Fifty μ L of anti-TGF- α rabbit IgG diluted to 5 μ g/ml in 10 mmol/L PBS containing 10% BA, 10 mmol/L ethylenediaminetetraacetic acid, and 0.25% polyoxyethylene-10-octylphenyl ether (BA buffer) was added to each well, and incubated overnight at 4°C. The well was washed six times with 350 μ L of BA buffer and 50 μ L of peroxidase-conjugated anti-rabbit IgG solution diluted 50-fold in BA buffer was added to each well. The wells were incubated for 1 hour at room temperature, followed by six washings. To each well, 100 μ L of o-phenylenediamine solution was added, incubated at room temperature for 20 minutes, followed by the addition of 100 μ L of 0.5 N oxalic acid solution. The optical density of the solution in each well was determined at 490 nm in an automated microplate reader (MR 700; Dynatech Laboratories, Inc., Chantilly, VA).

For the measurement of the total cellular protein by Bradford's method, the cells were solubilized in 100 μ L of 1 N NaOH at 65°C for 15 minutes and neutralized with the same amount of 1 N HCl.⁴¹ Mitochondria function of cultured hepatocytes was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay as previously described.⁴²

Assay for HGF and TGF- α in the Liver

Liver extracts were prepared according to the manufacturer's instructions of a HGF ELISA kit. Extracts were applied to HGF ELISA as previously described.³⁵ Hepatic TGF- α levels were determined by ELISA as previously reported.²⁵

Assay for Serum Rabbit IgG and IL-6 Levels in Rats

Serum rabbit IgG levels in rats dosed with rabbit IgG after partial hepatectomy were measured by sandwich ELISA as follows. In each well of a 96-well assay plate, 50 μ L of 5 μ g/ml anti-rabbit IgG preabsorbed with rat serum in 50 mmol/L of PBS (pH 7.4) was added, incubated overnight at 4°C, and removed by aspiration. After washing three times with 400 μ L of 50 mmol/L PBS (pH 7.2), the well was filled with 400 μ L of 70% (v/v) BA in 50 mmol/L PBS (pH 7.2), incubated overnight at 4°C, and removed by aspiration. The well was washed three times with 400 μ L of 10% BA in 50 mmol/L of PBS (pH 7.2). To each well, 100

μ l of assay samples diluted with 10% BA containing 10 mmol/L of ethylenediaminetetraacetic acid in 50 mmol/L of PBS (pH 7.2) was added, and incubated overnight at 4°C. The wells were washed eight times with 400 μ l of 0.05% polyoxyethylene-20-sorbitan monolaurate and 10% BA in 50 mmol/L PBS (pH 7.2) followed by the addition of 50 μ l of 1 μ g/ml peroxidase conjugated anti-rabbit IgG in the 10% BA to each well. The plate was incubated overnight at 4°C, followed by similar washings. To each well, 100 μ l of 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate(6)] solution was added, incubated at room temperature for 20 minutes, followed by the addition of 50 μ l of 0.25 mol/L oxalic acid solution. The optical density of the solution in each well was determined at 405 nm on an automated microplate reader. The lower limit of this assay was 160 pg/ml. Serum IL-6 levels in rats after partial hepatectomy were measured using a rat IL-6 ELISA according to the manufacturer's instructions.

Determination of the Reactivity of Anti-TGF- α Rabbit IgG

The reactivity of anti-TGF- α rabbit IgG to TGF- α , EGF, and HB-EGF was determined by an immunoblotting method. Various concentrations of growth factors in 50 mmol/L of PBS (pH 7.4) were distributed in each well of a 96-well assay plate, incubated overnight at 4°C, and removed by aspiration. Subsequent procedures were similar to those used to detect TGF- α in cultured hepatocytes as described above.

Histological Examination

Liver specimens were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The mitotic index of hepatocytes was determined by counting more than 3,000 hepatocytes in each liver specimen.

Experiments with Cultured Hepatocytes

Hepatocytes were isolated from rat livers according to Seglen's method.⁴³ They were plated on collagen-coated dishes at 5×10^4 cells/cm² in WE containing 5% (v/v) FCS, 1 mmol/L insulin, and 1 mmol/L dexamethasone. After 2 hours, the culture medium was replaced with WE supplemented with 5% FCS. After a further 24 hours of incubation, the medium was changed to WE containing 5% FCS and various concentrations of dHGF. After another 24 hours incubation, the medium was replaced with media supplemented with 2% FCS containing 1 mmol/L BrdU, instead of the 5% FCS. The cells were harvested 16 hours later for the determinations of cellular TGF- α contents and BrdU incorporation into cellular DNA.

To examine the effects of anti-sense oligonucleotides, hepatocytes cultured for 26 hours as described above were used. The medium was changed to WE containing 5% FCS and 10 ng/ml of dHGF. After 24 hours, the medium was replaced with WE containing 2% FCS, 10

ng/ml of dHGF, and 1 mmol/L of BrdU, along with various concentrations of either anti-sense TGF- α mRNA oligonucleotide or nonsense oligonucleotide. The cells were harvested 16 hours later for the determinations of cellular TGF- α contents and BrdU incorporation into cellular DNA.

To examine the effects of antibodies against TGF- α , hepatocytes cultured for 50 hours as described above were used. The medium was replaced with WE containing 5% FCS, 10 ng/ml of dHGF, and 1 mmol/L of BrdU, along with various concentrations of either anti-TGF- α rabbit IgG or normal rabbit IgG. After 16 hours, the cells were harvested for the determination of BrdU incorporation into cellular DNA.

In addition to these experiments, hepatocytes were cultured in serum-free WE containing 0.5 μ g/ml of aprolinin along with either anti-sense TGF- α mRNA oligonucleotides or antibodies for TGF- α to avoid potential effects of growth factors and cytokines in the serum.

Animal Experiments

Rats were subjected to a two-thirds resection of the liver under diethyl ether anesthesia. The rats were injected intraperitoneally with normal rabbit IgG (0.5 mg in 2.5 ml saline/kg body weight) at 0 and 12 hours after surgery. Rats were anesthetized with diethyl ether, and blood was collected through the inferior caval vein using an empty plastic syringe to prepare serum for the determination of serum rabbit IgG levels.

Similarly operated rats were injected intraperitoneally with either anti-TGF- α rabbit IgG or normal rabbit IgG (0.5 mg in 2.5 ml saline/kg body weight) at 0 and 12 hours after the operation. Twenty-four hours later, blood was collected through the inferior caval vein to prepare serum for the determination of serum IL-6 levels. After collecting blood, the liver was perfused with 25 ml of saline at 37°C through the portal vein after near-total exsanguination, and excised. A portion of the liver was immersed in 20% neutral-buffered formalin solution for histological examination. The remaining liver was frozen in liquid nitrogen, and stored at -80°C for use in the HGF assay.

Statistical Analyses

The differences between two unpaired samples were defined as significant when *P* values by both Student's *t*-test and Mann-Whitney *U* test were <0.05. Spearman's correlation test was used for analysis of correlations. The dose-related effects were tested by one-way analysis of variance followed by Spearman's correlation test.

Results

TGF- α Content and BrdU Incorporation in Cultured Rat Hepatocytes in the Presence of HGF

As shown in Figure 1, when dHGF was added to the medium at increasing concentrations, cultured rat hepatocytes increased their contents of TGF- α in a dose-

Figure 1. Effect of HGF on cellular TGF- α content and BrdU incorporation in cultured rat hepatocytes. **A:** Cellular TGF- α in the presence of increasing concentrations of HGF (expressed as percent of levels in the absence of HGF). Hepatocytes were plated on collagen-coated dishes in WE containing 5% FCS, 1 mmol/L insulin, and 1 mmol/L dexamethasone. After 2 hours, the culture medium was replaced with WE supplemented with 5% FCS. After a further 24 hours of incubation, the medium was changed to WE containing 5% FCS and dHGF. After another 24 hours incubation, the medium was replaced with media supplemented with HGF and 2% FCS instead of the 5% FCS. The cells were harvested 16 hours later. **B:** Same cultures as in **A** except that the medium supplemented with 2% FCS was containing 1 mmol/L BrdU, examined for BrdU incorporation. Data are mean $\pm$ SEM of six dishes. *, $P < 0.05$; **, $P < 0.01$ compared with the values in the absence of HGF.

related manner up to 10 ng/ml dHGF. The addition of dHGF at concentrations of 20 ng/ml or more resulted in a reduction of TGF- α content in hepatocytes compared to 10 ng/ml of dHGF addition. A similar dose-related response was observed in the BrdU incorporation into hepatocytes treated with dHGF.

Effect of Anti-Sense TGF- α mRNA Oligonucleotide on TGF- α Content and BrdU Incorporation in Cultured Rat Hepatocytes in the Presence of HGF

In the presence of 10 ng/ml of dHGF, anti-sense TGF- α mRNA oligonucleotide in the culture medium reduced TGF- α content in hepatocytes in a dose-related manner by up to 52% of the levels observed with the addition of control nonsense oligonucleotide ($F = 8.9$, $P < 0.01$; $r = -0.75$, $P < 0.01$) (Figure 2A). The addition of anti-sense oligonucleotide resulted in a similar decrease in BrdU incorporation, as determined by ELISA ($F = 76.7$, $P < 0.01$; $r = 0.87$, $P < 0.01$) (Figure 2, B-1). The anti-sense was effective in reducing BrdU incorporation even when

Figure 2. Effect of anti-sense TGF- α mRNA oligonucleotide on cellular TGF- α levels and BrdU incorporation in rat hepatocytes cultured in the presence of HGF. **A:** Hepatocytes cultured for 26 hours as described in the legend of Figure 1 were used. The medium was changed to WE containing 5% FCS and 10 ng/ml HGF. After 24 hours, the medium was replaced with WE containing 2% FCS, 10 ng/ml of dHGF along with either anti-sense TGF- α mRNA oligonucleotide or nonsense oligonucleotide. The cells were harvested 16 hours later for the determination of cellular TGF- α contents of hepatocytes. **B:** Similar cultures and conditions as in **A**, examined for BrdU incorporation. **B-1:** Hepatocytes were cultured in the same medium as in **A** except that the medium supplemented with 2% FCS was containing 1 mmol/L BrdU. **B-2:** Similar cultures as in **B-1**. However, the medium contained 0.5 μ g/ml of aprotinin instead of FCS. Data are mean $\pm$ SEM of six dishes. *, $P < 0.05$; **, $P < 0.01$ compared with the values in the presence of nonsense oligonucleotide at same concentrations.

hepatocytes were cultured in serum-free medium (Figure 2, B-2). The BrdU nuclear labeling index of hepatocytes was also reduced significantly by the addition of 80 μ mol/L of the anti-sense oligonucleotide, when compared to the addition of nonsense oligonucleotide (mean $\pm$ SD: 36.5 $\pm$ 1.4% versus 51.3 $\pm$ 0.5%, $P < 0.01$). The total cellular protein content and the mitochondrial function of the cells, as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay,

Figure 3. Reactivity of anti-TGF- α rabbit IgG to growth factors. **Closed circles, open circles, and squares** indicate the reactivity of anti-TGF- α rabbit IgG with TGF- α , EGF, and HB-EGF, respectively.

were not affected by the addition of either oligonucleotide (data not shown).

Effect of Anti-TGF- α Rabbit IgG on BrdU Incorporation in Cultured Rat Hepatocytes in the Presence of HGF

The anti-TGF- α rabbit IgG used was specific for TGF- α and did not recognize up to 500 ng of either EGF or HB-EGF by immunoblotting assays (Figure 3). BrdU incorporation by cultured hepatocytes in the presence of 10 ng/ml dHGF was decreased by 1 μ g/ml and 10 μ g/ml of anti-TGF- α rabbit IgG addition, as determined by ELISA ($F = 50.2$, $P < 0.01$; $r = -0.80$, $P < 0.01$) (Figure

Figure 4. Effect of anti-TGF- α rabbit IgG on BrdU incorporation in rat hepatocytes cultured in the presence of HGF. Hepatocytes cultured for 26 hours as described in the legend of Figure 1 were used. The medium was changed to WE containing 10 ng/ml of dHGF along with either 5% FCS or 0.5 μ g/ml of aprotinin. After another 24 hours incubation, the medium was replaced with WE containing either 5% FCS or 0.5 μ g/ml of aprotinin, 10 ng/ml of dHGF, and 1 mmol/L of BrdU, along with either anti-TGF- α rabbit IgG or normal rabbit IgG. After 16 hours, the cells were harvested for the determination of BrdU incorporation into cellular DNA. **Closed lines** indicate hepatocytes were cultured in medium containing 5% of FCS. **Dotted lines** indicate hepatocytes were cultured in medium without FCS. **Closed circles** denote rat hepatocytes cultured in the presence of 10 ng/ml dHGF with the addition of increasing concentrations of anti-TGF- α rabbit IgG. **Open circles** denote experiments using the control normal rabbit IgG. Data are mean $\pm$ SEM of six dishes. **, $P < 0.01$ compared with the values in the presence of normal rabbit IgG at the same concentrations.

Figure 5. Changes of serum rabbit IgG levels in rats after partial hepatectomy and dosed with rabbit IgG. Data are mean $\pm$ SEM of four rats.

4). A similar dose-related decrease in BrdU incorporation by addition of the antibody was observed even when hepatocytes were cultured in serum-free medium (Figure 4). The addition of 10 μ g/ml of anti-TGF- α rabbit IgG suppressed the BrdU nuclear labeling index of hepatocytes, when compared to that in the presence of normal rabbit IgG (mean $\pm$ SD: $34.9 \pm 1.9\%$ versus $50.7 \pm 1.0\%$, $P < 0.01$). The total cellular protein content and the mitochondrial function by hepatocytes were not affected by the addition of the rabbit IgGs (data not shown).

Effect of Anti-TGF- α Rabbit IgG on the Mitotic Index of Hepatocytes after Partial Hepatectomy in Rats

As shown in Figure 5, rats dosed with normal rabbit IgG after partial hepatectomy possessed serum rabbit IgG levels in excess of 1 μ g/ml at 2 hours after the surgery. These rats attained a plateau of ~ 10 μ g/ml at 12 hours. In addition, rats given either normal rabbit IgG or anti-TGF- α rabbit IgG after partial hepatectomy increased hepatic HGF levels comparable to that obtained when rats were dosed with the vehicle alone (Figure 6). However, the increased mitotic index of hepatocytes 24 hours after partial hepatectomy was suppressed by the administration of anti-TGF- α rabbit IgG to 20% of the mitotic index of rats given normal rabbit IgG (Figure 6). Hepatic TGF- α levels were significantly decreased in rats treated with anti-TGF- α IgG compared to controls (Figure 6). Serum IL-6 levels were not affected by administration of either IgG (data not shown).

Discussion

Our results indicate that the TGF- α production and DNA synthesis of rat hepatocytes in primary culture were enhanced in a dose-related manner by the addition of HGF to the medium. These enhancements were attenuated by the addition of anti-sense TGF- α mRNA oligonucleotide. Furthermore, anti-TGF- α rabbit IgG reduced DNA synthe-

Figure 6. Effect of anti-TGF- α rabbit IgG on the mitotic index of hepatocytes and hepatic HGF and TGF- α levels 24 hours after partial hepatectomy in rats. Data are mean $\pm$ SEM of four rats. "Before" means the levels of HGF or TGF- α and the mitotic index of hepatocytes before the operation; "Ve" indicates levels in rats dosed with vehicle alone; "IgG" labels the levels in rats dosed with normal rabbit IgG; and "Ab" indicates levels in rats dosed with anti-TGF- α rabbit IgG. **Left: Shaded and open columns** indicate hepatic levels of HGF and TGF- α , respectively. **, $P < 0.01$ compared with the levels before operation. #, $P < 0.05$ compared with the levels in rats dosed with vehicle alone or normal rabbit IgG. **Right: Circles** indicate mitotic index of hepatocytes. **, $P < 0.01$ compared with the levels in rats dosed with normal rabbit IgG.

sis in hepatocytes *in vitro* and *in vivo*, even in the presence of HGF.

We cultured hepatocytes in the medium containing FCS. Furthermore, we performed the experiments using serum-free medium to confirm the results. Serum deprivation, which causes apoptosis of many types of cultured cells, is liable to induce death of hepatocytes in culture, although the addition of aprotinin to the medium would prevent it.^{44–48} On the other hand, HGF has been reported to have a cytoprotective effect as well as a mitogenic effect on hepatocytes.^{49–51} Recently, it was reported that HGF may prevent apoptosis of hepatocytes.^{49,52} To minimize the possibility that HGF may act on hepatocytes as a cytoprotective factor, and not to cloud interpretation of the results, we performed experiments using the medium containing FCS as well as serum-free medium supplemented with aprotinin in hepatocyte culture. In addition, we used dHGF (a 5-amino acid deleted form of HGF) *in vitro* experiments. dHGF is known to be more potent than HGF in the stimulation of DNA synthesis in hepatocytes.⁵³

To examine DNA synthesis by cultured hepatocytes in the presence of the anti-sense oligonucleotides, we added BrdU to the culture medium. We used a BrdU concentration >10 times higher than that of the added oligonucleotides to avoid a reduced uptake of BrdU because of the presence of degraded oligonucleotides in the medium. Furthermore, a nonsense oligonucleotide containing the same numbers of G, C, A, and T as the anti-sense oligonucleotide was used as a control. The addition of oligonucleotides did not affect the mitochondrial function of the hepatocytes, as well as cellular protein content, suggesting that the addition of oligonucleotides did not influence the viability of hepatocytes. Suppressed TGF- α production by anti-sense TGF- α mRNA oligonucleotide in cultured hepatocytes resulted in a reduced DNA synthesis by these cells. These observations are consistent with a previous report describing

the inhibition of tumor growth in liver epithelial cells transfected with a TGF- α anti-sense gene.⁵⁴

Currently, there are six known ligands for the EGF/TGF- α receptor. At least three of them, EGF, TGF- α , and HB-EGF, have been found to stimulate DNA synthesis in cultured rat hepatocytes.^{1,55} Of these, hepatocytes produce only TGF- α in substantial quantities,^{1,10–16} although a possible transient production of a small amount of EGF by hepatocytes has been reported.^{1,56} Our results demonstrate that the addition of highly specific anti-TGF- α rabbit IgG to the culture medium inhibited DNA synthesis by hepatocytes, even though the antibody did not influence hepatocyte viability. These results suggest that the anti-TGF- α rabbit IgG may inhibit the action of TGF- α produced by hepatocytes, leading to a suppression of DNA synthesis by hepatocytes. Previously, several reports have described the suppression of TGF- α activity of various kinds of TGF- α -producing cells by the addition of monoclonal or polyclonal anti-TGF- α antibodies to the culture medium.^{57–61} In those studies, the effective concentration of the antibodies in the medium varied from 0.5 to 50 μ g/ml depending on the antibodies and cells used.^{57–61} In the present study, hepatocyte DNA synthesis was suppressed by the addition of 1 μ g/ml or more of anti-TGF- α IgG to the medium, consistent with the previous reports concerning antibody concentration.

To examine the activity of anti-TGF- α rabbit IgG on hepatocytes *in vivo*, we administered 0.5 mg/kg body weight of the anti-TGF- α rabbit IgG intraperitoneally to rats at 0 and 12 hours after partial hepatectomy. When we treated rats in a similar manner with normal rabbit IgG, serum rabbit IgG levels exceeded 1 μ g/ml at 2 hours, and were maintained at $\sim$ 10 μ g/ml at 12 hours or more after the operation. Previously, it has been reported that, in rats subjected to partial hepatectomy, the hepatic and serum TGF- α levels are significantly increased at 12 hours after the operation when compared to the preoperative levels.^{1,14,25} The TGF- α reaches maximal levels at 24 hours, coinciding with the maximum number of mitotic hepatocytes. These observations confirm that the rise in serum and hepatic levels of TGF- α in rats after partial hepatectomy can be accompanied by a rise in serum levels of anti-TGF- α rabbit IgG in rats dosed with the antibody. The concentration of antibody in these rats during this time should be comparable to the concentration of anti-TGF- α rabbit IgG that was effective in the suppression of DNA synthesis in cultured hepatocytes. Indeed, our results demonstrate that the antibody administration suppressed hepatocyte proliferation 24 hours after partial hepatectomy, even when the increase of HGF was not affected. In addition, serum levels of IL-6, which may play a critical role in liver regeneration, were not influenced by the antibody administration.^{62–68} As for hepatic TGF- α levels, the moderate but significant decrease was observed in rats treated with anti-TGF- α IgG compared to controls. The antibody may not suppress the synthesis of TGF- α by hepatocytes, whereas TGF- α trapped by the antibody may not be internalized into hepatocytes. The reduction of hepatic TGF- α contents might reflect these situations. Recently, Russell et al⁶⁹ reported no significant difference in hepatocyte prolifer-

ation after partial hepatectomy in TGF- α knockout mice and wild-type mice. As mentioned above, there are other ligands for the TGF- α /EGF receptor, such as EGF, which is abundantly produced by salivary glands in mice.^{1,15} The effects of the loss of TGF- α , in knockout mice, might be obviated by the opportunistic compensation of such ligands.⁶⁹ Furthermore, it has been demonstrated that the targeting of a gene can increase the expression of functionally related proteins.⁷⁰ Therefore, other proteins that have the potential to act as ligands to the TGF- α /EGF receptor may compensate for the loss of TGF- α . To clarify this point, studies using TGF- α /EGF receptor knockout mice will be required. In addition, it would be important to learn whether neutralizing antibodies to HGF would prevent the subsequent increase of TGF- α during liver regeneration to clarify the relationship between HGF and TGF- α *in vivo*.

The communication between HGF, TGF- α , and other growth factors has been a topic of study for several years. TGF- α secretion by cultured rat hepatocytes has been shown to be enhanced by EGF, TGF- α , or HGF.^{14,26} In addition, TGF- α mRNA expression is stimulated by TGF- α in human colon carcinoma cells and by HGF in human pancreatic cancer cells.^{71,72} The mitogenic effects of HGF and TGF- α have been shown to be additive in cultured fetal and adult hepatocytes.^{26,73,74} Recently, a communication between HGF and TGF- α /EGF receptor systems was reported in rat liver epithelial cells.^{75,76} The TGF- α /EGF receptor and *c-met*, the HGF receptor, are tyrosine kinases that autophosphorylate, as well as phosphorylate, to activate other enzymes. The src-homology regions of activated *c-met* and the TGF- α /EGF receptor are reported to bind different downstream signal transduction elements, suggesting that HGF and TGF- α , via their receptors, might stimulate unique targets.^{77,78} These observations, together with our results, highlight the significance of the communication between growth factors and underlie the need for further research.

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