Characterization of a Mouse Hepatocyte Growth-Stimulating Factor in Serum of Mice Treated with Carbon Tetrachloride

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The physicochemical and biological properties of a mouse hepatocyte growth-stimulating factor (mHGSF), whose amount in mouse serum increased markedly 24 h after carbon tetrachloride administration (E. Gohda et al., Life Sci. 46, 1801 (1990)), were examined. This factor was a heat-labile protein with a molecular weight of 75000. Its activity was sensitive to disulfide reduction. Maximal stimulation of deoxyribonucleic acid synthesis in cultured rat hepatocytes by this factor was greater than that by acidic fibroblast growth factor (acidic FGF) or mouse epidermal growth factor (EGF) and was comparable to maximal stimulation by human hepatocyte growth factor (hHGF), a heterodimer with a molecular weight of about 85000. The effect of mHGSF was additive to the maximal effects of acidic FGF and EGF and was synergistic with the maximal effect of insulin, but was neither additive nor synergistic with the maximal effect of hHGF. The mHGSF activity was not inhibited by a neutralizing anti-hHGF antiserum, which recognizes nonreduced hHGF but not reduced heavy and light chains of hHGF. mHGSF did not show any cross-reactivity to anti-hHGF monoclonal and/or polyclonal antibodies as measured by an enzyme-linked immunosorbent assay for hHGF. These results suggest that mHGSF is a hHGF-like factor with some structural difference from hHGF.

Keywords: mouse hepatocyte growth-stimulating factor; rat hepatocyte; primary culture; DNA synthesis; human hepatocyte growth factor; epidermal growth factor; acidic fibroblast growth factor; liver injury

Liver regeneration occurs rapidly after partial hepatectomy or hepatic injury. Its mechanism is not well understood, but many reports have suggested the presence of a humoral hepatotrophic factor(s). Some such factors have been isolated, mainly from the serum and plasma of partially hepatectomized rats. We found a hepatocyte growth factor, human hepatocyte growth factor (hHGF), in the plasma of patients with fulminant hepatic failure and we purified this factor from patients' plasma obtained during plasma exchange therapy. hHGF is a new factor, different from well-known growth factors, and is composed of two peptide chains, with molecular weights of 54000—65000 and 31500—34500, which are linked together by a disulfide bond(s). Nakamura et al. and Zarnegar and Michalopoulos also purified the same factor from rat platelets, normal human plasma, and rabbit serum. Our group and another group have recently deduced the complete primary structure of hHGF from the nucleotide sequence of hHGF complementary deoxyribonucleic acid (cDNA). However, it is not known at present how these humoral hepatotrophic factors, including hHGF, participate in hepatic regeneration.

We investigated whether a hHGF-like hepatocyte growth-stimulating factor is present in the blood of mice with liver injury, since it is difficult to perform extensive studies on the physiological roles of hHGF in human patients. We found that hepatocyte growth-stimulating activity in mouse serum increased markedly prior to the peak of liver DNA synthesis after carbon tetrachloride administration. In this paper we report the physicochemical and biological properties of this factor, mouse hepatocyte growth-stimulating factor (mHGSF), which was partially purified by heparin-Sepharose affinity chromatography from the serum of mice with liver injury.

Materials and Methods

Materials: The materials used for isolation and primary culture of rat hepatocytes have been described previously. Trypsin (type XII), x-chymotrypsin (type VII), dL-dithiothreitol, bovine serum albumin (fraction V), and bovine pancreas insulin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Mouse epidermal growth factor (EGF) and bovine acidic fibroblast growth factor (acidic FGF) were obtained from Collaborative Research (Bedford, Mass.) and R & D Systems (Minneapolis, Minn.), respectively. hHGF, purified from the plasma of patients with fulminant hepatic failure which was obtained during plasma exchange therapy, was donated by Drs. Y. Daikuhara and H. Tsoubouchi, Kagoshima University, Kagoshima, Japan. Rabbit anti-hHGF antiserum, which was prepared by immunizing New Zealand white rabbits with hHGF purified from the plasma of patients with fulminant hepatic failure, and an enzyme-linked immunosorbent assay (ELISA) kit for hHGF were kindly supplied by Otsuka Assay Laboratories, Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan. [Methyl-3H]thymidine was purchased from American Radiolabeled Chemicals (St. Louis, Mo.). Heparin-Sepharose CL-6B and molecular weight standards for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Pharmacia Japan (Tokyo).

Partial Purification of mHGSF from the Serum of Carbon Tetrachloride-Treated Mice: Male ddY mice, 5—9 weeks of age, were maintained on laboratory chow and water ad libitum. Carbon tetrachloride was administered as a 50% (v/v) solution in olive oil through an intragastric tube at a single dose of 0.4 ml/100 g body weight. While mice were under ether anesthesia, blood was collected from the inferior vena cava in the presence of 0.1 volume of 3.8% sodium citrate. Serum was prepared from platelet-poor plasma, as described previously. The serum was then dialyzed overnight against phosphate-buffered saline (PBS) at 4°C. mHGSF in this serum was partially purified by heparin-Sepharose chromatography, as described previously. All procedures were performed at 4°C. Concentrated Triton X-100 solution was added at a final concentration of 0.0313% to 4.5 ml of the dialyzed serum which was then dialyzed with 3 volumes of PBS containing 0.01% Triton X-100 (TPBS). This mixture was applied to a heparin-Sepharose column (1.2 × 1.8 cm), equilibrated with TPBS, at a flow rate of 10 ml/h. The column was washed successively with 7 ml each of TPBS and 0.5 m NaCl in TPBS. The mHGSF activity was then eluted with 7 ml of 1.75 m NaCl in TPBS, and the eluate was dialyzed overnight against TPBS. Protein was assayed by the method of Lowry et al. The specific activity of mHGSF increased by about 130-fold from the levels in the dialyzed serum.

Assay of mHGSF: The mHGSF activity was determined by measuring the stimulation of DNA synthesis in adult rat hepatocytes in primary culture after addition of the factor preparations, as described previously. Parenchymal liver cells, isolated from adult male Wistar rats (about 200 g), were plated in Nunc 24-well plastic dishes precoated with collagen (Cellmatrix-1P) at a density of 2.5 × 10⁴ cells/0.2 ml/cm² (0.38 ml/well) and were cultured essentially as described previously. The plating medium used was Williams medium E, supplemented with 5% fetal bovine serum, 10 μM dexamethasone, 100 U/ml of penicillin, and 100 U/ml of streptomycin.
μg/ml of streptomycin. The medium was replaced with serum-free Williams medium E containing 10 nm dexamethasone, 0.1 μg/ml of aprotinin, 2.5 mg/ml of bovine serum albumin (fraction V), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (basal medium) 4 and 22 h after plating. Samples of growth factors were added to the basal medium at each medium change. Cultures were then labeled with [3H]thymidine (74 kBq/ml, 37 GBq/mmol) for 18 h, between 28 and 46 h after plating, in the presence and absence of 10 μM hydroxyurea. After the labeling period, the cells were washed in situ 3 times each with cold PBS, 2% perchloric acid, and 95% ethanol. The cells were then air-dried and solubilized with 1 ml of 2% SDS containing 2 mM ethylenediaminetetra-acetic acid (EDTA) and 20 mM NaHCO3. Aliquots of this solution were used for measuring radioactivity and protein. Activity of replicating DNA synthesis was calculated as the difference between values with and without hydroxyurea and expressed as incorporated [3H]thymidine per μg of cellular protein, as described previously.36

SDS-PAGE and Elution of mHGSF from Gels SDS was added to an aliquot of the mHGSF preparation purified partially by heparin-Sepharose chromatography at a final concentration of 0.05%, and the mixture was concentrated to one-twentieth of its original volume with a Centricon-10 concentrator. The concentrated factor was then treated with an equal volume of 2-fold concentrated sample buffer consisting of 125 mM Tris–HCl (pH 6.8), 6% SDS, 20% glycerol, and 0.0025% bromphenol blue, in the absence of 2-mercaptoethanol, for 1 h at 25°C. SDS-PAGE was performed at room temperature by the method of Laemmli,11 using a 3% stacking gel and an 8% separating gel, both 1 mm thick, as described previously.36 Each of four lanes was loaded with 13.7 μg protein of the treated mHGSF preparation, and another lane with approximately 1 μg of each of the molecular weight standards. After electrophoresis, three lanes out of the four of the separating gel were cut into 1.5-mm slices with a razor blade. Each slice was minced, placed in a test tube, and incubated, with shaking, in 1 ml of TBS and 0.02% SDS for 18 h at room temperature. The mHGSF activity was then assayed by adding the eluates to the cultured hepatocyte medium, at a final concentration of 5% by volume. Other lanes were silver-stained by the method of Wray et al.40 Molecular weight standards used were phosphorylase b (94000), bovine serum albumin (67000), and ovalbumin (44000).

hHGF ELISA The sandwich ELISA for hHGF, which was recently developed using anti-hHGF monoclonal and polyclonal antibodies, was performed as described previously.35 The anti-hHGF monoclonal and polyclonal antibodies were prepared by immunizing BALB/c mice and New Zealand white rabbits with hHGF purified from the plasma of patients with fulminant hepatic failure and were partially purified as described previously.36 Both antibodies react with nonreduced hHGF, but not with reduced heavy and light chains of hHGF.4,7

**Results**

It has recently been reported that acidic FGF stimulates DNA synthesis in cultured adult rat hepatocytes.14 We compared the effects of mHGSF purified partially from mouse serum by heparin-Sepharose affinity chromatography with those of acidic FGF. As shown in Fig. 1, maximal effects of the partially purified factor and acidic FGF were observed at concentrations of 4700 and 3 ng/ml, respectively. Maximal stimulation induced by mHGSF was much greater than that induced by acidic FGF. The effects of mHGSF were also compared with those of hHGF and EGF (Fig. 1). Maximal stimulation by mHGSF was comparable to the maximal stimulatory effect of hHGF and was greater than that of EGF. The combined effects of mHGSF and each of the other known growth factors for hepatocytes are shown in Fig. 2. Optimal concentrations of growth factors were used in this experiment. The effect of mHGSF was additive to the maximal effects of acidic FGF and EGF, while addition of hHGF to hepatocyte cultures containing the optimal dose of mHGSF did not further stimulate DNA synthesis in these cells. The effect of the mouse factor, like the effect of hHGF,35 was synergistic with the effect of insulin.

Table I shows the effects of various treatments on the mHGSF activity. We found that this factor was completely destroyed by being heated for 10 min at 80°C. It was also sensitive to treatment with dithiothreitol, trypsin, and chymotrypsin, but was insensitive to 0.5 N acetic acid.

![Fig. 1. Dose-Response Curves for Stimulation of DNA Synthesis in Cultured Rat Hepatocytes by mHGSF, hHGF, EGF, and Acidic FGF](image1)

![Fig. 2. Effects of mHGSF Combined with Optimal Concentrations of hHGF, EGF, Acidic FGF or Insulin on DNA Synthesis in Cultured Rat Hepatocytes](image2)

**Table I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DNA synthesis (dpm/μg protein)</th>
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<tbody>
<tr>
<td>No growth factor</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>mHGSF</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>135 ± 20</td>
</tr>
<tr>
<td>Dithiothreitol⁴⁰</td>
<td>65 ± 1</td>
</tr>
<tr>
<td>Acetic acid⁴⁰</td>
<td>158 ± 31</td>
</tr>
<tr>
<td>Trypsin⁵</td>
<td>36 ± 6</td>
</tr>
<tr>
<td>Chymotrypsin⁴⁰</td>
<td>45 ± 5</td>
</tr>
<tr>
<td>Heat, 80°C, 10 min⁶</td>
<td>8 ± 1</td>
</tr>
</tbody>
</table>

a) The growth factor (28 μg protein/ml) was incubated with 10 mM dithiothreitol for 2 h at 20°C. b) The factor (28 μg protein/ml) was incubated with 0.5 N acetic acid for 16 h at 4°C and neutralized with 5 N NaOH. These treated preparations of the growth factor were diluted with 0.2% bovine serum albumin in PBS and dialyzed against PBS. c) The factor (28 μg protein/ml) was treated with trypsin (2.8 μg/ml) or chymotrypsin (2.8 μg/ml) for 30 min at 37°C, in a total volume of 325 μl, and these proteases were then inhibited by additions of 0.91 μg of soybean trypsin inhibitor and 0.91 μg of lima bean trypsin inhibitor, respectively. d) The factor (28 μg protein/ml) was heated for 10 min at 80°C. The factor which had been treated with proteases, heated or untreated was also diluted with 0.2% bovine serum albumin. All samples were sterilized on a 0.22-μm membrane filter and were assayed for their growth-promoting activity at a concentration of 0.4 μg of the factor/ml. Values are means ± S.D. for triplicate dishes.
mHGSF purified partially by heparin-Sepharose chromatography still showed many bands detectable with silver stain after nonreducing SDS-PAGE (data not shown), so that it is difficult to identify which one is the band of mHGSF. The molecular weight of mHGSF was then determined by measuring its activity in eluates of gel slices after SDS-PAGE of the partially purified factor under nonreducing conditions, since we found that this factor, like hHGF, retained its activity even after being treated with 3% SDS for 1 h at 25°C (data not shown). As shown in Fig. 3, a single peak of the activity with an approximate molecular weight of 75000 was detected. Table II shows the effects of a neutralizing anti-hHGF antiseraum on the mHGSF activity. As reported previously,41 the anti-hHGF antiseraum recognizes nonreduced hHGF, but not the reduced heavy and light chains of hHGF. The antiseraum had no effect on the mHGSF activity, although it completely inhibited the biological activity of hHGF. Moreover, this mouse factor did not show any cross-reactivity to anti-hHGF monoclonal and/or polyclonal antibodies as measured by a hHGF ELISA (Fig. 4).

Discussion

Mitogens reported so far for adult rat hepatocytes in primary culture are EGF, hepatocyte growth factor, transforming growth factor-α (TGF-α), and acidic FGF.15 The physicochemical and biological properties of mHGSF described in this report are different from those of EGF, TGF-α, and acidic FGF. Its molecular weight (75000) is much higher than those of the other three growth factors (all less than 20000). Maximal stimulation of DNA synthesis in cultured hepatocytes by mHGSF was greater than maximal stimulation by EGF and acidic FGF. The effect of mHGSF was additive to the maximal effects of EGF and acidic FGF, suggesting that receptors for the factor are different from EGF and FGF receptors. The effects of TGF-α are considered to be mediated through its binding to the EGF receptor.18 The properties of mHGSF are also different from those of hepatopoietin and biliprotein (isolated as liver growth factors from the plasma of partially hepatocemotized rats by Goldberg and Diaz-Gil et al.) with respect to molecular weight and heat stability. In contrast, the properties of mHGSF are quite similar to those of hHGF; the activity of both these factors being sensitive to heat, trypsin, chymotrypsin, and dithiothreitol treatment, and insensitive to acid treatment. In addition, both of these factors had high affinity to heparin.36 Moreover, the maximal effect of mHGSF on DNA synthesis in cultured hepatocytes was comparable to and not additive to that of hHGF. The maximal effects of both mHGSF and hHGF were synergistic with that of insulin. The molecular weight of mHGSF was found to be slightly lower than that of hHGF; the molecular weight of hHGF, determined by the same method as that described in this report, was about 85000. Therefore, it can be seen that mHGSF in the serum of carbon tetrachloride-treated mice is a hHGF-like factor. Its activity, however, was not inhibited by anti-hHGF antiseraum (Table II), which probably recognizes the quaternary and/or tertiary structure of hHGF and neutralizes hHGF activity.4 Moreover, this factor was not cross-reactive to anti-hHGF monoclonal and/or polyclonal antibodies as measured by the hHGF ELISA (Fig. 4). Both antibodies probably recognize the quaternary and/or tertiary structure of hHGF as well.4,13 Thus, mHGSF may not be structurally identical with hHGF.

We used mouse serum prepared from platelet-poor plasma in this study, as it has been reported by several groups7) that rat platelets contain large amounts of a hepatocyte growth factor. When hepatocyte growth-promoting activity in rat blood is assayed, special care should be taken to prevent release of the hepatocyte.
growth factor from platelets. Mouse platelets, however, appear not to contain such a factor(s), since hepatocyte growth-promoting activity in normal mouse serum is not higher than that in platelet-poor mouse serum.\(^7\)\(^\text{\textsuperscript{1,2}}\)\(^\text{\textsuperscript{7,10}}\)

The origin of the elevated mHGSF in the serum of carbon tetrachloride-treated mice is still unknown. Recently, Asami et al. and Kinoshita et al. reported marked increases in the amount of hepatocyte growth factor and of its messenger ribonucleic acid (mRNA) in the livers of rats treated with carbon tetrachloride.\(^8\)\(^\text{\textsuperscript{1,2}}\) We also found that hepatocyte growth-stimulating activity in liver extracts increased 24 h after carbon tetrachloride administration to mice, and we found that this elevated level lasted for at least 3 d (our unpublished data). Physicochemical, biological, and immunological properties of this mouse factor in the liver were indistinguishable from those of mHGSF (our unpublished data). Since the mHGSF activity in serum was maximal and dropped to the normal level 24 and 48 h after hepatotoxic administration, respectively,\(^7\)\(^\text{\textsuperscript{1}}\) it can be seen that a high level of the activity in liver is not directly linked to increase of the factor in serum. The time course of changes in serum mHGSF was parallel with that of changes in serum alanine and aspartate amino-transferases.\(^7\) Thus, mHGSF produced in liver at an accelerated rate may be released into the blood only as the liver tissue is degraded. Masumoto and Yamamoto\(^9\) also reported recently that a hepatocyte growth-promoting factor in rat serum increased 24 h after injection of carbon tetrachloride. Their data suggested that the factor was released into the blood from the degraded extracellular matrix in the liver. These factors, released prior to the increase of DNA synthesis, whose highest rate is reached about 48 h after administration of carbon tetrachloride, may play an important role in stimulating the proliferation of surviving hepatocytes subsequent to hepatic injury.

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References