Identification of a 79-kDa Heparin-binding Fibroblast Growth Factor (FGF) Receptor in Rat Hepatocytes and Its Correlation with the Different Growth Responses to FGF-1 between Hepatocyte Subpopulations*

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We reported previously that the potency of heparin-binding fibroblast growth factor-1 (FGF-1) as a mitogen for rat hepatocytes in primary culture is as high as that of epidermal growth factor (EGF) and hepatocyte growth factor. To gain insight into the pathophysiological significance of FGF-1 in hepatocyte growth, we analyzed the cooperative mitogenicity of FGF-1 and EGF. Results from a nuclear labeling assay using [3H]thymidine suggest that most hepatocytes in primary culture consist of two cell populations that differ in response to FGF-1: one is an FGF-1-responsive cell population, and the other is an EGF-responsive (but not FGF-1-responsive) cell population. On the other hand, autoradiographic analysis of 125I-FGF-1 binding demonstrated that high affinity FGF receptors were homogeneously distributed on the surface of all hepatocytes. Cross-linking 125I-FGF-1 to the nonstimulated hepatocyte surface indicated that the high affinity FGF receptors comprise two FGF receptors that differ in molecular mass (128 and 79 kDa). Furthermore, the 79-kDa receptor was preferentially down-regulated when the hepatocytes were stimulated with EGF or hepatocyte growth factor. These data suggest that the abundant expression of the 79-kDa FGF receptor on some populations of hepatocytes is involved in their lack of response to FGF-1. The 128- and 79-kDa FGF receptors were assigned as FGFR2 using an antibody specific to the ectodomain of FGFR2, whereas the 79-kDa receptor was not reactive to the antibody against the carboxyl terminus of FGFR2. This 79-kDa FGF receptor was not tyrosine-phosphorylated in response to FGF-1 stimulation, while the 128-kDa FGF receptor was recognized by anti-phosphotyrosine antibody under the same conditions. Also, the heterodimer of 79- and 128-kDa FGF receptors was less tyrosine-phosphorylated than the homodimer of 128-kDa FGF receptors. These data suggest that the 79-kDa FGF receptor inhibits the function of the 128-kDa FGF receptor through their heterodimerization. Thus, we surmise that the difference in response to FGF-1 between the cell populations of normal rat hepatocytes was caused by the different levels of the 79-kDa FGF receptor in each cell population.

Various growth factors, including transforming growth factor-α (TGF-α), 1 epidermal growth factor (EGF), and hepatocyte growth factor (HGF), have been identified as potent heparotrophic factors in primary-cultured hepatocytes (1–3). In agreement with the consequence in vitro, TGF-α (which acts through the EGF receptor) and HGF may contribute to the proliferation of liver parenchymal cells during regeneration in vivo (1, 2). We previously reported that the mitogenic activity of heparin-binding fibroblast growth factor-1 (FGF-1) in rat hepatocytes is comparable to that of EGF and HGF in vitro (4, 5). In addition, Kan et al. (6) reported that the expression of FGF-1 mRNA is immediately induced in the remnant of rat liver after partial hepatectomy, prior to the induction of TGF-α and HGF mRNAs (7, 8). These observations suggest that FGF-1 also promotes the growth of liver parenchymal cells during liver regeneration; however, the significance of FGF-1 in the proliferation of liver parenchymal cells has remained unknown. In this study, we examined the contribution of two potent mitogens, FGF-1 and EGF, to hepatocyte growth in vitro and found that the simultaneous addition of FGF-1 and EGF had a spatiotemporal additive effect upon DNA synthesis. These results suggest that the mitogenicity of FGF-1 in rat hepatocytes is mediated by an FGF-1-responsive cell population, which differs from an EGF-responsive cell population.

Furthermore, to clarify the cause of this difference in mitogenicity of FGF-1 between these hepatocyte subpopulations, we characterized the FGF receptors on rat hepatocytes in primary culture. The well-characterized FGF receptor family consists of two categories as follows: (i) four types of high affinity FGF receptors (FGFR1, FGFR2, FGFR3, and FGFR4) that are 110–150-kDa transmembrane protein-tyrosine kinases with two or three immunoglobulin-like loops and an acidic domain in the extracellular region (9, 10) and (ii) low affinity FGF-binding sites (synergistic with heparan sulfate proteoglycans) that play an important role in the binding of FGF to the high affinity FGF receptors (10, 11). Since exogenous heparin can mimic the function of the low affinity FGF-binding sites for FGF-1 (5) as well as FGF-2 (11), we concluded that the difference in mitogenicity of FGF-1 between hepatocyte subpopulations was not caused by the quantity and/or nature of the heparan sulfate chains on the cell surface when heparin was added to the culture medium. We therefore presume that the

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1 The abbreviations used are: TGF-α, transforming growth factor-α; EGF, epidermal growth factor; HGF, hepatocyte growth factor; FGF, heparin-binding fibroblast growth factor; FGFR, FGF receptor; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; RIPA, radiimmune precipitation assay; KGF, keratinocyte growth factor.
difference in mitogenicity of FGF-1 between hepatocyte subpopulations is due to signal transduction via the high affinity FGF receptor.

The high affinity FGF receptor is essential for growth promotion by FGF stimulation (9, 10, 12). After binding of FGFS, the high affinity FGF receptors form a dimeric complex (13, 14); tyrosine kinase in the FGF receptor is activated; and then the signal for growth promotion is transduced through the phosphorylation of some proteins including the high affinity FGF receptor itself (15). In this study, we detected two forms (128 and 79 kDa) of FGFR2 on rat hepatocytes. The 79-kDa FGF receptor was not activated by FGF-1 stimulation and inhibited the function of the 128-kDa FGF receptor. In addition, it was suggested that the 79-kDa FGF receptor was abundantly expressed in the cell population that lacked a responsiveness to FGF-1. Thus, the roles of the 79-kDa FGF receptor are discussed in relation to its involvement in the difference in mitogenicity of FGF-1 between rat hepatocyte subpopulations.

Experimental Procedures

Primary Culture of Rat Hepatocytes—Adult rat (7–9-week-old female Wistar) hepatocytes were isolated by perfusing the liver in situ with 0.7% collagenase A solution (10). After isolation, the hepatocytes constituted >95% of the preparation. These hepatocytes were plated at 2 × 10^6 cells/0.25 ml/cm² in collagen (type I)-coated 24-well plates with or without coverslips and cultured in Williams’ E medium supplemented with 10% fetal bovine serum (FBS) and 10^-7 M dexamethasone under 5% CO2 in air at 37°C (4, 5). Five hours after cell seeding, the medium was replaced with basal medium (Williams’ E medium supplemented with 10% FBS, 10^-7 M dexamethasone, and 10^-7 M insulin) with or without growth factors (FGF-1, HGF, and/or EGF).

DNA Synthesis Assay—DNA synthesis was determined by the incorporation of [3H]thymidine (ICN) into DNA. The hepatocytes were labeled for 24 h with [3H]thymidine (9.25 kBq/ml, 6.18 GBq/mmol) 30 h after cell seeding. The cultures were fixed, and DNA synthesis was determined in the presence of a 200-fold excess of nonradiolabeled FGF-1. Thus, the roles of the 79-kDa FGF receptor are discussed in relation to its involvement in the difference in mitogenicity of FGF-1 between hepatocyte subpopulations.

Iodination of Recombinant FGF-1—Recombinant human FGF-1 was labeled with 125I using immobilized lactoperoxidase/glucose oxidase (Enzymobeads, Bio-Rad) as described (5, 17). The specific activity of 125I-FGF-1 was ~2 × 10^11 cpm/pmol obtained by trichloroacetic acid precipitation (18), and the total 125I-FGF-1 recovery was >20%. 125I-FGF-1 was stored at 4°C and used within 2 weeks. Binding Assay of 125I-FGF-1—Rat hepatocytes were plated in collagen-coated 24-well plates and cultured as described above. Five hours after cell seeding, 125I-FGF-1 binding to the hepatocytes was assayed on ice as described (19), including heparin extraction to eliminate 125I-FGF-1 binding to low affinity FGF-binding sites. Finally, the cells were solubilized with 1 N NaOH, and the radioactivity of bound 125I-FGF-1 was determined using a γ-counter. Nonspecific binding was determined in the presence of a 200-fold excess of nonradiolabeled recombinant human FGF-1. Heparin was added to 125I-FGF-1 and nonradiolabeled FGF-1 at an FGF-1/heparin ratio of 1:500 (w/w). The density of the cell layer and the number of binding sites were determined according to Scatchard (20), and the protein concentration was estimated by the method of Bradford (21).

Detection of High Affinity Binding Sites for FGF-1 on Single Hepatocytes—Rat hepatocytes were cultured on collagen-coated polystyrene coverslips (Cell dish, Sumitomo Bakelite, Tokyo, Japan) in basal medium after seeding, the cells were washed with 500 µl of binding buffer (PBS, 0.1% bovine serum albumin, and 0.1% dithiothreitol). The cells were incubated on ice for 4 h in 250 µl of binding buffer containing 100 ng/ml 125I-FGF-1 and 50 µg/ml heparin and then washed four times with PBS containing 0.1% bovine serum albumin. To measure nonspecific binding, a 200-fold excess of nonradiolabeled recombinant human FGF-1 was added. After incubation in 500 µl of binding buffer containing 250 µg/ml heparin on ice for 1 h, the cells were fixed for 25% glutaraldehyde on ice for 30 min, washed, air-dried, and mounted on microscope slides. The slides were dipped in photographic emulsion (NR-M2, Konika, Tokyo, Japan) exposed for 4 weeks at ~80 °C developed with Kodak D-19, fixed, and stained with 0.2% eosin Y. The silver grains in single cells were scored under a light microscope, and the size of each cell was estimated using a microscopical cross-sectioned micrometer. The background silver grains were scored in an area where hepatocytes did not adhere. The significance of the difference in the number of nonspecifically bound grains by Welch’s t test because the variances of both types of binding differed in the F-test. The distribution of both categories of binding was analyzed by testing the goodness-of-fit to the normal distribution.

Cross-linking of 125I-FGF-1 to Rat Hepatocytes—Rat hepatocytes in basal medium were incubated in 6-cm tissue culture dishes coated with collagen. Thirty hours after cell seeding, the cells were incubated with 5 ml of binding buffer and incubated for 4 h on ice in 2 ml of binding buffer containing 10 ng/ml 125I-FGF-1 and 5 µg/ml heparin. After washing twice with PBS, the cells were incubated for 30 min in PBS containing the cross-linker disuccinimidyl suberate (1 µM). The cross-linking reaction was stopped with 0.2 ml of stop buffer (50 mM Tris-Cl, pH 7.3, 0.2 M glycine, 2 mM EDTA, 1 µg/ml aproptinin, and 10 µg/ml trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64)). After decanting the mixture, 1 ml of PBS, 1 µg/ml aproptinin, and 10 µM E-64 was added, and the cells were scraped and collected by centrifugation. Cell pellets were solubilized in 50 µl of 2× Laemmli’s sample buffer (0.12 M Tris-Cl, pH 6.8, 2% SDS, 20% glycerol, 0.001% bromphenol blue, and 10% mercaptoethanol) and boiled for 5 min. SDS-PAGE was carried out by Laemmli (22). After electrophoresis on 7% polyacrylamide gels, the gels were stained with Coomassie Brilliant Blue to verify that all samples contained approximately equal amounts of protein. After drying, the gels were autoradiographed and examined using a Fuji BA-100 radioimage analyzer.

Dimerization of FGF Receptors on Rat Hepatocytes—Rat hepatocytes were incubated in Williams’ E medium supplemented with 10% FBS and 10^-7 M dexamethasone in 6-cm culture dishes for 2 h; sodium orthovanadate (50 µM) was then added, and the cells were further cultured for another 2 h. After washing twice with cold Williams’ E medium (10 ml), the cells were incubated on ice for 4 h in basal medium supplemented with 30 µM HEPES, pH 7.4, 1 µM sodium orthovanadate, 10 µg/ml aprotinin, and 10 µg/ml E-64. After washing twice with PBS, the cells were incubated at 37°C for 5 min. After washing twice with 0.1 M sodium orthovanadate in PBS, the cells were incubated on ice for 30 min in PBS containing 1 mM disuccinimidyl suberate and phosphatase inhibitors (50 mM sodium fluoride, 0.1 M sodium orthovanadate, 30 mM sodium pyrophosphate, and 5 mM EDTA). The cross-linking reaction was terminated with stop buffer. The 125I-FGF-1-cross-linked immunocomplexes were precipitated with protein A-Sepharose beads. FGF-1-containing immunocomplexes were precipitated with protein A-Sepharose beads. The immunoprecipitates were washed twice in 2× Laemmli’s sample buffer and separated on a 5% SDS-polyacrylamide gel. Thereafter, autoradiographs were examined using a Fuji BAS-2000 radioimage analyzer.

Immunoprecipitation of FGF-FGF-1 Complex by Anti-FGF Receptor Antibody—Rat hepatocytes were cultured for 5 h and cross-linked with 125I-FGF-1 as described under “Cross-linking of 125I-FGF-1 to Rat Hepatocytes”. The cross-linked hepatocytes were lysed in 1 ml of RIPA buffer at 4°C for 30 min. The lysates obtained by centrifugation (10,000 × g, 30 min) was cleared with protein A-Sepharose beads (Pharmacia Biotech Inc.) at 4°C. After washing with PBS containing 1 M NaCl, the beads were washed in 2 ml of RIPA buffer containing phosphatase inhibitors. The proteins bound on the beads were boiled in 2× Laemmli’s sample buffer and separated on a 5% SDS-polyacrylamide gel. Thereafter, autoradiographs were obtained as described above.

Immunoprecipitation of FGF-FGF-1 Complex by Anti-FGF Receptor Antibody—Rat hepatocytes were cultured for 5 h and cross-linked with 125I-FGF-1 as described under “Cross-linking of 125I-FGF-1 to Rat Hepatocytes”. The cross-linked hepatocytes were lysed in 1 ml of RIPA buffer at 4°C for 30 min. The lysate obtained by centrifugation (10,000 × g, 30 min) was cleared with protein A-Sepharose beads (Pharmacia Biotech Inc.). Following incubation with 5 µg of anti-FGF-FGF polyclonal antibody (Santa Cruz Biotechnology Inc.) and protein A-Sepharose beads, the samples were dried, and the precipitated peptides consisting of 15–17 residues in each carboxyl-terminal region of FGFR1, FGFR2, FGFR3, and FGFR4 at 4°C for 4 h, the immunocomplexes were precipitated with protein A-Sepharose beads. The immunoprecipitates were washed five times with RIPA buffer and boiled in 2× Laemmli’s sample buffer before electrophoresis (7% SDS-polyacrylamide gel). Autoradiographs were obtained as described above.

Immunoprecipitation of FGF Receptor by Anti-FGF Receptor Antibodies—Rat hepatocytes were plated in collagen-coated 6-cm culture dishes, and the medium was replaced with methionine-free Williams’ E medium supplemented with 10% FBS, 10^-7 M insulin, and 10^-7 M dexamethasone 5 h after cell seeding. The cells were incubated with [35S]methionine (3.7 MBq/ml) 7 h after cell seeding. Thirty hours after cell seeding, the cells were washed twice with PBS and lysed in RIPA buffer at 4°C for 30 min. The cell lysate was obtained by centrifugation.
recombinant human FGF-1 supplemented with 5 μg/ml heparin (open triangles), or both growth factors (open squares). The cells were labeled for 3 h with \(^{3}H\)thymidine and then harvested. Each point represents DNA synthesis at the time of harvest with the mean ± S.E. of triplicate cultures.

at 10,000 × g at 4 °C for 30 min. The lysate containing 3 × 10^6 dpm of chloroacetate acid-precipitable counts was cleared with protein G-Sepharose (Pharmacia Biotech Inc.). After incubation at 4 °C for 10 h with 2 μl of anti-FGFR antisera (the kind gifts of Drs. M. Kan and W. L. McKeehan, Texas A & M University) that recognize each ectodomain of FGFR2 and FGFR4, the immunocomplexes were precipitated with protein G-Sepharose beads. The immunoprecipitates were washed five times with RIPA buffer and boiled in 2 × Laemmli's sample buffer. The immunoprecipitated proteins were resolved by SDS-PAGE (7% gel) and detected by fluorography using Enlightning (DuPont NEN).

Immunoprecipitation of FGFR:FGF-1 Complexes by Anti-phosphotyrosine Antibody—Rat hepatocytes were cultured for 5 h, cross-linked with \(^{125}I\)-FGF-1, and lysed as described under “Dimerization of FGFR Receptors on Rat Hepatocytes”. The cell lysates were cleared with protein-A Sepharose and then incubated with 5 μg of anti-phosphotyrosine polyclonal antibody (Upstate Biotechnology Inc.) at 4 °C for 8 h. The immunocomplexes were adsorbed to protein A-Sepharose beads and washed five times with RIPA buffer supplemented with phosphatase inhibitors. The complexes bound to the beads were boiled in 2 × Laemmli's sample buffer, separated on a 7% SDS-polyacrylamide gel, and autoradiographed as described above.

Materials—Human heparin-binding growth factor (HBGF)-1α was used as recombinant human FGF-1 (23). Recombinant human HGF was a gift of Dr. Yasufumi Sato (Oita Medical School, Oita, Japan). FBS was purchased from Irvine Scientific. Mouse EGF, insulin, dexamethasone, and heparin were from Sigma. Collagenase was from Wako Pure Chemicals (Osaka, Japan).

RESULTS

Additive Stimulation of DNA Synthesis by EGF and FGF-1 in Rat Hepatocytes—We previously reported that the mitogenic effects of EGF and recombinant human FGF-1 supplemented with heparin on the DNA synthesis of rat hepatocytes in primary culture are essentially equivalent (4). To clarify the significance of these two growth factors on hepatocyte growth, we examined the combinational effects of EGF and FGF-1 by measuring the time course of DNA synthesis by rat hepatocytes. In the presence of either FGF-1 or EGF, DNA synthesis by rat hepatocytes in primary culture became relatively synchronized (Fig. 1). Fig. 1 shows that the first and main peak of \(^{3}H\)thymidine incorporation appeared 30–54 h after seeding in both EGF- and FGF-1-stimulated cells and that some minor peaks appeared thereafter. The time courses of the response to each growth factor were similar, and the stimulation of DNA synthesis by each growth factor was comparable throughout the first S phase. Furthermore, the effect of EGF and FGF-1 on DNA synthesis in hepatocytes was additive at almost all points during the first peak.

The populations of hepatocytes that were stimulated by various concentrations of FGF-1 and/or EGF were determined by labeling the nuclei with \(^{3}H\)thymidine. As shown in Table I, EGF and FGF-1 individually induced ~55% labeling of hepatocyte nuclei at 10 ng/ml, the concentration at which each growth factor exerted their maximal activities as described (4). Even when the concentration of each growth factor was increased up to 100 ng/ml, the labeling indices were not increased. However, the number of nuclei labeled by a combination of these growth factors was enhanced to 80% at a 10 ng/ml concentration of each growth factor. The labeling indices were not changed by increasing the concentration of FGF-1 and/or EGF up to 100 ng/ml. These data indicated that 55% of the hepatocytes were responsive to FGF-1, although FGF-1 did not exert its mitogenic activity on the remaining hepatocytes.

High Affinity Binding Sites for FGF-1 on Single Hepatocytes—To determine the cause of the difference in mitogenicity of FGF-1 described above, we characterized FGF-1 binding to hepatocytes by Scatchard analysis and microautoradiography. Specific high affinity binding was saturable at concentrations of \(^{125}I\)-FGF-1 above 4 nM, and it resulted in a linear Scatchard plot (Fig. 2). The apparent dissociation constant and the number of binding sites were estimated as 150–400 fm and 25–35 fmol/mg of protein, respectively. On the other hand, when the cells were not extracted with heparin, specific FGF-1 binding with an apparent dissociation constant of >1.5 nM appeared (data not shown). These data indicated that the low affinity binding sites for FGF-1 were completely eliminated by heparin extraction even when \(^{125}I\)-FGF-1 was added up to 7.5 nM (~120 ng/ml).

For the high affinity binding sites for FGF-1 characterized above, we developed a method of detecting high affinity FGF-1-binding sites on single rat hepatocytes in primary culture. We added \(^{125}I\)-FGF-1 (100 ng/ml) to saturate the binding of FGF-1 to its high affinity receptor to cultured hepatocytes and then extracted them with heparin to eliminate the low affinity binding. After microautoradiography, grains on single cells were scored, and the cell area was measured under the microscope (Fig. 3, A and B). As shown in Fig. 3C, the grain density of total \(^{125}I\)-FGF-1 binding was distributed as one peak. This fits to the normal distribution, which had a mean value and standard deviation of 10.36 ± 2.2 grains/100-μm² cell area. The grain density of the background was 0.84 ± 0.27 grains/100-μm² cell area, and none of the cells corresponded to this background value. However, the grain density on the cells was decreased to 1.70 ± 0.72 grains/100-μm² cell area when excess nonradioabeled FGF-1 was added (Fig. 3D). The cell area associated with total binding was 2330 ± 830 μm², and that with nonspecific binding was 1830 ± 680 μm², indicating that the areas were essentially similar. Thus, the grain density of total binding was significantly greater than that of nonspecific binding (p < 0.01).

| Labeling index (%) | 0 ng/ml EGF | 10 ng/ml EGF | 100 ng/ml EGF |
|--------------------|-------------|--------------|--------------|
| FGF-1              | 3.2 ± 0.51  | 54.3 ± 1.55  | 55.0 ± 2.52  |
| 10 ng/ml           | 53.8 ± 0.69 | 80.1 ± 1.25  | 80.19 ± 1.75 |
| 100 ng/ml          | 53.03 ± 2.55| 82.69 ± 0.03 | 79.98 ± 0.65 |

The labeling index is defined as the percentage of nuclei labeled with \(^{3}H\)thymidine. At least 600 hepatocytes with normal morphology were counted for each sample. Recombinant human FGF-1 was added with heparin at a ratio of 1.500 (w/w). Experimental conditions are described under “Experimental Procedures.” Values represent the means ± S.D. of duplicate cultures.
Effects of EGF and HGF on Binding of FGF-1 to FGF Receptors in Hepatocytes—To confirm the effect of EGF or HGF on FGF-1 binding, we analyzed alterations in the FGF receptor on hepatocytes stimulated with EGF or HGF by Scatchard analysis and by cross-linking 125I-FGF-1 to its receptors. The binding of 125I-FGF-1 was not competed by EGF or HGF (Fig. 4A). It was also indicated that the FGF receptors corresponding to both bands were not shared by EGF or HGF since EGF or HGF did not inhibit the binding of FGF-1 (Fig. 4A). Stimulation with EGF (10 ng/ml) or HGF (10 ng/ml) for 25 h reduced the intensities of both bands (Table II). Whereas the radiointensity of the 95-kDa band was reduced to 65–75% by treatment with EGF or HGF, a greater decrease in that of the 95-kDa band was observed. As shown in Table II, the 95-kDa band was decreased ∼2-fold compared with the 144-kDa band by stimulation with EGF or HGF. These data indicated that the 95-kDa band was preferentially down-regulated by stimulation with EGF or HGF.

Characterization of FGF Receptors on Rat Hepatocytes—To clarify the molecular aspects of the two FGF receptors in rat hepatocytes, we immunoprecipitated the FGFR-FGF-1 complexes using anti-FGFR antibodies that recognize the respective carboxyl-terminal domains of FGFRs. Fig. 6A shows that the anti-FGFR2 antibody immunoprecipitated the 144-kDa FGFR-FGF-1 complex, while the 95-kDa FGFR-FGF-1 complex was not recognized by this antibody (Fig. 6A). In addition, no FGFR-FGF-1 complexes were detected by immunoprecipitation

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**Fig. 2. Specific binding of FGF-1 to rat hepatocytes in primary culture.** Thirty hours after cell seeding, the equilibrium binding of 125I-FGF-1 to hepatocytes was analyzed as described under "Experimental Procedures." The background in the presence of a 200-fold excess of nonradiolabeled FGF-1 was subtracted from the total binding in the absence of nonradiolabeled FGF-1. Inset, Scatchard plot of FGF-1 binding to rat hepatocytes.

**Fig. 3. Detection of high affinity binding sites for 125I-FGF-1 on rat hepatocytes in primary culture.** Micrograph of an intact single hepatocyte showing silver grains (A and B) and histograms of their grain density (C and D) are represented. Thirty hours after seeding, the binding of 100 ng/ml 125I-FGF-1 to hepatocytes in the presence of 50 μg/ml heparin proceeded without (A and C) or with (B and D) a 200-fold excess of nonradiolabeled FGF-1. After heparin extraction, the cells were fixed, and 125I-FGF-1 bound to the high affinity FGF receptor was visualized by microautoradiography. The silver grains on single hepatocytes were scored (50 cells), and the cell area was also measured. The bar (A and B) indicates a 10-μm scale. Vertical dashed lines (C and D) indicate the mean value of the grain density in the background area.

**Fig. 4. Effect of EGF or HGF on binding of FGF-1 to high affinity binding sites on rat hepatocytes in primary culture.** A, competitive FGF receptor binding with EGF or HGF. Rat hepatocytes were cultured in basal medium 5–30 h after cell seeding. The binding of 125I-FGF-1 (0.7 nM) to its high affinity binding sites was measured in the presence of nonradiolabeled FGF-1 (closed circles), EGF (40 nM; open triangles), or HGF (6 nM; ×). Data represent the means ± S.D. of duplicates. B, Scatchard analysis of FGF-1 binding to rat hepatocytes stimulated with EGF or HGF. Hepatocytes were incubated without (circles) or with 10 ng/ml EGF (triangles) or 10 ng/ml HGF (×) 5–30 h after cell seeding. 125I-FGF-1 binding to hepatocytes proceeded 5 h (open circles) and 30 h (closed circles, triangles, and ×) after cell seeding as described under "Experimental Procedure." B/F, bound/free.
with the carboxyl-terminal antibodies of FGFR1, FGFR3, and FGFR4 (Fig. 6A). These results indicated that the 128-kDa FGF receptor (corresponding to the 144-kDa FGF receptor) was an FGFR2 containing its carboxyl terminus. On the other hand, the 128- and 79-kDa proteins were specifically immunoprecipitated with antibodies to each carboxyl terminus of FGFR1, FGFR3, and FGFR4 (Fig. 6B). These data indicated that the 79-kDa protein was an FGFR2 containing its carboxyl terminus, whereas the carboxyl-terminal domain of FGFR2 was not included in this 79-kDa FGF receptor.

We examined the oligomerization and tyrosine phosphorylation of the two FGF receptors to clarify whether they were involved in signal transduction by FGF-1 stimulation. After the binding of [125I]-FGF-1 to cell-surface FGF receptors on ice for 4 h, the cells were incubated at 37°C for 5 min to potentially activate signal transduction pathways; [125I]-FGF-1 and FGF receptors were then cross-linked, and the formed FGFR-FGF-1 complexes were adsorbed to wheat germ agglutinin-Sepharose or immunoprecipitated by anti-phosphotyrosine antibody. These complexes were analyzed by SDS-PAGE followed by autoradiography. Fig. 7A shows that the dimeric form of the FGFR-FGF-1 complexes migrated as a 220–320-kDa band together with two monomeric forms (144- and 95-kDa bands). The broad band of the dimeric complex corresponded to a combination of a homodimer of the 144-kDa FGFR-FGF-1 complex and a heterodimer of the 144- and 95-kDa FGFR-FGF-1 complexes. It is likely that the radiointensities of the homodimer and heterodimer were comparable. The homodimeric form of

### Table II
Down-regulation of [125I]-FGF-1-cross-linked bands by treatment of rat hepatocytes with HGF or EGF
These data were obtained by a quantitative radiograms analysis of the raw data for Fig. 5.

| Growth factor | Intensitya | 144-kDa band | 95-kDa band | 144-kDa/95-kDa ratio |
|---------------|------------|--------------|-------------|---------------------|
| None          | 100.0      | 89.4         | 0.894       |
| None (+ competitor)b | 10.6     | 11.4         | 0.422       |
| HGF           | 65.0       | 27.4         | 0.422       |
| EGF           | 75.2       | 35.7         | 0.474       |

a The radiointensity of the 144-kDa band in nontreated cells is defined as 100%.
b The band intensities of [125I]-FGF-1-cross-linked bands in the presence of 200-fold nonradiolabeled FGF-1 are represented.
the 95-kDa FGF receptor (FGF-1 complex was not found. These bands disappeared in the presence of excess nonradiolabeled FGF-1 (Fig. 7A). The 144-kDa band of the FGF receptor complex was immunoprecipitated by anti-phosphotyrosine antibody, whereas the 95-kDa band of the FGF receptor complex was not detected by this antibody (Fig. 7B). In addition, a 280–300-kDa band corresponding to the homodimeric form of the 144-kDa FGF receptor complex was strongly tyrosine-phosphorylated. The heterodimer (220–280-kDa band) of the 144- and 95-kDa FGF receptor complexes was recognized as a weak band by anti-phosphotyrosine antibody. These bands were not detected when the incubation at 37 °C for 5 min was eliminated (data not shown).

**DISCUSSION**

Some mitogens, such as TGF-α, EGF, and HGF, contribute to the proliferation of hepatocytes (1–3). Recently, we demonstrated that FGF-1 is also a strong mitogen for hepatocytes in vitro (4, 5). However, the significance of FGF-1 in the cooperative effects with other hepatocyte mitogens has not been addressed. In this study, we demonstrated that two major hepatocyte mitogens, FGF-1 and EGF, additively stimulated DNA synthesis in rat hepatocytes as follows. (i) Each growth factor had a saturating level of stimulating DNA synthesis in hepatocytes. (ii) Even when the DNA synthesis was stimulated up to the maximal level by one of these growth factors, the stimulation of the other growth factor was additive. (iii) This stimulation was additive throughout the first S phase. (iv) The fractions of hepatocytes that responded to FGF-1, EGF, and both growth factors were about 55, 55, and 80%, respectively. These results indicate that 80% of the hepatocytes consist of FGF-1- and EGF-responsive cells. Furthermore, these results suggest that the mitogenities of FGF-1 and EGF are mediated by the different cell populations corresponding to each growth factor. This suggestion not only supports the hypothesis that the EGF- and HGF-responsive cell populations are contained in rat hepatocytes (24–27), but also adds a new FGF-1-responsive cell population that is detectable under the conditions that we used.

Kan et al. (6) have demonstrated that EGF can neutralize or mask the mitogenic effect of FGF-1 when the mitogenic activity of FGF-1 in hepatocytes is much lower than that of EGF in serum-free cultured hepatocytes. As we reported, however, the mitogenic activity of FGF-1 in hepatocytes is comparable to that of EGF and HGF under conditions that may better reflect the milieu of hepatocytes in vivo (4, 5). We demonstrated here that excess EGF does not inhibit the FGF-1 activity and that EGF acts additively with FGF-1. These data suggest the sequential proliferation of respective cell populations after liver injury is due to the sequential expression of FGF-1 and EGF/FGF-α, respectively, rather than the earlier speculation that expressed EGF/FGF-α inhibited liver parenchymal cell proliferation that is promoted by FGF-1 (6).

We investigated the cause of the difference in mitogenicity of FGF-1 between FGF-1-responsive and -unresponsive cell populations in rat hepatocytes. Using a modified method of the FGF-1 binding assay, high affinity binding sites on a single hepatocyte were detected, and the difference in the density of these sites on each hepatocyte was statistically analyzed. It was revealed that the silver grains indicate the actual high affinity binding sites for FGF-1 based on the following reasons. (i) The binding of FGF-1 was maintained after heparin extraction and had a dissociation constant of 150–400 pm, which is remarkably different from that of the low affinity binding of FGF-1 (K_{d} > 1.5 nM); and (ii) the grain density was significantly reduced to the background level in the presence of excess nonradiolabeled FGF-1 (p < 0.01). In addition, since the Scatchard analysis confirmed that >90% of the high affinity binding sites for FGF-1 were occupied by the radiolabeled ligands at the concentration of 125I-FGF-1 used here, we concluded that the silver grains represent most of the high affinity binding sites for FGF-1. If the difference in response to FGF-1 between hepatocyte subpopulations was caused by the presence or absence of the high affinity binding sites for FGF-1, two peaks would be observed in the histogram of the grain density. One peak would reflect the absence of the high affinity binding sites for FGF-1, and the second peak would reflect their presence. However, a peak corresponding to cells without the high affinity binding sites for FGF-1 was not observed, and the grain density of high affinity binding sites for FGF-1 was normally distributed. These results suggest that the grains are homogeneously distributed on each hepatocyte. Thus, we concluded that high affinity binding sites for FGF-1, which are regarded as high affinity FGF receptors, exist on the surface of all hepatocytes. It is suggested that the difference in mitogenicity of FGF-1 between FGF-1-responsive and -unresponsive cell populations is not due to the presence or absence of high affinity FGF receptors.

Despite the existence of high affinity FGF receptors in all hepatocytes, only 55% of the hepatocytes responded to FGF-1. We then examined the effect of EGF and HGF, which were thought to have other cell populations in hepatocytes (24–28), on the binding of FGF-1 to hepatocytes. We confirmed that EGF and HGF did not interfere with the binding of FGF-1 to FGF receptors on rat hepatocytes. After incubation with EGF or HGF for 25 h, however, the number of FGF receptors on hepatocytes decreased to half, but the FGF-1 binding affinity was not affected. Thus, FGF receptors were down-regulated by EGF and HGF. These results, in combination with the implications that the mitogenicity of EGF is mediated by the EGF-responsive cell population, which differed from the FGF-1-responsive cell population, suggest that FGF receptors in EGF-responsive cells are down-regulated by EGF. It is possible to speculate that HGF induces the down-regulation of FGF receptors in HGF-responsive cells by a similar manner to that of EGF since HGF and FGF-1 additively stimulate the DNA synthesis of rat hepatocytes (5). Furthermore, the down-regulation of FGF receptors by EGF or HGF was confirmed by cross-linking studies using 125I-FGF-1. A 79-kDa FGF receptor was abundantly found on the surface of nonstimulated hepatocytes together with the 128-kDa FGF receptor (Table II). However, the 79-kDa FGF receptor was preferentially down-regulated by EGF or HGF stimulation. As discussed above, these results suggest that this down-regulation of the 79-kDa FGF receptor occurred in EGF- and HGF-responsive cells. This raises the possibility that, under nonstimulating conditions, the 79-kDa FGF receptor is a dominant form in the EGF- or HGF-responsive cell populations; these populations also being synonymous with the FGF-1-unresponsive cell population. In contrast, the 128-kDa FGF receptor is likely to be dominant in the FGF-1-responsive cell population since the reduction level of the 128-kDa FGF receptor was less than that of the 79-kDa FGF receptor when the cells were stimulated with EGF or HGF. Therefore, it is suggested that the abundant expression of the 79-kDa FGF receptor is involved in the lack of responsiveness to FGF-1 in the FGF-1-unresponsive cell population.

The mRNA expression of FGR2, but not that of FGR1, has been confirmed in primary-cultured rat hepatocytes (29), and the FGR4 mRNA is not expressed in adult rat liver (30). In addition, a full-length FGR2 (135 kDa) and an amino-terminal truncated FGR2 (115 kDa) with a deleted first immunoglobulin-like loop and acidic domain have been described (9, 10), and it has been reported that both of these FGR2 receptors have similar activities in FGF-1 binding and that these...
FGF2 receptors are comparably activated by FGF-1 (31). Evidence has also been provided that FGF-1 binds to the second immunoglobulin-like loop of FGFR2 molecules (32). Furthermore, many species of truncated variants of FGFR2 in the carboxyl-terminal region have been reported (33). Here, we demonstrated that the 128- and 79-kDa FGF receptors were FGFR2 using an antibody against the ectodomain of FGFR2. The 128-kDa FGF receptor was also recognized by the antibody to the carboxyl terminus of FGFR2; however, this antibody did not react with the 79-kDa FGF receptor. Neither the 128- nor the 79-kDa FGF receptor reacted with any polyclonal antibodies against the carboxyl-terminal regions of FGFR1, FGFR3, and FGFR4. These results indicate that the 128-kDa FGF receptor is an FGFR2 with its carboxyl terminus intact, whereas the 79-kDa FGF receptor is a carboxyl-terminal truncated FGFR2. In addition, the molecular mass of the 79-kDa FGF receptor suggests that the cytoplasmic domain including the kinase domains is likely to be deleted in this form. This is further supported by the observation that when the cells were stimulated with FGF-1, the 79-kDa FGF receptor was not tyrosine-phosphorylated, whereas the phosphorylation of the 128-kDa FGF receptor was detected. Thus, the 79-kDa FGF receptor is not activated by FGF-1 stimulation upon FGF-1 binding to its extracellular domain. Two types of FGFR2 (BEK and keratocytokeratin growth factor (KGF) receptor) that differ in the third immunoglobulin-like loop have been identified in human, mouse, and rat (34–36). Although FGF-1 binds to both types of FGFR2, KGF reacts with only the KGF receptor (37).

Reverse transcription-polymerase chain reaction analysis of types of FGFR2, KGF reacts with only the KGF receptor (37). Tyrosine phosphorylation of the protein corresponding to the size of the homodimer of the 128-kDa FGF receptor was more intense, while that of the size of the heterodimer of the 128- and 79-kDa FGF receptors was less intense. The results suggest that the 79-kDa FGF receptor is an inactive FGF receptor, even when the heterodimers with the 128-kDa FGF receptor are formed. The data further imply that this 79-kDa receptor, even when the heterodimers with the 128-kDa FGF receptor are formed. The data further imply that this 79-kDa receptor is an inactive FGF receptor and suggest that it is controlled by dominant-negative FGF receptor.

In summary, we have demonstrated that there are FGF-1-responsive and -unresponsive cell populations in primary-cultured rat hepatocytes and that this difference in mitogenicity of FGF-1 is likely to depend on a difference in the expression level of an inactive 79-kDa FGF receptor in individual cells. Further investigations of the expression and function of the 79-kDa FGF receptor are necessary to elucidate the pathophysiological significance of FGF-1 in growth control of liver parenchymal cells in vivo.

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Identification of a 79-kDa Heparin-binding Fibroblast Growth Factor (FGF) Receptor in Rat Hepatocytes and Its Correlation with the Different Growth Responses to FGF-1 between Hepatocyte Subpopulations
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