Identification and Characterization of Receptor for Mammalian Hepatopoietin That Is Homologous to Yeast ERV1*

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Hepatopoietin (HPO) is a novel polypeptide mitogen specific for hepatocytes and hepatoma cell lines, which is derived from liver and supports its regeneration. To determine whether HPO acts via a receptor-based signal transduction, recombinant human hepatopoietin was labeled by iodination and used to characterize its binding activity by specific displacement test and Scatchard analysis in primarily cultured rat hepatocytes and human hepatoma Hep-G2 cells. The binding was saturable and specific because it was replaceable by HPO but not by epidermal growth factor, transforming growth factor-α, or insulin. Scatchard analysis indicated the presence of a single class of high affinity receptor with dissociation constant (Kd) of 2 and 0.7 μM, and a receptor density of about 10,000 sites/cell and 55,000 sites/cell in the rat hepatocytes and human hepatoma cells, respectively. The Kd values were consistent with the half-maximum dose of HPO activity. Affinity cross-linking of the receptor with 125I-HPO revealed a polypeptide of molecular mass approximately 90 kDa by SDS-polyacrylamide gel electrophoresis. Thus, the molecular mass of the HPO receptor was calculated to be about 75 kDa. These data demonstrated the existence of an HPO receptor in hepatocytes and hepatoma cells, which may account for biological effect.

Previous studies implicate that a small molecule derived from liver itself specifically stimulates hepatocytes proliferation and supports liver regeneration (1–3). In 1975, LaBrecque et al. (3) reported that in the liver of a weaning rat and the regenerating liver of a partially hepatectomized rat, there exists hepatic stimulator substance (HSS) that could specifically stimulate DNA synthesis in hepatic cells. Other groups have also carried out extensive research on HSS derived from other species (2). At the same time, experiments and clinical research on human fetal liver cells demonstrated its therapeutic effect on hematopoietic diseases and severe liver diseases (2, 4). Since the 1980s, we began to isolate and purify the effective component from fetal liver. We identified hepatic stimulatory activity in the fraction with molecular size ranging from 10 to 30 kDa of human fetal liver lysate (5–7). The activity was target-specific, which was different from various well known nonspecific hepatic stimulators such as insulin, EGF, insulin-like growth factor, and TGF-α. The characteristics of the effective component derived from human fetal liver were consistent with those of HSSs derived from other species, suggesting that the effective component could be the human-derived homologue of the animal’s HSS. Then, we purified this activity and demonstrated that the biological activity of its pure form is identical to those of the crude form and consistent with those of animal-derived HSSs, but evidently different from those of serum-derived hepatocyte growth factor (8). The factor was named as hepatopoietin (HPO). Later, we proved that HPO is encoded by mRNA of fetal liver (9) and further cloned (10) its full-length cDNA, encoding a 15.1-kDa protein from the cDNA library of human fetal liver, which is of 87% homology with rat augmenter of liver regeneration cDNA (11) and is identical to the human homologue of yeast ERV1 (essential for respiration and viability) cDNA (12). Recombinant human hepatopoietin (rhHPO) can stimulate proliferation of hepatocytes and hepatoma cells in vitro (13). Furthermore, in animal models, rhHPO promotes regeneration and recovery of damaged hepatocytes and rescues acute hepatic failure in vivo (13, 14). Thus, HPO is a growth factor important in liver regeneration. However, the signaling mechanism of HPO has been unclear. It remained unknown whether HPO bind to a specific receptor in cell membrane then initiate a corresponding cytoplasmic signal transduction pathway and mediate its biological effect on hepatocytes. We report here the identification and characterization of the receptor for HPO, which may give some insight into the mechanisms of its biological action.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were purchased from Sigma Chemical Company. Sodium-125I and hypercoat emulsions were purchased from Amersham Pharmacia Biotech. Reagents for SDS-polyacrylamide gel electrophoresis (PAGE) was obtained from Bio-Rad. Tissue culture reagents were purchased from Life Technologies, Inc. EGF and TGF-α were purchased from Earth Chemical Corp. rhHPO was expressed in Escherichia coli and prepared with high purity (≥95%) as described previously (15). HepG2 was from the human hepatoma cell line.

HPO Biological Activity Test—5 × 10⁴ HepG2 cells were plated on a 96-well plastic culture plate and cultured in Dulbecco’s modified Eagle’s medium containing 10% calf serum, 10 μg/ml insulin, and 10 μg/ml dexamethasone under 5% CO₂ and 30% O₂ in air at 37 °C. After 12 h, various concentrations of human HPO were added with EGF and TGF-α as the positive controls. The incubation time was for 24 h, then 0.5 μCi [3H]thymidine deoxyriboside was added to each plate well for 3 h. Radioactivity of cells was measured in a γ-counter after detachment of the monolayer by incubation for 10–20 min with 200 μl of 0.25% trypsin.
solution (16, 17).

Isolation and Primary Culture of Rat Hepatocytes—Parenchymal hepatocytes were isolated from adult male Wistar rats weighing 150–200 g by in situ perfusion of the liver with collagenase (18–20), further purified by Percoll density gradient centrifugation. Cell viability was measured by testing exclusion of trypan blue, and cell preparations showing over 90% viability were used to culture. Cells were plated at a density of 10^5 cells/ml and cultured in Dulbecco's modified Eagle's medium containing 10% calf serum, 10 mM insulin, and 10 mM dexamethasone under 5% CO₂ and 30% O₂ in air at 37 °C.

Radioiodination of HPO—rhHPO was iodinated by chloramine-T methods (21–24). Briefly, 15 µl of 50 mM sodium phosphate buffers (pH 7.0) and 0.5 µCi sodium-125I were added to a siliconized tube containing 5 µg of HPO. The reaction was started by adding 10 µl of chloramine-T solution (1 mg/ml) for 1 min under room temperature. After halting the reaction using 20 µl of ending solution (50 mM Na-acetyl-l-tirosine (Sigma), 0.01 mM sodium metabisulfite, 10% glycerol, 0.1% xylene cyanole, 0.1 mM sodium phosphate buffer), 125I-HPO was separated from free iodides by gel filtration on a column (20 × 1.0 cm) of Sephadex G-25 (Amersham Pharmacia Biotech) equilibrated with PBS and 0.1% bovine serum albumin (Sigma), and the fractions containing 125I-HPO were pooled.

125I-HPO Binding Assay—Adult rat hepatocytes and human hepatoma cells were cultured for 24 h then the monolayers were washed with binding buffer (20 mM HEPES, 0.1% bovine serum albumin/ Hanks, pH 7.0) and pre-incubated in the presence of the same buffer for 30 min at 25 °C. After equilibration, fresh ice-cold binding buffer containing various concentrations of 125I-HPO with or without excess amounts of unlabeled HPO was added as indicated. Incubation was run for 1 h at 25 °C with constant shaking. The monolayer was washed 5 times with ice-cold buffer. Radioactivity of 125I-HPO to cells was measured in a γ-counter after detachment of the monolayer with 0.25% trypsin solution (24–27). All binding experiments were done in triplicate.

Cross-linking of 125I-HPO to Its Receptor—After binding of 125I-HPO to hepatocytes, each well was washed 5 times with cold PBS. Freshly prepared bis-sulfosuccinimidyl suberate (Sigma) was then added to the final concentration of 0.25 mM to each well containing 1 ml of PBS (26–28). The dishes were incubated at 4 °C for 15 min on a shaker platform. After cross-linking, each plate was washed twice with cold PBS then placed in 1 ml of cold detachment buffer consisting of 10 mM Tris (pH 7.4), 1 mM EDTA, 0.25% sucrose, and 1 mM phenylmethylsulfonyl fluoride. The cells were scraped from the plates and pelleted in a microcentrifuge at 10,000 × g for 1 min. Cell pellets were solved in 50 µl of nonreducing SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, and 0.002% bromphenol blue) containing 1% Triton X-100, boiled for 5 min, and subjected to 12.5% SDS-PAGE. Gel were dried and exposed to Kodak film at −70 °C for 5–7 days.

RESULTS

Biological Activity of 125I-HPO—To rule out the effect of iodination on rhHPO, molecular weight and biological activity of 125I-HPO were detected. As shown in Fig. 1A, 125I-HPO had the similar molecular weight as the unlabeled HPO measured by SDS-PAGE and autoradiography. Otherwise, 125I-HPO retained the same biological activity (stimulation of hepatocyte proliferation) as the unlabeled human HPO as shown in Fig. 1B. Taken together, the results above demonstrated that iodination did not change the characteristics of natural human HPO, and also indicated the feasibility that 125I-HPO can be used for identification of HPO receptor.

Identification of a High Affinity Receptor for HPO—Fig. 2, A and C show typical saturation curves of 125I-HPO binding to cultured hepatocytes. Specific binding of HPO was saturated at about 1.5 pm. Scatchard analysis resulted in a rectilinear plot, thereby suggesting the presence of a single class of high affinity binding sites, i.e. the existence of a receptor of HPO. The Kd value and the number of HPO receptors calculated from the Scatchard plots were 2 pm and 50,000 sites/cell for primarily cultured rat hepatocytes, and 0.7 pm and 55,000 sites/cell for HepG2 cells, respectively.

Specificity of 125I-HPO Binding to the Receptor—Fig. 3 shows typical displacement curves of 125I-HPO binding to the HPO receptor. Only unlabeled HPO could replace the binding of 125I-HPO to the receptor in a concentration-dependent manner, and almost complete replacement was achieved with 100 times the concentrations of 125I-HPO. EGF, TGF-α, and insulin could not replace 125I-HPO to the receptor even at 100 times the concentrations (1 nM) of unlabeled HPO. These results confirmed the specificity of 125I-HPO binding to the receptor and indicated that HPO receptor is different from the receptors of EGF, TGF-α, or insulin.

Identification of 125I-HPO-Receptor Complex—To characterize the molecular weight of this receptor, hepatocytes were chemically cross-linked with 125I-HPO by the homobifunctional reagent bis-sulfosuccinimidyl suberate. The HPO receptor was specifically labeled with 125I-HPO and the complex of receptor-ligand could be identified by SDS-PAGE and autoradiography. This resulted in the labeling of one cross-linked species that migrated as the complex of HPO (molecular mass, 15 kDa) with the molecular mass of its receptor being about 90 kDa. This band was absent when an excess amount of unlabeled HPO were present. EGF does not compete with 125I-HPO for binding to its receptor and did not affect the labeling of the band with molecular mass of about 90 kDa, as shown in Fig. 4. Thus, the molecular mass of the HPO receptor was calculated to be about 75 kDa by subtracting molecular mass of 15 kDa of HPO from 90 kDa of the complex.
This study shows the presence of high affinity receptor for HPO on rat hepatocytes and human hepatoma cells. This is the first report about the existence of a cellular receptor for HPO. It seems likely that HPO stimulate hepatocyte proliferation by binding to the specific receptor on the cell surface. This finding might initiate further understanding of the molecular mechanism of the biological action of HPO and of its effect on liver regeneration.

HPO can obviously stimulate hepatocyte proliferation and liver regeneration. The biological effect of HPO on the stimulation of DNA synthesis in hepatocytes is half-maximal at 0.5–1.0 pM concentration (13). The half-maximum dosage for HPO activity was in good accord with the K_d value (0.8–2.0 pM) of the HPO receptor, thereby indicating that this high affinity receptor may play an important physiological role in the signaling system. 125I-HPO induced a time- and dose-dependent effect on binding to cell surface sites of the primarily cultured rat hepatocytes and human hepatoma cells. The binding reaction is reversible and saturable. When an excess amount of unlabeled HPO was added, it could replace the cell surface sites that HPO has been binding to. In addition, the binding is specific because a great amount of EGF, TGF-α, and insulin (Δ). Therefore, it is reasonable to conclude that the hepatocytes surface sites are just the receptors for HPO, which have typical characteristics of a receptor such as high affinity, high specificity, reversibility, and saturation.

By binding test and specific replacement test, we also found that the distribution of HPO receptor was certainly specific. Whether normal (primarily cultured rat hepatocytes, L02 cells, and primarily cultured human fetal hepatocytes) or abnormal
including hepatocytes (e.g., hepatoma cells such as HepG2, HTC, SMMC7721 cell lines) were derived from liver, all of them contained the receptor of HPO (data not shown). But for the nonhepatocytes cells derived from other tissue, such as COS-7 cells (kidney), GLC-82 cells (lung), K562 cells (hematopoietic tissue), Hep2 cells (larynx), and Chinese hamster ovary cells (ovary), none of them had a dose-dependent effect or receptor (data not shown). These results demonstrated that the tissue distribution and location of the cells of the HPO receptor was unique but correlated with the responsiveness of the unique target cells of HPO.

Although primarily cultured rat hepatocytes, human hepatoma cells, and human fetal hepatocytes all have the receptor for HPO, they are different in the number and the affinity of the receptor for HPO. Of primarily cultured rat hepatocytes and hepatoma cells, the receptor number of the former is less and the receptor affinity ($K_d$ value) is lower than that of the latter. The receptor number and affinity of fetal hepatocyte are median among the three kinds of cells above. The differences in HPO receptor number and affinity of those cells are in good accord with the degree of their response to HPO. The facts pointed out that HPO acts biologically on the target cells via its specific receptor and that the number and affinity of the HPO receptor represent the physiological modulation of its target cells.

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