Hepatocyte Growth Factor May Act as a Pulmotrophic Factor on Lung Regeneration after Acute Lung Injury*

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Hepatocyte growth factor (HGF) has been shown to have hepatotrophic and renotropic functions for regeneration of the liver and kidney through its mitogenic, motogenic, and morphogenic properties. To examine the involvement of HGF in lung regeneration after acute injury, we analyzed changes of HGF mRNA, HGF activity, and HGF receptors in the rat lung after lung insult and measured HGF concentration in sera of patients with various lung diseases. Following the onset of acute lung injury induced by intratracheal hydrochloride injection, a compensatory DNA synthesis occurred in the bronchial epithelium with a peak at 24 h and in the alveolar epithelium with a peak at 48 h. Expression of HGF mRNA in the rat lung remarkably increased only 3 h after the treatment and HGF activity in the lung also increased to about 3-fold at 6 h later. HGF receptors in the lung but not in the other noninjured organs were down-regulated 12 h later. These marked increases in HGF mRNA and HGF activity and the concomitant down-regulation of HGF receptor occurred before the marked compensatory DNA synthesis in bronchial and alveolar epithelial cells. HGF concentration in sera of patients with various lung diseases, as measured by radioimmunoassay, was much higher than that in healthy donors. These results suggest that HGF is newly produced in the lung after acute lung injury and may have a role in regeneration of the lung.

Hepatocyte growth factor (HGF)1 was first detected in the plasma of partially hepatectomized rats as a potent mitogen for plasma of partially hepatectomized rats as a potent mitogen for human lung fibroblasts in the pigeon medium of human lung fibroblasts and supports the thesis that HGF may be a "pulmotrophic factor" for regeneration of an injured lung.

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1 The abbreviations used are: HGF, hepatocyte growth factor; BrdU, 5-bromo-2′-deoxyuridine; EGF, epidermal growth factor; TGF, transforming growth factor; KGF, keratinocyte growth factor; IGF, insulin-like growth factor.
stained with hematoxylin. The labeling index was counted in cells among more than 1000 nuclei of bronchial epithelia and alveolar septa, but granulocytes and lymphocytes were excluded from counting. Preparation of Anti-rat HGF Antibodies—Recombinant rat HGF was purified from conditioned medium of Chinese hamster ovary (CHO) cells transfected with expression vector containing rat HGF cDNA (13). Twenty μg of total RNA was applied to electrophoresis on 1.0% formaldehyde-agarose gels and transferred to a Hyb-on-N membrane filter (Amersham). The EcoRI fragment (1.4 kilobase pairs) of rat HGF cDNA (RBC-1 clone, Ref. 13) was labeled, using the Megaprime DNA labeling system (Amersham), according to the manufacturer’s instruction. RBC-1 encodes the 5' portion including the fourth kringle domain of α-chain, the entire β-chain of HGF, and a part of 3'-noncoding region. Hybridization was performed at 42 °C for 20 h in solution composed of 50% (v/v) formamide, 5 × SSPE (1 SSPE consists of 0.15 M NaCl, 10 mM sodium phosphate buffer (pH 7.4) and 1 mM EDTA), 2 × Denhardt's solution (1), 0.1% SDS, and 0.02% Ficoll (Type 400, Pharmacia), 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin (Fraction V, Sigma), 0.5% SDS, and 50 μg/ml salmon sperm DNA. The filter was washed with 0.2 × SSPE, 0.1% SDS for 15 min at 65 °C, then was dried and autoradiographed on Fuji x-ray film. The gel was stained with ethidium bromide to estimate the amount of loaded RNAs.

Partial Purification of HGF from the Lung—HGF was partially purified from the lung, as described elsewhere (1, 4, 8). Briefly, the lung was homogenized with Polytron in 4 volumes of buffer composed of 10 mM Tris-HCl (pH 7.5), 0.3 M NaCl, and 0.01% Tween 80 containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM monoiodoacetate, and 1 mM EDTA). After centrifugation at 105,000 × g for 1 h, the supernatant was dialyzed against 100 volumes of the same buffer, using cellulose tube with molecular cut off >50,000, and applied to a heparin-Sepharose column equilibrated with the same buffer. The column was washed with 2 volumes of the same buffer and then eluted with buffer containing 2 M NaCl. The eluate was dialyzed against buffer composed of 2 M Hepes-NaOH (pH 7.2) and 20 mM NaCl, lyophilized, and then dissolved with H2O. Samples used for HGF activity assay were passed through a 0.22-μm pore size filter (Millipore).

To determine whether stimulatory effect of sample prepared from rat lung on DNA synthesis of hepatocytes was attributable to HGF, test sample was incubated with pre-immune control IgG or anti-rat HGF IgG at 4 °C for 1 h and assayed for HGF activity. Assay for HGF Activity—HGF activity was determined by measuring the stimulatory effect on DNA synthesis of rat hepatocytes in primary culture (4). Adult rat hepatocytes were isolated by the in situ collagenase perfusion method. The isolated cells were plated at a density of 6.25 × 10⁴ cells, 0.5 ml, 2 cm² on 24-well plastic dish (Corning) coated with dexamethasone for 3 h. The medium was changed to serum-free Williams’ E medium containing 5% calf serum, 0.1% FCS, and 0.5 μg/ml antibiotic (penicillin, streptomycin, and amphotericin B), and then incubated in a humidified atmosphere of 5% CO₂ in air at 37 °C. After appropriate time period, the cells were pulse-labeled with 1 μCi of [3H]deoxyuridine for 6 h, and [3H]deoxyuridine in the nuclei was measured in a γ-counter. One unit of HGF activity corresponds to the stimulatory activity which gives a half-maximal value of stimulatory effect on DNA synthesis of hepatocytes when 10 ng/ml EGF is added.

Assay for [125I]-HGF Binding to Plasma Membranes—Plasma membranes were prepared from lung, liver, kidney, and spleen of rats by density-gradient centrifugation on Percoll, as described elsewhere (36). Human recombinant HGF was radiiodinated by a chloramine-T method, as described elsewhere (37). The 125I-HGF had a specific radioactivity of 70–160 mCi/mg of protein. Fifty μg of plasma membranes were incubated with 45 pmol 125I-HGF for 1 h at 10 °C, with or without 6.4 nM unlabeled HGF in 100 μl of binding buffer (Hanks’ solution containing 5% calf serum, 2 mM β-glycerol phosphate, 50 μg/ml bovine serum albumin). Membranes were centrifuged for 10 min at 12,000 × g at 4 °C, resuspended with 10 ml of binding buffer, and transferred to fresh tubes. 125I-HGF specifically bound to membranes was counted in a γ-counter. All binding experiments were done in triplicate.

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1. 2 T. Kawamoto, H. Iwaki, K. Sekiguchi, and T. Nakamura, submitted for publication.

**RESULTS**

**Histological Change after Induced Acute Lung Injury**—To induce acute lung injury, 0.1 m HCl 2 ml/kg body weight was intratracheally administered into the lung of each rat. In the intact lung, the intima of the bronchus consisted of simple columnar epithelia and that of the thin alveolar septa consisted of simple squamous epithelia (Fig. 1A). Three hours after HCl injection, hemorrhage in the submucosal region of the bronchus and in the alveolar space was obvious, and infiltration of polymorphonuclear leukocytes was present in several regions (Fig. 1B). Twelve hours later, there was histological evidence of the onset of acute lung injury: some pulmonary edema, de-epithelialization of the bronchus and alveoli, a neutrophilic cellular response in the submucosal layer of the bronchus and perivascular area, and hemorrhage accompanying the formation of fibrin-net-forming (Fig. 1C). Thereafter, hyaline degeneration, infiltration of macrophages, and epithelial proliferation was observed at 24–48 h, and mesenchymal cell proliferation and fibrous thickening of alveoli were present 3–7 days later (not shown). Thus, intratracheal administration of HCl produced a severe acute lung injury in rats.

**DNA Synthesis in Bronchial Epithelia and Alveolar Septa following Lung Injury**—To determine the time course of regenerative cell proliferation after acute lung injury, cells undergoing DNA synthesis were identified by the incorporation of BrdU followed by immunochemical staining (Fig. 2). In the untreated rat lung, there were few cells undergoing DNA synthesis (0.95% in bronchus and 1.42% in alveoli). However, a marked increase in the number of cells undergoing DNA synthesis following HCl treatment. The cells undergoing DNA synthesis were predominantly simple columnar cells in the bronchial epithelia (Fig. 2A) and alveolar type II epithelial cells in alveolar septa, characterized by a round nucleus and a comparatively small cytosol protruding into the alveolar space (Fig. 2B). Several endothelial cells with a small flat nucleus and macrophages in the alveolar space also underwent DNA synthesis, although the number of these cells undergoing DNA synthesis was much fewer than in epithelial cells.

The number of cells undergoing DNA synthesis in the bronchial epithelia and the alveolar septa remarkably increased from 24 h following HCl treatment (Fig. 3). Maximum values of the labeling index were 13.0% at 24 h in bronchial epithelium and 11.7% at 48 h in alveolar septa. Then, labeling index decreased 72 h after HCl treatment and returned to almost normal levels within 1 week.

**Change in HGF mRNA in Lung after HCl-induced Lung Injury**—To determine whether or not HGF has a role in regeneration of the acutely injured lung, we examined HGF mRNA in the lung after HCl treatment (Fig. 4). HGF mRNA was detectable in the intact lung, and it markedly increased with a peak at only 3 h after HCl injection; the maximum level was 5-fold higher than normal. The high level of HGF mRNA expression in the lung continued for up to 12 h, and HGF mRNA levels reverted to normal at 24 h in two of three rats after HCl injection.

**HGF Activity in Lung**—HGF activity in the injured lung increased as early as 3 h after HCl injection and reached the maximum at 6 h (Fig. 5). The maximum value was 9–4-fold increased.
higher than that of the normal. HGF activity gradually decreased from 12 h after the treatment and reverted to normal within 48 h after the injection. Because HGF activity in the lung was almost completely abrogated by the addition of antiserum against recombinant rat HGF (Fig. 5) but not by preimmune serum (data not shown), HGF activity in the lung was attributable to HGF. These results suggested that HGF was newly synthesized in the injured lung following increase in HGF mRNA expression after the HCl-induced injury.

Change in Binding of $^{125}$I-HGF to the Plasma Membranes

-We reported that rapid down-regulation of cell surface HGF receptor was found only in the injured organs after liver or renal insult and not in intact organs (24, 25). Since down-

regulation of growth factor receptors occurs immediately after ligand binding, these results suggest that HGF immediately produced at the injured site and other intact organs such as lung, spleen, and kidney (29, 38, 39) exerts biological activities specifically at the injured site, as a trophic factor for regeneration. Therefore, to examine whether HGF newly synthesized in the lung subjected to insult is involved in lung regeneration, we
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preparied membranes from injured lungs and intact organs and analyzed HGF receptor. Specific binding of HGF was saturable at about 100 pm (not shown), and the amount of specific binding of HGF to plasma membranes from the lung decreased to 68% of that of normal lung at 3 h after HCl treatment, to 26% at 6 h, and almost completely disappeared during 12-48 h after HCl injection, as shown in Fig. 6A. The binding of 125I-HGF recovered to 26% of normal levels at 72 h after the treatment and to 88% 1 week after the insult. Scatchard analysis of 125I-HGF binding to plasma membranes prepared from normal and HCl-treated rat lung at 3 h after the injection revealed the $K_d$ value to be 58 pm and the number of binding sites ($B_{max}$) was 1530 sites/µg of plasma membranes protein in the normal lung, whereas 1050 sites/µg of plasma membrane protein with $K_d$ of 51 pm were present in lung 3 h after treatment (Fig. 6A, inset). Therefore, the decrease in specific binding of HGF after the induced injury was not due to change in affinity to the receptor, but rather to a decrease in the number of HGF receptors.

To determine whether down-regulation of HGF receptors was specific to the injured lung, we examined 125I-HGF binding to plasma membranes purified from liver, kidney, and spleen after lung insult. As shown in Fig. 6B, there was no significant change in 125I-HGF binding in the plasma membranes from these organs. These results clearly show that rapid down-regulation of the HGF receptor occurs specifically in the injured lung.

**HGF Concentration in Sera of Patients with Lung Diseases**

To examine changes in HGF levels in sera of patients with various lung diseases, we measured HGF concentration in sera from healthy donors and from patients with lung diseases, using a highly sensitive radioimmunoassay for HGF (Fig. 7). Elevated levels of HGF in sera of patients with lung diseases were found; HGF concentration in sera of 197 healthy donors was 0.33 ± 0.10 ng/ml, whereas HGF concentration in sera of 22 patients with lung diseases was 1.22 ± 1.01 ng/ml. Among patients with various lung diseases, HGF levels were 4.10 ng/ml in one with silicosis, 2.84 ng/ml in one with lung abscess, and in seven with pneumonia the level was 1.06-2.80 ng/ml.

**FIG. 4.** Northern blot analysis of HGF mRNA in rat lung after HCl-induced injury. RNA was extracted from the lung of each rat at various times after HCl treatment. Twenty µg of total RNA were electrophoresed on 1.0% formaldehyde-agarose gel, transferred to Hybond-N nylon membrane filter, and hybridized with 32P-labeled rat HGF cDNA: lane 1-3, normal; lane 4-6, 3 h after treatment with HCl; lane 7-9, 6 h after treatment with HCl; lane 10-12, 12 h after treatment with HCl; lane 13-15, 24 h after treatment with HCl; lane 16-18, 48 h after treatment with HCl; lane 19-21, 72 h after treatment with HCl; lane 22-24, 1 week after treatment with HCl. Ribosomal RNA (rRNA) are shown as an internal control after staining with ethidium bromide. Arrows indicate 28 and 18 S rRNAs.

**FIG. 5.** Change in HGF activity in lung after acute lung injury. HGF was partially purified from the lung using heparin-Sepharose. HGF activity was determined by measuring stimulatory effects on DNA synthesis of hepatocytes by adding 10 ng/ml EGF. Each value represents the mean ± S.D. of duplicate experiments on three rats in each group.

**FIG. 6.** Change in binding of 125I-HGF to the plasma membranes from lung, liver, kidney, and spleen after intratracheal HCl injection. A, change of specific binding of 125I-HGF to the plasma membranes (PM) from lung of each time point after HCl treatment (○). The inset panel shows a Scatchard analysis with plasma membranes from normal lung (○) and HCl-injured lung at 3 h (▲) and 12 h (■). B, changes of specific binding of 125I-HGF to the plasma membranes from spleen (○), kidney (■), and liver (▲).
Lung diseases were measured using sandwich radioimmunoassay. In alveolar injury, alveolar cells are stimulated by epidermal growth factor (EGF), transforming growth factor-α (TGF-α), keratinocyte growth factor (KGF), and insulin-like growth factor-I (IGF-I) (42). HGF is synthesized and secreted by mesenchymal cells, such as macrophages, endothelial cells, and fibroblasts, and controls proliferation and morphogenesis of a broad spectrum of epithelial cells. It has recently been revealed that HGF is a potent mitogen for rat alveolar type II cells in primary culture (46). Therefore, we hypothesized that HGF may have important roles in regulating growth of lung epithelium and in regeneration of the lung, as a paracrine factor. In the present study, we obtained evidence which suggests that HGF has a role in regeneration of the lung following acute lung injury.

Following intratracheal HCl injection, HGF mRNA and HGF activity in lung remarkably increased as early as 3–6 h later, and the HGF receptor on the plasma membranes of lung was markedly down-regulated to an undetectable level 12 h later. Since the down-regulation of HGF receptor is probably caused by internalization of the HGF receptor following HGF binding, our results suggest that HGF exerts biological activities in the lung. Following marked changes in HGF mRNA, HGF activity, and the receptor in lung, there was marked increase in DNA synthesis of bronchial epithelial cells and alveolar epithelial cells during 24–48 h after the onset of lung injury. Thus the rapid and sequential induction of HGF mRNA and HGF activity that precede pulmonary epithelial cell proliferation strongly suggests that HGF may have a “trophic” role for the regeneration of lung following acute lung injury. Based on the pleiotropic actions of HGF as mitogen, motogen (stimulation of cell motility), and morphogen, we propose that HGF may enhance lung regeneration through its multiple biological activities, e.g., not only by stimulating proliferation of bronchial and alveolar epithelial cells, but also by constructing normal tissue architecture of the bronchus and alveolus.

Although the HGF receptor on plasma membranes of the lung was markedly down-regulated, the HGF receptor in liver, kidney, and spleen did not change after lung injury. Thus HGF synthesized after lung injury specifically binds to the receptor in lung, but not to that in other intact organs. We reported that HGF mRNA is markedly induced in the intact lung following partial hepatectomy and unilateral nephrectomy in rats (39) and that the HGF receptor was specifically down-regulated in the injured organ but not in the intact lung (25). Our previous results suggested that HGF synthesized in the intact lung after hepatic or renal injury acts as a hepatotrophic or renotropic factor for regeneration, in an endocrine fashion. Therefore, taken together with present results, HGF synthesized in lung appears to contribute to the regeneration of the lung itself and also to regeneration of distal organs.

HGF concentrations in sera of patients with various hepatic and renal diseases were significantly higher than those in healthy donors. We reported direct evidence that HGF functions as a hepatotrophic and renotropic factor in vivo; administration of recombinant HGF into mice with hepatic or renal injury remarkably stimulated regeneration of the liver or kidney and prevented the onset of severe hepatic or renal dysfunction (30, 49). All these findings mean that HGF can be considered to treat subjects with hepatic and renal diseases, as well as being a diagnostic for these diseases. Since HGF concentration in sera of patients with various lung diseases were much higher than those in healthy donors, HGF seems to be involved in regeneration of lung also in human. Following acute or chronic lung injury, overgrowth of fibroblasts and overproduction of extracellular matrix often causes lung fibrosis which results in a decrease in respiratory functions. Since HGF is produced by mesenchymal cells and acts predominantly on epithelial cells, HGF is a putative “pulmotrophic factor” for regeneration of the lung, preventing onset of lung fibrosis. Our preliminary experiment using experimental animals has indeed proved that administration of HGF accelerates lung regeneration in vivo and plans are being designed to clinical administration of HGF to treat subjects with lung disorders.

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