Hepatocyte Growth Factor Suppresses Vascular Medial Hyperplasia and Matrix Accumulation in Advanced Pulmonary Hypertension of Rats
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Background—Pulmonary hypertension (PH) is a progressive disease characterized by raised pulmonary vascular resistance, thought to be curable only through lung transplantation. Pathophysiologically, proliferation of pulmonary artery smooth muscle cells triggers pulmonary arterial stenosis and/or regurgitation, especially in advanced PH.

Methods and Results—Using a rat model of advanced pulmonary vascular disease produced by injecting monocrotaline, we show that hepatocyte growth factor (HGF) targets pulmonary arterioles and blocks the progression of PH. In these rats, endogenous HGF production was dramatically downregulated during developing experimental PH, but c-Met/HGF receptor was abundant in the medial layers of pulmonary arterioles. HGF gene transfection 2 weeks after the monocrotaline injection resulted in milder medial hyperplasia in lung arterioles and inhibited overgrowth of pulmonary artery smooth muscle cells. Notably, exogenous HGF reduced lung expression levels of endothelin-1 and transforming growth factor-β, which are critically involved in PH-linked fibrogenic events. Overall, medial wall thickening of pulmonary arteries was almost completely prevented by HGF, and the total collagen deposition in the lung decreased; both effects contributed to the suppression of pulmonary artery hypertension.

Conclusions—Our results suggest that the loss of endogenous HGF may be a feature of the pathogenesis of PH and that HGF supplementation may minimize pathological lung conditions, even advanced PH. (Circulation. 2004;110:2896-2902.)

Key Words: gene therapy ■ pulmonary vasculature ■ growth factor ■ remodeling

Pulmonary hypertension (PH) is a progressive disease caused by a variety of pulmonary and/or cardiac disorders and characterized by an increase in pulmonary vascular resistance that leads to right ventricular failure. Histologically, the proliferation of pulmonary artery smooth muscle cells (PA-SMCs) and the progression of pulmonary arterial fibrosis are key features of primary and secondary PH, which can be caused by congenital heart disease, ingested substances, or hepatic dysfunction.

This background delineates a central role for PA-SMC overgrowth (ie, medial hyperplasia) in provoking the initial pathogenesis of PH. Several lines of evidence indicate that (1) platelet-derived growth factor is a key mitogen for eliciting PA-SMC proliferation both in vitro and in vivo, (2) transforming growth factor-β (TGF-β) is responsible for lung fibrogenesis, and (3) endothelin-1 (ET-1) is a potent mediator for blood hypertension via enhancing the vasoconstriction of PA-SMCs (as pericytes). Such previous studies focused on molecular roles in the initial pathogenesis, but little information is available about the body’s self-defense system, which should help block the PH-related pathological changes. Therefore, identification of a natural ligand(s) involved in an antipathogenic mechanism would shed light on how to develop a therapeutic strategy for advanced PH.

Hepatocyte growth factor (HGF), which was originally purified and cloned as a potent mitogen for hepatocytes, has mitogenic, motogenic, morphogenic, and antiapoptotic activities in various cell types. The pluripotent activities of HGF are mediated by a membrane-spanning tyrosine kinase receptor encoded by the c-Met proto-oncogene. Physiologically, HGF acts as an organotrophic factor for protection from injury and the regeneration of various organs. In the lung, biological and pulmotrophic roles for HGF have been well documented. In response to acute lung injury, HGF plays a role in lung regeneration and protection. Furthermore, research elucidating the pulmotrophic role of HGF has led to the development of therapeutic approaches for the treatment of chronic lung diseases. Despite the usefulness...
of these findings, few studies have discussed potential functions of HGF in regulating PH-related pathological conditions.

In the present study, we used monocrotaline-injected rats as an animal model to mimic human PH and investigated the roles of HGF and the \( \text{c-Met/HGF receptor} \) under pathological conditions.

### Methods

#### Animal Care

This study was performed under the supervision of the Animal Research Committee in accordance with the Guidelines on Animal Experiments of Osaka University Graduate School of Medicine and the Japanese Government Animal Protection and Management Law.

#### Constriction of Plasmid With Human HGF Gene

The plasmid encoding the human HGF gene was constructed according to our previous methods.\(^2^4\) We also constructed a LacZ expression vector as the control.

#### Preparation of Hemagglutinating Virus of Japan Envelope Vector

The preparation of the hemagglutinating virus of Japan (HVJ) envelope vector was described by Kaneda et al.\(^2^6\) Stored virus was suspended in 30 \( \mu \)L of TE solution (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA). The virus suspension was mixed with 200 \( \mu \)g of plasmid DNA and 5 \( \mu \)L detergents. The mixture was spun at 18 500 \( \times \)g for 15 minutes at 4°C. After the pellet had been washed with 1 mL balanced salt solution (10 mmol/L Tris-HCl, pH 7.5, 137 mmol/L NaCl, and 5.4 mmol/L KCl) to remove the detergent and unincorporated DNA, the envelope vector was suspended in 1 mL of PBS and used for subsequent experiments.

#### Experimental PH Model and Surgical Approach

Male Wistar rats (9 to 10 weeks old) were purchased from SLC Japan. Monocrotaline (MCT) (Sigma Chemical), 60 mg/kg, was injected subcutaneously into the backs of 30 rats. Six rats were killed at each time point on days 4, 7, 14, 21, and 28 to evaluate the effect of exogenous HGF, 14 rats that had received MCT were used for subsequent experiments.

#### Transfection of the Left Lung With the Human HGF Gene via the PA

The lung was transfected via the PA using the HVJ envelope containing the HGF gene or the LacZ gene as the control, according to our previously published methods.\(^2^4\) The HVJ envelope–plasmid complex (0.3 mL, including 60 \( \mu \)g of cDNA) was infused via a catheter. The rats in each group were examined 14 days after the transfection.

#### Evaluation of PH and Sample Preparation

The rats were anesthetized and ventilated. A small midline sternotomy was performed, and pressure measurements of the right and left ventricles were performed with a 24-gauge needle and a pressure transducer. Next, the heart and lungs were resected en bloc, and the lungs were cleared of blood. The weight of the right and left ventricles plus the septum was measured. Lung samples were frozen to a commercial kit for human HGF. Total RNA was extracted from the lungs by use of an RNeasy Mini kit and RNase-Free DNase Set (Qiagen). One microgram of total RNA was reverse-transcribed into first-strand cDNA with a random hexaprimer using the Superscript II reverse transcriptase (Invitrogen). TaqMan quantitative PCR was performed using the ABI PRISM 7700 Sequence Detector System (Perkin-Elmer Biosynthesis). The sequences for the primers and TaqMan fluorogenic probes were as follows: rat HGF, forward primer, 5'-AGGCGAGGAA-A CGCATAA-3', reverse primer, 5'-GATCAATCCGTTAGCGCC-CA-3'; probe, 5'-TGTCGGCTGGCAATCTGTGAA-3'; human HGF, forward primer, 5'-ATGTAGTCCACGGAAGAGGAG A-3'; reverse primer, 5'-CCTCAGTGTTTTCATCATGTTGA-3'; probe, 5'-TGCCAAAATCGTTTCATCATTCTCCAGC-3'; GAPDH, forward primer, 5'-CCATCATCTGGCAGTCAAGGAC G-3'; reverse primer, 5'-TGTTGAAGCACTGGTATCCT-3'; and probe, 5'-CATGCATGTGGCCTCATCTGGT-3'. Enzyme assays were matched to a standard curve generated by amplifying serially diluted products. To correct for variability in RNA recovery and the efficiency of the reverse transcription, GAPDH cDNA was amplified and quantified in each cDNA preparation.

#### Histological Analysis

The tissue specimens obtained from the hilum of the left lung were fixed in ethanol, embedded in paraffin, and sectioned. Tissue sections were stained with hematoxylin and eosin and immunohistochemically stained with a rabbit polyclonal antibody against human HGF, as we have described previously.\(^2^4\) Sections were also immunostained with antibodies against factor VIII, proliferating cell nuclear antigen (PCNA) (Dako), TGF-\( \beta \) (R&D Systems), and c-Met (SP260, Santa Cruz Biotechnology) in the same manner. The capillary density was determined as described previously.\(^2^3\) Mediawall thickening was assessed as the percent mediawall thickness using elastica van Gieson stain, as described elsewhere.\(^6\)

#### Enzyme Immunoassays

To measure the levels of TGF-\( \beta \), endothelin-1, rat HGF, and collagen in the lung, we made lung-tissue extracts as we described previously\(^1^6\) and applied them to a 96-well microplate for an ELISA analysis, using a commercial kit for TGF-\( \beta \) (Quantikine kit; R&D systems), endothelin-1 (Cayman Chemical Co), and rat HGF (Institute for Immunology). The levels of total collagen were analyzed with a Sircol collagen assay kit (Biocolor Assays; Biocolor Ltd). Plasma was purified from whole blood by centrifugation and applied to a commercial kit for rat HGF.

#### Statistical Analyses

All data were expressed as the mean\(\pm\)SEM. A Student unpaired \( t \) test or ANOVA test for parametric values and Mann-Whitney \( U \) test for nonparametric values were used to compare group means, with a value of \( P<0.05 \) accepted as statistically significant.

### Results

#### Pulmonary Vascular Disease in MCT-Induced PH

After MCT administration, the progression of PH (determined by the pressure and weight ratios of the right to left ventricle) was confirmed on days 14, 21, and 28 (Figure 1A), and the pulmonary arteries showed changes of typical PH (Figure 1B). The percent mediawall thickening of pulmonary arteries was significantly increased on days 14, 21, and 28 (Figure 1B). The capillary density of the lung was significantly decreased on days 14, 21, and 28 (Figure 1C). The expression levels of lung TGF-\( \beta \) and ET-1 and the total collagen significantly increased after MCT treatment (Figure 2A). Lung HGF mRNA and protein concentration decreased significantly after MCT administration, whereas plasma HGF level increased on days 4, 7, and 14 (Figure 2B).
Induction of the c-Met/HGF Receptor in MCT-Induced PH

In normal pulmonary arteries, the c-Met receptor expression was evident in cells that were presumably parenchymal and endothelial cells, given their localization and morphology. When c-Met immunostaining was analyzed in rats with MCT-induced PH, the PA-SMCs of the pulmonary arteries were almost all c-Met–positive (Figure 2C).

Expression of Exogenous Human HGF

Four days after the transfection, we found by RT-PCR a significant \((P<0.01)\) pulmonary expression of human HGF mRNA in the left lung (Figure 3A). Likewise, immunohistochemical examination using an anti-human HGF polyclonal antibody 4 days after the transfection showed expression of human HGF in the HGF group (Figure 3B, bottom). In contrast, human HGF was undetectable in lung tissues in the LacZ-transfected lung (Figure 3B, top).

Inhibitory Effects of HGF on PA-SMC Overgrowth

We evaluated the effect of exogenous HGF gene transfection in rats with MCT-induced PH. Two weeks after the HGF gene transfection, we found by histological examination a marked decrease in medial wall thickening in the HGF-transfected lung compared with the control (Figure 4A). The percent medial wall thickening of the pulmonary arteries revealed a significant decrease after the transfection (Figure 4B).

We next evaluated the effects of HGF on the PA-SMCs in the pulmonary arteries. Proliferating cells were detected immunohistochemically with an anti-PCNA antibody. A significant and marked decrease in the number of PCNA-positive SMCs was seen 14 days after the lung was transfected with HGF (Figure 4A). The percent PCNA-positive PA-SMCs in the HGF group was 12.0\(\pm\)1.2\%, whereas it was 25.0\(\pm\)3.0\% in the control group (Figure 4B). Furthermore, to evaluate smooth muscle apoptosis, apoptotic cells were immunohistochemically detected with terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining, and the percentage of TUNEL-positive PA-SMCs was determined. A significant and marked increase in the number of TUNEL-positive PA-SMCs was seen 14 days after the transfection (Figure 4B). The percentage of TUNEL-positive PA-SMCs in the HGF group was 9.0\(\pm\)0.8\%, whereas in the control group it was 3.0\(\pm\)0.3\%.

Angiogenic Effects of HGF on Endothelial Cells in PH Rats

To evaluate the effect of HGF on endothelial cells, we also performed an immunohistochemical examination with anti-
factor VIII. There was a significant difference in capillary density between the control and HGF cDNA groups. The number of PCNA-positive endothelial cells also increased significantly after the HGF gene transfection. In contrast, the number of TUNEL-positive endothelial cells decreased significantly after the transfection (Table).

**Changes in TGF-β, Total Collagen, and ET-1 Concentration in the Lung Tissue**

Immunostaining using a TGF-β antibody showed markedly decreased expression of TGF-β in the HGF cDNA-transfected lung compared with the control. The tissue concentration of TGF-β in the HGF-transfected lung was significantly lower than in the control (Figure 5A). Likewise, the total collagen content of the HGF-transfected lung was significantly lower than in the control (Figure 5B).

**Changes in PH After HGF Transfection**

To assess the effect on hemodynamic change induced by the exogenous HGF, we evaluated the tissue concentration of ET-1, which in the HGF-transfected lung was significantly lower than in the control (Figure 6A). The pressure and weight ratios of the right ventricle to the left were also significantly attenuated on day 14 after the transfection (Figure 6B).

**Discussion**

PH is histologically characterized by vascular sclerosis/stenosis and interstitial fibrosis, followed by impaired right ventricular output, but there has been no effective treatment except lung and heart transplantations. Using a rat model of PH, we first demonstrated that local supplementation with HGF cDNA diminished PH-linked pathological...
The key pathogenetic cascades for the development of PH include (1) vascular medial hyperplasia with sclerosis, (2) constriction of PA and/or arterioles, (3) interstitial fibrosis leading to lung consolidation, and (4) loss of pulmonary vascular beds, all of which worsen the PH-linked pathological events. Importantly, exogenous HGF supplement therapy counteracted all of these cascades. Here, we discuss the physiological and therapeutic value of the effects of HGF on PH-associated clinical and histological findings.

We first examined changes in HGF and c-Met expression levels as the experimental PH developed. Plasma HGF was upregulated like other injurious conditions, but the lung HGF was repressed in our model according to the progression of pulmonary vascular disease. In contrast, c-Met was upregulated, and its distribution was particularly concentrated on sclerotic vessels, and parenchymal and interstitial areas were also c-Met–positive (data not shown). We speculate that it is because of the irreversible lung damage in this lethal PH model. TGF-β is known to be upregulated in the MCT-induced PH rat model. Interestingly, this fibrogenic cytokine directly suppresses HGF production. Thus, we hypothesized that repression of the intrinsic HGF production by the upregulated TGF-β may alter the natural course of the developing experimental PH. To test our hypothesis, we administered HGF cDNA locally and obtained evidence that HGF is preventive, especially against vascular stenosis and sclerosis.

Intrapulmonary arteriovascular stenosis underlies the mechanisms whereby lung-to-heart arterial regurgitation occurs. Especially during the initial pathogenesis of vasostenosis, PA-SMC overgrowth is important for medial hyperplasia to develop. Here, we found in this model that HGF inhibits PA-SMCs proliferation. Because it is not clear that HGF directly targets PA-SMCs and arrests their overgrowth, we have performed an in vitro study that suggests that HGF inhibits platelet-derived growth factor–dependent PA-SMC proliferation (data not shown). These findings are in agreement with the role of HGF in renal mesangial cells. Furthermore, an increase in apoptotic SMCs was noted in our rat model concomitantly with suppressed proliferation of the pericyte layers. Although it is difficult to determine whether or not this effect is direct, we recently found that HGF induces apoptotic cell death in myofibroblast-like stromal cells (S. Mizuno and T. Nakamura, unpublished data). This apoptotic effect might be helpful in reducing the thickness of hyperplastic walls.

In addition to medial hyperplasia, vasoconstriction is important in promoting an increase in pulmonary arterial pressure during the progression of PH. In vitro studies demonstrate an important role for ET-1 as a vasoconstrictor. Furthermore, in vivo antagonism of ET-1 leads to suppressed PH, suggesting that ET-1 plays an essential part in the pathogenesis of PH, especially by enhancing vasoconstriction and hypertension. Interestingly, we obtained evidence that local supplementation of the lung with HGF cDNA led to a decrease in local ET-1 expression levels. Haug et al showed that HGF inhibited ET-1 release in cultured human coronary artery endothelial cells. How HGF inhibits ET-1 expression is still unclear, but the suppression of ET-1 by HGF may contribute in part to the improvement of PH in our model.

In the process of PH development, fibrotic events (such as interstitial fibrosis and arteriolar sclerosis) become

### Table: Angiogenic Effect of HGF on Endothelial Cells in Pulmonary Hypertension Model

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<th>MCT-CON</th>
<th>MCT-HGF</th>
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<tr>
<td>Capillary density</td>
<td>4.1±0.4</td>
<td>6.6±0.6</td>
</tr>
<tr>
<td>PCNA score</td>
<td>25±3</td>
<td>70±6</td>
</tr>
<tr>
<td>TUNEL score</td>
<td>6.5±0.6</td>
<td>3.0±0.3</td>
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Capillary density indicates number of factor VIII–positive capillaries per square meter; PCNA score, percent PCNA-positive endothelial cells; and TUNEL score, percent TUNEL-positive endothelial cells.
evident in humans as well as in rodent models. These fibrotic lesions cause pulmonary consolidation and may limit movement of the lung lobes, and the pathological conditions are further accelerated in turn. TGF-β is a key mediator of interstitial fibrosis in the lungs, and we found that the exogenous HGF suppressed an increase in lung TGF-β levels, as has been noted in the cirrhotic liver and renal fibrosis. The possible mechanisms underlying the reduction by HGF of TGF-β levels are (1) direct effects of HGF on TGF-β–producing fibroblasts and (2) a secondary effect caused by the suppression of the infiltration of macrophages/monocytes, an important source of TGF-β. Our results strengthen a previous hypothesis that in vivo suppression of TGF-β by HGF is key to explaining antifibrotic mechanisms.

In advanced PH, decreased pulmonary blood flow becomes evident and leads to lung hypoxia. Under ischemic states, parenchymal destruction is further aggravated and is associated with the expansion of interstitial fibrotic spaces. Therefore, a strategy to increase pulmonary blood flow should be considered for stopping these pathological cycles. We have established successful techniques for inducing angiogenesis in lung tissues via an HGF cDNA plus HVJ-liposome transfection. In addition, in this study, the number of lung vessels was significantly increased after the HGF supplementation, comcomitantly with the enhanced proliferation of endothelial cells. Thus, HGF-mediated angiogenesis in PH could be responsible not only for improved hypoxia but also for a decline in peripheral blood pressure.

Throughout the present experiments, we delineated the roles of HGF in PH to antagonize overgrowth of PA-SMCs as well as to suppress the lung expression of TGF-β and ET-1, major fibrogenic and hypertensive mediators. HGF is angiogenic for endothelial cells and possibly morphogenic for alveolar or bronchial epithelial cells. Thus, HGF is recognized as a regenerative factor through antifibrotic, pulmotrophic, and angiogenic effects in advanced PH. Collectively, many roles played by HGF could participate in lung reconstruction, even after the onset of PH, whereas impairment in endogenous HGF production might allow the onset and progression of PH. The potential therapeutic value of HGF for the treatment of patients with PH deserves attention.

Acknowledgments

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References


Figure 6. Effects of HGF on endothelin-1 concentration and PH. A, Tissue level of endothelin-1. B, Pressure and weight ratio of right to left ventricle. Each value represents mean±SEM (n=7). *P<0.05 vs control.


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