In Vivo Gene Transfection With Hepatocyte Growth Factor via the Pulmonary Artery Induces Angiogenesis in the Rat Lung

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Background—Recent studies have demonstrated that gene transfer with hepatocyte growth factor (HGF) induces angiogenesis for coronary and peripheral artery diseases. We investigated the ability of gene transfer with human HGF to induce angiogenesis in the rat lung.

Methods and Results—The left lung was selectively transfected with a cDNA encoding human HGF via the left pulmonary artery, using the HVJ-liposome method (H group); rats transfected with the same vector lacking the HGF gene served as controls (C group). HGF gene transfer significantly increased the capillary density in the left lung compared with the C group 7 days after transfection (15.0±1.3 versus 8.0±1.7 mm², P<0.01). The left to right average blood perfusion ratio detected by laser Doppler imaging increased significantly in the H group 14 days after transfection (1.12±0.09 versus 0.91±0.11, P<0.01). A right pulmonary artery clamp test, in which only the left lung received all the pulmonary blood flow from the right ventricle, revealed that the increase in right ventricular pressure was significantly attenuated in the H group compared with the C group 7 days after transfection (8.6±3.5 versus 15.3±2.8 mm Hg, P<0.01).

Conclusions—Trans-pulmonary arterial transfer of the human HGF gene into the left lung increased capillary density and blood perfusion, and decreased vascular resistance when blood flow increased. These results suggest therapeutic angiogenesis induced by HGF gene expression in the lung may be found suitable in treating subjects with decreased pulmonary vasculature or increased pulmonary vascular resistance. (Circulation. 2002;106[suppl I]:I-264-I-269.)

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

Although recent advances in cardiovascular surgery have improved clinical results in patients with decreased pulmonary vasculature or increased pulmonary vascular resistance, such as pulmonary infarction, pulmonary hypertension, or hypoplastic pulmonary vasculature associated with congenital heart disease, the prognosis of critical cases has remained poor.1–3 A growing body of evidence indicates that in vivo gene transfection with angiogenic factors can induce angiogenesis to treat ischemic coronary and peripheral artery disease.4,5 This is called therapeutic angiogenesis. If angiogenesis could be achieved for the pulmonary vasculature, the prognosis of critical cases have remained poor.1–3 A growing body of evidence indicates that in vivo gene transfection with angiogenic factors can induce angiogenesis to treat ischemic coronary and peripheral artery disease.4,5 This is called therapeutic angiogenesis. If angiogenesis could be achieved for the pulmonary vasculature, the mortality and morbidity of patients with severely decreased pulmonary vasculature could be minimized.

Hepatocyte growth factor (HGF), which was originally purified and cloned as a potent mitogen for hepatocytes,6 has mitogenic, motogenic, morphogenic, and antiapoptotic activities in various cell types.7,8 The pluripotent activities of HGF are mediated by a membrane-spanning tyrosine kinase receptor encoded by the c-met proto-oncogene.8 Physiologically, HGF acts as an organotrophic factor for the regeneration and protection of organs, including the liver,9,10,11 kidney,12,13 heart,14,15 and lung.16,17 Furthermore, HGF has an angiogenic effect and recent studies have demonstrated that HGF can potentially induce angiogenesis to alleviate coronary and peripheral artery disease.18,19 Clinical studies have begun for the treatment of patients with arteriosclerosis obliterans. HGF’s potent angiogenic effect, as well as its other multiple effects, is considered likely to be useful in cases of decreased pulmonary vasculature such as pulmonary infarction, pulmonary hypertension, or hypoplastic pulmonary vasculature of congenital heart disease. However, no study has yet reported angiogenesis in the lung, using in vivo transfection and expression of genes for any angiogenic factor, including HGF. Based on the above background, we hypothesized that in vivo gene transfection of the lung with HGF might possibly become a novel therapy in patients with severely decreased or hypoplastic pulmonary vasculature.

In this study, we determined whether in vivo gene transfection with HGF induces angiogenesis in the rat lung.

Materials and Methods

Animal Care

This study was carried out under the supervision of the Animal Research Committee in accordance with the Guidelines on Animal Care.
Experiments of Osaka University Medical School and the Japanese Government Animal Protection and Management Law.

Construction of Plasmid With Human HGF gene
To prepare an HGF expression vector, a human HGF cDNA was inserted into the Not I site of the pUC-SRα expression vector plasmid.20 In this plasmid, expression of the HGF cDNA is regulated under the control of the SRα promoter.21 We also constructed a control expression vector without the HGF gene.

Preparation of HVJ-Liposome
The preparation of the liposome complexed with the hemagglutination virus of Japan (HVJ) is described elsewhere.22 Briefly, 10 mg of a lipid mixture, prepared by mixing cholesterol, and a high mobility group 1 nuclear protein (64 g), was deposited on the side of a flask by removing tetrahydrofuran in a rotary evaporator. The dried lipid was hydrated in 200 μL of a balanced salt solution (137 mmol/L NaCl, 5.4 mmol/L KCl, 10 mmol/L Tris-HCl; pH 7.6) containing a DNA (200 μg)-HMGI (high mobility group 1 nuclear protein, 64 μg) complex. A liposome-DNA-HMGI complex suspension was prepared by thorough vortexing, sonication, and shaking. The liposome suspension was incubated with 30,000 HVJ particles, inactivated by ultraviolet irradiation, first at 4°C and then at 37°C. After a sucrose gradient centrifugation through, 4 mL of the layer containing HVJ-liposomes was collected and used for subsequent experiments.

Surgical Approaches
Wistar rats weighing 170 to 210 g were anesthetized by intraperitoneal injection of 50 mg/kg ketamine (Sankyo Co) and 5 mg/kg xylazine (Bayer Co). The rats were intubated and ventilated with a rodent ventilator (SN480–7, SHINANO Instruments). Breaths were administered at a rate of 80 per minute with a tidal volume of 2 mL.

Gene Transfection of the Left Lung via the Pulmonary Artery
Rats underwent occlusion of the left pulmonary artery and vein with infusion of liposomes into the left lung according to the method of Schachter et al.25 Briefly, a left thoracotomy was performed by entering the thoracic cavity at the third to fourth intercostal space. The single left lobe was reflected, and the left pulmonary artery and vein were isolated. After arterial inflow was occluded with a small vascular clip, a small arteriotomy was made in the left pulmonary artery distally, and a 28-gauge PI catheter (Argyle) was inserted. The catheter was secured in the left main pulmonary artery with a 3-00 suture. After clamping the distal left pulmonary artery with a third vascular clip, the catheter was removed and the arteriotomy was repaired with 9-0 sutures. After a 20-minute dwell period, the arterial and the venous clips were removed, and the pulmonary arterial flow was reestablished. The thorax was closed, and the soft tissues and the skin were closed in layers with 3 to 0 silk sutures. The animal was allowed to recover in a warm, oxygenated environment. The expression vector with HGF cDNA was transfected into 40 rats (H group) and the vector without HGF was transfected into another 40 rats, which served as the controls (C group) for the assessment of a pulmonary artery clamp test and histological analysis. Eight rats in each group were sacrificed 4, 7, 14, 21, and 28 days after the transfection, respectively. Another 60 rats (30 rats with HGF and 30 rats with control vector) were transfected for evaluation of pulmonary blood perfusion analysis and ELISA. Six rats in each group were sacrificed 4, 7, 14, 21, and 28 days after transfection, respectively.

Pulmonary Artery Clamp Test
To assess the change in pulmonary vascular resistance after HGF gene transfection, we performed a pulmonary artery clamp test that was designed based on our clinical experience23 and a preliminary result that rats subjected to a right pulmonary artery clamp tolerated for at least 5 minutes. Briefly, rats were anesthetized, intubated and ventilated again. A small mid-line thoracotomy was carefully performed with an electro-cautery to prevent bleeding. The pulmonary artery trunk was dissected out and taped. The proximal right and left pulmonary arteries were carefully dissected out. The right pulmonary artery was then clamped with a small clip just distal to the bifurcation. The right ventricular systolic pressure before and after clamping was measured by puncture with a 24G needle connected to a transducer (TERUMO) and a polygraph system (Nihon Kohden Co). This measurement was done 3 times, and the increase in right ventricular pressure (ΔRVP) was calculated. After collecting arterial blood from the ascending aorta, the heart and lungs were resected en bloc, and the lungs were cleared of blood by infusing cold phosphate-buffered saline (PBS) through a catheter positioned in the main pulmonary artery. All tissue samples subjected to histological analysis were fixed in ethanol.

Immunohistochemical Analysis
The tissue specimens obtained from the hilum of the left lung were fixed in ethanol, embedded in paraffin, and sectioned. Tissue sections were immunohistochemically stained with a rabbit polyclonal antibody against human HGF, using a standard indirect peroxidase-antiperoxidase method.24 Briefly, sections with 4-μm-thick were mounted on glass slides coated with 3-aminopropyltriethoxysilane, and air-dried overnight at room temperature. After deparaffinization, endogenous peroxidase was inactivated with methanol containing 0.3% hydrogen peroxide for 30 minutes. The sections were incubated at 4°C overnight with primary antibodies, and subsequently with biotinylated goat anti-rabbit IgG (DAKO) and biotinylated goat anti-mouse IgG (DAKO) at room temperature for 30 minutes. After incubation with the avidin-biotin-horseradish peroxidase complex (Vector Labs), enzymatic reaction was visualized with DAB, followed by incubation with DAB-enhancing solution (Vector Labs). The sections were counterstained with hematoxylin. Sections were also immunostained with antibodies against factor VIII and PCNA in the same fashion. The number of factor VIII-positive capillaries that were less than 100 μm in diameter was counted under microscopy for 10 randomly selected fields per specimen. The capillary density was determined as the average number of factor VIII-positive capillaries per 1 mm². The number of PCNA-positive endothelial cells per 1 mm² was also counted in the same fashion.

Laser Doppler Blood Flow Analysis
We measured the left/right lung blood perfusion ratio using a Laser Doppler Image (LDPI) analyzer (Moor Instruments, Cambridge, UK), with modified method used for rat hindlimb ischemia models.19,26 The blood flow measured by LDPI correlated well with capillary density.19 In this method, a color-coded image representing blood perfusion distribution is displayed. Low or no blood perfusion is displayed as dark blue, and the highest perfusion level is displayed as red to white. Before measurement, rats were anesthetized, intubated, and connected to a respirator. A midline thoracotomy was performed with an electro-cautery, the chest was opened wide using a set of clamps and the lung surface was exposed. The instrument was then placed above the rat so that the Laser beam scanned the lung surface vertically. After setting the condition of the scan area, ventilation was discontinued, and the lungs were inflated. We performed 2 consecutive LDPI scans over the same region of interest. After the scanning, the average perfusion values of the left (transfected) and right (nontransfected) lungs were calculated from histograms of the colored pixels. To minimize variations because of ambient light, calculated blood perfusion (relative units) was expressed as the left/right lung blood perfusion ratio.19,26

Enzyme-Linked Immunosorbent Assay
Human HGF in the left lung was measured by enzyme-linked immunosorbent assay (ELISA) using an HGF monoclonal antibody (Institute of Immunology, Tokyo, Japan). The human HGF ELISA kit specifically detects human HGF but not rat HGF.
Results

Expression of Human HGF Introduced by Gene Transfection
At 4 days after transfection of plasmid, an enzyme immunoassay demonstrated a significant \((P<0.01)\) pulmonary expression of human HGF protein in the H group compared with the C group (Figure 1A). Likewise, immunohistochemical examination with an anti-human HGF polyclonal antibody 4 days after transfection showed apparent and extensive expression of human HGF in the cytoplasm of the endothelium of the pulmonary arteries in the H group (Figure 1B). In contrast, human HGF was undetectable in lung tissues in the C group (Figure 1D). These results indicated that human HGF was specifically and predominantly expressed in endothelial cells in the lung transfected with HVJ-liposome containing expression vector for human HGF.

Pathological Evaluation of Angiogenesis by Immunohistochemistry
To evaluate angiogenesis in the lung, we performed an immunohistochemical examination with anti-factor VIII, a specific marker for endothelial cells. At 7 days after transfection, a marked increase in the number of factor VIII-positive pulmonary capillaries was observed in the H group (Figure 2A), compared with the C group (Figure 2B). Change in capillary density determined as Factor VIII-positive capillaries that were less than 100 \(\mu\)m in diameter per square millimeter, indicated that significant increase in capillary vessels was seen on day 4 after gene transfection (Figure 2C). The capillary density reached the maximum level by day 7 and the level was maintained within 28 days. In contrast, the capillary density in control lung transfected mock vector was not changed within 28 days after transfection. On day 7, the capillary density reached 15.0 \pm 1.3 in the H group, whereas it remained 8.0 \pm 1.7 \(\text{mm}^2\) in the C group. Therefore, angiogenesis in the lung was induced after HGF gene transfection, and capillary formation had apparently progressed by 7 days after transfection.

To evaluate endothelial proliferation, proliferating cells were immunohistochemically detected with an anti-PCNA antibody and PCNA-positive endothelial cells per \(\text{mm}^2\) was determined. The number of PCNA-positive cells did not change within 28 days in control lung transfected with mock vector (Figure 2D). In contrast, significant and marked increase in the number of PCNA-positive endothelial cells was seen as early as day 4 after transfection of expression vector for HGF. The number of PCNA-positive nuclei in capillaries 7 days after transfection in the H group was 4.8 \pm 0.4, whereas in the C group it was 0.8 \pm 0.3 \(\text{mm}^2\). Thus, the result indicates that HGF gene expression stimulated capillary endothelial proliferation in the lung.

Changes in Pulmonary Vascular Resistance of the Left Lung After HGF Transfection
To assess the hemodynamic change induced by HGF gene transfection, we performed a right pulmonary artery clamp test, in which the left lung received all the pulmonary blood flow from the right ventricle. This test measures changes in the pulmonary vascular resistance of the left lung. The increase in right ventricle pressure after right pulmonary artery clamp (\(\Delta\text{RVP}\)) was 16 mm Hg preoperatively, and it did not change in the C group after transfection. In contrast, a significant attenuation of the \(\Delta\text{RVP}\) was observed in the H group, 7, 14, 21, and 28 days after HGF gene transfection (Figure 3). The \(\Delta\text{RVP}\) value in the H group decreased to
9 mm Hg on day 7, the value was 60% of that in C group, and it remained similar levels within 28 days. Taken together with change in the capillary density induced by HGF gene transfection, these results strongly suggested that angiogenesis induced by HGF gene expression resulted in decreased right ventricle blood pressure after occlusion of right pulmonary artery.

Laser Doppler Analysis for Lung Blood Perfusion
To determine whether blood perfusion increased in the transfected left lung, we measured blood perfusion of the lung surface with a Laser Doppler Imager. Representative images of the lung blood perfusion 14 days after gene transfection are shown in Figure 4A (HGF-transfected) and Figure 4B (control-transfected). The ratio of left (transfected)/right (nontransfected) blood perfusion was 0.95 in both C and H groups before transfection, and it did not change within 28 days after transfection of mock vector. In contrast, the left/right blood perfusion ratio increased significantly 14, 21, and 28 days after transfection of HGF gene (Figure 4C). Thus, average blood perfusion values in the transfected left lung increased significantly by 14 days after the HGF gene transfection.

Discussion
In this study, we have demonstrated that in vivo transfection of HGF gene via the pulmonary artery induced angiogenesis in the lung, and that this angiogenic action might be exerted through the direct action of HGF on the endothelial cells of pulmonary capillaries. This conclusion is supported by the following evidence: (1) human HGF was expressed predominantly in the cytoplasm of endothelial cells; (2) the number of factor VIII-positive pulmonary capillaries significantly increased; (3) the blood perfusion of the left lung surface, as assessed by the Laser Doppler Imager, increased; and (4) left pulmonary vascular resistance, assessed by the pulmonary artery clamp test, decreased after HGF gene transfection. We previously demonstrated that in vivo transfection of HGF gene induced angiogenesis in the ischemic hindlimb29 and the infarcted heart.14,18 However, our present results are the first to show that in vivo transfection of HGF gene induces angiogenesis in the lung.

We evaluated capillary blood vessels in the lung after transfection and expression of HGF gene, both histologically and functionally. There was no histological abnormality in vascular structure such as pulmonary arterio-venous fistula29 and edema-like pathology because of immature vascularity by the experts of lung pathology. No significant decrease was seen in oxygen saturation in arterial blood after expression of HGF gene (data not shown). We could not see any general side effects during and after gene expression of HGF. Likewise, previous approaches on therapeutic angiogenesis with the similar delivery method of HGF gene in hind-limb
and ischemic heart models did not note abnormality in vascular structures and functions, and general side effects during the treatment. In stead, aberrant expression in c-Met receptor and HGF genes were noted in patients with lung cancer.27,28 However, neoplastic changes in various tissues of rats treated with HGF gene transfer were not seen in the present study, as well as previous studies on HGF gene therapy in experimental animals.18,19 Because HVJ-liposome-mediated HGF gene transfection allows local and transient but not persistent expression of HGF gene, and it did not allow systemic increase in blood HGF levels (not shown). HVJ-liposome-mediated HGF gene expression is likely to be safe and distinguished from aberrant expression and genetical alteration in c-Met receptor and HGF genes in cancer tissues. Nevertheless, because HGF enhances invasion of cancer cells, including lung carcinoma cells,27,28 careful analysis to evaluate potential involvement of HGF gene transfer in regulation of invasion and angiogenesis in lung cancer remains to be addressed.

Most recent studies of in vivo gene transfer to the lung used the intra-tracheal method of gene transfection.30–32 This method is technically easier than the trans-pulmonary arterial approach, but the transfected gene is expressed mainly in the alveolar epithelial cells or pneumocytes, and endothelial expression is difficult to obtain. In contrast, because the purpose of this study was to induce angiogenesis of the pulmonary arteries, we chose the method of transpulmonary arterial gene transfection. Our result showed the arterial transfection of HGF gene resulted in predominant expression in the pulmonary endothelial cells and proved our original hypothesis that the preferential expression of HGF gene in endothelial cells of the pulmonary arteries would sufficiently induce endothelial proliferation and subsequent vascular formation. In regard to technical details, Jeppesson et al13 first showed the efficacy of the trans-pulmonary venous approach in the lung transplantation model. However, their technique increased perfusion of the bronchial circulation, which was deleterious for the induction of pulmonary arterial angiogenesis. We used a 20-minute dwell time to permit liposome contact with endothelial cells. Mechanical ventilation may help improve the transfer efficiency.

HGF is a potent angiogenic factor, and could be useful as a clinical agent.18,19 We believe that promoting the endogenous angiogenic system through genetic engineering is a promising advanced strategy for regenerating the pulmonary vasculature. One of the advantages of gene transfer over recombinant protein administration is a persistent, localized delivery of the protein.34 Systemic delivery of recombinant protein has limitations that include the necessity of repeated injections of a large amount of peptide with possible side effects in other organs. Local drug delivery also has to be repeated with special equipment to sustain a high level of HGF in the lung. In contrast, gene transfection could effectively express the protein in the lung for the necessary time without repeated treatment. Thus, enhancing pulmonary HGF expression through gene transfection could be a promising therapeutic strategy.

Our strategy of gene therapy with angiogenic factors for the treatment of the lung is intended to combine with surgery. From a clinical perspective, the trans-pulmonary approach is feasible when it is combined with a conventional surgical procedure such as endarterectomy or a systemic-pulmonary shunt. This combined therapy may increase both pulmonary blood flow and the pulmonary vascular bed. This is important because even the gene expression obtained by transfection is transient. The safety of the HVJ liposome method has been reported,35 and a clinical trial of therapeutic angiogenesis with HGF gene for peripheral artery disease has begun with good short-term results. Therefore, our method may become a novel strategy for patients with severely decreased pulmonary vasculature.

The main limitation of this study is that our data were obtained using the normal rat lung. There are some differ-

Figure 4. Changes in the blood perfusion of the left lung after gene transfection. Blood perfusion of the lung was assessed using Laser Doppler Imager. (A, B) Representative Laser Doppler Image analysis of lung blood perfusion at 14 days after transfection of HGF gene (A) or mock vector (B). RU indicates relative units. (C) Change in the Left/right lung blood perfusion ratio after transfection of HGF gene (HGF) or control mock vector (CON). Left/right lung blood perfusion ratio was calculated using the average values of blood perfusion in the left (transfected) and the right (nontransfected) lung. Rats were measured on day 0 (before surgery), 4, 7, 14, 21, and 28 days after gene transfection. Each value represents the mean ± SEM of values obtained using 6 rats at each time point in each group. *P<0.01 versus CON (ANOVA).
ences in the condition of pulmonary vasculature in normal and diseased lungs, including the tissue level of angiogenic factors and endothelial function. Further investigation is required under conditions that represent the damaged or underdeveloped lung, including pulmonary infarction or pulmonary hypertension models, to assess the potential response of the damaged endothelium to HGF and to assess its other effects in the damaged pulmonary vasculature.

In summary, in vivo gene transfection of the lung with HGF by means of the pulmonary arterial injection of HVJ-liposomes caused pulmonary vascular endothelial-cell proliferation, ameliorated the increase in pulmonary vascular resistance when pulmonary blood flow increased, and increased pulmonary blood flow in the left lung, suggesting pulmonary angiogenesis. These data indicate a direct role for HGF angiogenesis in the lung, suggesting the possibility that gene transfection with HGF may become a novel gene therapy for patients with severely decreased pulmonary vasculature.

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