Transfection of Human Hepatocyte Growth Factor Gene Ameliorates Secondary Lymphedema via Promotion of Lymphangiogenesis

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Background—Lymphedema is a disorder of the lymphatic vascular system characterized by impaired lymphatic return and swelling of the extremities. Treatment for this disabling condition remains limited and largely ineffective. The goal of the present study was to investigate the therapeutic efficacy of hepatocyte growth factor (HGF) in animal models of lymphedema.

Methods and Results—Immunofluorescent analysis demonstrated that canine primary lymphatic endothelial cells (cLECs) were positive for lymphatic-specific markers (vascular endothelial growth factor receptor-3, LYVE-1, podoplanin, and Prox1) and the HGF receptor c-Met. Treating cLECs with human recombinant HGF resulted in a dose-dependent increase in cell growth and migration and increased activity of extracellular signal-regulated kinase and Akt. In human LECs, c-Met also was expressed, and treatment with HGF increased cell growth and migration in a dose-dependent manner. Transfection of human HGF plasmid DNA in cLECs also increased the c-fos promoter activity. Furthermore, weekly HGF gene transfer in a rat tail lymphedema model by disruption of lymphatic vessels resulted in a decrease in lymphedema thickness. Although expression of the endothelial cell marker PECAM-1 was increased in both HGF- and vascular endothelial growth factor 165–injected groups, expression of LEC markers (LYVE-1 and Prox1) was increased only in the HGF-injected group.

Conclusions—These data demonstrate that expression of HGF via plasmid transfer improves lymphedema via promotion of lymphangiogenesis. Further studies to determine the clinical utility of this approach would be of benefit to patients with lymphedema. (Circulation. 2006;114:1177-1184.)

Key Words: gene therapy ■ hepatocyte growth factor ■ lymphangiogenesis

The lymphatic vascular system maintains tissue fluid homeostasis and plays a role in the affterent immune response.1,2 Anatomic or functional obstruction in the lymphatic system after radical surgery or radiotherapy3,4 can result in the progressive accumulation of protein-rich fluid in the interstitial spaces (lymphedema). Despite substantial advances in surgical and conservative techniques, therapeutic options for management of lymphedema are limited.4,5

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Recent studies suggest that lymphangiogenesis that can be stimulated by various cytokines,6,7 for example, vascular endothelial growth factor (VEGF)-C and -D promote lymphangiogenesis by activating the VEGF receptor-3 (VEGFR-3), which is expressed on lymphatic endothelial cells (LECs).8 Furthermore, VEGF-C–deficient mice fail to develop a functional lymphatic system,9 transgenic expression of soluble VEGF-R-3 results in pronounced lymphedema,10 and gene transfer of VEGF-C effectively reduces lymphedema in an animal model.11 Another study reported that angiopoietin-1 also promotes lymphatic vessel formation through Tie2.12

Hepatocyte growth factor (HGF) is a mesenchyme-derived pleiotropic factor that regulates growth, motility, and morphogenesis of various types of cells and thus is considered a humoral mediator of the epithelial-mesenchymal interactions responsible for morphogenic tissue interactions during embryonic development and organogenesis.13,14 Moreover, the presence of a local HGF system (HGF and its specific receptor, c-Met) has been characterized in vascular cells in vitro and in vivo.15 Our group and others have demonstrated that HGF treatment results in an increase in aortic endothelial...
cell proliferation and migration and that intramuscular injection of “naked” human HGF plasmid resulted in a significant increase in blood flow and capillary density in a rat and rabbit ischemic model. Furthermore, a recent study reported that c-Met was highly expressed in LECs and that HGF was a strong promoter of lymphatic vessel formation. Therefore, the goal of the present study was to investigate the therapeutic efficacy of HGF in animal models of lymphedema.

Methods

Cell Culture

Canine LECs (cLECs), canine aortic endothelial cells (cAECs), and canine venous endothelial cells (cVECs) were isolated from canine thoracic ducts, aorta, or vein as previously described with some modifications. Briefly, adult mongrel female dogs (8.0 to 10.0 kg body weight; Nihon Nosan Inc, Yokohama, Japan) were anesthetized with thiamylal sodium (50 mg/kg IV) and then intubated. For visualization of the thoracic duct, 20 mL IP of 0.5% patent blue was injected. Ten to 15 cm of the thoracic duct, aorta, or vein was isolated and placed in Hanks’ buffered salt solution at 4°C and then skeletonized rapidly. Vessels were flushed with cold Hanks’ buffered salt solution and incubated with collagenase solution (500 U/mL in Hanks’ buffered salt solution, Worthington Biochemical Co, Lakewood, NJ) for 10 minutes at 37°C. Cells were harvested by washing vessels and subsequent centrifugation, and cells were maintained in endothelial basal medium supplemented with 20% FBS and endothelial growth supplement on 0.1% gelatin-coated culture dishes. Cultures were incubated at 37°C in a humidified atmosphere of 95% air/5% CO₂ with exchange of medium every 2 days. These cells were further purified with magnetic microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) attached to an anti–von Willebrand factor or an anti–LYVE-1 antibody as previously described with slight modifications. These cells were used at passages 5 to 8 for subsequent experimental protocols.

Human LECs were from AngioBio Co (Del Mar, Calif) and were used at passages 5 to 8 for subsequent experimental protocols.

Immunofluorescent Analysis

Cells on glass coverslips and 8-μm cryostat sections of rat tail tissue were fixed in 4% paraformaldehyde or ethanol for 10 minutes. The samples were permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 5 minutes. After blocking in 5% skim milk, the samples were stained with primary antibodies, including polyclonal rabbit anti-human von Willebrand factor (Dako, Glostrup, Denmark), polyclonal goat anti-human PECAM-1 (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), polyclonal rabbit anti-human VEGFR-3 (CHEMICON, Temecula, Calif), LYVE-1, podoplanin, Prox1 (Research Diagnostics, Inc, Flanders, NJ), or polyclonal rabbit anti–c-Met (Santa Cruz Biotechnology, Inc) for 1 hour at room temperature. Corresponding secondary antibodies were labeled with AlexaFluor488 or AlexaFluor546 (Molecular Probes, Eugene, Ore).

MTS Assay and c-fos Promoter Assay

Cell proliferation of LECs or endothelial cells seeded in 6-well plates was measured with the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay at 48 hours after treatment with recombinant HGF (a kind gift from K. Matsumoto, Osaka University, Osaka, Japan), as previously described. Approximately 100 μL CellTiter 96 One Solution Reagent (Promega, Madison, Wis) in 500 μL of Dulbecco’s modified Eagle’s medium was added to each well, and absorbance was measured at 490 nm. The c-fos promoter assay was performed in cLECs by cotransfection with the c-fos–luciferase reporter gene (p2FTL) using Lipofectamine2000 Transfection Regent (Invitrogen, Carlsbad, Calif). The c-fos–luciferase reporter gene consisted of 2 copies of the c-fos 5′-regulated enhancer element (–357 to –276); the herpes simplex virus thymidine kinase gene promoter (–200 to 70); and the luciferase gene. At 1 hour after transfection, transfected cells were incubated with serum-free medium for 24 hours. Cells were washed with PBS and lysed for 15 minutes with 200 μL cell lysis buffer at room temperature. Then, 20 μL cell extract was mixed with 100 μL luciferase assay reagent, and the light produced was measured for 30 seconds with a luminometer.

Cell Migration

Migration of LECs was estimated in a modified Boyden chamber as previously described. In brief, polivinylpyrrolidone-free polycarbonate membranes (Neuro Probe Inc, Gaithersburg, Md) with 5-μm pores were coated with 0.1% gelatin overnight and then washed with PBS to remove excess coating. Next, 28 μL Dulbecco’s modified...
Eagle's medium with or without recombinant HGF (10 or 50 ng/mL) was placed in the lower chamber. The membrane was positioned above the lower chamber, and 10^6 cells/mL were suspended in 50% Dulbecco's modified Eagle's medium with or without recombinant HGF (10 or 50 ng/mL) and then added to the upper chamber. The Boyden chamber was incubated at 37°C for 4 hours. After incubation, the membrane was removed, and the cells on the upper side of the membrane were scraped off. The cells on the lower side of the membrane were stained with Diff-Quick (Sysmex, Hyogo, Japan), and the cells were counted in 5 randomly chosen fields under 200 magnification.

Western Blotting
Western blotting was performed for analysis of extracellular signal-regulated kinase (ERK) and Akt expression using a phospho-specific antibody as previously described. After treatment, cells were extracted with a lysis buffer (50 mmol/L Tris(hydroxymethyl)methylamino chloride, 2.5 mmol/L ethyleneglycoltetraacetic acid, 1 mmol/L ethylenediaminetetraacetic acid, 10 mmol/L sodium fluoride, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L Na_2VO_4). Samples containing 20 μg protein were separated on 10% SDS polyacrylamide gels, transferred to nitrocellulose membranes (Hybond ECL, Amersham), and incubated with a polyclonal antibody against phospho-specific or total ERK (polyclonal rabbit immunoglobulin G [IgG], 1:1000, Cell Signaling Technology, Beverly, Mass) or phospho-specific or total Akt (polyclonal rabbit IgG, 1:1000, Cell Signaling Technology) at 4°C overnight. The membranes were then washed and incubated with a 1:2000 dilution of anti-rabbit IgG horseradish peroxidase–conjugated antibody (Amersham). Bound antibodies were detected by enhanced chemiluminescence (Amersham) and Hyperfilm-MP (Amersham).

Inhibition of ERK and Akt
cLECs were pretreated with a mitogen-activated protein kinase (MEK) inhibitor, U0126 (50 μmol/L; Calbiochem, San Diego, Calif) or PD98059 (30 μmol/L; Calbiochem), and an inhibitor of phosphatidylinositol 3-kinase (PI3K), LY294002 (50 μmol/L; Calbiochem) or wortmannin (100 μmol/L; Calbiochem) for 1 hour. Then, the cells were treated by the inhibitors and recombinant HGF (50 ng/mL) for 24 hours in preparation for assays.

Gene Transfer Protocol in a Rat Tail Model
A rat lymphedema model was created as previously described with slight modification. Briefly, an 8-week-old male Sprague-Dawley rat (Oriental Bio Science Co, Ltd, Kyoto, Japan) was anesthetized with pentobarbital sodium (50 mg/kg IP), and 0.2 mL of 0.5% patent blue was injected into the tip of the tail for visualization of lymphatic vessels. At the base of the tail, a circumferential incision was made through the dermis to sever the superficial lymphatic network. The blue lymphatic vessels were ligated with 6-0 silk suture or cauterized. Cauterization was applied to the edges of the circumferential wound, which resulted in a 2- to 4-mm gap between the skin edges, followed by secondary healing at the site of ligation.

In total, 60 rats were randomly assigned to 1 of 6 groups: human HGF plasmid (200 μg/0.1 mL; inserted into pVAX),25 human VEGF165 plasmid (200 μg/0.1 mL; inserted into pCAGGS),25 green fluorescent protein (GFP) plasmid (200 μg/0.1 mL), saline, operation only, and no operation (n=10 in each group). HGF, VEGF165, GFP plasmid, or 0.1 mL saline was injected intramuscularly at the distal operated site with a 30-gauge needle on days 1, 7, and 14 after surgery. Lymphatic vessel quantification was performed by assessing positive immunofluorescent staining for c-Met in cLECs as previously described.11
All animal protocols were approved by the Animal Ethics Committee of Asahikawa Medical University and by the Osaka University Committee on Animal Research.

**Measurement of Human and Rat HGF**

Tissue samples were harvested from the operative site of the rat tail at the first time point. Human HGF expression was measured by real-time reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was extracted with ISOGEN (Nippon Gene, Toyama, Japan). Complementary DNA was synthesized with the ThermoScript RT-PCR System (Invitrogen, Carlsbad, Calif). Relative gene copy numbers of HGF and glyceraldehyde phosphate dehydrogenase were quantified by real-time RT-PCR with TaqMan Gene Expression Assays (HS.00300159, Applied Biosystems, Foster City, Calif). The absolute number of gene copies was normalized with glyceraldehyde phosphate dehydrogenase and standardized by a sample standard curve.

Rat endogenous immunoreactive HGF concentration also was measured by an enzyme immunoassay using anti-rat HGF antibody. In brief, 96-well plates (Corning, NY) were coated with rabbit anti-rat HGF IgG at 4°C for 15 hours. After blocking with 3% bovine serum albumin in PBS, the conditioned medium was added to each well, and the preparation was incubated for 2 hours at 25°C. The wells were washed 3 times with PBS containing 0.025% Tween 20 (PBS-Tween); biotinylated rabbit anti-rat HGF IgG was added; and the preparation was incubated for 2 hours at 25°C. After washing with PBS-Tween, the wells were incubated with horseradish peroxidase–conjugated streptavidin-biotin complex in PBS-Tween. The enzyme reaction was initiated by adding substrate solution composed of 2.5 mg/mL p-phenylenediamine, 100 mmol/L citric acid, and 0.015% H2O2. The enzyme reaction was terminated by adding 1 mol/L H2SO4, and absorbance was measured at 490 nm.

**Statistical Analysis**

Statistical analysis was performed with StatView 5.0 software (SAS Institute, Inc, Cary, NC). All results are expressed as mean±SEM. Data were compared using ANOVA, followed by the Dunnett test for pairwise comparisons against control and by the Tukey test for multiple comparisons. Rat tail thickness data also were analyzed by repeated-measures ANOVA. To evaluate the effect of each treatment further, we used the mixed-effect model from day 1 to 35.

The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.

**Results**

**Development of LEC Culture System**

cLECs were isolated from canine thoracic ducts (Figure 1A) and grown in culture until confluence on day 14 (Figure 1B). Immunofluorescent staining of these cells was positive for the endothelial cell markers von Willebrand factor and PECAM-1 (Figure 1C and 1D) and for the lymphatic-specific markers VEGFR-3, LYVE-1, podoplanin, and Prox1 (Figure 1E through 1H). Because all cells were positive for these markers, the purities of cLEC cultures were considered to be >99%.

**HGF Promotes LEC Proliferation and Migration**

To investigate whether HGF promotes cLEC proliferation and migration, the cell was treated with several concentrations of human recombinant HGF. Treating cLECs with human recombinant HGF resulted in a dose-dependent increase in cellular proliferation (no treatment, 0.227±0.014 cells; 2 ng/mL, 0.303±0.023 cells; 10 ng/mL, 0.368±0.033 cells; 50 ng/mL, 0.367±0.031 cells; absorbance optical density (OD), 490 nm; *P<0.05; Figure 2A) and migration (no treatment, 4.333±0.882 cells; 10 ng/mL, 228.000±5.033 cells; 50 ng/mL, 277.333±15.624 cells; *P<0.05; Figure 2B). Furthermore, cells cotransfected with the c-fos–luciferase reporter gene and plasmid encoding human HGF showed increased c-fos promoter activity compared with cells transfected with GFP plasmid (as control) or with VEGF165 plasmid (P<0.05; Figure 2C).

Expression of c-Met was demonstrated by immunofluorescent staining (Figure 3A), suggesting that cLECs are responsive to HGF. Furthermore, ERK or Akt was phosphorylated from 5 to 15 minutes after the addition of HGF in cLECs, whereas total ERK or Akt protein levels were not altered by treatment with recombinant HGF (Figure 3B).

To investigate the role of ERK and Akt in the proliferative or migratory pathway of HGF, cLECs were pretreated with an MEK inhibitor (U0126 or PD98059) and a PI3K inhibitor (LY294002 or wortmannin). Treating cLECs with both inhibitors completely attenuated cellular proliferation (P<0.0001; Figure 4A). In cellular migration, both inhibitors significantly attenuated HGF-induced migration, whereas the effect of the PI3K inhibitors was more potent (P<0.0001; Figure 4B).

![Figure 4. Inhibition of ERK and Akt in LECs. A, B, Effect of the MEK inhibitors U0126 (50 μmol/L) or PD98059 (30 μmol/L) and PI3K inhibitors LY294002 (50 μmol/L) or wortmannin (100 nmol/L) in the proliferative or migratory pathway of HGF on cLECs as demonstrated by the MTS and migration assay. n=8. *P<0.0001 vs control; †P<0.0001 vs HGF; ‡P<0.05 vs control.](image-url)
To examine the specificity of cLECs, we also examined the effect of HGF on aortic and venous endothelial cellular growth obtained from dog. Immunofluorescent staining of these cells was positive for the endothelial cell marker von Willebrand factor (Figure 5A) and the HGF receptor c-Met (Figure 5B). Treatment with human recombinant HGF resulted in dose-dependent increases in cellular growth (cAECs: no treatment, 1.132±0.011; 2 ng/mL, 1.156±0.016; 10 ng/mL, 1.249±0.024; 50 ng/mL, 1.268±0.033; cVECs: no treatment, 0.814±0.008; 2 ng/mL, 0.910±0.010; 10 ng/mL, 0.945±0.032; 50 ng/mL, 1.001±0.032; absorbance OD, 490 nm; P<0.05; Figure 5C), which were consistent with the previous results of human aortic endothelial cells.26 Moreover, we also used human LECs to confirm the effect of HGF. As shown in Figure 5D and 5E, immunofluorescent analysis demonstrated that human LECs were positive for endothelial cell marker (von Willebrand factor), lymphatic-specific markers (VEGFR-3 and Prox1), and the HGF receptor c-Met. Treating human LECs with recombinant HGF resulted in an increase in cellular proliferation by MTS assay (no treatment, 0.810±0.035 cells; 2 ng/mL, 0.919±0.056 cells; 10 ng/mL, 1.278±0.035 cells; 50 ng/mL, 1.081±0.052 cells; absorbance OD, 490 nm; P<0.05; Figure 5F) and migration by modified Boyden chamber method (no treatment, 5.800±0.663 cells; 2 ng/mL, 55.200±6.127 cells; 10 ng/mL, 208.400±36.405 cells; 50 ng/mL, 166.600±7.991 cells; P<0.05; Figure 5G) in a dose-dependent manner. These results support the idea that HGF would induce LEC growth and migration.

Local Human HGF Gene Transfer Improves Lymphedema in a Rat Tail Model

Given the in vitro data, we hypothesized that overexpression of HGF would stimulate the growth of the lymphatic vascular system and alter the lymphedema. To investigate the effect of HGF gene transfer on the lymphedema, an operative model of rat tail lymphedema was created, and tail thickness was measured over the subsequent 35 days. Human HGF expression was detected by RT-PCR only in the human HGF-injected group, and maximal expression was detected 4 days after the operation. Of note, endogenous rat HGF concentrations were significantly upregulated in the human HGF-injected group at 4, 10, and 17 days after the operation (P<0.001; Figure 6A), which was consistent with a previous report.27 Tail thickness began to increase 1 day after the operation and was stable at 7 days in all animal groups, except for...
those that did not undergo operation (Figure 6B). However, on days 14, 21, 28 and 35, tail thickness was decreased from the thickness noted on day 7 only in the HGF group compared with the control group \( (P < 0.0001; \text{Figure 6C}) \). To analyze the total amount of HGF-induced effect on tail thickness during the recovery process of tail thickness, we performed repeated-measures ANOVA, including group, days, and interaction between group and days as factors. There are significant differences in group, days, and interactions between group and days \( (P < 0.0001) \). To evaluate the effect of each treatment further, we repeatedly compared the curve in a mixed-effect model from day 1 to 35. Indeed, the curve of the HGF group was significantly different from that of the control group \( (P < 0.0001; \text{Figure 6C}) \).

Discussion

Chronic lymphedema is a disabling condition characterized by thickening of the skin resulting from fibrofatty deposition in underlying tissues and disfiguring swelling of affected limbs. Most cases of secondary lymphedema in humans are due to disruption or depletion of lymphatic vessels.\(^3\) The present study demonstrated that human HGF gene transfer promoted local lymphangiogenesis and attenuated the phenotypic changes associated with secondary lymphedema.

The present study used an adult LEC system for various experimental protocols. Although most previous studies that investigated lymphangiogenesis have used fetal or neonatal LECs,\(^2\) expression levels of lymphatic markers such as Prox1 or LYVE-1 in those studies have been variable. Because LECs differentiate and bud from embryonic veins during early embryogenesis, these lymphatic markers also are expressed in early venous endothelial cells, and LECs can transdifferentiate into venous endothelial cells.\(^7\) In the adult LEC system used in the present study, LECs were purified with LYVE-1–conjugated magnetic beads, and lymphatic markers were stably expressed in this system. Moreover, the
HGF receptor c-Met also was expressed in the adult LEC, and ERK and Akt phosphorylation increased with HGF exposure, suggesting that these adult LECs were HGF responsive, similar to the manner in which aortic endothelial cells respond to HGF. Because the inhibitors of MEK and PI3K significantly attenuated the cellular viability and migration, we speculate that ERK and Akt could be important in lymphangiogenesis and arterial angiogenesis. Interestingly, the effect of the PI3K inhibitors was more potent in cellular migration, which was consistent with that in aortic endothelial cells. These data demonstrate that HGF promotes arterial and lymphatic angiogenesis after surgical disruption of these vessels.

In terms of therapeutic angiogenesis using human HGF plasmid, the safety and efficacy of HGF plasmid DNA in patients with critical limb ischemia have been investigated in an ongoing prospective, open-label, clinical trial. Preliminary data suggest that HGF treatment results in a reduction in pain scale, an increase in the ankle pressure index, and a decrease in lower extremity ulcer size. Furthermore, there were no signs of systemic or local inflammatory reactions and no development of tumors or progression of diabetic retinopathy in this population. Of note, no edema was observed in this trial, in contrast to the transient lower-extremity edema reported with clinical gene therapy using the VEGF-A gene.

In terms of lymphedema therapy, VEGF-C also activates VEGFR-2, which may induce angiogenesis and local edema through increases in vascular permeability. In contrast, HGF treatment has not been associated with edema in human trials or animal experiments. Moreover, a recent study reported that HGF promotes lymphatic vessel formation by mechanisms that are independent of the VEGF-C pathway. These data suggest that HGF is a promising therapeutic agent for the treatment or prevention of lymphedema.

Many cancer patients develop lymphedema after the dissection of lymph nodes that is part of the staging and treatment process of their disease. However, clinical trials of HGF therapy have excluded patients with preexisting cancer because of theoretical concerns about tumor progression in the context of an angiogenic stimulus. We previously reported that local overexpression of HGF in tumor-bearing mice did not stimulate distant tumor growth despite local elevations in HGF concentrations. Further investigation to characterize the risks of HGF therapy in patients with preexisting cancer would be of benefit.

In conclusion, the present study demonstrated that genetic transfer of HGF plasmid DNA–attenuated lymphedema via the promotion of lymphangiogenesis. Further studies to determine the clinical utility of this approach would be of benefit to patients with lymphedema.

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Disclosures
None.

References
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