Critical limb ischemia is estimated to develop in 500 to 1000 individuals per million per year.\(^1\) In a large proportion of these patients, the anatomic extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization. Thus the disease frequently follows an inexorable downhill course.\(^2,3\) Of importance, there is no optimal medical therapy for critical limb ischemia, as the Consensus Document of the European Working Group on Critical Limb Ischemia concluded.\(^1\) Therefore novel therapeutics are required to treat these patients. In the pathophysiology of the disease, in the presence of obstruction of a major artery, blood flow to the ischemic tissue is often dependent on collateral vessels. When spontaneous development of collateral vessels is insufficient to allow normal perfusion of the tissue at risk, residual ischemia occurs. Preclinical studies have demonstrated that angiogenic growth factors can stimulate the development of collateral arteries in animal models of peripheral and myocardial ischemia,\(^4-9\) a concept called therapeutic angiogenesis. Most of the studies have used vascular endothelial growth factor (VEGF), also known as vascular permeability factor as well as a secreted endothelial-cell mitogen. The endothelial cell specificity of VEGF has been considered to be an important advantage for therapeutic angiogenesis, as endothelial cells represent the critical cellular element responsible for new vessel formation.\(^10,11\) More recently, the efficacy of...
therapeutic angiogenesis with VEGF gene transfer has been reported in human patients with critical limb ischemia.\textsuperscript{12,13} Thus the strategy for therapeutic angiogenesis with angiogenic growth factors should be considered for the treatment of patients with critical limb ischemia.

On the other hand, we and others have previously reported that hepatocyte growth factor (HGF) exclusively stimulated the growth of endothelial cells without replication of vascular smooth muscle cells (VSMC), thereby indicating it to be a potential angiogenic growth factor.\textsuperscript{14–17} Unexpectedly, the mitogenic activity of HGF is more potent than that of VEGF in human aortic endothelial cells.\textsuperscript{15,17} Moreover, HGF and its specific receptor, c-met, have been shown to be expressed in the heart and blood vessels including endothelial cells and VSMC.\textsuperscript{18,19} Therefore we reasoned that HGF may be a potential therapeutic angiogenic growth factor, in addition to VEGF. Indeed, activation of the HGF system promoted angiogenesis in a Matrigel system,\textsuperscript{20} but this system provides far from physiological conditions. Thus there is no direct in vivo evidence that HGF produces therapeutic angiogenesis in ischemic disease. In this study, we addressed 2 specific questions: (1) how endogenous HGF is regulated in the blood vessels of patients with ischemic limb disease and (2) whether it is possible to promote therapeutic angiogenesis by means of HGF in the rabbit ischemia model to examine the feasibility of therapy for critical limb ischemia.

\section*{Methods}

\subsection*{Experiment 1}

\textbf{Subjects}

For the study of serum and vascular HGF concentration, diseased segments of vessels from 7 patients with arteriosclerosis obliterans (ASO) (65.6±12.2 years old) and 6 disease-free segments from the same patients were obtained at the time of operation. Patients did not have the following disorders: cardiac valvular disease, congestive heart failure, arrhythmia, or hepatic, renal, or pulmonary dysfunction. The study protocol was approved by the Ethics Committee of Osaka University Hospital.

\textbf{Measurement of HGF Concentration in Blood Vessels}

Vascular HGF concentration was assayed with a recently developed enzyme immunoassay for use in humans.\textsuperscript{18,19,21} On the day of extraction, the tissue was thawed at 4°C, weighed, and homogenized by polytron in assay solution. Each specimen was centrifuged at 20,000 \(g\) for 30 minutes at 4°C to remove the lysates. The concentration of HGF in blood vessels was determined by enzyme immunoassay with anti-human HGF antibody, as described previously.\textsuperscript{21} The antibody against human HGF reacts only with human HGF and not with rat HGF.\textsuperscript{21} For the organ culture experiment, rat HGF concentration was measured with the use of anti-rat HGF antibody.\textsuperscript{21}

\textbf{Reverse Transcription–Polymerase Chain Reaction}

RNA was extracted from blood vessels by treatment with RNAzol (Tel-Test Inc). Levels of HGF and G3PDH mRNA were measured by reverse transcription–polymerase chain reaction (RT-PCR).\textsuperscript{18,19} The HGF 5′ primer (nucleotides 1409 to 1426 of human sequence) was 5′-ATG-CTC-ATG-GAC-GCT-GGT-3′; the 3′ primer (nucleotides 1797 to 1814 of human sequence) was 5′-GCC-TGA-GAA-GCT-TCA-TTA-3′ (423 bp). Extreme care was taken to avoid contamination of tissue samples with trace amounts of experimental RNA. Aliquots of RNA (0.5 \(\mu\)g) derived from cultured cells were amplified simultaneously by PCR (30 cycles), by individuals who were blinded to the identity of the samples, and compared with a negative control (primers without RNA). In preliminary experiments, the number of amplification cycles for each gene was determined by performing RT-PCR for 20, 25, 30, 35, and 40 cycles. PCR products were within the linear logarithmic phase of the amplification curve until 40 cycles. To ensure that the RT-PCR amplified product reflects transcribed HGF RNA without significant DNA contamination, RNA samples treated with RNase A or amplified without RT were simultaneously amplified as negative controls. These samples did not result in a visual band. Moreover, PCR products were cut by restriction enzymes, and the fragments were identical to the theoretical bands.

\subsection*{Experiment 2}

\textbf{Rabbit Ischemic Hind Limb Model}

The physiological response to administration of rhHGF was investigated in the rabbit ischemic hind limb model, described in previous reports.\textsuperscript{5,6} Male New Zealand White rabbits (3.5 to 4.0 kg) (Kitayama Rabes) were anesthetized with a mixture of xylazine (5 mg/kg) and ketamine (50 mg/kg). A longitudinal incision was then made, extending inferiorly from the inguinal ligament to a point just proximal to the patella. Through this incision, with the use of surgical loupes, the operator dissected free the left femoral artery along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric arteries, were also dissected free. After dissection of the popliteal and saphenous arteries distally, the external iliac artery and all of the mentioned arteries were ligated with 4-0 silk (Ethicon). Finally, the left femoral artery was completely excised to create the ischemic limb model, from its proximal origin as a branch of the external iliac artery to the point distally where it bifurcates to form the saphenous and popliteal arteries. Excision of the femoral artery results in retrograde propagation of thrombus and occlusion of the external iliac artery. Excision of the femoral artery 1 cm below the peritoneum created a moderate ischemia model; excision 1 cm above the peritoneum created a severe ischemia model. The operative procedure was similar in these 2 models. However, only a difference in excision points produced a marked difference in ischemic symptoms. The calf blood pressure ratio in the moderate ischemia model was determined 0, 10, and 30 days after operation (0 days: 0.46±0.05, 10 days: 0.61±0.08, 30 days: 0.69±0.06). In contrast, it was impossible to measure calf blood pressure in the severe ischemia model both at 0 and 10 days after operation (0 days: not detected, 10 days: not detected). In addition, probably because of some blood flow in the moderate ischemia model, we did not find any signs of necrosis of the nails and muscle, whereas severe necrosis was found in all severe ischemia animals. We therefore used the relatively moderate ischemia model in this study. Consequently, blood flow to the ischemic limb was dependent on collateral vessels developing from the internal iliac artery.

\textbf{Design 1}

To ensure the maximum effect of rhHGF, we administered 2 separate injections of 500 \(\mu\)g rhHGF locally (intra-arterially into the ischemic limb): the first dose on day 10 and the second on day 12 after the operation. The dose of rhHGF used in the present study was chosen on the basis of previous experiments.\textsuperscript{5,6} Ten days after surgery (day 10) and after measurement of baseline body weight as well as baseline noninvasive and invasive measurements of hemodynamic parameters, the animals received the first intra-arterial bolus of rhHGF (500 \(\mu\)g/animal) or vehicle (3 mL saline with 0.1% rabbit serum albumin; Sigma) administered over 1 minute through a 3F end-hole infusion catheter (Terumo) positioned in the internal iliac artery of the ischemic limb. On day 12, the same dose of drug was administered intra-arterially.

\textbf{Design 2}

In design 2, rhHGF (3 mg) was intravenously administered for 5 days from day 10 to day 14 after the operation. Animals received rhHGF (3 mg in 6 mL vehicle/animal) or vehicle (6 mL saline with 0.1% rabbit serum albumin) intravenously administered over 1 hour with an infusion pump.
Quantitative Angiography
The angiographic luminal diameter of the internal iliac artery in the ischemic limb at baseline and after drug infusion was determined on days 0, 10, and 30 by previously described techniques. Briefly, morphometric analysis of collateral vessel development in the ischemic limb was performed in 4-second angiograms recorded after injection of contrast medium into the internal iliac artery. A grid with 20-mm spaces was placed over the angiogram in the region of the medial thigh. The number of contrast-opacified arteries crossing over circles and the total number of lines encompassing the medial thigh area were counted in a blinded fashion. The angiographic score was calculated as the ratio of overlying opacified arteries divided by the total number of lines in the ischemic thigh. This angiographic score reflects vascular density in the medial thigh.

Materials
Human recombinant HGF was purified from the culture medium of Chinese hamster ovary cells or C-127 cells transfected with an expression plasmid containing human HGF cDNA.

Statistical Analysis
All values are expressed as mean±SEM. ANOVA with subsequent Duncan’s test was used to determine the significance of differences in multiple comparisons. Differences with a value of P<0.05 were considered significant.

Results
Decreased Endogenous HGF Production in Ischemic Limb in Humans
As shown in Figure 1, vascular HGF concentration in the diseased segments of blood vessels from patients was significantly decreased as compared with that in disease-free segments of blood vessels from the same patients as control vessels. The decrease in vascular HGF concentration was accompanied by a decrease in HGF mRNA in the diseased segments of blood vessels (Figure 1c). In contrast, serum HGF concentration in patients with peripheral arterial disease was significantly higher than that in healthy volunteers (patients, 0.43±0.03 ng/mL vs control 0.35±0.03 ng/mL, P<0.01), probably in response to damaged vasculature. Downregulation of the vascular HGF system may be related to the pathogenesis of peripheral arterial disease, since HGF showed an antiapoptotic action on endothelial cells, in addition to its mitogenic activity. The decrease in local HGF production in the vessels of patients with ASO might be due to transforming growth factor-β or hypoxia, which are strong suppressors of vascular HGF in vitro as well as in vivo. Alternatively, HGF belongs to the family of kringle proteins that mediate protein/protein and protein/cell interactions, which suggests a potential role in the regulation of thrombosis. Therefore decrease in local HGF production may also be responsible for disturbance of local microcirculation.

Angiogenesis Induced by Intra-Arterially Injected rhHGF
Given the significant decrease in endogenous HGF production in the ischemic limb, we hypothesized that administration of rhHGF into the ischemic limb might result in a beneficial effect in hypoxia. Therefore rhHGF was intra-arterially administered through the internal iliac artery of rabbits in which the femoral artery was excised to induce unilateral hind limb ischemia.

There was no significant difference in body weight between the rabbits treated with rhHGF and vehicle on day 40 after surgery (vehicle, n=7: 3.63±0.13 kg, rhHGF: n=5: 3.86±0.07 kg). Administration of rhHGF into the ischemic limb on days 10 and 12 after surgery produced significant augmentation of collateral vessel development as assessed by angiography on day 30 in the moderate ischemia model, as shown in Figure 2 (P<0.01). Serial angiograms revealed...
progressive linear extension of collateral arteries from the origin stem artery to the distal point of the reconstituted parent vessel in HGF-treated animals (Figure 2). Moreover, we evaluated a single administration of rhHGF in the ischemia model and found that it also caused a significant increase in angiographic score as compared with vehicle-treated rabbits.

Angiogenesis Induced by Intravenously Injected rhHGF

Finally, we evaluated the efficacy of intravenous administration of rhHGF in the rabbit ischemia model to examine the clinical feasibility of rhHGF for the treatment of ASO patients. Consistent with the angiogenic activity of intra-arterially injected rhHGF, repeated intravenous administration of rhHGF (3 mg/d/animal) for 5 days from day 0 to day 4 also resulted in a significant increase in the angiographic score as compared with vehicle treatment ($P < 0.01$, Figure 3). Consistent with other experiments, there was no significant difference in body weight between the rabbits intravenously injected with rhHGF and vehicle on day 40 after surgery (data not shown).

Discussion

In patients with critical limb ischemia, since there is no pharmacological treatment. Amputation, despite its associated morbidity, mortality rates, and functional implications,$^{1,27,28}$ is often recommended as a solution to the disabling symptoms, in particular excruciating ischemic rest pain.$^{29-31}$ Indeed, a second major amputation is required in nearly 10% of such patients. Consequently, the need for alternative treatment strategies in patients with critical limb ischemia is compelling. A novel therapeutic strategy that uses angiogenic growth factors to expedite and/or augment collateral artery development has recently entered the realm of treatment of ischemic diseases. Indeed, the clinical utility of gene therapy with VEGF, an endothelium-specific growth factor gene, has been recently reported for the treatment of critical limb ischemia.$^{12,13}$ The present study raises the possibility of a new strategy, therapeutic angiogenesis with HGF instead of VEGF, for the treatment of patients with critical limb ischemia. HGF is a mesenchyme-derived pleio-
tropic factor that regulates cell growth, cell motility, and morphogenesis of various types of cells and is thus considered a humoral mediator of epithelial-mesenchymal interactions responsible for morphogenic tissue interactions during embryonic development and organogenesis.\textsuperscript{12-13} On the basis of our recent finding that HGF exclusively stimulated the growth of endothelial cells without VSMC replication, we further studied the capability of HGF to stimulate angiogenesis.

Although an angiogenic response to HGF has been reported in an experimental model system of implanted reconstituted basement membrane (Matrigel),\textsuperscript{20} few reports have examined the angiogenic activity of HGF in physiological conditions in normal tissue without a supplement such as Matrigel, and controversial reports have described the inability of HGF to stimulate angiogenesis.\textsuperscript{34} Here we demonstrated direct in vivo evidence of angiogenesis induced by recombinant HGF, consistent with previous findings.\textsuperscript{14,20} Notably, a single intra-arterial administration of rhHGF was sufficient to induce angiogenesis in the rabbit hind limb ischemia model. As previously reported, HGF has been postulated to promote angiogenesis as a result of a combination of direct effects on endothelial cells and indirect effects, including paracrine upregulation of VEGF, on VSMC.\textsuperscript{35} Of importance, the promotion of angiogenesis by HGF prevented necrosis of muscle and nails in a critical limb ischemia model, accompanied by a significant increase in angiographic score (data not shown). These results demonstrate the utility of therapeutic angiogenesis induced by recombinant HGF. In this study we used the moderate ischemia model because this model appears to be more similar to human ischemic disease (see Methods).

Finally, we investigated the feasibility of intravenous administration of rhHGF in the ischemia model to examine the clinical utility of the therapeutic angiogenesis induced by HGF because there is no report of the effects of intravenous administration of rhHGF. Importantly, intravenous injection of rhHGF sufficiently increased angiogenesis, as shown in Figure 3. Although further studies are necessary, the present data suggest the clinical utility of intravenous administration of rhHGF for the treatment of ischemic disease. In contrast, endogenously expressed HGF was markedly decreased in the diseased segments of blood vessels from patients with critical ischemic limb as compared with disease-free segments of vessels, which suggests an insufficient level of HGF to salvage damaged vessels. Our in vitro experiments suggest that the decrease in vascular HGF production is caused by hypoxia (unpublished observation). Therefore a sufficient supply of recombinant HGF would be expected to enhance collateral formation in patients with critical limb ischemia (see Figure 4). What is the clinical relevance of therapeutic angiogenesis induced by HGF as compared with VEGF? First, HGF may not cause edema as a side-effect, as it does not increase permeability, different from VEGF. Second, c-met (the specific receptor for HGF) has been reported to be upregulated in response to hypoxia in a myocardial ischemia model,\textsuperscript{36} probably enhancing the angiogenic activity of HGF. Third, previous reports showed elevated levels of cell-associated matrix degrading enzymes (MMP-1, and so on) and enhanced plasmin-generating ability (uPA) by HGF.\textsuperscript{37–39} Interestingly, uPA activates pro-HGF in vitro, and activation of pro-HGF involves the formation of a stable complex between pro-HGF and uPA,\textsuperscript{38} which suggests that the biological effects of HGF can be titrated in vivo by the level of uPA activity. Increased amounts of uPA locally induced by HGF may condition the tissue microenvironment by rendering HGF bioavailable to its target cells. Although further studies are necessary to distinguish the mechanisms of HGF and VEGF, the present study at least demonstrated the potential utility of rhHGF for therapeutic angiogenesis.

Overall, the present studies suggest a novel therapeutic strategy that might reduce the symptoms of critical limb ischemia by use of the angiogenic properties of recombinant HGF. It is noteworthy that the supply of HGF into the ischemic limb supplements the downregulated endogenous vascular HGF expression. In addition, stimulation of new vessel formation by HGF is likely to create new therapeutic options in angiogenesis-dependent conditions such as wound healing, inflammatory diseases, ischemic heart disease, myocardial infarction, and peripheral arterial disease.

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References

Therapeutic Angiogenesis Induced by HGF


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