Fibroblast Growth Factor-2 Gene Transfer Can Stimulate Hepatocyte Growth Factor Expression Irrespective of Hypoxia-Mediated Downregulation in Ischemic Limbs

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Abstract—Hepatocyte growth factor (HGF) is a potent angiogenic polypeptide that stimulates angiogenesis. Transcriptional regulation of HGF, however, has not been fully defined, with the exception of the hypoxia-mediated downregulation in cultured cells. In the present study, we report that angiogenic growth factors, including HGF, were upregulated in a murine model of critical limb ischemia in vivo, a finding that was in conflict with previous in vitro data. Mice deficient in basic fibroblast growth factor-2 (FGF-2) showed reduced induction of HGF protein in ischemic muscles, and overexpression of FGF-2 via gene transfer stimulated endogenous HGF, irrespective of the presence of ischemia. In culture, FGF-2 rapidly stimulated HGF mRNA, and a sustained expression was evident in the time course in vascular smooth muscle cells and fibroblasts. FGF-2–mediated induction of HGF was fully dependent on the mitogen-activated protein kinase pathway yet was not affected by either hypoxia or a protein kinase A inhibitor. In the early expression, FGF-2 directly stimulated HGF mRNA without the requirement of new protein synthesis, whereas sustained induction of HGF in the later phase was partly mediated by platelet-derived growth factor-AA. Furthermore, in vivo overexpression of FGF-2 significantly improved the blood perfusion, and the effect was abolished by systemic blockade of HGF in ischemic limbs. This is the first demonstration of a regulational mechanism of HGF expression via FGF-2 that was independent of the presence of hypoxia. The harmonized therapeutic effects of FGF-2, accompanied with the activity of endogenous HGF, may provide a beneficial effect for the treatment of limb ischemia. (Circ Res. 2002;91:923-930.)

Key Words: fibroblast growth factor-2 ■ hepatocyte growth factor ■ ischemia

Early clinical studies have suggested that gene transfer of angiogenic growth factors, including vascular endothelial growth factor (VEGF), may provide a strategy for the treatment of patients with critical limb ischemia. The notion of “therapeutic angiogenesis” has been supported by a number of laboratory studies that showed improved blood flow via enhanced neovascularization by the gene transfer of angiogenic factors. Less information is available, however, concerning the complex actions and relationships among exogenous and endogenous angiogenic factors. For instance, we do not know the extent to which exogenous gene expression may be helpful in treating patients with limb ischemia in which endogenous VEGF may already be upregulated. In addition, the regulation of other angiogenic factors via overexpression of the therapeutic gene under hypoxic conditions is not well understood. Clearly, more biological studies of gene transfer during tissue ischemia are needed to clarify these important considerations.

Angiogenesis, the formation of capillaries from preexisting blood vessels, occurs in a variety of physiological and pathological settings, and a number of factors are known to modulate angiogenesis through autocrine and/or paracrine modes of action. VEGF, basic fibroblast growth factor-2 (FGF-2), and hepatocyte growth factor (HGF) are potent angiogenesis inducers. HGF, an antiapoptotic cytokine for endothelial cells (ECs), stimulates enhanced paracrine angiogenic responses by inducing VEGF, which means that it may play a critical role in angiogenesis in vivo. The regulatory mechanisms of HGF in vivo are, however, not fully understood. Because HGF expression in cultured cells is downregulated under hypoxia, cytokine supplementation with HGF may benefit patients with limb ischemia.

In the present study, we provide evidence that FGF-2 is a potent inducer of HGF through a hypoxia-independent mechanism in ischemic limbs both in vitro and in vivo.
Materials and Methods

Animals and Surgical Procedures

Ninety-nine male C57BL/6 mice (KBT Oriental Co, Ltd, Charles River grade, Tosu, Saga, Japan) were used in this study. Twenty-four mice were used for protein measurement to investigate the isch- 
aemia-related expression of VEGF, HGF, and FGF-2; 36 mice were treated with Ras inhibitory peptide, rapamycin, U0126, or wortman-
ning to determine the consistency of surgery-induced ischemia using laser Doppler imager (the technical details are given below), showing that there was a marked decrease in flow in operated limbs soon after surgery (untreated versus ischemia: 104.0±14.6 versus 21.2±3.6 pixels/muscle; *P<0.001), a finding that was consistent with our several years of experience on >500 animals. A needle-type oxy- 
gen pressure meter simultaneously confirmed that the oxygen levels in the operated limbs decreased markedly, in most cases to 
<2 mm Hg versus 80 to 90 mm Hg in the untreated limbs.

For gene transfer, 25 μL of vector solutions was injected into 2 portions of the thigh muscle soon after completion of the surgical procedures. For protein measurements, the posterior thigh muscles were excised and subjected to immunoassay as described below.

Gene Transfer Vectors

The stocks of recombinant Sendai viruses (SeVs), including SeV encoding murine FGF-2 (SeV-mFGF2) and SeV encoding firefly luciferase (SeV-luciferase) used in this study were prepared as described previously. The virus titer was determined by hemag- 
glutination assay using chicken red blood cells and was kept at 
−80°C until use. Murine FGF-2 cDNA for constructing SeV- 
mFGF2 was the kind gift of Dr T. Imamura (National Institute of 
Bioscience and Human Technology, Tsukuba, Japan).

Cells and Reagents

Human aortic smooth muscle cells (HSMCs; Kurabo Co, Ltd, 
Tokyo, Japan) and murine immortalized fibroblasts, NIH3T3 (Amer- 
ican Type Culture Collection, Manassas, Va), were purchased. 
Bovine SMCs (BSMCs) were isolated as described previously. Anti-PDGF-AA neutralizing goat antibody and control goat IgG 
were from Sigma-Aldrich Japan (Tokyo, Japan). The following intracellular signal inhibitors were used at the indicated working concentrations on SMCs, fibroblasts, and ECs:11-14 Ras: Ras inhibi-
tory peptide (50 μmol/L; Alexis Japan, Tokyo, Japan); p70S6K: 
rapamycin (100 ng/mL; Sigma); PKC: bisindolylmaleimide (100 
nmol/L; Sigma); PKA: PKA inhibitor peptide (1 μmol/L; Calbiochem, San Diego, Calif); NF-κB: NF-κB inhibitor ALLN (5 μmol/L; Roche Diagnostics, 
Tokyo); and CHX: cycloheximide (5 μmol/L; Sigma). To confirm 
whether these concentrations were functional in our HSMCs 
and BSMCs, a pilot Western blot study was performed using cells 
treated with Ras inhibitory peptide, rapamycin, U0126, or wortman-
nin. Each of these inhibitors specifically inhibited the phosphoryla-
tion in both cell types (data not shown). In addition, a pilot gel-mobility shift assay for NF-κB using nuclear extracts from 
BSMCs treated with ALLN showed nearly complete inhibition of 
nuclear translocation of NF-κB (data not shown).

Imunoassays

Protein contents in murine thigh muscles and culture medium were 
determined using Quantikine immunoassay systems for murine 
VEGF (recognizes both the 164 and 120 amino acid residue forms of 
mouse VEGF), FGF-2, and murine and human HGF (R&D Systems 
Inc, Minneapolis, Minn) according to the manufacturer’s instruc-
tions, as previously described.9

RT-PCR

The gene expressions of ischemia-induced murine VEGF isoforms 
were determined using primer sets for rat VEGF on exons 1 to 8,13 the sequences of which corresponded to those murine VEGF 
isoforms. The RT-PCR conditions were as described elsewhere.13 
Three animals were used.

Northern Blotting

Total cellular RNA, isolated using the ISOGEN system (Wako Pure 
Chemicals, Osaka, Japan), was electrophoresed and transferred onto 
a nylon membrane. The filters were hybridized overnight at 42°C 
with random [α-32P]dCTP-labeled probes, obtained from full-length 
human HGF cDNA (the kind gift of Prof T. Nakamura, Osaka 
University). The bands were then visualized and subjected to 
densitometric analysis using a photoimager.

Measurement of Cellular cAMP

Cellular cAMP content was measured using cell lysates after 
cultivation for 48 hours under a hypoxic condition (2% O2) using 
a commercially available cAMP enzyme immunoassay system accord-
ing to the manufacturer’s instructions (BioTrak cAMP ELIA System; 
Amersham Pharmacia Biotech UK, Ltd, Buckinghamshire, UK)

Western Blotting

Fifty micrograms of nuclear protein was separated on SDS-PAGE 
and transferred to the membrane. The band of cAMP-response 
element binding protein (CREB) was visualized by chemolumines- 
cence, using a CREB PhosphoPlus CREB antibody kit (Cell Signal-
ing Technology Inc, Beverly, Mass).

In Vivo Inhibition of Endogenous HGF

In vivo endogenous HGF activity was diminished using anti-HGF 
neutralizing goat polyclonal IgG (R&D Systems). One day before 
surgery, a disposable micro-osmotic pump (model 1007D; ALZA 
Co, Mountain View, Calif) with 200 μL of either nonimmunized 
rabbit IgG or anti-HGF IgG (1 mg/mL, respectively) was implanted 
into the peritoneal cavity.8 This pump continuously released these 
solutions at the rate of 0.5 to 1.0 μL/h for about 7 days. Soon after 
the surgery, an additional bolus administration of these antibodies 
(100 μg, respectively) was given via the pinel vein.

Laser Doppler Perfusion Images (LDPIs)

Measurements of the ischemic (left)/normal (right) limb blood flow 
ratio were made using a LDPI analyzer (Moor Instruments, Devon, 
UK). To minimize data variables due to ambient light and temper-

ture, the LDPI index was expressed as the ratio of the left (ischemic) to the right (normoxic) limb blood flow.

Statistical Analysis

All data are expressed as mean±SEM and were analyzed by one-
way ANOVA with Fisher’s adjustment. A value of *P<0.05 was 
considered to be statistically significant.

Results

Typical Angiogenic Growth Factors Are 
Upregulated in Murine Ischemic Hind Limbs

Although the surgically induced tissue ischemia was successful 
in muscles of C57BL/6 mice, there was initially no clear
evidence of increased expression of angiogenic growth factors. To further explore the ischemic tissue response, we first examined by RT-PCR analysis the expression of murine splicing isoforms of VEGF, a typical hypoxia-inducible factor. Two days after the initial surgery, the enhancement of VEGF mRNA was seen in only 164 amino acid isoforms (data not shown). An ELISA for murine VEGF protein in ischemic muscle 2 days after surgery also showed a significant upregulation of VEGF (P<0.01, n=8), thereby indicating the successful induction of tissue ischemia in murine limbs (Figure 1A).

Next, protein quantification of other typical angiogenic polypeptides, FGF-2 and HGF, was done using the same tissue samples. Unexpectedly, both HGF and FGF-2 were significantly upregulated in the ischemic muscles of C57BL/6 mice, as shown in Figure 1A (HGF and FGF-2; P<0.01, respectively, n=8 in each group). Similar results were also seen in the C3H and Balb/c strains (Figure 1A, n=8 in each group; P<0.01, respectively). Thus, the results disagreed with those reported previously, particularly in the case of HGF; it has been reported that hypoxia reduced HGF expression in cultured SMCs via a cAMP-dependent mechanism. Because FGF-2 can stimulate HGF expression in osteoblasts, we performed a scatter-plot analysis to determine the relationship between HGF and FGF-2 protein levels in untreated (○) or ischemic (●) muscles. Plots contain data from all strains (n=8 each, total 24). The correlation was significant in ischemic muscles but not in untreated muscles. C, Ischemia-induced FGF-2 (left) and HGF (right) expression in FGF-2–deficient mice. D, Ischemia-independent induction of endogenous HGF via FGF-2 gene transfer. Two days after gene transfer, the posterior portion of the thigh muscles was subjected to protein quantification. The luciferase group included 6 animals and the FGF-2 group 12 animals. Data are presented as mean±SEM. *P<0.01; #P<0.001.

Figure 1. Expression of angiogenic growth factors in murine thigh muscles in response to limb ischemia. Two days after operation, a posterior portion of the thigh muscles was subjected to protein quantification. A, Increase of the intramuscular protein level of murine VEGF, HGF, and FGF-2 in the presence of induced limb ischemia in 3 different strains (C57/BL6, C3H, and Balb/c). Each group included 8 animals. Data are presented as mean±SEM. *P<0.01. B, Scatter-plot analysis of the relationship between HGF and FGF-2 protein levels in untreated (○) or ischemic (●) muscles. The correlation was significant in ischemic muscles but not in untreated muscles. C, Ischemia-induced FGF-2 (left) and HGF (right) expression in FGF-2–deficient mice. D, Ischemia-independent induction of endogenous HGF via FGF-2 gene transfer. Two days after gene transfer, the posterior portion of the thigh muscles was subjected to protein quantification. The luciferase group included 6 animals and the FGF-2 group 12 animals. Data are presented as mean±SEM. *P<0.01; #P<0.001.
FGF-2 Stimulates HGF Transcription and Protein Secretion

p42/44 MAPK (MEK1/2) Pathway Is a Major Signal for FGF-2–Mediated HGF Expression

We next tested the hypothesis that FGF-2 might play a role in the regulation of HGF expression, using 3 independent cells in vitro. No significant secretion of FGF-2 was detected by ELISA (detection limit: <5 pg/mL), even when a 50× concentrated medium was used for the analysis (data not shown). Northern blot analysis demonstrated that FGF-2 dose-dependently upregulated HGF mRNA in HSMCs at 3 hours after stimulation, similar to findings with BSMCs and NIH3T3 cells (data not shown). Hence, FGF-2 may stimulate the HGF transcript irrespective of differences among mesenchymal lineage or species.

Next, to obtain information regarding critical signals on FGF-2–mediated HGF expression in the early phase (3 hours after stimulation), Northern blots were performed using various inhibitors for cytoplasmic signals (Figure 2). We used BSMCs in these triplicate experiments because these cells proliferate efficiently and are readily obtained, whereas it is more difficult to obtain sufficient amounts of HSMCs with their lower proliferative activity. Three independent experiments demonstrated upregulation of HGF mRNA via FGF-2, an event not affected by CHX, thereby indicating that new protein synthesis is not required for this process. An MEK1/2-specific inhibitor completely abolished the FGF-2–mediated upregulation of HGF, whereas other inhibitors were without significant effects. Similar results were obtained using HSMCs in a single experiment (data not shown).

Sustained Expression of HGF in the Later Phase via FGF-2 Is Partially Mediated by the Enhanced Expression of Endogenous PDGF-AA

Next, we checked the time course of HGF expression. All 3 cell types showed a sustained (BSMCs: top panel) or nearly biphase (HSMCs: bottom panel and NIH3T3: data not shown) expression pattern at early (3 to 6 hours after stimulation) and late (12 to 48 hours) phases (Figure 3A). To assess the mechanisms in the later phase, we measured HGF secretion at 24 hours in culture medium using several inhibitors and HSMCs. As shown in Figure 3B, an MEK inhibitor abolished HGF expression, as seen in the mRNA form in the earlier phase. On the other hand, at this time, a Ras inhibitory peptide and p70S6K inhibitor rapamycin also significantly suppressed HGF secretion. Other inhibitors had no effect, thereby indicating that HGF expression via FGF-2 at the late phase might be induced indirectly, via Ras and p70S6K signals, by cellular factor(s) regulated by FGF-2.

Because the mitogenic activity of PDGF has been known to depend both on Ras and p70S6K,18 we hypothesized that one of the major indirect cellular factors might be PDGF. We thus assessed the secretion of PDGF-AA into the medium and the effect of anti–PDGF-AA neutralizing antibody (anti–PDGF-Ab) on HGF secretion in HSMCs (PDGF-B chain was not detected by ELISA; data not shown). As shown in Figure 3C, FGF-2 significantly upregulated PDGF-A secretion into the culture medium, and an anti–PDGF-Ab significantly inhibited FGF-2–mediated HGF secretion in a dose-dependent manner at 48 hours of cultivation. Therefore, the enhanced secretion of endogenous PDGF-AA plays a significant role in the FGF-2–mediated HGF expression in SMCs.
cAMP-Related Hypoxia Signaling Does Not Affect FGF-2–Mediated HGF Expression

Because we also had data indicating that a PKA inhibitory peptide did not inhibit dose increases in FGF-2–mediated HGF secretion (Figure 4A), we next assessed the effect of hypoxia on HGF expression using HSMCs (Figure 4B). Cells were preincubated for 48 hours under conditions of normoxia (21% O₂) or hypoxic (2% O₂) conditions with serum-free medium, the culture medium was replaced with or without human recombinant FGF-2 (10 ng/mL), and the medium was subjected to ELISA 24 hours later. Endogenous HGF secretion was impaired by hypoxia without FGF-2, whereas FGF-2–mediated HGF secretion was not affected by hypoxia. *P<0.01. C. Western blot analysis indicates that phosphorylation of CREB was not found by FGF-2, whereas nuclear extracts from control HSMCs treated with forskolin gave a positive result.

Figure 3. A, Time course of HGF mRNA expression in the presence of FGF-2 in BSMCs (top) and HSMCs (bottom). After 48 hours of cultivation under a serum-free condition, the culture medium was replaced with human recombinant FGF-2 (10 ng/mL), and cells were harvested at each time point. Data were standardized by each optical density of 28S on autoradiography and expressed as a relative fold of increase compared with that without FGF-2. Note the sustained (BSMCs) or nearly biphasic (HSMCs) expression patterns. B, Effects of various intracellular signal inhibitors on HGF protein secretion into the culture medium. After 48 hours of cultivation under a serum-free condition, the culture medium was replaced with or without PKA inhibitors. After 30 minutes of cultivation, stimulation of human recombinant FGF-2 (10 ng/mL) was applied. The medium was subjected to ELISA 24 hours later. The graph contains data from 3 independent experiments, respectively. Data are presented as mean±SEM. *P<0.01. C, Western blot analysis to detect secretion of PDGF-A protein into culture medium in the presence or absence of FGF-2. After 48 hours of cultivation under a serum-free condition, the culture medium was replaced with or without PKA inhibitors. Ten microliters of the concentrated medium was subjected to Western blot analysis using human PDGF-A–specific antibody. Right, Dose-dependent neutralization of PDGF-AA on HGF protein secretion via FGF-2. The experimental conditions were similar to those in panel B (n=3 in each group). Data are presented as mean±SEM. *P<0.01; #P<0.05.

Figure 4. FGF-2–mediated HGF expression is not affected by hypoxia-related signals. The graphs (A and B) contain data from 3 independent experiments, respectively. Data are presented as mean±SEM. *P<0.01. A, No significant effect of a PKA inhibitory peptide on FGF-2–mediated HGF secretion. B, Effect of cultivation under hypoxia. After 48 hours of cultivation under normoxic (21% O₂) or hypoxic (2% O₂) conditions with serum-free medium, the culture medium was replaced with or without human recombinant FGF-2 (10 ng/mL), and the medium was subjected to ELISA 24 hours later. Endogenous HGF secretion was impaired by hypoxia without FGF-2, whereas FGF-2–mediated HGF secretion was not affected by hypoxia. *P<0.01. C, Western blot analysis indicates that phosphorylation of CREB was not found by FGF-2, whereas nuclear extracts from control HSMCs treated with forskolin gave a positive result.

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whereas the FGF-2–mediated enhancement of HGF expression was not significantly affected by the hypoxia.

We next examined the phosphorylation of the cAMP-dependent transcription factor CREB. As shown in Figure 4C, Western blot analysis revealed that HSMC nuclear extracts obtained with or without the potent cAMP inducer, forskolin, showed positive and negative results, respectively, as controls. Simultaneous experiments using nuclear extracts of HSMCs, with or without FGF-2 exposure, gave negative results, thus confirming that FGF-2–mediated HGF expression does not occur through the cAMP-PKA-CREB pathway.

**HGF Plays a Critical Role in FGF-2–Mediated Restoration of Blood Flow in Ischemic Limbs**

Finally, to confirm the role of HGF in FGF-2–mediated therapeutic effects, we assessed the recovery of blood perfusion using LDPI analysis. As shown in Figure 5, ischemic hind limbs treated with FGF-2 gene transfer and nonimmunized goat IgG showed a significant improvement in blood flow (n=9), compared with the findings in PBS-injected limbs (n=6), whereas administration of an anti-HGF neutralizing antibody significantly abolished the effect of FGF-2 gene transfer (n=8). The time course of recovery of blood flow in the ischemic limbs treated with PBS was comparable to that of the recovery in limbs treated with empty SeV or SeV-luciferase, as noted during our several years of experience (data not shown). Interestingly, 4 of 13 control animals treated with PBS and with anti-HGF neutralizing antibody lost their limbs during the experimental course (n=1 on day 5 and n=3 on day 8), and thus these animals were excluded from LDPI measurements on day 7 and day 10.

These findings strongly suggest that endogenous HGF expression mediated by FGF-2 may play a critical role in the accelerated blood perfusion and therapeutic properties of FGF-2 gene transfer.

**Discussion**

In the present study, we have provided evidence that FGF-2 mediates regulation of HGF expression in vitro and in vivo. Key findings in this study are as follows: (1) Limb ischemia upregulated in vivo the local concentration not only of a well-known hypoxia-inducible angiogenic factor VEGF but also HGF and FGF-2, despite reports indicating that the latter two factors were shown to be stimulated by hypoxia in vitro. (2) FGF-2 stimulated HGF mRNA expression and protein secretion via the MEK signal transduction pathway throughout the time course in the in vitro experiments. (3) Both Ras and p70S6K signals were shown to be important in the later phase of FGF-2–mediated HGF expression, which was partly regulated by the accelerated expression of endogenous PDGF-AA. (4) FGF-2–mediated upregulation of HGF was independent of hypoxia-related signal transduction in vitro and in vivo. To our knowledge, this is the first report to describe the FGF-2–mediated signal transduction mechanism that evokes HGF. These findings strongly suggest the potential utility of FGF-2 as a therapeutic tool for patients with limb ischemia.

Angiogenesis is a complex process that involves a variety of regulatory molecules, including angiogenic factors. Previous in vitro studies suggested not only angiogenic properties in each molecule but also possible related associations among these during the angiogenic process. Because in vivo data are sparse in this regard, it is important to explore the endogenous regulation of angiogenic polypeptides in the tissue response to ischemia and under conditions of overexpression of a specific factor via gene transfer. Indeed, our previous and present studies have demonstrated that upregulation of both VEGF and HGF plays a critical role in the FGF-2–mediated angiogenic process in limb ischemia in vivo.

**Possible Role of Endogenous HGF Expression in the Effect of FGF-2 Gene Transfer**

Although we and others reported that FGF-2 gene transfer stimulated VEGF in vitro and in vivo, the mechanism
of induction seems quite different from that seen in HGF. In the present study, we demonstrated that FGF-2 stimulated endogenous HGF expression irrespective of the presence or absence of hypoxia, whereas FGF-2–mediated VEGF expression was markedly enhanced under ischemia. The present study also showed that blockade of endogenous HGF activity by neutralizing antibody diminished the therapeutic effect of FGF-2 gene transfer in ischemic limbs, which was similar to a result obtained previously by administration of anti-VEGF neutralizing antibody. Furthermore, FGF-2 also upregulated the endothelial expression of both VEGF and its functional receptor Flk-1/KDR, suggesting that FGF-2 mediates the enhancement of autocrine loop for angiogenesis via leading upregulation of other angiogenic factors and their receptors. These advantageous actions of FGF-2 utilizing HGF and VEGF under conditions of hypoxia may well explain the synergistic angiogenic effects under the combination of FGF-2 and hypoxia.

**Possible Role of Endogenous HGF in the Tissue Response to Ischemia**

HGF is likely to be an important factor not only for exogenously transferred FGF-2–mediated angiogenesis but also for physiological tissue response to ischemia. This may be supported by the finding that blockade of endogenous HGF activity not only abolished the FGF-2–dependent increase of blood perfusion but also resulted in major limb loss above the knee under the condition of ischemia in 4 of 13 animals (30.1%), a result that was not seen in the PBS-injected controls. Although the exact molecular mechanisms are still unclear, this suggests that HGF may play a unique role in the maintenance of the vasculature in ischemic tissue, because such an effect was not seen in mice treated with anti-VEGF neutralizing antibody (unpublished observation, Y. Yonemitsu, M. Tanii, M. Hasegawa, K. Sueishi, 2002). Wang et al. recently indicated that Met, a tyrosine kinase receptor specific for HGF that upregulated by HGF, directly interrupts death signaling via Fas-Fas ligand (Fas-FasL). A more recent study showed that Fas-FasL–mediated endothelial death is an important mechanism for endogenous angiogenic inhibitors, including thrombospondin-1 and pigment epithelium-derived factor, suggesting that the increased activity of HGF-Met signaling may protect the newly formed vessels evoked by tissue ischemia from an endogenous pool of antiangiogenic factors. According to this notion, blockade of the HGF-Met system may also result in regression of the vasculature. We are currently investigating this hypothesis.

**PDGF-AA: A Non–Endothelium-Targeting Growth Factor That Modulates Angiogenesis?**

In the present study, we demonstrated that FGF-2–mediated expression of PDGF-AA indirectly leads to sustained upregulation of HGF expression. These findings suggest an important role of PDGF-AA during the angiogenic process; however, little information is available in this regard. Fruttiger et al. reported that transgenic mice overexpressing PDGF-AA in the developing retina exhibited not only extensive neuron-astrocyte network but also increased vascular channels, suggesting that PDGF-AA could be an important modulator of angio-/vasculogenesis. Thus, we are now assessing the role of PDGF-AA in the maturation of capillaries during the angiogenic process.

**Ischemia-Induced Expression of FGF-2: Discrepant Findings Between In Vitro and In Vivo**

It remains uncertain why endogenous FGF-2 was upregulated in ischemic muscles, because hypoxia itself cannot induce FGF-2 in some cell species, including SMCs. An increase in the local concentration of FGF-2 in ischemic muscle in vivo was due to either transcriptional upregulation or migration of FGF-2–expressing cells, but not to stimulated protein secretion, because we already confirmed an apparent increase of mRNA of FGF-2 in ischemic muscles (data not shown). Not only bioactive substances, including transforming growth factor-β1, but also the tissue environment, including such conditions as acidosis, can stimulate the expression of FGF-2, suggesting that such factors may play a role of induction of FGF-2 in response to tissue ischemia.

**Conclusion**

Taken together, FGF-2, which has the potential to induce other angiogenic factors including VEGF and HGF, is likely to be a major conductor of the “angiogenic orchestra.” Use of FGF-2 for therapeutic angiogenesis may prove to be a reasonable strategy for treatment of patients with critical limb ischemia.

**Acknowledgments**

This work was supported by a Grant of Promotion of Basic Scientific Research in Medical Frontier of the Organization for Pharmaceutical Safety and Research.

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_Circ Res._ 2002;91:923-930; originally published online October 24, 2002; doi: 10.1161/01.RES.0000043281.66969.32

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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