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IV. 研究成果の刊行物・別冊

本研究と密接に関係する以下の論文を抜粋する

1. Fujii T, Yonemitsu Y, Tanii M, Nakano T, Egashira K, Takehara T, Inoue M, Hasegawa M, Kuwano H, Sueishi K.
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Nonendothelial Mesenchymal Cell–Derived MCP-1 Is Required for FGF-2–Mediated Therapeutic Neovascularization: Critical Role of the Inflammatory/Arteriogenic Pathway

Takaaki Fujii, Yoshikazu Yonemitsu, Mitsuho Onimaru, Mitsugu Tanii, Toshiaki Nakano, Kensuke Egashira, Takako Takehara, Makoto Inoue, Mamoru Hasegawa, Hiroyuki Kuwano and Katsuo Sueishi

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Nonendothelial Mesenchymal Cell–Derived MCP-1 Is Required for FGF-2–Mediated Therapeutic Neovascularization

Critical Role of the Inflammatory/Arteriogenic Pathway

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Objective—Monocyte chemoattractant protein-1 (MCP-1) is a C-C chemokine that is known as an inflammatory/arteriogenic factor. Angiogenesis contributes to the inflammatory process; however, the molecular and cellular mechanisms of the links among the inflammatory pathway, arteriogenesis, and angiogenesis have not been well elucidated.

Methods and Results—Using murine models of fibroblast growth factor-2 (FGF-2)–mediated therapeutic neovascularization, we here show that FGF-2 targets nonendothelial mesenchymal cells (NEMCs) enhancing both angiogenic (vascular endothelial growth factor [VEGF]) and arteriogenic (MCP-1) signals via independent signal transduction pathways. Severe hindlimb ischemia stimulated MCP-1 expression that was strongly enhanced by FGF-2 gene transfer, and a blockade of MCP-1 activity via a dominant negative mutant as well as a deficiency of its functional receptor CCR2 resulted in the diminished recovery of blood flow attributable to adaptive and therapeutic neovascularization. Tumor necrosis factor (TNF)-α stimulated MCP-1 expression in all cell types tested, whereas FGF-2–mediated upregulation of MCP-1 was found only in NEMCs but not in others, a finding that was not affected by VEGF in vitro and in vivo.

Conclusions—These results indicate that FGF-2 targets NEMCs independently, enhancing both angiogenic (VEGF) as well as inflammatory/arteriogenic (MCP-1) pathways. Therefore, MCP-1/CCR2 plays a critical role in adaptive and FGF-2–mediated therapeutic neovascularization. ***(Arterioscler Thromb Vasc Biol.* 2006;26:2483-2489.)**

Key Words: MCP-1 ■ FGF-2 ■ arteriogenesis ■ angiogenesis ■ mesenchymal cells

It has been widely accepted that angiogenesis is required for the progression and maintenance of physiological reactions to injury (ie, wound healing) as well as pathophysiological conditions associated with the inflammatory process (ie, cancers, atherosclerosis, rheumatoid arthritis, etc).[1] Various cell types, including circulating mononuclear cells, fibroblasts, endothelial cells, etc, are involved in the recruitment to inflammatory foci, and these cells express angiogenic substances inducing neovascularization for the maintenance of the inflammatory reaction.[2] The regulatory mechanisms and molecular/cellular network underlying the link of angiogenesis to the inflammatory reaction, however, have not been well elucidated.

Monocyte chemoattractant protein-1 (MCP-1), the murine homologue of which is known as JE, is a member of the C-C chemokines, which promotes the recruitment and activation of monocytes/macrophages, critically contributing to the process of inflammatory reaction in various diseases.[3–5] Extensive studies done by Schaper and his colleagues have revealed that, in addition to its role in inflammatory foci, MCP-1 is the most potent enhancer of collateral vessel growth in ischemic tissue, known as arteriogenesis.[6,7] They revealed that monocytes played a major role in both angiogenesis and collateral artery growth,[8] and that depletion of monocytes/macrophages by cytotoxic liposomes almost completely abolished arteriogenesis induced by MCP-1.[9,10] Furthermore, their recent study demonstrated that mice deficient in C-C chemokine receptor-2 (CCR2), a major functional receptor for MCP-1, exhibited impaired arteriogenesis, indicating that the MCP-1/CCR2 pathway is responsible for the

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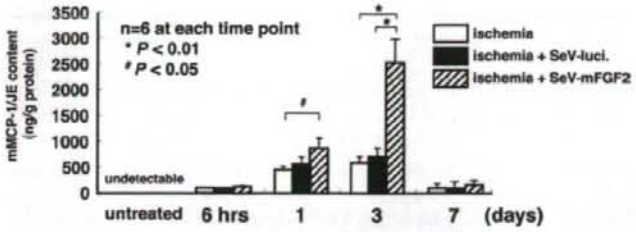

Figure 1. Adaptive expression of mMCP-1/JE in surgically induced hindlimb ischemia of C57BL/6 mice and its enhancement by FGF-2 gene transfer. #$P<0.01$, *$P<0.05$. Time course of endogenous mMCP-1/JE protein expression in murine limb ischemic muscles with or without gene transfer. Animals treated with mFGF-2 showed marked enhancement of mMCP-1/JE expression on day 3. Each group contained 6 mice at each time point, including all data from experiments repeated at least twice.

recruitment of monocytes during the early phase of arteriogenesis.[11] From these results, it is clear that monocyte lineage cells and MCP-1 have an essential role in tissue circulation.

In the last several years, we have focused on the role of nonendothelial mesenchymal cells (NEMCs: mural cells, vascular smooth muscle cells, and fibroblasts) during the angiogenic process. We previously demonstrated that boosted overexpression of fibroblast growth factor-2 (FGF-2) by gene transfer consistently showed highly therapeutic potential against murine severe hindlimb ischemia compared with the effect induced by vascular endothelial growth factor (VEGF).[12] While seeking the molecular and cellular mechanisms of the limb-salvaging effect of FGF-2, we found that the function of FGF-2 in ischemic limbs highly depended on the endogenous expression of VEGF and the hepatocyte growth factor (HGF),[13] which are strictly regulated and maintained by NEMCs via the autocrine system of the platelet-derived growth factor-AA (PDGF-AA)/PDGF receptor-α (PDGFRα)/p70S6 kinase (p70S6K) signal transduction pathway.[13,14] At the initial stage of these series of studies, we also found that FGF-2 gene transfer, but not VEGF, increased not only the number of capillaries but also those with pericyte lining, indicating that FGF-2 has the potential to stimulate the mature phenotype of the neovasculature, in contrast to VEGF.[12] However, information regarding that role of FGF-2 in the context of inflammatory/arteriogenic pathways is sparse at present.

In this study, therefore, we examined the role of the inflammatory/arteriogenic chemokine MCP-1 during FGF-2–mediated therapeutic neovascularization using murine critical limb ischemia models. We here demonstrate that FGF-2 targets NEMCs to enhance not only the angiogenic pathway (VEGF) but also the inflammatory/arteriogenic pathway (MCP-1), resulting in efficient recovery of blood flow, via divergent signal transduction pathways.

Materials and Methods

Animal Experiments

Male C57BL/6 (6 to 7 weeks old) and balb/c *nu/nu* mice (5 weeks old) were purchased from KBT Oriental Co, Ltd (Charles River Grade, Tosu, Saga, Japan). These mice were used for the "limb salvage model" and "autoamputation model," respectively, as previously described.[12] Mice deficient in CCR2 and their controls (wild genotype $CCR2^{+/+}$) were generated from the same genetic background (hybrids of C57BL/6 and 129/svjae).[15,16] All animal experiments were performed according to approved protocols and in accordance with recommendations for the proper care and use of laboratory animals by the Committee for Animals, rDNA, and Experiments Using Infectious Pathogens at Kyushu University, and according to law and notification of the Japanese Government.

Details of the surgical treatment and evaluation of limb prognosis have been described previously[12–14]; specifically, the excision of both the left femoral artery and vein and their branches from the inguinal ligament up to and including the saphenous-popliteal bifurcation. In vivo suppression of endogenous VEGF activity was performed using VEGF-specific neutralizing antibody via bolus injection coupled with continuous release administration using a disposable micro-osmotic pump (Model 1007D, ALZA Co), as previously described.[12–14]

Laser Doppler Perfusion Images

Measurements of the ischemic (left) and normal (right) limb blood flow were performed on a warm plate at body temperature using a laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, UK).[12–14,17,18] To minimize data variables attributable to ambient light and temperature, the LDPI index was expressed as the ratio of left (ischemic) to right (nonischemic) limb blood flow.

Enzyme-Linked Immunosorbent Assay

Protein contents in murine limb muscles and culture medium were determined using Quantikine Immunoassay systems for murine and human VEGF-A, murine tumor necrosis-α (TNF-α), human MCP-1, and murine MCP-1/JE (R&D Systems Inc).

Statistical Analysis

All data were expressed as means±SEM and were analyzed by one-way ANOVA with Fisher adjustment. The survival rate, expressed by limb salvage score, was analyzed using Kaplan–Meier method as previously described.[12–14] The statistical significance of the limb survival was determined using the log-rank test, and $P<0.05$ was considered to be statistically significant.

Results

Endogenous MCP-1 Is Expressed After Induced Hindlimb Ischemia, Which Is Strongly Enhanced by FGF-2 Gene Transfer, Inducing Arteriogenesis

To examine the role of MCP-1 in the ischemic hindlimb, we first examined the expression of MCP-1 using a murine model of severe hindlimb ischemia, namely the "limb salvage model," in C57 BL/6 mice, using SeV-mFGF2. Murine MCP-1/JE (mMCP-1/JE) protein expression, which was not detected in muscles without ischemia, was strongly upregulated soon after ischemia induction, and its expression level was strongly enhanced via overexpression of FGF-2 (Figure 1). Similar results were found in the case of mMCP-1/JE mRNA by quantitative real-time polymerase chain reaction (PCR) (date not shown). Both protein and mRNA expressions had their peak on day 3 after surgery, and similar protein expression patterns to those seen in FGF-2 were found in the same tissue samples (data not shown).

Next, we performed immunohistochemical examination for the accumulation of monocyte/macrophages in ischemic muscles to determine whether the upregulated mMCP-1/JE ex-

pression was really functional (please see the supplemental materials, available online at http://atvb.ahajournals.org). A monoclonal antibody against murine monocytes/macrophages, BM8, rarely labeled cells in adductor muscles of untreated control mice. In contrast, apparent infiltration of BM8-positive cells was found in ischemic muscles of mice treated with SeV-luciferase, a finding enhanced by the gene transfer of FGF-2.

We also performed immunohistochemistry for the αSMA to determine whether the upregulated mMCP-1/JE expression was arteriogenic[6] (please see the supplemental materials). Apparent increase of number of vessels circumvented by αSMA was evident in ischemic adductor muscles of mice treated with SeV-luciferase compared with those with untreated mice, a finding enhanced by the gene transfer of FGF-2. Quantitative analysis confirmed these results, indicating that both adaptation to tissue ischemia and FGF-2 provide arteriogenic signals.

MCP-1/CCR2 Is Essential for the Adaptive and FGF-2–Mediated Recovery of Blood Flow in Murine Ischemic Limbs

To assess the biological role of the upregulated expression of mMCP-1/JE after induced ischemia, preinjection of either a plasmid expressing the dominant negative mutant of MCP-1 (7ND MCP-1)[15] or a control gene (luciferase) was performed 2 days before operation (day −2). This procedure was then followed by induced ischemia and SeV-mediated gene transfer of mFGF-2 or of luciferase. As shown in Figure 2a, 7ND MCP-1 gene transfer diminished both adaptive and FGF-2–mediated recovery of blood flow, indicating that mMCP-1/JE may play a significant role in both conditions.

For further assessment, we examined the role of endogenous expression of mMCP-1/JE in both models of murine autoamputation (balb/c *nu/nu*) for FGF-2–mediated therapeutic angiogenesis[12–14] using 7ND MCP-1 as well as CCR2-deficient mice, assessing their role for adaptive angiogenesis.[9]

As shown in Figure 2b, balb/c *nu/nu* mice pretreated with SeV-luciferase frequently lost their hind limb irrespective of the injected plasmids. In contrast, SeV-mFGF2 significantly rescued the ischemia-induced autoamputation in mice pretreated with plasmid-luciferase, a finding representative of our previous studies.[12–14] This therapeutic effect was completely abrogated by 7ND MCP-1 gene transfer, indicating the essential role of endogenous mMCP-1/JE in the FGF-2–mediated therapeutic angiogenesis.

In the case of the $CCR2^{+/+}$ C57BL/6 to 129/svjae hybrid strain, induced severe ischemia did not result in autoamputation of hindlimbs, a similar finding to that seen in the C57BL/6 strain.[12–14] CCR2-deficient mice of the same genetic background, however, occasionally lost their limbs at ≈40% (Figure 2b), indicating the impaired tolerance against severe limb ischemia in $CCR^{-/-}$ mice. SeV-mediated gene transfer significantly but partially restored the limb survival of $CCR2^{-/-}$ mice.

These findings were clear evidence of the essential role of endogenous expression of mMCP-1/JE in both adaptive recovery of blood flow and FGF-2–mediated therapeutic angiogenesis in mouse models of limb ischemia.

FGF-2 Targets NEMCs To Stimulate MCP-1

To assess the source of the MCP-1 expression in vivo, we firstly performed immunohistochemistry against same tissue samples tested in Figure 1b and 1c in use of a goat anti-mouse MCP-1/JE polyclonal antibody. Comparable staining in serial sections using anti–PECAM-1 (for endothelial cells), anti–α-smooth muscle cell actin (for smooth muscle cells), BM8 (for monocytes/macrophages), and vimentin (for fibroblasts), suggested that these cells were expressing mMCP-1/JE; however, no clear difference of cell sources between muscles treated with control vector and SeV-mFGF2 (data not shown).

We next examined the induction of MCP-1 via FGF-2 using cultured human cells, including NEMCs (MRC5 and HSMC), monocyte/macrophage linage cells (THP-1), and endothelial cells (HUVEC and HPAEC) in vitro (please see the supplemental materials). RT-PCR analyses confirmed that these cells expressed a typical high-affinity receptor for FGF-2, FGFR1 (data not shown). A typical proinflammatory cytokine TNF-α, which is known as a strong inducer of MCP-1,[19] but not angiogenic polypeptides including VEGF, placental growth factor (PlGF), and FGF-2, stimulated MCP-1 in the culture medium of HUVEC, HPAEC, or THP-1. In contrast, similar upregulated expression of MCP-1 was seen not only under TNF-α but also FGF-2 in NEMCs, a finding that was confirmed by the mRNA level evaluated by quantitative real-time PCR (data not shown). To exclude the possibility of species-specific induction of MCP-1, we further assessed TNF-α and FGF-2–dependent induction of mMCP-1/JE using murine NEMCs (NIH3T3) and peritoneal macrophages (Mϕ). FGF-2–mediated induction of mMCP-1/JE was seen only in NIH3T3 cells, indicating that FGF-2 may target NEMCs to stimulate the expression and secretion of MCP-1.

FGF-2 Stimulates MCP-1 Expression via PKC, MEK, and NF-κB–Related Pathways, Similar to Those Associated With TNF-α, but not via Hypoxia

Considering the in vivo situation of surgically induced hindlimb ischemia, hypoxia, proinflammatory signals, and exogenously overexpressed FGF-2 should be encountered as stimuli for mMCP-1/JE. To seek the possible mechanism, therefore, we next examined the effect of hypoxia on NEMCs to the expression of MCP-1 in vitro (please see the supplemental materials).

In case of MRC-5 cells, FGF-2–mediated upregulation of hMCP-1 was not affected even under hypoxic cultivation at 5% O_2, a condition showing marked upregulation of VEGF in same-medium samples (data not shown). No significant change of hMCP-1 expression was found irrespective of FGF-2 or hypoxia, as expected in the case of HUVECs. Subsequent signal inhibition experiments revealed that PKC, classical MAPK (MEK), and NF-κB–related pathways were essential in stimulating hMCP-1 expression in both FGF-2 and TNF-α (please see the supplemental materials).

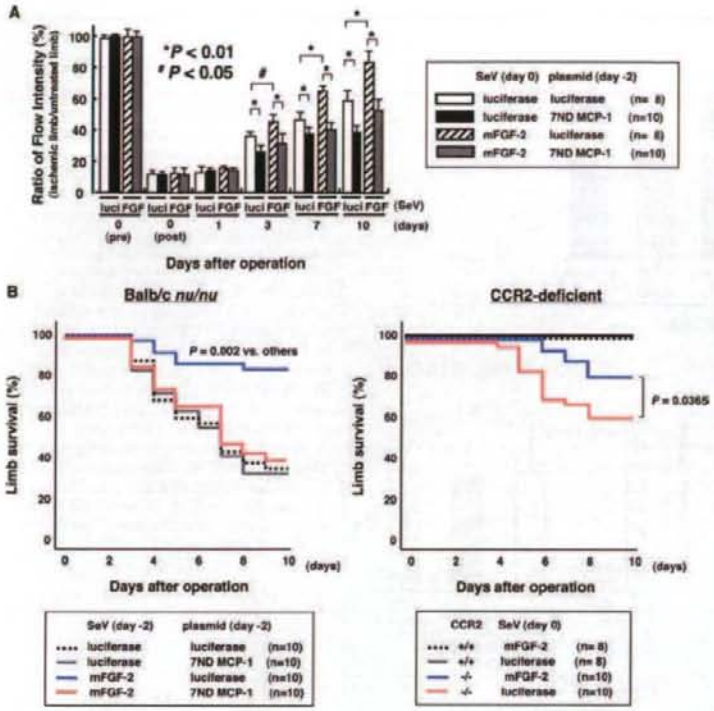

Figure 2. Biological role of endogenous mMCP-1/JE expression for adaptive and FGF-2–mediated neovascularization in hindlimb ischemia of C57BL/6 mice. The following results include all data from experiments repeated at least twice. #$P<0.01$, *$P<0.05$. a, Impact of the blockade of endogenous mMCP-1/JE activity using 7ND MCP-1 (a dominant negative inhibitor for MCP-1) on the recovery of blood flow during adaptive and FGF-2–mediated therapeutic neovascularization in a limb-salvaging model (C57BL/6). Computer-assisted and laser Doppler–mediated quantitative analyses of blood flow in ischemic hindlimb (left), standardized by that of untreated (right) hindlimb, are shown. Plasmid-based gene transfer of 7ND MCP-1 abrogated both adaptive and FGF-2–mediated recovery of blood flow. b, Impact of 7ND MCP-1 on the limb prognosis of autoamputation model (balb/c *nu/nu*; left) or of genetic deficiency in the functional receptor for mMCP-1/JE (CCR2) (C57BL/6 to 129/svjae hybrid strain; right). These curves were obtained using Kaplan–Meier method, and data were analyzed using the log-rank test.

These findings were also tested in vivo using a murine hindlimb ischemia model in the C57BL/6 strain. Each inhibitor compound for the signal transduction pathway (bis-I for pan-PKC, bis-V as a negative control compound for bis-I, U0126 for MEK, and rapamycin for p70S6K) was intraperitoneally administered daily from the day before limb ischemia and SeV-mFGF2, and each thigh muscle on day 2 was subjected to ELISA to measure the expression level of mMCP-1/JE protein. All mice receiving ALLN, an inhibitor for NF-κB, died in the course of the experiment because of toxicity and were thus excluded. As shown in Figure 3a, only bis-I and U0126 significantly reduced the expression of mMCP-1/JE, indicating the significant contribution of PKC and MEK during FGF-2–mediated MCP-1 expression in ischemic hind limbs in vivo.

Next, we examined the possible link between FGF-2 and TNF-α for the induction of endogenous mMCP-1/JE in induced ischemia of hind limb of C57BL/6 mice. At the physiological condition, FGF-2 was suggested to be expressed by endothelial cells, smooth muscle cells, and interstitial cells, and be stored on basement membrane.[20] Induced ischemia resulted in a modest increase of endogenous FGF-2, probably because of the previously known mechanism including acidosis in endothelial cells,[13,21] and SeV-mediated gene transfer markedly elevated the local concentration of FGF-2 (Figure 3b, left). However, increase at the local level of FGF-2 did not contribute to TNF-α expression in the same samples, whereas ischemia itself strongly induced TNF-α (Figure 3b, right). These results strongly suggest that FGF-2–mediated stimulation of mMCP-1/JE expression in ischemic hind limbs is an additive effect with proinflammatory signals including TNF-α using similar signal transduction pathways.

Proinflammatory/Arteriogenic FGF-2/MCP-1 Pathway Is Independent of the Angiogenic Signal of FGF-2/VEGF

Finally, we investigated the possible direct link of MCP-1 and VEGF, which were suggested as stimulators of each other.[22–24] Our in vitro experiments showed that FGF-2 but not MCP-1 stimulated VEGF expression in human NEMCs, a finding that was not seen among the monocyte-lineage cell (THP-1) findings confirmed in murine cells (please see the supplemental materials).

Similar findings were also obtained in vivo. Neither the induced ischemia- nor the FGF-2 gene transfer–mediated upregulation of VEGF was affected by inhibition of endogenous mMCP-1/JE activity by sufficient amount of 7ND MCP-1 (Figure 4, left graph), which exhibited a significant biological effect as shown in Figures 1 and 2. Furthermore, both the induced ischemia- and FGF-2 gene transfer–mediated upregulation of mMCP-1/JE expression demonstrated no significant alteration by inhibition of endogenous VEGF activity by sufficient amount of anti-VEGF neutralizing antibody (Figure 4, right graph), which exhibited a significant biological effect in our previous studies.[12–14]

These results thus indicate that the FGF-2/MCP-1 axis for inflammatory/arteriogenic signaling is independent of the FGF-2/VEGF sequence for angiogenesis in vitro and in vivo.

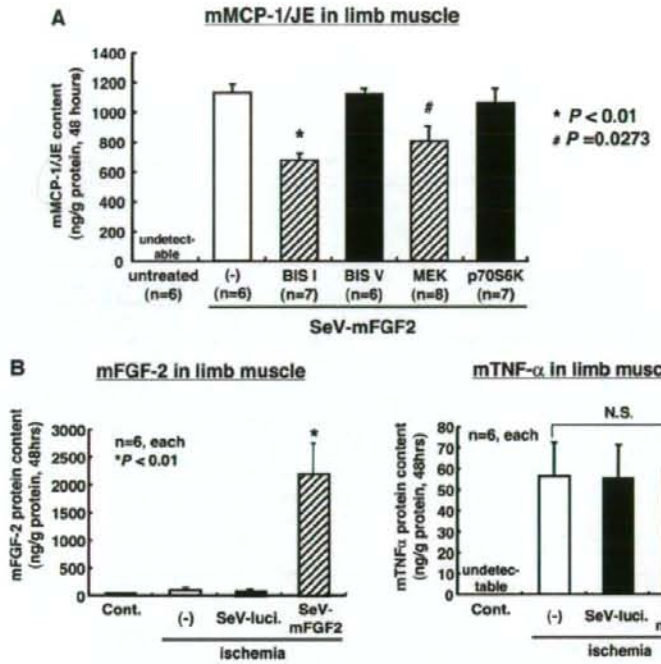

Figure 3. FGF-2–mediated upregulation of MCP-1 depends on PKC and MAPK, but is not sensitive to TNF-α. *$P<0.01$ and #$P<0.05$. a, Effect of various inhibitors for intracellular signal transduction pathways on FGF-2–mediated mMCP-1/JE expression in vivo using the hindlimb ischemia model of C57BL/6. Each inhibitor compound (bis-I for pan-PKC, bis-V as a negative control for bis-I, U0126 for MEK, and rapamycin for p70S6K) was intraperitoneally administered daily from the day before induced limb ischemia and gene transfer, and each thigh muscle was subjected to ELISA on day 2. All mice receiving ALLN, an inhibitor for NF-κB, died in the course of the experiment and were thus excluded. b, mTNF-α expression was not affected by FGF-2 in the ischemic limb. Soon after surgery inducing hindlimb ischemia, SeV-mFGF2 or SeV-luciferase was injected intramuscularly. Two days later, limb muscles were subjected to ELISA.

Discussion

We here demonstrated the independence between the inflammatory/arteriogenic (MCP-1) and angiogenic (VEGF) pathways, both downstream signals of FGF-2, in a murine hindlimb ischemia model. Key observations obtained in the present study were as follows: (1) MCP-1 expression was strongly stimulated by FGF-2 gene transfer, and MCP-1/CCR2 played a critical biological role both in adaptive and FGF-2–mediated recovery of blood flow; (2) FGF-2 stimulated MCP-1 expression via NEMCs but not endothelial or monocyte-lineage cells, in a hypoxia-independent manner; (3) FGF-2–mediated stimulation of MCP-1 was independent of a proinflammatory cytokine, ie, TNF-α, using similar signal transduction pathways, PKC-, MEK-, and probably NF-κB–dependent signaling; and (4) FGF-2/MCP-1 for the arteriogenic axis is independent of the FGF-2/VEGF sequence for angiogenic signaling.

These results are the first demonstration of the critical role of MCP-1/CCR2, an inflammatory/arteriogenic stream, for FGF-2–mediated neovascularization for blood perfusion in

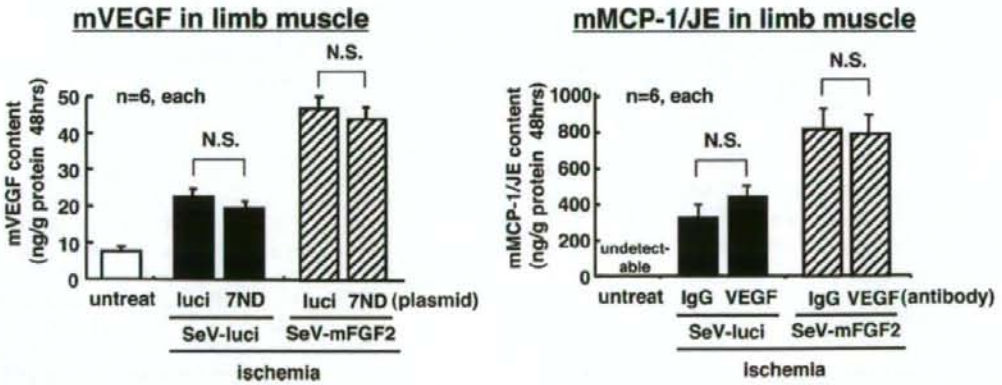

Figure 4. Both inflammatory/arteriogenic (MCP-1) and angiogenic (VEGF) pathways are downstream of FGF-2 in vivo, but are independent of each other. Plasmid-based 7ND MCP-1 was intramuscularly injected 2 days before surgery, and surgical ischemia was induced on day 0. At that time, 10^7 pfu of SeV-luciferase or SeV-mFGF2 was intramuscularly injected. Two days later, limb muscles were subjected to ELISA. VEGF-neutralizing antibody was administered by intraperitoneal continuous release (~28.6 μg/d) via peritoneal implantation of a disposable osmotic pump and an additional intravenous injection bolus (100 μg) soon after induced ischemia. *$P<0.01$ and #$P<0.05$.

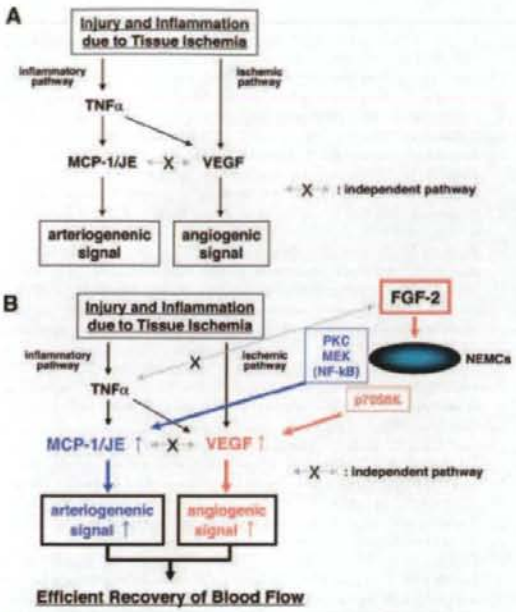

Figure 5. Schematic representation of the role of MCP-1 and NEMCs in adaptive and FGF-2–mediated neovascularization. a, The role of inflammatory/arteriogenic (MCP-1) and ischemia/angiogenic (VEGF) pathways during adaptive recovery of blood flow in limb ischemia is shown. Injury and inflammation attributable to tissue ischemia stimulate both inflammatory (TNF-α/MCP-1) and ischemic (VEGF) pathways. TNF-α but not MCP-1 also contributes VEGF expression resulting in an enhanced angiogenic pathway. b, The essential role of NEMCs during FGF-2–mediated therapeutic neovascularization is shown. FGF-2 stimulated MCP-1 expression via NEMCs but not other cells, via hypoxia-independent and PKC-, MEK-, and probably NF-κB–dependent signaling. This was independent of the proinflammatory cytokine, ie, TNF-α. In addition, FGF-2 targets NEMCs to induce VEGF via the p70S6K signal transduction pathway independent of FGF-2/MCP-1, as we demonstrated previously (ref. 13 and 14). Importantly, FGF-2/MCP-1 for the inflammatory/arteriogenic axis is independent of the FGF-2/VEGF sequence for the angiogenic signal. Simultaneous enhancement of the inflammatory/arteriogenic (MCP-1) and angiogenic (VEGF) pathways is essential to FGF-2–mediated recovery of blood flow.

ischemic tissue, and suggest the multiple functions and utility of FGF-2 overexpression for therapeutic angiogenesis, which is strictly regulated by NEMCs (the conceptual scheme is demonstrated in Figure 5a and 5b).

One important advance of the current study is to clarify the critical role of MCP-1 during adaptive and FGF-2–mediated recovery of tissue perfusion. As we previously reported, the limb-salvaging effect of FGF-2 was abolished by neutralization of endogenous VEGF activity,[12] as well as by inhibition of MCP-1/CCR2 signaling when using the dominant-negative inhibitor 7ND MCP-1 and CCR2-deficient mice, as shown in the present study. In addition, we here showed that MCP-1 was also necessary for spontaneous recovery of blood flow, assessed by LDPI, after induced hind limb ischemia. These results thus suggest that simultaneous enhancement of both systems may be absolutely required for both adaptive and FGF-2–mediated recovery of blood flow in ischemic limbs.

On the other hand, whereas these results are clear evidence of the tight link between MCP-1 and VEGF during FGF-2–mediated therapeutic angiogenesis, we here also showed that the regulatory pathways for their expression by FGF-2 was completely different each other in vitro and in vivo. Together with our previous and current findings, PKC, MEK, and probably NF-κB are important for MCP-1 expression, while p70S6K is the critical regulator for VEGF,[12–14] as shown in Figure 5a and 5b. Because both are essential for the biological activity of FGF-2, either FGFR agonists or a compound that simultaneously stimulates these signal transduction pathways may be a highly effective drug for therapeutic angiogenesis.

It has previously been demonstrated that MCP-1 is expressed by various cell types, including fibroblasts, smooth muscle cells, endothelial cells, and monocytes/ macrophages, in response to proinflammatory cytokines including interleukin (IL)-1 and TNF-α,[25–27] findings corroborated by our current study. We are the first to demonstrate that FGF-2 induces MCP-1 only in NEMCs but not in other cell types, emphasizing the essential role of NEMCs during FGF-2–mediated therapeutic neovascularization; however, the complex angio-/arteriogenic network between NEMCs and monocytes/macrophages via MCP-1/FGF-2 has not been fully clarified. Interestingly, previous studies demonstrated that MCP-1–mediated collateral artery growth was associated with an increased expression of FGFR1,[28] and in turn, that FGF-2 promoted monocyte accumulation and angiogenesis in a rabbit model of limb ischemia.[8] These studies strongly suggest the positive feedback loop between NEMCs and monocytes/macrophages when using MCP-1 and FGF-2 for enhancing both arteriogenesis and angiogenesis.

Our current findings suggest that FGF-2 might mediate inflammatory responses via MCP-1 and VEGF at its downstream; however, some studies suggested that FGF-2 is an antiinflammatory factor. During the review process of this manuscript, an important study has been published, indicating that dermal administration of FGF-2 itself induced neither inflammatory nor vascular reactions at the local site, ie, vascular permeability and hyperemia; however, in turn, FGF-2 synergistically enhanced leukocyte/monocyte recruitment induced by proinflammatory cytokines.[29] Their findings are in accordance with our previous findings obtained in use of a rat model of adjuvant-induced arthritis,[30] and importantly, they also demonstrated that is suggested to be an adhesion molecule-dependent mechanism. These conflicting results might be explained as follows: transient increase of local concentrations of downstream players of FGF-2, namely MCP-1 and VEGF, may be beneficial, but prolonged upregulation of these can be deleterious, during wound repair and limb ischemia. This hypothesis can well explain the following paradoxical findings related to VEGF; plasmid-based transient expression of VEGF increased the blood flow in hindlimb ischemia model, as shown by several laboratories, although implantation of fibroblasts continuously expressing VEGF by retroviral vector induced tissue edema, inflammation and hemangiomas.[31] This may also be a case of MCP-1; because the prolonged expression of MCP-1 is a cause of an

experimental model of atherosclerosis,[32] although bolus administration of MCP-1 is beneficial for collateral development in limb ischemia.[6] Further study should be called for to investigate which parameter is the determinant of the biological effects of MCP-1 as well as VEGF.

In conclusion, we here demonstrated that FGF-2 targets NEMCs to stimulate expression of VEGF and MCP-1 via independent signal transduction pathways, resulting in an efficient limb-salvaging effect. Therefore, the MCP-1/CCR2 system, known as proinflammatory/arteriogenic pathway, plays a critical role in FGF-2–mediated therapeutic neovascularization.

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Disclosures

Dr Yonemitsu is a member of Scientific Advisory Board of DNAVEC Corporation..

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VEGF function for upregulation of endogenous PlGF expression during FGF-2-mediated therapeutic angiogenesis

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Abstract

Vascular endothelial growth factor (VEGF) is a major positive angiogenic factor. Using a murine hindlimb ischemia model, we previously showed that fibroblast growth factor-2 (FGF-2) enhances the expression of endogenous VEGF which highly contribute to the therapeutic effect of FGF-2 gene transfer. Recently, placental growth factor (PlGF) has been shown to play an important role in angiogenesis. However, the molecular mechanism of endogenous PlGF during FGF-2-mediated angiogenesis has not been elucidated. Severe hindlimb ischemia stimulated PlGF expression that was more strongly enhanced by FGF-2 gene transfer, and a blockade of PlGF activity diminished the recovery of blood flow by FGF-2-mediated angiogenesis. The PlGF expression in cultured endothelial cells was significantly enhanced by VEGF stimulation, but not by FGF-2. The upregulation of endogenous PlGF expression was significantly decreased by the inhibition of endogenous VEGF activity *in vivo*. Subsequent signal inhibition experiments revealed that the PKC, MEK, and possibly NF-κB-related pathways were essential in stimulating PlGF expression with VEGF, while p70S6K is the regulator for VEGF expression. These results indicate that the FGF-2-mediated enhancement of PlGF expression was dependent on VEGF function, and the FGF-2/VEGF axis participates in FGF-2-mediated angiogenesis indirectly via PlGF as well as directly.

Keywords: Ischemia; PlGF; VEGF; FGF-2; Therapeutic angiogenesis

1. Introduction

Vascular endothelial growth factor (VEGF) is a major potent positive regulator of angiogenesis [1,2]. A well-documented function of VEGF is the ability to directly promote the growth of vascular endothelial cells (ECs) [2]. VEGF regulates angiogenesis mainly via two interacting tyrosine kinase receptors, vascular endothelial growth factor receptor 1 (VEGFR-1) and vascular endothelial growth factor 2 (VEGFR-2), and this signal transduction and biological responses in ECs are mediated primarily via VEGFR-2 [2,3]. The role of VEGFR-1 for angiogenesis has been reported as a negative regulator during embryogenesis acting as a 'decoy' receptor, resulting in the modulation of VEGFR-2 activity [1,4]. Currently, however, placental growth factor (PlGF), a VEGF homologue and the specific ligand for VEGFR-1, has been shown to play an important role in angiogenesis by signaling through its receptor VEGFR-1 on ECs [5]. Recent studies demonstrated that mice deficient in PlGF or the inhibition of VEGFR-1 exhibited impaired collateral artery growth in mouse limbs and the neovascularization of tumors, choroids and ischemic retinas, and that exogenous

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PlGF delivery promoted angiogenesis or collateral artery growth in ischemic limb, hearts and skin [5–13]. The mechanism of PlGF function during angiogenesis has been already reported as direct stimuli on ECs, monocyte-mediated mechanism, enhancing EPC recruitment, and synergistically by amplifying VEGF [5–13].

We previously demonstrated that the boosted overexpression of fibroblast growth factor-2 (FGF-2) by gene transfer consistently showed highly therapeutic potential against murine severe hindlimb ischemia [14]. While seeking the molecular and cellular mechanisms of the limb-salvaging effect of FGF-2, we found that the function of FGF-2 in ischemic limbs highly depended on the endogenous expression of vascular endothelial growth factor [14], which is strictly regulated and maintained by non-endothelial mesenchymal cells (NEMCs) via the autocrine system of the platelet-derived growth factor-AA (PDGF-AA)/PDGF receptor-α (PDGFRα)/p70S6 kinase (p70S6K) signal transduction pathway [15,16]. However, information regarding the role of FGF-2 in the context of PlGF is sparse at present.

In this study, therefore, we examined the role of the PlGF during FGF-2-mediated therapeutic neovascularization using a murine critical limb ischemia model. We here demonstrate that VEGF, but not the direct stimuli of FGF-2, enhanced the expression of endogenous PlGF *in vitro* and *in vivo*. Our results suggest that VEGF also functions indirectly via the expression of PlGF during FGF-2-mediated angiogenesis, resulting in the efficient recovery of blood flow.

2. Materials and methods

2.1. Cells and reagents

HUVECs (human umbilical vascular endothelial cells) and HPAECs (human pulmonary artery endothelial cells) were purchased from Kurabo Co. Ltd., Tokyo, Japan, and MRC-5 (human fetal lung fibroblasts) and THP-1 (monocyte/macrophage linage cells) were from the American Type Culture Collection. The following intracellular signal inhibitors were used at each of the following working concentrations, as previously described [15–18]: classical MAP kinase (MEK) inhibitor, U0126 (10 mmol/L, Promega K.K., Tokyo, Japan); NF-κB, ALLN (5 mmol/L, Roche Diagnostics, Tokyo, Japan); Ras, Ras-inhibitory peptide (50 mmol/L, Alexis Japan, Tokyo, Japan); p70S6K, rapamycin (100 ng/mL, Sigma–Aldrich, Tokyo, Japan); PKC, bisindolylmaleimide-I (bis-I, 100 nmol/L, Sigma); PI3K, wortmannin (120 nmol/L, Sigma); and PKA, PKA-inhibitory peptide (1 mmol/L, Calbiochem, San Diego, CA). The neutralizing antibody (anti-VEGF from rabbit) was from NeoMarkers Co. Ltd. (Fremont, CA). Stocks of recombinant Sendai virus vectors (SeVs: SeV-mouseFGF-2 (mFGF-2) and SeV-luciferase) were prepared as previously described [14–16,18].

2.2. Animals

Male C57BL/6 (6–7 weeks old) were purchased from KBT Oriental Co., Ltd. (Charles River Grade, Tosu, Saga, Japan). These mice were used for the "limb salvage model", respectively, as previously described [14]. All animal experiments were performed according to approved protocols and in accordance with recommendations for the proper care and use of laboratory animals by the Committee for Animals, Recombinant DNA, and Experiments Using Infectious Pathogens at Kyushu University, and according to law No. 105 and notification No. 6 of the Japanese government.

2.3. Murine severe hindlimb ischemia

Details of the surgical treatment and evaluation of limb prognosis have been described previously [14–18]: specifically, the excision of both the left femoral artery and vein and their branches from the inguinal ligament up to and including the saphenous–popliteal bifurcation was performed. For gene transfer, 25 μL of vector solutions were injected into two portions of the thigh and calf muscles, respectively, soon after the completion of surgery. The *in vivo* suppression of endogenous VEGF or PlGF activity was performed using VEGF-specific or PlGF-specific neutralizing antibody via bolus injection coupled with continuous release administration using a disposable micro-osmotic pump (Model 1007D, ALZA Co.), as previously described [14–16].

2.4. Laser doppler perfusion images

Measurements of the ischemic (left) and normal (right) limb blood flow were performed on a warm plate at body temperature using a laser doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, UK) [14–18]. To minimize data variables due to ambient light and temperature, the LDPI index was expressed as the ratio of left (ischemic) to right (non-ischemic) limb blood flow.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Time course of endogenous PlGF, and VEGF protein contents in murine limb ischemic muscles with or without gene transfer and culture medium were determined using Quantikine Immunoassay systems for murine and human VEGF-A, murine PlGF-2, and human PlGF (R&D Systems Inc., Minneapolis, MN). All thigh and calf muscles were subjected to ELISA for murine VEGF and PlGF. Values were standardized total protein of each muscles.

2.6. Statistical analysis

All data were expressed as means ± S.E.M. and were analyzed by one-way ANOVA with Fisher's adjustment, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. The expressions of endogenous VEGF and PlGF are upregulated after hindlimb ischemia and strongly enhanced by FGF-2 gene transfer

To examine the role of PlGF in the ischemic hindlimb, we first examined the expression of PlGF and VEGF using a murine model of hindlimb ischemia, namely the "limb salvage model," in C57 BL/6 mice, using SeV-mFGF-2 [14]. The murine PlGF protein expression, which was not detected in muscles under the non-ischemic condition, was strongly upregulated soon after ischemia induction, and its expression level was further enhanced by the overexpression of FGF-2 (Fig. 1a). Similar results were found in the case of PlGF mRNA by quantitative real-time PCR (data not shown). Both the protein and mRNA expressions had their peak on day 1 after ischemia-inducing surgery, and these protein expression patterns were similar to those of VEGF in the same tissue samples (Fig. 1a and b).

3.2. VEGF, but no FGF-2, targets ECs to stimulate PlGF expression

To assess the source of the PlGF expression, we examined the induction of PlGF via FGF-2 using cultured human cells, including NEMCs (MRC5), monocyte/macrophage linage cells (THP-1) and endothelial cells (HUVEC and HPAEC). As shown in Fig. 2a, VEGF, which was reported as an inducer of PlGF [19,20] but not FGF-2, stimulated to produce and secrete PlGF in the culture medium of HUVEC and HPAEC, a finding that was confirmed by the mRNA level evaluated by quantitative real-time PCR (data not shown). In contrast, the protein expression of PlGF was not detected in MRC5 and THP-1 by ELISA (data not shown). We also examined the effect of inflammation on the expression of PlGF in ECs *in vitro*. In the cases of HUVECs and HPAECs, the expression of PlGF was not affected by typical proinflammatory cytokine TNF-α (Fig. 2a).

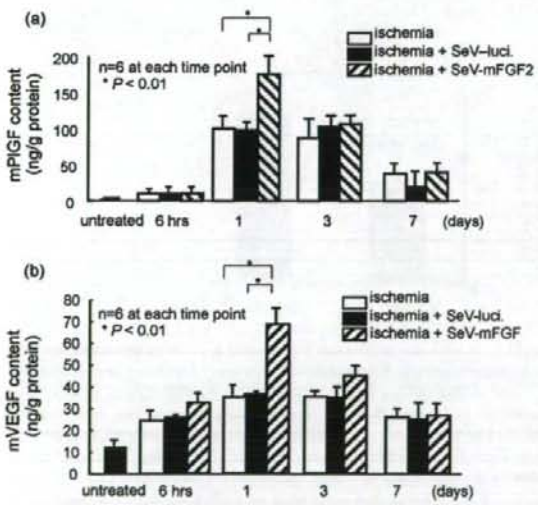

Fig. 1. Endogenous PlGF and VEGF expressions for FGF-2-mediated angiogenesis in hindlimb ischemia of C57BL/6 mice. The following results include all data from experiments repeated at least twice. *$P < 0.01$. (a) Time course of endogenous PlGF protein expression in murine limb ischemic muscles with or without gene transfer. The animals treated with mFGF-2 showed marked enhancement of PlGF expression on day 1. Each group contained 6 mice at each time point. (b) Time course of endogenous VEGF protein expression in murine limb ischemic muscles with or without gene transfer. The animals treated with mFGF-2 showed marked enhancement of VEGF expression on day 1. Each group contained 6 mice at each time point.

3.3. Signaling of FGF-2/VEGF for PlGF expression in vivo

Next, we investigated the possible link of PlGF and VEGF expressions *in vivo*. As shown in Fig. 1a and b, both the expressions of PlGF and VEGF were enhanced by ischemia and additionally by SeV-mFGF-2 gene transfer. The ischemia-induced and FGF-2 gene transfer-mediated upregulation of endogenous PlGF expression was significantly decreased by the inhibition of endogenous VEGF activity with a sufficient amount of anti-VEGF neutralizing antibody (Fig. 2b), which exhibited a significant suppression of VEGF-related biological effects *in vivo* in our previous studies [14–16,18]. These results thus suggest that the FGF-2/PlGF sequence is dependent on the FGF-2-mediated VEGF expression *in vitro* and *in vivo*.

3.4. FGF-2/p70S6K/VEGF axis stimulates PlGF expression via PKC, MEK, and NF-κB-related pathways

Considering the *in vivo* situation of surgically induced hindlimb ischemia, hypoxia signal and exogenously overexpressed FGF-2 should be encountered as stimuli for VEGF expression, followed by the promotion of PlGF expression. To seek this possible mechanism, we next examined the effect of hypoxia on the expression of PlGF in ECs *in vitro*. In the case of HUVECs, the expression of PlGF was not affected even under hypoxic cultivation at 2.5% O_2 (Fig. 2c), a condition inducing a marked upregulation of VEGF in the case of MRC5 samples (Fig. 2c).

Subsequent signal inhibition experiments revealed that PKC, classical MAPK (MEK), and NF-κB-related pathways were essential in stimulating PlGF expression with VEGF (Fig. 3a), and the results concerning PKC and MEK are consistent with those of a previous study [19]. These findings were also tested *in vivo* using a murine hindlimb ischemia model. Each inhibitor compound for the respective signal transduction pathway (bis-I for pan-PKC, bis-V as a negative control compound for bis-I, U0126 for MEK, and rapamycin for p70S6K) was intraperitoneally administered daily from the day before limb ischemia, and each thigh muscle on day 2 was subjected to ELISA to measure the expression levels