## 10. 研究発表

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|---|---|
| 口頭発表： | １８件 |
| 原著論文による発表： | ０件 |
| それ以外（レビュー）などによる発表： | １０件 |

【2. 国外】

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|---|---|
| 口頭発表： | ９件 |
| 原著論文による発表： | １３件 |
| それ以外（レビュー）などによる発表： | ０件 |

## 11. 知的財産の出願・登録状況

本研究に直接該当するもの無し。

# III. 研究の成果の刊行に関する一覧表

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## IV. 研究成果の刊行物・別冊

**本研究と最も密接に関係する以下の論文を抜粋する**

1. Fujii T, Yonemitsu Y, Onimaru M, Inoue M, Hasegawa M, Kuwano H, Sueishi K.
VEGF Function for Upregulation of Endogenous PlGF Expression during FGF-2-mediated Therapeutic Angiogenesis.
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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.

Please cite this article in press as: Fujii T, et al., VEGF function for upregulation of endogenous PlGF expression during FGF-2-mediated therapeutic angiogenesis, Atherosclerosis (2008), doi:10.1016/j.atherosclerosis.2007.12.012

## 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,

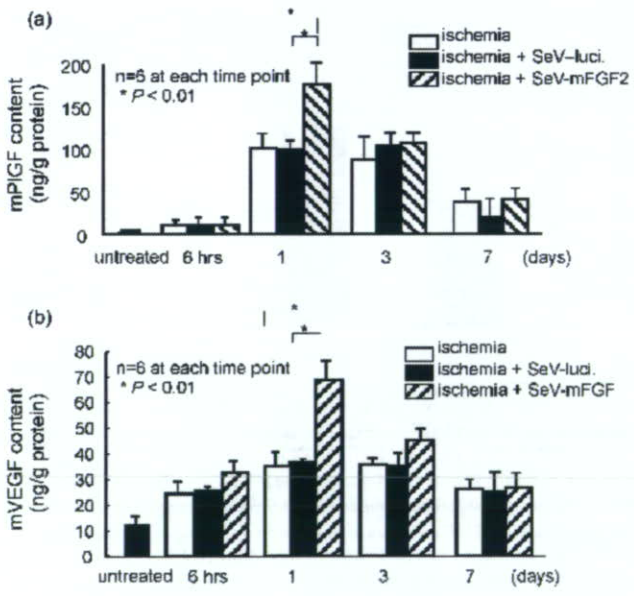


Fig. 1. Endogenous PlGF and VEGF expressions for FGF-2-mediated angiogenesis in hindlimb ischemia of C57BL/6 mice. The following results include all data from experiments repeated at least twice. $^{*}P < 0.01$. (a) Time course of endogenous PlGF protein expression in murine limb ischemic muscles with 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.

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).

### 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

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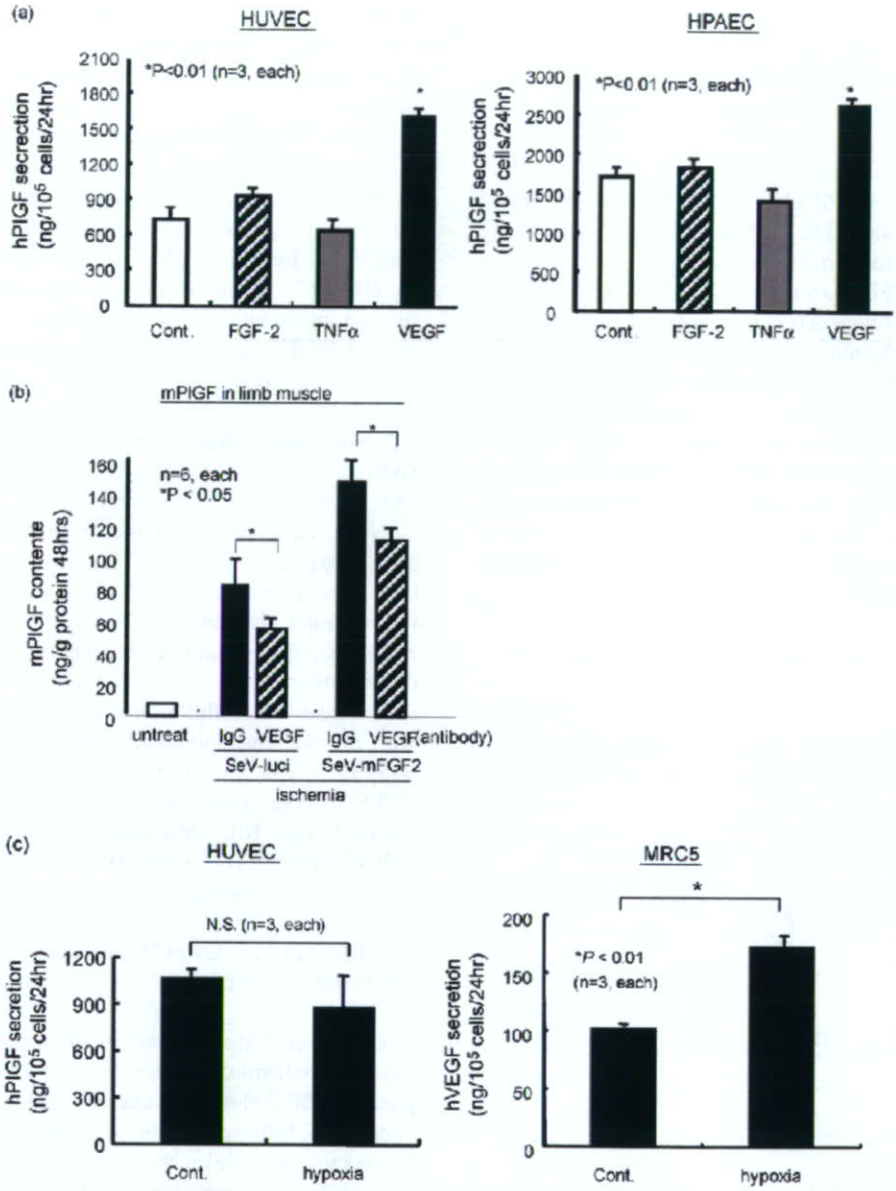


Fig. 2. VEGF, but not FGF-2 or hypoxia, stimulates PlGF in endothelial cells (ECs) *in vitro* and *in vivo*. (a) Twenty-four hours after preincubation with 5% FBS, HUVECs and HPAECs were stimulated with recombinant proteins (10 ng/ml, respectively, VEGF and FGF-2). Twenty-four hours later, the culture medium was subjected to ELISA. $^{*}P<0.01$. VEGF stimulated PlGF in ECs, while FGF-2 did not induce PlGF in ECs. (b) FGF-2/PlGF pathway was through VEGF *in vivo*. Surgical ischemia was induced, and $10^7$ pfu of SeV-luciferase or SeV-mFGF-2 was intramuscularly injected. Two days later, the limb muscles were subjected to ELISA. VEGF-neutralizing antibody was administered by intraperitoneal continuous release (approximately 28.6 μg/day) via peritoneal implantation of a disposable osmotic pump and an additional intravenous injection bolus (100 μg) soon after induced ischemia. (c) No contribution of hypoxia on the basal expression of PlGF on HUVEC was found (left), whereas the expression of VEGF was promoted on NMECs (MRC5) in the hypoxia condition (right). After preincubation for 24 h with or without hypoxia (2.5% $O_2$). Twenty-four hours later, the culture medium was subjected to ELISA.

of PlGF and VEGF proteins. All mice receiving ALLN, an inhibitor for NF-kB, died from toxicity in the experimental course and were thus excluded. As shown in Fig. 3b, bis-I, U0126, and rapamycin significantly reduced the *in vivo* expression of PlGF, while p70S6K signaling did not contribute to the PlGF expression in HUVEC *in vitro* (Fig. 3a). In contrast, as shown in Fig. 3c, U0126 and rapamycin significantly reduced the *in vivo* expression of VEGF, indicating the significant contribution of the p70S6K and MEK pathways for FGF-2-mediated VEGF expression, but PKC, p70S6K and MEK for the FGF-2-mediated PlGF expression in ischemic hindlimbs *in vivo*. These results strongly sug-

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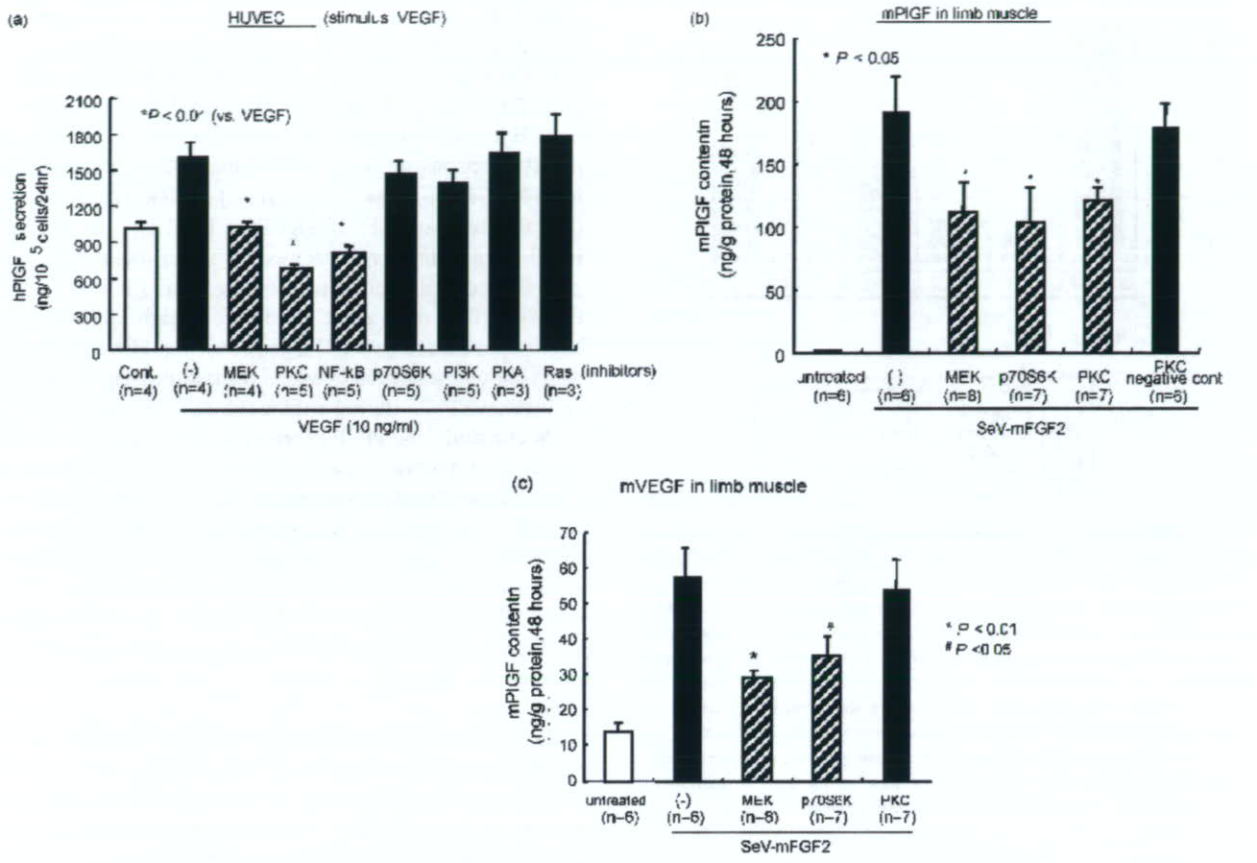


Fig. 3. FGF-2/p70S6K/VEGF axis stimulates PlGF expression via PKC, MEK, and NF-κB-related pathways in FGF-2-mediated angiogenesis. $^{*}P < 0.01$ and $^{\#}P < 0.05$. (a) VEGF-mediated PlGF secretion in HUVECs depended on PKC, MEK, and NF-κB *in vitro*. After preincubation without serum for 24 h, the cells were stimulated by 10 ng/mL of human recombinant VEGF with or without various inhibitors. Twenty-four hours later, the culture medium was subjected to ELISA. (b and c) Effect of various inhibitors for intracellular signal transduction pathways on FGF-2-mediated PlGF and VEGF 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 during the course of the experiment and were thus excluded.

gest that VEGF plays a critical role in the FGF-2-mediated stimulation of PlGF expression in ischemic hindlimbs.

### *3.5. Endogenous PlGF is essential for the FGF-2-mediated recovery of blood flow in murine ischemic limbs*

To assess the biological role of the upregulated expression of PlGF in ischemic hindlimbs, we tested the effect of PlGF function on the therapeutic effect of FGF-2 gene transfer using a murine model of severe hindlimb ischemia mice treated with PlGF-specific neutralizing antibody. As shown in Fig. 4, the inhibition of endogenous PlGF activity by a sufficient amount of anti-PlGF neutralizing antibody significantly reduced the FGF-2-mediated recovery of blood flow, indicating that PlGF may play a significant role in FGF-2-mediated angiogenesis. These findings were clear evidence that the endogenous expression of PlGF plays an essential role during FGF-2-mediated therapeutic angiogenesis in a mouse model of limb ischemia.

## 4. Discussion

We here demonstrated that the expression of PlGF is partly dependent on the FGF-2/VEGF sequence in a murine hindlimb ischemia model. The key observations obtained in the present study were: (1) PlGF expression was stimulated by FGF-2 gene transfer in a murine hindlimb ischemia model and played an important role in FGF-2-mediated therapeutic angiogenesis in this murine model; (2) VEGF, but not FGF-2, stimulated PlGF expression by ECs *in vitro*; (3) the FGF-2-mediated stimulation of PlGF was dependent on the signaling pathway via FGF-2/p70S6K/VEGF/PKC-, MEK- and possibly the NF-kB-dependent signal transduction pathways. We have already demonstrated that FGF-2 upregulated the endogenous expression of VEGF *in vivo* and *in vitro* [14,16]. These results are the first demonstration of the critical role of PlGF in a cooperative manner with VEGF for FGF-2-mediated neovascularization, resulting in the functional blood perfusion into ischemic tissue.

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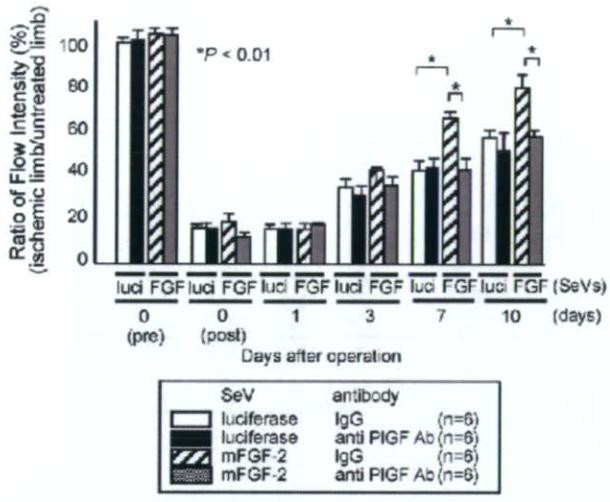


Fig. 4. Impact of the blockade of endogenous PlGF activity on the recovery of blood flow during FGF-2-mediated therapeutic angiogenesis. The blockade of endogenous PlGF activity using PlGF-neutralizing antibody on the recovery of blood flow during FGF-2-mediated therapeutic angiogenesis was assessed using a limb-salvaging model (C57BL/6). Computer-assisted and laser Doppler-mediated quantitative analyses of blood flow in the ischemic hindlimb (left), standardized by that of the untreated (right) hindlimb, are shown. Inhibition of endogenous PlGF activity abrogated FGF-2-mediated recovery of blood flow. PlGF-neutralizing antibody was administered by intraperitoneal continuous release (approximately 28.6 μg/day) via peritoneal implantation of a disposable osmotic pump and an additional intravenous injection bolus (100 μg) soon after induced ischemia.

One important advance of the current study is to clarify the necessity of PlGF during the FGF-2-mediated recovery of blood perfusion in ischemic tissues. The limb-salvaging effect of FGF-2 is reduced by not only neutralization of endogenous VEGF activity as previously reported [14], but also partly by inhibition of endogenous PlGF activity, as shown in the present study. We here showed that the upregulation of PlGF, the indirect pathway of VEGF for angiogenesis, was also necessary for FGF-2-mediated spontaneous recovery of blood flow assessed by LDPI after induced hindlimb ischemia. These results thus suggest that VEGF is absolutely required indirectly via PlGF function as well as directly for the FGF-2-mediated recovery of blood flow in ischemic limbs. In other words, these results provide clear evidence that both the FGF-2 and PlGF functions tightly link with each other during FGF-2-mediated therapeutic angiogenesis, and that PlGF overexpression by FGF-2 gene transfer is mediated by VEGF function *in vivo* as well as *in vitro*.

PlGF has been reported to play an important role in the angiogenesis of tumor or ischemic tissues by signaling through its receptor VEGFR-1 on ECs as well as by an inflammatory macrophage/monocyte-mediated mechanism or enhancing EPC recruitment [5–13]. Our current study showed that PlGF played an important role for FGF-2-mediated angiogenesis, however, the definitive mechanism of endogenous PlGF during FGF-2-mediated angiogenesis was not fully elucidated. We previously reported that neutralization of endogenous VEGF activity significantly impaired in adaptive and the FGF-2-mediated recovery of blood flow [14]. In contrast, as shown Fig. 4, the inhibition of endogenous PlGF reduced the FGF-2-mediated recovery of blood flow, but not reduced adaptive recovery of blood flow. The possible reasons are as follows: (1) the biological signal activity of VEGFR-1 is low, and high level of PlGF is necessary for the effectiveness of PlGF signaling [2,21]; (2) endogenous PlGF would play a role to modify VEGF-mediated angiogenesis because inhibition of endogenous PlGF is reduced the blood flow only at the condition of high level of VEGF expression caused by FGF-2 gene transfer. However, further study should be needed to assess the definitive mechanism of endogenous PlGF during angiogenesis.

In our study, the PlGF expression in ECs was not directly dependent on a hypoxic condition, and PlGF protein was not detected in fibroblast and monocyte/macrophage cell lines with ELISA. However, it has previously been demonstrated that PlGF is expressed by fibroblasts under the non-hypoxic state and upregulated under the hypoxic condition [22]. In this study, we showed that VEGF expression was promoted by hypoxia in NEMCs, indicating the possibility that VEGF induced by hypoxia in NEMCs is concerned with PlGF expression.

In addition, VEGF stimulated PlGF expression via ECs via hypoxia-independent and PKC-, MEK-, and probably NF-kB-dependent signaling, resulting in the indirect pathway of the FGF-2/VEGF sequence for angiogenesis, while p70S6K is the critical regulator for VEGF [14–16]. As shown in Fig. 1, the endogenous VEGF expression was detected to some extent, but PlGF expression was not detected in untreated condition *in vivo*. From these findings, the FGF-2/PlGF axis is dependent on the expression of VEGF in part, however, another mechanism for regulation of endogenous PlGF might be also present.

In conclusion, we here demonstrated that FGF-2 targets NEMCs to stimulate the expression of VEGF and this upregulation of VEGF promotes the expression of endogenous PlGF, resulting in an efficient limb-salvaging effect. Therefore, VEGF plays a critical role directly and indirectly via PlGF in FGF-2-mediated therapeutic angiogenesis.

## Competing interest statement

The authors declare that they have no competing financial interests.

## Acknowledgments

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# サイトカインによる末梢動脈閉塞性疾患に対する血管新生療法：その現状と将来

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**要　旨**：難治性末梢動脈閉塞性疾患(PAD)に対する新しい治療法として血管新生療法が注目され，国内外で臨床的評価が行われている。しかしながら，初期において有望な成績が示唆されていたにもかかわらず，多施設二重盲検による第II相試験では，多くのプロトコールが失敗に終わっている。本稿では，血管新生療法の現状と臨床試験の問題点，そしてわれわれが進めているまったく新しい組換えセンダイウイルスベクター(rSeV/dF)による臨床研究の現状を概括する。
(J Jpn Coll Angiol, 2007, 47: 491–497)

Key words: peripheral arterial diseases, intermittent claudication, critical limb ischemia, angiogenesis, recombinant Sendai virus

## 疾患の背景および現行の治療的血管新生療法の問題点

### (1)疾患の特性

閉塞性動脈硬化症やバージャー病を代表とする末梢動脈閉塞性疾患(peripheral arterial disease: PAD)は，軽度な場合は，冷感やしびれ感(いわゆるFontaine I度)を，症状が進行するにつれ間歇性跛行(同II度，intermittent claudication: IC)を，さらには安静時疼痛(Fontaine III度)，潰瘍形成(同IV度)を呈するようになる。ICはQOLを著しく損なわない初期段階では保存的な薬物療法(シロスタゾールなど)，運動療法など，すでにエビデンスが蓄積された治療法が第一選択として行われる。さらに進行し，患者が歩行可能距離の短縮にQOLの低下を相当なレベルで感じる場合(一般に200m以下)には，血管内治療あるいは外科手術の適応となる。進行症例(Fontaine III・IV度，重症虚血肢:critical limb ischemia: CLI)は，リスクファクターの除去，現行の薬物療法，外科的療法に反応せず，下肢切断を余儀なくされる場合が多い。下肢切断は患者のQOL(quality of life)を著しく低下させるばかりでなく生命予後にも大きく関わり，下肢切断を行った患者の2年生存率は50%以下，特に透析患者では5年生存率は10%を下回ることが報告されており，したがって，これら重症のCLIに対し，下肢切断を回避(limb salvage)する治療法の確立が望まれている。

以上，PADの中でも積極的治療の対象となる病態は進行したICとCLIであることを述べたが，この両者は血行動態に基づく病態がまったく異なっている。両者は「虚血性疾患」としてよく混同されることがあるが，ICはあくまで相対的虚血であり，安静時では血流低下は存在するものの組織虚血ではない(虚血性の生体反応は見られない)。労作時に血液需要量が増加した場合に初めて組織虚血が誘発され，それが痛みとなって発現する。一方でCLIは絶対的虚血であり，安静時にも痛みを含む慢性的な虚血性反応が認められ，これがICとCLIの治療効果に対する臨床的評価を複雑なものにしている(**Table 1**)。

### (2)血管新生療法臨床的評価の現状

疾患の病態が虚血による組織障害である以上，治療の目指すところは虚血組織局所に有効な血流回復を誘

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2007年3月22日受理

**Table 1** The current standard management of PAD

| Claudication | CLI |
|---|---|
| Pathophysiology:<br>ischemia, exercise-induced | Pathophysiology:<br>ischemia, sustained at rest |
| Current management:<br>1. Removal of risk factors<br>- smoking secession, statins<br>2. Excercise<br>3. Revascularization<br>- most effective<br>4. Cilostazol<br>- evidence level A for IC | Current management:<br>1. Revascularization<br>- most effective, bypass surgery, catheter intervention<br>2. Amputation<br>・No effective drug available<br>・Loss of QOL, poor survival |

導し，虚血状態を改善することにある。そのための治療戦略の一つとして，血管新生活性を有する細胞増殖因子を外来性に投与し積極的に血管新生を誘導することで血流回復，虚血改善を目指す"治療的血管新生療法"確立への模索が開始され，すでに約10年が経過しようとしている。

TASC(TransAtrantic Inter-Society Consensus)より発行されている「Management of Peripheral Arterial Disease (PAD)，邦訳：下肢閉塞性動脈硬化症の診断・治療指針(日本脈管学会編)」によれば，特にCLIに対する有効性に関するエビデンスの確立した薬物療法はなく，また手術適応が限定されているため，治療的血管新生療法には多くの期待が寄せられている。治療的血管新生療法への初期の試みは血管新生因子蛋白を用いたものであり，数年後より血管新生因子を用いた遺伝子治療が開始，そして最近では骨髄単核球細胞や血管前駆細胞を比較的多く含むと考えられているCD34陽性細胞などによる細胞療法も試みられている。特に蛋白療法については，虚血性心疾患を含めすでに第II・III相試験の成績が公表されているが，初期試験では「安全かつ有効」とされてきた試験プロトコールが，後期相試験ではすべて「無効」と判定されている。遺伝子治療も同様で初期にはすべてのものが「安全かつ有効」とされてきたにもかかわらず，すでにVEGF(vascular endotherial growth factor) 121やDel(developmentally regulated endothelial locus)-1を用いた第II相試験では「無効」と判定されている。

このような初期試験と後期試験の成績の解離の原因としていくつかの要因が考えられる。具体的には，①対象となる病態の選択(ICか，CLIか？)，②本疾患に特有の高いプラセボ効果，③前治療や禁煙等の生活指導・歩行訓練などの有無，④副次評価項目(いわゆるsurrogate markers)測定の不安定性，⑤特にCLIについて，現行の主要評価項目の妥当性，など多くの因子が関与している。

以下に，これらの詳細について考察を試みる。

1)対象となる病態の選択(ICか，CLIか？)

すでに多くのエビデンスが蓄積されているように，ICに対して運動療法は有効であり，指導下で実施された運動療法により跛行誘発歩行距離・歩行可能距離は2倍以上になる。これは運動療法による運動耐容能と側副血行の増加によるものと考えられている。前述のごとくICの病態は相対虚血であり，したがって理論的に考えて，通常虚血状態ではない下肢に対して血管新生療法により血管数を増やすことが可能であったとしても，増加した血管が機能的にも維持されるか否かについては疑問が残る。また新しく血管が再生されたとして，そちらにドミナントに血流が流れてしまい，本来必要とされる部分の血流がスチールされる可能性も否定できない。前述したいわゆるnegative trialでは，対象がすべてICであることから，この考察は妥当性があるかもしれない。

一方でCLIでは絶対的に血流が足りない状態であるため，わずかに血管が再生したとしても，その結果がダイレクトに治療効果として結びつきやすいのではな

いかと理論的に考えられる。CLIに関して第II相試験結果が報告されているのは，Sanofi-Aventis社による欧州でのNV1-FGF(ヒトFGF-1を発現するプラスミドベクター)による遺伝子治療，ならびにAnGes MG社による米国でのTREAT-HGF(ヒトHGFを発現するプラスミドベクター)による遺伝子治療の2件である。前者ではCLIの客観的臨床評価に最も重要な，「下肢切断率の低下」が得られており，後者では下肢切断率，潰瘍治癒率などでは有意ではないものの，皮膚血行動態を示すsurrogate markerの一つである$tcPO_2$で用量依存性の改善が得られている。これらの第II相試験結果から予測しても，血管新生療法の効能を評価する対象という観点からは，CLIのほうがより適していると考えられる。

2)本疾患に特有の高いプラセボ効果

PADに対する薬剤の有効性判定で最も困難を伴うのは，いかにしてプラセボ効果を凌駕するかという問題である。例えば，前述のDel-1による遺伝子治療でも，プラセボ群が歩行距離などの指標で35%程度の改善を示しており，被験薬はこれを超えることができていない。本疾患の臨床試験にプラセボ効果がつきものである以上，これを超える効果を示す薬剤・治療法の開発が必要である。

3)前治療や禁煙等の生活指導・歩行訓練などの有無

PADの日常診療を行っている血管外科医はよく経験するが，慢性のFontaine IV度の患者の潰瘍は，禁煙などを含むリスクファクターの除去と入院・生活指導，投薬，そして運動療法等である程度改善することが知られており，特にこの傾向は若年者のバージャー病に強い。血管外科医からの血管新生療法への批判として，「distal bypassを含めて，これら前治療がしっかりと行われたうえで難治性となっているCLIで同様の効果が出ているならば認められるが，バージンケースで効果があったと言われても，一体血管新生療法の結果なのか，入院やそれによる禁煙の効果なのか判定できない」と述べられるゆえんである。したがって，臨床的有効性評価を行うという観点からは，しっかりとしたプラセボ群をおき，これらの前治療に配慮した試験デザインを採用する必要がある。

4)副次評価項目(いわゆるsurrogate marker)測定の不安定性と問題点

ICの基本病態(相対虚血かつ生存率)から判断し，現在のICに対する主要エンドポイントは跛行出現距離と最大歩行時間・距離であり，これはQOLの維持・向上を最大の目的としたものである。この測定法はすでに十分に確立しており，かつ試験担当施設間でのばらつきも少ないため，ICの臨床試験は比較的実施しやすい。

一方CLIの場合，基本的な臨床像が「死亡率・心血管イベント率の増加，下肢切断」であることから，欧米での主要エンドポイントは「心血管系イベント発生率の低下，肢切断の回避状態での生存率+肢切断回避率：amputation-free survival」が客観性・安定性の高いゴールドスタンダードである。しかし，これが血管新生療法によるベネフィットを検出できる感度を持つか否かについては，議論が残るところである(後述)。

以上の背景から，血管新生療法の基本コンセプトを間接的に示唆する副次エンドポイント(パラメータ)として，血行動態の変化に関するパラメータ(ankle-brachial index: ABI，$tcPO_2$など)が使用されている。しかし，これらのパラメータの改善が，治療のベネフィットと繋がるか否か，については，今なお否定的見解が多数を占める。これは例えば抗悪性腫瘍剤の臨床評価と同等の問題をはらんでおり，「腫瘍が小さくなる」というパラメータが「生存率が延長する」というベネフィットに必ずしも寄与しないのと同様，「多少の血行の改善」が「心血管イベント発生率の低下と肢切断率の低下」に必ずしも寄与しないのは明らかである。

また血行動態パラメータは，施設間におけるデータのばらつきが大きいことも，重大な問題である。NV1-FGFの第I相試験ではABIの有意な上昇が観察されていたが，第II相試験ではプラセボと有意差が得られなかったことなどは，その代表的な例である。一方で，大阪大学で実施されたTREAT-HGFの成績では$tcPO_2$の変動が大きく，一定の傾向は見られなかったにもかかわらず，米国での第II相試験では30mmHgという基準値を超える症例数が用量依存性に得られていることから考察しても，これら血行動態検査に十分な経験を持つ施設を集めて試験を行うことの重要性が示唆される。

5)特にCLIについて，現行の主要評価項目の妥当性

前述のごとく，CLIに関する欧米での主要エンドポ

**Table 2** Regulatory process of SeVAT trial at Kyushu University Hospital

| | | |
|---|---|---|
| IRB Processing (1 yr & 1 mo) | | |
| 2001 | 8/17 | Clinical study plan has been submitted to IRB |
| 2002 | 9/26 | Approved by IRB |
| Processing in MHLW (3 yrs & 3 mo) | | |
| 2002 | 8/17 | Clinical study plan has been submitted to MHLW |
| 2003 | 2/17 | 1st Review and Hearing |
| | 3/25 | Revision suggested |
| | 9/26 | Revised study plan has been submitted to MHLW |
| 2004 | 3/15 | 2nd revision suggested |
| | 6/15 | Revised study plan has been submitted to MHLW |
| | 6/17 | 2nd Review and Hearing |
| | 6/30 | Revision suggested |
| | 10/18 | Revised study plan has been submitted to MHLW |
| 2005 | 2/7 | 3rd Review and Hearing |
| | 2/15 | Review board: 'review has been almost completed' |
| | | Revised study plan has been submitted to MHLW |
| | 6/15 | Revision suggested |
| | 10/12 | Review board: 'application has been approved' |
| 2006 | 1/31 | Official letter for approval from Minister of MHLW |

IRB: institutional review board, MHLW: Ministry for Health, Labor, and Welfare

イントは「心血管系イベント発生率の低下，肢切断の回避状態での生存率+肢切断回避率：amputation-free survival」が客観性・安定性の高いゴールドスタンダードである。しかし，これが血管新生療法によるベネフィットを検出できる感度を持つか否か，については議論が残るところである。

これまで薬剤の試験では，CLIに対するプロトコールはすべて失敗に終わっている。しかし現行の血行再建に関しても，成功事例では有効であることは間違いないが，失敗例における肢切断率は60%を超えるため，その適応には厳密である必要がある。その意味からも血管新生療法の要求性は高いため，今後の進展が望まれる。

## ヒトFGF-2遺伝子発現非伝搬型組換えセンダイウイルスベクターによる遺伝子治療臨床研究

### (1)本臨床研究に至る経緯

このような研究の歴史は，虚血性疾患に対する血管新生療法の確立の困難さを示している。しかしながら，われわれは戦略の限界を示唆するものであるとは考えていない。特に蛋白療法，遺伝子治療の場合，外来性に投与される細胞増殖因子は，もともと内因性に存在するものがほとんどで，元来半減期が短い血管新生因子について，焼け石に水程度の外来性因子を投与しても有効性につながりにくいことは容易に想像がつく。つまり内因性レベルと比較した有効域の決定は極めて大切な検討項目であるにもかかわらず，現行の治療的血管新生療法においてその基礎データを踏まえて行われているものはほとんどない。さらに，用いる治療因子の科学的必然性にも乏しい。したがって，治療的血管新生療法の確立を目指した研究はいまだ発展途上である。その戦略の妥当性は多くの動物実験において世界中が確認している以上，今後，有効性を示すレベルに至る可能性を十分秘めていると考える。

以上の背景のもと，われわれは「圧倒的な治療効能を示す血管新生療法の確立」を目指し，独自に開発を進めている非伝搬型組換えセンダイウイルスベクター(recombinant Sendai virus: rSeV/dF)による遺伝子治療臨床研究を立案，治療遺伝子としてヒト塩基性線維芽細胞増殖因子(hFGF-2)との組合せが既存法と比較して最も高い治療効果を示すことを明らかにし，施設倫理委員会の承認後，厚生科学審議会へ実施申請書を提出した。rSeV/dF-hFGF2の優れた治療効能とその分子メカニズムに関する基礎研究の成果は他に譲るが[1~10]，合計4年半の経過を経て，本臨床研究プロトコールが正式に大臣承認を得た(2006年1月31日付，**Table 2**)

### (2)本臨床研究の内容

本臨床研究は，世界初の遺伝子治療ベクターを用いた臨床研究であることを鑑み，抗癌剤と同様のオープンラベル，4段階の用量漸増式第I・IIa相試験として計画されている(**Fig. 1**)。動物実験の結果から推測して，最低用量はプラスミドレベルの発現と同等と考えら