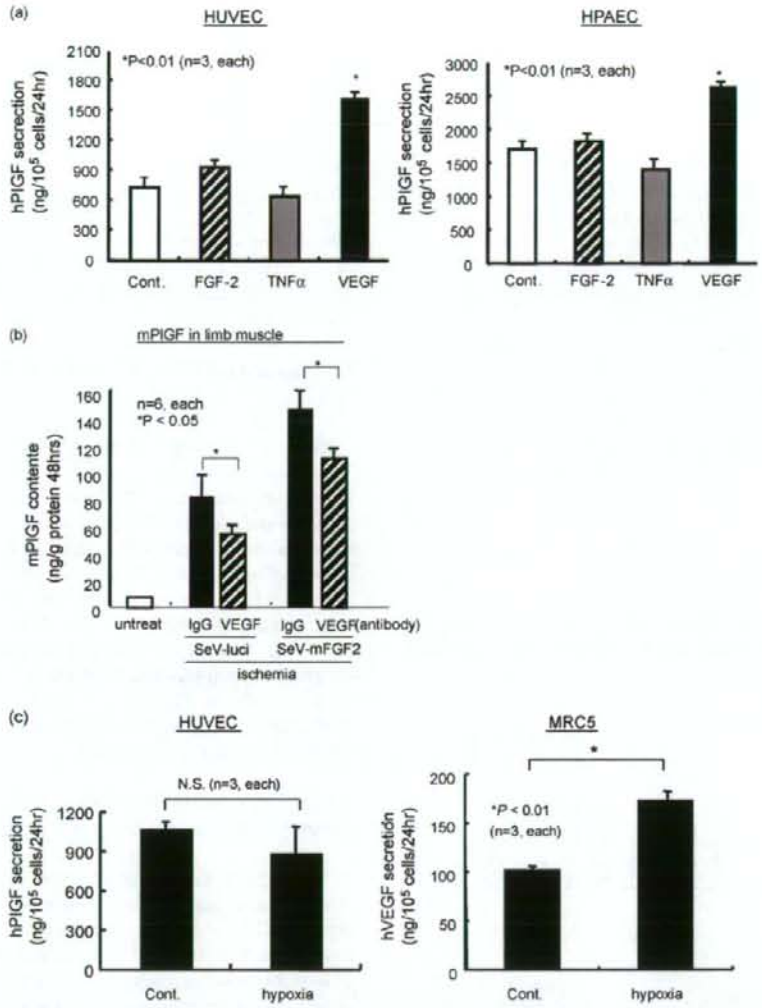

Fig. 2. VEGF, but not FGF-2 or hypoxia, stimulates PlGF in endothelial cells (ECs) *in vitro* and *in vivo*. (a) Twenty-four hours after preincubation with 5% FBS, HUVECs and HPAECs were stimulated with recombinant proteins (10 ng/ml, respectively, VEGF and FGF-2). Twenty-four hours later, the culture 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-

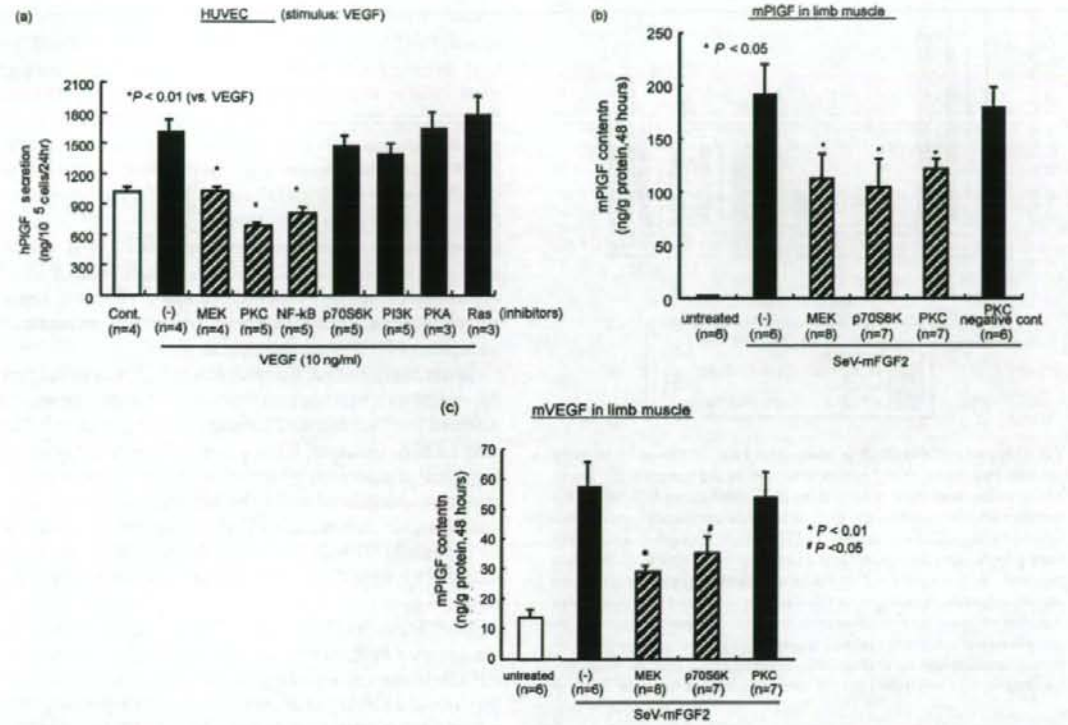

Fig. 3. FGF-2/p70S6K/VEGF axis stimulates PlGF expression via PKC, MEK, and NF-κB-related pathways in FGF-2-mediated angiogenesis. $^{*}P < 0.01$ and $^{\#}P < 0.05$. (a) VEGF-mediated PlGF secretion in HUVECs depended on PKC, MEK, and NF-κB *in vitro*. After preincubation without serum for 24 h, the 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.

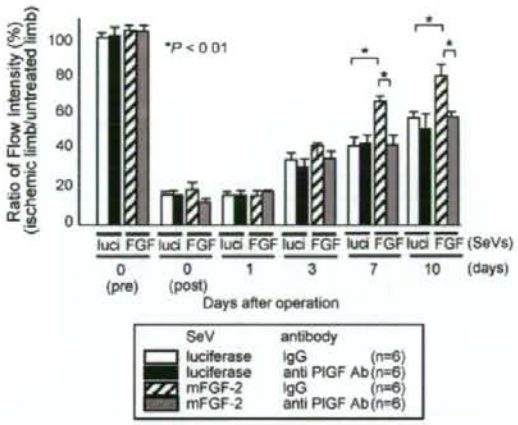

Fig. 4. Impact of the blockade of endogenous PlGF activity on the recovery of blood flow during FGF-2-mediated therapeutic angiogenesis. The blockade of endogenous PlGF activity using PlGF-neutralizing antibody on the recovery of blood flow during FGF-2-mediated therapeutic angiogenesis was assessed 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欠性跛行(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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Table 1 The current standard management of PAD

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

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

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

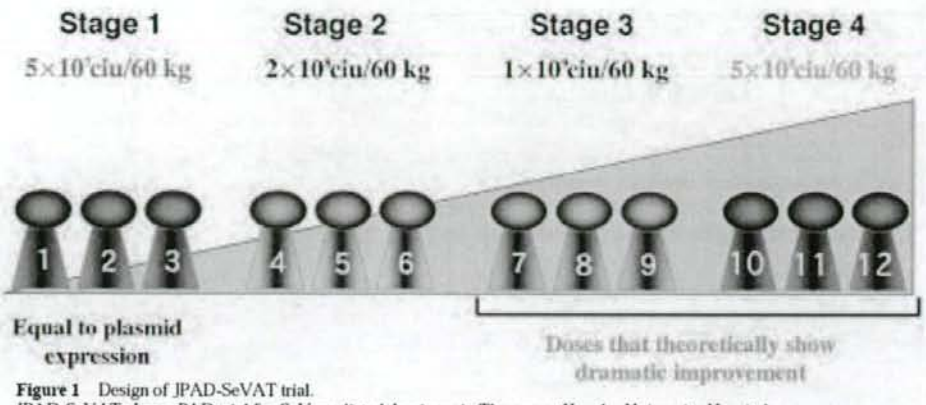


Figure 1　Design of JPAD-SeVAT trial.
JPAD-SeVAT: Japan PAD trial for SeV-mediated Angiogenic Therapy at Kyushu University Hospital

れ，高用量ではrSeV/dFの最大のパフォーマンスが発揮できると期待される。将来的に製剤開発を前提にしていることから，本臨床研究は外部contract research organization（CRO）のデータマネージメントによる新good clinical practice（GCP）準拠試験として実施されており，第三者委員会による厳密な適応決定を実施していることから，複数回の血行再建術の結果，他に治療法がない症例が選択される傾向にある。被験者の正式なリクルートは2006年4月より開始されている。

(3)症例提示

CROによりデータ仮固定が終了した第1例目の症例を提示する（**Fig. 2A**，1カ月目まで）。

症例（登録番号102）は59歳男性で，右のCLIであり安静時疼痛が存在するが，虚血性潰瘍なし。3度のバイパス手術が実施されているが，いずれも閉塞しており，遺伝子治療の適応評価のため紹介となった。血管造影上，右総腸骨動脈高位より腸骨ー大腿動脈全長が閉塞し，右下肢の血流は腰動脈からの側副血行路により唯一開存する深大腿動脈を経由して下肢を栄養。右膝窩動脈も閉塞しており，側副血行路により右腓骨動脈の末梢1/2のみ開存が認められる（**Fig. 2B**）。血管造影上はさらなる血行再建も不可能ではないと考えられたが，4度目の閉塞の可能性が高く，バイパス閉塞時には下肢切断に至る可能性も高いため，遺伝子治療の適応とされた。2006年7月4日に投与が実施された（**Fig. 2C**）。

投与後の経過は順調であり，重篤な有害事象の発生は認めていない。データ仮固定が終了している1カ月後のデータでは，最大歩行距離は約50%改善し，血管造影上，新たに描出される血管を認める（**Fig. 3**）など，最低用量にも関わらず10種の評価項目中4項目で改善が得られている。

ただし現時点ではまだ症例がわずかであり，観察期間も短いため，特に安全性に留意しつつ慎重かつ速やかに臨床研究を進めていく計画である。

おわりに

初期にセンセーショナルに喧伝された血管新生療法は，臨床評価を受けることにより，その問題点も明らかになってきた。しかしそれは，ようやく地に足を付けた評価が始まったということであり，今後次第にその効果と限界，そして明確な適応が明らかになってくるものと考えられる。

特にCLIは，血行再建不能例に関しては悪性腫瘍と同等な死亡率を示すことからも，米国では「un-met medical needs」として捉えられている。その意味からも血管新生療法への期待は大きい。蛋白治療，遺伝子治療だけでなく細胞移植療法も，この観点から厳密なプラセボ対照試験を行い，臨床現場での意味づけを明確にすることによって，初めてその真価が明らかになるであろう。

最近TASC IIが発表されたが，今後の改訂において，血管新生療法がエビデンスレベルAのfirst line治療とし

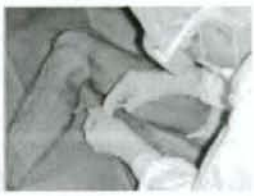
Case 102 59 yo, male

Diagnosis : Arteriosclerosis Obriterans of Rt. Lower extremity
- Fontaine III, Rutherford II-4

History : IC has been conscious since 1998.
He underwent bypass surgery 3 times; however, all grafts were obstructed, and followed as an outpatient with medication.
Recently, he feel rest pain, and therefore, admitted to Kyushu University Hospital to be involved as a possible candidate of SeVAT trial.

Past history :
1999 Aorto-rt. Femoro- popliteal (BK) bypass
2000 Rt. Femoro- popliteal (BK) bypass
2002 Rt. Femoro- peroneal bypass

Smoking : 30/day, 40 yrs (already secessed)
Hypertension



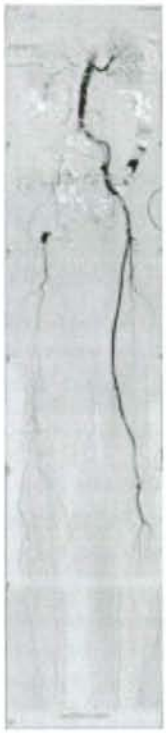


Figure 2 Case report of the first patient (Case 102) for SeVAT trail.
A: Summary of clinical history
B: Angiogram (pretreatment)
C: Panels indicating vector preparation (left), preparation for injection (middle), injection procedures (right).

A
B
C

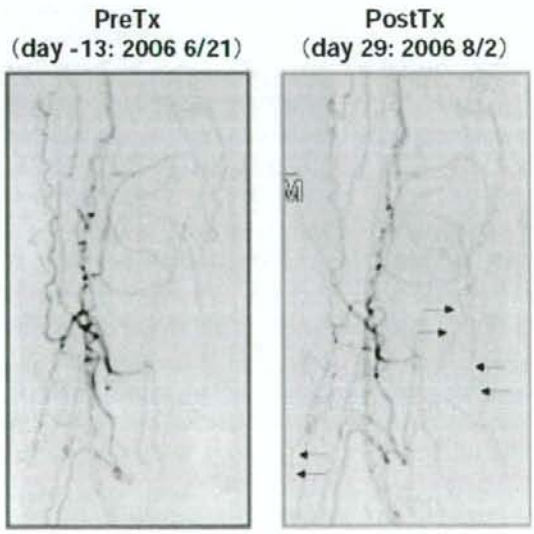


Figure 3 Angiograms pre- and post-treatment by SeV/dF-hFGF2. Arrows indicate newly visible blood vessels.

て認知される日が来るのは，そう遠くないかもしれない．

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Cytokine-induced Angiogenesis as a Therapeutic for PAD: Current Status and Future Directions

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

Therapeutic angiogenesis has been an attractive option to treat patients with intractable peripheral arterial diseases (PAD). A number of clinical studies have been performed, yet limited outcomes have been reported in phase II multicenter, placebo-controlled studies, even though phase I studies showed promising results. We summarize the current status of the clinical studies for cytokine-induced therapeutic angiogenesis, and discuss what needs to be addressed for success in this field.

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Statins restore ischemic limb blood flow in diabetic microangiopathy via eNOS/NO upregulation but not via PDGF-BB expression

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Fujii T, Onimaru M, Yonemitsu Y, Kuwano H, Sueishi K. Statins restore ischemic limb blood flow in diabetic microangiopathy via eNOS/NO upregulation but not via PDGF-BB expression. *Am J Physiol Heart Circ Physiol* 294: H2785–H2791, 2008. First published April 25, 2008; doi:10.1152/ajpheart.00149.2008.—3-Hydroxy-3-methyl-glutaryl CoA reductase inhibitors, or statins, have pleiotropic effects and can protect the vasculature in a manner independent of their lipid-lowering effect. The effectiveness of statins in reducing the risk of coronary events has been shown even in patients with diabetes, and their effects on diabetic complications have been reported. Using a model of severe hindlimb ischemia in streptozotocin-induced diabetic mice (STZ-DM), we investigated the effects and mechanisms of statin therapy in diabetic angiopathy in ischemic hindlimbs. As a result, STZ-DM mice frequently lost their hindlimbs after induced ischemia, whereas non-DM mice did not. Supplementation with statins significantly prevented autoamputation. We previously showed that diabetic vascular complications are caused by impaired expression of PDGF-BB, but statin therapy did not enhance PDGF-BB expression. Statins helped enhance endogenous endothelial nitric oxide (NO) synthase (eNOS) expression. Furthermore, the inhibition of NO synthesis by the administration of N^{ω}-nitro-L-arginine methyl ester impaired the ability of statins to prevent STZ-DM mouse limb autoamputation, indicating that the therapeutic effect of statins in hindlimb ischemia in STZ-DM mice occurs via the eNOS/NO pathway. A combination therapy of statins and PDGF-BB gene supplementation was more effective for diabetic angiopathy than either therapy alone. In conclusion, these findings indicate that statin therapy might be useful for preventing intractable diabetic foot disease in patients with diabetic angiopathy.

endothelial nitric oxide synthase; nitric oxide; platelet-derived growth factor-BB; diabetes mellitus

DIABETES MELLITUS (DM) is characterized by a chronic state of hyperglycemia and is increasing to epidemic proportions throughout the world. The morbidity and mortality associated with diabetes is primarily due to macro- and microangiopathy occurring in multiple organs (2, 20). The diabetic foot is an intractable disease categorized by DM-related vascular complications, and patients with it have a much higher risk of gangrene and the need for consequent amputation of the lower extremities (20). Collateral vessel development is insufficient to compensate with the reduced blood flow through occluded arteries in patients with peripheral vascular disease, especially DM (10). Furthermore, surgical and catheter interventions are usually difficult to treat limb ischemia of DM patients with DM, because vascular diseases are located in small vessels.

3-Hydroxy-3-methyl-glutaryl CoA reductase inhibitors or statins are potent inhibitors of cholesterol biosynthesis that have become more widely used in greater numbers of patients with hypercholesterolemia (13, 31). Furthermore, recent studies suggest that statins have pleiotropic effects in a manner independent of their lipid-lowering effect and can protect the vasculature (8, 27, 29). Several trials have demonstrated the beneficial effects of statins in lowering cardiovascular-related morbidity and mortality in patients with coronary artery disease. The effectiveness of statins in reducing the risk of coronary events has been established in patients with and without diabetes, and it has been suggested that patients with diabetes benefit more than patients without in both primary and secondary prevention (6).

Recent studies have also revealed the modulatory effects of statins in diabetic microangiopathy (7). The effectiveness of statins appears to involve restoring or improving endothelial function through the attenuation of high glucose-induced or diabetes-induced oxidative stress, thereby increasing the bioavailability of nitric oxide (NO) or inhibiting inflammatory responses (7).

On the other hand, using a model of severe hindlimb ischemia in streptozotocin-induced diabetic mice (STZ-DM), we previously showed that the diabetic foot is a disease involving the disturbance of PDGF-BB expression but not of the disturbance of the responses of angiogenic factors (32). Screening of angiogenesis-related factors revealed that the expression of PDGF-BB was impaired in STZ-DM mice at baseline as well as over a time course after limb ischemia. Increased expression of PDGF-BB prevented autoamputation in the STZ-DM mice (32).

In this study, we investigated the effect of and the mechanism underlying statin therapy in hindlimb ischemia under chronic hyperglycemia using STZ-DM. We here demonstrate that statins show significant therapeutic effects in hindlimb ischemia in STZ-DM mice via the endothelial NO synthase (eNOS)/NO pathway but not via PDGF-BB expression. Our results suggest that statin therapy would be useful for preventing intractable diabetic foot disease in patients with diabetic angiopathy.

MATERIALS AND METHODS

Cells and reagents. Human umbilical vascular endothelial cells (HUVECs) were purchased from Kurabo, Tokyo, Japan. Two intracellular signal inhibitors were used as previously described (11, 12,

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23, 32, 33): bis-I (a PKC inhibitor) and its inactive control, bis-V (100 mmol/l, Sigma-Aldrich, Tokyo, Japan).

Animals. Male C57BL/6J (7 wk old) were purchased from KBT Oriental (Charles River Grade, Tosu, Saga, Japan). 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 Japanese government Law No. 105 and Notification No. 6. Experimental diabetes was induced in mice by daily intravenous injection of STZ in citrate buffer (1.5 mg/body) for 5 days (*days −5* to *0*) for the Type 1 diabetic model. As for Type 2 diabetic models, 10-wk-old male *ob/ob* (C57BL/6J-*Lep^{ob}/Lep^{ob}*) mice (4) and normal control homozygous (+/+) mice (KBT Oriental) were also used to confirm eNOS and PDGF-BB expression. For statin therapy, pravastatin and pitavastatin (0.2 mg/g body wt; injected volume, 0.02 ml/g body wt) were administered by intraperitoneal injection during *days 14* to *28*. To inhibit NO synthesis, N^{ω}-nitro-L-arginine methyl ester (1 mg/ml in drinking water; Sigma) was orally administered during *days 14* to *28*.

Murine severe hindlimb ischemia. Details of the surgical treatment and evaluation of limb prognosis have been described previously (11, 12, 18, 23, 32, 33); 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.

Gene transfer vectors. The plasmid-based gene of human PDGF-BB was prepared as previously described (32). Human full-length cDNA of PDGF-B (GenBank: No. BC029822) was amplified by PCR using specific primers (forward: 5′-AAGGTACCATGAATCGCTGCTGG-GCGCTC-3′ and reverse: 5′-TTCTCGAGCTAGGCTCCAAGGGTC-TCCTTC-3′) and subcloned into a TA cloning vector (Invitrogen, San Diego, CA). The whole sequence was then determined using the CEQ 2000 Sequence Detection System (Beckman Coulter, Fullerton, CA). The amplicon was transferred to the *KpnI-XhoI* sites of the mammalian expression vector pCEP4 (Invitrogen).

ELISA. The protein contents in the limb muscles and culture medium were determined using Quantikine Immunoassay systems for human eNOS (available for both humans and mice; R&D Systems, Minneapolis, MN), murine PDGF-BB (R&D Systems) and human PDGF-BB (specific for humans; R&D Systems) according to the manufacturer's instructions, as previously described (11, 12, 18, 23, 32, 33).

Biochemical analysis. Serum levels of LDL cholesterol and advanced glycation end product (AGE) were measured in serum samples when all animals were euthanized. Concentrations of LDL cholesterol and AGE were determined by an automatic analyzer (Research Testing Department, SRL, Hachiohji-shi, Tokyo, Japan).

Laser-Doppler perfusion images. Measurements of the ischemic (left)/normal (right) limb blood flow ratio were made using a laser-Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, UK) as previously described (11, 12, 18, 23, 32, 33). To minimize data variability due to ambient light and temperature, the LDPI index was expressed as the ratio of the left (ischemic) to the right (nonischemic) limb blood flow.

Statistical analysis. All data except for those of limb survival were expressed as means ± SE and were analyzed by one-way ANOVA with Fisher adjustment. For the survival analysis, the survival rate expressed by the limb salvage score was analyzed using Kaplan-Meyer's method (11, 18, 23, 32, 33). The statistical significance of the survival experiments was determined using the log-rank test. $P < 0.05$ was considered statistically significant in all analyses.

RESULTS

Effects of statins on serum glucose, LDL cholesterol, and AGE in DM mice. Serum levels of glucose, LDL cholesterol (LDL), and AGE were measured in serum samples of a relevant model for Type 1 diabetes, STZ-DM mice, and a well-accepted model of Type 2 diabetes, namely *ob/ob* mice that are leptin-deficient C57BL/6 (4). Serum concentrations of glucose, LDL, and AGE were increased in the DM mice. Statin treatment did not affect the increases in glucose and AGE, whereas the increased LDL concentration was relatively decreased (Table 1). In the following experiments, STZ-DM mice were used after we confirmed a significant upregulation of the serum glucose.

Therapeutic effects of statins in hindlimb ischemia in STZ-DM mice. The STZ-DM mice frequently lost their hindlimbs at various levels after surgically induced severe limb ischemia, although the non-DM mice did not. Quantitative analysis of the degree of autoamputation using the limb salvage score (11, 18, 23, 32, 33) demonstrated impaired limb survival in the STZ-DM mice. Administration of statins (pravastatin and pitavastatin) by intraperitoneal injection daily on *days 14* to *38* resulted in the significant prevention of autoamputation in the STZ-DM mice (Fig. 1), indicating that the statin therapy effectively restored tolerance against hindlimb ischemia in STZ-DM mice.

eNOS/NO is important for the therapeutic effects of statins, but endogenous PDGF-BB expression is not. We previously showed that the diabetic foot is a disease involving the disturbance of PDGF-BB expression (32). To explain the mechanism underlying the therapeutic effect of statins on the tolerance of limb ischemia in STZ-DM mice, we measured the expression of murine (m)PDGF-BB protein in thigh muscles of STZ-DM. Downregulated expression of PDGF-BB was evident in both the STZ-DM, and statin therapy did not influence the impaired expression of PDGF-BB (Fig. 2*A*). In addition, the expression of mPDGF-BB protein was not influenced by statin therapy

Table 1. *Serum LDL cholesterol, AGE, and glucose concentration are significantly increased in C57BL/6J strain-based 10-week-old Type 1 (STZ-DM) and Type 2 (Lep^{ob}/Lep^{ob}; ob/ob) mice*

		STZ-DM (Type1)			*ob/ob* (Type2)		
	Non-DM	Nontreatment	PRA	PTA	Nontreatment	PRA	PTA
n	6	6	6	6	6	6	6
Glucose, mg/dl	113±4.2	621±6.6*	622±17.9*	598±14.2*	213±4.8*	205±6.6*	198±9.3*
LDL cholesterol, mg/dl	11.0±0.47	31.5±2.77*	25.2±2.59*	24.9±3.87*	24.4±0.20*	17.3±0.65*	16.5±0.96*
AGE, mU/ml	1.0±0.12	1.6±0.17*	1.7±0.23*	1.8±0.14*	1.6±0.15*	1.6±0.34*	1.8±0.27*

Data are means ± SE; *n*, animals/group. These increased glucose and advanced glycation end product (AGE) levels were not influenced by treatment with statins, whereas the increased LDL concentration was relatively decreased by statins administration. STZ-DM, streptozotocin-induced diabetes mice; PRA, pravastatin, hydrophilic statin; PTA, pitavastatin, lipophilic statin; *$P < 0.01$ vs. non-DM.

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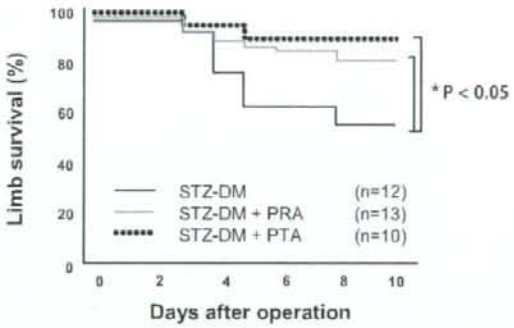

Fig. 1. Limb prognosis curve according to the limb salvage score in streptozotocin-induced diabetes (STZ-DM) C57/BL6 mice. These curves were obtained using Kaplan-Meyer's method, and data were analyzed using the log-rank test. Administration of statins, pravastatin (PRA, hydrophilic statin) or pitavastatin (PTA, lipophilic statin), by daily intraperitoneal injection on *days 14* to *38*, resulted in the significant prevention of autoamputation in the STZ-DM mice (n = animals/group).

even in *ob/ob* mice, a model of Type 2 diabetes. To confirm the modulatory effect of statins on PDGF-BB expression, we examined the induction of PDGF-BB via statins using cultured HUVECs. As shown in Fig. 2*B*, the statins did not stimulate the production or secretion of PDGF-BB in the culture medium of HUVECs. These results suggest that the effectiveness of statins in hindlimb ischemia in DM mice may be independent of the endogenous expression of PDGF-BB.

We previously reported that PDGF-B gene expression was downregulated in the limb muscles of STZ-DM mice among the factors tested (VEGF-A and -C, hepatocyte growth factor, FGF-2, PDGF-A and -B, and angiopoietin-1 and -2), as well as their receptors (tie-2, flk-1, fibroblast growth factor receptor 1, flt-4, and platelet-derived growth factor receptor-A and -B) (32). Recent important studies indicated that statins activated and upregulated the expression of endogenous eNOS, which has angiogenic potency (1, 21). We next investigated the expression of eNOS and whether or not eNOS/NO is important for the ability of statins to prevent the impairment of limb survival. Statin therapy significantly restored or increased the expression of eNOS in thigh muscles of both STZ-DM and *ob/ob* DM mice (Fig. 3*A*). As in previous studies (17, 19, 22, 24, 34), statin stimuli induced the upregulation of endogenous eNOS in the lysate of the cultured cells (HUVECs). Furthermore, the daily oral administration of N^{ω}-nitro-L-arginine methyl ester, an inhibitor of NO synthesis, prevented the autoamputation rate for ischemic limb amputation of STZ-DM mice (Fig. 4), indicating that the therapeutic effect of statins on

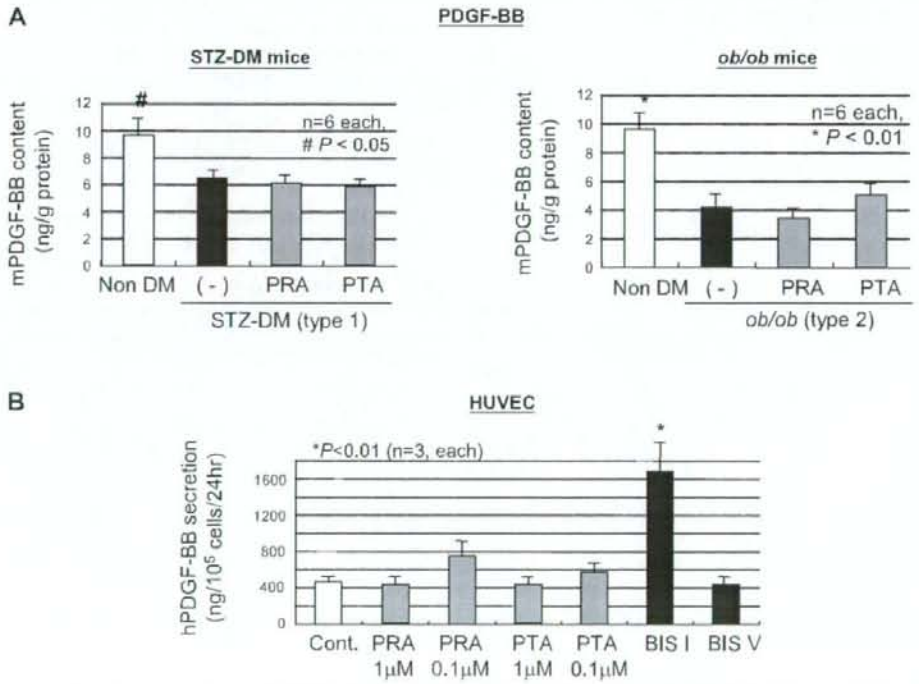

Fig. 2. *A*: comparison of protein expression of murine PDGF-BB in the thigh muscles of non-DM or DM mice assessed by ELISA. Impaired expression of murine PDGF-BB (mPDGF-BB) protein in C57BL/6J strain-based 10-wk-old Type 1 (STZ-DM; *left*) and Type 2 (*Lep*^{ob}/*Lep*^{ob}; *ob/ob*; *right*) mice was not restored by statin treatment (n = 6 animals/group). *P < 0.01; #P < 0.05 vs. non-DM. *B*: statins did not contribute to the PDGF-BB expression in human umbilical vascular endothelial cells (HUVECs). Twenty-four hours after preincubation with 5% FBS, HUVECs were stimulated with statins (0.1 or 1 mM, respectively). Twenty-four hours later, the culture medium was subjected to ELISA. BIS-I, PKC inhibitor; BIS-V, PKC inhibitor control compound; Cont, control; hPDGF-BB, human PDGF-BB. *P < 0.01.

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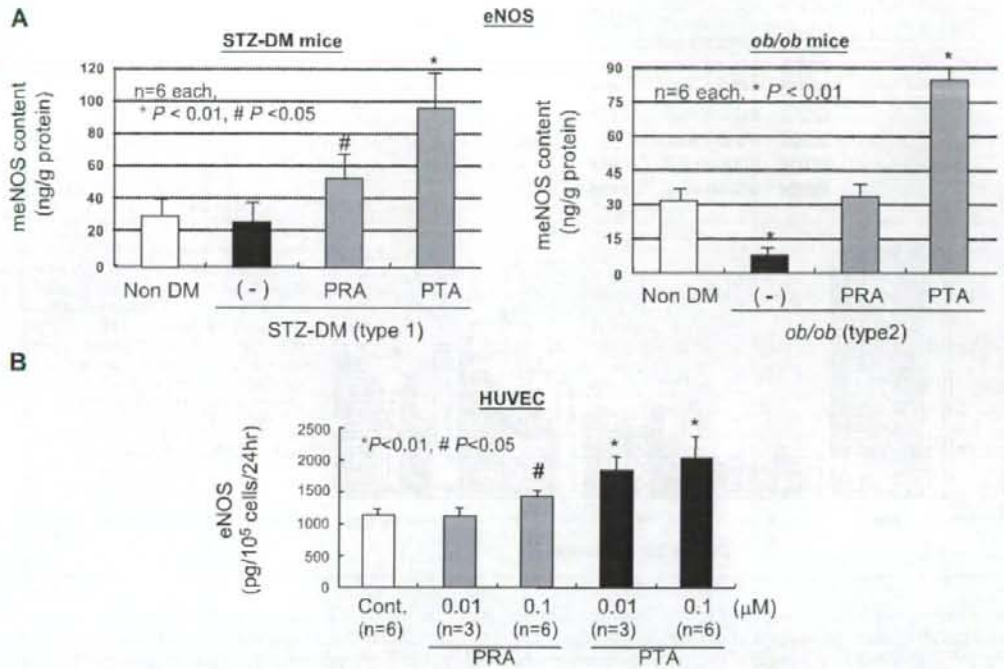

Fig. 3. *A*: endothelial nitric oxide (NO) synthase (eNOS) protein expression in the thigh muscles of non-DM or DM mice assessed by ELISA. Statin therapy significantly restored or increased the expression of eNOS in both STZ-DM (*right*) and *ob/ob* (*left*) DM mice (n = 6 animals/group). $*P < 0.01$; $\#P < 0.05$ vs. non-DM. *B*: eNOS expression was increased by statin stimulation in HUVECs. Twenty-four hours after preincubation with 5% FBS, HUVECs were stimulated with statins (0.01 or 0.1 mM, respectively). Twenty-four hours later, the lysate of the cultured HUVECs was subjected to ELISA. $*P < 0.01$; $\#P < 0.05$.

tolerance against limb ischemia in the diabetic foot might occur at least partly via the eNOS/NO pathway.

Combination therapy of statins and PDGF-BB gene supplementation for diabetic angiopathy. These results may suggest that the therapeutic effect of statins occurs via eNOS/NO but not via PDGF-BB expression in diabetic vascular dysfunction. As a final assessment, to confirm whether or not statin therapy is independent of the endogenous expression of PDGF-BB, we administered a combination therapy of statins and PDGF-BB gene supplementation and found that this combination is more effective for diabetic angiopathy than either treatment alone. A supplementation study on the plasmid-based intramuscular gene transfer of human PDGF-B (pCEP4-hPDGFB) was previously described (32). In the present study we assessed the recovery of blood flow evaluated by LDPI, because the PDGF-B gene transfer resulted in the complete prevention of autoamputation in STZ-DM mice. As shown in Fig. 5, both statin therapy and the preinjection of pCEP4-hPDGFB significantly improved the disturbed blood perfusion in STZ-DM mice; furthermore, the combination therapy had an effect on

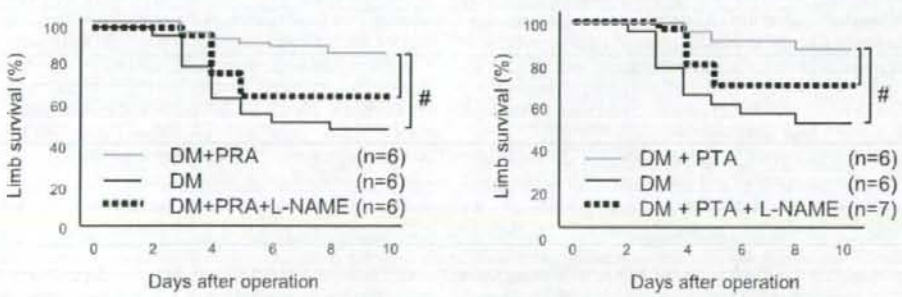

Fig. 4. Limb prognosis curve according to the limb salvage score after the daily oral administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthesis in STZ-DM mice. These curves were obtained using Kaplan-Meyer's method, and the data were analyzed using the log-rank test. Administration of L-NAME prevented the therapeutic effect for ischemic limb autoamputation in STZ-DM mice by treatment with either statin: PRA (*left*) and PTA (*right*). $\#P <$ 0.05 vs. others.

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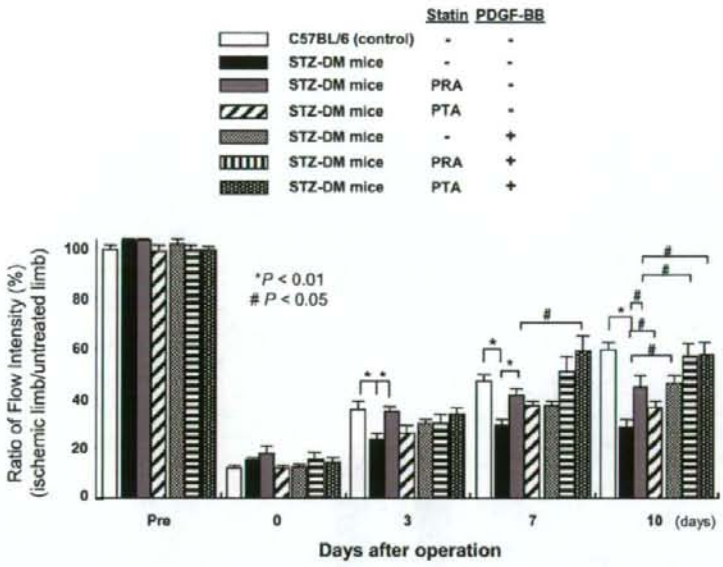

Fig. 5. Effects of statin therapy and/or plasmid-based PDGF-B gene transfer on recovery of blood flow in ischemic limbs of STZ-DM mice. Administration of PRA or PTA by daily intraperitoneal injection on *days 14* to *38* significantly prevented autoamputation in the STZ-DM mice. pCEP4-hPDGFB and control plasmid (pCEP4-empty) were injected into the thigh muscle 2 days before induced limb ischemia. After assessment of blood flow before or after ischemic operation, recovery of blood flow was assessed by laser-Doppler perfusion imaging at each time point. Data were standardized by data related to the untreated right limb and expressed as the ratio of flow intensity (in %). $*P < 0.01$; $\#P < 0.05$.

diabetic angiopathy that was superior to that by either therapy alone, indicating that the statin therapy might work independently from PDGF-BB expression and that the combination therapy was sufficient to restore the tolerance against hindlimb ischemia in STZ-DM mice.

DISCUSSION

The key observations made in this study are summarized as follows: *1*) the administration of statin was effective for preventing autoamputation of the ischemic limb of STZ-DM mice; *2*) statin therapy could not restore the disturbed expression of PDGF-BB in DM mice, suggesting that the effect of statins is independent of PDGF-BB expression; *3*) statins could restore or increase eNOS expression in the limb of DM mice; *4*) supplementation with the NO inhibitor inhibited the ability of statins to prevent autoamputation, indicating that the therapeutic effect of statins in hindlimb ischemia in STZ-DM mice might occur via the eNOS/NO pathway; and *5*) the combination therapy of statins and PDGF-BB supplementation was more effective than either therapy alone. These findings suggest that the statin therapy is effective against the formation of hyperglycemia-related vascular complications, and they imply that statin therapy improves limb survival and perfusion via a mechanism involving eNOS/NO pathway in Type 1 DM, and probably in Type 2 DM microangiopathy.

Over the last several years, it has been already demonstrated that statin treatment improved and promoted angiogenesis in a hindlimb ischemia of non-DM mouse model (16, 26). In the case of DM mouse model, we previously demonstrated that the disturbed tolerance against severe limb ischemia under hyperglycemia was due to the disturbance of PDGF-BB expression and not to the angiogenic responses and that the supplementation of PDGF-B gene expression was sufficient to prevent autoamputation due to limb ischemia in STZ-DM mice (32). The reduction of PDGF-BB expression, which was not dependent on the level of hyperglycemia (32), was critical to inducing functional and morphological vascular change, which is the dissociation of pericytes from the capillaries in muscles of STZ-DM mice, indicating that impaired PDGF-BB expression disturbed vessel maturation. In the present study, statin administration did not influence PDGF-BB expression, which is concerned with vessel maturation. We postulated that the mechanism underlying the therapeutic effect of statins might be due to improved endothelial function because of the lower PDGF-BB expression. Many diabetic vascular complications involve endothelial cell dysfunction characterized by reduced NO-dependent phenomena, including vasodilation and protection against leucocyte-endothelial interactions. Several studies have shown that hyperglycemia impairs NO production and have demonstrated impaired endothelium-dependent vasorelaxation in diabetic humans and in experimental diabetic animals (5, 14, 15, 30). A hallmark of endothelial dysfunction is reduced bioavailability of NO, which could be caused by reduced expression of eNOS, impairment of eNOS activation, or increased inactivation of NO by oxidative stress. Upregulation of the activity or expression of eNOS is considered to be effective in diabetic angiopathy, and statins can increase eNOS expression and activation in addition to their lipid-lowering effect (8, 17, 19, 22, 24, 27, 34). Furthermore, statins have also been reported to promote angiogenesis via eNOS (16, 26). In our current study, statin therapy was effective for preventing autoamputation of the ischemic limb under hyperglycemia at least partly via eNOS, and those previous reports essentially support our findings that statins could restore or increase the eNOS expression in the ischemic limb of DM mice.

In addition to eNOS/NO dependent pathway, it has been revealed that statins have additional effects of growing interest that include the ability to recruit endothelial progenitor cells (3, 8, 9) or activate the protein kinase Akt, which leads to angiogenesis and prevents apoptosis in endothelial cells (9,16). As

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shown Fig. 4, our current findings indicate that the therapeutic effects of statins on tolerance against limb ischemia in the diabetic foot might occur at least partly via the eNOS/NO pathway. Therefore, there is a possibility that the clinical benefits of statin therapy might be caused partly by these NO-independent pathways; however, further extensive studies should be carried out to determine this hypothesis.

Although statins have the favorable effect of restoring blood flow in the ischemic limb of DM mice (16, 26), they prevented autoamputation of the ischemic limb of STZ-DM mice to some extent, but not completely. In turn, this result also suggests that the therapeutic effect of statins is due to improved endothelial function and is independent of the restoration of the impairment PDGF-BB expression, which was sufficient to prevent autoamputation due to limb ischemia in STZ-DM mice. Furthermore, the results of the combination therapy of statins and PDGF-BB supplementation confirmed that the favorable effect of statin therapy on the diabetic foot occurs by mechanisms other than the upregulation of PDGF-BB expression.

Some clinical trials have suggested that the vasculature effects of hydrophilic statins are similar in extent to those of lipophilic statins, but it is still controversial whether lipophilic or hydrophilic statins have more clinical benefits (23a, 25). An important advance of our current study was to determine that there are clearly differences between lipophilic and hydrophilic statins, although both types are beneficial to ischemic limbs under hyperglycemia. Limb survival was similar in mice receiving lipophilic and hydrophilic statins; however, eNOS expression was more upregulated by the administration of lipophilic statins than by that of hydrophilic statins (Fig. 3). Therefore, the present study indicates that clinically lipophilic statins may be more useful for ischemic diabetic foot.

In conclusion, we demonstrated that statin therapy restored the disturbed blood flow of severe limb ischemia under hyperglycemia by increasing eNOS/NO expression and not by increasing PDGF-BB expression and that the inhibition of NO reduced the ability of statins to prevent autoamputation due to limb ischemia in STZ-DM mice. Therefore, statin therapy is expected to be useful for preventing intractable diabetic foot disease in patients with diabetic angiopathy via eNOS/NO.

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重症虚血肢に対する血管新生治療の役割

Role of therapeutic angiogenesis for critical limb ischemia : current status and future directions

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Summary

近年，末梢動脈閉塞性疾患に対する蛋白や遺伝子を用いた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，初期において有望な成績が示唆されていたにも関わらず，多施設二重盲検試験の多くが失敗に終わっている。一方で，骨髄や末梢血単核球を用いた細胞治療の研究は，その手軽さからわが国を中心に多くの施設で進められているものの，その臨床的評価は定まっていない。ところが，不適切な適応評価により本来標準的治療で救肢できるにも関わらず，血管新生療法を先行した結果肢切断に至ったのではないか，と考えられる症例も目立ってきた。

本稿では，特に血管新生治療の臨床試験の問題点を明確にし，われわれが進めている臨床研究の位置付けを概括する。

Key words

●重症虚血肢
●血管新生療法
●センダイウイルスベクター
●FGF-2

1 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Buerger病に代表され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(Fontaine分類Ⅰ度)を，症状が進行するにつれ間歇性跛行(intermittent claudication；IC)(Fontaine分類Ⅱ度)，さらには安静時疼痛(Fontaine分類Ⅲ度)，潰瘍形成(Fontaine分類Ⅳ度)を呈するようになる。ICに対しては，生活の質(quality of life；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)や運動療法など，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ICが進行し，患者が歩行距離の短縮により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。一方で，進行症例(重症虚血肢(critical limb ischemia；CLI))(Fontaine分類Ⅲおよび Ⅳ度)ではリスクファクターの除去や現行の薬物療法，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を著しく低下させる