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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. Tanii M, Yonemitsu Y, Shikada Y, Kohno R, Fujii T, Onimaru M, Okano S, Hasegawa M, Onohara T, Maehara Y, Sueishi K.
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Diabetic Microangiopathy in Ischemic Limb Is a Disease of Disturbance of the Platelet-Derived Growth Factor-BB/Protein Kinase C Axis but Not of Impaired Expression of Angiogenic Factors

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Abstract—Diabetic foot is caused by microangiopathy and is suggested to be a result of impaired angiogenesis. Using a severe hindlimb ischemia model of streptozotocin-induced diabetic mice (STZ-DM), we show that diabetic foot is a disease solely of the disturbance of platelet-derived growth factor B-chain homodimer (PDGF-BB) expression but not responses of angiogenic factors. STZ-DM mice frequently lost their hindlimbs after induced ischemia, whereas non-DM mice did not. Screening of angiogenesis-related factors revealed that only the expression of PDGF-BB was impaired in the STZ-DM mice on baseline, as well as over a time course after limb ischemia. Supplementation of the PDGF-B gene resulted in the prevention of autoamputation, and, furthermore, a protein kinase C (PKC) inhibitor restored the PDGF-BB expression and also resulted in complete rescue of the limbs of the STZ-DM mice. Inhibition of overproduction of advanced-glycation end product resulted in dephosphorylation of PKC-α and restored expression of PDGF-BB irrespective of blood sugar and HbA1c, indicating that advanced-glycation end product is an essential regulator for PKC/PDGF-BB in diabetic state. These findings are clear evidence indicating that diabetic vascular complications are caused by impairment of the PKC/PDGF-B axis, but not by the impaired expression of angiogenic factors, and possibly imply the molecular target of diabetic foot. (*Circ Res.* 2006;98:55-62.)

Key Words: diabetic microangiopathy ■ PDGF-BB ■ PKC ■ advanced-glycation end product ■ pericyte

Critical limb ischemia is often caused by severe stenosis of feeder arteries or occlusion of a remaining below-knee artery. Bypass surgery and transluminal angioplasty are the efficient treatments of critical limb ischemia, and the focus of these interventions in patients with critical limb ischemia is to either restore arterial blood flow to at least 1 tibial vessel or to amputate the limb when tissue loss or pain becomes intractable.

Diabetes mellitus (DM), affecting 135 million people worldwide, is characterized by a chronic state of hyperglycemia. DM accompanies macro- and microangiopathy in multiple organs and is 1 of the major causes of morbidity and mortality of patients with the disease.[1] Diabetic foot is an intractable disease categorized by DM-related vascular complications, and patients with it have a much higher risk of gangrene and amputation of the lower extremities.[1] Collateral vessel development is insufficient to support the loss of blood flow through occluded arteries in patients with peripheral vascular disease, and the problem is exacerbated in DM patients.[2] Furthermore, surgical and catheter interventions are usually difficult to treat limb ischemia of DM patients because vascular diseases are located at small vessels.

Therapeutic angiogenesis has been expected as a novel approach to treat patients with limb ischemia, including diabetic foot, without indication for bypass surgery or angioplasty. Current clinical results of therapeutic angiogenesis, however, have shown relatively limited outcome[3–5]; therefore, further studies of the basic mechanisms of therapeutic angiogenesis should be performed.

With this point of view, we have performed some important studies assessing the levels and genes of angiogenic factors to be delivered to show the optimized therapeutic effect for murine and rabbit critical limb ischemia models using highly efficient gene transfer vector, namely recombinant Sendai virus.[6–8] These studies revealed the following: (1) more than 2-fold overexpression of vascular endothelial growth factor (VEGF) compared with its baseline level accelerated limb loss, whereas, in contrast, basic fibroblast

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From the Division of Pathophysiological and Experimental Pathology (M.T., Y.Y., T.F., Y.S., R.-i.K., M.O., S.O., K.S.), Department of Pathology, and Department of Surgery and Science (M.T., Y.S., T.O., Y.M.), Graduate School of Medical Sciences, Kyushu University, Fukuoka; and DNAVEC Corporation (M.I., M.H.), Tsukuba, Ibaraki, Japan.

Correspondence to Yoshikazu Yonemitsu, MD, PhD, Division of Pathophysiological and Experimental Pathology, Department of Pathology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail yonemitu@patho11.med.kyushu-u.ac.jp

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growth factor (bFGF/FGF-2) gene transfer constantly showed therapeutic effect inducing morphologically matured capillary vessels[9]; and (2) therapeutic effect of FGF-2 gene transfer depended on the harmonized induction of multiple endogenous angiogenic factors, including VEGF[9,10] as well as hepatocyte growth factor (HGF),[11] in pericytes and stromal cells via PDGF-AA/PDGF α-receptor (PDGFRα)/p70S6 kinase pathway. These results indicate FGF-2 gene transfer as a therapy via multiple angiogenic factors in vivo; however, no information is now available indicating whether or not these cascade-like system for therapeutic angiogenesis function under chronic diabetic state.

DM-related vascular complications have been widely explained by disorganized expression of angiogenic factors including VEGF. For instance, it has been demonstrated that advanced-glycation end products (AGEs) induced by chronic hyperglycemia regulates the expression of VEGF in retinal epithelial cells, suggesting the possible link between chronic DM state and hypervascularization of diabetic retinopathy.[12] As reviewed in a recent article,[13] however, DM is a paradoxical disease associated with hypervascularization in the retina and, inversely, with impaired collateral development in the ischemic limbs and hearts.

As such, because less information is available on the molecular mechanisms of the diabetic foot, particularly under limb ischemia, we investigated the essential factors related to angiogenic responses under chronic hyperglycemia using streptozotocin-induced diabetic mice (STZ-DM).

Materials and Methods

Reagents and Antibodies

The following intracellular signal inhibitors were dissolved at each concentration, which was previously demonstrated to sufficiently work in vivo[10,11,14–16]: protein kinase C (PKC) inhibitor bisindolylmaleimide (bis)-I and its inactive control bis-V (100 mmol/L, Sigma-Aldrich, Tokyo, Japan); p70S6K inhibitor rapamycin (1 mmol/L; Sigma); and mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor U0126 (10 mmol/L; Promega, Madison, Wis). One hundred microliters of these inhibitors were intraperitoneally administered daily from day −1 to 10. The antibodies used to detect PKCs in the thigh muscles by Western blotting were as follows: anti–phospho-PKCs antibody (rabbit polyclonal, no. 9371, Sigma), which recognizes PKC-α, -βI, -βII; and anti–nonphospho-PKC-α antibody (rabbit polyclonal, P4434; Sigma).

Gene Transfer Vectors

Recombinant SeVs (SeV–FGF-2 and SeV-luciferase) were prepared as previously described.[5–8] Human full-length cDNA of PDGF-B (GenBank accession no. BC029822) was amplified by PCR using specific primers (forward: 5′-AAGGTACCATGAATCGCTGCTGGGCGCTC-3′; reverse: 5′-TTCTCGAGCTAGGCTCCAAGGGTCTCCTTC-3′) and subcloned into the TA cloning vector (Invitrogen, San Diego, Calif). The entire sequence was then determined using the CEQ 2000 Sequence Detection System (Beckman Coulter, Fullerton, Calif). The amplicon was transferred into the *Kpn*I–*Xho*I sites of the mammalian expression vector pCEP4 (Invitrogen).

Animals

Male C57BL/6J (7 weeks old) were purchased from KBT Oriental Co Ltd (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 the law (No. 105) and notification (No. 6) from the Japanese government. The details of the surgical treatment and evaluation of limb prognosis were described previously.[9–11] Experimental diabetes was induced in mice by daily intravenous injection of streptozotocin (STZ) in citrate buffer (1.5 mg/body) for 5 days (day −5 to 0) for type 1 diabetic model. As models for type 2 diabetes, 10-week-old male *ob/ob* (C57BL/6J-*Lep*^{ob}/*Lep*^{ob}) mice[17] and normal control homozygous (+/+) mice (KBT Oriental Co, Ltd) were also used for confirming platelet-derived growth factor B-chain homodimer B (PDGF-BB) expression. For AGE inhibition, aminoguanidine hydrogen carbonate (AMG) (1 g/L in drinking water; Fluka Chemie GmbH, Buchs, Switzerland) was orally administered during day 0 to 28.

Enzyme-Linked Immunosorbent Assay

The protein contents in the limb muscles and culture medium were determined using Quantikine Immunoassay systems for human FGF-2 (available for both humans and mice; R&D Systems Inc, Minneapolis, Minn), murine VEGF-A (recognizes both 164 and 120 amino acid residue forms; R&D Systems), human PDGF-BB (specific for humans; R&D Systems), rat HGF (available as murine HGF; Institute of Immunology Inc, Tokyo, Japan), and murine PDGF-BB (BioSource International Inc, Camarillo, Calif) according to the instructions of the manufacturer, as previously described.[9–11]

Real-Time PCR

The procedure was described previously.[10,11] The total RNA was extracted from the ischemic limb muscles followed by treatment with RNase-free DNase I. The RNA was then reverse-transcribed and amplified with the TaqMan EZ RT-PCR kit and a Sequence Detection System, model 7000 (PE Biosystems, Foster City, Calif). The nucleotide sequences of the PCR primers and TaqMan probes are listed in the supplementary Table in the online data supplement available at http://circres.ahajournals.org. The murine GAPDH was used as the internal standard. The target quantity was determined from the relative standard curves constructed with serial dilutions of the control total RNA (PE Biosystems), according to the instructions of the manufacturer.

Transmission Electron Microscopy

Each harvested thigh muscle was fixed with 3% glutaraldehyde. After postfixation with 2% osmium tetroxide, the tissues were dehydrated in a graded series of ethanol and embedded in Epon 812. The ultra-thin sections were cut, stained with uranyl acetate, and examined under a JEOL 1200 EX transmission electron microscope (Nippon Denshi Ltd, Tokyo, Japan) at 80 kV.

Laser Doppler Perfusion Images

Measurements of the ischemic (left)/normal (right) limb blood flow ratio were made using a laser Doppler perfusion images (LDPI) analyzer (Moor Instruments, Devon, UK), as previously described.[9,11] To minimize data variables caused by ambient light and temperature, the LDPI index was expressed as the ratio of the left (ischemic) to the right (nonischemic) limb blood flow.

Western Blotting

Each harvested thigh muscle was homogenized, the supernatant was separated on a 10% SDS-PAGE, and the proteins were transblotted. After blocking using 3.0% nonfat dried milk, the membrane was reacted with anti–phospho-PKCs antibody (rabbit polyclonal, no. 9371, Sigma), which recognizes PKC-α, -βI, -βII, or anti–nonphospho-PKC-α antibody (rabbit polyclonal, P4434, Sigma). Immunoreactivity for pPKC-α or PKC-α was visualized using the ECL Plus (Amersham Biosciences, Buckinghamshire, UK), and the expression level was determined by densitometry.

Statistical Analysis

All data except for limb survival were expressed as mean±SEM and were analyzed by 1-way ANOVA with Fisher's adjustment. For the survival analysis, the survival rate expressed by the limb salvage score was analyzed using the Kaplan–Meyer method.[9–11] The statistical significance of the survival experiments was determined using the log-rank test. $P<0.05$ was considered to be statistically significant in all analyses.

Results

Tolerance Against Induced Hindlimb Ischemia Is Impaired in STZ-DM Mice

C57BL/6 mice were induced with DM by receiving an intraperitoneal injection of STZ (1.5 mg/body) for 5 days. In the following experiments, all animals were used after confirming significant upregulation of both the free blood sugar (pre-STZ: 161.4±17.2 mg/dL; 4 weeks later: 585.0±55.9 mg/dL) and HbA1c (pre-STZ: 1.78±0.9%; 4 weeks later: 5.20±1.0%) at 4 weeks.

We first discovered that the STZ-DM mice frequently lost their hindlimbs at various levels after surgically induced severe limb ischemia, whereas the non-DM mice did not. Quantitative analysis of the degree of autoamputation using the limb salvage score[9–11] demonstrated impaired limb survival in the STZ-DM mice (Figure 1a).

FGF-2–Mediated Angiogenic Responses Are Not Impaired in STZ-DM Mice

Next, to investigate whether impairment of limb survival might be related to the expression of angiogenic factors, we examined the expression of typical angiogenic factors, namely FGF-2, VEGF, and HGF, following the administration of recombinant SeV-mediated murine FGF-2 gene transfer.[9–11] Similar to our previous findings,[9–11] a boost in FGF-2 resulted in the upregulation of endogenous VEGF and HGF, irrespective of the diabetic state (Figure 1b). Furthermore, the FGF-2 gene transfer prevented limb amputation in the STZ-DM mice by significantly restoring the blood flow (data not shown), suggesting that angiogenic responses were not seriously impaired in the STZ-DM mice.

PDGF-BB Is the Essential Factor That Determines the Tolerance Against Hindlimb Ischemia of STZ-DM Mice

To explain the causes of disturbed tolerance of limb ischemia in STZ-DM mice, we conducted an extensive assessment of the baseline gene expression of angiogenesis-related factors and their receptors. Quantitative analysis by real-time RT-PCR revealed that the PDGF-B gene expression was solely downregulated in the limb muscles of STZ-DM mice among the genes tested (VEGF-A and -C, HGF, FGF-2, PDGF-A and -B, angiopoietin-1 and -2) (Figure 2a), as well as their receptors (tie-2, flk-1, FGFR1, flt-4, PDGFRα, and -β) (data not shown). The disturbed expression of the PDGF-B gene in the STZ-DM mice was sustained after induced limb ischemia (Figure 2b), a finding that was not observed in the other genes tested (data not shown). These results possibly suggested that impaired expression of PDGF-BB might be a contributor for the diabetic vascular dysfunction, at least, in the case of STZ-DM mice, a relevant model for type 1 diabetes.

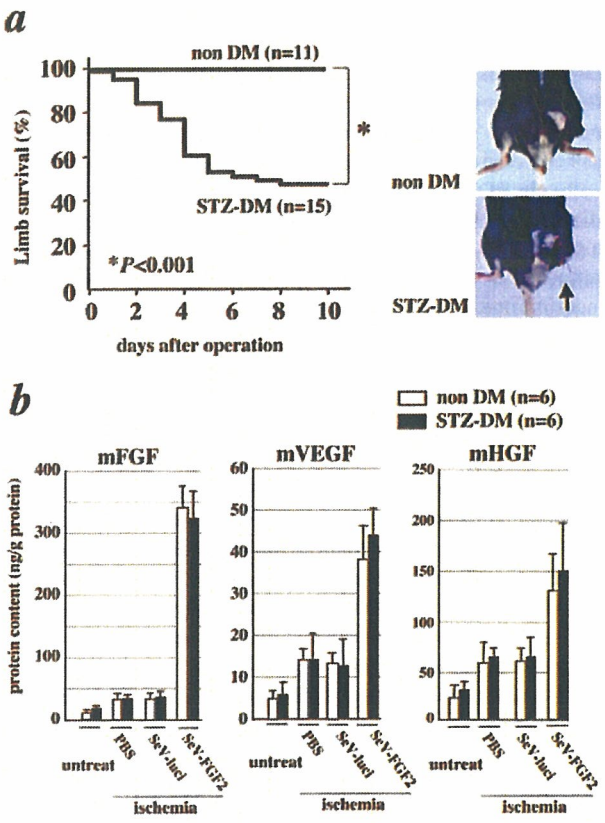


Figure 1. a. Limb prognosis curve according to the limb salvage score in non-DM or STZ-DM C57/BL6 mice. These curves were obtained using the Kaplan–Meyer method, and data were analyzed using the log rank test. The right panels are representative gross observations of the non-DM (top) and STZ-DM mice (bottom) 10 days after surgically induced ischemia. Note limb loss in the STZ-DM mice (arrow). b, Comparison of expression of typical angiogenic growth factors in the non-DM (open bar) or STZ-DM (closed bar) mice. Soon after surgery for ischemia, buffer (PBS), control vector (SeV-luciferase: 10^7 pfu) or SeV-expressing murine FGF-2 (SeV-FGF2: 10^7 pfu) was injected into the thigh muscle. Two days later, the thigh muscles were subjected to the ELISA for murine FGF-2, VEGF, and HGF. Each group contained 6 animals.

To confirm that the disturbed expression of PDGF-BB might be common in diabetic state, baseline expression mPDGF-BB protein in thigh muscles of a well-accepted model of type 2 diabetes, namely *ob/ob* mice, which are leptin-deficient C57BL6.[17] Downregulated expression of PDGF-BB was evident in both DM mice, STZ-DM and *ob/ob* (Figure 2c, right graph), whereas the elevation of serum blood sugar and HbA1c in *ob/ob* mice was rather milder than those seen in STZ-DM mice (Figure 2c, left). These results suggest that the disturbed expression of PDGF-BB may be common in both types of diabetic states, at least, of C57BL6 strain.

Next we examined the impact of impaired expression of PDGF-BB on the ultrastructure of vessels in thigh muscles of STZ-DM mice. Electron microscopic examination occasionally demonstrated the apparent dissociation of pericytes from the capillary tube (Figure 2d, middle; 1 to 3 vessels/each tissue section containing 32 to 128 capillaries in 5 animals) in nonischemic limb muscles (left adductor muscles) of STZ-DM mice, a finding that was not seen at all in the

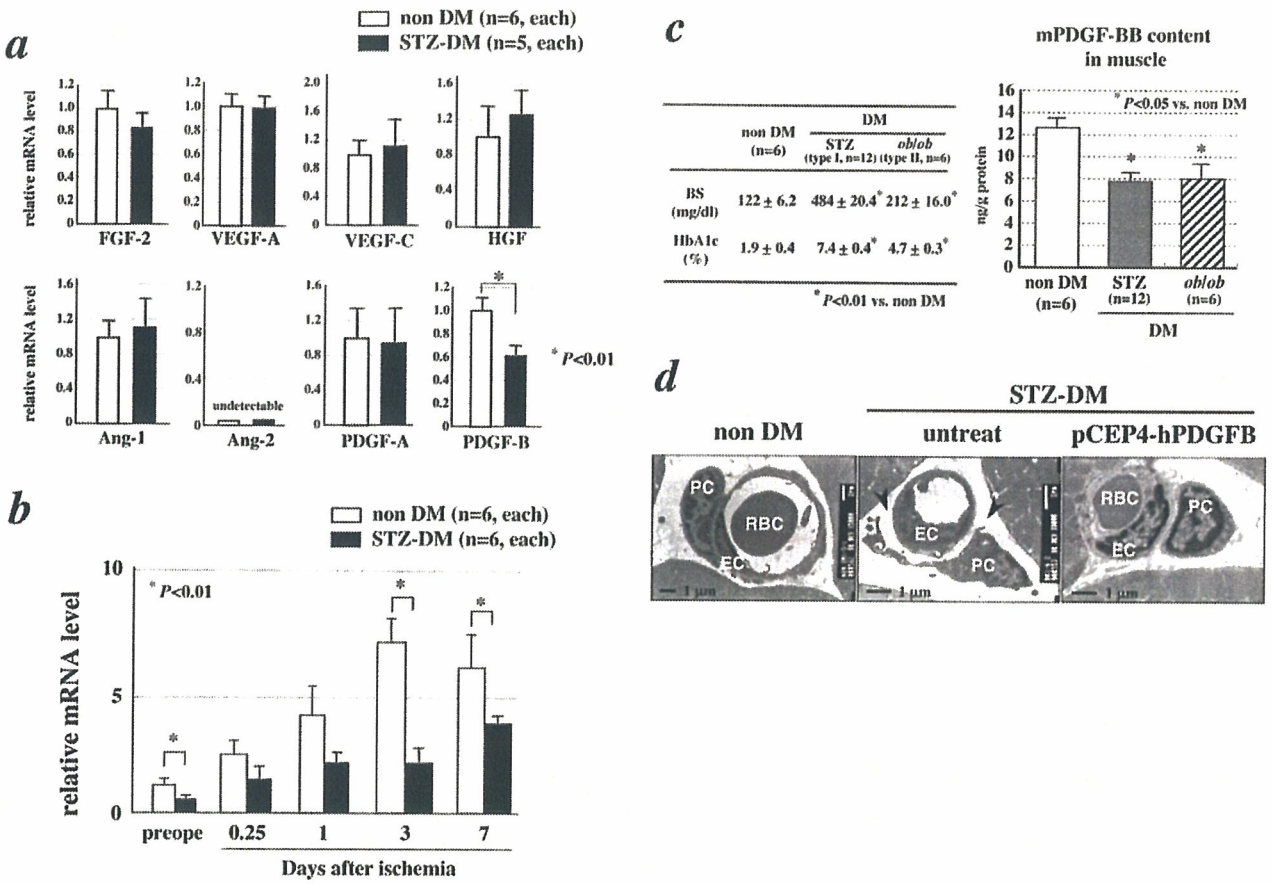
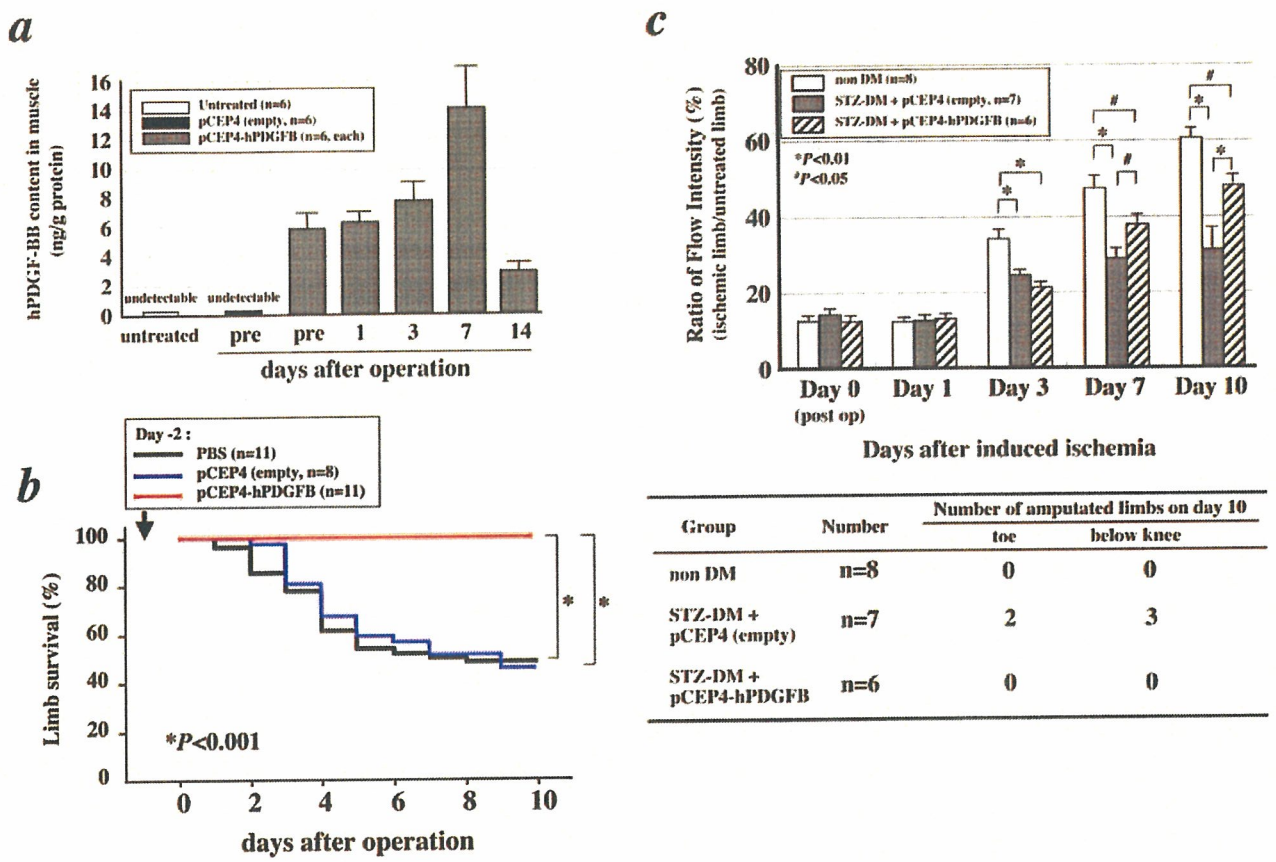


Figure 2. a, Baseline mRNA expressions of typical angiogenesis-related factors in the thigh muscles in non-DM or STZ-DM mice assessed by real-time RT-PCR. Data were standardized by the expression level of GAPDH in each sample, and the data were presented as the relative expression of those from the non-DM mice. b, Time course of relative PDGF-B mRNA expression in ischemic thigh muscles in non-DM or DM mice. After surgery inducing hindlimb ischemia, the thigh muscles were subjected to real-time RT-PCR at each time point. Data were standardized by the mRNA level of GAPDH in each sample, and the data represented as the relative expression of those from untreated control mice. c, Impaired expression of murine PDGF-BB protein (mPDGF-BB) in C57BL/6J strain-based 11-week-old type 1 (STZ-DM) and type 2 (Lep^{ob}/Lep^{ob}: ob/ob) mice. Blood sugar was significantly increased, and inversely, HbA1c was decreased in both mice compared with those of control non-DM mice (left) at death. mPDGF-BB protein content in whole thigh muscles of these mice. d, Typical electron microscopic findings of capillaries in the thigh muscles of non-DM (left) and STZ-DM mice without any treatment (middle) or with intramuscular injection of plasmid DNA expressing human PDGF-B gene (right, pCEP4-hPDGFB). The dissociation of pericytes from the capillary channels were occasionally seen in the adductor muscles of the STZ-DM mice (middle, arrowheads) but not in those of the non-DM mice (left) as well as STZ-DM mice supplemented with human PDGF-B gene (right). PC indicates pericytes; EC, endothelial cells; RBC, red blood cells.

non-DM mice (5 animals; Figure 2d, left) as well as STZ-DM mice intramuscularly treated with plasmid DNA expressing human PDGF-B gene (pCEP4-hPDGFB, 5 animals; Figure 2d, right), suggesting that the impaired expression of PDGF-BB could be 1 cause for the morphological abnormality of the capillaries, namely, the pericyte loss seen in the PDGF-B–deficient mice.[18] Inversely, these results suggest that the supplementation of PDGF-BB is sufficient to rescue the DM-based abnormality of ultrastructure of capillaries. Whereas irregular dilatation and microaneurysm formation can be observed in the capillaries of the brain of PDGF-B–deficient mice[18,19] and the quadriceps muscles of diabetic individuals,[20] it was not observed in the adductor muscles of the STZ-DM mice. In the ultra-thin sections of these 5 animals, larger vessels, ie, arterioles, muscular arteries and veins, showed no apparent abnormal finding in their ultrastructure examined by an electron microscope (data not shown). Furthermore, we could not detect pericyte detachment in retinal vasculature of these 5 animals under electron microscopy, a reasonable result that was similar to a previous observation showing rare drop off of pericyte in the same condition by the other group.[21]

To investigate the functional role of the disturbed expression of PDGF-BB in STZ-diabetic mice, we performed a supplementation study by the plasmid-based intramuscular gene transfer of human PDGF-B (pCEP4-hPDGFB). The PDGF-B gene transfer at day −2 resulted not only in the sustained expression of human PDGF-BB for at least 2 weeks (Figure 3a) but also in the complete prevention of autoamputation in the STZ-DM mice (Figure 3b). Next, we performed another set of experiment assessing the effect of hPDGF-B gene transfer to the recovery of blood flow evaluated by LDPI. As shown in Figure 3c, preinjection of pCEP4-hPDGFB significantly improved the disturbed blood



Group	Number	Number of amputated limbs on day 10	
		toe	below knee
non DM	n=8	0	0
STZ-DM + pCEP4 (empty)	n=7	2	3
STZ-DM + pCEP4-hPDGFB	n=6	0	0

Figure 3. a, Time course of human PDGF-BB protein expression in ischemic thigh muscles in STZ-DM mice. Two days before surgery, 100 μg of plasmid expressing human PDGF-B (pCEP4-hPDGFB) or empty vector (pCEP4) was injected into the left thigh muscle. After surgery inducing limb ischemia, the thigh muscles were subjected to ELISA at each time point. The untreated mice were also used as a control group. b, Limb prognosis curve according to the limb salvage score after the plasmid-based intramuscular gene transfer of PDGF-B and empty or PBS injection in STZ-DM mice. These curves were obtained using the Kaplan–Meyer method, and the data were analyzed using the log rank test. c, Top, Effect of plasmid-based PDGF-B gene transfer on recovery of blood flow in ischemic limbs of 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 LDPI at each time point. Data were standardized by data related to the untreated right limb and expressed as the ratio of flow intensity (percentage). *$P<0.01$, #$P<0.05$. Bottom, Summary of the fate of limbs subjected to LDPI. Five animals of STZ-DM+pCEP4-empty group lost their limbs at toe or below knee level, whereas no animal in other groups showed autoamputation.

perfusion in STZ-DM mice, indicating that the supplementation of PDGF-BB was sufficient to restore the tolerance against hindlimb ischemia in STZ-DM mice.

Spontaneous Phosphorylation of PKC Is the Determinant of Impaired Expression of PDGF-BB in STZ-DM Mice

We further investigated the essential intracellular signals that are related to endogenous PDGF-B expression as well as limb salvage in diabetic mice. Among some of the inhibitors tested (MAPK: U0126; p70S6K: rapamycin; phosphatidylinositol 3-kinase: wortmannin; PKC: bis-I), only bis-I restored the PDGF-BB protein level in the ischemic muscles of diabetic mice, a finding not seen in the use of a control compound (bis-V) (Figure 4a). The spontaneous phosphorylation of PKC-$\alpha+\beta$ ($\alpha \gg \beta$: assessed by RT-PCR, data not shown) and its inhibition by bis-I treatment at that concentration administered in vivo were confirmed by Western blotting (Figure 4b). Furthermore, the daily intraperitoneal administration of bis-I prevented the ischemic limb amputation of STZ-DM mice (Figure 4c), indicating that the PKC signal-transduction pathway is essential for PDGF-BB expression as well as for the tolerance against limb ischemia in the diabetic foot.

AGE Is Important to Spontaneous Phosphorylation of PKC-α As Well As Impaired Expression of PDGF-BB in STZ-DM Mice

A recent important study indicated that AGE directly activated PKC, particularly PKC-α, among various PKC isoforms in diabetic kidney, which could be inhibited by an AGE cross-breaker ALT-711 and an inhibitor of AGE formation, AMG.[22] As the final assessment, we tested whether AGE/PKC-α pathway might be a major stream for PKC/PDGF-BB axis in STZ-DM mice.

One month after induced DM by STZ, plasma AGE level was significantly increased (non-DM versus DM: 1.8 ± 0.1 versus 2.3 ± 0.5 U/mL, n=10 and 9, respectively; $P<0.05$)

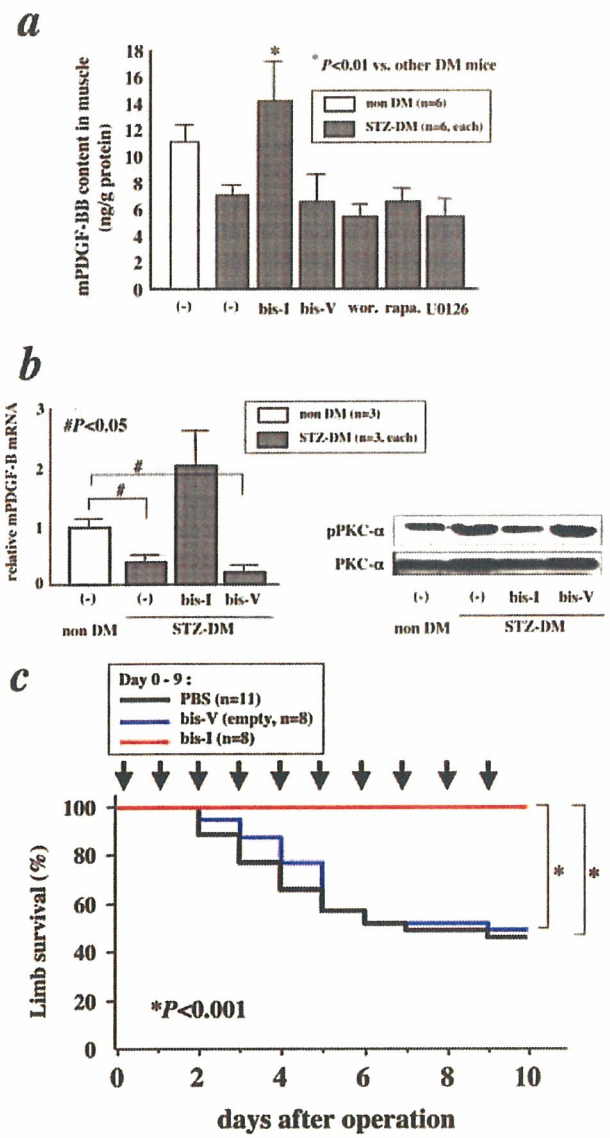


Figure 4. a, Effects of typical inhibitors for signal transduction (PKC: bis-I; phosphatidylinositol 3-kinase: wortmannin; p70S6K: rapamycin; and MAPK/MEK: U0126) on PDGF-BB expression in the thigh muscles of STZ-DM mice. The day before tissue sampling, 100 μL of inhibitor solution was intraperitoneally injected. The thigh muscles were subjected to the ELISA. A control compound for bis-I, bis-V, was also used. b, Effect of broad PKC inhibitor (bis-I) or its control compound (bis-V) on the PDGF-BB mRNA expression and confirmation of spontaneous PKC phosphorylation in thigh muscles in STZ-DM mice. The PKC inhibitor was intraperitoneally injected on the day before tissue sampling, and the thigh muscles were subjected to quantitative real-time RT-PCR (n=3, each group) or Western blot analysis, which was done in triplicate, and showed similar results. c, Limb prognosis curve according to the limb salvage score after intraperitoneal injection of bis-I, bis-V, or buffer (PBS) in STZ-DM C57/BL6 mice. These curves were obtained using the Kaplan–Meyer method, and the data were analyzed using the log rank test.

without significant alteration in free blood sugar and HbA1c (data not shown). The increase of AGE was significantly inhibited by AMG (1 g/L in drinking water during day 0 to 28) (Figure 5a). As expected, both the spontaneous phosphorylation of PKC-α and impaired expression of PDGF-BB

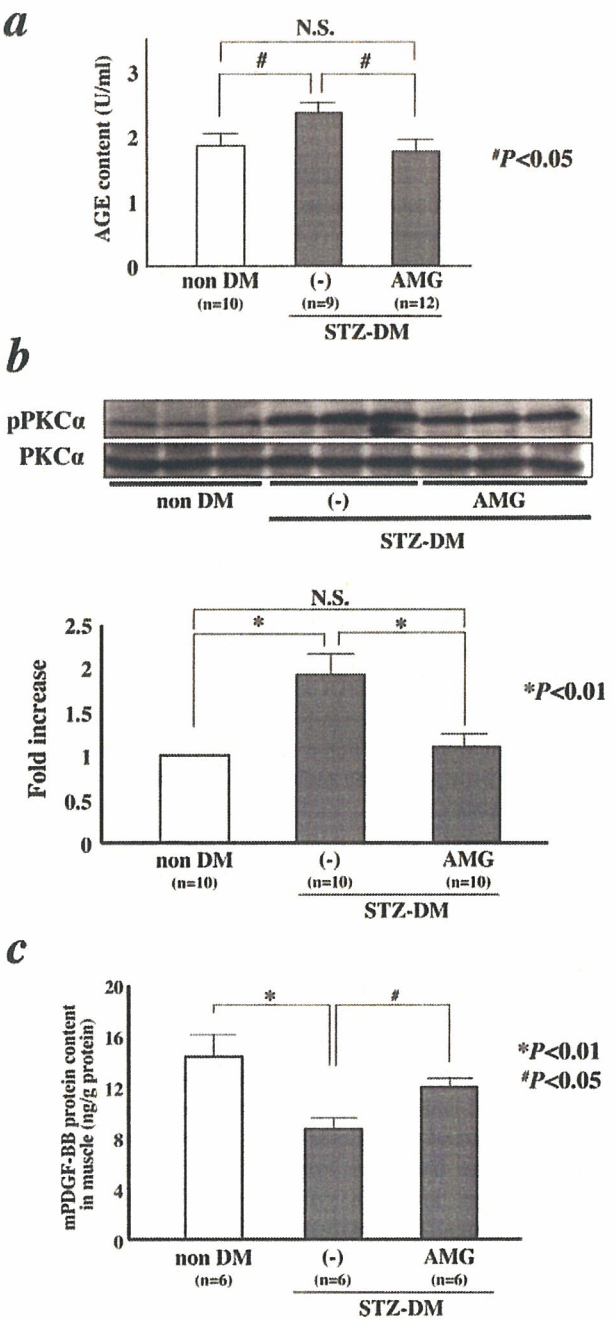


Figure 5. a, Bar graph indicating that daily and oral administration of aminoguanidine (AMG, 1 g/L, day 0 to 28) diminishes the increase of plasma AGE level in STZ-DM mice. #$P<0.05$. b, Effect of AMG on activation of PKC-α. The panels indicate the results of Western blot analysis for phosphorylated (pPKC-α) (top) and nonphosphorylated (bottom) PKC-α in thigh muscles of non DM or STZ-DM mice. The panels present the results of 3 animals in each group. The bar graph indicates the relative levels of pPKC-α/PKC-α in thigh muscles of STZ-DM mice by densitometric analysis standardized by that in non DM mice. AMG treatment significantly reduced the relative level of pPKC-α/PKC-α in STZ-DM mice. *$P<0.01$. c, Bar graph indicating that daily and oral administration of AMG attenuated the expression level of PDGF-BB protein in thigh muscles of STZ-DM mice. #$P<0.05$, *$P<0.01$.

protein were significantly restored by AMG treatment (Figure 5b and 5c), suggesting that impairment of PDGF-BB in diabetic state might be largely attributable to AGE/PKC-α–related pathway.

Discussion

The key observations made in this study are summarized as follows. (1) Although the STZ-DM mice showed disturbed tolerance against hindlimb ischemia, the baseline expression of angiogenesis-related genes as well as the FGF-2–mediated responses of the induction of VEGF and HGF were not impaired, except for the PDGF-B expression, suggesting that the angiogenic responses were preserved, even under the diabetic state. (2) The disturbed expression of PDGF-BB might relate to the morphological abnormality of the capillaries, namely, the drop off of pericytes from the endothelial tubes. (3) Supplementation of the PDGF-B gene expression was sufficient to prevent autoamputation of the ischemic limb of diabetic mice. (4) The PKC inhibitor was also effective in preventing autoamputation and restored the expression of endogenous PDGF-BB. (5) AGE is probably an upstream regulator of PKC/PDGF-BB axis. These findings are clear evidence that the PKC/PDGF-BB axis is essential to the formation of hyperglycemia-related vascular complications and imply that the PKC/PDGF-BB signal-transduction pathway could be a promising molecular target for treating DM microangiopathy.

In our current study, it was surprising for us that an approximately 40% to 50% reduction of PDGF-B expression (Figures 2a and 2c and 4a) was critical to inducing functional and morphological vascular changes, namely, the dissociation of pericytes from the capillaries in muscles, in STZ-DM mice. This seems to be paradoxical, because brain capillaries in PDGF-$B^{+/-}$ mice showed no significant reduction in the number of pericytes, unlike in the homozygous PDGF-$B^{-/-}$ mice.[18,19] In a more recent study, however, both the PDGF-$B^{+/-}$ and STZ-DM mice revealed not so frequent, but significant, reduction of the pericyte lining in the retinal capillaries, a finding that had been synergistically enhanced by the coexistence of low PDGF-B and DM,[21] supporting our current findings. The absence of microaneurysm formation and the relatively small number of pericyte-dissociated capillaries in the current study, a subacute model (−4 weeks), might be explained by the more recent study referred to above, in which a chronic model (−6 months) was used.[21]

Although PKCs have been shown to be activated spontaneously under hyperglycemic conditions, and the favorable effects of PKC inhibitors against diabetic complications have been reported,[23–26] very little knowledge is available regarding the target molecules that are downstream from PKCs in diabetic disease. An important advance of our current study, therefore, was in identifying PDGF-BB not only as an essential regulator for the function of capillary vessels but also as a target of spontaneously activated PKC in ischemic limbs under hyperglycemia. Our current study thus scientifically supports the results of a multicenter clinical trial indicating that treatment using gel that contained human recombinant PDGF-BB was highly effective (95% healing rate in 9 weeks) for treating diabetic ulcers in type 2 DM patients.[27]

Limitations of the current study include the fact that, even though AGE/PKC-α pathway was suggested to mediate the impairment of PDGF-BB expression in STZ-DM mice, a typical type 1 DM model, other PKCs may still be involved in diabetic vascular complications. Furthermore, we have not assessed yet whether disturbance of AGE/PKC/PDGF-BB is the common pathway in vascular disorder in any types of diabetes. We here demonstrated that a C57BL6-based well-accepted type 2 DM model, namely leptin-deficient *ob/ob* mice, exhibited disturbed expression of PDGF-BB as well (Figure 2c). A previous report, however, demonstrated a conflicting result to our current findings; baseline and ischemia-induced expression of VEGF was considerably disturbed in nonobese diabetic mice.[28] In addition, our preliminary study using *db/db* mice, an alternative and well-used mouse model of type 2 DM, demonstrated very low level of PDGF-BB expression at baseline that was equal level to that seen in control *db/+* mice (Y.Y. and T.F., unpublished data, 2005). These paradoxical results suggest that other factors, including genetic status, may also affect the angiogenic responses under DM state. Further extensive studies, therefore, should be carried out to determine the exact role of AGE/PKC/PDGF-BB in each type of diabetic state.

In addition to PKC/PDGF-BB axis, it has been revealed that AGE also impaired the angiogenic process via the other pathway. A recent important study by Tamarat et al using STZ-DM mouse model showed that AGE-related disturbance of ischemia-induced angiogenesis via inhibition of activity of matrix metalloproteinases (MMPs).[29] Although the direct interaction system between PKC/PDGF-BB and MMPs has not been well understood, MMPs are possibly downstream players of PKC[30] and PDGF-BB via membrane type 1–MMP, which activates MMPs.[31] This notion may be reasonable, because data obtained in the current study showed the clear limb salvaging effect of PKC inhibitor as well as hPDGF-B gene transfer that could activate MMPs.

Over the last several years, we demonstrated that boost of FGF-2 constantly showed highest limb salvaging effect to non-DM mouse model of severe hindlimb ischemia[9] and that such efficacy of FGF-2 was guaranteed by the downstream expression of VEGF and HGF, indicating that FGF-2 gene transfer is a multiple angiogenic therapy.[9–11] Whether is FGF-2 sole therapy effective to diabetic foot too? It is still premature to draw the conclusion, but this may be possible because we obtained the data indicating the sustained upregulation of PDGF-BB by FGF-2 gene transfer in ischemic muscles of STZ-DM mice (data not shown). In turn, it has been demonstrated that the synergistic effect on vascular stability by a combination of PDGF-BB and FGF-2,[32] suggesting that this combination may show far superior effect to diabetic foot to that by each sole therapy. Again, further study should be done to determine such synergism under diabetic state.

In conclusion, we demonstrated that disturbed tolerance against severe limb ischemia under hyperglycemia was solely attributable to the disturbance of the PKC/PDGF-BB axis, not of the angiogenic responses, and that the supplementation of

the PDGF-B gene expression was sufficient to prevent autoamputation caused by limb ischemia in STZ-DM mice. Therefore, PKC/PDGF-BB could be an attractive molecular target for treating intractable diabetic foot disease in patients with diabetic vascular complications.

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Molecular Basis of Angiopathy in Diabetes Mellitus

Jean-Sébastien Silvestre and Bernard I. Lévy

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Molecular Basis of Angiopathy in Diabetes Mellitus

Jean-Sébastien Silvestre, Bernard I. Lévy

Cardiovascular complications are the leading cause of morbidity and mortality in patients with diabetes mellitus; up to 80% of deaths in patients with diabetes are closely associated with vascular disease. The ability of the organism to form a collateral network of blood vessels constitutes an important response to vascular occlusive disease and determines to a large part the clinical consequences and severity of tissue ischemia. The development of new vessels is significantly reduced in diabetic patients with coronary or peripheral artery disease.[1,2] This probably contributes to the severe course of limb ischemia in diabetic patients, in which peripheral artery disease often results in foot ulceration and lower extremity amputation.

Diabetic retinopathy remains one of the major causes of acquired blindness in developed nations. This is true despite the development of laser treatment, which can prevent blindness in the majority of those who develop macular edema or proliferative diabetic retinopathy. The hallmark of diabetic retinopathy is the lack of microvessels in the macula, leading to hypoxia, associated with peripheral retinal neovascularization that may ultimately cause severe vitreous cavity bleeding and/or retinal detachment. The factors that stimulate retinal blood vessel growth have not been fully defined, but there is accumulating evidence that the renin-angiotensin-bradykinin system (RAKS) may be involved in a number of retinal vascular disorders, including retinopathy of prematurity and proliferative diabetic retinopathy[3,4]

Only a few studies have specifically evaluated the effect of diabetes on angiogenesis in ischemic vascular disease and in the retina. Moreover, the mechanisms by which diabetes could both limit the formation of new blood vessels in most organs and simultaneously induce proliferative diabetic retinopathy remain largely undefined.

Main Molecular Mechanisms of Ischemia-Induced Neovascularization

After acute ischemia, hypoxia and inflammation are believed to be the major stimuli causing neovascularization.[5] Cells exposed to hypoxia respond by increasing the level of hypoxia-inducible factor-1 (HIF-1). This factor then activates a number of genes by binding to hypoxia response elements in their promoter regions. A second hypoxia-responsive factor, HIF-2, can activate many of the same genes as HIF-1, such as the vascular endothelial growth factor (VEGF).[6] VEGF is a major proangiogenic factor activating phosphatidylinositol-3′-kinase/Akt and thus the cell survival, migration, and proliferation.[7] Akt has been shown to phosphorylate endothelial nitric oxide synthase (eNOS) leading to a persistent calcium-independent enzyme activation and enhanced endothelial NO synthesis and thereby influences the long-term regulation of vessel growth. A large body of literature indicates an essential role of endothelial NO for postnatal neovascularization.[8] The downstream effector pathways, by which NO mediates its effects, are less clear but may involve integrin-linked signal transduction processes.

Inflammatory processes are also necessary in the ischemia-induced neovascularization process. Activated monocytes and macrophages have been evidenced in ischemic tissues; these cells adhere to the vascular wall during angiogenesis and activate the production of both proangiogenic cytokines such as basic fibroblast growth factor (bFGF), VEGF, IL-18 binding protein, tumor necrosis factor (TNF)-α, and matrix metalloproteinases (MMPs), a family of enzymes that proteolytically degrade various components of the extracellular matrix.[9–12]

Ischemia-induced neovascularization also involves circulating vascular progenitor stem cells. After tissue ischemia, progenitor endothelial cells are mobilized from the bone marrow to the blood stream, and then home to ischemic tissues where they promote neovascularization through the paracrine production of growth factors and possible also by incorporation into neovessels.[13–15]

Impaired Ischemia-Induced Neovascularization in Diabetes

In diabetic patients, collateralization and angiogenesis are insufficient to overcome the loss of blood flow through narrowed or occluded arteries leading to ischemia and often nontraumatic limb amputation. However, only few studies have focused on the identification of factors that may affect neovascularization in the setting of ischemia in diabetes. It has been suggested that alteration in VEGF expression and signalization participate to neovascularization abnormalities in diabetes mellitus.[2] Attenuation of the ability of monocytes to migrate has also been reported in diabetic patients because of a downstream signal transduction defect. The abrogation of the resulting inflammatory reaction might be critical to the formation of new blood vessels in this context.[16] Increased formation of advanced glycation end-products (AGEs) is also regarded as one of the main mechanisms responsible for vascular damage in patients with diabetes. Glycation of extracellular matrix is a consequence of prolonged elevated glucose levels that react with proteins by a nonenzymatic posttranslational modification process called nonenzymatic

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From the Centre for Cardiovascular Research Lariboisière; INSERM Unit 689, Paris, France.

Correspondence to Bernard I. Lévy, Centre for Cardiovascular Research Lariboisière; INSERM Unit 689, 41 Bd de la Chapelle, 75475 Paris Cedex 10, France. E-mail: levy@larib.inserm.fr

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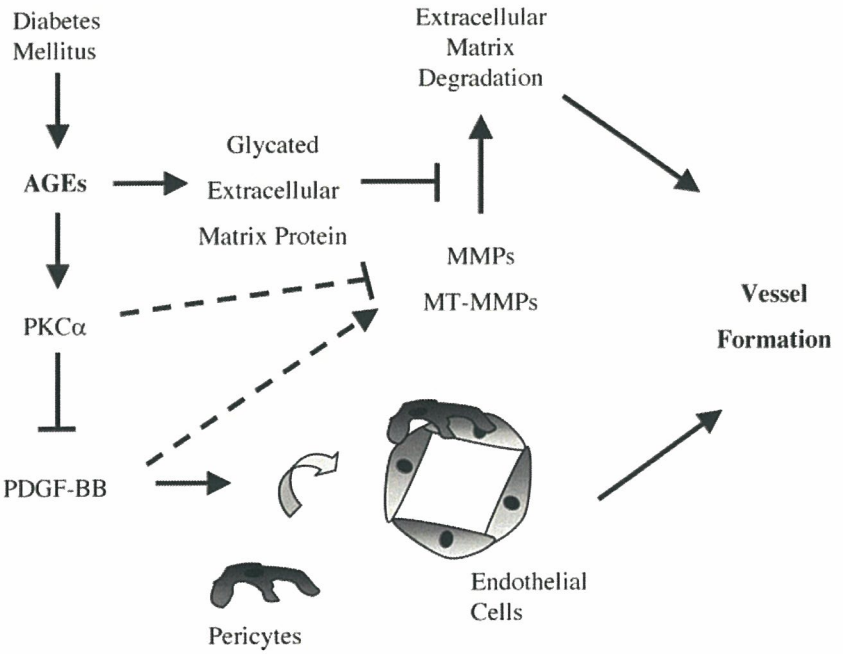

Graphical representation of the proposed mechanisms leading to neovascularization abnormalities in diabetes mellitus. Diabetes-induced AGE accumulation promotes PKC activation and subsequent reduction in PDGF-BB gene expression. At the same time, AGEs hamper extracellular matrix degradation. Supplementation of the PDGF-B gene expression and PKC inhibitors restored the tissue levels of PDGF-B and are effective in increasing neovascularization and preventing auto-amputation in diabetic mice.

glycation. This process is purely adventitious and therefore is likely to be more important in proteins possessing a long biological half-life such as collagen.[17] In experimental models of diabetes, the ability to inhibit these pathways prevented diabetic retinopathy.[18] AGE formation has also been shown to reduce the proteolysis of the glycated proteins and therefore may affect the angiogenic reaction. Hence, pharmacological inhibition of AGE formation is able to restore the ischemia-induced revascularization process in mice hindlimb.[19] Finally, reduction in the mobilization, differentiation, and incorporation of endothelial progenitor cells at the level of the neo-vessels might participate to the angiogenic deficit in a diabetic context. In a diabetic type-1 mouse model, it has been reported that the ability of bone marrow mononuclear cells to differentiate into endothelial progenitor cells was strongly affected, resulting in reduced proangiogenic potential.[20] In the same way, EPCs from type-1 or -2 diabetic patients evidenced a impaired proliferation and adhesion and incorporation into vascular structures, supporting the hypothesis of altered EPCs in diabetes[21,22]

Tanii et al[23] in this issue of *Circulation Research* used a model of severe hind limb ischemia to further investigate the mechanisms of microangiopathy in streptozotocin-induced diabetic mice (STZ-DM). Diabetic mice frequently lost their hind limbs at various levels after ischemia, whereas the nondiabetic mice did not. The authors showed a disturbance of the PDGF-BB/PKC axis, but not of impaired expression or efficiency of angiogenic factors. Hence, VEGF-A, VEGF-C, HGF, FGF-1, PDGF-A, and their receptors flk-1, flt-1, PDGFRα, and -β gene expression were unaffected in STZ-DM. In addition, FGF-2 gene transfer resulted in the upregulation of endogenous VEGF and HGF, prevented limb amputation, and restored limb blood flow, suggesting that angiogenic responses were minimally impaired in the STZ-DM model. In contrast, the PDGF-B expression was reduced in the STZ-DM in correlation with AGEs accumulation and morphological abnormalities of newly formed capillaries (dissociation of pericytes from the capillaries in ischemic muscles). Supplementation of the PDGF-B gene expression and PKC inhibitors restored the tissue levels of PDGF-B and were effective in preventing auto-amputation (Figure).

Many questions remain unanswered. Is PKC/PDGF-BB axis affected in other models of diabetes; how does a 50% reduction in PDGF-BB expression lead to pericytes dissociation; and what is the nature of the intracellular signaling involved in PKC-induced downregulation of PDGF-BB gene expression? How does an alteration in the expression of a single growth factor can induced abnormalities in neovascularization? With a disease as complex as diabetes, other factors are likely to be involved as well. However, these results strongly suggest that defects and impairments in the main proangiogenic factors are not obligatorily involved in the complication of severe ischemia occurring in this model of STZ-induced diabetes. In addition, the PKC/PDGF-BB axis could be a new molecular target for treating severe ischemic peripheral lesions in patients with diabetic complications.

Acknowledgments

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KEY WORDS: diabetes ■ Ischemia ■ angiogenesis ■ PDGF-BB

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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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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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From the Division of Pathophysiological and Experimental Pathology, Departments of Pathology (T.F., Y.Y., M.O., T.N., T.T., K.S.) and Cardiovascular Medicine (K.E.), Graduate School of Medical Science, Kyushu University, Fukuoka; the Department of General Surgical Science (T.F., H.K.), Graduate School of Medicine, Gunma University, Gunma; and DNAVEC Corporation (M.I., M.H.), Tsukuba, Ibaraki, Japan. Present address for Y.Y.: Department of Gene Therapy at the 21Century Center of Excellence Program, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan.

Correspondence to Yoshikazu Yonemitsu, MD, PhD, FAHA, Division of Pathophysiological and Experimental Pathology, Department of Pathology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail yonemitu@med.kyushu-u.ac.jp

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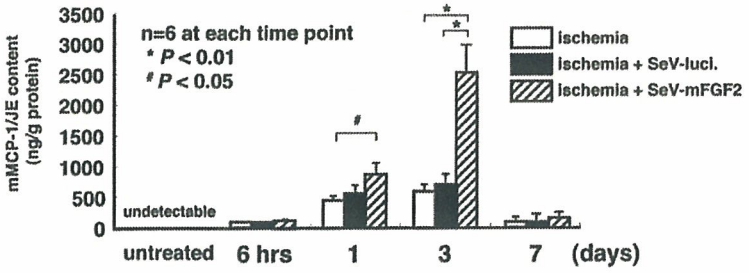

Figure 1. Adaptive expression of mMCP-1/JE in surgically induced hindlimb ischemia of C57BL/6 mice and its enhancement by FGF-2 gene transfer. #$P<0.01$, *$P<0.05$. Time course of endogenous mMCP-1/JE protein expression in murine limb ischemic muscles with or without gene transfer. Animals treated with 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-