Sustained Release of Prostaglandin E₁ Potentiates the Impaired Therapeutic Angiogenesis by Basic Fibroblast Growth Factor in Diabetic Murine Hindlimb Ischemia

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Background  Basic fibroblast growth factor (bFGF) is a potent mitogen; however, diabetes mellitus might impair its angiogenic property. Prostaglandin E₁ (PGE₁) is a potent vasodilator and improves endothelial function. Thus, PGE₁ could potentiate the angiogenic properties of bFGF in patients with diabetes mellitus.

Methods and Results  Streptozotocin-induced diabetic mice with unilateral hindlimb ischemia were randomly treated as follows: no treatment, 0.2 μg of PGE₁, 10 μg of bFGF, and combined administration of PGE₁ and bFGF. Blood perfusion was evaluated by the ratio of ischemic- to normal-limb blood perfusion. Four weeks after the treatment, the combined administration of bFGF and PGE₁ increased the blood perfusion ratio as compared with single bFGF or PGE₁ (77±10% vs 56±10% and 58±10%; p<0.05, respectively). A histological evaluation showed that vascular density in the combined therapy was higher than single bFGF or PGE₁ (418±59 vs 306±69 and 283±71 vessels/mm²; p<0.01, respectively); the maturity in combined therapy was also higher than single bFGF or PGE₁ (46±14 vs 30±14 and 28±6 vessels/mm²; p<0.01, respectively).

Conclusions  PGE₁ potentiates the impaired angiogenic properties of bFGF in diabetic murine hindlimb ischemia. This new strategy might contribute to more effective therapeutic angiogenesis for ischemic limb in patients with diabetes.  (Circ J 2008; 72: 1693–1699)

Key Words:  Angiogenesis; Blood flow; Diabetes mellitus; Ischemia; Peripheral vascular disease

The regeneration therapy with angiogenic cytokines has shown encouraging results for severe peripheral artery disease (PAD). Among them, basic fibroblast growth factor (bFGF) is one of the potent mitogen for vascular and other mesenchymal cells in vitro and in vivo. We have shown the efficacy of bFGF for therapeutic angiogenesis in several experimental and clinical studies.1–8

Endothelial dependent vasodilatation and collateral development are impaired in patients with diabetes mellitus, and it is associated with higher risks of developing PAD.9–12 Regarding therapeutic angiogenesis, to establish stable and functional blood vessel network is a complex process that requires several angiogenic factors aimed at stimulating vessel sprouting and remodeling of the pre-existent collateral network.13,14 Thus, the combination of different strategies might be useful to achieve sufficient angiogenesis in patients with diabetes.15,16

Prostaglandin E₁ (PGE₁) is a potent vasodilator that increases peripheral blood perfusion, followed by improvement of endothelial function.17,18 We hypothesized that the angiogenic properties of bFGF might be impaired in an ischemic limb of streptozotocin (STZ)-induced diabetic mouse, and that simultaneous administration of PGE₁ might potentiate the impaired angiogenesis of bFGF. Because the bioactivity of bFGF and PGE₁ rapidly disappear in solution form, we have used a sustained-release system using gelatin hydrogel for bFGF and poly lactide-co-glycolid (PLGA) for PGE₁, respectively.

Methods  Six-week-old male C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). To generate a diabetic model, the mice were randomized to receive intraperitoneal injection with 150 mg/kg of STZ dissolved in 1 mol/L sodium citrate buffer. Two weeks after injection, the blood glucose concentration was measured with an Accuchek II Advantage Glucometer (Roche Diagnostics). A blood glucose concentration <300 mg/dl were excluded from the study. The Kyoto University Animal Experiment Committee approved the experiment protocol. The animals were cared for in compliance with the Guidelines for the Care and Use of Laboratory Animals published by USA National Institutes of Health (NIH Publication No. 85-23, revised 1996).

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Preparation of Gelatin Hydrogel Microspheres Incorporating bFGF

Gelatin hydrogel microspheres containing bFGF were prepared as previously described. Briefly, gelatin hydrogel microspheres were prepared by crosslinking glutaraldehyde of gelatin in the dispersed state. Human recombinant bFGF was incorporated into the gelatin microspheres by dropping 20 μl of bFGF solution at various concentrations into 2 mg of freeze-dried gelatin microspheres; the microspheres were then left at room temperature for 3 h. At use, the bFGF-containing gelatin hydrogel microspheres were dispersed in 100 μl of phosphate buffer saline, aspirated into a 1-ml syringe attached with a 27-G needle (TERUMO, Tokyo, Japan), and injected into the 5 sites of ischemic thigh muscles of mice hindlimb.

Preparation of PLGA Microsphere Containing PGE1

Instead of repeated intravenous injection, we used sustained-release system of PGE1 using PLGA. PLGA microspheres containing PGE1 was supplied from Ono Pharmaceutical Company (Osaka, Japan). Microspheres were prepared by the emulsion-solvent evaporation method. Briefly, PGE1 ester (10 mg) and PLGA75-65 (90 mg) were dissolved in 1 ml of dichloromethane as oil phase. The emulsion was continuously stirred gently with a magnetic stirrer for 3 h to remove dichloromethane. Then the PLGA microsphere was suspended in the PVA solution after organic solvent evaporation. Washed microsphere precipitate was lyophilized to remove residual organic solvent and water, and then dried solid PGE1 ester microsphere was recovered. PGE1 is released from the PLGA approximately for 4 weeks. At use, PLGA incorporating PGE1 was intramuscularly administered in the same manner of bFGF.

Measurement of Platelet Count and Aggregation

Because PGE1 has a potent ability of anti-aggregation, we evaluated the influences of PGE1 on platelet count and aggregation. The platelet count of whole blood was measured at FALCO Biosystems (Kyoto, Japan). Under anesthesia by intraperitoneal sodium pentobarbital, blood was collected by cardiac puncture with a 21-G needle into plastic syringes containing a final concentration of 0.313% sodium citrate (TERUMO). Platelet-rich plasma (PRP) was prepared by centrifugation of blood at 200 g at 25°C for 10 min and platelet-poor plasma (PPP) was prepared by centrifugation at 2,000 g at 25°C for 10 min. Blood was pooled from 4 to 5 mice for each PRP and PPP sample. PRP samples were diluted to 4×10^5 cells/μl with PPP. PRP aggregation was measured by the addition of adenosine diphosphate (Sigma, St Louis, MO, USA) at a final concentration of 3 μmol/L while constantly stirring at 37°C using a light transmission aggregometer, MCM HEMA TRACER 313M (MC Medical, Tokyo, Japan). PRP aggregation was measured during 60 to 120 min after blood collection. The degree of light transmission of the PRP was defined as an aggregation rate of 0% and that of the PPP as 100%. To evaluate the influences of PGE1 on platelet aggregation, mice with limb ischemia were treated with 100 μl of saline (control, n=12) or 0.2 μg of PGE1 (n=9), and maximal rates of PRP aggregation was measured 2 weeks after the treatment.

Experimental Protocols

Hindlimb ischemia was created in 8-week-old male non-diabetic (wild-type) and STZ-induced diabetic mice (DM). Briefly, the mice were anesthetized with an intraperitoneal sodium pentobarbital (60 mg/kg) injection, the right groin area was shaved and prepped with povidone-iodine. The entire right saphenous artery and the right external iliac artery with along deep femoral and circumflex arteries were ligated, cut, and excised to obtain a murine model of severe hindlimb ischemia. The blood glucose concentration and body weight were measured at the point of entry both in non-DM (NDM) and DM.

Study 1: Restoration of the Blood Perfusion in NDM vs DM

Both NDM and DM were randomly divided into groups (n=8 to 12 per group) and ischemic hindlimb was intramuscularly treated as follows: no treatment, gelatin hydrogel microsphere alone, gelatin hydrogel microsphere incorporating 10 or 40 μg of FGF. Four weeks after the treatment, hindlimb blood perfusion was measured in each group.

Study 2: Single Therapy vs Combined Therapy in DM

DM were randomly divided into groups and ischemic hindlimb intramuscularly treated as follows: no treatment (Group N), PLGA incorporating 0.2 μg PGE1 (Group P), gelatin hydrogel microsphere incorporating 10 or 40 μg of bFGF (Group F10 or F40), and combination of 0.2 μg of PGE1 and 10 μg of FGF (Group FP). Four weeks after the treatment, the animals were anesthetized with intraperitoneal pentobarbital, subjected to the blood perfusion measurement described below, and then killed with an overdose of pentobarbital for the histological examination.

Study 3: Effects of Combined Therapy in NDM vs DM

To compare the effects of combined therapy between NDM and DM, NDM and DM were treated with the combination of 0.2 μg of PGE1 and 10 μg of bFGF and evaluated.

Measurement of Hindlimb Blood Perfusion

Hindlimb blood perfusion was scanned by a laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, UK) on the first day of treatment (2 days after surgery) and 4 weeks after the treatment as previously described. To eliminate the influence of the surgical procedure, the average blood perfusion of the bilateral feet was evaluated. To minimize influential variables including ambient light and temperature, perfusion was expressed as the ratio of the blood perfusion in the right (ischemic) limb to that in the left (non-ischemic) limb of the same mouse, that is, in terms of the LDPI index (LDPII).

Immunohistochemistry

Four weeks after the initiation of treatment, the mice were euthanized and perfusion-fixed with 4% paraformaldehyde. The ischemic calf muscles were embedded in optimal cutting temperature compound (Sakura Finetechical, Tokyo, Japan) and frozen at ~80°C. Cryostat sections (5-μm thick) of the tissues were stained with rabbit polyclonal anti-human von Willebrand factor antibody (vWF; Dako Japan, Kyoto, Japan) or mouse monoclonal anti-human smooth muscle actin antibody (SMA; Sigma-Aldrich Japan K.K., Tokyo, Japan). For the negative control, rabbit normal immunoglobulin fraction (Dako Japan) was used to show antibody specificity.

From each mouse, 8 random fields on 2 different sections (approximately 3 mm apart) were photographed with a digital camera (Olympus, Tokyo, Japan). The number of vWF (endothelial marker)-positive (capillaries and arterioles) or...
SMA (vascular smooth muscle marker)-positive vessels (arterioles) was counted manually in a blind fashion. Vascular density was calculated by counting the number of vWF-positive vessels. The mature vessel density was calculated by counting the number of SMA-positive vessels.

**Statistical Analysis**

Results are expressed as the mean±standard deviation. Differences among groups were determined by one-way analysis of variance followed by multiple comparisons by the Bonferroni/Dunn’s test. Differences between groups were determined with the Student’s t-test. All statistical analyses were performed using Statview software (Abacus, MI, USA). A p-value <0.05 was considered significant.

**Results**

**General Features**

At the point of entry, blood glucose concentration was higher in DM than that in NDM (500±99 mg/dl vs 149±27 mg/dl, p<0.0001). Body weight was lower in DM than NDM (22.4±1.1 g vs 21.3±1.8 g, p<0.01).

**Influence of PGE1 on Platelet Count and Aggregation**

Two weeks after the treatment, there was no difference in platelet count between mice treated with PGE1 and saline (3.4×10^4±1.2×10^4/μl vs 3.5×10^4±1.7×10^4/μl). There was also no difference in maximum rates of PRP aggregation (55.2±3.1% vs 55.8±4.2%).

**Study 1**

Among the NDM and DM which were treated with 10 μg or 40 μg of bFGF, the LDPIIIIs in the DM were lower than those in the NDM (58±±10% vs 73±13% for 10 μg bFGF and 73±±9% vs 85±10% for 40 μg bFGF; p<0.01, respectively; Fig 1). These results indicated that angiogenic property of bFGF was impaired in DM. There were no significant differences in LDPIII between the diabetic and NDM which had no treatment or vehicle.

**Study 2**

Restoration of blood perfusion (Fig 2A): There were no significant differences in LDPIII among the groups N, P and F10. However, the LDPIII in group FP was significantly higher than those in group N, P and F10 (77±±10% vs 73±±10%, 56±±10%, and 58±±10%; p=0.36); whereas mature vessel density was higher in the NDM (64±±13 vessels/mm^2 vs 46±±14 vessels/mm^2, p=0.01). Furthermore, the LDPIII in group FP was as high as that in group F40 (77±±10% vs 73±±8%), which indicated that combination therapy with bFGF and PGE1 recovered higher blood perfusion than either bFGF or PGE1 alone, and recovered equivalent blood perfusion as higher dose of bFGF did.

Vascular density: Representative photographs of histological sections are shown in Fig 2B. The vascular density in group FP was higher than those in groups P and F10 (418±±59 vessels/mm^2 vs 306±±69 vessels/mm^2 and 28±±71 vessels/mm^2, p<0.01, respectively). Furthermore, the vascular density in group FP was also higher than that in group F40 (418±±59 vessels/mm^2 vs 345±±82 vessels/mm^2, p<0.05).

Mature vessel density: Representative photographs of histological sections are shown in Fig 2C. Mature vessel density in group FP was significantly higher than those in group P, F10 and F40 (46±±14 vessels/mm^2 vs 30±±14 vessels/mm^2, 28±±6 vessels/mm^2, and 32±±14 vessels/mm^2; p=0.01, respectively). These results indicated that combination administration of bFGF and PGE1 increased more capillaries and arterioles than higher dose of bFGF alone.

**Study 3**

Four weeks after the combined treatment, the blood perfusion in NDM was higher than that in DM (91±±6% vs 77±±10%, p<0.01; Fig 3). Vascular density was not different between them (418±±59 vessels/mm^2 vs 383±±58 vessels/mm^2, p=0.36); whereas mature vessel density was higher in the NDM (64±±13 vessels/mm^2 vs 46±±14 vessels/mm^2, p<0.01). These results indicated that the combined therapy could increase number of vessels in the DM as much as the NDM. However, the number of mature vessels was lower in the DM, which might result in lower blood perfusion recovery in the DM.

**Discussion**

**Key Findings**

The present study showed the efficacy of simultaneous administration of bFGF and PGE1 for the treatment of diabetic ischemic limb. In the present study, we have shown that angiogenic property of bFGF was impaired in hindlimb ischemia of DM as compared to that of NDM. However, simultaneous administration of sustained release of bFGF and PGE1 achieved equivalent blood perfusion recovery and more mature vessels than higher dose of bFGF alone. The combined therapy was more effective in NDM. These results indicate that PGE1 is the effective adjuvant to therapeutic angiogenesis with bFGF. To the best of our knowledge, this is the first to show the efficacy of a combined treatment with sustained release of bFGF and PGE1 on the neovascularization in diabetic hindlimb ischemia. Our results can shed new light on the treatment of peripheral arterial disease.
Fig 2. Single vs combined therapy with bFGF and PGE₁ in diabetic mice. (A) Restoration of the blood perfusion. (B) Vascular density. (C) Mature vessel density. bFGF, basic fibroblast growth factor; PGE₁, prostaglandin E₁; Control, no treatment; P, poly lactide-co-glycolide incorporating 0.2 μg of PGE₁; F10 and F40, gelatin hydrogel microsphere incorporating 10 and 40 μg of bFGF; FP, combined administration of 0.2 μg of PGE₁ and 10 μg of bFGF. Scale bar =100 μm. Magnification ×100.
Therapeutic Angiogenesis and Diabetes Mellitus

The function of the endothelium is important to develop collateral vessels. A high concentration of glucose causes endothelial cell dysfunction and diabetes mellitus has been identified as one of the first negative predictors of collateral vessels development. Impaired monocyte migration, alterations in vascular endothelial growth factor (VEGF) expression and signalization, and increased formation of advanced glycation end-products are reported to be the cause of impaired neovascularization in the setting of ischemia in diabetes. Regarding therapeutic angiogenesis to limb ischemia, angiogenic potential of bone marrow mononuclear cells transplantation was impaired in DM as compared with NDM. bFGF glycation by high glucose reduces its angiogenic activities in murine hindlimb ischemia. Thus, some method to enhance the therapeutic angiogenesis is desirable in patients with diabetes mellitus.

Sustained Release of bFGF From Biodegradable Gelatin Hydrogel

Therapeutic angiogenesis with growth factors has been established to enhance neovascularization with limb ischemia. Of the growth factors, bFGF is one of the most potent mitogens regulating the proteins that induce the proliferation of a variety of cells and promote the growth and regeneration of organs and tissues both by a direct and indirect effect in vivo. We have shown the efficacy of sustained release of bFGF in several animal and clinical studies. We used gelatin hydrogel as a sustained-release carrier for bFGF instead of genetic materials. There have been concerns about the unpredictable duration and level of gene expression or immunity of inflammatory responses of viral vectors. In addition, this system did not need stem cell collection under general anesthesia or granulocyte-stimulating factor administration, which is beneficial particularly in high-risk patients.

PGE1 and Angiogenesis

PGE1 is a potent pulmonary and systemic vasodilator with important endogenous-flow regulating activity for maintaining the blood perfusion to vital organs. In addition, inhibition of plate aggregation, reduction in blood viscosity, and fibrinolysis have been observed. PGE1 is also known to stimulate angiogenesis in several animal and clinical studies. The angiogenic action of PGE1 seems to be mediated by an angiogenic factor such as VEGF and hepatocyte growth factor (HGF) or by endothelial nitric oxide synthase. In the present study, we used sustained release system of PGE1 to avoid systemic repeated injection of PGE1. Systemic and repeated injection of PGE1 needs hospitalization and sometimes causes hypotension and severe phlebitis. Single intramuscular injection of PLGE incorporating PGE1 achieves dose-reduction and did not need hospitalization. This method could spread an application of PGE1 to several situations.

Platelet Aggregation and Angiogenesis

Platelet aggregation plays an important role in angiogenesis. Aggregation of platelets is mediated by molecules of fibrinogen or von Willebrand factor, which connect platelets by bridging the complex of glycoprotein IIb/IIIa on adjacent platelets, forming a platelet aggregate. In a mouse model of hypoxia-induced retinal angiogenesis, the inhibition of platelet aggregation by acetyl salicylic acid (Aspirin) resulted in approximately 40% reduction in retinal neovascularization, compatible with a significant contribution of blood platelets aggregation in angiogenesis. However, PGE1 is a multifactorial eicosanoid and has a potent ability of anti-aggregation of platelet. In the present study, PGE1 administration did not influence the platelet aggregation, which indicated that local sustained release system of PGE1 might minimize the systemic effects of PGE1 including the influences on platelet aggregation.

Combined Treatment of bFGF and PGE1

Prolonged exposure of skeletal muscle or myocardium to high local levels of angiogenic cytokines, such as VEGF and bFGF, can cause hemangioma-like tumors, vascular malformations, and neoimtimal development. However, combined treatment enhances collateral vessels development and vascular maturation with lower-dose of angiogenic cytokines. PGE1 is a potent vasodilator. In addition, it is a potent stimulator of angiogenesis via upregulation of VEGF and endothelial nitric oxide synthase. Upregulation of HGF improves endothelial function and blood perfusion. PGE1 also downregulates monocyte chemotactant protein-1 (MCP-1) which leads to the suppression of the development of atherosclerosis. However,
bFGF by itself stimulates angiogenesis and arteriogenesis, and synergistically enhances angiogenesis with VEGF, basic HGF, platelet-derived growth factor-BB, and heparin and salopregulate.

Taken together, in addition to functions of bFGF and PGE1 per se, we speculate that the functional mechanisms of the combination of bFGF and PGE1 are composed of: (1) synergistic angiogenesis with bFGF and PGE1-induced VEGF/HGF; (2) improvement of endothelial function via upregulation of HGF and downregulation of MCP-1; and (3) enhancement of arteriogenesis by PGE1-induced vasodilatation. Although the degree of synergistic effects with bFGF and PGE1 depends on the amount of upregulated VEGF, we speculate that the combination of bFGF and PGE1 can be more effective than that of bFGF and VEGF because bFGF, when combined with PGE1, can cooperate with multiple cytokines besides VEGF.

**Clinical Relevance and Study Limitation**

Clinical trials of growth-factor therapies for ischemia have shown several problems, such as immature vasculature, adverse effects of high-dose growth factors, or immune and inflammatory responses of viral vectors. The present study shows that the sustained dual release of lower-dose bFGF and PGE1 from biodegradable gelatin hydrogel and PLGA can achieve equivalent blood perfusion recovery and more mature vasculature in ischemic limb than higher-dose bFGF alone. In addition, PGE1 is already clinically used. Thus, this approach might be a highly promising strategy for the future treatment of PAD.

As a study limitation, we did not evaluate the STZ-mediated metabolic effects, such as insulin level, although they are important angiogenic mediators.

**Conclusions**

Combined administration with sustained release of bFGF and PGE1 enhances neovascularization in the hindlimb ischemia of DM with impaired angiogenic responses to the ischemic stimulus.

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**References**


30. Lederman RJ, Mendelsohn FO, Anderson RD, Saucedo JF, Tenaglia
bFGF and PGE1 to Potentiate Angiogenesis


