Local Sustained Release of Prostaglandin E₁ Induces Neovascularization in Murine Hindlimb Ischemia

Jiro Esaki, MD; Hisashi Sakaguchi, MD; Akira Marui, MD; Shyamal Chandra Bir, MBBS; Yoshio Arii, MA; Yuhong Huang, MD; Hideki Tsubota, MD; Toshiya Kanaji*; Tadashi Ikeda, MD; Ryuzo Sakata, MD

Background: Although intravenous administration of prostaglandin E₁ (PGE₁) is commonly used in the treatment of peripheral arterial disease, it rapidly becomes inactivated in the lung. Whether local administration of sustained-release (SR) PGE₁ enhances neovascularization in murine hindlimb ischemia was investigated.

Methods and Results: Poly lactic-co-glycolic acid (PLGA) microspheres were the 4-week SR carrier of PGE₁. C57BL/6 mice with unilateral hindlimb ischemia were randomly treated as follows: no treatment (Group N); single administration of 100 μg/kg PGE₁ solution (Group L) into the ischemic muscles; daily systemic administration of PGE₁ for 2 weeks at a total dose 100 μg/kg (Group S); and single administration of PGE₁ 100 μg/kg-loaded PLGA (Group P100) into the ischemic muscles. The blood perfusion in Group P100 was higher than in Groups N, L and S (ischemic/nonischemic blood perfusion ratio 88±11% vs 73±1% (P<0.01), 77±9% (P<0.05), 73±11% (P<0.05), respectively). Vascular density and αSMA-positive-vessel density in Group P100 were higher than in Groups N, L and S (vascular density (vessels/m²): 241±39 vs 169±39 (P<0.01), 169±54 (P<0.01), 201±42 (P<0.05), respectively; αSMA-positive-vessel density (vessels/m²): 34±10 vs 18±6 (P<0.01), 21±11 (P<0.01), 22±10 (P<0.01), respectively).

Conclusions: Local administration of a single dose of SR PGE₁ enhances neovascularization in mice hindlimb ischemia more efficiently than daily systemic administration. (Circ J 2009; 73: 1330–1336)

Key Words: Angiogenesis; Peripheral vascular disease; Poly lactide-co-glycolide; Prostaglandin E₁; Sustained release

With the aging population and an increase in the prevalence of atherosclerotic diseases such as diabetes mellitus, hyperlipidemia and hypertension, there is a growing number of patients with associated lower limb ischemia. The number of patients with severely diseased arteries that cannot be effectively treated with the current revascularization techniques has increased and because of the severe ischemia they lose the limb, thereby negatively affecting quality of life.

Editorial p 1201

Prostaglandin E₁ (PGE₁) is a vasodilator that increases peripheral blood perfusion¹ and improves endothelial function² and also has an angiogenic properties³–⁵. Therefore, PGE₁ has been used for the treatment of peripheral arterial disease (PAD), especially critical limb ischemia. However, PGE₁ infusion has to be administered repeatedly for several weeks because it has a short half-life related to rapid and extensive inactivation in the lung. Therefore, local sustained administration has been used, utilizing poly lactide-co-glycolide (PLGA), which has excellent biocompatibility and biodegradability⁶. The ability of local administration of a single dose of sustained-release (SR) PGE₁ to improve blood perfusion and enhance neovascularization for mouse hindlimb ischemia was investigated in the present study.

Methods

Animals
Six-week-old male C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). 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).

Preparation of PGE₁-Loaded PLGA Microspheres
PLGA was used as the SR carrier for PGE₁. PGE₁-loaded-PLGA microspheres were prepared by the emulsion–solvent evaporation method. Briefly, 10 mg of PGE₁ ester prodrug, ONO-AE5-507, and 90 mg of PLGA75-65 were dissolved in 1 ml of dichloromethane as the oil phase. The oil phase was gradually added to aqueous polyvinyl alcohol (PVA) 0.1% solution while stirring with a turbine-shaped mixer (Homomixer) at 6,000 rpm to obtain o/w emulsion. The emulsion was continuously stirred gently with a magnetic stirrer for 3 h to remove the dichloromethane. The PLGA microspheres were suspended in the PVA solution after the organic solvent had evaporated.
In order to remove the free form of PGE1 ester and collect the solid microspheres, the microspheres were washed repeatedly by centrifugation with distilled water containing 0.2%ween 80. the washed microsphere precipitation was lyophilized to remove the residual organic solvent and water and then the dried solid PGE1 ester microspheres were recovered.

**Measurement of Microsphere Particle Size**

PGE1-loaded-PLGA microspheres were suspended in distilled water and dispersed by sonication for 1 min. The particle size was measured using a laser diffraction particle size analyzer (SALD-2100, Shimadzu, Kyoto, Japan).

**Morphologic Studies by Scanning Electron Microscopy**

After lyophilization, the microspheres were mounted on an aluminum stub and coated with a thin layer (200 A°) of gold by ion sputter (model E-1010; Hitachi, Tokyo, Japan). The morphology of the PGE1-loaded-PLGA microspheres was visualized with a scanning electron microscope (model S-2460N; Hitachi).

**In Vitro Release Profile of PGE1 From PLGA Microspheres**

PGE1-loaded-PLGA microspheres were suspended in phosphate-buffered saline (PBS) containing 0.2%ween 80 to adjust the concentration of PGE1 to 100μg/ml and completely dispersed by mixing with a vortex and sonicator. The solution was divided into 1 ml samples and incubated at 37°C. At various time intervals, the aliquots were centrifuged for 5 min at 12,000 rpm (n=3 for each). The supernatant was discarded and the pellet was dissolved with p-hydroxy benzoic acid n-nonylvester in acetonitrile. The concentration of PGE1 in the solution was analyzed by high performance liquid chromatography (HPLC).

**In Vivo Release Profile of PGE1 From PLGA Microspheres**

PGE1-loaded-PLGA microspheres were suspended in PBS containing 0.2%ween 80 to adjust the concentration of PGE1 to 500μg/ml and completely dispersed by mixing with a vortex and sonicator. PGE1-100μg/kg-loaded-PLGA was injected into the thigh muscle of C57BL/6 mice under ether anesthesia.

At 14 days and 28 days after injection, mice were euthanized with an overdose of pentobarbital (n=6 for each). The thigh muscles were resected and homogenized in 15 ml of dichloromethane. The homogenates were centrifuged at 3,000 rpm for 5 min: 750 μl of the lower phase was transferred into a glass test tube and 10 μl of p-hydroxy benzoic acid n-nonyl ester in acetonitrile was added. The solution was dried and dissolved again with LC mobile phase. The concentration of PGE1 in the solution was analyzed by a Liquid Chromatograph (LC)/Mass Spectrometer (MS)/MS (API3200, Applied Biosystems/MDS SCIEX, Carlsbad, CA, USA).

**Murine Hindlimb Ischemia Model**

Hindlimb ischemia was created in 6-week old male C57BL/6 mice as previously reported. Briefly, the mice were anesthetized with intraperitoneal sodium pentobarbital (60 mg/kg) injection. The proximal right femoral artery and the distal portion of the saphenous artery were ligated. All branches between these 2 sites were ligated or cauterized and the entire femoral artery was excised.

**Dose–Response Study of Single Administration of PGE1-Loaded-PLGA Microspheres**

Two days after femoral arterial ligation, the mice were randomly divided into groups (n=20 for each) and drugs were injected in the ischemic thigh muscles as follows: no treatment (Group N); PLGA (Group PLGA); PGE1-10μg/kg-loaded PLGA (Group P10); PGE1-30μg/kg-loaded PLGA (Group P30); and PGE1-100μg/kg-loaded PLGA (Group P100). In all groups except N, PLGA or PGE1-loaded PLGA was dissolved in 100μl PBS containing 0.2%ween 80 and then injected directly into the ischemic thigh muscles.

**Single Local Administration of PGE1-Loaded-PLGA Microspheres vs Repeated Systemic Administration of PGE1 Solution**

Two days after femoral arterial ligation, the mice were randomly divided as follows (n=20 for each): no treatment (Group N); single local intramuscular injection of 100μg/kg of PGE1 solution (Group L); daily subcutaneous injection of 7.14 μg/kg of PGE1 for 14 days (total dose 100μg/kg, Group S); and single local intramuscular injection of PGE1-100μg/kg-loaded PLGA (Group P100). In Group L, 100μg/kg of PGE1 (alprostadil, alfadex, Ono Pharmaceutical Co Ltd, Osaka, Japan) was dissolved in 100μl PBS and injected directly into the ischemic thigh muscles. In Group P100, PGE1-100μg/kg-loaded PLGA was dissolved in 100μl PBS containing 0.2%ween 80 and then injected directly into the ischemic thigh muscles. In Group S, 7.14 μg/kg of PGE1 was dissolved in 150μl PBS and then injected subcutaneously in the dorsum.

**Hindlimb Blood Perfusion**

Hindlimb blood perfusion was scanned using a laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, UK) on the first day of the treatment (ie, 2 days after femoral artery ligation) and at 4 weeks after treatment. The stored images were subjected to computer-assisted quantification and the average blood flow in the ischemic and non-ischemic limbs was calculated to minimize influential variables, including ambient light and temperature, perfusion was expressed as the ratio of the blood perfusion in the ischemic limb to that in the non-ischemic limb of the same mouse; that is, the LDPI index (LDPI). For statistical analysis, 15 different sections were photographed with a digital camera (Olympus, Tokyo, Japan). The number of vWF-positive vessels and SMA-positive vessels were counted manually in a blind fashion.

**Immunohistological Analysis**

At 4 weeks after the initiation of treatment, the mice were euthanized and perfused-fixed with 4% paraformaldehyde. The ischemic gastrocnemius muscles were embedded in OCT and cut into 5-mm thick sections. Cryostat sections of 5 mm thick of the tissues were stained with primary antibody (vWF; Dako Japan, Osaka, Japan) or mouse monoclonal anti-human smooth muscle actin antibody (SMA; Sigma-Aldrich Japan K.K., Tokyo, Japan). Rabbit normal immunoglobulin fraction (Dako Japan) was used as the negative control to show antibody specificity. For each mouse, 15 different sections were photographed with a digital camera (Olympus, Tokyo, Japan). The number of vWF-positive vessels and SMA-positive vessels were counted manually in a blind fashion.

**Statistical Analysis**

The results are expressed as the mean±standard deviation. Differences among groups were determined by 1-way
Results

Particle Size and Release Profile of PGE1-Loaded-PLGA Microspheres

The particle sizes of most PGE1-loaded-PLGA microspheres were between 15 and 40 μm (Figure 1).

In the in vitro release study of PGE1, the percentage of remaining PGE1 in PLGA was 71.8±5.9% at 1 week, 64.5±6.2% at 2 weeks and 30.6±1.3% at 4 weeks. PGE1 was gradually released from PLGA for more than 4 weeks (Figure 1).

In the in vivo release study of PGE1, 2 weeks after intramuscular injection of PGE1-loaded PLGA, the remaining PGE1 in the injection site at 2 weeks after injection was 64.7±16.6% and 27.7±4.6% at 4 weeks after injection, which showed that PGE1 was gradually released from PLGA for more than 4 weeks in vivo as well.

Dose–Response Study of Single Administration of PGE1-Loaded-PLGA Microspheres

The LDPII at 4 weeks in Group P100 was significantly higher than that in Group N and Group PLGA (Group P100 88±11% vs Group N 73±11% (P<0.01), Group PLGA 74±15% (P<0.05), Group P10 76±10%, Group P30 81±13%; Figure 2).

Both the vascular density and αSMA-positive vessel density in Group P100 were significantly higher than in Groups N, PLGA and P10 (vascular density (vessels/m²): Group P100 241±39 vs Group N 169±49 (P<0.01), Group

analysis of variance followed by multiple comparisons using the Tukey test. All statistical analyses were performed using Dr SPSS II software (SPSS Inc, Chicago, IL, USA). P<0.05 was considered to be significant.

Figure 1. (A) Distribution of particle diameter of PGE1-loaded-PLGA. (B) Morphology of PGE1-loaded-PLGA studied by scanning electron microscopy. Scale bar=100 μm. (C) In vitro release profiles of PGE1-loaded-PLGA. PGE1, prostaglandin E1; PLGA, poly lactide-co-glycolide.

Figure 2. Comparison of the laser Doppler perfusion image index at 4 weeks after treatment. Group N, no treatment; Group PLGA, treatment with intramuscular injection of PLGA; Group P10, treatment with intramuscular injection of PGE1-10 μg/kg-loaded PLGA; Group P30, treatment with intramuscular injection of PGE1-30 μg/kg-loaded PLGA; Group P100, treatment with intramuscular injection of PGE1-100 μg/kg-loaded PLGA. *P<0.01 vs Group N, §P<0.05 vs Group PLGA. PGE1, prostaglandin E1; PLGA, poly lactide-co-glycolide.
PLGA 178±49 (P<0.01), Group P10 180±53 (P<0.01), Group P30 215±46; αSMA-positive vessel density (vessels/m²): Group P100 34±10 vs Group N 18±6 (P<0.01), Group PLGA 22±11 (P<0.05), Group P10 22±11 (P<0.05), Group P30 24±7; Figure 3. The vehicle (Group PLGA) had no effect on the LDPII, vascular density or αSMA-positive vessel density.

Figure 3. (A) Vascular density in the ischemic gastrocnemius muscles 4 weeks after femoral artery ligation. (B) αSMA-positive vessel density in the ischemic gastrocnemius muscles 4 weeks after femoral artery ligation. (C) Representative photomicrographs of ischemic gastrocnemius muscles stained with anti-human von Willebrand factor antibody or anti-human SMA antibody 4 weeks after femoral artery ligation. Original magnification, ×200; scale bars=100μm. Group N, non treatment; Group PLGA, treatment with intramuscular injection of PLGA; Group P10, treatment with intramuscular injection of PGE1-10μg/kg-loaded PLGA; Group P30, treatment with intramuscular injection of PGE1-30μg/kg-loaded PLGA; Group P100, treatment with intramuscular injection of PGE1-100μg/kg-loaded PLGA. *P<0.01 vs Group N, †P<0.01 vs Group PLGA, ‡P<0.01 vs Group P10, ∫P<0.05 vs Group PLGA, †P<0.05 vs Group P10. SMA, smooth muscle actin; PGE1, prostaglandin E1; PLGA, poly lactide-co-glycolide.

Single Local Administration of PGE1-Loaded-PLGA Microspheres vs Repeated Systemic Administration of PGE1 Solution

The LDPII at 4 weeks in Group P100 was significantly higher than that in Group N, Group L and Group S (Group P100 88±11% vs Group N 73±11% (P<0.01), Group L 77±9% (P<0.05), Group S 79±11% (P<0.05); Figure 4).
The LDPII in Group S was not significantly higher than that in Group N.

The vascular density in Group P100 was significantly higher than that in Group N, Group L and Group S (Group P100 241±39 vessels/m² vs Group N 169±49 (P<0.01), Group L 169±54 (P<0.01), Group S 201±42 (P<0.05); Figure 5). The vascular density in Group S was not significantly different from that in Group N.

The aSMA-positive vessel density in Group P100 was significantly higher than that in Group N, Group L and Group S (Group P100 34±10 vessels/m² vs Group N 18±6 (P<0.01), Group L 21±11 (P<0.01), Group S 22±10 (P<0.01); Figure 5). The aSMA-positive vessel density in Group S was not significantly different from that in Group N.

**Discussion**

**Key Findings**

This study showed that (1) PGE₁ was gradually released from PLGA for more than 4 weeks in vitro and in vivo and (2) local administration of a single dose of SR PGE₁ improved blood perfusion and induced angiogenesis and arteriogenesis in murine hindlimb ischemia more efficiently than repeated systemic administration of PGE₁ solution.

**SR System of PGE₁**

PGE₁ is used as treatment for PAD because it has a systemic and pulmonary vasodilating effect and also inhibits platelet aggregation. PGE₁ is usually repeatedly administered intravenously for several weeks, but approximately 70% is metabolized by the lung during the first pass immediately after injection. In addition, the serum concentrations of PGE₁ and its biologically active metabolites rapidly decrease after intravenous injection. Therefore, it is assumed that intermittent intravenous administration of PGE₁ raises the concentration of PGE₁ and its metabolites only for a short period of time. The efficacy of local SR of PGE₁ utilizing PLGA as the carrier was evaluated in the current study in order to improve the efficiency of PGE₁ therapy. PLGA has excellent biocompatibility and biodegradability and has been approved by the Food and Drug Administration for human clinical use. The present study showed that PGE₁ is gradually released from PLGA for more than 4 weeks. Local SR of PGE₁ is assumed to maintain the tissue concentration of PGE₁ in ischemic tissue at a certain level for a long time without inactivation by the lung, which may induce effective neovascularization. Local SR of several growth factors is effective for myocardial ischemia and limb ischemia without systemic side-effects. These treatment strategies improve efficacy without systemic side-effects.

**PGE₁ and Angiogenesis**

PGE₁ has pro-angiogenic properties, although others have reported anti-angiogenic properties. Mehrabi et al. reported that PGE₁ pretreatment increased CD34+ cells, vWF+ cells and VEGF-positive capillaries in explanted tissue.
hearts for transplantation. The present study showed that local administration of PGE1-loaded PLGA microspheres increased vascular density and αSMA-positive vessel density in ischemic hindlimbs, which indicated that a local SR system of PGE1 induced the formation of not only capillaries but also mature vessels. Therefore, local administration of SR PGE1 has both angiogenic and arteriogenic properties.

The angiogenic effect of PGE1 has been reported to be mediated by upregulation of eNOS, VEGF, and HGF. PGE1 has also been reported to increase the number of endothelial progenitor cells in the bone and blood marrow in a NO-dependent manner. In contrast, administration of SR PGE1 did not increase the concentrations of VEGF and HGF in the ischemic muscles in this study (data not shown). Therefore, the mechanism of neovascularization is unknown in this system. Some vasodilators induce angiogenesis and have a vasodilating effect and vasodilation increases fluid shear stress, which has been reported to induce angiogenesis and reduced type-1 plasminogen activator inhibitor and D-dimer. Those properties may attenuate the progression of atherosclerosis and make PGE1 a suitable drug for PAD.

Clinical Study of PGE1

In a clinical study of limb ischemia, intravenous administration of PGE1 increased the blood flow as assessed by a laser Doppler imager and increased transcutaneous oxygen pressure values. However, in randomized trials for limb ischemia, the long-term efficacy of PGE1 is controversial. The ICAI study group reported a randomized controlled trial for chronic critical leg ischemia (CLI). Daily intravenous infusion of 60 μg of alprostadil for up to 28 days was effective with respect to the resolution of CLI for 6 months. Brass et al and Nehler et al reported other randomized, multicenter, double-blind and controlled studies that showed that the intravenous administration of 60 μg of lipo-eicaprost, a PGE1 analog, 5 days per week for 8 weeks had no benefit in the reduction of major amputation or death at 180 days for patients with CLI. Further studies are therefore needed to elucidate the efficacy of PGE1 for critical limb ischemia.

Clinical Implications

This study showed that local administration of a single dose of SR PGE1 improved neovascularization more efficiently than repeated systemic administration of PGE1 solution. Therefore, local injection of SR PGE1 could be a treatment option for patients with CLI. This system does not require daily injections for several weeks and can be repeated if the effect is diminished several months later.

Study Limitation

The current study only evaluated the effect of PGE1 at 4 weeks and did not evaluate any longer term effects. Because the duration of this SR system of PGE1 is more than 4 weeks, the angiogenic properties after 4 weeks may thus decrease, so a long-term study is needed.

Conclusions

Local administration of SR PGE1 was found to enhance neovascularization in mouse hindlimb ischemia more efficiently than repeated systemic administration of PGE1.

Acknowledgment

The authors thank Ms Fumiyo Kataoka for her meticulous assistance with the experiment.

References

blasts via a cAMP-dependent mechanism. *J Mol Cell Cardiol* 2004; 36: 539 – 546.


