Efficacy of fragmin/protamine microparticles containing fibroblast growth factor-2 (F/P MPs/FGF-2) to induce collateral vessels in a rabbit model of hindlimb ischemia

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Objectives: **The localized delivery of exogenous, angiogenic growth factors such as fibroblast growth factor (FGF)-2 has become a promising alternative treatment of peripheral artery disease (PAD) and critical limb ischemia (CLI). The present study describes the efficacy of fragmin/protamine microparticles containing FGF-2 (F/P-MPs/FGF-2) to promote vessel growth in a rabbit model of hindlimb ischemia.**
Methods: **A total of 24 rabbits were used to construct a model of hindlimb ischemia by resection of the left femoral artery. The rabbits were randomly divided into four groups 10 days after surgery (day 0); group A: control (non-treated; 1 mL of phosphate-buffered saline [PBS]); group B: FGF-2 (100 μg FGF-2 in 1 mL PBS)-treated; group C: F/P-MPs (12 mg dried F/P MPs in 1 mL PBS)-treated; and group D; F/P MPs/FGF-2 (100 μg FGF-2 and 12 mg dried F/P MPs in 1 mL PBS)-treated (n = 6 each). The drugs were administered intramuscularly to each group. Blood flow and blood pressure were measured in each group on days 0, 14, and 28. Angiography was performed to assess arteriogenesis on day 28. The number of capillaries on day 28 was determined by direct counting $CD31^-$ and α-smooth muscle antibody (α-SMA)-positive vessels.**
Results: **Neither death nor wound infection was observed throughout the experiment. The F/P MPs/FGF-2-treated group showed marked improvement in the blood flow ratio, blood pressure ratio, and capillary number in comparison to the control group, FGF-2-treated group, and F/P MPs-treated group. The F/P MPs-treated group showed intermediate improvement in blood flow ratio and capillary number in comparison to the control group and FGF-2-treated group.**
Conclusions: **The F/P MPs/FGF-2-treated group strongly induced functional collateral vessels in the rabbit model of hindlimb ischemia, indicating a possible therapy for PAD. (J Vasc Surg 2011;54:791-8.)**

Clinical Relevance. **PAD due to atherosclerotic vascular disease is a major health problem. Despite recent advances in surgical and radiologic vascular techniques, certain patients with CLI are not suitable for revascularization. A variety of strategies have been tried to promote development of collateral vessels. F/P MPs can act as carriers for controlled release of FGF-2. The purpose of this study was to evaluate the efficacy of F/P MPs/FGF-2 to induce functional collateral vessels in a rabbit model of hindlimb ischemia. This study will lead to F/P MPs/FGF-2-therapy which is an effective therapeutic strategy for treating PAD patients in clinic.**

Peripheral arterial disease (PAD) is a common cause of disability and morbidity.[1] Despite recent advances in surgical and radiologic vascular techniques, certain patients with critical limb ischemia (CLI) are not eligible for those revascularization procedures, because of the anatomical location of the lesions, the extent of the disease, or extensive comorbidity,[2,3] and no pharmacologic therapy has been established.[4]

Amputation, despite its associated morbidity, mortality rates, and functional implications, is occasionally recommended as a solution to the disabling symptoms, especially for those patients with CLI experiencing excruciating ischemic pain.[5] A variety of other strategies have also been tried for the creation of collateral vessels as an alternative approach to the management of severe ischemia, with the hope of preventing amputation from nonreconstructable vascular diseases.[6] Those strategies included uses of numerous angiogenic factors, including fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF), and

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hepatocyte growth factor (HGF), which are known to promote collateral vessel formation in animal models of limb and myocardial ischemia.[7,8] In addition, introductions of bone marrow cells or adipose-derived stromal cells and direct injection of naked plasmid DNA encoding human VEGF or recombinant growth factors have a potential to induce angiogenesis.[1,9] Although some of those studies have revealed good results, their performance is limited by high cost.[7,9]

Two forms of vessel growth "angiogenesis and arteriogenesis" are observed after birth.[10,11] Angiogenesis is defined as the formation of new capillaries by sprouting and arteriogenesis as the growth of collateral arteries. Arteriogenesis refers to the maturation and stabilization process of preexisting arterioles, which includes smooth muscle cell (SMC) recruitment and vessel enlargement through vascular remodeling.[6] Arteriogenesis increases blood flow 10- to 20-fold, whereas angiogenesis increases 1.5- to 1.7-fold.[11] Therefore, the growth factor used for therapy should potently induce arteriogenesis to develop large-conductance collateral vessels.

FGF-2 is one of the most suitable growth factors for angiogenic therapy that can induce more mature vessels and promote more collateral vessel development.[7,8] The difficulty with the use of FGF-2 is the low accumulation in the ischemic tissue and rapid inactivation. In addition, FGF-2 itself has an acute vasodilatory effect[12] and the potential to induce nephropathy in a dose-related manner.[13] Therefore, studies have investigated carriers for controlled release of FGF-2. Recently collagen,[14] gelatin,[15-17] fibrin,[18] and alginate[19] have been evaluated as carriers for controlled release of FGF-2. Two clinical trials in Japan have evaluated the use of gelatin hydrogel as a control releasing carrier for FGF-2.[20,21] One study used 100 μg of FGF-2[20] and the other used 200 μg.[21] Application of the appropriate carrier for controlled release enabled dose reduction and vessel growth at target site. These trials showed a certain level of efficacy, but there were some patients who showed allergy symptoms to gelatin hydrogel.[22]

Fragmin/protamine microparticles (F/P MPs) have been evaluated as carriers for controlled release of FGF-2.[8] Low-molecular-weight heparin (fragmin) mixed with protamine formed water-insoluble microparticles (about 0.5-3 μm in diameter), and F/P MPs containing FGF-2 (F/P MPs/FGF-2) induced temporal vascularization and fibrous tissue formations in normal mice.[8] Cytokines, extracellular matrix components, adhesion molecules, and heparin could be useful therapeutic agents for various pathologic conditions in which such functional proteins are involved, because heparin and fragmin interact with a variety of functional proteins such as growth factors.[22-24] However, high-dose heparin cannot be used because of the excessive risk of bleeding.[25] In contrast, fragmin has pharmacologic and practical advantages in comparison to heparin. Fragmin induces a low, stable, and predictable anticoagulant response, obviating the need for laboratory monitoring to adjust dosage. In addition, one or two subcutaneous injections per day are sufficient to maintain therapeutic concentrations in blood because of the longer plasma half-life of fragmin.[26,27] We previously reported that fragmin has minor prolonged activated partial thromboplastin time and prothrombin time, compared with native heparin,[8] and injection of fragmin and F/P MPs never caused bleeding at injection sites.

On the other hand, protamine, a purified mixture of proteins obtained from fish sperm, neutralizes heparin by forming a stable complex that lacks anticoagulant activity.[28] Protamine is also in clinical use as an antidote to heparin to reverse its anticoagulant activity following cardiopulmonary bypass and in cases of heparin-induced bleeding.[28] Fragmin without bleeding potency and protamine was used to prepare F/P MPs as an FGF-2-carrier for promotion of vessel growth in a rabbit model of hindlimb ischemia. F/P MPs used in this study were about 1 to 3 μm in diameter with a degradation time of 2 weeks.[8] This report describes the efficacy of F/P MPs/FGF-2 to induce functional collateral vessels in a rabbit model of hindlimb ischemia in comparison to F/P MPs and FGF-2 alone.

METHODS

Preparation of F/P MPs.[8] It is well-known that the mixing of anionic and cationic macromolecules in solution leads to the formation of colloidal anionic molecules/chitosan aggregation complexes (polyelectrolyte complexes [PECs]). Protamine solution (0.6 mL) (10 mg/mL; Mochida Pharmaceutical, Tokyo, Japan) was added dropwise to 1.4 mL of fragmin solution (6.4 mg/mL; Kissei Pharmaceutical, Tokyo, Japan) with vigorous mixing using Vortex Genie-2 mixer (Scientific Industries, Inc, Bohemia, NY) for approximately 2 minutes to produce the colloidal PECs. Protamine and fragmin were mixed in a ratio at 3:7 (vol: vol) to maximize the productions of MPs in this study. The solution of F/P MPs (2 mL) was then washed twice with phosphate-buffered saline (PBS) to remove unreacted components, and then the volume was increased to 1 mL with PBS. About 12 mg of the dry F/P MPs was obtained from 2 mL of the F/P MPs solution. The F/P MPs used in this study were about 0.5 to 3 μm in diameter, and the F/P MPs (200 μL) subcutaneously injected into mice visually disappeared within 2 weeks.[8,9]

Animal model of hindlimb ischemia. The present study used a rabbit model of hindlimb ischemia in all experiments. Male New Zealand White rabbits weighing 3.0 to 3.5 kg (Kitayama Rabes Co, Ltd, Nagano, Japan) were anesthetized with an intramuscular injection of a mixture of 50 mg/kg ketamine (Daiichi-Sankyo Co, Ltd, Tokyo, Japan) and 5 mg/kg xylazine (Bayer Ltd, Tokyo, Japan). The hindlimb ischemia model was created by resecting the left femoral artery from the inguinal ligament to the popliteal fossa and bifurcation of the saphenous artery. The deep femoral artery was preserved (Fig 1). The rabbits were then maintained for 10 days on a normal diet to avoid mild inflammation occurred by the surgery in the models.[29,30] All animal experiments were performed under sterile conditions and in accordance with the guidelines of

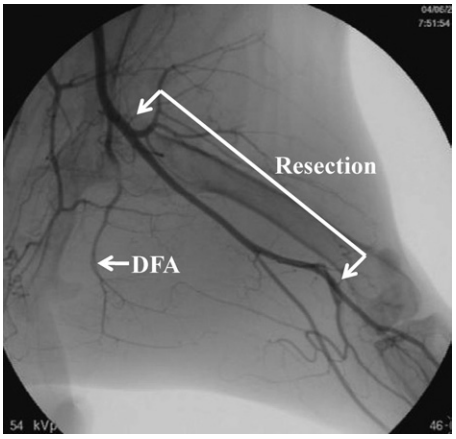

Fig 1. The rabbit acute hindlimb ischemia model was created by resecting the left femoral artery from the inguinal ligament to the popliteal fossa and bifurcation of the saphenous artery.

the Institutional Animal Care and Use Committee of National Defense Medical College (Tokorozawa, Japan).

Study protocol. Rabbits were randomly divided into four groups 10 days after surgery (day 0). Group A: control (non-treated: 1 mL phosphate-buffered saline [PBS]) group; group B: FGF-2 (100 μg FGF-2 in 1 mL PBS)-treated group; group C and F/P MPs (12 mg dried F/P MPs in 1 mL PBS)-treated group; and group D and F/P MPs/FGF-2 (100 μg FGF-2 and 12 mg dried F/P MPs in 1 mL PBS)-treated group (n = 6 each). Rabbits in groups A, B, C, and D were administered the drugs intramuscularly into two different sites in the adductor muscle and three different sites in the semimembranous muscle of ischemic hindlimb with a 27 G needle and a 1 mL syringe (0.2 mL each into five separate injections). Collateral vessel development was evaluated by blood flow, blood pressure, angiography, and pathologic analyses. Calf blood flow ratios and blood pressure ratios were determined 10 days after the creation hindlimb ischemia (day 0), day 14, and day 28. Angiograms were taken on day 28.

Laser Doppler perfusion imaging. Hindlimb perfusion was assessed using laser Doppler perfusion imaging (LDPI) (LSFG; Advance Co, Ltd, Tokyo, Japan) on days 0, 14, and 28. LDPI provides a noninvasive measurement of blood flow by determining the Doppler frequency shift due to light reflecting off the moving red blood cells. The Doppler perfusion in the limb was measured from the thigh, including femoral adductors to knee. Color images were obtained and blood flow ratio was determined as the ratio of perfusion of the ischemic left hindlimb to that of the intact right hindlimb.

Measurement of calf blood pressure. Calf blood pressure was measured on days 0, 14, and 28, using noninvasive blood pressure monitor (SurgiVet V6004; Smiths Medical PM, Inc, Waukesha, Wisc) and 25-mm-wide cuff. In brief, both hindlimbs were shaved and cleaned on the day before measurement. The rabbits were anesthetized, and a 25-mm-wide cuff was wrapped around the calf along the course of the posterior tibial artery to determine the systolic, diastolic, and mean blood pressures. The calf blood pressure ratio (L/R ratio) was determined as the ratio of systolic pressure of the ischemic left hindlimb to that of the intact right hindlimb.

Angiography and quantification of collateral growth. Angiography was performed to visualize collaterals (>200 μm in diameter) on day 28 just before killing. The jugular vein was cannulated for continuous infusion of lactated Ringer's solution and occasional injections of pentobarbital to maintain an appropriate level of anesthesia. A 5F introducing sheath (Medikit, Tokyo, Japan) was inserted into the right common carotid artery. Rentogenographic imaging (Series 9800; A GE Medical Systems, Inc, Salt Lake, Utah) was used to guide the sheath into the descending aorta. A 3F catheter was placed in a proximal position in the common iliac artery. The manufacturer revealed that the angiography system has resolution capacity of >200 μm in diameter. Ten milliliters of contrast medium (320 mg iodine/mL; Omnipaque, Amersham, Nydalen, Norway) were manually injected at the rate of 2 mL/s. Serial images were recorded at the rate of 8 images per second for 6 seconds, and the image representing the best arterial filling was chosen for analysis. The resulting angiograms were assessed in a single-blinded fashion under stereoscopic viewing for quantification of the visible collateral arteries. Only vessels unquestionably showing a defined stem-, mid-, and re-entrant zone according to the Longland classification[31] were counted and marked to confirm that no vessel was counted twice.

Histology and capillary vessel measurements. Open thoracotomy was performed after angiography on day 28, and the inferior vena cava was dissected and cannulated with a 16-gauge (G) catheter. Rabbits were heparinized with 2000 IU heparin, and sacrificed with overdose injections of pentobarbital (Dainippon Sumitomo Pharma, Tokyo, Japan). The organs were fixed by perfusion with 10% neutral-buffered formaldehyde at 120 cm H_2O (88.2 mm Hg) through the abdominal aorta. Excessive blood was left out through the vena cava. Semimembranous muscles of the ischemic limb were excised and immersed another 24 hours in 10% neutral-buffered formaldehyde, and embedded in paraffin and cut into 4-μm transverse tissue sections. The tissues were stained by hematoxylin-eosin and Masson's trichrome for pathologic assessment. Immunohistochemical staining was carried out with a monoclonal antibody against human CD31 (JC/70A; Dako, Carpinteria, Calif) to detect vascular endothelial cells. Those sections were also immunostained for α-SM actin (H0708; Nichirei Bioscience Corporation, Tokyo, Japan) to detect vascular smooth muscle cells. The capillary number represents total vessels counted from 10 different fields randomly selected at ×200 magnification in a blinded manner attained by CD31 and α-SM actin double-immunostaining superimposed upon the same field images stained with Masson's trichrome reagent.

Statistical analyses. Statistical analyses were performed using the State Mate Version III software package (Win-

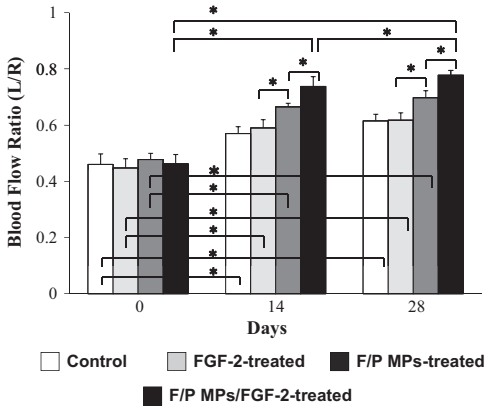

Fig 2. Laser Doppler perfusion imaging. No significant differences in blood flow ratio of the control (*white bar*), FGF-2-treated (*light gray bar*), F/P MPs-treated (*dark gray bar*), and F/P MPs/FGF-2-treated (*black bar*) groups were observed on day 0. The blood flow ratio showed significant improvement in the F/P MPs-treated and F/P MPs/FGF-2-treated groups on days 14 and 28. Data represent the mean ± SD of six determinations. **P* < .05.

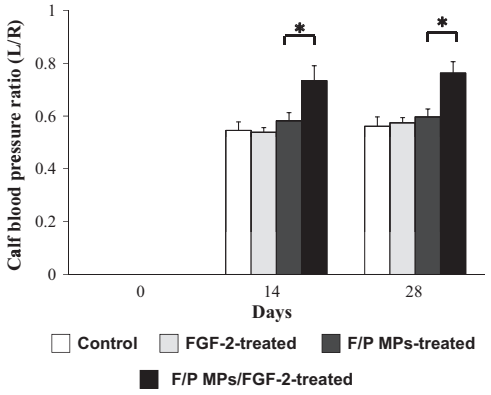

Fig 3. Measurement of calf blood pressure. While calf blood pressure was not detected in the ischemic limbs of the four groups before the initiation of therapy (*day 0*), they showed significant improvement in the F/P MPs/FGF-2-treated (*black bars*) group on days 14 and 28 in comparison to the control (*white bars*), FGF-2-treated (*light gray bars*), and F/P MPs-treated groups (*dark gray bars*). Data represent the mean ± SD of six determinations. **P* < .01.

dows version; ATOMS, Tokyo, Japan). Statistical significance was evaluated by one-way analysis of variance (ANOVA), followed by Dunn-test as *post hoc* test. Values are presented as the mean ± SD. *P* values less than .05 were considered statistically significant.

RESULTS

Laser Doppler perfusion imaging. Thus, F/P MPs/FGF-2 has no side effects in at least rabbits. Calf blood flow ratios were determined 10 days after hindlimb ischemia creation (day 0). There were no significant differences observed among four groups. The blood flow ratio showed significant improvement in the F/P MPs/FGF-2-treated group in comparison to the control, FGF-2-treated, and F/P MPs-treated groups on days 14 and 28. On the other hand, the blood flow ratio in the F/P MPs-treated group also showed significant improvement in comparison to the control group and FGF-2-treated groups on days 14 and 28 (Fig 2). The values of blood flow (intact limbs) were 81.3 ± 14.9. The hindlimb blood flow determined immediately prior to surgery was identical to the value of the intact limb.

Measurement of calf blood pressure. The calf blood pressure in the ischemic limbs was below the level of detection (30 mm Hg) in all four groups before the initiation of therapy (day 0). However, the calf blood pressure ratio on days 14 and 28 showed significant improvement in the F/P MPs/FGF-2-treated group in comparison to the control, FGF-2-treated, and F/P MPs-treated groups (Fig 3) while there were no significant differences among the control, FGF-2-treated, and F/P MPs-treated groups. On the other hand, the values of blood pressure (intact limb) were 105.6 ± 13.7, and the calf blood pressure determined immediately prior to surgery was identical to the value.

Angiography and quantification of collateral growth. Fig 4 show angiograms of the rabbits on day 28. Quantification of visible collateral arteries has been performed with the radiographic appearances under stereoscopic viewing. The number of angiographically visible collateral arteries in the control group, FGF-2-treated, F/P MPs-treated, and F/P MPs/FGF-2-treated groups were 7 ± 1, 7 ± 1, 11 ± 1, and 16 ± 2, respectively (Fig 5). Therefore, significant increases of detectable collateral arteries were observed in the F/P MPs/FGF-2- and F/P MPs-treated groups and those in the F/P MPs/FGF-2-treated groups were significantly higher than that in F/P MPs-treated groups ($P < .001$).

Histology and capillary vessel measurements. F/P MPs/FGF-2-treatment induced marked improvement on the number of mature capillaries in comparison to the control, FGF-2-, and F/P MPs-treatments on day 28 (Fig 6). The F/P MPs/FGF-2-treated group showed mature enlarged vessels (>200 μm in diameter) with thick medial layers containing SMCs. On the contrary, the control- and FGF-2-treatments did not induce such enlarged vessels, and the F/P MPs-treatment induced large vessels poorly. Furthermore, those in the F/P MPs-treated group possessed only thin medial layers containing SMCs. Therefore, the enlarged vessels with a medial layer containing SMCs in the F/P MPs/FGF-2-treated and F/P MPs-treated groups appeared to reflect the improvement of blood flow (Fig 6).

The number of histologically visible capillaries (>50 μm in diameter) counted from 10 different fields randomly selected at ×200 magnification in a blinded manner attained by CD31 and α-SM actin double-immunostaining superimposed upon the same field images stained with Masson's trichrome reagent in the FGF-2-treated, F/P MPs-treated, and F/P MPs/FGF-2-treated groups were 4 ±

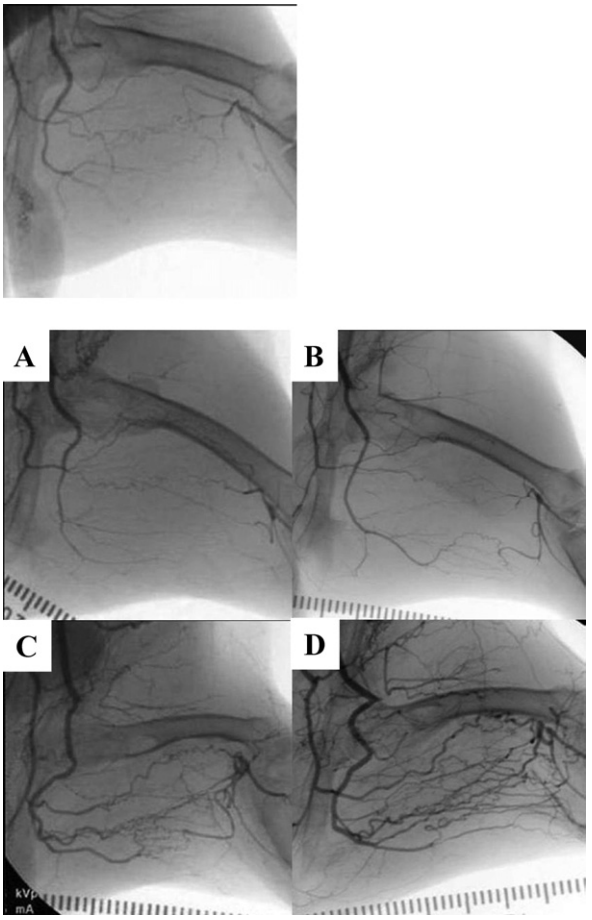

Fig 4. Angiograms of ischemic limb on day 28. **A,** control group. **B,** FGF-2-treated group. **C,** F/P MPs-treated group. **D,** F/P MPs/FGF-2-treated group. The top angiogram shows a representative for intact limb. Photographs are representative of six angiograms in each of four groups.

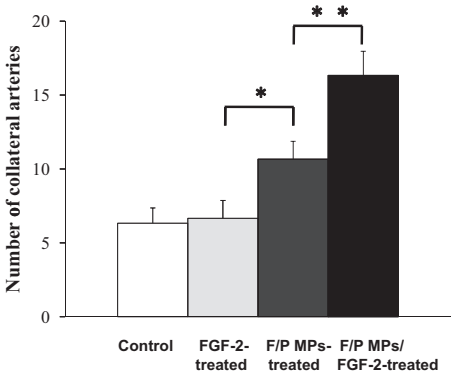

Fig 5. Quantification of visible collateral arteries under stereoscopic viewing on day 28. Data show the number of angiographically visible collateral arteries in the control group (*white bar*), FGF-2-treated (*light gray bar*), F/P MPs-treated (*dark gray bar*), and F/P MPs/FGF-2-treated (*black bar*) groups. Data represent the mean ± SD of six determinations. $*P < .01$, $**P < .001$, *NS,* not significant.

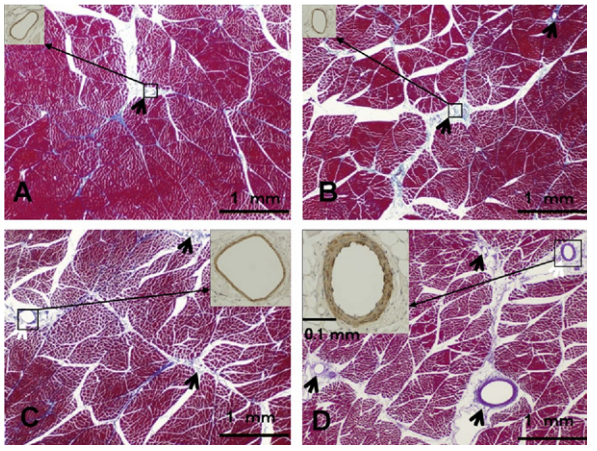

Fig 6. CD31 and α-SM actin double-immunostaining histology. The sections were double-immunostained for CD-31 and α-SM actin to detect vascular smooth muscle cells upon the same field images stained with Masson's trichrome reagent. **A,** control group. **B,** FGF-2-treated group. **C,** F/P MPs-treated group. **D,** F/P MPs/FGF-2-treated group. Each photograph is a representative of six histologic samples in each group. *Black arrows* indicate visible blood vessels, and square areas immunostained with α-SM actin alone were magnified in each photograph.

1, 8 ± 1, 15 ± 1, and 23 ± 2, respectively (Fig 7). The number of enlarged vessels (>200 μm in diameter) that possessed a medial layer containing SMCs among those capillary in the control group, FGF-2-treated, F/P MPs-treated, and F/P MPs/FGF-2-treated groups were 0, 0, 4 ± 1, and 8 ± 1, respectively.

DISCUSSION

The principal goal of angiogenic therapy is to develop collateral vessels that can provide sufficient blood flow to the pre-existing vascular network in ischemic tissue.[31] Newly formed capillaries lack a medial layer containing SMCs and, therefore, have little effect on total vascular resistance. In contrast, arteriogenesis involves the growth, development, and remodeling of pre-existing vessels (mainly arterioles) into larger collateral arteries of sufficient diameter (>200 μm in diameter) to be visualized by angiography that can serve as conduit arteries and influence vascular resistance.[32,33] Results obtained in this study demonstrated clearly that the F/P MPs/FGF-2-treatment could stimulate arteriogenesis as well as angiogenesis at a target site in a rabbit model of hindlimb ischemia in comparison to the control-, FGF-2-, and F/P MPs-treated groups.

The methods used for the evaluation of calf blood pressure, hindlimb perfusion, angiography, and histology performed in this study have been used frequently to assess collateral vessel development.[15,32,33] Although CD-31-positive vessels can be considered parameters of new blood

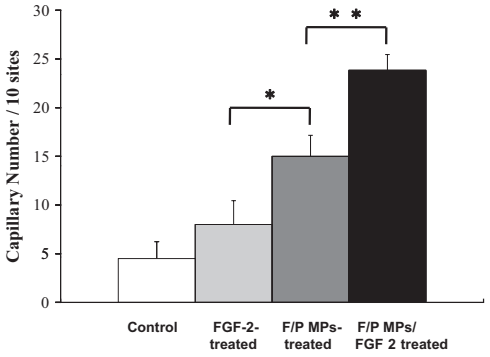

Fig 7. The number of capillaries was counted from 10 different fields randomly selected at ×200 magnification in a blinded manner determined by CD31 and α-SM actin double-immunostaining superimposed upon the same field images stained with Masson's trichrome reagent. Data represent the mean ± SD of six determinations. $*P < .05$, $**P < .01$.

vessels, including both angiogenesis and arteriogenesis, SMC-positive vessels are considered to be parameters of arteriogenesis. Both the hemodynamics and histology in the current study demonstrated that F/P MPs/FGF-2-treatment induced remarkable improvement of blood flow and pressure. The histology demonstrated dilated blood vessels in conjunction with medial hypertrophy, and numerous inflammatory cells, possibly macrophages, were observed in the medial layer only in the F/P MPs/FGF-2-treated group. FGF-2 may play an important role to induce SMCs and macrophages migration as a mediator since SMCs and macrophages are activated by numerous cytokines and growth factors such as MCP-1, TNF-α, FGF-2, and matrix metalloproteinases involved in arteriogenesis,[6] which act as mediators via autocrine or paracrine mechanisms. Those results suggested that the F/P MPs/FGF-2-treatment could stimulate arteriogenesis as well as angiogenesis at a target site in a rabbit model of hindlimb ischemia.

F/P MPs-treatment also showed significant improvements in blood flow ratio and capillary number in comparison to the control- and FGF-2-treated groups on day 28 but those efficacies were lower than that of F/P MP/FGF-2. We previously showed that various growth factors in platelet-rich plasma (PRP) effectively bind to F/P MPs, and F/P MPs maintain and enhance their biological activities in vitro and in vivo.[34] The result suggested that the activities of various endogenous angiogenic growth factors produced at the wound areas accompanied by inflammation may be enhanced with their bindings to F/P MPs and in turn F/P MPs allow their stabilization, local accumulation, and controlled release due to high affinity of F/P MPs for the matrix. Similarly, F/P MPs may bind various endogenous (heparin-binding) angiogenic growth factors accompanied by the removal of the femoral artery and may allow their local accumulation and controlled release.

The most widely studied techniques have been local delivery of angiogenic growth factors such as VEGF, and many researchers have confirmed that exogenous angiogenic growth factors can induce the formation of new blood vessels and increase collateral blood flow in ischemic tissues.[35-37] However, although VEGFs markedly induce angiogenesis, administration of large doses causes edema and induces the development of functionally abnormal blood vessels. In contrast, FGF-2 leads to the formation of granulation tissue, and thereafter, induction of microvessels. Furthermore, FGF-2 can enhance SMC recruitment which leads to arteriogenesis. However, one difficulty in the use of FGF-2 lies in its low accumulation in the ischemic tissues and its rapid inactivation.[38] Therefore, application of the appropriate carrier for controlled release enables dose reduction and vessel growth at target site, since the activity of FGF-2 is stabilized and enhanced,[8,34] and since F/P MPs/FGF-2 rapidly binds to various cell surfaces and extracellular matrix components,[9] F/P MPs may allow their stabilization, local accumulation, and controlled release.

It has been also reported that introductions of bone marrow cells or adipose-derived stromal cells have a potential to induce angiogenesis.[1,9] In addition, clinical studies have revealed good results following direct injection of naked plasmid DNA encoding human VEGF or recombinant growth factors. However, their performance is limited by high cost.[7,9]

F/P MPs have a high affinity for FGF-2 molecules and protect FGF-2 from heat and proteolytic inactivation and enhance the FGF-2-activity.[8] In addition, FGF-2 is gradually released from F/P MPs/FGF-2 (6 mg/mL F/P MPs and 50 μg/mL FGF-2, injection of 100 μL in each site), and local delivery of the F/P MPs/FGF-2 leads to induction of local angiogenesis and fibrous tissue formation in normal mice.[8] However, the previous study did not evaluate the induction of arteriogenesis. In this study, we tested several different dosages for this rabbit hindlimb ischemia model, and we adopted the optimum dosage of F/P MPs/FGF-2 (12 mg/mL F/P MPs and 100 μg/mL FGF-2, injection of 200 μL in each site) for injections. Although the efficacy of F/P MPs/FGF-2 for arteriogenesis was maintained rabbit model of hindlimb ischemia for at least 6 weeks, we speculate that maintenance of a collateral bed required repeated injections of FGF-2 every 2 months or longer. Neither death, wound infection, nor any morbidity was observed using the optimal dosage of F/P MPs/FGF-2 throughout the experiment.

No bleeding complications were observed in the animals injected with F/P MPs in the previous study.[8,9] Although all components for F/P MPs (fragmin and protamine) are in clinical use and FGF-2 was already used in clinical trial, their safety needs further evaluation.[8,9] In addition, the F/P MPs/FGF-2 can be made quickly and easily by simply mixing the three components at the time of use. This study clearly demonstrated that F/P MPs/FGF-2 can stimulate local arteriogenesis as well as angiogenesis in a rabbit model of hindlimb ischemia. Therefore, F/P MPs/FGF-2 may be a promising new biomaterial to induce arteriogenesis as well as angiogenesis in ischemic limbs.

CONCLUSION

The current study evaluated the ability of F/P MPs/FGF-2 to induce both arteriogenesis and angiogenesis in ischemic limbs. The primary conclusion is that F/P MPs/FGF-2-treatment effectively induces the development of collateral vessels, which can provide sufficient blood flow to the pre-existing vascular network in ischemic tissue. Thus, F/P MPs may be a promising effective carrier for FGF-2-therapy for PAD patients. However, since sufficient data are not yet available on the complete toxicity profile of the F/P MPs/FGF-2, standard toxicologic studies need to be completed prior to using the F/P MPs/FGF-2 in human subjects.

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AUTHOR CONTRIBUTIONS

Conception and design: TH, MF, MI, SK, SN
Analysis and interpretation: TH, YT, SK, SN, KH, TM
Data collection: TH, MF, YT, SN
Writing the article: TH, MF, MI
Critical revision of the article: TH, MF, MI
Final approval of the article: MF, MI
Statistical analysis: TH, MF, MI
Obtained funding: MF, MI, KH, TM
Overall responsibility: MF, MI, KH, TM

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