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ORIGINAL ARTICLE
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Angiogenesis Using Recombinant Basic Fibroblast Growth Factor With Atelocollagen in Normal and Hind Limb Ischemia Models

Atsushi Kotani; Shin Watanabe, MD, PhD; Takao Kato, MD, PhD; Takayuki Kikuchi, MSc; Keiji Toya; Katsuhiko Hori; Noriko Minobe; Kaori Musumi; Yasuko Kimura, PhD; Yoji Nagai, MD, PhD; Jun Yoshimura, MD; Hirofumi Kawamata, MD, PhD; Kenji Yanishi, MD, PhD; Satoaki Matoba, MD, PhD

Background: Basic fibroblast growth factor (bFGF) is an angiogenic factor with a short half-life. Because recombinant bFGF is in clinical use, we hypothesized that the localization of recombinant bFGF with atelocollagen would have angiogenic effects at the injection site in normal and hind limb ischemic animal models.

Methods and Results: We administered the recombinant bFGF with atelocollagen intramuscularly to hind limbs in normal rabbits or in a mouse model of femoral artery ligation to explore the pharmacological action for ischemia. We evaluated blood flow in the ischemic/normal limb using laser speckle perfusion imaging and the density of blood vessels by pathological examination. At the administration site in normal rabbits, a significant increase in the number of blood vessels was noted at 14 days post-administration of recombinant bFGF with atelocollagen compared with saline or atelocollagen alone. In mice with femoral artery ligation, blood flow and vessels in the ischemic hind limb increased at 2 weeks after injection and more at 4 weeks after injection, and the effect was most significant in mice administered 100 μg of recombinant bFGF with 3% of atelocollagen.

Conclusions: Intramuscular administration of recombinant bFGF with atelocollagen induced angiogenesis between 2 and 4 weeks in both normal and ischemic hind limbs.

Key Words: bFGF; Limb ischemia; Regeneration

With the rise in lifestyle-related diseases and an aging population, there has been a notable increase in the number of patients diagnosed with peripheral arterial disease (PAD) affecting the lower limbs.[1] PAD primarily develops due to the progression of atherosclerosis, which results in the narrowing or complete occlusion of the peripheral arteries, leading to tissue ischemia. In the lower limbs, symptoms typically manifest as intermittent claudication characterized by leg pain during walking. As foot ischemia progresses, patients may experience pain at rest, ulcers, or gangrene, a condition known as critical limb-threatening ischemia (CLTI). Despite advancements in PAD treatment, revascularization is often difficult in patients with poor distal runoff or no vascular beds below the ankle, and even when revascularization is conducted, restenosis is frequent, especially in small vessels and calcified lesions in the distal limb. Consequently, a significant number of patients with CLTI remain at a high risk of amputation and are categorized as having non-optional CLTI, and there is a pressing need for simple and non-invasive therapeutic angiogenesis approaches.

Fibroblast growth factors (FGFs) are potent mitogens in vascular and capillary endothelial cells in vitro.[2] Owing to its short half-life and instability in vivo,[3] basic FGF (bFGF) typically does not exhibit significant long-term angiogenic effects when dissolved in water or injected

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FEF Pharmaceutical Co., Ltd., Tokyo (A.K., K.T.); Research Center, EPS Innovative Medicine Co., Ltd., Tokyo (A.K.); Innovation Research Center, EPS Holdings, Inc., Tokyo (A.K.); Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto (S.W., T. Kato); Department of Clinical Research Facilitation, Institute for Advancement of Clinical and Translational Science, Kyoto University Hospital, Kyoto (T. Kato, Y.K., Y.N.); Learning Health Society Institute, Nagoya (T. Kikuchi); Shiga Laboratory, NISSEI BILIS Co., Ltd., Shiga (K.H., N.M., K.M.); and Department of Cardiovascular Medicine, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto (J.Y., H.K., K.Y., S.M.), Japan

Mailing address: Takao Kato, MD, PhD, Department of Clinical Research Facilitation, Institute for Advancement of Clinical and Translational Science, Kyoto University Hospital, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto, Kyoto 606-8507, Japan. email: tkato75@kuhp.kyoto-u.ac.jp

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intramuscularly, and recombinant bFGF is clinically used only for topical skin applications. To address these challenges, a gelatin hydrogel has been developed as a potential solution,[4,5] demonstrating efficacy in clinical trials involving patients with arteriosclerosis obliterans of the lower extremities and Berger's disease.[6–8] However, recombinant bFGF with gelatin hydrogels has not been commercialized because of problems with industrialization.

Atelocollagen is the most commonly utilized material in tissue engineering owing to its reduced immunogenicity and enhanced biocompatibility. At room temperature (approximately 25°C), atelocollagen exists as a clear, colorless, and viscous solution. On reaching body temperature (approximately 36°C), atelocollagen molecules undergo polymerization in an organized manner, forming a gel that remains localized at the site of administration. This property renders atelocollagen particularly useful for medical applications where the sustained release or localization of therapeutic agents is desired. Thus, we hypothesized that the persistent localization of recombinant bFGF with atelocollagen at the injection site causes angiogenic effects at the injection site in normal and hindlimb ischemia models.

Methods

Preparation of bFGF With Atelocollagen

In this study, the genetically recombinant bFGF, trafermin (Fiblast ® Spray 250, Kaken Pharmaceutical Co., Ltd), and atelocollagen (Koken Atelocollagen Implant, Koken Co., Ltd) were procured and utilized. The recombinant bFGF solution was prepared by reconstituting the lyophilized product with water for injection to achieve a concentration of 250 μg/mL. For the bFGF with atelocollagen formulation, 0.6 mL of the prepared recombinant bFGF solution was mixed with 3 mL of 3% atelocollagen to 200 μg/4.8 mL (2.5% of atelocollagen) in the final dose. Mixing was performed according to the method described by Ono et al.[9]

Animals

All animal experiments were conducted in accordance with the institutional guidelines of NISSEI BILIS Co., Ltd, and were approved by the Animal Research Committee (0810-10, 2021-199, 2021-210, 2022-027). This study followed the guidelines of the National Center for the Replacement, Refinement, and Reduction of Animals. Male rabbits, aged 18 weeks, from Japan SLC, Inc., were purchased and used to analyze the kinetics of bFGF with atelocollagen. Male mice (C3H/He, aged 33–34 weeks at grouping; Japan SLC, Inc.) were purchased and used for the analysis of angiogenesis in a murine model of femoral artery ligation.

Protocol 1: Analysis of Angiogenesis and Kinetics of Recombinant bFGF With Atelocollagen in Normal Rabbits

Rabbit experiments were conducted concurrently with the local irritation test (data not shown) performed before administration to humans. The 3 rabbits received an intramuscular injection of a recombinant bFGF with atelocollagen, where the dosage of recombinant bFGF administered was 4 μg per kilogram of the rabbit's body weight. The injections were administered to the lateral thighs of the rabbits. In the 3 rabbits administered with recombinant bFGF with atelocollagen, we serially measured the serum concentrations of bFGF before injection and 30 min, 1, 3, 6, 12, and 24 h after injection into the rabbits. Tissue samples were collected after euthanasia at each time point of 1, 2, 7, and 14 days post-injection (n=3 at each time point). Tissue samples were collected from 4 specific locations: the injection site containing gelatinized atelocollagen, and sites located 5, 10, and 15 mm away from the injection site. Photographs of the injection sites were acquired prior to tissue collection. We measured the concentration of bFGF in the tissue homogenates. To measure the number of blood vessels, we harvested tissue 1 mm from the area where the atelocollagen was gelatinized towards the fascia within 5 mm of the injection site, as atelocollagen becomes gelatinized when administered intramuscularly. We counted the number of blood vessels determined using anti-CD31 antibody immunostaining 2 and 14 days after injection. In this examination, we used controls as the rabbits administered atelocollagen (3%, 96 μL/kg) or normal saline (96 μL/kg). We also visually and morphologically measured the vessel number density using a specimen examiner.

Protocol 2: Analysis for the Angiogenesis in the Murine Model of Femoral Artery Ligation

The experimental procedure involved ligating the proximal and distal femoral arteries as well as the distal saphenous artery of the right thigh using sutures. First, we administered a total of 100 μg of recombinant bFGF with 0%, 1%, and 3% of atelocollagen or phosphate buffer (0.1 mol/L) at 5 sites to explore the appropriate concentration of atelocollagen for angiogenesis. Subsequently, a recombinant bFGF with 3% of atelocollagen was administered intramuscularly at doses of 0, 50, 100, and 200 μg of recombinant bFGF per animal at 5 sites. The injection site was located on the thigh of the right hind limb. Injections were administered immediately after surgery on the day of model preparation. Blood flow was measured using Laser Speckle Perfusion Imaging, and the ratio of blood flow in the ischemic hind limb to that in the normal hind limb was calculated on the day of pre-injection, and also at 2 and 4 weeks post-injection.

Measurement of bFGF Concentrations in Tissue and Blood Samples Using Enzyme-Linked Immunosorbent Assay (ELISA)

A Human FGF Basic Immunoassay ELISA Kit (Quantikine, R&D Systems) was used. Serum was obtained by placing the blood in a Venoject II vacuum blood collection tube (plain, with coagulation accelerator film; Terumo Corporation) and allowing it to stand at room temperature for 30 min, followed by centrifugation at room temperature (1,000 g, 15 min). Homogenate supernatant was obtained from a surrounding area of 5 mm^3 around the 4 sites mentioned above by adding 1,000 μL of PBS solution to 1 g of tissue fragments and performing homogenization (Biomasher II, Nippi Corporation), followed by centrifugation (4°C, 10,000 g, 20 min). The PBS solution was prepared from PBS (−) powder (Fujifilm Wako Pure Chemical Industries, Ltd) and a protease inhibitor cocktail set (Fujifilm Wako Pure Chemical Industries, Ltd). The bFGF concentration in the tissue was calculated by subtracting the concentration in the supernatant containing atelocollagen from the average value after the intramuscular injection of atelocollagen as the baseline.

Laser Speckle Perfusion Imaging in Mice

Mice were anesthetized with 0.5–4% isoflurane and fixed in a dorsal position 2 and 4 weeks after femoral artery ligation.

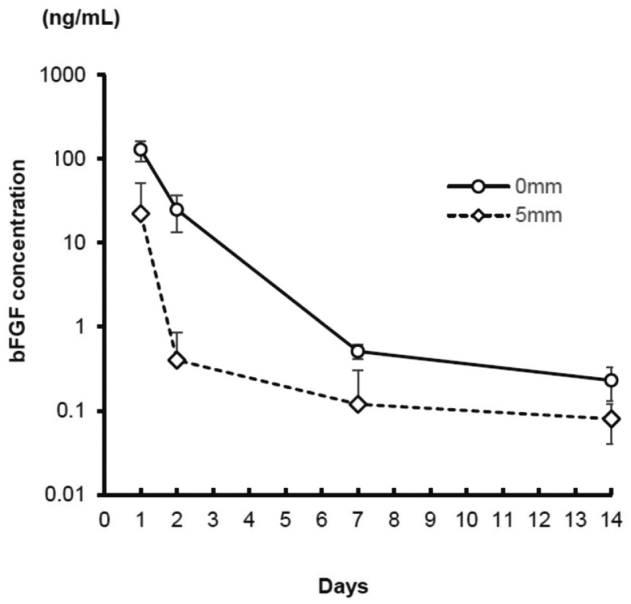

Figure 1. Time course of basic fibroblast growth factor (bFGF) concentration at the injection site. A recombinant bFGF with atelocollagen (bFGF content of 4 μg/kg) was injected intramuscularly into the thighs of rabbits (n=3). The concentrations of bFGF in the supernatant of tissue homogenates at both the injection site and 5-mm distance from the injection site gradually decreased, although the concentration of bFGF was detected at the injection site 14 days after injection. The concentrations at 10 and 15 mm distances from the injection site were below the enzyme-linked immunosorbent assay (ELISA) detection limit and are not shown in the figure.

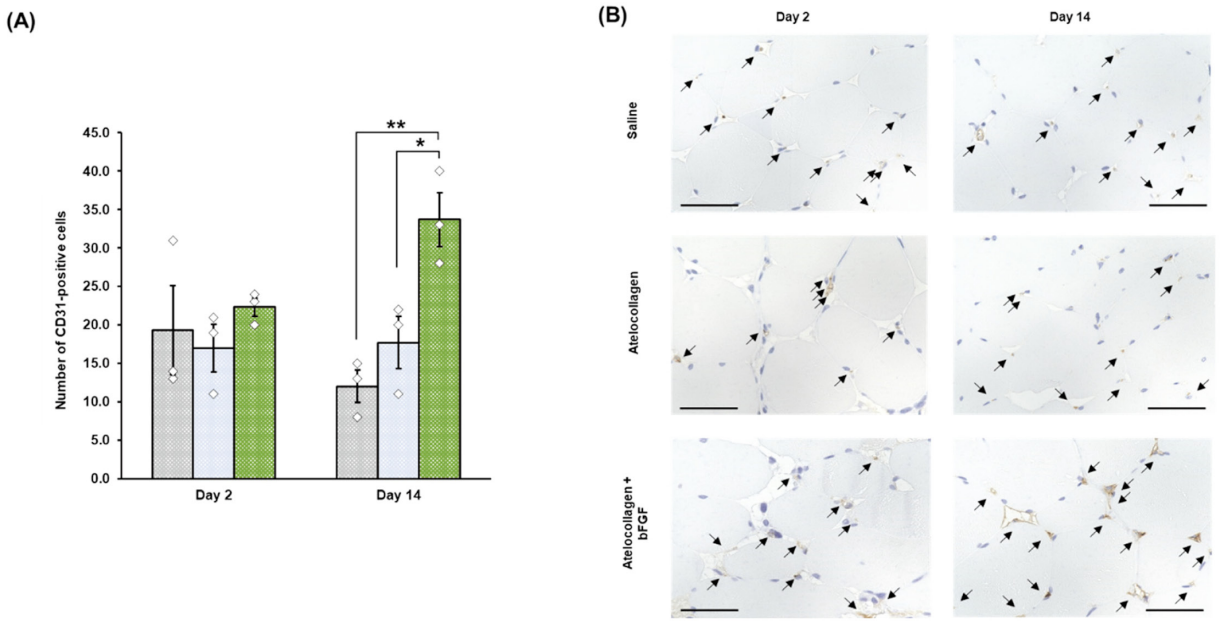

Figure 2. Time course of blood vessel number in normal rabbits. (**A**) Measurement of blood vessel number by immunostaining with anti-CD31 antibodies. *P<0.05, **P<0.01 vs. basic fibroblast growth factor (bFGF) with atelocollagen numbers in parentheses are P values (Student's t-test). (**B**) Representative images. Arrows indicate the blood vessels counted. Scale bar=50 μm.

Laser speckle perfusion imaging (OZ-1, Omega-Wave Co., Ltd) was used to measure hind limb circulation volumes in the right (ischemic) and left (normal) hind limbs and to calculate the ischemic limb/normal limb blood flow ratio. The biological data statistical analysis system (EXSAS Ver. 7.16, Arm Sysmex Co., Ltd) linked to SAS System Release 8.2 (TS2M0) for Windows (SAS Institute Inc.) was used for statistical processing.

Pathological Examination

For anti-CD31 antibody immunostaining, rabbit thighs were extracted and fixed in 10% neutral-buffered formalin to prepare paraffin-embedded specimens. Digital images of CD31 immunostained slides were captured using an all-in-one fluorescence microscope BZ-X800 (Keyence Corporation). The number of blood vessels near the injection site was quantified at 2 sites per slide using a 40× objective lens in

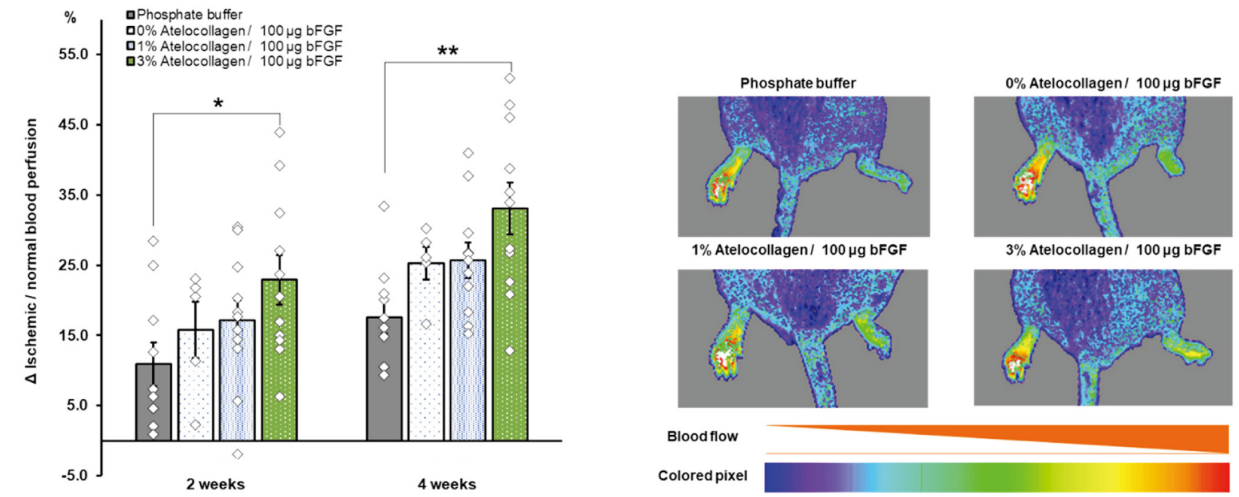

Figure 3. Effect of atelocollagen concentration on blood flow in a mouse femoral ligation artery model. (**A**) Percentage increase of the ratio of ischemic limb/normal limb blood flow from Week 0. Data are expressed as mean±SE. *P<0.05, **P<0.01 vs. the phosphate-buffered group. (**B**) Representative photographs of laser speckle perfusion imaging at 4 weeks. bFGF, basic fibroblast growth factor.

the atelocollagen (n=3), saline (n=3), and atelocollagen with bFGF (n=3) groups.

In the hindlimb ischemia mouse model, muscle samples from the ischemic hind limb (right thigh) were harvested, frozen in OCT compound, and sectioned for immunostaining with anti-CD31 antibodies. Each immunostained slide was imaged at 3 sites (area per site: 37,000 μm^2) using a CCD camera through a microscope with a 40× objective lens and 3.3× imaging lens. Capillary density was quantified by dividing by the number of myofibers in the tissue.

Statistics

Data are presented as mean±standard error (SE). The bars and lines represent the means and SE of the samples, respectively. A paired t-test was used to compare the capillary counts between the atelocollagen (n=3) and saline groups (n=3). Dunnett's multiple-comparison test was used for multiple comparisons. For serial measurements, Dunnett's test was performed on the change from Week 0 in the phosphate-buffered group or the control group. When assessing the effect of doses of bFGF and atelocollagen or weeks, changes from the value at Week 0 were calculated and compared using 3-way ANOVA. Statistical significance was defined as a 2-tailed P value of <0.05. Statistical analyses were performed using SAS 9.4 TS1M6 (SAS Institute Inc.) and SAS 8.2 (TS2M0; SAS Institute Inc.).

Results

Serum Concentrations of Recombinant bFGF in Normal Rabbits Administered With Recombinant bFGF With Atelocollagen

Serum bFGF concentrations in normal rabbits at all time points (30 min, and 1, 3, 6, 12, and 24 h after injection) were below the ELISA detection limit (<10 pg/mL).

Appearance of the Injection Sites and Tissue Concentrations of bFGF in Normal Rabbits Administered With Recombinant bFGF With Atelocollagen

At the injection site, the atelocollagen formed a gel (**Supplementary Figure**). The concentrations of bFGF in the supernatant of tissue homogenates at both the injection site and 5-mm distance are shown in **Figure 1**; however, the concentrations at 10 and 15 mm distances from the injection site were below the ELISA detection limit (<10 pg/mL). Although the bFGF concentration in the tissue decreased, the concentration of bFGF was 0.23±0.10 ng/mL at the injection site 14 days after injection.

Angiogenesis in Normal Rabbits Administered With bFGF With Atelocollagen or Controls

There was no difference in the number of blood vessels 2 days after injection between rabbits administered saline, atelocollagen, or recombinant bFGF with atelocollagen (**Figure 2**). The number of blood vessels was significantly increased in the recombinant bFGF with atelocollagen group on day 14 (**Figure 2A**).

Effect of Atelocollagen Concentrations on Angiogenesis in Mice With Femoral Artery Ligation Administered With 100 μg of Recombinant bFGF With Atelocollagen

When we administered phosphate buffer (n=10) or a 100 μg of recombinant bFGF with 0% (n=5), 1% (n=11), and 3% (n=11) atelocollagen to mice with femoral artery ligation to explore the most effective concentration of atelocollagen for angiogenesis, blood flow increased at 2 weeks after injection and more at 4 weeks after injection in a dose-dependent manner (**Figure 3**).

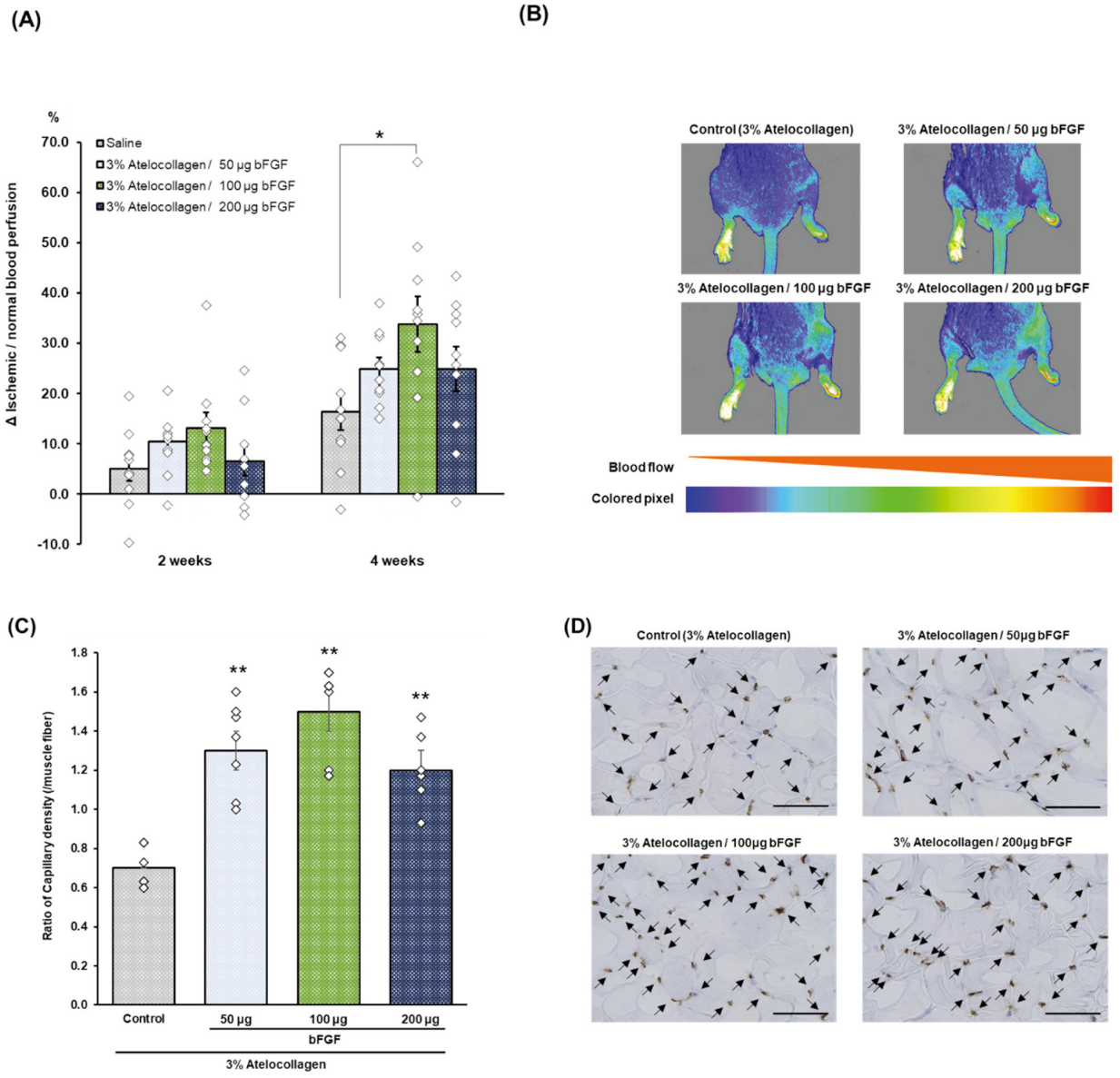

Figure 4. Effect of recombinant basic fibroblast growth factor (bFGF) concentration on blood flow in a mouse model of femoral artery ligation. (**A**) Percentage increase of the ratio of ischemic/normal blood flow from Week 0. Data are expressed as mean±SE. *P<0.05 vs. control group. (**B**) Representative photographs of laser speckle perfusion imaging at 4 weeks. (**C**) Measurement of vessel number density by immunostaining with anit-CD31 antibody and (**D**) representative images. Arrows indicate the blood vessels counted. Scale bar=50 μm.

Effect of Recombinant bFGF Concentrations on Angiogenesis in Mice With Femoral Artery Administered With Recombinant bFGF With 3% Atelocollagen

When we administered 50, 100, and 200 μg of recombinant bFGF or saline with 3% of atelocollagen (n=10 in each group), blood flow increased up to 4 weeks after injection (**Figure 4A**,**B**), and there was no correlation between blood flow and the amount of recombinant bFGF (**Figure 4A**). Blood flow increased the most in the 100 μg of recombinant bFGF groups at 4 weeks after ligation (the ratio of ischemic limb/normal limb blood flow: 74.1±5.1% in recombinant bFGF, 56.4±3.2% in the control; P<0.05 vs. control; **Figure 4A**). The capillary densities investigated histologically (n=7 in each group) demonstrated a maximal increase in the 100 μg of recombinant bFGF group (1.3±0.1, 1.5±0.1, 1.2±0.1 in 50, 100, and 200 μg of recombinant bFGF groups, respectively; P<0.01 vs. control: 0.7±0.0; **Figure 4C**,**D**).

Discussion

The study findings are as follows. (1) The concentration of bFGF retained in the tissue at the administration site decreased over time; however, bFGF remained for up to 2 weeks post-administration in normal rabbits. Conversely,

bFGF was not detected in the serum. (2) At the administration site in normal rabbits, a significant increase in the number of blood vessels was observed 2 weeks after administration of recombinant bFGF with atelocollagen compared with saline or atelocollagen alone. (3) Blood flow in the hind limb with femoral artery ligation increased at 2 weeks after injection and more at 4 weeks after injection, and its effect was most significant in the femoral artery ligation model mice administered 100 μg of recombinant bFGF with 3% of atelocollagen.

These findings suggest that recombinant bFGF with atelocollagen maintained a concentration at the administration site and its vicinity that was sufficient to promote the proliferation of vascular endothelial cells for at least 2 weeks. This supports the sustained local angiogenic effect of recombinant bFGF, although we did not have data on other concentrations of bFGF in the rabbit model compared with those in the clinical study, and the hardness of the gel increased the diffusion distance of bFGF. Additionally, there appears to be no or minimal transfer into the bloodstream, indicating that intramuscular administration minimizes the potential for systemic side-effects, such as accelerated tumor growth due to angiogenesis. When the effect of atelocollagen concentration was examined in a mouse model of femoral artery ligation, blood flow and vessel number increased only in the group receiving the high concentration of 3% atelocollagen/100-μg recombinant bFGF. In contrast, when bFGF concentration was examined under 3% atelocollagen, the blood flow and vessel number increased the most in the group treated with 3% atelocollagen/100-μg recombinant bFGF, independent of the recombinant bFGF dose. This indicates that high concentrations of atelocollagen are warranted for bFGF to remain localized and that a certain concentration of bFGF, when it remains localized, exerts its angiogenic effects efficiently.

Clinical trials on topical bFGF have been conducted; however, its short half-life[3] and adverse reactions to high-dose systemic administration[10] preclude its practical use. In a single-arm clinical study conducted by Ono et al.[9] using recombinant bFGF with atelocollagen, no adverse events associated with the recombinant bFGF with atelocollagen were noted. Improvement in the visual analog scale scores was observed and was noted for 1 year. Given the increase in the number of blood vessels and blood flow in the femoral artery ligation model in the present study, the administration of recombinant bFGF with atelocollagen promoted angiogenesis and the formation of collateral blood vessels in the limb with ischemia. The promotion of angiogenesis and formation of collateral blood vessels may contribute to a reduction in pain due to limb ischemia. The findings of the present study and clinical trials suggest that recombinant bFGF with atelocollagen may lead to the medical treatment of limb ischemia through an increase in collateral circulation.

Current angiogenic therapies under development include methods for expressing target proteins through gene therapy and administration of vascular endothelial progenitor cells. Although a hepatocyte growth factor gene therapy drug for CLTI was conditionally approved in Japan ahead of the rest of the world with a 5-year time limit, the approval was not pursued primarily because of the lack of expected efficacy based on comparative studies. Cell therapy using autologous cells from the bone marrow or peripheral blood is promising,[11–15] although these methods require harvesting cells from patients, which is time consuming, invasive, and costly. Recombinant bFGF combined with atelocollagen is a potential solution to the problems faced by current angiogenic therapies. In Japan, recombinant bFGF is approved for the treatment of bedsores and skin ulcers, whereas atelocollagen is approved as a medical device for soft tissue injection into skin irregularities. Both studies have accumulated extensive safety data for humans. Furthermore, they are less invasive because they do not require collection of autologous cells. Consequently, recombinant bFGF with atelocollagen is expected to emerge as a new treatment for atherosclerosis obliterans in the lower extremities, bridging the gap between existing medical, surgical, and endovascular therapies. At present, a randomized controlled trial is underway to evaluate the efficacy and safety of recombinant bFGF with atelocollagen in patients with lower-extremity atherosclerosis obliterans, with the goal of clinical application.

Study Limitations

The present study has several limitations. First, we could not examine how bFGF was dispersed around the injected tissue, for example, the degradation mode of atelocollagen. We hypothesized that atelocollagen is degraded and bFGF is gradually released into the tissue, which is supported by the tissue homogenates of bFGF concentrations. Second, we did not note the long-term effects on angiogenesis or determine the time course at which the increase in blood flow ceased in mice with femoral artery ligation. Additionally, we did not have the data regarding angiographic validation for angiogenesis and/or collateral formation in vivo, nor pharmacokinetic data in mice with femoral artery ligation. Third, there are no data on dosing with gels that are stiffer than 3%. Higher concentrations of atelocollagen are more viscous and difficult to administer. We did not have data on the intact lumen and functional vessels. Fourth, the patent was first established and published (PCT/JP2011/073632) and included animal experimental data. In this study, we conducted additional experiments comprehensively and strengthened the novelty of angiogenesis using recombinant bFGF with atelocollagen.

Conclusions

Intramuscular administration of recombinant bFGF with atelocollagen appeared to induce angiogenesis between 2 and 4 weeks in both normal and ischemic hind limbs.

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Disclosures

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References

1. Fowkes FG, Rudan D, Rudan I, Aboyans V, Denenberg JO, McDermott MM, et al. Comparison of global estimates of prevalence and risk factors for peripheral artery disease in 2000 and 2010: A systematic review and analysis. *Lancet* 2013; **382:** 1329–1340.
2. Montesano R, Vassalli JD, Baird A, Guillemin R, Orci L. Basic fibroblast growth factor induces angiogenesis in vitro. *Proc Natl Acad Sci U S A* 1986; **83:** 7297–7301.

3. Edelman ER, Nugent MA, Karnovsky MJ. Perivascular and intravenous administration of basic fibroblast growth factor: Vascular and solid organ deposition. *Proc Natl Acad Sci U S A* 1993; **90:** 1513–1517.
4. Hirose K, Fujita M, Marui A, Arai Y, Sakaguchi H, Huang Y, et al. Combined treatment of sustained-release basic fibroblast growth factor and sarpogrelate enhances collateral blood flow effectively in rabbit hindlimb ischemia. *Circ J* 2006; **70:** 1190–1194.
5. Arai Y, Fujita M, Marui A, Hirose K, Sakaguchi H, Ikeda T, et al. Combined treatment with sustained-release basic fibroblast growth factor and heparin enhances neovascularization in hypercholesterolemic mouse hindlimb ischemia. *Circ J* 2007; **71:** 412–417.
6. Marui A, Tabata Y, Kojima S, Yamamoto M, Tambara K, Nishina T, et al. A novel approach to therapeutic angiogenesis for patients with critical limb ischemia by sustained release of basic fibroblast growth factor using biodegradable gelatin hydrogel: An initial report of the phase I-IIa study. *Circ J* 2007; **71:** 1181–1186.
7. Hashimoto T, Koyama H, Miyata T, Hosaka A, Tabata Y, Takato T, et al. Selective and sustained delivery of basic fibroblast growth factor (bFGF) for treatment of peripheral arterial disease: Results of a phase I trial. *Eur J Vasc Endovasc Surg* 2009; **38:** 71–75.
8. Kumagai M, Marui A, Tabata Y, Takeda T, Yamamoto M, Yonezawa A, et al. Safety and efficacy of sustained release of basic fibroblast growth factor using gelatin hydrogel in patients with critical limb ischemia. *Heart Vessels* 2016; **31:** 713–721.
9. Ono K, Yanishi K, Ariyoshi M, Kaimoto S, Uchihashi M, Shoji K, et al. First-in-man clinical pilot study showing the safety and efficacy of intramuscular injection of basic fibroblast growth factor with atelocollagen solution for critical limb ischemia. *Circ J* 2018; **83:** 217–223.
10. Lederman RJ, Mendelsohn FO, Anderson RD, Saucedo JF, Tenaglia AN, Hermiller JB, et al. Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): A randomised trial. *Lancet* 2002; **359:** 2053–2058.
11. Kondo K, Yanishi K, Hayashida R, Shintani S, Shibata R, Murotani K, et al. Long-term clinical outcomes survey of bone marrow-derived cell therapy in critical limb ischemia in Japan. *Circ J* 2018; **82:** 1168–1178.
12. Kawamoto A, Katayama M, Handa N, Kinoshita M, Takano H, Horii M, et al. Intramuscular transplantation of G-CSF-mobilized CD34(+) cells in patients with critical limb ischemia: A phase I/IIa, multicenter, single-blinded, dose-escalation clinical trial. *Stem Cells* 2009; **27:** 2857–2864.
13. Fujita Y, Kinoshita M, Furukawa Y, Nagano T, Hashimoto H, Hirami Y, et al. Phase II clinical trial of CD34+ cell therapy to explore endpoint selection and timing in patients with critical limb ischemia. *Circ J* 2014; **78:** 490–501.
14. Horie T, Yamazaki S, Hanada S, Kobayashi S, Tsukamoto T, Haruna T, et al. Outcome from a randomized controlled clinical trial: Improvement of peripheral arterial disease by granulocyte colony-stimulating factor-mobilized autologous peripheral-blood-mononuclear cell transplantation (IMPACT). *Circ J* 2018; **82:** 2165–2174.
15. Ohtake T, Mochida Y, Ishioka K, Oka M, Maesato K, Moriya H, et al. Autologous granulocyte colony-stimulating factor-mobilized peripheral blood CD34 positive cell transplantation for hemodialysis patients with critical limb ischemia: A prospective phase II clinical trial. *Stem Cells Transl Med* 2018; **7:** 774–782.

Supplementary Files

Please find supplementary file(s);
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