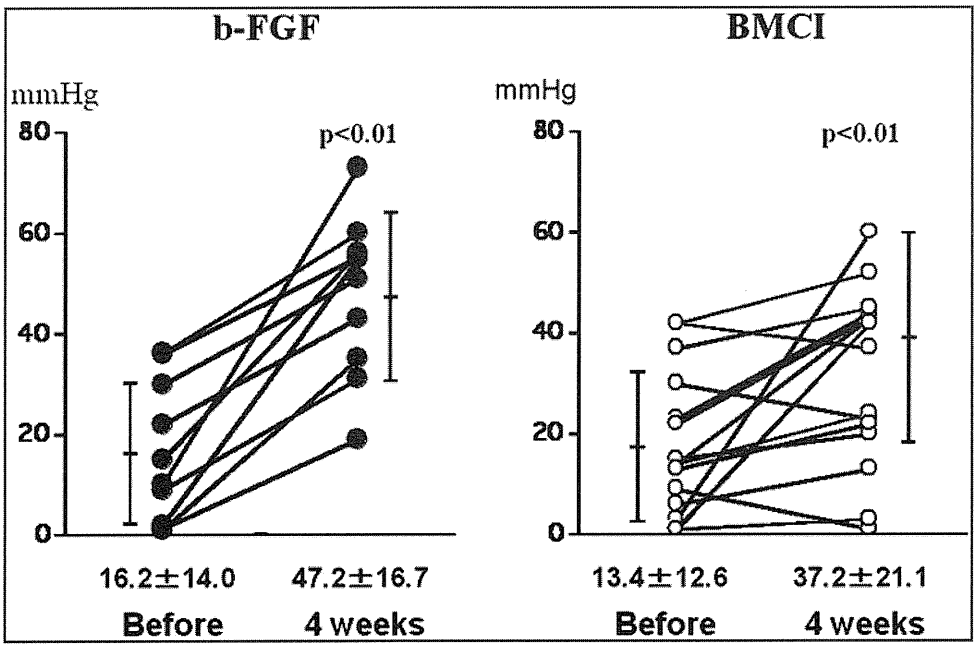

FIG. 5. The effect of controlled-release b-FGF therapy to transcutaneous oxygen tension. b-FGF therapy (left) and BMCI therapy (right).

pain scale (VAS). It was significantly decreased in both groups after treatment (b-FGF 67±32 to 4±5, $p<0.01$, BMCI 67±42 to 5±9 mm, $p<0.01$). Also, skin perfusion, determined by $TcPO_2$, was significantly increased 4 weeks after b-FGF treatment in both groups (b-FGF 16±14 to 47±17, $p<0.01$, BMCI 13±13 to 37±21 mmHg, $p<0.01$; Fig. 5). Serum b-FGF level was slightly elevated immediately after injection in two patients (Fig. 6, left), but it then returned to the normal range in the b-FGF group until 4 weeks. Urine protein level was not elevated after b-FGF treatment (Fig. 6, right). Figure 7 indicates the representative picture of foot ulcer before and after the therapy.

Two-year prognosis after administration was not different between the groups (b-FGF 91%, BMCI 80% $p=0.502$; Fig. 8). One patient died because of ischemic heart disease, but there was no amputation case in the b-FGF group. Three patients died because of pneumonia ($n=2$) and sepsis ($n=1$), including three amputation cases in the BMCI group. No limb amputation was found in survived patients in the BMCI group. Rest pain scale remains zero for 2 years in all patients except for event cases in both groups.

Discussion

Even though recent medical progress has achieved effective vascular regenerative therapy, the underlying ischemic condition or disease status is not simple and sometimes results in an undesirable clinical outcome. For example, on clinical observation, intractable ischemic ulcers occur in patients with vasculitis or auto-immune disease. The effects of BMCI for ischemic condition were confirmed; however, it is unknown whether this autologous bone marrow, which containing immune-related cells, may worsen auto-immune related ischemia by immune modulation. Thus, one of the

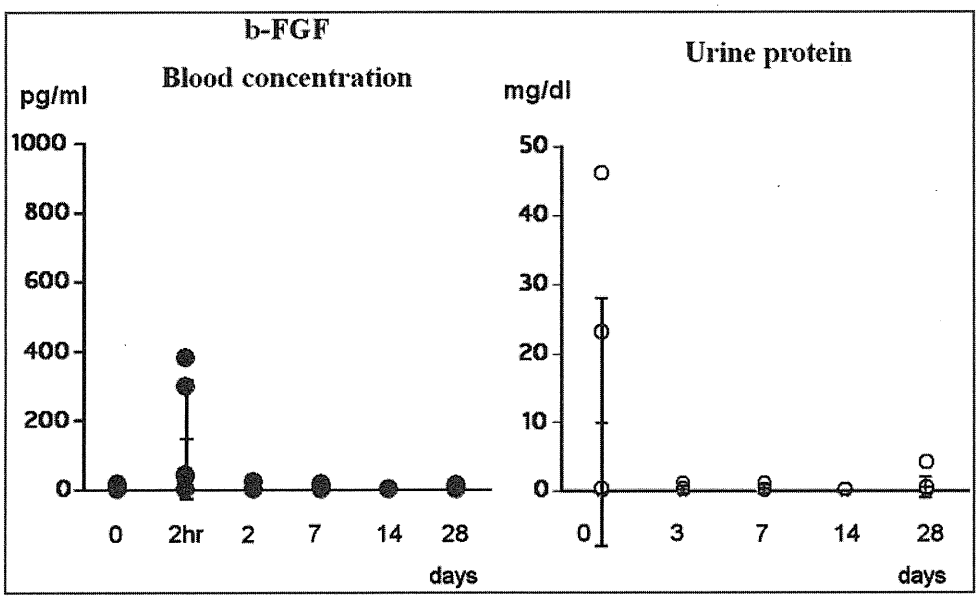

FIG. 6. Serum b-FGF concentration and urine protein level: b-FGF blood concentration (left) and urine protein level (right).

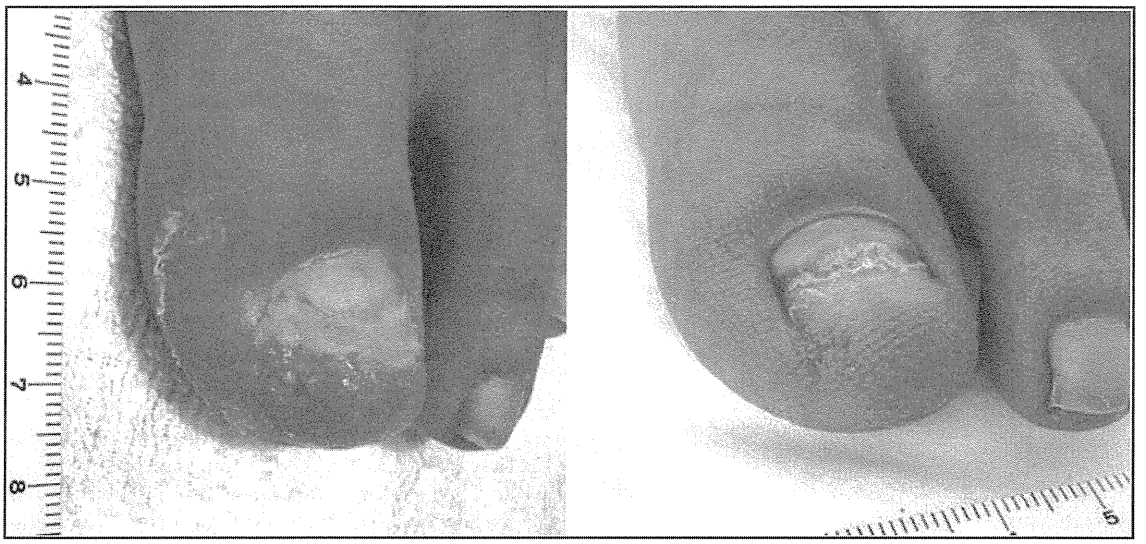

FIG. 7. Representative picture. Digitus primus ulceration of the left leg. Before treatment on the left and after treatment on the right.

new concepts is to establish alternative angiogenesis therapy for many disease states. We experienced a case in which this controlled-release b-FGF protein had potential clinical benefit, in a patient with allergic vasculitis due to Churg-Strauss syndrome under no systemic immune modulation.[15] To examine this concept, a phase I to II study in arteriosclerosis obliterans or Buerger's disease was designed as a pilot study at this investigation. We also achieved standardization, the purification profile, and a controlled delivery system of b-FGF protein as a therapeutic angiogenesis. The major findings of this study were restoration of blood flow by b-FGF, confirmed by skin perfusion examinations in the ischemic area, and safety, confirmed until 4 weeks after

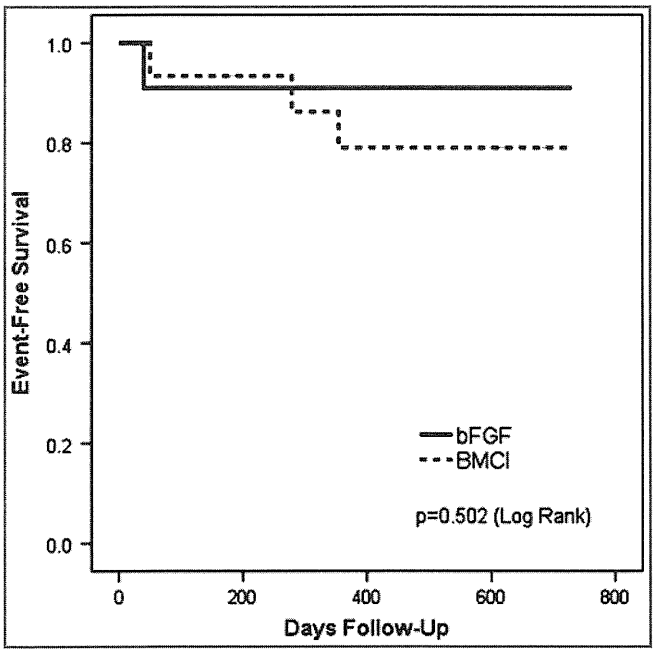

FIG. 8. Event-free survival. Data represent Kaplan–Meier curves for all cause mortality during 2-year follow-up in each group.

administration, resulted in better 2 years prognosis. Also, the effectiveness showed the same tendency as that of BMCI. A clinical study utilizing b-FGF showed that bolus administration was preferable in terms of efficacy.[18–23] On the other hand, because b-FGF is water soluble, unbound free b-FGF, administered directly into the artery, resulted in an extremely high b-FGF blood concentration such as 60,000 pg/mL.[23] It has also been reported that intra-arterial injection was associated with the development of glomerulosclerosis in an experimental model.[24–27] A clinical double-blind placebo-controlled trial reported that gross proteinuria occurred after b-FGF administration.[28] To resolve this problem, Tabata *et al.* developed a novel technique, which incorporated a variety of substances with biodegradable gelatin hydrogel enabling b-FGF to be released at the site of action for 2–4 weeks.[29,30] When this gelatin incorporated with bFGF was administered intramuscularly, it minimized side effects, avoided loss to the systemic circulation, and yielded a higher local concentration and maintained it compared with arterial administration,[31] maximizing clinical effectiveness. Our results indicated that b-FGF blood concentration was very low (< 10 pg/mL). Thus, our data support the safety and efficacy of controlled-release b-FGF administration in clinical situations even in small sample size. A recent basic science report suggested that controlled-release b-FGF combined with stem cells is an effective approach.[32] Thus, this investigation may provide important clinical information to progress future vascular regenerative therapy.

Limitations

Because of ethical and clinical considerations, treatment was not randomized, and this bias reflects to worse Fontaine classification in BMCI group and the data favor of b-FGF group. Also, from the methodological aspect, the study was open labeled, which reflects to the VAS value, but not to ABI, $TcPO_2$, and Tc-TF value, because automated scoring was made by equipments. Because injection amount was standardized between the groups, different injection points may affect the result. Even with careful injection, the injection needle hit vessels on administration in two patients, which

was confirmed by the b-FGF blood concentration at first pass (Fig. 2). Thus, technical failure may occur.

In summary, controlled-release b-FGF protein therapy was performed in 10 patients with PAD. Four weeks after therapy, pain scale and $TcPO_2$ were significantly improved to similar extent as that with BMCI, and 2-year prognosis was similar to BMCI.

Conclusion

Controlled-release b-FGF protein therapy was found to be safe, and its efficacy appeared to be comparable to that of BMCI. Thus, this therapy may be able to become an alternative to BMCI.

Acknowledgments

The authors wish to thank Harumi Ohtsubo for technical assistance with protein preparation. This work was supported by a Grant-in-Aid for Scientific Research (C, No. 20590844).

Disclosure Statement

There is no disclosure except the **above funding source.** This investigation was an academia-initiated exploratory phase I–IIa study. There was no external sponsor involved in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in this study and had final responsibility for the decision to submit the article for publication.

References

1. Carmeliet, P. Angiogenesis in health and disease. Nat Med **9,** 653, 2003.
2. Folkman, J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov **6,** 273, 2007.
3. Moreno, P.R., Sanz, J., and Fuster, V. Promoting mechanisms of vascular health: circulating progenitor cells, angiogenesis, and reverse cholesterol transport. J Am Coll Cardiol **53,** 2315, 2009.
4. Alpert, J.S., Larsen, O.A., and Lassen, N.A. Exercise and intermittent claudication. Blood flow in the calf muscle during walking studied by the xenon-133 clearance method. Circulation **39,** 353, 1969.
5. Kutryk, M.J., and Stewart, D.J. Angiogenesis of the heart. Microsc Res Tech **60,** 138, 2003.
6. Lundgren, F., Dahllof, A.G., Lundholm, K., Schersten, T., and Volkmann, R. Intermittent claudication—surgical reconstruction or physical training? A prospective randomized trial of treatment efficiency. Ann Surg **209,** 346, 1989.
7. Nagy, J.A., Dvorak, A.M., and Dvorak, H.F. VEGF-A(164/165) and PlGF: roles in angiogenesis and arteriogenesis. Trends Cardiovasc Med **13,** 169, 2003.
8. Cao, Y., Hong, A., Schulten, H., and Post, M.J. Update on therapeutic neovascularization. Cardiovasc Res **65,** 639, 2005.
9. Simons, M., and Ware, J.A. Therapeutic angiogenesis in cardiovascular disease. Nat Rev Drug Discov **2,** 863, 2003.
10. Kamihata, H., Matsubara, H., Nishiue, T., *et al.* Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation **104,** 1046, 2001.
11. Uemura, R., Xu, M., Ahmad, N., and Ashraf, M. Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. Circ Res **98,** 1414, 2006.
12. Tateno, K., Minamino, T., Toko, H., *et al.* Critical roles of muscle-secreted angiogenic factors in therapeutic neovascularization. Circ Res **98,** 1194, 2006.
13. Tabata, Y., Nagano, A., and Ikada, Y. Biodegradation of hydrogel carrier incorporating fibroblast growth factor. Tissue Eng **5,** 127, 1999.
14. Miyamoto, M., Yasutake, M., Takano, H., *et al.* Therapeutic angiogenesis by autologous bone marrow cell implantation for refractory chronic peripheral arterial disease using assessment of neovascularization by 99mTc-tetrofosmin (TF) perfusion scintigraphy. Cell Transplant **13,** 429, 2004.
15. Kawanaka, H., Takagi, G., Miyamoto, M., *et al.* Therapeutic angiogenesis by controlled-release fibroblast growth factor in a patient with Churg-Strauss syndrome complicated by an intractable ischemic leg ulcer. Am J Med Sci **338,** 341, 2009.
16. Marui, A., Tabata, Y., Kojima, S., *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 **71,** 1181, 2007.
17. Tabata, Y., and Ikada, Y. Vascularization effect of basic fibroblast growth factor released from gelatin hydrogels with different biodegradabilities. Biomaterials **20,** 2169, 1999.
18. Simons, M., Annex, B.H., Laham, R.J., *et al.* Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial. Circulation **105,** 788, 2002.
19. Laham, R.J., Sellke, F.W., Edelman, E.R., *et al.* Local perivascular delivery of basic fibroblast growth factor in patients undergoing coronary bypass surgery: results of a phase I randomized, double-blind, placebo-controlled trial. Circulation **100,** 1865, 1999.
20. Schumacher, B., Pecher, P., von Specht, B.U., and Stegmann, T. Induction of neoangiogenesis in ischemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease. Circulation **97,** 645, 1998.
21. Schumacher, B., Stegmann, T., and Pecher, P. The stimulation of neoangiogenesis in the ischemic human heart by the growth factor FGF: first clinical results. J Cardiovasc Surg (Torino) **39,** 783, 1998.
22. Lederman, R.J., Mendelsohn, F.O., Anderson, R.D., *et al.* Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): a randomised trial. Lancet **359,** 2053, 2002.
23. Lazarous, D.F., Unger, E.F., Epstein, S.E., *et al.* Basic fibroblast growth factor in patients with intermittent claudication: results of a phase I trial. J Am Coll Cardiol **36,** 1239, 2000.
24. Floege, J., Hudkins, K.L., Eitner, F., *et al.* Localization of fibroblast growth factor-2 (basic FGF) and FGF receptor-1 in adult human kidney. Kidney Int **56,** 883, 1999.
25. Floege, J., Burg, M., Hugo, C., *et al.* Endogenous fibroblast growth factor-2 mediates cytotoxicity in experimental mesangioproliferative glomerulonephritis. J Am Soc Nephrol **9,** 792, 1998.
26. Floege, J., Kriz, W., Schulze, M., *et al.* Basic fibroblast growth factor augments podocyte injury and induces glomerulosclerosis in rats with experimental membranous nephropathy. J Clin Invest **96,** 2809, 1995.

27. Francki, A., Uciechowski, P., Floege, J., von der Ohe, J., Resch, K., and Radeke, H.H. Autocrine growth regulation of human glomerular mesangial cells is primarily mediated by basic fibroblast growth factor. Am J Pathol **147,** 1372, 1995.
28. Cooper, L.T., Jr., Hiatt, W.R., Creager, M.A., *et al.* Proteinuria in a placebo-controlled study of basic fibroblast growth factor for intermittent claudication. Vasc Med **6,** 235, 2001.
29. Tabata, Y., Miyao, M., Yamamoto, M., and Ikada, Y. Vascularization into a porous sponge by sustained release of basic fibroblast growth factor. J Biomater Sci Polym Ed **10,** 957, 1999.
30. Tabata, Y., Hijikata, S., Muniruzzaman, M., and Ikada, Y. Neovascularization effect of biodegradable gelatin microspheres incorporating basic fibroblast growth factor. J Biomater Sci Polym Ed **10,** 79, 1999.
31. Hashimoto, T., Koyama, H., Miyata, 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 **38,** 71, 2009.
32. Takehara, N., Tsutsumi, Y., Tateishi, K., *et al.* Controlled delivery of basic fibroblast growth factor promotes human cardiosphere-derived cell engraftment to enhance cardiac repair for chronic myocardial infarction. J Am Coll Cardiol **52,** 1858, 2008.

Address correspondence to:
Gen Takagi, M.D., Ph.D.
Division of Regenerative Medicine and Cardiology
Department of Internal Medicine
Nippon Medical School
1-1-5, Sendagi, Bunkyo-ku
Tokyo 113-8603
Japan

E-mail: gen52@nms.ac.jp

Received: September 03, 2010
Accepted: June 17, 2011
Online Publication Date: July 29, 2011

Geriatrics Gerontology 1st Impact Factor in June 2010 Medline Indexed

Geriatr Gerontol Int 2011; **11:** *527–530*

LETTER TO THE EDITOR

Novel approach to ischemic skin ulcer in systemic lupus erythematosus: Therapeutic angiogenesis by controlled-release basic fibroblast growth factor

Shuhei Tara,[1] Gen Takagi,[1] Masaaki Miyamoto,[1] Sonoko Kirinoki-Ichikawa,[1] Teppei Yamamoto,[1] Hitoshi Takano,[1] Ikuyo Takagi,[1] Masahiro Yasutake,[1] Yasuhiko Tabata[2] and Kyoichi Mizuno[1]

[1]Division of Cardiology and Regenerative Medicine, Department of Internal Medicine, Nippon Medical School, Tokyo, and [2]Department of Biomaterials, Frontier Medical Sciences, Kyoto University, Kyoto, Japan

Dear Editor,

Systemic lupus erythematosus (SLE) frequently involves the vascular system, and the acquired inflammatory process is known to accelerate atherosclerosis. We report a 65-year-old woman with SLE complicated by an ischemic skin ulcer of the superior portion of the right lateral malleolus which was untreatable by local therapy. The ankle–brachial index was reduced to 0.72 in the right leg, and transcutaneous oxygen tension ($TcPO_2$) at the dorsum of the right foot was 1.0 mmHg (normal limit: >30 mmHg) with no increase after pure oxygen inhalation. Angiography revealed total occlusions of all the below-the-knee arteries with poor distal collateral circulation, indicating an Inter-Society Consensus type D lesion. Because the patient's SLE disease activity index was 11 points under the administration of prednisolone and methotrexate, invasive therapy by percutaneous or surgical revascularization was not preferable. Thus, we decided to perform therapeutic angiogenesis by administration of controlled-release basic fibroblast growth factor (bFGF)-incorporated biodegradable gelatin hydrogel. Four weeks after the therapy, there was no significant improvement in ankle–brachial index (ABI) and angiographic findings. Instead, basal $TcPO_2$ was increased to 10 mmHg with an additional increase up to 39 mmHg by oxygen inhalation. Furthermore, the ulcer healed completely without any complications. This result suggests that bFGF protein therapy using the drug delivery system can be a promising approach to intractable skin ulcers complicating SLE.

Systemic lupus erythematosus is manifested by an interaction between susceptible genes and environmental risk factors, which leads to autoimmune reactions. As the next step, generated autoantibodies and immune complexes deposited in tissue activate complement, induce inflammation and lead to irreversible organ damage.[1] SLE frequently involves the vascular system, and the occurring inflammation accelerates atherosclerosis. Roman *et al.* reported that the prevalence of atherosclerosis was significantly higher in patients with SLE, and the accelerated atherosclerosis was not attributable to traditional cardiovascular risk factors.[2] Furthermore, progressive ischemic skin ulcers, requiring limb amputation, occasionally complicate SLE.

Previously, we demonstrated that i.m. administration of controlled-release bFGF-incorporated biodegradable gelatin hydrogel successfully improved leg ischemia and cured a skin ulcer in a patient with Churg–Strauss syndrome.[3] In the present case, we applied this novel treatment approach to a patient with SLE complicated by an unhealed skin ulcer, and achieved successful improvement of the local skin blood perfusion and healing of the skin ulcer 4 weeks after the therapy.

A 65-year-old woman, diagnosed with SLE at 42 years old, was referred to our hospital with resting pain of her right foot complicated by a skin ulcer of the superior portion of her right lateral malleolus (Fig. 1a), which was not improved by topical and systemic medication for 1 month. As a conventional therapy for peripheral arterial disease, cilostazol and prostaglandin agents were used before admission, and

doi: 10.1111/j.1447-0594.2011.00695.x

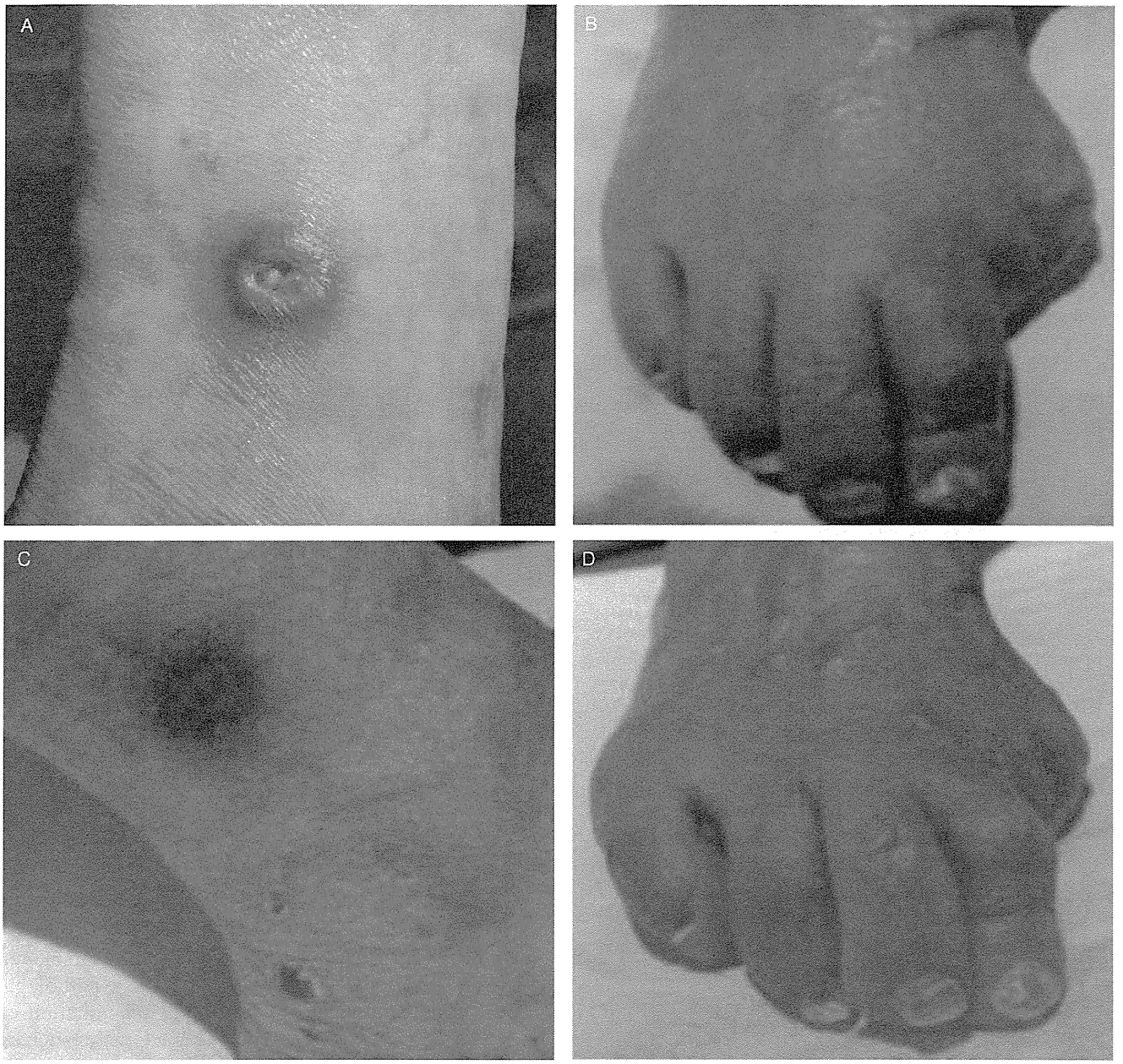

Figure 1 (a) Skin ulcer of superior portion of right lateral malleolus and (b) cyanosis of right toes and edema of dorsum of right foot before therapy. Four weeks after therapy, (c) the skin ulcer had healed completely, and (d) cyanosis and edema had improved.

that was continued through the follow-up period. She had been given prednisolone as standard treatment since 42 years of age and maintained on 5 mg until now. Cyanosis of the right toes and edema of the dorsum of the right foot were also noted (Fig. 1b). Blood examination showed elevated C-reactive protein (0.75 mg/dL), immunoglobulin G (2193 mg/dL), anti-centromere antibody (68.4 index) and anti-double-stranded DNA antibody (23 IU/mL) levels. The SLE disease activity index was 11 points, indicating an active state.[4] Angiography revealed total occlusions of the right anterior tibial artery, right posterior tibial artery and right peroneal artery, and the dorsalis pedis artery was barely supplied by collateral circulation (Fig. 2). Severe ischemia was shown by blood flow examinations, reduced ABI in the right leg (0.72), severely reduced $TcPO_2$ at the dorsum of the right foot (1.0 mmHg; normal limit: >30 mmHg) and absence of $TcPO_2$ response to pure oxygen inhalation. From this clinical profile and angiographic findings, the advisory committee (consisting of cardiologists, vascular surgeons, plastic surgeons, radiologists and anesthetists)

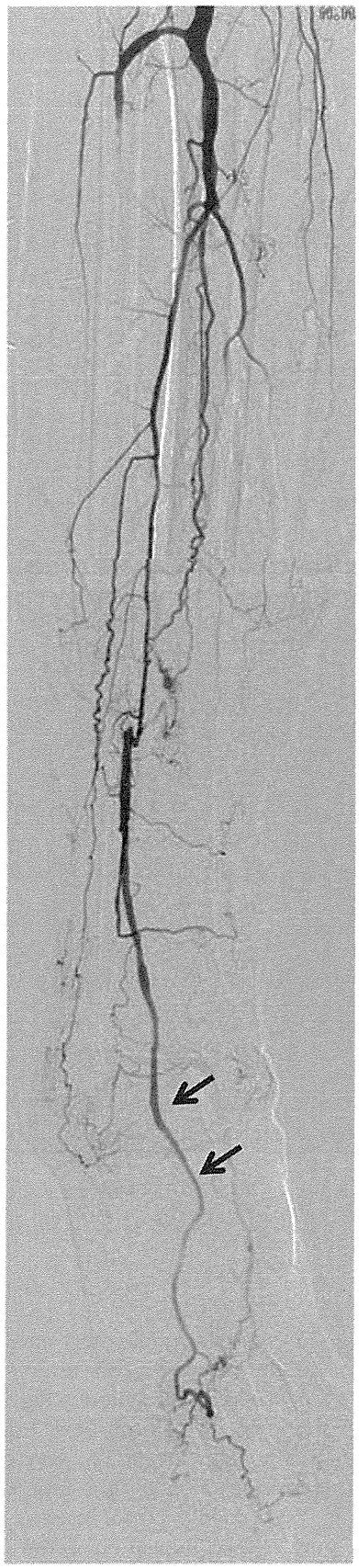

Figure 2 Digital subtraction angiography revealed total occlusions of right anterior tibial artery, right posterior tibial artery and right peroneal artery. The dorsalis pedis artery was enhanced by collateral circulation (arrow).

determined that these vascular lesions were unsuitable for percutaneous or surgical revascularization. We then decided to apply therapeutic angiogenesis by administration of controlled-release bFGF-incorporated gelatin hydrogel to improve local blood perfusion as an alternative treatment for limb salvage.

Gelatin hydrogel was prepared as previously described,[5] and impregnated with an aqueous solution containing recombinant human bFGF, followed by leaving it at 4°C for over 12 h to obtain bFGF-incorporated gelatin hydrogel. Under general anesthesia, we injected bFGF (600 μg)-incorporated gelatin hydrogel directory into the calf muscle at 20 sites around the ulcer.

Four weeks after the therapy, resting pain had disappeared, and the ulcer had healed completely (Fig. 1c). Cyanosis of the right toes and edema of the right dorsal foot were also improved (Fig. 1d). Local skin perfusion determined by $TcPO_2$ was improved (10 mmHg) and maximum $TcPO_2$ was enhanced (39 mmHg), although angiographic findings and ABI (0.66) were not changed. Dosage of prednisolone (5 mg/day) and methotrexate (2 mg/week) for SLE were not altered during the period. Serological findings reflecting SLE activity, such as serum complement level, serum albumin level and white blood cells count, and SLE disease activity index did not change after the therapy.

In cases of peripheral arterial disease associated with connective tissue diseases such as SLE, we have experienced that those patients are especially unsuitable for catheter and surgical revascularization due to pathological characteristics such as continuous inflammation of the vascular bed, and anatomical characteristics such as distal arterial obstruction. For such patients, there are some reports that therapeutic angiogenesis by implantation of autologous mononuclear cells obtained from bone marrow or peripheral blood into the ischemic lesion is effective.[6,7] On the other hand, there is a report that endothelial progenitor cell function is inhibited, especially in SLE patients treated with corticosteroids,[8] and also production of angiogenic factors from bone marrow cells is suppressed.[9,10] Thus, the effects of implantation of bone marrow mononuclear cells as therapeutic angiogenesis are inconclusive and still under debate. Here, we propose a novel protein therapy using bFGF that is simpler and less invasive than the cell transplantation.

First, we focused on a single growth factor, bFGF, which is known to play a major role in angiogenesis. This factor modulates proliferation and migration of

ndothelial cells in the early phase, and contributes to vessel maturation by matrix deposition and pericyte recruitment in the late phase.[11] Thus, bFGF is considered to be suitable for therapeutic angiogenesis.[12] Second, because bFGF plays an important role in granulation tissue formation, re-epithelialization and tissue remodeling,[13] administration of bFGF in ischemic tissue is thought to be effective in ulcer healing. Third, human recombinant bFGF is clinically available in Japan. Fourth, a drug delivery system has been utilized in order to maximally potentiate the drug effect, by means of a prolonged clinical half-life of the drug through slow and stable release.[14,15] As mentioned earlier, the therapeutic effect was confirmed in a patient with Churg–Strauss syndrome who developed an intractable leg ulcer with a similar pathogenic background to SLE.[3] Thus, we thought this therapy would be safe and effective also in this elderly patient with SLE.

In the present case, we found that $TcPO_2$ reflecting local microcirculation was increased after the therapy, whereas angiographic findings and ABI were not changed. It can be inferred that the therapeutic angiogenesis might increase regional microperfusion sufficient to heal a wound by enhancing mainly neocapillary formation rather than arteriogenesis which may result in an elevation of leg blood pressure.[16]

In summary, application of controlled-release bFGF successfully improved local skin blood flow, and eventually cured the ischemic skin ulcer in this case. Our findings suggest a novel clinical approach to ischemic skin ulcers associated with SLE.

Acknowledgments

We thank Ms Harumi Ohtsubo for technical assistance for preparing gelatin hydrogel. The authors have no conflict of interest to declare.

References

1 Sitia S, Atzeni F, Sarzi-Puttini P *et al.* Cardiovascular involvement in systemic autoimmune diseases. *Autoimmun Rev* 2009; **8**: 281–286.
2 Roman MJ, Shanker BA, Davis A *et al.* Prevalence and correlates of accelerated atherosclerosis in systemic lupus erythematosus. *N Engl J Med* 2003; **349**: 2399–2406.
3 Kawanaka H, Takagi G, Miyamoto M *et al.* Therapeutic angiogenesis by controlled-release fibroblast growth factor in a patient with Churg-Strauss syndrome complicated by an intractable ischemic leg ulcer. *Am J Med Sci* 2009; **338**: 341–342.
4 Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. *Arthritis Rheum* 1992; **35**: 630–640.
5 Tabata Y, Yamada K, Miyamoto S *et al.* Bone regeneration by basic fibroblast growth factor complexed with biodegradable hydrogels. *Biomaterials* 1998; **19**: 807–815.
6 Kamata Y, Takahashi Y, Iwamoto M *et al.* Local implantation of autologous mononuclear cells from bone marrow and peripheral blood for treatment of ischaemic digits in patients with connective tissue diseases. *Rheumatology* 2007; **46**: 882–884.
7 Takahashi M, Izawa A, Ishigatsubo Y *et al.* Therapeutic neovascularization by the implantation of autologous mononuclear cells in patients with connective tissue diseases. *Curr Pharm Des* 2009; **15**: 2778–2783.
8 Grisar J, Steiner CW, Bonelli M *et al.* Systemic lupus erythematosus patients exhibit functional deficiencies of endothelial progenitor cells. *Rheumatology* 2008; **47**: 1476–1483.
9 Nauck M, Karakiulakis G, Perruchoud AP, Papakonstantinou E, Roth M. Corticosteroids inhibit the expression of the vascular endothelial growth factor gene in human vascular smooth muscle cells. *Eur J Pharmacol* 1998; **341**: 309–315.
10 Greenberger S, Boscolo E, Adini I, Mulliken JB, Bischoff J. Corticosteroid suppression of VEGF-A in infantile hemangioma-derived stem cells. *N Engl J Med* 2010; **362**: 1005–1013.
11 Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev* 2005; **16**: 159–178.
12 Doi K, Ikeda T, Marui A *et al.* Enhanced angiogenesis by gelatin hydrogels incorporating basic fibroblast growth factor in rabbit model of hind limb ischemia. *Heart Vessels* 2007; **22**: 104–108.
13 Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen* 2008; **16**: 585–601.
14 Tabata Y, Nagano A, Ikada Y. Biodegradation of hydrogel carrier incorporating fibroblast growth factor. *Tissue Eng* 1999; **5**: 127–138.
15 Tabata Y, Hijikata S, Muniruzzaman M, Ikada Y. Neovascularization effect of biodegradable gelatin microspheres incorporating basic fibroblast growth factor. *J Biomater Sci Polym Ed* 1999; **10**: 79–94.
16 Matoba S, Tatsumi T, Murohara T *et al.* Long-term clinical outcome after intramuscular implantation of bone marrow mononuclear cells (Therapeutic Angiogenesis by Cell Transplantation [TACT] trial) in patients with chronic limb ischemia. *Am Heart J* 2008; **156**: 1010–1018.

Enhanced Vascularization by Controlled Release of Platelet-Rich Plasma Impregnated in Biodegradable Gelatin Hydrogel

Jiro Kurita, MD, Masaaki Miyamoto, MD, FACS, Yosuke Ishii, MD, Junya Aoyama, MD, Gen Takagi, MD, Zenya Naito, MD, Yasuhiko Tabata, PhD, DMedSci, Masami Ochi, MD, and Kazuo Shimizu, MD, FACS

Department of Biological Regulation and Regenerative Surgery, Cardiovascular Surgery, Nippon Medical School Graduate School of Medicine, and Departments of Regenerative Medicine and Pathology, Nippon Medical School, Tokyo; and Institute for Frontier Medical Science, Kyoto University, Kyoto, Japan

Background. Platelet-rich plasma (PRP) contains numerous growth factors that have angiogenic activities. However, the PRP-induced angiogenesis is limited by the short half-life period of growth factors. A new drug delivery system of biodegradable gelatin hydrogel was designed to achieve the controlled release of growth factors in PRP. The purpose of this study is to demonstrate the therapeutic efficacy of slow-release of PRP in the inducing of angiogenesis for critical ischemia.

Methods. The PRP was prepared from the whole blood of inbred rats. Thirty-two rats underwent excision of the left femoral artery and its branches to create critical limb ischemia. The rats were randomized into four groups (n = 8 each): no treatment (control), intramuscular injection of platelet-poor plasma (PPP), PRP only, or a combination of PRP and gelatin hydrogel (PRP+Gel). Four weeks after the treatment, angiogenesis was evaluated by laser doppler, microangiogram, and immunohistology.

Results. The resultant number of platelets for PRP was higher than that of PPP ($p < 0.01$). The concentrations of vascular endothelial growth factor, transforming growth factor-β1, and platelet-derived growth factor-BB were significantly higher in PRP animals than in PPP ($p < 0.01$). Although the PRP group improved tissue blood flow (82.7% ± 6.2%) compared with the control group or PPP group (69.6 ± 12.2 or 72.2 ± 11.8%, $p < 0.05$), the improvement of blood flow in the PRP+Gel group was significantly better (95.1% ± 8.0%, $p < 0.05$) than in the PRP group. Angiographic score in the PRP+Gel group was significantly higher than that in the control, PPP, and PRP groups (8.6 ± 2.1 versus 3.8 ± 0.8, 3.7 ± 0.6, and 5.6 ± 1.5, respectively; $p < 0.01$). Capillary density also increased immunohistologically in the PRP+Gel group when compared with the control, PPP, and PRP groups ($p < 0.01$).

Conclusions. A controlled release system of PRP was effective in inducing angiogenesis for critical ischemia. The biodegradable gelatin hydrogel incorporating PRP as applicable could possibly be used to treat for patients with ischemic cardiomyopathy.

(Ann Thorac Surg 2011;92:837–44)

In recent years, angiogenesis research has made remarkable progress in the fields of ischemic heart and limb diseases. Various therapeutic angiogenesis techniques including gene therapy using potent growth factors have been reported, some with positive results in animal studies or clinical trials [1-3]. However, most of these approaches were based on the biological effect of a single recombinant angiogenic molecule. For the critical ischemic cases, it seems to have be limited because the establishment of stable and functional blood vessel networks is a complex process and requires several angiogenic factors to stimulate the vessels sprouting and remodeling the primitive vascular network [4]. Moreover, there are some major concerns about adverse effects of high-dose single recombinant growth factors, as well as immune and inflammatory reactions shown in previous studies [5].

Conversely, it had been described previously that a combination of growth factors synergistically induced angiogenesis and long-lasting functional vessels compared with single growth factor [6, 7]. Platelet-rich plasma (PRP) is an autologous concentration of platelets in a small volume of plasma and contains biologically determined ratios of various autologous growth factors in high concentrations [8]. It is also cost saving, biologically safe, and has conventional availability. Moreover, it has been already used clinically in the dentistry, maxillofacial surgery, and plastic surgery fields for the application of regenerating damaged tissue [8, 9].

However, application of PRP is currently limited because of its short half-life period [8, 10]. Since the PRP

Accepted for publication April 22, 2011.

Presented at the Forty-seventh Annual Meeting of The Society of Thoracic Surgeons, San Diego, CA, Jan 31–Feb 2, 2011.

Address correspondence to Dr Miyamoto, Department of Regenerative Medicine, Nippon Medical School Graduate School of Medicine, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan; e-mail: miyamoto-m@nms.ac.jp.

Published by Elsevier Inc

0003-4975/$36.00
doi:10.1016/j.athoracsur.2011.04.084

Abbreviations and Acronyms

IEP	= isoelectric point
LDPI	= laser doppler perfusion imaging
PBS	= phosphate-buffered saline
PDGF-BB	= platelet-derived growth factor-BB
PPP	= platelet-poor plasma
PRP	= platelet-rich plasma
TGF-β1	= transforming growth factor-β1
VEGF	= vascular endothelial growth factor
vWF	= von Willebrand factor

and growth factors dissolve into blood stream in just a couple of days after injection, it is difficult to maintain its presence in the tissue long enough for the promotion of effective vascularization [8]. To resolve this disadvantage, we employed a novel drug delivery system utilizing biodegradable gelatin hydrogel [11]. The biodegradable gelatin hydrogel can control the timing of the release of the growth factors to ischemic regions. Because platelets are activated by impregnation of the PRP into the biodegradable gelatin hydrogel, growth factors are secreted from the platelets [12]. The secreted growth factors are immobilized in the hydrogel through electrical and physicochemical interaction with gelatin molecules (Fig 1). Degradation of the hydrogel impregnated with growth factors allows for slow release of growth factors into the tissue over a period of about 2 weeks after the injection of PRP and biodegradable gelatin hydrogel into the ischemic tissue, resulting in effective angiogenesis [12, 13].

The purpose of this study was to verify the effectiveness of the controlled release of PRP using the biodegradable gelatin hydrogel in inducing angiogenesis for critical limb ischemia.

Material and Methods

Experimental Animals

All animals received humane care in compliance with the "Principles of Laboratory Animal Care," formulated by the National Society for Medical Research, and the "Guide for the Care and Use of Laboratory Animals," prepared by the National Academy of Science and published by the National Institute of Health (NIH Publication 86-23, revised 1985). In addition, this study protocol was approved by the Animal Ethics Committee of Nippon Medical School (approval number 21-145), and was performed according to the "Guidelines for Regulation of Animal Experiments" of Nippon Medical School. Male Wistar rats (Sankyo Labo Service, Tokyo, Japan), weighing 380 to 400 g and aged 10 to 12 weeks were used for the experiments. Animals were maintained in a room controlled for temperature and light and were provided food and water ad libitum. These isogenic rats were used as donors and recipients of PRP to simulate autologous implantation.

Preparation of PRP and Platelet-Poor Plasma

Whole blood (7 to 10 mL) was preoperatively drawn from an inbred male Wistar rat through cardiac puncture with an 18G needle (Terumo, Tokyo, Japan) into tubes containing 3.8% sodium citrate, under general anesthesia

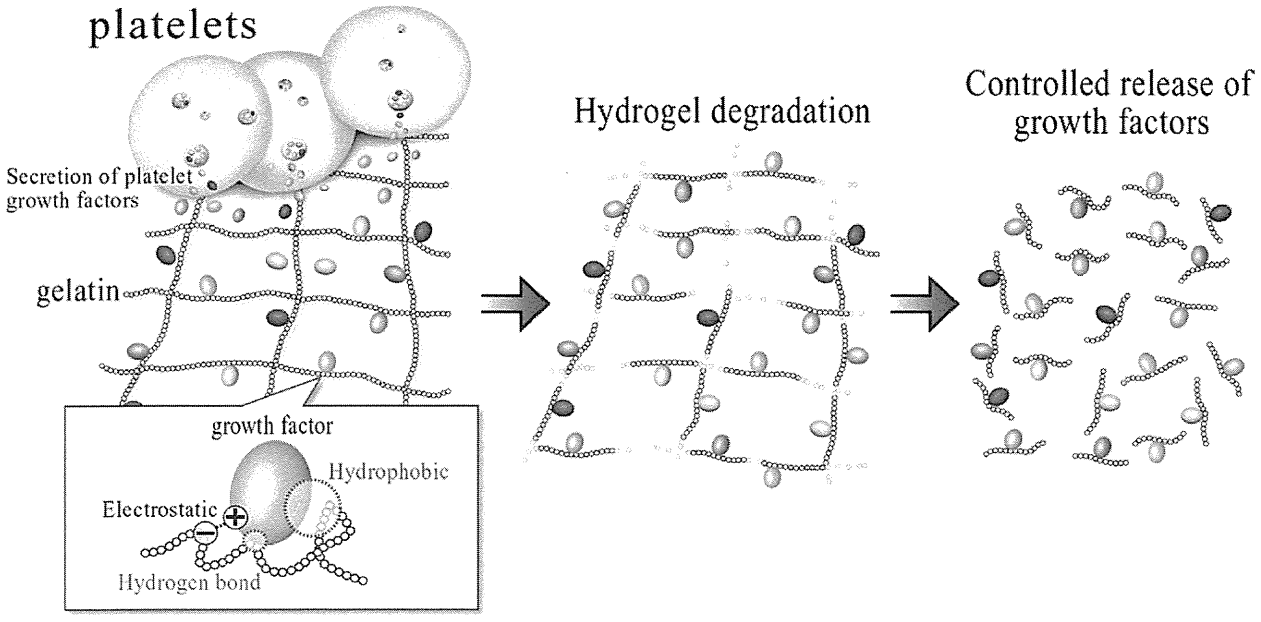

Fig 1. Schematic illustration of the controlled release of growth factors contained in platelet-rich plasma (PRP). When platelets are activated by contact with gelatin hydrogel molecules, growth factors are released from the platelets. The growth factors are immobilized in the gelatin hydrogel through electrical and physiochemical interaction with gelatin molecules. The immobilized growth factors are released as a result of hydrogel degradation.

with pentobarbital sodium at 50 mg/kg. The blood was first centrifuged in a standard laboratory centrifuge machine (Kubota 2700, Tokyo, Japan) for 10 minutes at 1,700 rpm (450*g*). The supernatant plasma was collected along with the buffy coat, which consists of platelets and leukocytes, into a neutral tube using a long cannula. A second centrifugation at 2,300 rpm (850*g*) was performed for 15 minutes to concentrate the platelets. The infranatant fluid containing the buffy coat at the bottom of the centrifuge tube was collected with 300 μL of its supernatant, platelet-poor plasma (PPP) to yield the final PRP product. The final platelet concentrations in whole blood (n = 15), PPP (n = 15), and PRP (n = 15) were analyzed in an automatic counter (LC-152; Horiba, Kyoto, Japan). Smears of whole blood and PRP were also observed by light microscopy after Wright-Giemsa staining.

Measurement of Growth Factors in PRP and PPP

Growth factors such as vascular endothelial growth factor (VEGF [n = 8]), transforming growth factor-β1 (TGF-β1 [n = 8]), and platelet-derived growth factor-BB (PDGF-BB [n = 8]) in PRP and PPP were quantified by the enzyme-linked immunosorbent assay (ELISA) using VEGF, TGF-β1, and PDGF-BB ELISA Kits (R&D Systems, Minneapolis, MN). Samples of the PRP and PPP, and standards were added to 96 growth factor antibody-coated well plates. After the incubation and removal of unbound substances, an enzymes-coupled secondary antibody was added to the well plates. Optical densities of each product were measured at the appropriate wavelength after the color reactions.

Incorporation of PRP Growth Factors to Gelatin Hydrogel Microspheres

A gelatin sample with an isoelectric point (IEP) of 5.0 (Nitta, Osaka, Japan) was extracted from porcine skin (type I collagen) by an alkaline process. The result, named "acidic gelatin," was then used to prepare gelatin hydrogel through glutaraldehyde cross-linking in an aqueous solution, as reported previously [13]. Preheated at 40°C, 20 mL 10 wt% acidic gelatin aqueous solution was added to 600 mL olive oil preheated at 40°C for 1 hour while stirring at 500 rpm for 30 minutes. The emulsion was cooled down to less than 4°C, and stirring was continued at 500 rpm for 45 minutes. After addition of 200 mL cold acetone, the emulsion was further stirred at 200 rpm for 10 minutes. The resulting gelatin hydrogels were washed once with acetone at 4°C, collected by centrifuge (2,500 rpm, for 10 minutes, at 4 °C), and a lump of gelatin hydrogels was homogenized for 1 minute. Furthermore, the gelatin hydrogels were washed twice with acetone at 4°C. The washed gelatin hydrogels were passed through sieves that had aperture of 32 μm, and air dried at 4°C to obtain noncrosslinked gelatin hydrogels. The noncrosslinked hydrogels were placed in a glutaraldehyde aqueous solution containing 0.1% wt Tween 80 (the ratio of gelatin hydrogel to 0.1% wt Tween 80 to glutaraldehyde is 20 mg to 20 mL to 100 μL), and gelatin crosslinking proceeded at 4°C for 24 hours. The crosslinked hydrogels were placed in glycine aqueous solution (100 mM) to block unreacted residue of glutaraldehyde, and left in it for 1 hour at room temperature. The resulting gelatin hydrogels were washed with distilled water, centrifuged at 5,000 rpm three times at 4°C, followed by freeze-drying. Immediately after preparation, 200 μL PRP was dropped onto 10 mg of the freeze-dried and sterilized gelatin microspheres and incubated for 1 hour at 37°C to electrically bind the PRP into the gelatin microspheres.

Creation of Hind Limb Ischemia

Critical hind limb ischemia was created in the rats as described previously [14]. Rats were anesthetized with 50 mg/kg of pentobarbital given intraperitoneally. The rat was transferred to a heated table, and body temperature was maintained at 37°C. After making a vertical longitudinal incision in the left inguinal and femoral areas, the entire left femoral arterial vasculature was excised. The major arteries from the iliac to superficial and deep femoral arteries were completely excised, and the other small branches of the external iliac and femoral arteries were thermocoagulated. Because excision of entire femoral arteries is not enough to create critical ischemia at rest in rats, the arteries distal from the external iliac artery were embolized by injection of 10,000 microspheres (Cytodex3; Amersham Bioscience AB, Uppsala, Sweden) measuring 150 μm in diameter. The skin was then sutured in layers. The contralateral intact right hind limb served as a nonischemic limb control. Anesthesia was discontinued, and animals were allowed to recover completely before returning them to their cages. All animals were housed under clean conditions and survived at the Institute of Laboratory Animals, Nippon Medical School.

Treatment of Angiogenesis

On the day after the creation of critical hind limb ischemia, the rats were randomized into four groups (n = 8 each): intramuscular injection of platelet-poor plasma (PPP group), platelet-rich plasma only (PRP group), a combination of PRP and gelatin hydrogel (PRP+Gel group), or phosphate-buffered saline (PBS) solution (control group). The PPP, PRP, PRP+Gel, and control groups were intramuscularly injected with 300 μL PPP, 200 μL PRP plus 100 μL PBS, 200 μL PRP plus 10 mg gelatin hydrogel microspheres plus 100 μL PBS, or 300 μL PBS, respectively, at six different sites (50 μL each) in the skeletal muscle of the left ischemic thigh by a 26G needle. At 4 weeks after the initiation of treatment, the rats were reanesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg), subjected to the measurements of angiogenesis and collateral vessel formation in the ischemic limb tissues described below, and then sacrificed with an overdose of pentobarbital for histologic examinations.

Blood Flow Measurement

Blood flow in the hind limbs was measured by a laser Doppler perfusion imaging (LDPI) system (Moor Instruments, Devon, UK) using a 12-mW helium-neon laser

beam that can sequentially scan a 5 × 5 cm surface area. During the scanning procedure, the LDPI system detects frequency shifts of the laser caused by blood cells moving through the vasculature according to the Doppler principle. The LDPI system transforms these shifts into voltage variations that represent blood perfusion in the scanned area. This perfusion signal is split into 14 different intervals, and each is displayed as a separate color. Low or no blood perfusion was displayed as dark blue, whereas the highest perfusion was displayed as red. Hind limb blood perfusion was scanned before and immediately after the creation of leg ischemia, on the first day of treatment, and then every week during the 4 weeks after the treatment. Excess hairs were removed by depilatory cream from the limb before imaging, and rats were placed on a heating plate set at 37°C to minimize temperature variation. After blood flow was scanned twice, the average blood perfusions of the bilateral legs and feet were evaluated. The average flow values were calculated by computer-assisted quantification using stored images. To minimize influential variables including ambient light and temperature, blood perfusion was expressed as the ratio of the blood flow value (LDPI index) in the left (ischemic) limb to that in the right (nonischemic) limb of the same rat [15].

Microangiography of Hind Limb

Six rats of each group were anesthetized and evaluated angiographically with a contact radiograph 4 weeks after the treatment. To evaluate the angiogenesis, microangiography was performed (C-60; Softex, Kanagawa, Japan) under the conditions of 10 mA, 40 kV (peak), and 25 s exposure after infusion of 2.0 mL 50% barium sulfate (Maruishi Pharmaceutical, Osaka, Japan) from a 22G injection needle (Terumo) inserted in the abdominal aorta [16]. Rats were euthanized before the evaluation. Radiographs were taken using a microfocus setting and a 50-cm tube-film distance from the legs of the rats. Images were scored independently by two observers blinded to treatment, who counted the number of vessels that crossed a standardized 1 × 1 cm^2 grid placed over the ischemic area of the image within an area of interest defined as the greater trochanter to the distal femur. The number of vessels was divided by the lines of the grid in the area of interest to produce an angiographic score. The angiographic score in each film was calculated as the ratio of grid intersections crossed by opacified vessels divided by the total of grid intersections in this area [17].

Histologic and Immunohistologic Assessment of Angiogenesis

After blood flow measurements and angiography were completed, the medial thigh muscles of the ischemic limbs were excised and perfusion fixed with 4% paraformaldehyde in each animal 4 weeks after the initiation of treatment. Each sample was embedded in paraffin, cut into slices 5 μm thick, and stained with hematoxylin and eosin. For immunohistochemical staining, the ischemic thigh muscles were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen at −80°C. Cryostat sections (5 μm thick) of the tissue were stained with monoclonal antihuman von Willebrand factor (vWF) antibody (Dako Japan, Kyoto, Japan). Ten fields from a calibrated graticule measuring 0.0625 mm^2 at a magnification of ×400 on two different sections photographed with a digital camera (Olympus, Tokyo, Japan) were randomly selected from each sample [18]. The number of vWF (endothelial marker)-positive vessels was counted manually in a blind fashion by two observers. Capillary density was determined by the counted number of vWF-positive vessels. The mean capillary density (capillary/mm^2) was reported.

Statistical Analysis

All experimental values were expressed as the mean ± SD. Differences among groups were determined by one-way analysis of variance followed by multiple comparisons using the Bonferroni/Dunn's test for more than two groups. Two-tail unpaired Student t test was used to check the significance difference between two groups. All statistical analyses were performed using the Statview software (Abacus, Grand Rapids, MI). Statistical significance was defined as a p value of less than 0.05.

Results

Platelet Concentration in Whole Blood, PPP, and PRP

The platelets in whole blood, PPP, and PRP were counted by an automatic counter. The number of platelets was 65 ± 17 × 10^4, 5 ± 4 × 10^4, and 1,483 ± 671 × 10^4 /mL, respectively. The purified PRP contained about 23 times the number of platelets found in the whole blood sample ($p < 0.01$). When compared with the whole blood sample, more numerous platelets were observed by light microscopy (Wright-Giemsa staining) in the PRP sample.

Growth Factors in Whole Blood, PPP, and PRP

The PRP was confirmed to contain many more growth factors than PPP. Although VEGF was not detected in the PPP, PRP contained a significant amount of VEGF (77.5 ± 50.4 pg/mL; $p < 0.01$). The concentration of TGF-β1 in PPP and PRP was 1.8 ± 0.4 and 132.9 ± 24.6 ng/mL, respectively; $p < 0.01$). The concentration of PDGF-BB in PPP and PRP was 118.2 ± 71.3 and 10,272.2 ± 888.9 pg/mL, respectively; $p < 0.01$; Table 1).

Tissue Blood Flow in Hind Limb

None of the subject animals experienced side effects such as infection, paralysis, or necrosis during the 4 weeks of

Table 1. Concentration of Various Growth Factors

	PPP	PRP	p Value
VEGF, pg/mL	ND	77.5 ± 50.4	0.001606
TGF-β1, ng/mL	1.8 ± 0.4	132.9 ± 24.6	0.000010
PDGF-BB, pg/mL	118.2 ± 71.3	10272.2 ± 888.9	0.000005

ND = no detection; PDGF-BB = platelet-derived growth factor BB; PPP = platelet-poor plasma; PRP = platelet-rich plasma; TGF-β1 = transforming growth factor-β1; VEGF = vascular endothelial growth factor.

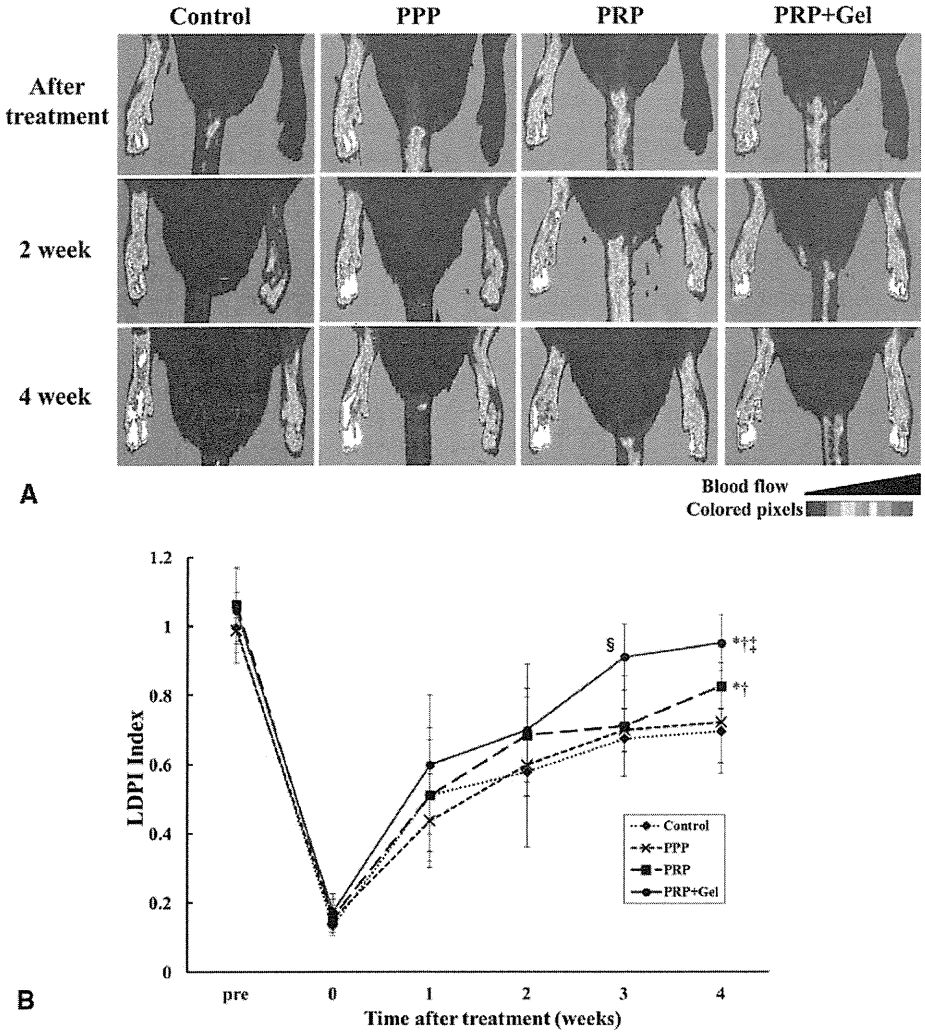

*Fig 2. (A) Hind limb blood flow monitored serially in vivo by laser Doppler perfusion imaging (LDPI). Immediately after angiogenesis treatment, marked reduction in blood flow of left hind limb in all groups is depicted in blue. Blood perfusion of ischemic hind limb significantly increased in the platelet-rich plasma plus gelatin (PRP+Gel) group at 4 weeks after the treatment compared with the other groups. (B) Serial changes of LDPI index in the control group (dotted line), platelet-poor plasma (PPP) group (dashed line), PRP group (long-dashed line), and PRP+Gel group (solid line) during the 4 weeks after the treatment. *$p < 0.05$ versus the control group. †$p < 0.05$ versus the PPP group; §$p < 0.01$ versus the control, PPP, and PRP groups; and ‡$p < 0.05$ versus the PRP group.*

the study. Blood perfusion in both the ischemic and unaffected hind limbs was measured by LDPI. Immediately after the creation of hind limb ischemia, low blood perfusion (dark blue) was observed in all ischemic limbs (Fig 2A). Tissue blood flow was observed serially during the 4 weeks after angiogenesis treatment in the PPP, PRP, PRP+Gel, and control groups. Blood perfusion progressively improved from 1 week after treatment in all groups. The blood flow in the PRP+Gel group was significantly superior to that of the other groups 3 weeks after treatment. The LDPI index of the PRP+Gel group versus the PRP, PPP, and control groups was 91.0% ± 10.2% versus 71.0% ± 15.6%, 70.0% ± 6.5%, and 67.4% ± 4.1%, respectively; $p < 0.01$) at 3 weeks after treatment. The LDPI indices of the PRP+Gel and PRP groups were significantly higher than those of the PPP and control groups 4 weeks after the treatment (95.1% ± 8.0%, 82.7% ± 6.2% versus 72.2% ± 11.8%, 69.6% ± 12.2%, respectively; $p < 0.05$). Moreover, the blood flow improvement of the PRP+Gel group was significantly better than that of the PRP group ($p < 0.05$; Fig 2B).

Microangiography of Ischemic Hind Limb

Representative microangiograms from all groups at 4 weeks after angiogenesis treatment are shown in Figure 3A. While the control and PPP groups showed little collateral vessel formation, it was visually confirmed that the PRP and PRP+Gel groups had massive collateral arteries in their thigh muscle 4 weeks after treatment. Because the collateral vessels of the PRP+Gel group were markedly augmented compared with those of the PRP group, the peripheral vessels of the lower thigh in the PRP+Gel group were filled with more contrast than those of the PRP group.

Angiographic Score

Angiographic score was calculated to quantify the angiogenesis in the all groups (Fig 3B). There was no significant difference between the control and PPP groups (3.8 ± 0.8 and 3.7 ± 0.6, respectively; $p = 0.82$). However, the angiographic scores of the PRP+Gel and PRP groups were significantly higher than those of the control and

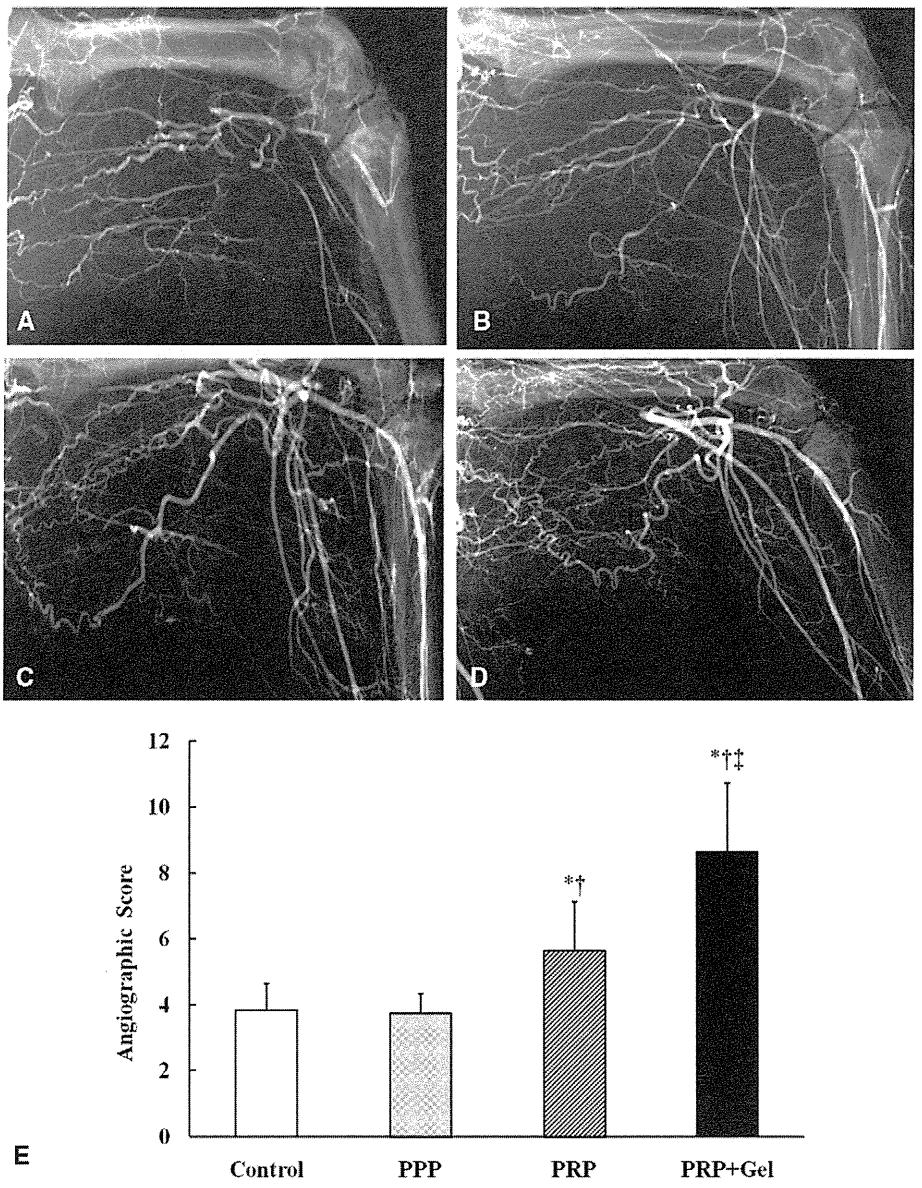

*Fig 3. (A) Microangiograms of the ischemic hind limb 4 weeks after the treatment in all groups: (a) control, (b) platelet-poor plasma (PPP), (c) platelet-rich plasma (PRP), and (d) PRP plus gelatin (PRP+Gel). (B) The angiographic score in the control, PPP, PRP, and PRP+Gel groups at 4 weeks after angiogenesis treatment is shown as a quantitative analysis. The collateral vessels of the PRP+Gel group were markedly augmented compared with those of the other groups. *$p < 0.05$ versus the control group; †$p < 0.05$ versus the PPP group; ‡$p < 0.01$ versus the PRP group.*

PPP groups (8.6 ± 2.1 and 5.6 ± 1.5, respectively; $p < 0.05$); the PRP+Gel group had the highest angiographic score of the all groups.

Histologic and Immunohistologic Findings of Angiogenesis in Ischemic Hind Limbs

Little vascular vessels were histologically observed in the thigh muscles of the control and PPP groups. In the PRP and PRP+Gel groups, there was massive neovascular formation in the thigh muscles 4 weeks after the treatment. The control group remarkably had severe diffuse ischemia, muscle atrophy, disappeared muscle nuclei, and inflammatory cell infiltration. The thigh muscle of the PRP+Gel group had preserved normal muscle architecture at 4 weeks after the treatment. Moreover, immunohistology proved fewer endothelial cells in the control and PPP groups than in the PRP and PRP+Gel groups. Capillary density was calculated by the number of vWF-positive vessels in each group (Table 2). The capillary densities of the PRP and PRP+Gel groups were significantly higher than those of the control and PPP groups (540 ± 109 and 884 ± 94 capillary/mm^2 versus 210 ± 54

Table 2. Capillary Density at 4 Weeks After Angiogenesis Treatment

Groups	Control	PPP	PRP	PRP+Gel
Capillary density (capillary/mm^2)	210 ± 54	190 ± 52	540 ± 109*	884 ± 94*†

* $p < 0.01$ versus the control group, the platelet-poor plasma (PPP) group.

† $p < 0.01$ versus the platelet-rich plasma (PRP) group.

and 190 ± 52 capillary/mm^2, respectively; $p < 0.01$). Furthermore, the capillary density had significantly increased in the PRP+Gel group when compared with the PRP group ($p < 0.01$).

Comment

In this animal study, it was shown that highly concentrated PRP from whole blood contained numerous growth factors such as VEGF, TGF-β1, and PDGF-BB. A controlled release system of PRP (the combination of PRP and biodegradable gelatin hydrogel) was markedly effective in encouraging angiogenesis in critical limb ischemia. In addition, there were no apparent side effects such as infection, paralysis, or necrosis resulting from this treatment.

Platelet-rich plasma initially attracted attention as a new application of tissue engineering in the field of oral maxillofacial surgery, plastic and cosmetic surgery, and orthopedic surgery [8, 19]. It has been previously reported that PRP contains VEGF, TGF-β1, and PDGF [20, 21], and in the present study, significantly higher amounts of VEGF, TGF-β1, and PDGF were found in the PRP group compared with the PPP and control groups. The growth factors contained in PRP act on healing capable cells to increase their numbers (mitogenesis) and stimulate vascular ingrowth (angiogenesis) [22]. Several growth factors become intricately involved in angiogenesis. The VEGF, TGF-β1, and PDGF-BB play an important role as proangiogenic stimulators. Studies have reported that TGF-β1 promotes cell mitosis, and that PDGF-BB has a potent arteriogenic effect that promotes differentiation of endothelial cells [4], and VEGF is well known to stimulate angiogenesis after ischemia [23]. There is a possibility that the abundance of these growth factors included in PRP affect the formation of a vascular network. The lifetime of PRP is 5 to 7 days after the purification [8], and some studies have reported that PRP alone is not enough to induce angiogenesis because of its short lifetime of growth factors included [10]. Also, the therapeutic effects of growth factors are limited because they are normally excreted rapidly. However, in the present study, the PRP group showed significant improvement in blood perfusion at 4 weeks after treatment much more than the control or PPP groups. One reason for this might be that the PRP group in this study had more numerous growth factors than that used in the other studies.

It is well known that, in living tissue, various growth factors are generally stored, interacting with extracellular matrix components, such as acidic polysaccharides, through various intermolecular interaction forces [11]. Furthermore, this physiochemical interaction enables the growth factors to maintain and enhance their biological functions in vivo. In addition to the physiochemical interaction, growth factors are immobilized in the gelatin hydrogel through electrical interaction with gelatin molecules (Fig 1). The IEP of gelatin can be changed by preparation conditions from collagen. Gelatin hydrogel prepared by an alkaline process of collagen has an IEP of 5.0 and is of negative charge at the physiologic pH. The acidic gelatin hydrogel with negative charge can ionically interact to form a poly-ion complex with basic growth factors, such as TGF-β1 [13], PDGF [24], and VEGF [25] with IEPs higher than 8.5. For VEGF with an IEP of 8.6, acidic gelatin (IEP = 5) was chosen as the best carrier [25]. Moreover, the electrical binding of TGF-β1 and PDGF with the acidic gelatin hydrogels is stronger than that of VEGF. That may be due to differences in IEPs, sizes, or tertiary structures [26]. Hydrogel degradation can succeed in slowly releasing the growth factors binding the gelatin hydrogel (Fig 1). The immobilized growth factors in the gelatin hydrogels are reported to be released over a period of 2 weeks as a result of hydrogel degradation [27]. In the present study, the PRP+Gel group had significantly better angiogenesis than the PRP group from 3 weeks after the treatment. This observation suggests that the combination of PRP and gelatin hydrogels facilitates slower release of the growth factors, which results in better angiogenesis. Therefore, the controlled release of PRP using biodegradable gelatin hydrogel is effective to induce angiogenesis in critical limb ischemia.

This research demonstrates the effectiveness of the controlled release of PRP using biodegradable gelatin hydrogel in inducing angiogenesis in critical limb ischemia. Platelet-rich plasma has already been used in the clinical setting. Recent research indicates favorable effects of injecting PRP into areas of myocardial infarction [28]. It is also demonstrated that no side effects of autologous PRP were observed [29]. Some gene therapies have revealed that unregulated growth protein can lead to the formation of many disorganized vessels and the development of hemangioma instead of functional vessels [5]. The use of autologous PRP, however, is simple and safe to induce angiogenesis without introducing the toxicity or immunologic rejection reported with other therapeutic methods of angiogenesis with human recombinant protein. Because the biodegradable gelatin hydrogel was made from porcine collagen, there was also no concern about bovine spongiform encephalopathy. We believe that using PRP impregnated with biodegradable gelatin hydrogel is a safer, less invasive, and more promising compared with previous therapeutic angiogenesis techniques. This is a preliminary study in a rat model for only 4 weeks after the treatment. A longer term study will be needed to evaluate the chronic efficacy or function for angiogenesis.

We acknowledge the excellent technical assistance of Shuhei Tara, Harumi Otsubo, Mitsuhiro Kudo, Shin-ichiro Kumita, Yoshimitsu Fukushima, Takahiko Mine, Yoshihiro Suzuki, Miyuki Takatori, and Hidemasa Takafuji.

References

1. Hashimoto T, Koyama H, Miyata 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;38:71–5.

2. Kang HJ, Kim HS, Zhang SY, et al. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. Lancet 2004;363:751–6.
3. Tateishi-Yuyama E, Matsubara H, Murohara T, et al. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. Lancet 2002;360: 427–35.
4. Carmeliet P. Angiogenesis in health and disease. Nat Med 2003;9:653–60.
5. Carmeliet P. VEGF gene therapy: stimulating angiogenesis or angioma-genesis? Nat Med 2000;6:1102–3.
6. Cao R, Bråkenhielm E, Pawliuk R, et al. Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. Nat Med 2003;9:604–13.
7. Marui A, Kanematsu A, Yamahara K, et al. Simultaneous application of basic fibroblast growth factor and hepatocyte growth factor to enhance the blood vessels formation. J Vasc Surg 2005;41:82–90.
8. Marx RE. Platelet-rich plasma: evidence to support its use. J Oral Maxillofac Surg 2004;62:489–96.
9. Man D, Plosker H, Winland-Brown JE. The use of autologous platelet-rich plasma (platelet gel) and autologous platelet-poor plasma (fibrin glue) in cosmetic surgery. Plast Reconstr Surg 2001;107:229–37.
10. Sugimori E, Shintani S, Ishikawa K, Hamakawa H. Effects of apatite foam combined with platelet-rich plasma on regeneration of bone defects. Dent Mater J 2006;25:591–6.
11. Tabata Y. Tissue regeneration based on growth factor release. Tissue Eng 2003;9(Suppl 1):5–15.
12. Hokugo A, Ozeki M, Kawakami O, et al. Augmented bone regeneration activity of platelet-rich plasma by biodegradable gelatin hydrogel. Tissue Eng 2005;11:1224–33.
13. Yamamoto M, Ikada Y, Tabata Y. Controlled release of growth factors based on biodegradation of gelatin hydrogel. J Biomater Sci Polym Ed 2001;12:77–88.
14. Luyt CE, Meddahi-Pellé A, Ho-Tin-Noe B, et al. Low-molecular-weight fucoidan promotes therapeutic revascularization in a rat model of critical hindlimb ischemia. J Pharmacol Exp Ther 2003;305:24–30.
15. Iwase T, Nagaya N, Fujii T, et al. Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia. Cardiovasc Res 2005;66:543–51.
16. Padilla L, Krötzsch E, Schalch P, et al. Administration of bone marrow cells into surgically induced fibrocollagenous tunnels induces angiogenesis in ischemic rat hindlimb model. Microsurgery 2003;23:568–74.
17. Gale LT, David SC, Rajabrata S, Rong W, Louis MM. The effect of gradual or acute arterial occlusion on skeletal muscle blood flow, arteriogenesis, and inflammation in rat hindlimb ischemia. J Vasc Surg 2005;41:312–20.
18. Lucy SB, David SC, Gale LT, Rajabrata S, Louis MM. Overexpression of endothelial nitric oxide synthase increases skeletal muscle blood flow and oxygenation in severe rat hind limb ischemia. J Vasc Surg 2003;38:820–6.
19. Nagae M, Ikeda T, Mikami Y, et al. Intervertebral disc regeneration using platelet-rich plasma and biodegradable gelatin hydrogel microspheres. Tissue Eng 2007;13 :147–58.
20. Okuda K, Kawase T, Momose M, et al. Platelet-rich plasma contains high levels of platelet-derived growth factor and transforming growth factor-beta and modulates the proliferation of periodontally related cells in vitro. J Periodontol 2003;74:849–57.
21. Weibrich G, Kleis WK, Hafner G, Hitzler WE. Growth factor levels in platelet-rich plasma and correlations with donor age, sex, and platelet count. J Craniomaxillofac Surg 2002;30: 97–102.
22. Marx RE. Platelet-rich plasma (PRP): what is PRP and what is not PRP? Implant Dent 2001;10:225–8.
23. Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. Am J Physiol Cell Physiol 2001;280:C1358–66.
24. Kanematsu A, Yamamoto S, Ozeki M, et al. Collagenous matrices as release carriers of exogenous growth factors. Biomaterials 2004;25:4513–20.
25. Patel ZS, Ueda H, Yamamoto M, Tabata Y, Mikos AG. In vitro and in vivo release of vascular endothelial growth factor from gelatin microparticles and biodegradable composite scaffolds. Pharm Res 2008;25:2370–8.
26. Muller YA, Li B, Christinger HW, Wells JA, Cunningham BC, de Vos AM. Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. Proc Natl Acad Sci USA 1997;94: 7192–7.
27. Tabata Y, Miyao M, Ozeki M, Ikada Y. Controlled release of vascular endothelial growth factor by use of collagen hydrogels. J Biomater Sci Polym Ed 2000;11:915–30.
28. Li XH, Zhou X, Zeng S, et al. Effects of intramyocardial injection of platelet-rich plasma on the healing process after myocardial infarction. Cor Artery Dis 2008;19:363–70.
29. Nikolidakis D, Jansen JA. The biology of platelet-rich plasma and its application in oral surgery: literature review. Tissue Eng Part B Rev 2008;14:249–58.

DISCUSSION

DR SI MAI PHAM (Miami, FL): In this kind of study, usually the angiogenesis is transient, with improvement in the first month, but it doesn't persist. Do you have any data longer than 1 month after the injection of this platelet-rich plasma impregnated hydrogel? Do you have any long-term data at 2 months, 3 months, or 6 months?

DR KURITA: Unfortunately, we don't have any data available since after 4 weeks. Some investigators reported about the histological findings that almost all gelatin hydrogels were indistinct at 8 weeks after injection. So, it is potential to keep slow releasing of growth factors with the gelatin hydrogel degradation at least for 8 weeks. We must check about the long-term data in the future.

Journal of Surgical Research **167,** 166–172 (2011)
doi:10.1016/j.jss.2009.05.042

The Controlled Release of Basic Fibroblast Growth Factor Promotes a Rapid Healing of Pancreaticojejunal Anastomosis with Potent Angiogenesis and Accelerates Apoptosis in Granulation Tissue

Akira Katsuno, M.D.,* Takayuki Aimoto, M.D.,*,[1] Eiji Uchida, M.D.,*
Yasuhiko Tabata, Ph.D., D.Med.Sci., D.Pharm.,‡ Masaaki Miyamoto, M.D.,† and Takashi Tajiri, M.D.*

**Department of Surgery, Nippon Medical School, Tokyo, Japan; †Department of Internal Medicine, Nippon Medical School, Tokyo, Japan; and ‡Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan*

Submitted for publication March 31, 2009

Background. **Our previous study demonstrated that bFGF-GH promoted healing of the pancreaticojejunostomy (PJ) in an animal model. We examined the healing process in detail to investigate the significance of treatment with basic fibroblast growth factor (bFGF) incorporated in gelatin hydrogel (GH) microspheres for anastomotic healing.**

Materials and Methods. **The optimal dose of bFGF was determined by administering bFGF concentrations of 1, 10, and 100μg in six beagle dogs and assessing the results on d 7. Next, 28 dogs received a jejunal subserosal injection of 10μg bFGF-GH or GH alone. The healing process was sequentially analyzed on d 4, 7, 21, and 28. The following types of assessment were performed: breaking strength test, pathologic examination, and calculations of collagen content, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) index, and microvessel density (MVD).**

Results. **The administration of a bFGF dose of more than 10μg induced a significantly higher breaking strength and more abundant granulation tissues. Histologic observations of the bFGF-GH group on d 7 and the GH-alone group on d 21 revealed abundant granulation tissue with migrating fibroblasts, inflammatory cells, and capillaries. Marked neovascularization and dense collagen deposition were detected in both groups on d 28. The collagen content and breaking strength did not significantly differ between both groups on d 28. A significantly higher TUNEL index and a rapid decline in the number of vimentin-positive cells were detected in the bFGF-GH group from d 21 onward. The MVD in the bFGF-GH group was significantly higher from d 7 onward**

Conclusions. **Basic FGF-GH administration can promote the rapid completion of PJ anastomosis and may help improve the quality of the healing of granulation tissue by conferring potent angiogenesis and accelerating apoptosis.**

Key Words: **pancreatic fistula (PF); pancreaticojejunostomy (PJ); pancreaticoduodenectomy (PD); basic fibroblast growth factor (bFGF); gelatin hydrogel (GH); microvessel density (MVD); regenerative medicine; anastomotic healing; quality of healing.**

INTRODUCTION

Pancreatic fistula (PF) after pancreaticoduodenectomy (PD) is an important morbidity and a potential cause of serious secondary complications [1, 2]. Modified anastomotic techniques have been proposed for the prevention of PF, but thus far they have only been successful in reducing the fistulas rather than eliminating them altogether [3, 4]. A novel approach capable of achieving zero PF is awaited.

Basic fibroblast growth factor (bFGF) can attract and stimulate the growth of endothelial cells and fibroblasts [5, 6]. The administration of recombinant bFGF to skin wound healing has been shown to accelerate wound healing by promoting fibroblast proliferation and neovascularization [7, 8]. There have also been reports that bFGF treatment for skin wounds induces the apoptosis of inflammatory cells and may contribute to a reduction of excess scarring in the latter phase of healing [9, 10].

[1] To whom correspondence and reprint requests should be addressed at Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan. E-mail: aimoto@nms.ac.jp.

0022-4804/$36.00

Tissue engineering is a new biomedical technology designed to create an environment for tissue regeneration [11, 12]. Growth factors released from gelatin controlled release systems retain their biological activity for longer than growth factors delivered immediately [12]. Tabata [12] developed a method for administering bFGF incorporated in gelatin hydrogel microspheres. This bFGF-incorporated gelatin hydrogel microsphere (bFGF-GH) has since been applied as a controlled release system in several studies on organ reconstruction [13, 14].

In a previous study by our group, bFGF-GH administration in an animal model of pancreaticojejunostomy (PJ) induced significant increases of granulation tissue at the anastomotic site on d 7 after PJ, compared with controls [15]. This novel controlled release strategy is expected to be adapted for clinical use in the future. Little has been done, however, to carefully examine the healing process following bFGF-GH treatment. In this study, we examined the level of apoptosis, collagen content, and microvessel density (MVD) as indexes of healing in order to assess the significance of bFGF-GH treatment in this model.

MATERIALS AND METHODS

Preparation of the bFGF-Incorporated Gelatin Hydrogel Microspheres

"Acid gelatin," gelatin with an isoelectric point (IPEs) of 5.0, was prepared through an alkaline process using pig skin collagen (Nitta Gelatin Co., Osaka, Japan). Gelatin hydrogels were then prepared through glutaral-dehydro crosslinking of the acidic gelatin in an aqueous solution. Basic FGF-GH was obtained by dropping 200 μL of distilled water with bFGF (100 μg) onto 20 mg gelatin hydrogels and leaving the treated hydrogels overnight at 4°C.

Animals

Thirty-four beagle dogs weighing 10–15 kg were obtained from the Institute for Animal Reproduction (Ibaragi, Japan) and housed in an accredited facility on a 12 h:12 h, light:dark cycle with access to food and water *ad libitum*.

All experimental studies were performed in accordance with the guidelines for Animal Experiments of the Nippon Medical School and Nippon Veterinary and Animal Science University, and the aforesaid guidelines conformed to the Legal Guidance on the Care and Use of Laboratory Animals in Japan.

Experimental Design and Surgical Procedure

All animals were fasted for 24 h preoperatively. The procedure began with intubation *via* the trachea under general anesthesia commenced by a subcutaneous injection of medetomidine hydrochloride (20 μ/Kg) (Meiji, Tokyo, Japan), meloxicam (Boehringer Ingelheim, Burlington, Canada), and midazolam (0.3 mg/Kg) (Astellas, Tokyo, Japan). The anesthesia was maintained during the procedure by mechanical ventilation with a mixture of isoflurane and oxygen, together with epidural anesthesia (10 μg/Kg of 0.5% bupivacaine hydrochloride). Intravenous lactated Ringer's solution was also administered during the operation. After making a midline incision, the pancreas was divided into two parts at the mid-body. The main pancreatic duct of the right lobe was then ligated distally, and the right lobe was left *in situ*. A Roux-en-Y side-to-side PJ to the left lobe was constructed as a one-layer anastomosis with a stent (sheath of a 24 G polyethylene needle). The pancreatic parenchyma was sutured to the jejunal seromuscular layer by placing four stitches with an interrupted suture (6-0 nonabsorbable monofilament suture). Before the completion of construction, either bFGF-GH or GH alone was injected into a jejunal subserosal layer at the anastomotic site.

First, bFGF doses ranging from 1 to 100 μg (1, 10, 100 μg) were applied to select the optimal dose of bFGF incorporated in the GH. Six dogs were divided into three groups and administered 1, 10, and 100 μg doses of bFGF, respectively. These dogs were sacrificed on d 7 and autopsied. Next, 28 dogs were divided into two groups, a bFGF-GH-treated group ($n = 16$) and GH-alone group ($n = 12$), to examine the healing process following bFGF-GH administration. These dogs were sacrificed on d 4, 7, 21, and 28. The anastomoses were removed immediately after sacrifice for macroscopic observation, assessment by the breaking strength test, histologic evaluation, and immunohistochemical analysis.

Breaking Strength Test

The breaking strength was measured by a spring balance. The tissues of anastomotic regions were cut into 5 × 5 mm squares and secured at two ends by binder clips. A force was applied across the anastomotic site at a constant speed (1 cm/s). The breaking strength was expressed as the point of maximal stress (g/25 mm^2) before separation of the anastomosis.

Microscopic Observance

Resected regions were fixed in 10% formalin, embedded in paraffin wax, and examined by hematoxylin and eosin (HE) and elastica van Gieson (EVG) staining. The percentage of collagen content at the anastomotic site on each slide stained with EVG was calculated by a previously reported method [16] with Photoshop hardware (version 5) (Adobe, Tokyo, Japan). In brief, the slides were scanned at a ×200 field to identify the three areas (0.78 mm^2 per field) with the most granulation tissue in the border zone of the anastomotic site; then the digitized images thus obtained were stored in a personal computer. The edges of the areas with dense collagen were accurately margined, a histogram was generated by clicking the image icon, and the pixel value of the area was calculated. Results were expressed as the percentage of the collagen content (the pixel value of the area with dense collagen/the pixel value of the whole area ×100%).

Immunohistochemistry

The sections were stained for factor VIII-related antigen and vimentin by the avidin-biotin-peroxidase complex (ABC)-immunoperoxidase method previously described [17]. In brief, the sections were incubated in a rabbit polyclonal anti-factor-VIII-related antigen (DAKO Japan Co., Kyoto, Japan) at a 1:200 dilution and a mouse monoclonal anti-vimentin antigen (DAKO Japan Co., Kyoto, Japan) at a 1:100 dilution at 4°C in a humid chamber. Peroxidase hydroxychloride activity was visualized with diaminobenzidine tetrahydrochloride solution (DAKO Japan Co., Kyoto, Japan) as substrate. The sections were counterstained lightly with Mayer's hematoxylin.

The slide was scanned systemically at ×40 magnification to identify the four areas with the greatest microvessel density, the so-called hot spots. The microvessel density (MVD) (/mm^2) was calculated by counting the microvessels in a single ×200 field (0.78 mm^2 per field) in each hot spot. The number of vimentin-positive cells per field was counted at ×400 magnification.

TUNEL Method and TUNEL Index

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method was performed using a polyclonal rabbit anti-single stranded DNA antibody (ssDNA; IBL, Tokyo, Japan) with the DAKO Envision + Rabbit/HRP system (DAKO, Copenhagen, Denmark). Negative controls had TdT or biotinylated substrate omitted from the buffer solution. TUNEL-positive cells were counted in four randomly selected fields in the border zone at ×400 magnification, and results were expressed as a TUNEL index [number of positively stained cells/total number of cells × 100%].

The investigators conducting the pathological evaluations were blinded to the treatment in this study.

Statistical Analysis

All data are given as mean ± standard deviation (SD). Statistical significance was tested using the Mann-Whitney U test for unpaired of paired comparisons between groups. Statistical significance was accepted at $P < 0.05$.

RESULTS

Evaluation of the Optimal Dose of bFGF (Fig. 1)

The dose-response effect on the anastomotic healing on d 7 was investigated to select the optimal dose of bFGF incorporated in the GH. The breaking strength was significantly higher in the bFGF-GH groups administered the 100 and 10 μg bFGF doses than in the GH-alone group. A dose-dependent effect was not discernable among the bFGF-GH groups administered doses higher than 10 μg (Fig. 1A). Histologically, treatment with doses higher than 10 μg induced more abundant granulation tissues than an injection of a 1 μg dose or GH alone (Fig. 1B). Therefore, we selected 10 μg as the optimal dose of bFGF incorporated in GH and applied this dose in the studies that followed.

Sequential Analysis of Healing at the Anastomotic Site (Fig. 2)

We examined the healing of the anastomosis following bFGF-GH treatment, in comparison with the healing in the animals injected with GH alone. In a preliminary study, we injected 10 μ of free bFGF into the jejunal subserosal layer at the anastomotic site ($n = 2$). The findings on d 7 for this group were very similar to those for the GH group and, therefore, we treated the GH group as the control group. In this study, the incidence of PF in the bFGF-GH group and the GH group was 0% (0/16) and 8% (1/12), respectively. Conservative treatment was successful in one animal with PF.

On d 4, both groups exhibited massive tissue necrosis, together with hemorrhaging and inflammatory cells, at the anastomotic site. No remarkable differences were found between the groups (Fig. 2A).

On d 7, the border zone of the anastomosis in the bFGF-GH group exhibited granulation tissue composed of collagen, with migrating fibroblasts, inflammatory

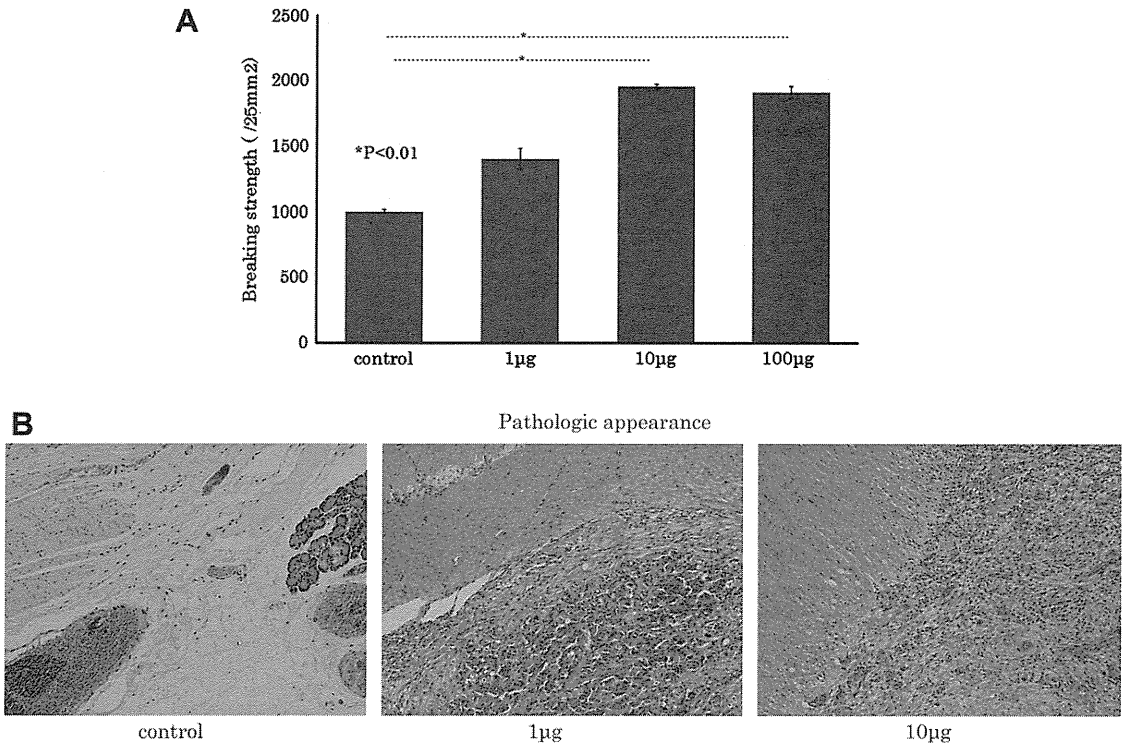

FIG. 1. Evaluation of the optimal dose of bFGF incorporated in GH. (A) Dose-response effect on breaking strength on d 7. (B) Microscopic appearance of the anastomotic site at different bFGF doses (0, 1, 10 μg) on d 7, HE, ×200. (Color version of figure is available online.)

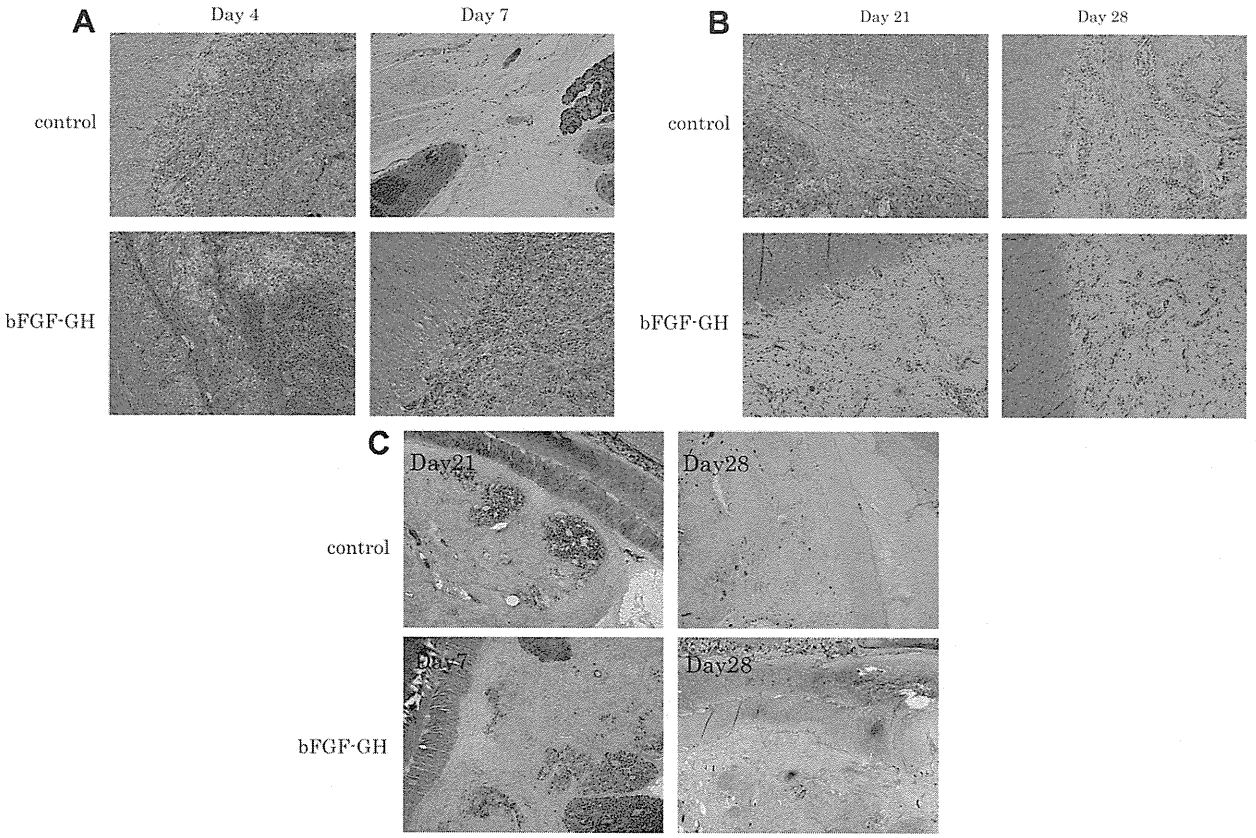

FIG. 2. Sequential study of microscopic findings in the bFGF-GH-treated group and GH-alone group from d 4 to 28. (A) Microscopic finding with HE staining on d 4 and 7, ×200. (B) Microscopic finding with HE staining on d 21 and 28, ×200. (C) Microscopic finding with EVG staining on days 7, 21, and 28, ×100. (Color version of figure is available online.)

cells, and capillaries. In contrast, only small amounts of granulation tissue were seen in the GH-alone group (Fig. 2A). No partial pancreatic necrosis was detected in any animals in the bFGF-GH group. In contrast, small necrotic areas remained in two animals in the GH group.

On d 21, remarkable neovascularization and dense collagen deposition accompanied by destruction of acinar cells were detected in the bFGF-GH group. In the GH-alone group, the anastomotic region consisted of granulation tissue with fibroblasts, inflammatory cells, and collagen. The microscopic appearance in the GH-alone group resembled that in the bFGF-GH group on d 7 (Fig. 2B).

On d 28, no significant difference between the two groups was detected pathologically. The microscopic appearance in both groups was similar to that in bFGF-GH group on d 21 (Fig. 2B).

We also analyzed the pattern of collagen distribution by EVG staining. The distribution pattern in the bFGF-GH group on d 7 was very similar to that in the GH-alone group on d 21. Collagen deposition was seen mainly at the borderline, and intralobular fibrosis was also detected. On d 28, diffuse collagen distribution was found in both groups (Fig. 2C).

Evaluation of the Quality of Anastomotic Healing

Breaking Strength Test

On d 7 and 21, the breaking strength was significantly higher in the bFGF-GH group than in the GH-alone group ($P < 0.01$). In contrast, there was no difference between the groups on d 4 or 28 (Fig. 3).

Semiquantitative Analysis of Collagen Content

The collagen content was significantly higher in the bFGF-GH group than in the GH-alone group on d 7 and 21 ($P < 0.01$). No significant difference between the groups was found on d 28 (Fig. 4).

Time Course of Vimentin Expression and Apoptosis During Healing

We quantified the expression of vimentin and the TUNEL index in granulation tissue at the anastomotic site to investigate the apoptosis of fibroblasts induced by bFGF administration during healing. Vimentin was positively expressed in the cytoplasm of fibroblasts and inflammatory cells. The time course of the number of vimentin-positive cells in both groups showed