of molecular therapy for ischemia, recent experimental studies have also indicated the effectiveness of combination strategies such as VEGF and platelet-derived growth factor (PDGF)-BB or basic FGF (bFGF) and PDGF-BB to establish more stable vasculature.[9-12]

Hepatocyte growth factor (HGF) is reported to improve endothelial cell survival when it is administered with bFGF.[13] In a murine limb ischemia model, exogenous bFGF not only upregulates endogenous HGF, but the blockade of endogenous HGF activity also diminishes the effect of exogenous bFGF.[14] Therefore, these interactions between bFGF and HGF may play an important role in growth-factor therapy.

In the present study, we hypothesized that the combination of bFGF and HGF might enhance blood vessel formation in a lower dose than would a higher dose of either single agent. To test this hypothesis, we first used an in vitro endothelial cell tubulogenesis assay to confirm the in vitro synergistic effect of these growth factors. Next, we examined the therapeutic benefit of this combination in a murine hindlimb ischemia model. Because the bioactivity of growth-factor proteins such as bFGF rapidly disappears in solution form, we have used a sustained-release system of growth-factor proteins in various cardiovascular animal models.[15-26] In the present study, we used collagen microspheres (CM) as a release carrier biomaterial to enable the simultaneous sustained release of bFGF and HGF and examined the therapeutic effect of this novel dual-release system.

MATERIALS AND METHODS

Collagen sheet and microsphere. Type I collagen sponge sheet, used in an in vivo release study, was provided by Gunze (Kyoto, Japan). The sponge sheet was prepared by dehydrothermal treatment (140°C, 6 hours) and subsequent ultraviolet irradiation cross-linking of porcine dermal type I collagen. It was then vacuum dried, sterilized by ethylene oxide gas, and packaged until use. The sheet was cut before use under aseptic condition to prepare 5-mg square sheets.

Type I CM used in an in vivo ischemic limb model was prepared as previously described.[27]

Growth factors and reagents. Recombinant human bFGF protein was provided by Kaken Pharmaceutical (Tokyo, Japan). Recombinant human HGF was provided from Snow Brand (Osaka, Japan). Radio labeling of growth factors by $Na^{125}I$ for tracing studies was done by the conventional chloramine T method.[17,18,26]

Animals. Six-week-old female ddY mice and 8-week-old male C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). The Kyoto University Animal Experiment Committee approved the experiments. Animals were cared for in compliance with the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council.

Capillary network formation assay. To evaluate the combined effects of bFGF and HGF on endothelial cell tubulogenesis in vitro, capillary network formation on Matrigel (BD Biosciences, Franklin Lakes, NJ) was assessed with human umbilical vein endothelial cells (HUVECs).[28-30] HUVECs (Clonetics, Walkersville, Md) were grown in basic medium (EBM2, Clonetics) containing growth supplement (EGM2, Clonetics). At the second or third passage, they were seeded in Matrigel-coated Cellware (BD Biosciences) 12-well plates (4×10^4 cells/well) and incubated for 1 hour in 200 μL of endothelial cell basal medium-2 containing 10% fetal bovine serum (FBS). After the FBS was removed, 1000 μL of serum-free medium, containing bFGF or HGF, or bFGF and HGF combined at the indicated concentrations, was added. The cells were incubated for 8 hours and then fixed with 10% buffered formalin.

Five random fields of view in six replicate wells were visualized, and images were captured with a digital camera (Olympus, Tokyo, Japan). NIH Image was use to quantify the total area covered with capillary networks in each field and quantification of network formation was performed by an observer blinded to the treatment groups. For each experiment, each group consisted of six replicate wells, and all experiments were repeated twice.

Release profile of bFGF and HGF from collagen. The in vivo release of bFGF from collagen was evaluated as previously described.[17,18,26,27] Briefly, 5-mg pieces of dry collagen sheet were re-swollen with ^{125}I-labeled bFGF and HGF solution (approximately 50 μg/mL). After incubation at 37°C for 1 hour, each sheet was implanted into a subcuticular pocket created in the backs of 6-week-old female ddY mice under pentobarbital anesthesia (1.5 mg/mouse).[17,18,26,30] The loading efficacy of bFGF or HGF to the collagen sheet was more than 95% by radioactive assay of ^{125}I (data not shown). The persistence of the growth-factor activity in vivo was confirmed as previously described.[18,27]

The sheets were retrieved from animals sacrificed at 1, 3, 7, 14, and 28 days (n = 6 for each time point) and the remaining radioactivity of the sheet and local tissue was measured with a gamma counter (Aloka, Tokyo).[31] The released and remaining radioactivity percentages were evaluated as previously described.[17,18,26,27] We also measured the in vivo degradation of the collagen matrices by implanting a 5-mg piece of collagen sheet, radio-iodinated with the Bolton-Hunter reagent, into the mouse subcutis and measuring the remaining radioactivity.[17,18,26,27] A separate group of mice were administered with the same amount of radio-iodinated bFGF or HGF solution (n = 6 for each) by bolus subcuticular injection.

Preparation of CM incorporating growth factors. For the sustained release of bFGF from CM in the hindlimb muscle, 4 mg of lyophilized microspheres were rehydrated with 20 μL of bFGF solution at various concentrations, followed by incubation at 37°C for 1 hour. The microspheres containing bFGF were expressed as "bFGF/CM". The microspheres containing 5 μg of bFGF were expressed as "5-μg bFGF/CM". The solution form of 5 μg of bFGF was expressed as "5-μg bFGF/solution". At use, the

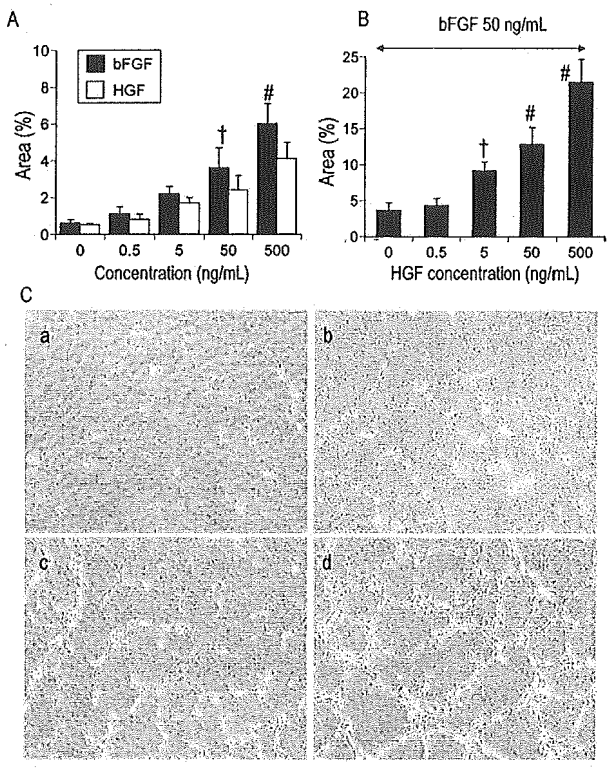

Fig 1. Capillary network formation assay with human umbilical vein endothelial cells (HUVECs). Capillary network formation was assessed on Matrigel in the presence of basic fibroblast growth factor (*bFGF*) or hepatocyte growth factor (*HGF*), or bFGF and HGF together. **A,** Dose-response of capillary network formation area (%) of HUVECs in the presence of bFGF (*closed bar*) or HGF (*open bar*). $^{\dagger}P < .05$ vs HGF 50 ng/mL; $^{\#}P < .01$vs HGF 500 ng/mL. **B,** Capillary network formation area in the presence of a fixed concentration of bFGF (50 ng/mL) and the indicated concentrations of HGF (0.5 to 500 ng/mL). $^{\dagger}P < .05$, $^{\#}P < .01$ vs 0 ng/mL of HGF. **C,** Enhancement of capillary network formation of HUVEC in Matrigel by the combination of bFGF and HGF. HUVECs were cultured in Matrigel in the presence of (*a*) control (no growth factor added); (*b*) HGF alone (50 ng/mL); (*c*) bFGF alone (50 ng/mL); (*d*) or combination of bFGF and HGF (50 ng/mL each) (×10).

bFGF/CM were dispersed in 100 μL of phosphate-buffered saline (PBS), aspirated into a 1 mL syringe with an attached 27-gauge needle (Terumo, Tokyo), and injected into murine thigh muscle. The bFGF/solution was also dispersed in 100 μL of PBS and injected.

The HGF-incorporated CM (HGF/CM) and the solution form of HGF (HGF/solution) were prepared in the same manner. For dual release, bFGF and HGF were incorporated into the CM in mixed solution. Sustained dual release was expressed as "dual/CM".

Restoration of blood perfusion in the murine hindlimb ischemia model. Hindlimb ischemia was created in 8-week old male C57BL/6 mice. After being anesthetized with 80 mg/kg of intraperitoneal pentobarbital, the entire right saphenous artery and vein and the right external iliac artery and vein with deep femoral and circumflex arteries and veins were ligated, cut, and excised to obtain a murine model of severe hindlimb ischemia.[13,28,32,33]

Hindlimb blood perfusion was scanned with a laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, UK) the day of surgery and every 7 days after the surgery.[32] To avoid the influence of the surgical area, the average blood perfusion of the foot and below the knee was bilaterally calculated for evaluation. To account for variables, including ambient light and temperature, calculated perfusion was expressed as a ratio of right (ischemic) to left (nonischemic) limb blood perfusion of the same mouse.[32]

Animals were randomly divided into groups (n = 7 to 8 per group) and treated as follows: bFGF/CM (1, 5, 20, and 80 μg), HGF/CM (1, 5, 20, and 80 μg), bFGF/solution (80 μg), HGF/solution (80 μg), dual/solution (solution form of 80 μg of bFGF and 80 μg of HGF), dual/CM, (combined doses are described in the *Results* section), or CM only. The CM incorporating growth factors or the solution form of growth factors were administered into the ischemic limb. The injection procedure was performed by 5 intramuscular injections into the thigh muscles of the ischemic hindlimb using a 27-gauge needle at the time of surgery. The injections were both along the operative site and deep into the muscle.

Immunohistochemistry and analysis of vascular density. Four weeks after surgery, the mice were euthanized and perfusion fixed with 4% paraformaldehyde. The ischemic calf muscles were embedded in optimal cutting temperature compound (Sakura Finetechnical, Tokyo) and frozen at −80°C. Cryostat sections (5-μm thick) of the tissues were stained with mouse antimouse von Willebrand factor (vWF) (DAKO Japan, Kyoto) and mouse anti–α-smooth muscle actin (SMA, Sigma, St. Louis, Mo). As the negative control, normal Ig fraction (DAKO) was used to show antibody specificity.

Eight random fields on two different sections (approximately 3 mm apart) from each mouse were photographed with a digital camera (Olympus, Tokyo). The number of vWF (endothelial marker) positive or α-SMA (vascular smooth muscle marker) positive vessels was counted manually in a blind fashion. Vascular density was calculated as the mean number of vessels stained with vWF or α-SMA.

Analysis of inflammation. Additional mice with the same growth factor treatments were prepared (n = 4 to 5 each) for assessment of focal inflammation in the ischemic tissue. Three days after surgery, cryostat sections of the calf muscles were obtained in the same manner and stained with rat-antimouse CD45 (PharMingen, San Diego, Calif). The mean number of infiltrating CD45-positive leukocytes was counted as the assessment of inflammation by computer-assisted analysis using NIH Image.[28]

Statistical analysis. Results are presented as means ± standard deviation. Normality of distribution was assessed by the Shapiro-Wilk test. The laser Doppler data were tested with repeated measures of analysis of variance

(ANOVA) between the control and each treatment group. In multiple comparisons among independent groups in which ANOVA indicated significant differences, the statistical value was determined according to the Bonferroni/Dunn method. Differences between groups were determined with the Student *t* test. The correlation of degradation profiles was analyzed by simple regression. All statistical analyses, except the Shapiro-Wilk test, were performed by Statview software (Abacus). A *P* value < .05 was considered significant.

RESULTS

Capillary network formation assay. HUVECs were cultured on Matrigel in serum-free medium supplemented with bFGF alone, HGF alone, or bFGF and HGF combined (from 0.5 to 500 ng/mL, each). Alone, bFGF dose-dependently potentiated capillary network formation of HUVECs (Fig 1, *A*). Although HGF similarly promoted tubulogenesis in a dose-dependent manner, the effect was lower than bFGF at concentrations of 50 ng/mL (3.6% ± 1.1% vs 2.4% ± 0.8%, $P < .05$) and 500 ng/mL (6.0 % ± 1.1% vs 4.1% ± 0.9%, $P < .01$). When the bFGF concentration was fixed at 50 ng/mL, the addition of HGF at concentrations of 5, 50, and 500 ng/mL increased the capillary network formation (9.1% ± 1.3%, 12.8% ± 2.4%, and 21.5% ± 3.2%, for 5, 50, and 500 ng/mL of HGF; $P < .05$ at 5 ng/mL and $P < .01$ at 50 and 500 ng/mL, respectively) compared with 0 ng/mL of HGF (3.6% ± 1.1%) (Fig 1, *B*). Similar data were obtained when the HGF concentration was fixed at 50 ng/mL and the bFGF concentration varied from 5 to 500 ng/ml (data not shown). The combination of bFGF and HGF (50 ng/mL each) increased the capillary network formation compared with bFGF alone or HGF alone (Fig 1, *C*). These results demonstrate that synergism with the bFGF and HGF endothelial mitogens potentiates capillary network formation of HUVECs.

Release profile of bFGF and HGF from collagen. In the back subcutis of the mice, the remaining bFGF radioactivity released from the collagen sheet gradually decreased over 4 weeks, while the radioactivity of the solution form of bFGF applied in a bolus subcutaneous injection disappeared within 3 days after administration (Fig 2, *A*). The release profile of bFGF closely correlated with the degradation profile of the collagen sheet (R^2 = 0.974, $P < .0005$, simple regression analysis) (Fig 2, *A*). Similarly, the remaining HGF radioactivity also gradually decreased over 4 weeks nearly in accordance with the collagen degradation (R^2 = 0.864, $P < .05$). The bolus injection of the solution form of HGF also rapidly disappeared within 3 days (Fig 2, *B*).

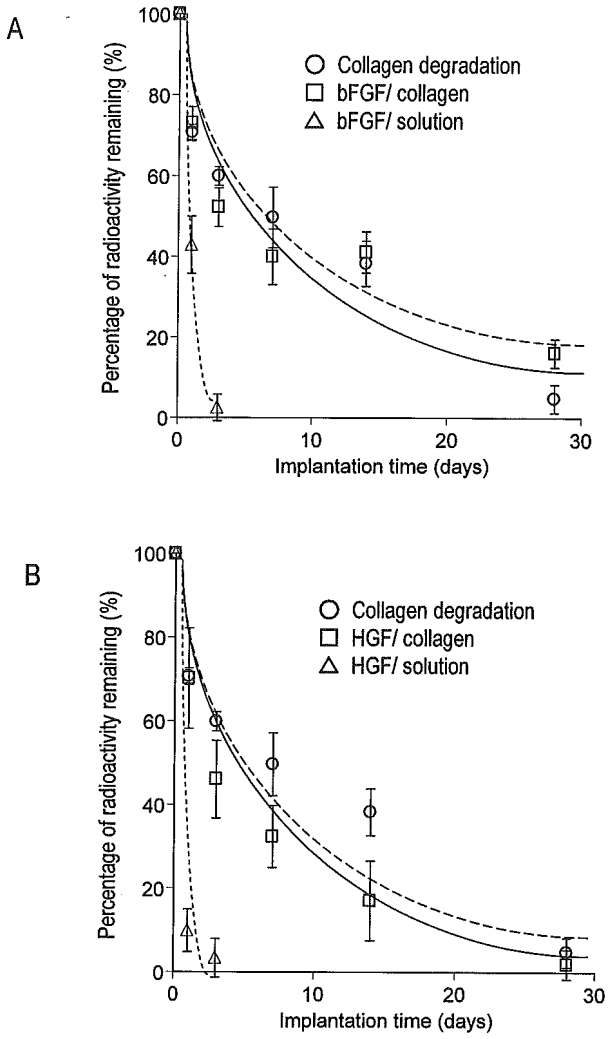

Fig 2. In vivo release profile of basic fibroblast growth factor (bFGF) or hepatocyte growth factor (HGF) from collagen. In vivo release profile of the radioactivity remaining after subcutaneous implantation of the collagen sheet incorporating ^{125}I-labeled bFGF or HGF (n = 6 each). The lines on the graphs are approximately growth factor/collagen (*solid line*), collagen degradation (*dashed line*), and growth factor/solution (*dotted line*). **A,** Sustained release of bFGF (*square*) from collagen was observed over 4 weeks, and the time profile correlates with that of the collagen degradation (*circle*). R^2 = 0.974, $P < .0005$, simple regression analysis. The bolus injection of bFGF solution (*triangle*) disappeared within 3 days. **B,** Sustained release of HGF (*square*) was observed over 4 weeks. The bolus injection of HGF solution (*triangle*) disappeared within 3 days.

Restoration of the blood perfusion in the murine hindlimb ischemia model

Sustained release versus bolus injection. A single 80-μg intramuscular injection of bFGF/CM increased the blood perfusion of the ischemic limb compared with control (no treatment) 4 weeks after the injection (95.1% ± 7.6% vs 46.1% ± 5.5%, $P < .01$) (Fig 3, *A*). A single 80-μg intramuscular injection of bFGF/solution did not significantly increase the blood perfusion (46.3% ± 5.3% vs 46.1% ± 5.5%). Similarly, 80 μg of HGF/CM increased the blood perfusion compared with the control (92.8% ± 7.6% vs 46.1% ± 5.5%, $P < 0.01$), but 80 μg of HGF/solution did not (45.4% ± 3.3% vs 46.1% ± 5.5%) (Fig 3, *B*). More than

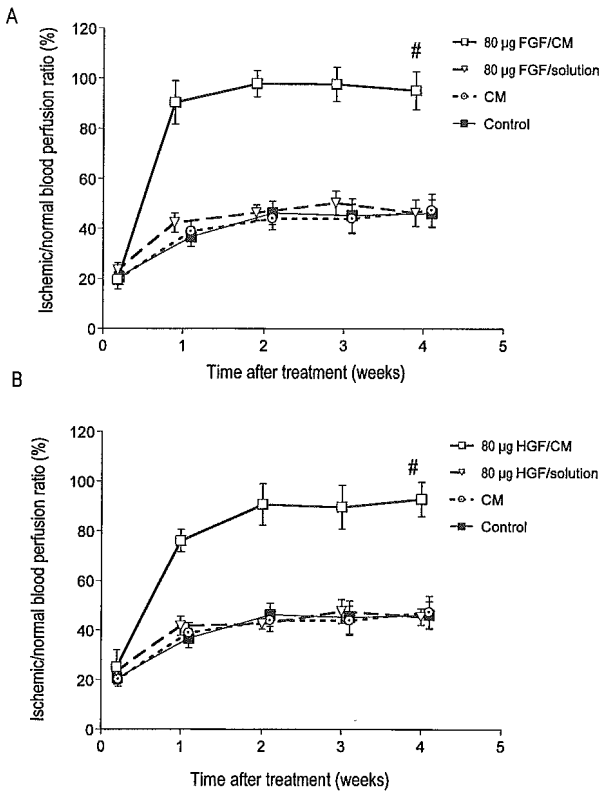

Fig 3. Restoration of the blood perfusion with basic fibroblast growth factor (bFGF) or hepatocyte growth factor (HGF) by sustained release vs bolus injection. Restoration of the blood perfusion in the ischemic limb with bFGF or HGF by sustained release or bolus injection was evaluated. Hindlimb blood perfusion was assessed by a ratio (%) of ischemic (*right*) to normal (*left*) hindlimb blood perfusion measured by laser Doppler perfusion imaging (LDPI) analyzer (n = 7 to 8). **A,** Serial LDPI measurements in ischemic limb treated with 80 μg of bFGF/CM, 80 μg of bFGF/solution, collagen microspheres (*CM*), and the control (no treatment) ($^{\#}P < .01$ vs. control). **B,** Serial LDPI measurements in ischemic limb treated with 80 μg of HGF/CM, 80 μg of HGF/solution, CM, and the control. $^{\#}P < .01$ vs. control.

80 μg of bFGF/solution or HGF/solution (up to 160 μg each) did not significantly increase the blood perfusion (data not shown). These results demonstrate that sustained release of bFGF or HGF from CM, but not bolus injection, is effective for restoration of the blood perfusion in this murine hindlimb ischemia model.

Threshold of restoration of the blood perfusion with bFGF or HGF. A single 20- or 80-μg intramuscular injection of bFGF/CM increased the blood perfusion compared with the control 4 weeks after the injection (93.8% ± 8.0% and 95.1% ± 7.6% vs 46.1% ± 5.5%; $P < .01$, respectively) (Fig 4, *A*). Restoration of the blood perfusion of 20 μg of bFGF/CM was equivalent to that of 80 μg of bFGF/CM. However, 1 or 5 μg of bFGF/CM injected into the ischemic limb did not significantly increase the blood perfusion (49.2% ± 4.3% and 51.2% ± 5.8% vs 46.1% ± 5.5%). Similarly, 80 μg of HGF/CM increased the blood perfusion compared with the control (92.8% ± 7.6% vs 46.1% ± 5.5%, $P < .01$), while 1, 5, or 20 μg of HGF/CM did not increase the blood perfusion (47.8% ± 4.3%, 48.2% ± 5.8%, 52.5% ± 8.0% vs 46.1% ± 5.5%) (Fig 4, *B*). These results demonstrate that 5 μg or less of bFGF/CM alone or 20 μg or less of HGF/CM alone is ineffective for restoration of the blood perfusion in the murine ischemic hindlimb model.

Sustained dual release of bFGF and HGF. To evaluate combined effects of bFGF and HGF, we simultaneously applied ineffective doses of bFGF/CM (5 μg) and HGF/CM (20 μg). The dual release (CM incorporating 5 μg of bFGF and 20 μg of HGF) was compared with either the single release of 5 μg of bFGF/CM alone or 20 μg of HGF/CM alone. Restoration of the blood perfusion in the dual release (94.2% ± 10.9%) was higher than the either single release (51.2% ± 5.8% or 52.5% ± 8.0%, respectively; $P < .01$) (Fig 4, *C*). Moreover, the restoration in the dual release was equivalent to that with 80 μg of bFGF/CM alone (95.1% ± 7.6%) or 80 μg of HGF/CM alone (92.8% ± 7.6%), namely lower-dose bFGF and HGF combined was as effective as higher-dose bFGF or HGF alone (Fig 4, *D*).

Immunohistochemistry

Capillary density. The number of capillaries was increased 4 weeks after the sustained single release of bFGF/CM at 20 μg (741 ± 155 vessels/mm^2) and 80 μg (893 ± 132 vessels/mm^2) compared with the control (168 ± 69 vessels/mm^2, $P < .01$ each) (Fig 5, *A*). Only 80 μg of HGF/CM (823 ± 132 vessels/mm^2) increased capillary density compared with the control ($P < .01$).

Capillary density in the dual release (CM incorporating 5 μg of bFGF and 20 μg of HGF) was higher at 868 ± 173 vessels/mm^2 than in the either the single release of 5 μg of bFGF/CM alone (204 ± 68 vessels/mm^2) or 20 μg of HGF/CM alone (185 ± 98 vessels/mm^2, $P < .01$). Capillary density in the dual release was equivalent to either single release at the higher dose (80 μg bFGF/CM or 80 μg HGF/CM).

Capillary density in the bolus injection groups (80 μg of bFGF/solution, 80 μg of HGF/solution, or their combination) did not increase compared with the control.

Vascular maturity. Because α-SMA marker is expressed in both pericytes and smooth muscle cells associated with endothelial cells in larger, mature blood vessels, we evaluated maturity of newly formed vessels by the percentage of α-SMA–positive vessels compared with total vessels counted by anti-vWF antibody staining[11,34](Fig 5, *C*). Vascular maturity was also higher in the dual release (43.8% ± 7.8%) than in either single release (9.5% ± 3.0% or 11.7% ± 3.8%, $P < .01$). Although capillary densities were equivalent between the dual release and the high-dose single release (80 μg of bFGF/CM or HGF/CM), vascular maturity in the dual release was higher than in the high-dose single release of bFGF (30.8% ± 8.8%, $P < .05$) or HGF (22.4% ± 4.9%, $P < .01$) (Fig 5, *C*).

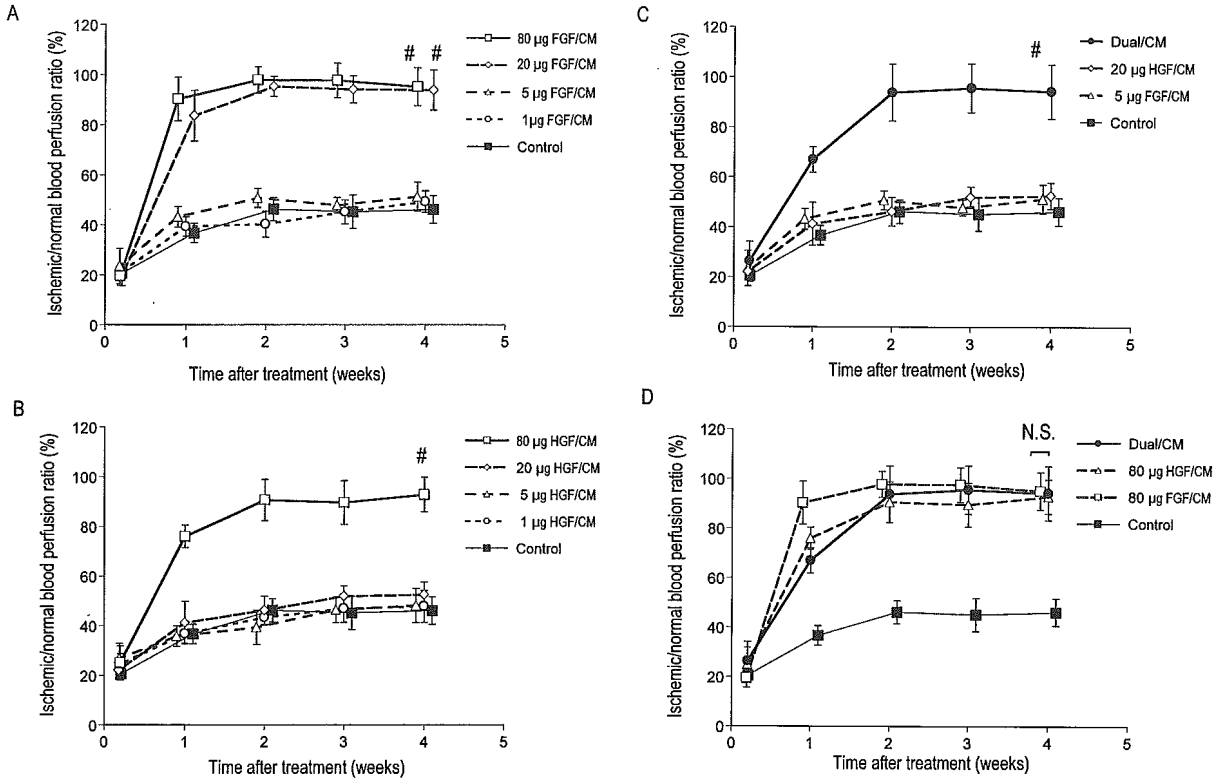

Fig 4. Restoration of the blood perfusion by single or dual release of basic fibroblast growth factor (bFGF) or hepatocyte growth factor (HGF). Quantitative analysis of ischemic/normal perfusion ratio (%) in ischemic hindlimb measured by laser Doppler perfusion image (LDPI) analyzer (n = 7 to 8). Serial LDPI measurements in ischemic limb treated with indicated doses. **A,** Threshold of restoration of the blood perfusion of bFGF/CM (1, 5, 20, and 80 μg) and the control. $^{\#}P < .01$ vs control. **B,** Threshold of restoration of the blood perfusion of HGF/CM (1, 5, 20, and 80 μg) and the control ($^{\#}P < .01$ vs control). **C,** Restoration of the blood perfusion with dual/CM (CM incorporating 5 μg of bFGF and 20 μg of HGF), 20 μg of HGF/CM alone, 5 μg of bFGF/CM alone, and the control. $^{\#}P < .01$ vs every other group. **D,** Restoration of the blood perfusion with dual/CM (CM incorporating 5 μg of bFGF and 20 μg of HGF), 80 μg of bFGF/CM, 80 μg of HGF/CM, and the control. *CM*, collagen microspheres; *NS*, not significant.

Focal inflammation. Three days after the treatment, number of CD45-positive infiltrating leukocytes in the ischemic limb did not significantly increase in all groups compared with the control; however, the number of leukocytes in the ischemic limbs treated with 80 μg of bFGF/ solution and 80 μg each of bFGF and HGF combined (*k* and *m* in Fig 5, *E*) tended to increase compared with the control (1342 ± 455/mm^2 and 1305 ± 440/mm^2; vs 1061 ± 254/mm^2; $P = .072$ and $P = .082$) (Fig 5, *E*). The number of infiltrating leukocytes showed no difference both among the bFGF/CM-treated groups (Fig 5, *E b-e*) and among the HGF/CM-treated groups (Fig 5, *E f- i*).

DISCUSSION

The present study demonstrates the utility of the simultaneous application of bFGF and HGF for the restoration of blood perfusion in limb ischemia. The combination of bFGF and HGF potentiates capillary network formation of HUVECs compared with either single growth factor alone. The sustained dual release of bFGF and HGF from biodegradable CM reproduces such synergism in vivo, resulting in an increase in the number of blood vessels and the promotion of vascular maturation in the murine limb ischemia model. The simultaneous application of lower-dose bFGF and HGF restored the blood perfusion equivalent to higher-dose bFGF or HGF alone.

In addition, the sustained release of these growth factors does not further aggravate focal inflammation. These results demonstrate that simultaneous application of bFGF and HGF released from CM can provide a more effective strategy for successful growth-factor therapy.

In the present study, the restoration of blood perfusion in the control or the CM-injected legs was lower than that reported by Couffinhal et al.[32] To obtain a severe hindlimb ischemia model, we excised the entire right saphenous artery and vein and the right external iliac artery and vein with deep femoral and circumflex arteries, according to several previous studies.[13,28,33] In such a severe ischemic model, the simultaneous application of lower-dose bFGF and HGF achieved equivalent blood perfusion recovery and more vascular maturity than did the higher-dose bFGF or HGF alone.

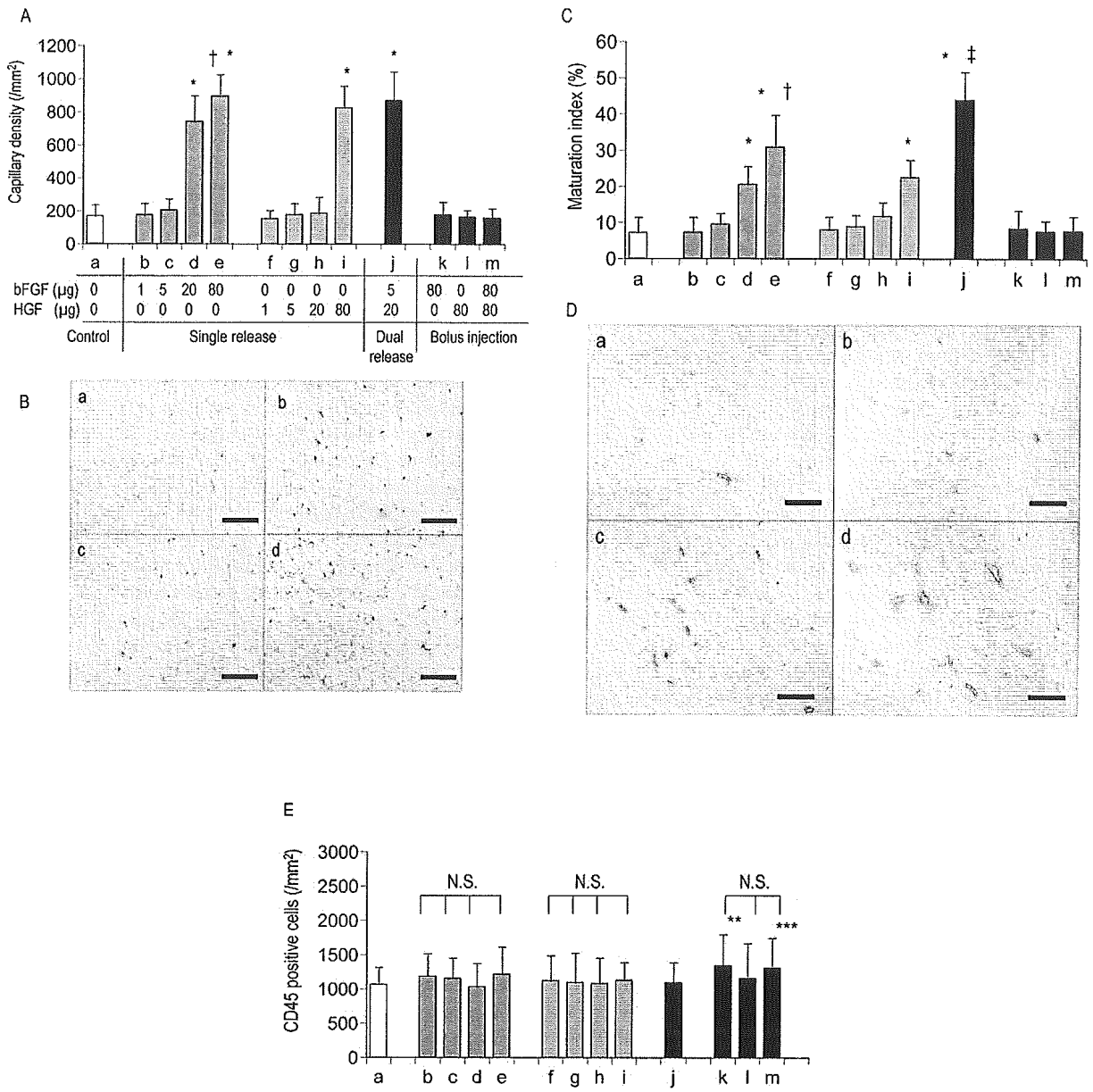

Fig 5. Immunohisotchemical analysis in the ischemic limb after treatments. **A,** Capillary density: quantitative analysis of capillary density (anti-vWF-positive vessels/mm^2) in the ischemic limb. $*P < .01$ vs control, $^{\dagger}P < .05$ vs (*d*) 20 μg of bFGF/CM. *Table, a-m,* Several treatments in the ischemic hindlimb: *a,* control; *b-e,* sustained single release of 1, 5, 20, and 80 μg of bFGF; *f-l,* sustained single release of 1, 5, 20, and 80 μg of HGF; *j,* sustained dual release of 5 μg of bFGF and 20 μg of HGF; *k-m,* bolus injection of *(k)* 80 μg of bFGF, *(l)* 80 μg of HGF, and *(m)* 80 μg of bFGF and HGF. **B,** Immunostaining of the ischemic hindlimb tissue with anti-vWF 4 weeks after the treatment: *a,* control; *b,* 20 μg of HGF/CM; *c,* 5 μg of bFGF/CM; *d,* dual/CM (CM incorporating 5 μg of bFGF and 20 μg HGF 5) (×100; scale bars, 100 μm). **C,** Maturation index: maturity of newly formed vessels was evaluated by a percentage of α-SMA–positive vessels compared with total vessels counted by anti-vWF antibody staining. $*P < .01$ vs *a*, $^{\dagger}P < .05$ vs *d*, $^{\ddagger}P < .01$ vs *i,* and $P < .05$ vs *e.* **D,** Immunostaining of the ischemic hindlimb tissue with α-SMA 4 weeks after the treatment: (*a*) Control, (*b*) 20 μg of HGF/CM, (*c*) 5 μg of bFGF/CM, (*d*) dual/CM (CM incorporating 5 μg of bFGF and 20 μg of HGF) (×100; scale bars, 100 μm). **E,** Focal inflammation: Number of infiltrating leukocytes in ischemic limb obtained from immunostaining with anti-CD45 antibody 3 days after the treatment (n = 4 to 5).$*P <$ .01, $**P = .072$, $***P = .83$ vs control, *vWF,* von Willebrand factor; *bFGF,* basic fibroblast growth factor; *HGF,* hepatocyte growth factor; *CM,* collagen microspheres; *SMA,* smooth muscle actin.

Although both bFGF and HGF recovered the blood perfusion in the ischemic limb as a single agent in this model, the recovery required substantial dose escalation. More than 20 μg of bFGF/CM sufficiently increased the blood flow, but vascular maturity was lower than in the dual release. To obtain significant increase in the blood flow by HGF alone, the highest dose of HGF (80 μg) was required, which was four times as much as that of bFGF; however, vascular maturity was also lower than the dual release even at the highest dosage. Although the long-term study of our system has not yet been accomplished, the higher maturity observed here might be a promising sign for longer durability of therapeutic effects.

The α-SMA–positive vessels in the calf muscles that were observed in the dual therapy could be interpreted either as mature new vessels resulting from angiogenesis or as dilated collaterals due to arteriogenesis or mature arterioles. Capillaries tend to form in areas of ischemia, while collateral arteries that are undergoing remodeling are typically not surrounded by ischemic tissue.[35-37] From this point of view, α-SMA–positive vessels in the ischemic calf muscles observed in the dual therapy could be mainly interpreted as mature new vessels resulting from angiogenesis. The origin of these vessels could not be precisely determined, but dual therapy resulted in both histologically and functionally better vasculature. If they were newly formed capillaries, maturing benefit of the dual growth-factor treatment on angiogenesis could be suggested; if they were dilated pre-existing vessels, the growth factors might have promoted remodeling.

The combination of bFGF and HGF is promising for several reasons. The administration of HGF alone or bFGF alone partially attenuated endothelial cell death mediated by serum deprivation, whereas co-incubation with bFGF and HGF completely abolished endothelial cell death.[14] Onimaru et al[13] reported that HGF plays a critical role in bFGF-mediated angiogenesis in ischemic limbs. bFGF stimulated endogenous HGF expression irrespective of the presence or absence of hypoxia. Furthermore, blockade of endogenous HGF activity by neutralizing antibody not only abolishes the bFGF-dependent increase in blood perfusion but also results in major limb loss.

The mechanisms underlying the angiogenic synergy induced by bFGF and HGF may be complex[13]: bFGF stimulates HGF mRNA expression and protein secretion mainly by the mitogen-activated protein kinase/extracellular signal-regulated kinase signal-transduction pathway. Both Ras and p70S6K signals are shown to be important in the later phase of bFGF-mediated HGF induction, which is partly regulated by the accelerated expression of endogenous PDGF-AA. Although endogenous HGF may play a significant role in bFGF-mediated angiogenesis, the level of induced HGF may be far less than optimal. To attain such synergism in vivo, the administration of two exogenous growth factors may be the only solution.

Past studies for therapeutic angiogenesis with multiple growth factors reported on the combination of PDGF-BB with VEGF or bFGF because PDGF-BB was expected to promote the maturation of vessels by recruitment of smooth muscle cells to the endothelial lining of nascent vasculature.[9,10] Dual delivery of VEGF and PDGF-BB from a polymer scaffold results in the rapid formation of a mature vascular network.[11] Single angiogenic factors, including bFGF, VEGF, or PDGF-BB, released slowly from carrier polymers were unable to establish stable vascular networks; however, the combination of bFGF and PDGF-BB synergistically induced vascular networks that remained stable for more than a year, even after the angiogenic factors were depleted.[12] Although the evaluation of combined therapy by VEGF and PDGF-BB was different, these reports suggest the impact of combinations of growth factors for therapeutic angiogenesis.

Therapeutic strategies with the gene transfer of angiogenic growth factors have shown promising results and safety in phase I and II clinical trials, although there are still concerns about unpredictable duration and level of gene expression and the immune or inflammatory responses of genetic materials.[1-6] The sustained release of growth-factor proteins from CM may solve these problems. By changing the cross-linking extent, each growth factor could be released at a desirable rate and duration for tissue regeneration.

Sustained release of growth factors from CM did not aggravate focal inflammation, which was also observed in the control group, while bolus injection of high-dose bFGF aggravated focal inflammation. Collagen is commonly resorbed via inflammatory phagocytosis or proteolysis by matrix metalloproteases. Meanwhile, both bFGF and HGF also have been shown to increase or be chemotactic for inflammation, or both.[38,39] However, such phagocytosis or inflammation induced by bFGF or HGF was not so significant as to cause a marked change in histology.

We have demonstrated the effectiveness of bFGF protein released from biodegradable acidic gelatin hydrogel in various animal models such as acute myocardial infarction, prevascularization for cardiomyocyte transplantation to the heart, and bone regeneration of the sternum.[19-26] We used CM instead of gelatin hydrogel in the present study because the release profile of bFGF from the collagen sheet was almost equal to that from gelatin hydrogel, whereas the release profile of HGF from collagen was superior to that from gelatin hydrogel. As a carrier biomaterial, collagen may be suitable for clinical use in terms of easy processability and versatile controlled release of various growth factors. Collagen is easily processed to microspheres, sheets, or disks. Microspheres are easily dispersed in the water and can be injected to various organs, whereas sheets can be put on the heart, bone, and other tissues. They enable sustained release by a single administration for various fields of regenerative medicine. The multiple-release system of growth factors from collagen may find broad utility in the regeneration of a variety of other tissues as well.

CONCLUSIONS

This study demonstrates that the sustained dual release of bFGF and HGF from a CM enhances the blood vessels formation compared with bFGF or HGF released alone. A therapeutic strategy with multiple growth factors released

from carrier biomaterial would be a highly promising regimen for near future.

We are very grateful to Ms. Kataoka and Ms. Toyoda for their meticulous work on the histologic studies.

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Effects of bFGF incorporated into a gelatin sheet on wound healing

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Abstract—Basic fibroblast growth factor (bFGF) is well known to promote the proliferation of almost all cells associated with wound healing. However, as the activation duration of bFGF is very short *in vivo*, we incorporated bFGF into an acidic gelatin hydrogel and studied the sustained release of bFGF *in vivo*. In addition, we investigated the effects of the acidic gelatin sheet containing bFGF on wound healing. To distinguish wound contraction from neoepithelialization, we measured both the wound area and neoepithelium length. Other histological parameters such as thickness of granulation tissue and number of capillaries were also determined as indices of wound healing. Fibrous tissue was assessed using an Elastica van Gieson and Azan stain. A skin defect (1.5 × 1.5 cm) of full thickness was created on the back of each test mouse and the wound was covered with an acidic gelatin hydrogel, referred to as a gelatin sheet in this study (2 × 2 cm), with bFGF (100 μg/site) (A) or without bFGF (B). 1, 2, 3, 5, 7 and 14 days after covering, mice were killed and an enzyme-linked immunosorbent assay (ELISA) was performed to estimate the concentration of bFGF in the plasma. In another experiment, each wound was covered with (A), (B) or a hydrogel dressing (control group, C) and the wound area was measured 1 or 2 weeks postoperatively with a computer planimeter. The histological parameters, as mentioned above, were assessed using a light microscope. Sustained release of bFGF from the gelatin sheet was observed and the gelatin sheet containing bFGF promoted neoepithelialization, granulation, neovascularization and wound closure. This gelatin sheet containing bFGF was concluded to be effective for wound healing and promising for clinical use.

Key words: Basic fibroblast growth factor; gelatin; sustained release; neoepithelialization; wound contraction; wound healing.

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INTRODUCTION

Basic fibroblast growth factor (bFGF), a cytokine, was first reported by Gospodarowicz in 1974 [1] and is known to proliferate ectoderm and mesoderm cells both *in vivo* and *in vitro*. For example, bFGF was originally characterized *in vivo* as a potent mitogen and a chemo-attractant for a wide range of cells, and *in vitro* as a growth factor for fibroblasts and capillary endothelial cells [2–6]. Recombinant human bFGF has already been used clinically for the treatment of bedsores and ulcers in Japan. However, as bFGF has a very short half-life in the body because of rapid diffusion and enzymolysis, repeated administration is required for clinical use. Therefore, we attempted to develop a new wound dressing with sustained release of bFGF over a long period of time. Matrices studied to date for the sustained release of cytokines include chitosan [7], poly L-lactic acid (PLLA) and its co-polymers with glycolic acid (PLGA) [8–10], polycyanoacrylates [11], polyethylene-co-vinylacetate [12, 13], hyaluronic acid derivatives [14], alginate [15, 16] and gelatin [17–20].

Gelatin is a biopolymer used in many medical applications because of its non-toxic nature. It is a water-soluble, denatured protein originating from collagen, which is the main protein component of connective tissues like bone and skin. Collagen as a biomaterial is mainly produced from cow bones, cowhide and pigskin. Gelatin has many advantageous characteristics including sol–gel conversion at a suitable temperature, water solubility at body temperature and good stability for emulsification. Gelatin can produce a membrane which is comparatively strong under dry conditions and easily cut according to necessity. Furthermore, a variety of gelatins with different isoelectric points (IEPs) can be obtained by alkaline or acidic production processes [21]. In neutral water, gelatin with an IEP of 5.0 (acidic gelatin), produced by an alkaline process, becomes negatively charged, while gelatin with an IEP of 9.0 (basic gelatin), produced by an acid process, becomes positively charged [22]. It has been confirmed that hydrogel made from acidic gelatin with an IEP of 5.0 can be used as a matrix for sustained release of bFGF, in contrast to the situation with basic gelatin [22–24]. The gelatin hydrogel with sustained release of bFGF induced neovascularization and tissue granulation more than bFGF alone [18]. Polyion complexations between gelatin (acidic or basic) and bFGF were evaluated using turbidimetric titration, which is a simple method for such evaluation. By this method, it was indicated that a polyion complex of bFGF was formed with the acidic gelatin but not with the basic gelatin, and the time-course of bFGF release was in accordance with the rate of hydrogel degradation [23]. In this report, bFGF-incorporating gelatin hydrogel was implanted subcutaneously onto the backs of mice. We used an acidic gelatin hydrogel sheet containing bFGF as a dressing material and investigated whether it could slowly release bFGF when placed on full-thickness skin defects of normal mice. In addition, we studied its effect on wound healing using diabetic mice by measuring the wound area, epithelium length, thickness of granulation tissue and capillary number. The biosynthesized collagen fibers were observed

using histological specimens with Elastica van Gieson and Azan staining. As the healing of wounds proceeds in three overlapping phases (inflammation, granulation tissue formation and matrix formation/remodeling [25]), clinically ideal wound healing requires quick wound closure brought about by epithelialization and wound contraction. Since the latter sometimes causes clinically unfavorable results like scar contracture, we distinguished epithelialization from reduction of the wound area in the evaluation.

MATERIALS AND METHODS

Experimental animals

Experiments were performed using 10-week-old normal and genetically diabetic male mice (Jcl:ICR and C57BL/KsJ-*dbdb*, respectively, CLEA Japan, Osaka, Japan). All mice were handled according to the Guidelines of the Committee on Animal Care and Use of Kagawa University School of Medicine.

Preparation of gelatin hydrogels containing bFGF

Gelatin hydrogel was prepared by cross-linking it with glutaraldehyde. Briefly, 5 wt% aqueous solutions of gelatin containing 0.05 wt% glutaraldehyde were spread on a sheet of polypropylene (14×14 cm^2) for 12 h at 4°C. Following the cross-linking reaction, the resulting sheet of gelatin hydrogel (2 mm thick) was dissected into 2×2 cm pieces, referred to as a 'gelatin sheet' in the present study. The gelatin sheet was immersed in 100 mM of glycine solution for 3 h at 37°C to block residual glutaraldehyde, washed twice with distilled water, freeze-dried and sterilized using ethylene oxide gas. The water content of the gelatin sheets was calculated from their weight before and after swelling in phosphate-buffered saline solution (PBS, pH 7.4) for 24 h at 37°C and expressed as the weight ratio of water in the gelatin sheet to the whole wet gelatin sheet. The weight of the dry gelatin sheet and the water content of the gelatin sheet were 75 mg and 95 wt%, respectively.

Full-thickness wound model

Each mouse was anesthetized with pentobarbital (50 mg/kg intraperitoneally (i.p.)) and a full-thickness wound (1.5×1.5 cm) was excised with scissors after shaving the mouse's back. The membrane-like tissue under the skin was retained.

Evaluation of concentration of bFGF in plasma

Mice (Jcl:ICR) were used to assess the controlled release of bFGF contained in the gelatin sheet. Recombinant human bFGF (100 μg) was dissolved with PBS and was immediately dropped onto a freeze-dried gelatin sheet with a pipette. The sheet was kept for 3 h at 4°C to get impregnated with bFGF by polyion complexation. The

wound was covered with the sheet, and the sheet was sutured to the adjacent normal skin with 6-0 prolene® thread. 1, 2, 3, 5, 7 and 14 days after covering, plasma was collected to measure bFGF using ELISA. As a control, a gelatin sheet containing PBS was used.

Measurement of wound area and histological observation

Genetically diabetic mice (C57BL/KsJ-*db*/*db*) were used for this measurement. A full-thickness wound was made and covered with a gelatin sheet containing bFGF (100 μg/site) (bFGF(+) group), a gelatin sheet without bFGF (bFGF(−) group), or a hydrogel dressing (NU-GEL®) (control group), and these covering materials were sutured to the adjacent normal skin with 6-0 prolene® thread. The size of the wounds was measured 1 and 2 weeks postoperatively. A computer planimeter (Placom KP-90) was used to analyze the rate of wound area change.

The length of neoepithelium from either side of a sagittal section of the central slice of each wound was measured using a light microscope. Samples were fixed with formalin and paraffin-embedded, then stained with H and E. The neoepithelium length of each specimen was measured twice, from one edge and from the other edge, and the average of these two lengths was used for statistical analysis. We also stained the specimens with Elastica van Gieson and Azan to assess the formation of granulation tissue at the central area of the wound.

The thickness of granulation tissue was measured at both wound margins using a light microscope for each sample, and the mean thickness was evaluated.

Neoformed capillaries in the wounds were stained with rat monoclonal antibody to mouse CD34. The number of capillaries at the central area of the wound was counted using a light microscope at $\times 100$ magnification.

Reagents

The following reagents were used (from Sigma, St. Louis, MO, USA, unless otherwise stated): recombinant human bFGF with an isoelectric point of 9.6 (Kaken Pharmaceutical, Tokyo, Japan), gelatin isolated *via* an alkaline process from bovine bone with an isoelectric point of 5.0 and an average molecular mass of 99 kDa (Nitta Gelatin, Osaka, Japan), glutaraldehyde, glycine, PBS, pentobarbital (Abbott, North Chicago, IL, USA), hydrogel dressing (NU-GEL®) and 6-0 prolene® (Johnson and Johnson Medical, Tokyo, Japan), human FGF basic immunoassay (Cosmo Bio, Tokyo, Japan), rat monoclonal antibody to mouse CD34 (HyCult Biotechnology, Uden, The Netherlands) and a computer planimeter (Placom KP-90, Koizumi Sokki, Tokyo, Japan). bFGF was dissolved in PBS. The control study (bFGF(−) group and control group) was performed using PBS. The concentrations of the reagents used were expressed as final molar concentrations.

Statistical analysis

Data are given as means±SEM. Two-way and one-way ANOVA followed by a multiple comparison test with a Fisher's Protected Least Significant Difference (PLSD) adjustment was used for evaluation of the wound area and concentration of bFGF in plasma, and the one-way ANOVA was used for the other evaluations. $P < 0.05$ was considered significant.

RESULTS

The gelatin sheet, which was used for covering the wounds, kept its form for 14 days.

Concentration of bFGF in plasma

The concentration of bFGF in plasma 1 day after grafting gelatin sheets containing bFGF was 297.5 ± 26.2 pg/ml. After that, the concentration in the bFGF(+) group gradually decreased until 14 days, but was statistically greater than that of the bFGF(−) group (non-detectable) for 7 days ($P < 0.005$, Fig. 1).

Measurement of wound area and histological observation

There was no significant difference in the wound area among the groups at day 7 (Fig. 2), but the wound area in the bFGF(+) group (6.7%) was significantly smaller than that of the control (21.3%, $P < 0.001$) and bFGF(−) groups (18.7%, $P < 0.005$) at day 14. There was no significant difference in the wound area between the bFGF(−) group and the control group on day 14.

Group	concentration (means±SEM)
bFGF(-)	non-detectable
bFGF(+)	
Day 1	297.5±26.2
Day 2	103.3±20.3
Day 3	105.7±32.5
Day 5	89.7±22.8
Day 7	56.5±10.5
Day 14	26.4±10.6

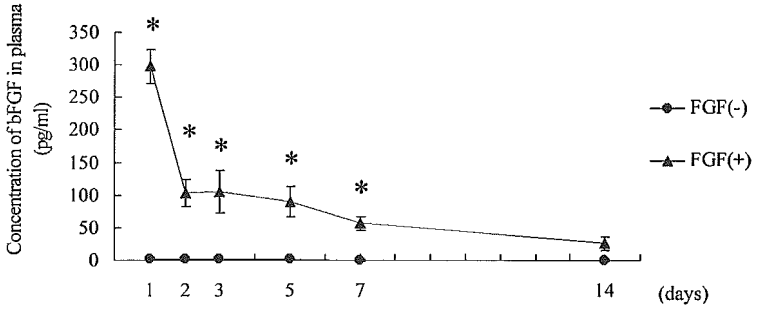


Figure 1. The concentration change in bFGF in plasma after covering the full-thickness wounds with a gelatin sheet containing bFGF (100 μg/site, bFGF(+), ▲) or not containing bFGF (bFGF(−), ●). Data are presented as means±SEM, and statistical analysis was done by two-way ANOVA; one independent variable was time-course and the other was with or without bFGF. When significance was indicated, comparisons between all groups were done by one-way ANOVA, followed by a multiple comparison test with a Fisher's Protected Least Significant Difference (PLSD) adjustment. $^*P < 0.005$ *versus* bFGF(−); $n = 3 - 6$ in each group. Error bars in bFGF(−) group fall within the circles.

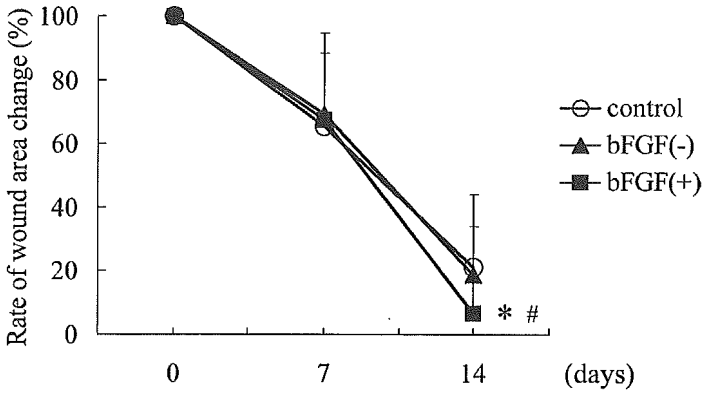


Figure 2. The change rate of wound area was calculated from the area before and after covering the wounds (expressed as the ratio of the healing wound area to the original wound area). Data are presented as means±SEM, and statistical analysis was done by two-way ANOVA; one independent variable was time-course, and the other was with or without bFGF or control. When significance was indicated, comparisons between all groups were done by one-way ANOVA, followed by a multiple comparison test with a Fisher's PLSD adjustment. $^{*}P < 0.001$ *versus* control, $^{\#}P < 0.005$ *versus* without bFGF; $n = 3 - 6$ in each group.

Histological findings at day 7 showed many fibroblasts growing in the dermis granulation tissue with little inflammatory cell infiltration in the bFGF(+) and bFGF(−) groups. Particularly in the bFGF(+) group, more capillaries were observed with increased fibroblasts around the capillaries. In the control group, invasion of neutrophils and lymphocytes was prominent, but there were fewer fibroblasts and capillaries than in the bFGF(+) and bFGF(−) groups. 14 days postoperatively, the neoepithelium from the edge of the wound in the bFGF(+) and bFGF(−) groups was longer than that of the control group. In the bFGF(+) group, the neoepithelium extended almost to the center of the wound. Many capillaries grew toward the superficial layer of subepidermis tissue, and scar contracture was small. In the control group, a crust containing neutrophils and lymphocytes covered the dermis granulation tissue and capillaries were distributed at the deep layer of subepidermal tissue in the control group (Fig. 3). 14 days postoperatively, the neoepithelium was longest in the bFGF(+) group (2.1 ± 0.2 mm), second longest in the bFGF(−) group (1.5 ± 0.2 mm) and shortest in the control group (1.1 ± 0.2 mm). The neoepithelial length was statistically longer in the bFGF(+) group compared with that in the control group ($P < 0.01$) (Fig. 4a).

Figure 4b shows the thickness of granulation tissue in each group at day 14. The granulation was thickest in the bFGF(+) group (1.4 ± 0.1 mm). Both the bFGF(+) group and the bFGF(−) group (0.9 ± 0.1 mm) showed thicker granulation tissue than the control group (0.4 ± 0.1 mm) ($P < 0.005$, $P < 0.01$, respectively), and the differences ($P < 0.01$) were significant among the three groups.

The capillary number of the bFGF(+) group (56.2±13.9) was significantly greater than that of the bFGF(−) group (22.5 ± 7.0) and control group (18 ± 7.6) (Fig. 4c and 4d). However, no significant difference was found between the bFGF(−) and

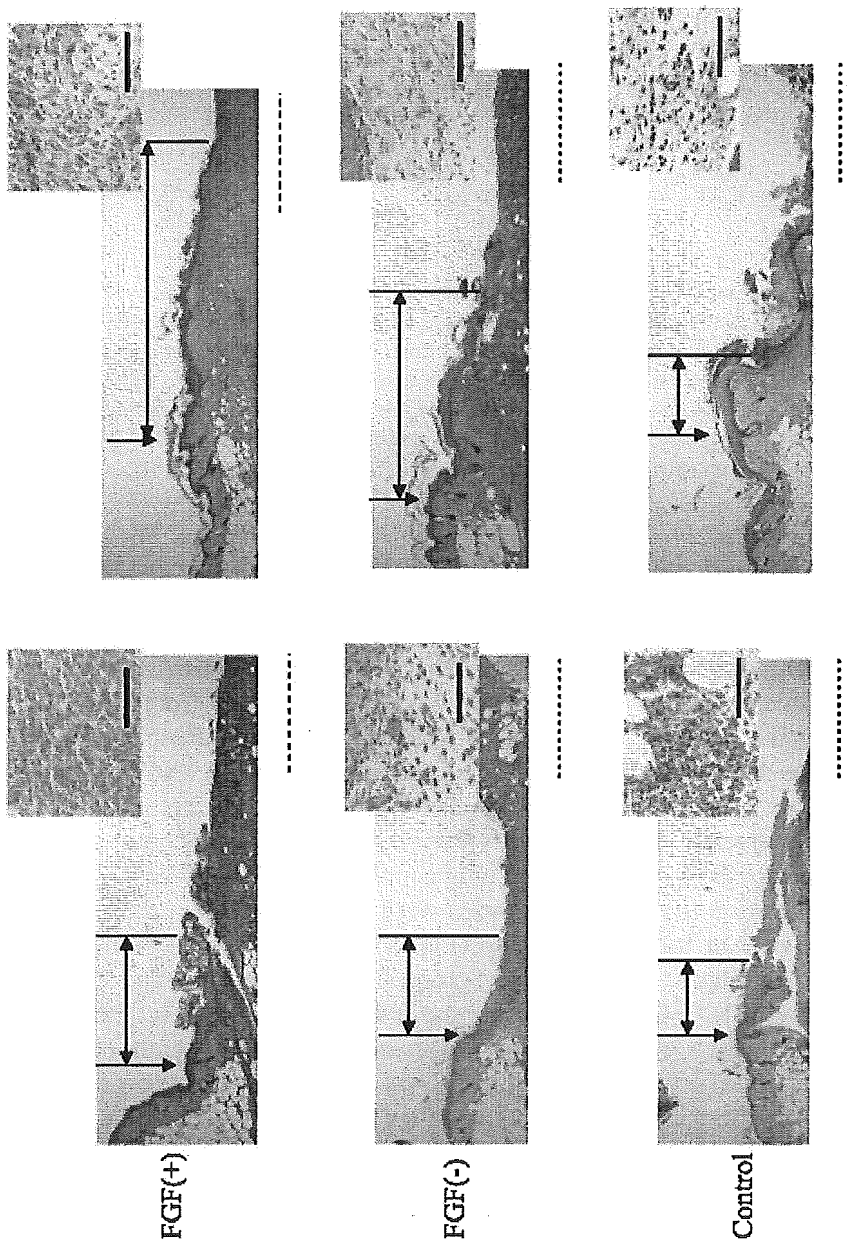


Figure 3. Photographs showing the wound healing process at days 7 and 14. Formation of neoepithelium was promoted well in the bFGF(+) and bFGF(−) groups. At day 14, the bFGF(+) group was almost repaired, with the wound nearly closed by neoepithelium. Arrows (→) show each wound edge and double arrows (↔) show neoepithelium length. Dotted line (—) is 1 mm, bar (–) is 100 μm.

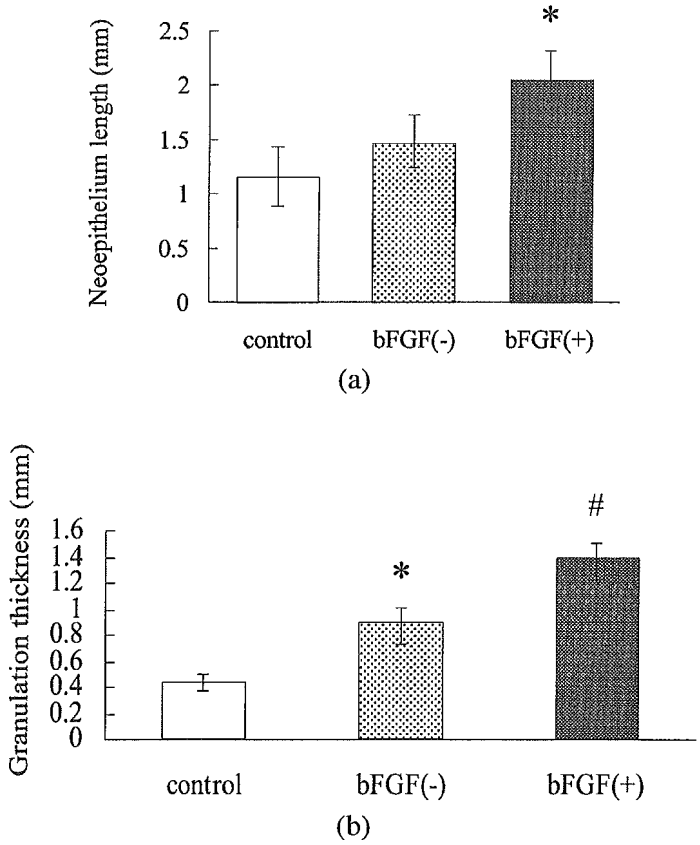
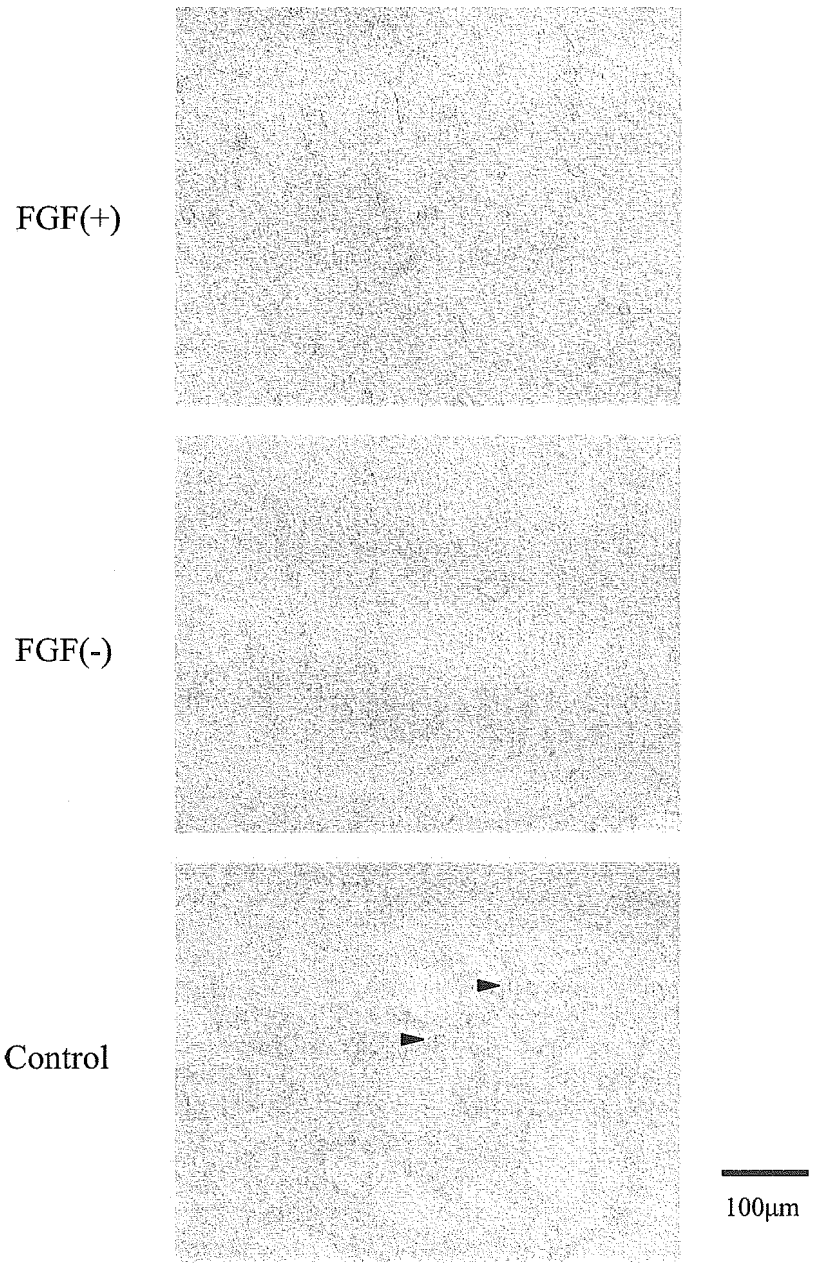


Figure 4. (a) Comparison of the neoepithelium length from the wound edge at 14 days after covering the full-thickness wounds. Data are presented as means±SEM and statistical analysis was done by one-way ANOVA; an independent variable was with or without bFGF or control. When significance was indicated, comparisons between all groups were done by a multiple comparison test with a Fisher's PLSD adjustment. $^{*}P < 0.01$ *versus* control; $n = 6$ in each group. (b) Comparison of the granulation thickness at the wound edge at 14 days after covering the full-thickness wounds with each material. Data are presented as means±SEM and statistical analysis was done by one-way ANOVA; an independent variable was with or without bFGF or control. When significance was indicated, comparisons between all groups were done by a multiple comparison test with a Fisher's PLSD adjustment. $^{*}P < 0.01$ *versus* control, $^{\#}P < 0.005$ *versus* control and without bFGF; $n = 6$ in each group.

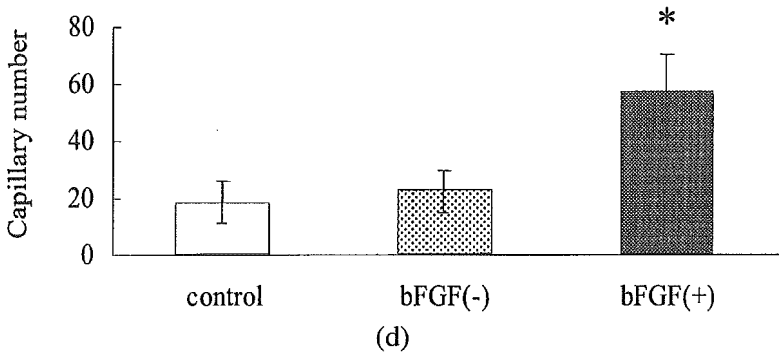
control groups. These results confirmed the new vessel growth in the wound healing process by bFGF.

Elastica van Gieson and Azan staining showed thick granulation tissue with both collagen fibers and cellular components at the center of the wound 14 days postoperatively, especially in the bFGF(+) group. In the control group, rough collagen fibers were observed in the thin granulation layer with few cellular components at the center of the wound (Fig. 5).



(c)

Figure 4. (c) Photographs showing mouse CD34-stained capillaries in the central areas of the wounds at 14 days after covering full-thickness wounds with each material. In the bFGF(+) group, many capillaries were seen in the upper layer, but in the control group, capillaries were observed around the fat tissue layer (arrowhead). This figure is published in colour on http://www.ingenta.com



(d)

Figure 4. (d) Comparison of the number of mouse CD34-stained capillaries in the central areas of the wounds at 14 days after covering full-thickness wounds with each material. Data are presented as means±SEM, and statistical analysis was done by one-way ANOVA; an independent variable was with or without bFGF or control. When significance was indicated, comparisons between all groups were done by a multiple comparison test with a Fisher's PLSD adjustment. $^*P < 0.05$ *versus* control and without bFGF; $n = 6$ in each group.

DISCUSSION

Since genetically diabetic and steroid-treated animals are known to be impaired wound healing models [26, 27], genetically diabetic mice may be advantageous in studies for distinguishing the wound healing difference, but normal mice can be used for measuring the bFGF concentration in plasma. The pharmacokinetics of bFGF in rat blood have been studied and rapid clearance of bFGF from blood was reported [28]. Indeed, the half-life of bFGF was about 2.4 h under conditions in which bFGF at 100 μg was applied as a bolus in solution to the surface of a full-thickness wound (2 cm^2). It was also pointed out that local injection of bFGF was almost resolved in the local area before escaping into the blood. These results indicate that, in our study, the bFGF detected in plasma was released from the gelatin sheet. Figure 1 indicates an initial surge of bFGF on day 1 post-surgery to be followed by slow release of bFGF from the gelatin sheet until 7 days. In a similar experiment, it was reported that a certain amount of bFGF was released from the acidic gelatin hydrogels within one day, probably because the impregnation conditions were not sufficient to completely form a polyion complex between bFGF and gelatin [23]. It was likely that bFGF molecules that are not complexed with gelatin were released from gelatin hydrogels during the initial period. This can be explained in terms of the initial release of non-complexed bFGF, and that all bFGF molecules were not ionically complexed with the acidic gelatin sheet. The effects of the initial higher dosage administration of bFGF were reported by Ono [29]. They demonstrated that bFGF administered immediately after making incisional wounds promoted vascularization and significantly increased the breaking strength and collagen fibers from the fifth week, and later after the operation, in a dose-dependent manner. These results lead us to believe that in Fig. 1 an initial surge of bFGF on day 1 post-surgery might be effective for wound healing. To assess

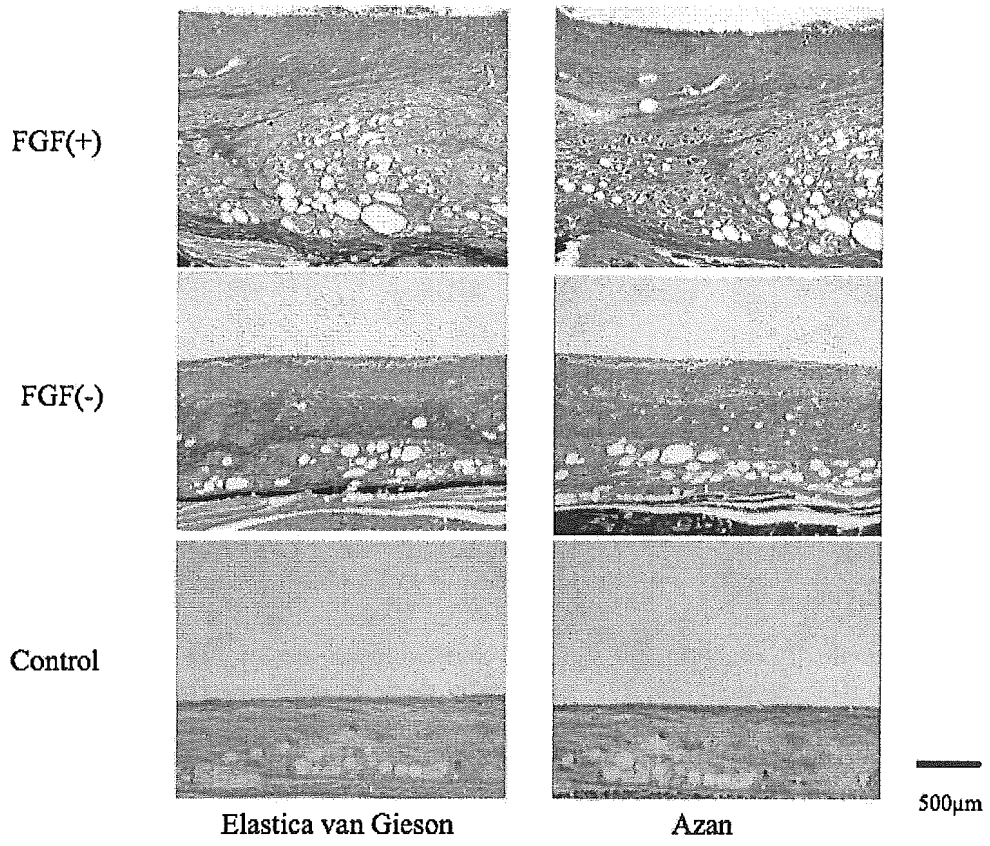


Figure 5. Elastica van Gieson and Azan stain at 14 days after covering the full-thickness wounds. Collagen fibers were stained red with Elastica van Gieson and blue with Azan stain. At the center of the wound, both collagen fibers and cellular components (red) increased in the bFGF(+) and bFGF(−) groups and thicker connective tissue was found, compared to the control group.

the *in vivo* profile of bFGF release from acidic gelatin hydrogels, acidic gelatin hydrogels containing ^{125}I-labeled bFGF were implanted subcutaneously onto the backs of mice and residual radioactivity was measured [22, 23]. The decrement pattern of bFGF radioactivity in the hydrogel was in agreement with that of gelatin radioactivity for 14 days. It was also pointed out that in the subcutaneous injection of ^{125}I-labeled bFGF in aqueous solution, about 80% of bFGF disappeared within 1 day [22]. These results lead us to believe that the gelatin sheet as a dressing material can release bFGF effectively for 7 days. Various studies on acidic gelatin hydrogel incorporating bFGF have reported the acceleration of normal sternal bone regeneration and healing after bilateral internal thoracic artery (BITA) removal in normal rats [30] and even in diabetic rats [31], as well as the promotion of corneal neovascularization in rabbits [32]. Gelatin and bFGF are combined by polyion complexation, so the bFGF is emitted slowly. Regarding the mechanism of bFGF release from gelatin hydrogel, it has been reported that biologically active bFGF

is released as a result of *in vivo* hydrogel degradation [18, 22–24, 32]. In the living body, heparin and heparin sulfate proteoglycans in the extracellular matrix bind bFGF, and this stored bFGF has recently been recognized as an important mechanism for the modulation of cell activity [33–37]. Furthermore, it has been reported that soluble heparin sulfates which bind bFGF enable bFGF to diffuse [38]. We think that heparin sulfates and acidic gelatin may have a similar ability to bind and sustain the release of bFGF. The association of gelatin and bFGF resemble physiological conditions.

The gelatin sheet containing bFGF accelerated not only the reduction of the wound area but also epithelialization. Both epithelialization and wound contraction reduce the wound area. Epithelialization is known to be completed by the proliferation and migration of epidermal cells from the wound edge. Wound contraction is based on a full-thickness afferent shift [39] and favorably accelerates wound healing, but it can sometimes cause functional disturbance. Thus, an appropriate balance of epithelialization and wound contraction for the region is required. The acceleration of wound area reduction and epithelialization observed in the bFGF(+) group suggests that the gelatin sheet incorporating bFGF is promising for future clinical applications.

The fact that wound reduction observed in the bFGF(+) group on day 7 was poor compared with that on day 14 may be partially due to bFGF being a potent mitogen and chemoattractant for endothelial cells as well as fibroblasts. In other words, bFGF is likely to mainly promote the second half of the wound healing process, i.e., granulation, vascularization and epithelialization, which are important factors in wound reduction. Actually, in the present study, granulation, vascularization and epithelialization were significantly accelerated by bFGF on day 14, as observed in the bFGF(+) group. Figure 5 may indicate that thick granulation tissue with both fibrous tissues and cellular components is relevant for epithelialization, vascularization and wound reduction.

Wound healing was promoted in both the bFGF(+) group and the bFGF(−) group, compared with the control group. The granulation tissue of both the bFGF(+) group and the bFGF(−) group was significantly thicker than that of the control group. This accelerated wound repair by the gelatin sheet might be attributed to its advantages. First, the gelatin sheet has a high water content, as it can maintain the absorption of excessive exudates and wet conditions for a long time. Second, the gelatin sheet may be biocompatible because it originates from collagen. Histological findings showed that mild inflammatory cell infiltration was observed in the bFGF(+) and bFGF(−) groups in comparison with the control group. This result also supports the possibility that the gelatin sheet is biocompatible. Indeed, some degree of inflammation may be necessary for the wound healing process, but strong and delayed inflammation may be undesirable for rapid wound repair. Leukocytes in the wound exudates were mainly neutrophils, but their infiltration might not be essential to the healing process [40]. The gelatin sheet seemed to help early inflammatory reactions suitably. Third, the gelatin sheet is biodegradable. bFGF is probably