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ORIGINAL ARTICLE

Collagen–poly glycolic acid hybrid matrix with basic fibroblast growth factor accelerated angiogenesis and granulation tissue formation in diabetic mice

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ABSTRACT

Because poor skin wound healing associated with diabetes is thought to be partly a result from impaired angiogenesis, treatments that improve angiogenesis could have important clinical applications. We herein report the effects of novel developed material, collagen–poly glycolic acid fiber hybrid matrix, being used together with basic fibroblast growth factor to promote wound healing of full-thickness skin defects on the back of type 2 diabetic Lepr^{db} mice. Our data indicates that this therapeutic approach markedly promotes angiogenesis and granulation tissue formation in comparison with other conditions 14 days after wounding.

Key words: angiogenesis, basic fibroblast growth factor (bFGF), collagen, poly glycolic acid (PGA), wound contraction.

INTRODUCTION

Normal wound healing requires both re-epithelization and ingress of collagen. The former occurs by migration and proliferation of keratinocytes from the wound edges and by differentiation of stem cells residing at the hair bulge. The latter is initiated by an influx of growth factors secreted by macrophages, platelets and fibroblasts, and is followed by fibroblast proliferation and subsequent synthesis and remodeling of the collagenous matrix. Because these processes are defective in the case of chronic wounds, new technologies are being developed to improve the healing in these conditions.

In order to restore impaired wound healing, a good circumference environment such as a scaffold of cells or growth factors is indispensable.[1] Artificial dermis, which is put to practical use, supplies a matrix of moist condition that enables fibroblasts and vascular endothelial cells to proliferate in wounds, and becomes filled with vascular-rich granulation tissue 2–3 weeks after being placed on the wound. Furthermore, advances in tissue repair depend partly on newly introduced recombinant growth factors such as basic fibroblast growth factor (bFGF), which is characterized as a growth factor for fibroblasts and vascular endothelial cells and as a potent mitogen for mesenchymal cells.[2] Therefore, topical application of recombinant bFGF is expected to be useful for acceleration of wound healing by promoting granulation and inducing angiogenesis.

Herein, we report the results of an *in vivo* animal trial of the novel developed scaffold, collagen (COL)–poly glycolic acid (PGA) fiber hybrid matrix (COL + PGA) being used together with the bFGF (COL + PGA/bFGF) on wound healing of type 2 diabetic mice. The data provides new insights into a therapy to improve outcomes for patients with deficient wound repair.

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MATERIALS AND METHODS

Scaffold and growth factor

Type I porcine collagen sponges (Nitta Gelatin, Osaka, Japan) incorporating poly glycolic acid fiber (Gunze, Kyoto, Japan) at weight ratio of 0.8 were prepared by the conventional freeze-drying method followed by dehydrothermal cross-linking as described previously.[3] Recombinant human bFGF protein was kindly provided by Kaken Pharmaceutical, Tokyo, Japan.

Animal surgery

Mutant diabetic mice, C57BLKS/J Iar^{-} + Lepr^{db}/ + Lepr^{db}, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained on a standard laboratory diet and water ad libitum, and were used at 8 weeks of age. The treatment of the animals was in accordance with the guidelines on animal experiments of Akita University and the Japanese Government Animal Protection and Management Law.

Mice were anesthetized with sodium ketamine solution (0.8 mg/g, i.p.), and two or three full-thickness round wounds were made on the upper back of each mouse using a punch biopsy instrument (6 mm diameter; Kai Industries, Osaka, Japan). Either COL + PGA or COL was implanted into the above-mentioned wounds, and open wounds were used as a control. Furthermore, 100 μl of bFGF solution (100 μg/ml) was dispersed evenly across the matrix, and saline was used as control. The wounds were covered with a transparent occlusive dressing (Bioclusive, Johnson and Johnson, Arlington, TX, USA) during the experiment. Five samples were studied for each experimental group.

Histological evaluation and morphometry

After 4 h fixation in methanol, the tissue was trimmed, cut through at the widest margin, and embedded in paraffin. The sections were made perpendicular to the anterior–posterior axis and perpendicular to the surface of the wound. Every section was stained with hematoxylin and eosin (HE) for wound analysis. To visualize vascular endothelial cells, the adjacent sections were deparaffinized and immunolabeled with anti-CD31 (DAKO, Carpenteria, CA, USA).

Images of HE and immunolabeled sections were captured digitally with an Olympus BH2 microscope (Tokyo, Japan) and Nikon coolpix880 camera (Tokyo, Japan). Lateral wound boundaries were determined by the presence of intact hair follicles and organized epidermis and dermis as compared to a lack of hair follicles, altered epidermal/dermal organization, and disorganization of collagen fibers within the wound. The blood vessels were traced digitally to determine the area of each in the wound using Scion Corporation software (Scion Image, Scion Corporation, Frederick, MD, USA). Statistical analysis of the wound area (mean area of wound granulation), vascular number (mean number of blood vessels) and vascular area (mean area of blood vessels) were calculated using a Kruskal–Wallis test and post-hoc analysis, with $P < 0.05$ being considered significant.

RESULTS

To evaluate the effect of bFGF and PGA on tissue generation, cross-linked collagenous matrices with or without PGA and with or without bFGF were initially implanted in full-thickness skin defects on the back of type 2 diabetic Lepr^{db}. Because control wounds were completely epithelized within 14 days of wounding, wound conditions were evaluated on day 14. We next performed two sets of experiments: (i) wounds were morphologically observed; and (ii) the area of wounds and the area and number of blood vessels were analyzed statistically.

Wound morphology

To characterize wound morphology better, we observed wounds 2 weeks after implantation. COL + PGA being used together with bFGF (COL + PGA/bFGF) resulted in significant morphological changes in the Lepr^{db} wounds in comparison with other conditions (Fig. 1a), namely, in that the tissue response involved high cellular infiltrations throughout the entire matrix. Matrix structures were filled with some fibroblasts, macrophages, lymphocytes and a large number of foreign body giant cells, although polymorphonuclear neutrophils (PMN) were only sporadically observed. In contrast, foreign body giant cells were observed occasionally in matrices without PGA such as COL with or

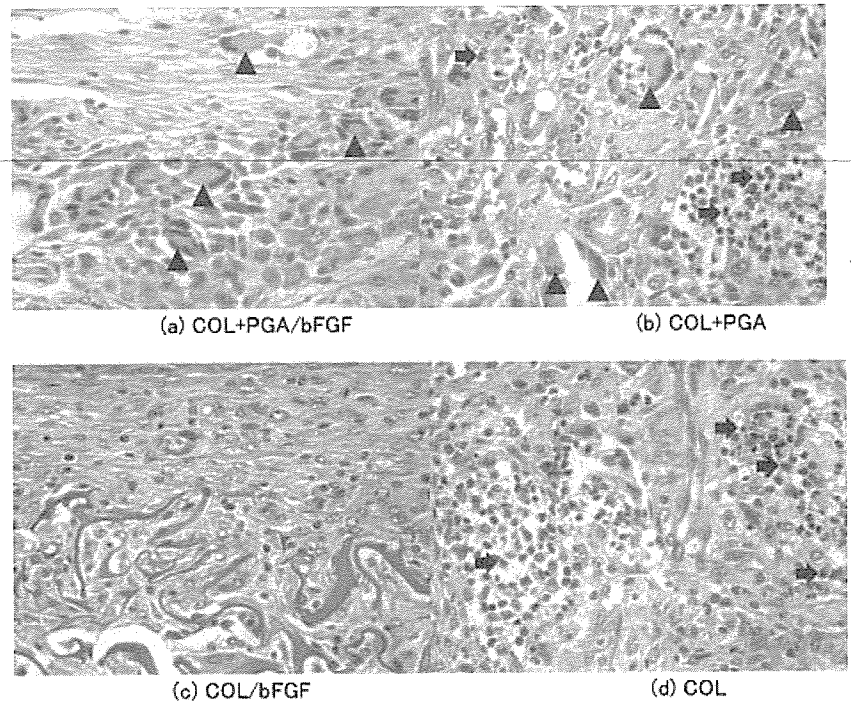

Figure 1. Histology 2 weeks after implantation of various matrices. Many foreign body giant cells were present in the wound implanted with matrices with poly glycolic acid (PGA) such as collagen (COL) + PGA/basic fibroblast growth factor (bFGF) (a) and COL + PGA (b) in comparison with matrices without PGA such as COL/bFGF (c) or COL alone (d). In addition, polymorphous nuclear cells were sporadically present in the wound implanted with matrices with bFGF such as COL + PGA/bFGF (a) and COL/bFGF (c) in comparison with matrices without bFGF such as COL + PGA (b) and COL alone (d).

without bFGF (Fig. 1c,d), and quite a number of PMN were observed in matrices without bFGF such as COL + PGA or COL alone (Fig. 1b,d). These findings suggested that bFGF suppressed chemotactic stimuli for PMN and that the implantation of matrices with PGA induced foreign body reaction.

Wound healing

We previously demonstrated the advantageous properties of PGA fiber that reinforce the collagen sponge.[3] With this in mind, to confirm the physiological properties of PGA, we then attempted to compare the area of wounds treated with various conditions on day 14 after implantation (Fig. 2). Post-hoc analysis of the wound area revealed that the implantation of COL + PGA/bFGF led to a strong inhibition of wound contraction compared with both COL with bFGF (COL/bFGF) ($P < 0.05$) and control wounds ($P < 0.01$). In addition, the COL + PGA alone inhibited wound contraction to a degree that was statistically significant compared with the control wounds ($P < 0.05$). These findings suggested that the implantation of COL + PGA/bFGF prevented wound contraction exceedingly, whereas wound contraction was observed in other conditions.

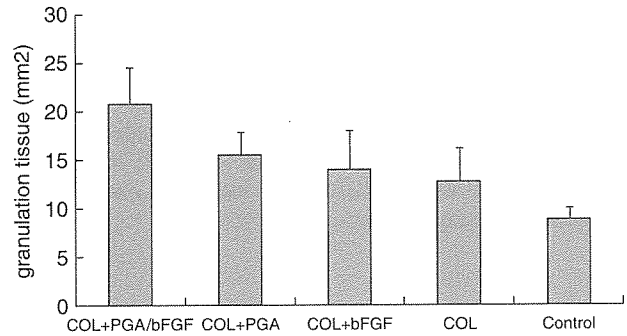

Figure 2. Statistical analysis of the wound area (mean area of wound granulation). Note that the implantation of COL + PGA/bFGF markedly prevented wound contraction in comparison with other conditions.

Wound vascularization

To determine whether bFGF cause angiogenesis in various matrices, we next statistically analyzed the number and area of blood vessels. Initially, a series of histological sections of wounded skin was immunolabeled with anti-CD31 antibodies to delineate blood vessels in a fully distinct fashion (Fig. 3a). The vessels were then traced digitally to determine vascular number and vascular area in injured skin on

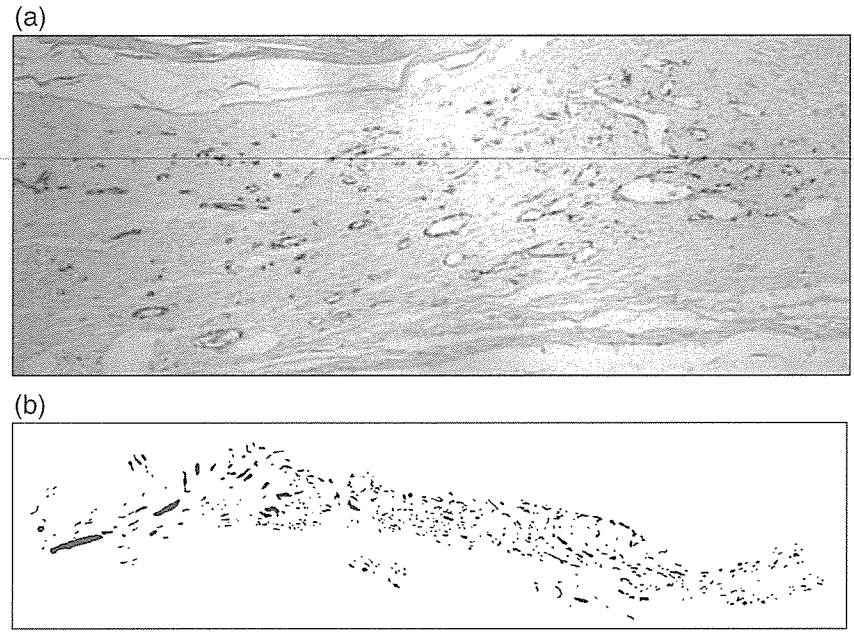

Figure 3. Images of CD31 immunolabeled section of various matrices such as COL + PGA/bFGF (a) were traced digitally to determine the number and the area of blood vessels (b).

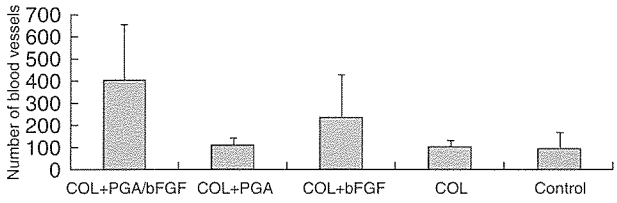

Figure 4. Statistical analysis of the vascular number (mean number of blood vessels). Note that the implantation of COL + PGA/bFGF led to an exceptional increase of vascular number.

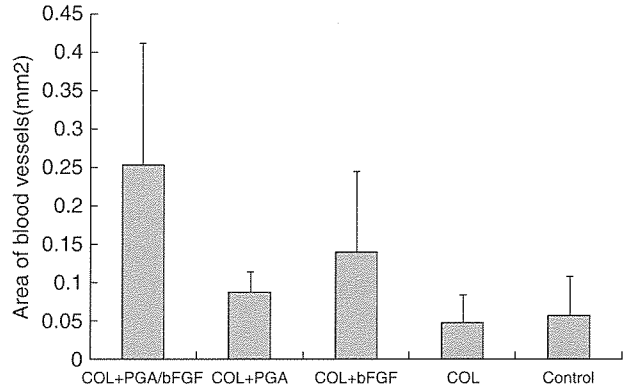

Figure 5. Statistical analysis of the vascular area (mean area of blood vessels). Note that the implantation of COL + PGA/bFGF led to an exceptional increase of vascular area.

day 14 after implantation of various matrices (Fig. 3b). Post-hoc analysis revealed that the implantation of COL + PGA/bFGF led to an increase in vascular number relative to COL + PGA, COL and control wounds ($P < 0.05$, respectively), and the vascular number of COL + PGA/bFGF were 4.4-fold greater than that of controls (Fig. 4). However, although there was a trend toward increased vascular numbers between COL + PGA/bFGF and COL/bFGF, it did not reach statistical significance. Similarly, post-hoc analysis of the vascular area revealed that the implantation of COL + PGA/bFGF led to an increase of vascular area in comparison with COL + PGA, COL and control wound ($P < 0.05$, respectively), and the vascular area of COL + PGA/bFGF were 4.4-fold greater than that of controls (Fig. 5). However, the vascular area between COL + PGA/bFGF and COL/bFGF did not reach statistical significance. These findings suggested that the increased vascular number is a major factor in the observed increase in vascular area.

DISCUSSION

There has been great progress in the development of artificial skin replacement products in recent years. Several artificial dermis including Pelnac (Gunze,

Kyoto, Japan) are available for impaired wound healing. The artificial dermis consists of fibers arranged in a lattice that act as a template for the formation of new tissue. Fibroblasts, blood vessels, nerve fibers and lymph vessels from surrounding healthy tissue grow into the collagen lattice, which eventually dissolves as these cells and structures build a new dermis. To develop the artificial dermis, naturally-derived biodegradable polymers such as collagen have been used as scaffolds of cells. Although collagen-based scaffolds have been the most popular for skin tissue engineering, they are compromised by the problematic effects of their scaffolds having rapid absorption and poor mechanical strength. Therefore, it has been necessary for the scaffold to maintain the desired shape and the inside space has been indispensable for the formation of new tissue via the proliferation and differentiation of cells or their extracellular matrix secretion. Recently, we have developed the collagen sponge reinforced with PGA (COL + PGA) with the advantageous properties of both collagen and PGA fiber. Moreover, we have determined that the novel scaffold enhances their compression strength without impairing their cellular affinities and maintains the construction of the forming tissue.[3] Thus, because the COL + PGA has been shown to provide a potential solution to physiological problems of collage-based scaffolds, this raises the possibility that the COL + PGA have therapeutic implications particularly for impaired wound healing such as for diabetic ulcers. However, it might be difficult to apply the COL + PGA to a diabetic ulcer, because they are usually accompanied by impaired angiogenesis that prevent graft fixation. To overcome these difficulties, we have attempted to develop a novel procedure to accelerate angiogenesis.

While the normal wound healing process entails a complex interplay between connective tissue formation, cellular activity and growth factor activation, all three of these physiological processes are altered in diabetic ulcers.[1] In particular, the diabetic ulcer is stalled in the inflammation phase of the normal wound healing process. During this delay, there is a cessation of epidermal growth and migration over the wound surface, and collagen synthesis is markedly decreased, resulting in chronic connective tissue complications. The defect in collagen metabolism in diabetes is present at both the collagen peptide production level as well as the post-translational modification of collagen degradation. Furthermore, growth factors such as bFGF or hepatocyte growth factor influence the wound healing process on the local wound environment and are known to be integral in the chemotaxis, migration, stimulation, and proliferation of cells and matrix substances necessary for wound healing.[2,4] Therefore, the altered secretion or absence of these growth factors in diabetic ulcers can potentially impair wound healing.

With the above findings in mind, we therefore reasoned that it would be interesting to use diabetic mice as an appropriate animal model to evaluate the effects of the artificial dermis being used together with growth factors. In this regard, it was currently known that angiogenesis and granulation tissue formation were reduced and that topical application of bFGF to diabetic mice reversed both responses to a significant level.[5] Furthermore, a recent report showed that bFGF inhibits wound contraction and that the combination of bFGF and collagen matrix is more effective than bFGF alone.[6] Therefore, to determine whether COL + PGA/bFGF suppressed wound contraction and promoted angiogenesis of the diabetic mice, collagenous matrices with or without covalently-attached PGA and with or without bFGF were implanted in full-thickness skin defects on the back of type 2 diabetic $Lepr^{db}$ mice. Consequently, the histological findings provided supporting evidence that COL + PGA/bFGF acts as a scaffold for reconstruction of blood vessels and granulation structures. In particular, the physiological characteristics of PGA and the angiogenic properties of bFGF were exploited by markedly suppressed wound contraction and promoted angiogenesis granulation tissue formation (summarized in Fig. 6).

However, the biological roles of PGA and bFGF in tissue repair were controversial. First, the PGA itself may induce a foreign body reaction that also contributes to the induction of subsequent cellular influxes and formation of capillaries through the release of various cytokines and growth factors. Second, the blood vessel formation and tissue generation may not only be due to the direct mitotic properties of bFGF but also provide chemotactic stimuli for macrophages. The recruited macrophages may release growth factors with chemotactic,

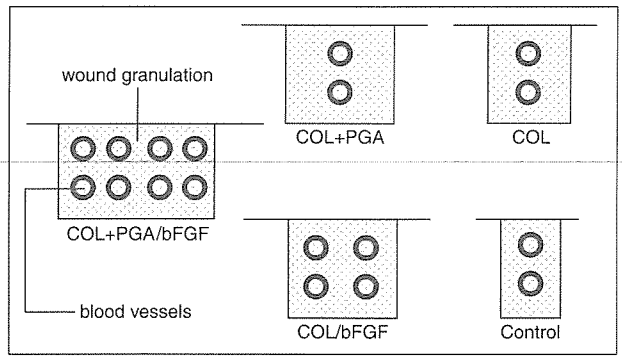

Figure 6. The schema of the results of animal trial of collagenous matrices with/without PGA and with/without bFGF on wound healing of type 2 diabetic mice. Note that COL + PGA/bFGF promoted angiogenesis and suppressed wound contraction.

angiogenic and mitogenic activity. Although the former possibility may seem less possible, the latter possibility remains to be determined.

Furthermore, while our findings that PMN were only sporadically observed in COL + PGA/bFGF support previous reports demonstrating that the deficiency of PMN had no effect on bFGF-induced angiogenesis in mice,[7] these results contradict those of other investigators, which reported that PMN play a key role in facilitating angiogenesis using an *in vitro* corneal pocket assay.[8] Several factors may account for the contradictory results between our study and the latter one. A first explanation may be that the two models of wound healing, one using the back skin of diabetic mouse and the other using the avascular mouse cornea, may elicit differences in the intensity of the inflammatory response. A second explanation may be that our use of a single administration of bFGF differs from the prior study that relied on bFGF pellets, which slowly disperse the reagent to induce corneal angiogenesis. Further studies are warranted to elucidate whether PMN play a role to facilitate angiogenesis in wound healing.

Together, COL + PGA/bFGF may be of additional value for those tissue-engineering applications that require enhanced tissue generation and angiogenesis. We therefore predict that this approach will become a promising therapy in future to improve outcomes for patients with deficient wound repair.

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Original Article

Effects of Basic Fibroblast Growth Factor on Experimental Diabetic Neuropathy in Rats

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Basic fibroblast growth factor (bFGF) stimulates angiogenesis and induces neural cell regeneration. We investigated the effects of bFGF on diabetic neuropathy in streptozotocin-induced diabetic rats. Diabetic rats were treated with human recombinant bFGF as follows: *1*) intravenous administration, *2*) intramuscular injection into thigh and soleus muscles with cross-linked gelatin hydrogel (CGH), and *3*) intramuscular injection with saline. Ten or 30 days later, the motor nerve conduction velocity (MNCV) of the sciatic-tibial and caudal nerves, sensitivity to mechanical stimuli, sciatic nerve blood flow (SNBF), and retinal blood flow (RBF) were measured. Delayed MNCV in the sciatic-tibial and caudal nerves, hypoalgesia, and reduced SNBF in diabetic rats were all ameliorated by intravenous administration of bFGF after 10, but not 30, days. Intramuscular injection of bFGF with CGH also improved sciatic-tibial MNCV, hypoalgesia, and SNBF after 10 and 30 days, but caudal MNCV was not improved. However, intramuscular injection of bFGF with saline had no significant effects. bFGF did not significantly alter RBF in either normal or diabetic rats. These observations suggest that bFGF could have therapeutic value for diabetic neuropathy and that CGH could play important roles as a carrier of bFGF. *Diabetes* 55:1470–1477, 2006

Diabetic neuropathy is one of the most common and important complications in diabetic patients. About one-half of patients with diabetes have some degree of diabetic neuropathy, and the progression of diabetic neuropathy causes various problems in the daily life and may affect the prognosis of diabetic patients (1). Therefore, it is important to prevent the development of diabetic neuropathy and to treat it at an early stage. Although strict glycemic control can prevent the onset and progression of diabetic neuropathy (2), the effectiveness has not been satisfactory. Then, additional treatment based on the pathogenic mechanisms becomes necessary.

The effects of various agents on diabetic neuropathy based on the pathogenic hypotheses, including increased polyol pathway activities (3,4), enhanced nonenzymatic glycation (5), altered protein kinase C activities (6), and increased oxidative stress (7,8) have been experimentally and clinically investigated. Most of these agents have demonstrated promising results in animal studies but have failed to deliver convincing data in clinical trials. In terms of prevention or cessation of diabetic neuropathy, there are some promising data showing efficacy in some clinical trials with aldose reductase inhibitors or α-lipoic acid; however, therapeutic or reparative effects on advanced diabetic neuropathy could not be exerted by these agents. Neural cell degeneration and decreased nerve blood flow (NBF) (6,8) have been recognized as pathophysiologically characteristic features of diabetic neuropathy. Therefore, agents that can act as both a neurotrophic and an angiogenic factor may be useful for treatment of diabetic neuropathy, even at an advanced stage.

Basic fibroblast growth factor (bFGF) is a single-chain polypeptide composed of 146 amino acids. It was originally isolated from bovine brain and pituitary gland and found to have stimulatory actions on fibroblast proliferation (9–11). With recent advances in molecular biology, bFGF has been recognized as a multifunctional growth factor that stimulates angiogenesis, acts as a vasodilatator (12,13), has antiapoptotic effects (14,15), and induces proliferation in various kinds of cells (10,11,16). Actually, the effects of bFGF have been investigated in the field of wound healing (17), bone regeneration (18,19), acute ischemic models (20,21), and myocardial infarction (22,23), both experimentally and clinically. The half-life time of bFGF is relatively short (24), and cross-linked gelatin hydrogel (CGH) has been used to maintain the bioactivity of locally administered bFGF for an extended period (25,26). Furthermore, it has been reported that bFGF has effects on the central nervous system (27,28) and peripheral nervous system (29,30). However, the effects of bFGF on diabetic neuropathy have not been investigated.

This study was conducted to investigate the effects of human recombinant bFGF administered intravenously or intramuscularly with CGH or saline on diabetic neuropathy in streptozotocin (STZ)-induced diabetic rats. The

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bFGF, basic fibroblast growth factor; CGH, cross-linked gelatin hydrogel; HGF, hepatocyte growth factor; MCT, mean circulation time; MNCV, motor nerve conduction velocity; NBF, nerve blood flow; NGF, nerve growth factor; RBF, retinal blood flow; SNBF, sciatic NBF; STZ, streptozotocin; VEGF, vascular endothelial growth factor; VFA, video fluorescein angiography.

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TABLE 1
Body weights and plasma glucose concentrations of normal and diabetic rats (study 1)

Group	*n*	Body weight (g)	Plasma glucose (mmol/l)
10 days			
Normal + saline	12	413 ± 23	3.6 ± 0.2
Normal + bFGF (2 μg/100 g body wt)	11	404 ± 14	3.7 ± 0.2
Normal + bFGF (20 μg/100 g body wt)	10	416 ± 21	3.7 ± 0.3
Diabetic + saline	11	214 ± 20*	22.9 ± 2.3*
Diabetic + bFGF (2 μg/100 g body wt)	10	212 ± 18*	23.3 ± 2.4*
Diabetic + bFGF (20 μg/100 g body wt)	10	212 ± 25*	22.8 ± 2.0*
30 days			
Normal + saline	6	410 ± 23	4.1 ± 0.3
Normal + bFGF (20 μg/100 g body wt)	6	413 ± 10	4.0 ± 0.5
Diabetic + saline	6	210 ± 18†	22.5 ± 1.4†
Diabetic + bFGF (20 μg/100 g body wt)	6	216 ± 23†	22.8 ± 1.5†

Data are means ± SD. *$P < 0.05$ vs. normal rats at 10 days; †$P < 0.05$ vs. normal rats at 30 days.

effects of bFGF on retinae in normal and diabetic rats were also evaluated. This is the first report to demonstrate therapeutic effects of bFGF on diabetic neuropathy.

RESEARCH DESIGN AND METHODS

Human recombinant bFGF and CGH were kind gifts from Kaken Pharmaceutical (Tokyo, Japan). CGH was made of glutaraldehyde cross-linking of acidic gelatin with an isoelectric point of 5.0, as previously reported (25,26,31).

Eight-week-old male Wistar rats (Chubu Kagakushizai, Nagoya, Japan) with an initial body weight of 210–240 g were allowed to adapt to the experimental animal facility for 7 days. They were housed in an aseptic animal room at a temperature of 20–24°C and a humidity of 40–70%, with a 12-h light cycle and 12 fresh air changes per hour, and were allowed free access to rat chow and water. Diabetes was induced by intraperitoneal injection of STZ (60 mg/kg) (Sigma, St. Louis, MO). Control rats received an equal volume of citric acid buffer. One week after STZ administration, rats with plasma glucose concentrations of >16 mmol/l were selected as the diabetic group. Control and diabetic rats had free access to rat chow and water. After 8 weeks, normal and diabetic rats were randomly divided into experimental groups and treated with bFGF as described below. The Nagoya University Institutional Animal Care and Use Committee approved all protocols.

In study 1, to evaluate the effects of intravenous administration of bFGF on diabetic neuropathy and to determine the appropriate dose of bFGF, saline alone, 2 μg/100 g body wt bFGF, or 20 μg/100 g body wt bFGF diluted with saline (total volume 400 μl) were injected into the tail veins of normal and diabetic rats for 3 consective days. Ten or 30 days later, the parameters described below were measured.

In study 2, to determine whether local treatment with bFGF has therapeutic effects on diabetic neuropathy, 20 μg/100 g body wt bFGF in 1.25 ml CGH or saline were injected into the right thigh and soleus muscles and 1.25 ml CGH or saline alone injected into the left thigh and soleus muscles of normal and diabetic rats. Ten or 30 days later, the following parameters were bilaterally measured.

Measurement of motor nerve conduction velocity. Rats were placed on a heated pad in a room maintained at 25°C to ensure a constant rectal temperature of 37°C. After intraperitoneal injection of sodium pentobarbital (5 mg/100 g), the sciatic-tibial motor nerve conduction velocity (MNCV) between the ankle and sciatic notch and the caudal MNCV were determined with a Neuropak NEM-3102 instrument (Nihon-Koden, Osaka, Japan), as previously described (5,6,32).

Measurement of sensitivity to mechanical stimuli. During the testing, rats were standing on a metal grid and the paw of the hindlimb stimulated with a series of calibrated monofilaments (Stoelting, Wood Dale, IL). A series of monofilaments of ascending force (0.5–21 g) were applied to the middle plantar surface of the hind paw. Monofilaments were applied in ascending order for a duration of 1 s. The response threshold was noted as the lowest force that elicited a 50% withdrawal response (5 of 10 applications).

Measurement of sciatic endoneurial nutritive blood flow. After anesthesia with sodium pentobarbital (5 mg/100 g), rats were placed on a heated pad in a room maintained at 25°C to ensure a constant rectal temperature of 37°C. Sciatic nerve blood flow (SNBF) was measured by the hydrogen clearance technique with an analog recorder (BW-4; Biochemical Science, Kanazawa, Japan) and an electrolysis tissue blood flow meter (RBA-2; Biochemical Science), as previously described (6,32), and calculated with the equation of Koshu et al. (33).

Measurements of retinal vessel diameters, mean circulation time (MCT), and retinal blood flow. Video fluorescein angiography (VFA) was performed to measure these parameters, as previously described (34,35). Twenty-four hours before the measurements, the animals underwent catheterization with a polyvinyl catheter inserted into the right jugular vein under anesthesia with sodium pentobarbital (5 mg/100 g). The catheter was flushed with 0.1 ml of 1,000 units/ml sodium heparin before and after implantation. It was positioned subcutaneously along the shoulder, and the distal end was externalized to the back of the neck. On the day of measurement, after the left eye was dilated with 1% tropicamide under anesthesia, a 100-μl syringe containing 10% sodium fluorescein was connected to the catheter and positioned on a platform attached to the imaging camera. Rats were maintained on a heated pad during the course of the measurements. The optic disc was centered and focused in the field of view, the VFA recording sequence initiated, and a 5-μl bolus of fluorescein dye rapidly injected into the jugular vein catheter. The injection time was marked on the video recording. The recorded fluorescein angiograms were digitized on a frame-by-frame basis and analyzed densitometrically to determine the retinal vessel diameters and MCT. Sample sites were chosen using primary retinal vessels at a fixed (one optic disc diameter) radical distance from the center of the optic disc. Vessel diameters in units of pixels were determined during peak fluorescein arterial and venous filling times at the defined vessel sample sites using a boundary-crossing algorithm. The average diameter for each vessel was measured for each sample site. The average vessel diameters for each eye represent the average of the individual vessel diameters for that eye. At the fixed-vessel sites, the average vessel fluorescence within a sample area defined by the vessel width was measured on a frame-by-frame basis to generate temporal fluorescence intensity or dye dilution curves. The resultant artery and vein fluorescence data were fit to a log–normal distribution function, from which average arterial appearance time of the dye bolus, defined as the time between dye injection and the first detectable appearance (vessel fluorescence intensity greater than background level by two times the SD of the average background intensity) of dye in the retinal artery, represents an assessment of systemic circulation times. The average MCT was calculated as the difference between the average retinal mean arterial and venous filling times for all primary arteries and veins. Retinal blood flow (RBF) was calculated by dividing the sum of the squares of the arterial and venous diameters by the MCT. Data establishing the sensitivity of this technique have been previously reported (36).

Statistical analyses. Results are presented as means ± SD. Differences among experimental groups were detected by ANOVA, and the significance of differences between groups was evaluated by Scheffe's *S* test. Significance was defined as a *P* value <0.05.

RESULTS

Body weight and plasma glucose concentration. Diabetic rats demonstrated no body weight gain and remarkable hyperglycemia compared with normal rats. Neither treatment with intravenous (Table 1) nor intramuscular (Table 2) administration of bFGF with or without CGH or saline altered the body weights or plasma glucose concentrations in normal and diabetic rats.

TABLE 2
Body weights and plasma glucose concentrations of normal and diabetic rats (study 2)

Group	*n*	Body weight (g)	Plasma glucose (mmol/l)
10 days			
Normal without treatment	10	415 ± 25	3.7 ± 0.2
Normal + bFGF with CGH (R) and CGH alone (L)	10	406 ± 21	3.7 ± 0.3
Normal + bFGF with saline (R) and saline alone (L)	8	404 ± 16	3.9 ± 0.3
Diabetic without treatment	10	216 ± 20*	23.1 ± 2.1*
Diabetic + bFGF with CGH (R) and CGH alone (L)	10	215 ± 14*	22.8 ± 2.4*
Diabetic + bFGF with saline (R) and saline alone (L)	8	208 ± 15*	22.7 ± 2.4*
30 days			
Normal + bFGF with CGH (R) and CGH alone (L)	12	419 ± 20	3.8 ± 0.6
Normal + bFGF with saline (R) and saline alone (L)	10	420 ± 29	4.1 ± 0.3
Diabetic + bFGF with CGH (R) and CGH alone (L)	10	217 ± 23*	22.7 ± 2.4*
Diabetic + bFGF with saline (R) and saline alone (L)	10	211 ± 11*	22.0 ± 2.5*

Data are means ± SD. *$P < 0.05$ vs. normal rats. L, left leg; R, right leg.

Effects of intravenous administration of bFGF on MNCV of sciatic-tibial nerves and tail nerves and sensitivity to mechanical stimuli. The MNCV of the sciatic-tibial nerves and tail nerves in the diabetic rats treated with saline alone (37.7 ± 3.4 and 27.1 ± 3.4 m/s, respectively) was significantly delayed compared with that in normal rats (54.0 ± 4.5 and 35.4 ± 4.0 m/s, respectively). Intravenous administration of bFGF did not significantly ameliorate these delays at a dose of 2 μg/100 g body wt but almost normalized the MNCV at a dose of 20 μg/100 g body wt after 10 days (sciatic-tibial 47.7 ± 6.9, tail 32.6 ± 3.8 m/s) (Fig. 1*A* and *B*). However, intravenous administration of bFGF at a dose of either 2 or 20 μg/100 g body wt did not alter the MNCV of the sciatic-tibial nerves and tail nerves in the normal rats. On the other hand, beneficial effects of intravenous administration of bFGF on the MNCV of sciatic tibial nerves were not observed after 30 days (untreated normal 56.2 ± 3.5, untreated diabetic 43.5 ± 1.4, and 20 μg bFGF–treated diabetic 45.1 ± 1.7 m/s) (Fig. 1*C*). Effects of intravenous administration of bFGF on sensitivity to mechanical stimuli were similar to those on MNCV. Untreated diabetic rats (13.0 ± 2.7 g) required a significantly greater force to elicit a 50% withdrawal response compared with untreated normal rats (8.4 ± 0.9 g). This reduced sensitivity to mechanical stimuli was ameliorated by 20 μg bFGF (9.4 ± 0.5 g) after 10 days (Fig. 2*A*). However, this effect was not maintained for 30 days (Fig. 2*B*).

Effects of intravenous administration of bFGF on SNBF. There were no differences in the SNBF between any of the groups of normal rats. The SNBF in diabetic rats treated with saline alone (6.5 ± 1.1 ml · min^{-1} · 100 g body wt^{-1}) was significantly reduced compared with that in normal rats (16.1 ± 2.1 ml · min^{-1} · 100 g body wt^{-1}). Intravenous administration of bFGF at a dose of 2 μg/100 g body wt (11.6 ± 2.3 ml · min^{-1} · 100 g body wt^{-1}) partially but significantly improved the decreased SNBF in diabetic rats, and this effect was more prominent at the high dose of 20 μg/100 g body wt (14.1 ± 2.5 ml · min^{-1} · 100 g body wt^{-1}) after 10 days (Fig. 3*A*). After 30 days, however, the effects of intravenous administration of bFGF on the SNBF were not observed (untreated normal 16.2 ± 1.8, untreated diabetic 8.3 ± 2.3, and 20 μg bFGF–treated diabetic 11.3 ± 1.7 ml · min^{-1} · 100 g body wt^{-1}) (Fig. 3*B*).

Effects of intravenous administration of bFGF on retinal vessel diameters, MCT, and RBF. There were no significant differences in the arterial or venous diameters between the normal and diabetic rats with or without intravenous administration of bFGF. The prolonged MCT and reduced RBF in the diabetic rats were not significantly altered by the intravenous administration of bFGF (Table 3). Moreover, fluorescein angiography showed no abnormality, including aneurism, occlusion, or neovascularization in retinal arteries, veins, or capillaries.

Effects of intramuscular administration of bFGF with saline or CGH on MNCV of sciatic-tibial nerves and tail nerves, as well as sensitivity to mechanical stimuli. Intramuscular administration of CGH or saline alone did not affect the MNCV of the sciatic-tibial nerves in normal or diabetic rats, and bFGF with CGH or saline had no effects on the MNCV of the sciatic-tibial nerves in normal rats. The decreased MNCV of the sciatic-tibial nerves in the untreated diabetic rats (untreated normal 55.1 ± 4.5, untreated diabetic 40.2 ± 3.1 m/s) was significantly ameliorated by intramuscular administration of bFGF with CGH (49.3 ± 4.6 m/s) after 10 days but not by that of bFGF with saline (Fig. 4*A*). On the other hand, the delayed MNCV of the tail nerves in diabetic rats was not improved by local injection of bFGF with CGH or saline into the thigh and soleus muscles (Fig. 4*B*). In addition, the effect of intramuscular administration of bFGF with CGH

TABLE 3
MCT, RBF, and diameters of retinal arteries and veins in normal and diabetic rats (study 1)

Group	MCT (s)	RBF (pixel²/s)	Arterial diameter (pixel)	Venous diameter (pixel)
Normal + saline	1.17 ± 0.28	378 ± 82	11.7 ± 0.9	16.5 ± 0.5
Normal + bFGF (20 μg/100 g body wt)	1.24 ± 0.25	382 ± 94	12.5 ± 1.1	17.0 ± 0.4
Diabetic + saline	1.58 ± 0.26*	301 ± 32*	12.7 ± 0.8	17.3 ± 1.1
Diabetic + bFGF (20 μg/100 g body wt)	1.33 ± 0.35	338 ± 80	12.7 ± 0.7	16.1 ± 1.2

Data are means ± SD. *$P < 0.05$ vs. normal rats.

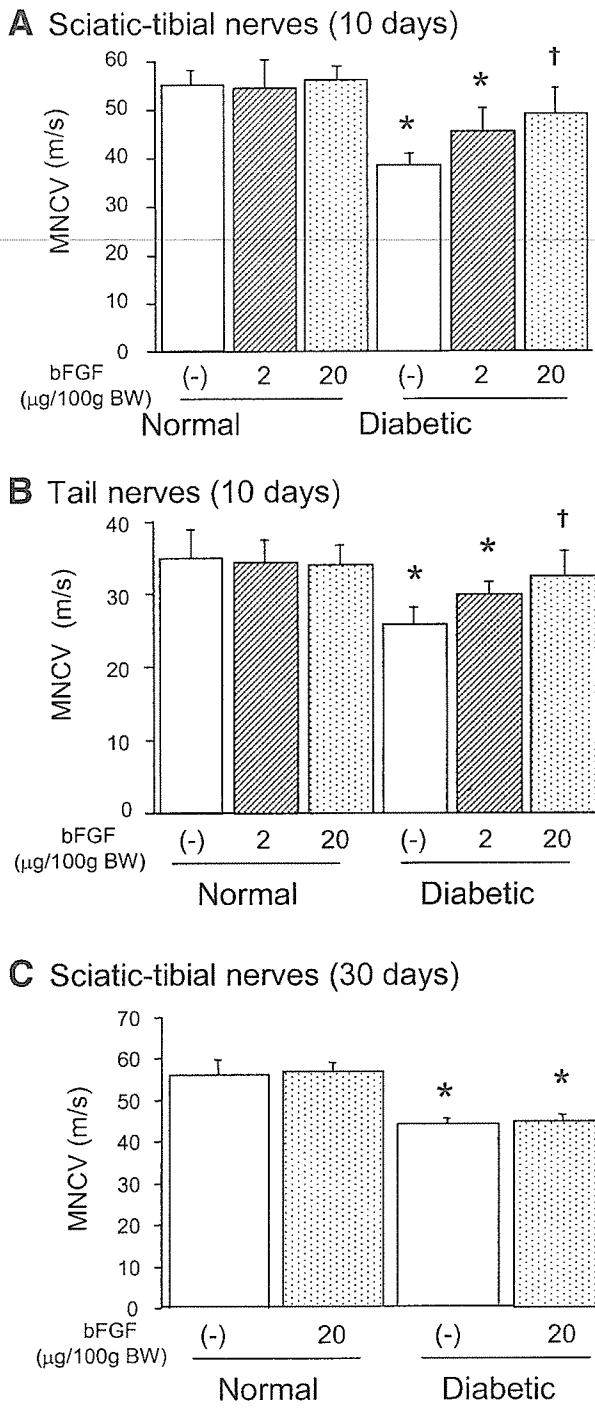

FIG. 1. MNCV of sciatic-tibial (*A*: 10 days; *C*: 30 days) and tail (*B*: 10 days) nerves in normal and diabetic rats treated with intravenous injection of saline or bFGF (study 1). □, rats treated with saline; ▨, rats treated with 2 μg/100 g body wt (BW) bFGF; ▩, rats treated with 20 μg/100 g body wt bFGF. Results are means ± SD. *$P < 0.05$ vs. normal rats; †$P < 0.05$ vs. diabetic rats treated with saline.

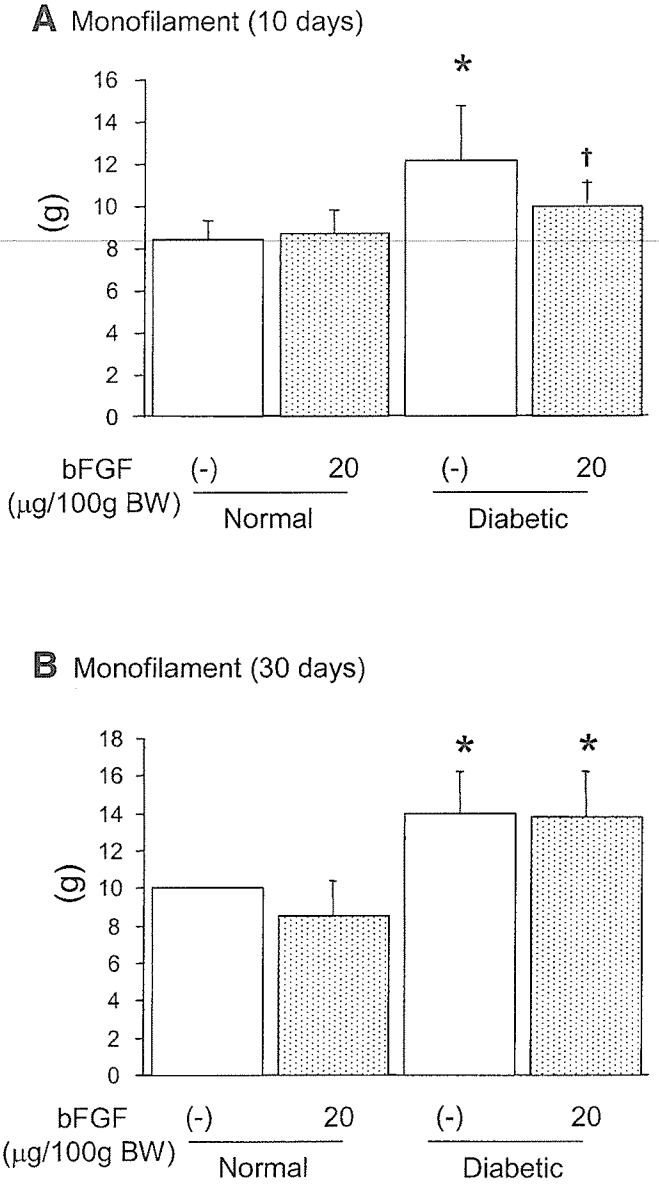

FIG. 2. Sensitivity to mechanical stimuli (*A*: 10 days; *B*: 30 days) in normal and diabetic rats treated with intravenous injection of saline or bFGF (study 1). □, rats treated with saline; ▨, rats treated with 20 μg/100 g body wt (BW) bFGF. Results are means ± SD. *$P < 0.05$ vs. normal rats; †$P < 0.05$ vs. diabetic rats treated with saline.

on the MNCV of the sciatic-tibial nerves was maintained for 30 days (Fig. 6*A*). The reduced sensitivity to mechanical stimuli in untreated diabetic rats (12.9 ± 2.7 g) compared with that in untreated normal rats (8.4 ± 1.6 g) was significantly ameliorated by intramuscular administration of bFGF with CGH (9.3 ± 1.0 g) after 10 days (Fig. 4*C*), and this effect was maintained for 30 days (Fig. 6*B*). However, intramuscular administration of bFGF with saline showed no significant effects.

Effects of intramuscular administration of bFGF with saline or CGH on SNBF. The effects of intramuscular injection of bFGF with saline or CGH on the SNBF were similar to those on the MNCV of the sciatic-tibial nerves. No significant differences were found in the SNBF between any of the groups of normal rats. The reduced SNBF in the diabetic rats (7.9 ± 0.8 ml · min^{-1} · 100 g body wt^{-1}) was almost normalized by the intramuscular administration of bFGF with CGH (14.7 ± 1.9 ml · min^{-1} · 100 g body wt^{-1}) to the level of the untreated normal rats after 10 days (15.7 ± 1.5 ml · min^{-1} · 100 g body wt^{-1}) (Fig. 5), but bFGF with saline did not significantly improve the reduction (Fig. 4). The beneficial effect of bFGF with CGH on SNBF was also observed after 30 days (Fig. 6*C*).

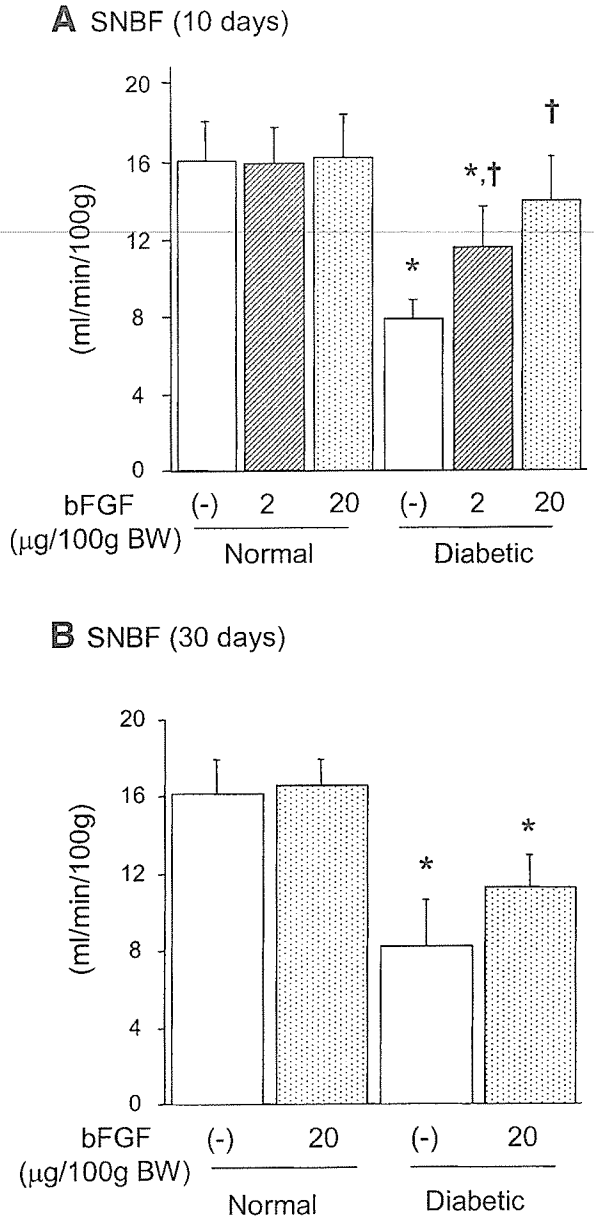

FIG. 3. SNBF (*A*: 10 days; *B*: 30 days) in normal and diabetic rats treated with intravenous injection of saline or bFGF (study 1). □, rats treated with saline; ▨, rats treated with 2 μg/100 g body wt (BW) bFGF; ▩, rats treated with 20 μg/100 g body wt bFGF. Results are means ± SD. **P* < 0.05 vs. normal rats; †*P* < 0.05 vs. diabetic rats treated with saline.

Effects of intramuscular administration of bFGF with saline or CGH on retinal vessel diameters, MCT, and RBF. No significant differences in the arterial and venous diameters between any of the experimental groups were observed after 10 days. The delayed MCT and decreased RBF in the diabetic rats were not altered by the local injection of bFGF with CGH or saline into the thigh and soleus muscles (data not shown). Because of the development of severe cataracts, the VFA measurements were not performed after 30 days.

DISCUSSION

The present study demonstrated that intravenous administration of bFGF has therapeutic effects on diabetic

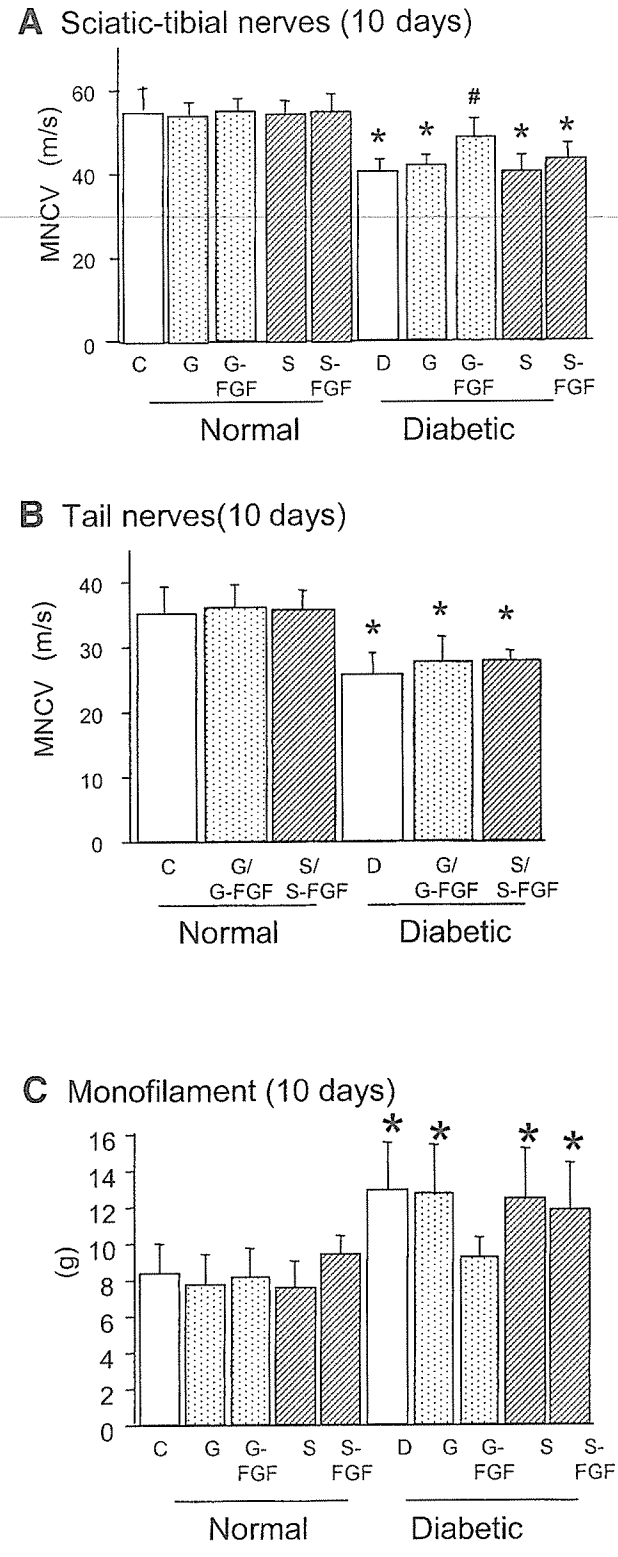

FIG. 4. MNCV of sciatic-tibial (*A*) and tail (*B*) nerves and sensitivity to mechanical stimuli (*C*) in normal and diabetic rats treated with intramuscular injection of bFGF with CGH or saline (study 2; 10 days). □, nerves without treatment (C, normal; D, diabetic); ▩, rats treated with CGH and bFGF (G, right nerves treated with CGH alone; G-FGF, left nerves treated with bFGF and CGH); ▨, rats treated with saline and bFGF (S, right nerves treated with saline alone; S-FGF, left nerves treated with bFGF and saline). Results are means ± SD. **P* < 0.05 vs. normal groups; #*P* < 0.05 vs. D, G, and S in diabetic rats.

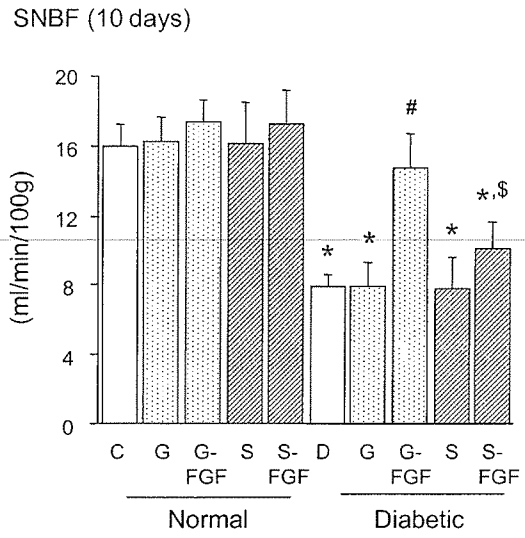

FIG. 5. SNBF in normal and diabetic rats treated with intramuscular injection of bFGF with CGH or saline (study 2, 10 days). □, nerves without treatment (C, normal; D, diabetic); ▨, rats treated with CGH and bFGF (G, right nerves treated with CGH alone; G-FGF, left nerves treated with bFGF and CGH); ▨, rats treated with saline and bFGF (S, right nerves treated with saline alone; S-FGF, left nerves treated with bFGF and saline). Results are means ± SD. *P < 0.05 vs. normal groups; #P < 0.05 vs. D, G, and S in diabetic rats; \$$P$ < 0.05 vs. G-FGF in diabetic rats.

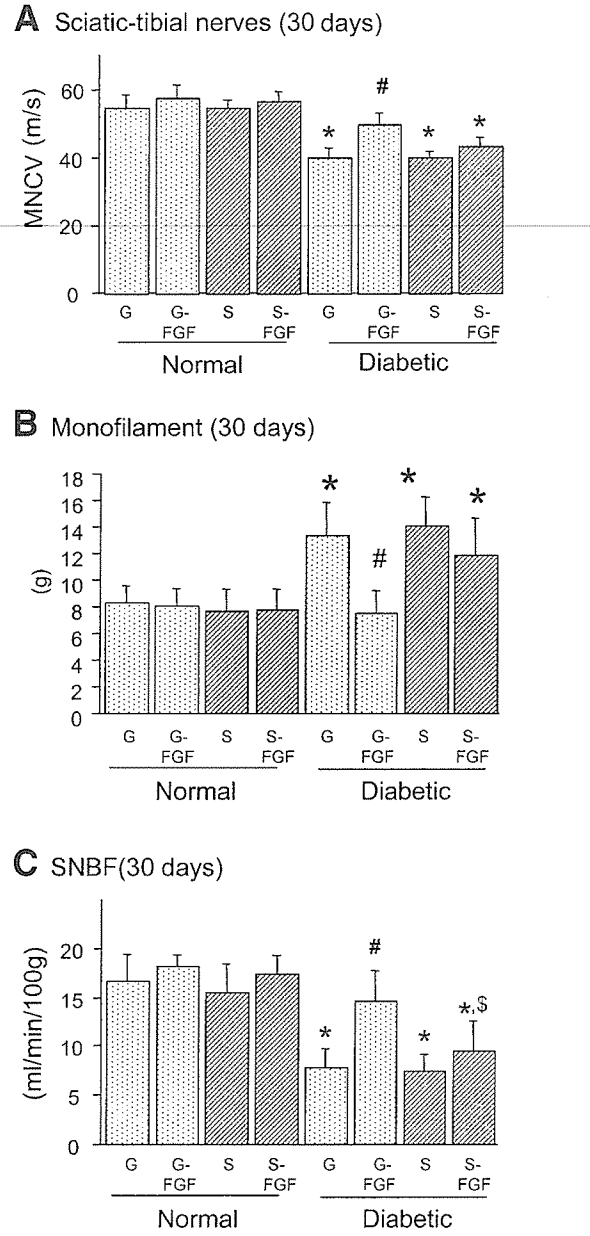

FIG. 6. MNCV of sciatic-tibial nerves (*A*), sensitivity to mechanical stimuli (*B*), and SNBF (*C*) in normal and diabetic rats treated with intramuscular injection of bFGF with CGH or saline (study 2, 30 days). ▨, rats treated with CGH and bFGF (G, right nerves treated with CGH alone; G-FGF, left nerves treated with bFGF and CGH); ▨, rats treated with saline and bFGF (S, right nerves treated with saline alone; S-FGF, left nerves treated with bFGF and saline). Results are means ± SD, *P < 0.05 vs. normal groups; #P < 0.05 vs. G and S in diabetic rats; \$$P$ < 0.05 vs. G-FGF in diabetic rats.

neuropathy and does not affect the retinal circulation and that local treatment by intramuscular injection of bFGF with CGH, a unique drug-delivery system, ameliorated hypoalgesia and the decreased MNCV and SNBF in diabetic rats without demonstrating systemic actions.

In the present study, sensitivity to mechanical stimuli was measured to evaluate a sensory nerve function. Our preliminary experiment demonstrated that diabetic rats develop hyperalgesia at 2–4 weeks after induction of diabetes and hypoalgesia at 7–8 weeks, which is consistent with a previous report by Calcutt et al. (37). However, deficits in MNCV and SNBF of diabetic rats are clearly demonstrated in 8 weeks. Therefore, therapeutic intervention with bFGF was performed 8 weeks after induction of diabetes.

Previous studies have shown that bFGF has various functions both in vitro and in vivo. In in vitro studies, the induction of proliferation activities by bFGF in various types of cells, including vascular cells (10,11,16), and the antiapoptotic action of bFGF on neural cells (14) have been reported. In in vivo studies, the effects of bFGF have been investigated in the fields of central and peripheral nervous systems (27–30), skin (17), bone (18,19), heart (22,23), and vasculatures (20,21), and beneficial effects have been reported both experimentally and clinically. In addition to these effects, bFGF induces NO production through endothelial NO synthase activation, resulting in vasodilation (12,13,38).

Several neurotrophic factors and growth factors such as nerve growth factor (NGF) (39), vascular endothelial growth factor (VEGF) (40), and hepatocyte growth factor (HGF) (41) have been recognized to ameliorate diabetic neuropathy in animal models. Although the effects of NGF on diabetic neuropathy were most extensively investigated experimentally, a clinical trial failed to establish the usefulness of NGF for diabetic neuropathy (42,43). The therapeutic effects of VEGF (40) and HGF (41) through their angiogenic actions on diabetic neuropathy have been reported. On the other hand, the induction of HGF and VEGF by treatment with bFGF in vitro and in vivo has been demonstrated (44–46). Interestingly, according to these studies (44–46), under ischemic conditions, bFGF exerts its angiogenic effects by not only inducing but also harmonizing with these endogenous angiogenic factors. Moreover, it has been confirmed that the vasculatures induced by bFGF consist of both endothelial cells and smooth muscle cells, which act as functional vessels. Considering these reports, bFGF may be a plausible an-

giogenic growth factor and therapeutic agent for diabetic neuropathy.

In this study, we compared the effects of bFGF administered through three different delivery systems on diabetic neuropathy. Despite its short half-life, intravenous administration of bFGF dose dependently ameliorated the reduced SNBF and delayed MNCV of both the sciatic-tibial and tail nerves in diabetic rats after 10 days (24). These effects may not be mediated through direct actions of bFGF on nerve tissues, but through systemic actions on other tissues, and bFGF might trigger the activation of a cytokine cascade, causing its effect to continue even after its inactivation. However, further long-term effects of bFGF cannot be expected by intravenous injection. In fact, beneficial effects of intravenous administration of bFGF on hypoalgesia, sciatic-tibial MNCV, and SNBF after 10 days were not observed after 30 days in this study. In addition, systemic administration of bFGF by intravenous injection might cause cytotoxicity in various tissues, including retinae and kidneys, through its angiogenic and cell-proliferative actions. In the present study, no beneficial or adverse effects of intravenously administered bFGF on the retinae were observed. The reason for this result remains unclear. Differences may exist in the distribution of exogenously administered bFGF among tissues, which might cause the inconsistent effects on nerve tissues and retinal tissues. To clarify the action mechanisms and establish long-term safety, histological analyses, including angiogenesis and measurement of the distribution of exogenously administered bFGF, would be required. Although the effects on renal functions were not investigated in this study, previous studies have in fact demonstrated toxic actions of bFGF on kidneys, such as podocyte injury (47), epithelial injury (48), and mesangial cell proliferation and matrix accumulation (49). Therefore, delivery systems that can be locally administered and retain the biological activity of bFGF for a long period would be required for the clinical application of bFGF.

Recently, local injection of VEGF gene (40) or HGF gene (41) and transplantation of endothelial progenitor cells (32) to muscles around peripheral nerves have been reported to improve experimental diabetic neuropathy through the induction of local angiogenesis. Therefore, we explored the usefulness of local treatment of bFGF using two drug delivery systems, CGH and saline. CGH is considered an excellent and unique carrier of bFGF (25,26). CGH was prepared through cross-linking of acidic gelatin with an isoelectric point (IEP) of 5.0 and bFGF with an IEP of 9.0. This difference in the IEP enabled bFGF to maintain its bioactivity for an extended period. bFGF administered with CGH can function when released by the biodegradation of the hydrogel (25,26,31). In this study, bFGF administered with saline did not improve the decreased MNCV and SNBF in diabetic rats, but bFGF injected with CGH brought about a remarkable amelioration of the sciatic-tibial MNCV and SNBF in the diabetic rats, and its effects continued for at least 30 days. In addition, this local treatment did not improve the MNCV or SNBF of the opposite side, the tail nerve MNCV, or the retinal circulation in diabetic rats. These findings support the efficiency and safety of local treatment of bFGF with CGH.

Intravenous administration of bFGF at a low dose significantly improved SNBF but not MNCV, and long-term effects of bFGF were not observed by intravenous injection. In addition, intramuscular injection of bFGF with saline tended to increase SNBF without changing MNCV. These observations suggest that the primary action sites of bFGF would be the endoneurial microvasculature, that partial correction of SNBF would be insufficient to improve MNCV, and that the nonvascular actions of bFGF may account for little of its therapeutic efficacy.

In clinical practice, it is recognized that treatment of advanced symptomatic diabetic neuropathy patients with intravenous administration of vasodilatory agents, such as prostaglandin E1, ameliorates their symptoms. However, this effect cannot be maintained for a long period. Long-term efficacy of intramuscular administration of bFGF with CGH was clearly demonstrated in this study, suggesting a clinical benefit of this treatment.

In summary, intravenous administration of bFGF demonstrated a systemic action on the nerve functions of diabetic rats, including amelioration of hypoalgesia and delayed MNCV in both the sciatic-tibial and tail nerves. On the other hand, intramuscular injection of bFGF with CGH, but not that with saline, improved hypoalgesia, sciatic-tibial MNCV, and SNBF only in the treated side, and these effects were maintained for at least 30 days. Although the direct effects of bFGF on neural cells remain unclear and further studies, including histological analyses, will be required, these results strongly suggest that bFGF could have therapeutic value for diabetic neuropathy through the improvement of microvascular blood flow. Given its apparent safety and long-term efficiency, intramuscular administration of bFGF with CGH could be suitable for clinical application.

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Enhanced Bone Regeneration at a Segmental Bone Defect by Controlled Release of Bone Morphogenetic Protein-2 from a Biodegradable Hydrogel

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ABSTRACT

The objective of this study is to investigate the feasibility of a biodegradable hydrogel of gelatin as the controlled release carrier of bone morphogenetic protein-2 (BMP-2) suitable for enhancement of bone regeneration at a segmental bone defect. Hydrogels with three different water contents were prepared through glutaraldehyde crosslinking of gelatin with an isoelectric point of 9.0 under varied reaction conditions. Segmental critical-sized defects (20 mm) were created at the ulnar bone of skeletally mature New Zealand white rabbits, and gelatin hydrogels incorporating BMP-2 (17 μg/hydrogel) were implanted into the defects. When bone regeneration was evaluated by soft x-ray observation and bone mineral density (BMD) measurement, the gelatin hydrogels incorporating BMP-2 exhibited significantly high osteoinduction activity compared with that of free BMP-2, although the activity depended on the water content of the hydrogels. Significantly higher BMD enhancement was observed in the gelatin hydrogel with a water content of 97.8 wt% than that with the lower or higher water content. We concluded that the biodegradable gelatin hydrogel is a promising controlled release carrier of BMP-2 for bone regeneration at the segmental bone defect.

INTRODUCTION

BONE REGENERATION is an attractive area of research within tissue engineering because of the demanding clinical requirements of bone repair. It is widely recognized that various osteogenic growth factors, such as bone morphogenetic protein (BMP), transforming growth factor-β1 (TGF-β1), and basic fibroblast growth factor (bFGF), regulate the proliferation and differentiation of osteogenic cells and enhance bone formation.[1] Thus, if one can accelerate bone regeneration using osteogenic growth factors in a suitable manner, this regeneration technology will provide a new clinical procedure to promote bone repair and be a substitute for autogenous and allogenous bone grafts or biomaterial implants.

BMPs, with their potential to promote bone formation *in vivo*, have been used for bone regeneration to repair bone injuries and defects.[2] The current DNA technologies have enabled the production of enough recombinant human BMPs for basic and applied research. BMP-2 and BMP-7 (OP-1) have already been clinically applied to accelerate bone regeneration, both in fracture healing and spinal fusion.[3] On the other hand, several preclinical experiments have demonstrated that BMP administrated in the solution form does not always induce the expected efficacy in bone regeneration.[4] To tackle this problem, BMP has been combined with various biodegradable carriers, such as collagen,[5,6] β-tricalcium phosphate,[7] lactide-glycolide copolymers,[8,9] and ethylene glycol-lactic acid copolymer[10] for controlled release. It is conceivable

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that these combinations were effective in enhancing the *in vivo* BMP retention to induce bone formation. However, little has been investigated on the ability of BMP for bone regeneration from the viewpoint of the *in vivo* release profile.

Gelatin is a denatured collagen and commercially available as a biodegradable polymer. It has been extensively utilized for pharmaceutical and medical purposes, and its biosafety has been proven through long clinical applications.[11] Other advantages of gelatin are ease of chemical modification and commercial availability of samples with different physicochemical properties. We have prepared hydrogels of different biodegradabilities from gelatin and succeeded in inducing the biological activities of bFGF,[12] TGF-β1,[13] and hepatocyte growth factor (HGF)[14] through their controlled release with the biodegradable hydrogel, although application with the growth factors in the solution form was not effective. In addition, we have demonstrated that the *in vivo* profile of BMP-2 release could be changed only by altering the biodegradability of gelatin hydrogels and affected the enhancement of BMP-2-induced ectopic bone formation at the subcutis of mice.[15]

The objective of this study is to explore feasibility of the gelatin hydrogel as a controlled release carrier of BMP-2 in enhancement of bone regeneration at a segmental bone defect. Hydrogels with different biodegradabilities were prepared by changing the concentration of gelatin and glutaraldehyde in preparation. We examined the effect of the biodegradability of hydrogels on the promotion of bone regeneration in a rabbit model with ulna defect.

MATERIALS AND METHODS

Materials

A gelatin sample with an isoelectric point of 9.0 and human recombinant bone morphogenetic protein-2 were kindly supplied by Nitta Gelatin (Osaka, Japan) and Yamanouchi Pharmaceutical (Tokyo, Japan), respectively. Glutaraldehyde, glycine, and other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) and used without further purification.

Preparation of BMP-2 incorporated gelatin hydrogels

Hydrogels were prepared through the chemical crosslinking of aqueous gelatin solution with glutaraldehyde, according to the method described previously.[15] Briefly, an aqueous gelatin solution mixed with glutaraldehyde was cast into a polypropylene dish (138 × 138 mm^2, BIO-BIK) at various concentrations of glutaraldehyde and gelatin (Table 1), followed by the crosslinking reaction at 4°C for 12 h. The prepared crosslinked hydrogel was cut into rod shape (20 × 5 × 5 mm^3). The hydrogel samples were stirred in 100 mM aqueous glycine solution at 37°C for 1 h to block the residual aldehyde groups of glutaraldehyde. Following washing three times with double-distilled water (DDW), the hydrogel samples were freeze-dried and sterilized with ethylene oxide gas. The hydrogel weight was measured before and after complete drying at 70°C under vacuum, and the water content, the weight percentage of water in the wet hydrogel, was calculated from the two weight values.[15]

To prepare the gelatin hydrogel incorporating BMP-2, 100 μL of phosphate-buffered saline solution (PBS, pH 7.5) containing 17 μg of BMP-2 was dropped onto a freeze-dried gelatin hydrogel and left overnight at 4°C. Similarly, 100 μL of BMP-2-free PBS was dropped onto a freeze-dried hydrogel to obtain the BMP-2-free empty hydrogel.

Surgical procedure

The *in vivo* bone regeneration experiment was performed by use of a segmental ulna bone defect of skeletally mature New Zealand white rabbits (20-week-old, male, 3.5 kg; Shimizu Laboratory Supply, Japan), according to the surgical procedure previously reported.[6,8,9] A 4 cm length superomedial incision was made and the tissue overlying the diaphysis of the ulna was dissected. A segmental defect (20 mm) was prepared in the ulna of 24 rabbits with a surgical oscillating saw supplemented by copious sterile saline water irrigation. Our previous study demonstrated that the BMP-2 dose per volume (34 μg of BMP-2/cm^3 of hydrogel) was sufficient to induce bone formation at ectopic sites of mice.[15] Based on the volume of hydrogel implanted and the BMP-2 dose per volume, the amount of BMP-2 incorporated was calculated. The gelatin hydrogels incorporating 17 μg of BMP-2, empty gelatin hydrogels, and 100 μL of free BMP-2 solution (170 μg/mL) were placed in the defect (Fig. 1), while the empty defect was left without free BMP-2. Each experimental group was composed of three rabbits. Fixation of the ostectomized bone was unnecessary due to the fibro-osseous union between the ulna and radius located distal and proximal to the surgical site. The soft tissue was approximated with interrupted 4-0 Vicryl (Ethicon, Somerville, NJ) and the skin was closed with 3-0 silk sutures. After different time intervals, the radius-ulna complex containing the defect was taken out and fixed in 10 wt% neutral phosphate-buffered formalin solution for assessment of bone regeneration.

TABLE 1. PREPARATION OF GELATIN HYDROGELS WITH DIFFERENT WATER CONTENTS

Concentration of gelatin (wt%)	*Concentration of glutaraldehyde (wt%)*	*Water content (wt%)*
5	0.83	93.8
3	0.16	97.8
3	0.06	99.7

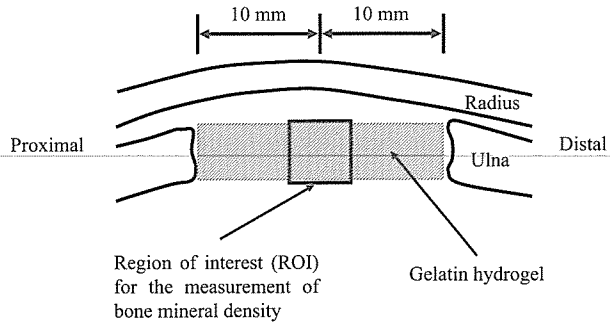

FIG. 1. Schematic illustration of the gelatin hydrogel implantation into an ulnar defect of rabbits and the region of interest for the DEXA measurement.

Assessment of bone regeneration

Bone regeneration at the bone defect was assessed by soft x-ray observation and dual-energy x-ray absorptometry (DEXA) 2, 4, and 6 weeks after surgery. Histological examinations were carried out 6 weeks after application of the BMP-2-incoporated gelatin hydrogel with a water content of 97.8 wt%. Bone regeneration was radiographically examined by soft x-ray (Hitex-100, Hitachi, Japan) at 54 KVP and 2.5 mA for 20 s. The bone mineral density (BMD) of each bone defect was measured at the 5×5 mm^2 region of interest (Fig. 1) by using the DEXA with a bone mineral analyzer (DSC 600EX-III, Aloka, Tokyo, Japan). This instrument was calibrated with a phantom of known mineral content according to the manufacture's instruction. Each scan was performed at a speed of 20 mm/s and the scanning length was 1 mm.

Bone specimens were placed into 10% neutral phosphate-buffered formalin solution, decalcified with 10% formic acid, and processed for the paraffin embedding. Sections with a thickness of 3 μm were prepared and stained with hematoxylin and eosin to view by a light microscopy (AX80T, Olympus, Tokyo, Japan).

Statistical analysis

All data were statically analyzed using Fisher's least significant difference test for multiple comparisons and statistical significance was established to be less than 0.05. The experimental results were expressed as the mean $\pm$ the standard deviation of the mean.

RESULTS

Osteoinductive effects of BMP-2 released from gelatin hydrogels

Figure 2 shows soft x-ray photographs of the ulna defects 6 weeks after application with gelatin hydrogels incorporating BMP-2. It is apparent that bone regeneration was detected radiographically in the ulna bone defect (Fig.

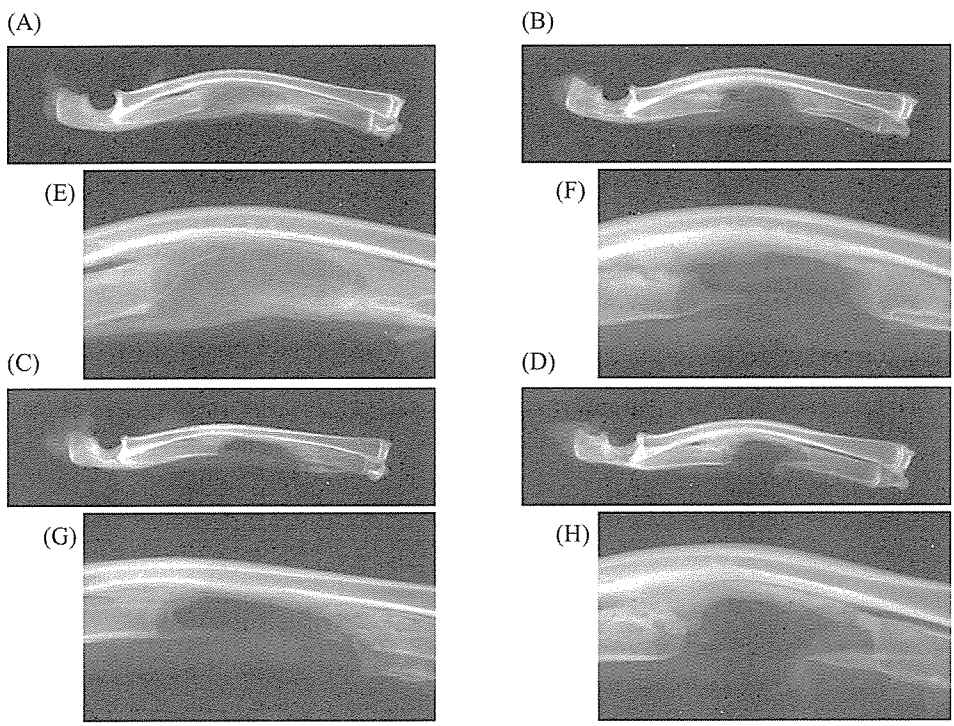

FIG. 2. Radiographic appearance of ulna defects 6 weeks after application with a gelatin hydrogel incorporating BMP-2 (**A and E**), an empty gelatin hydrogel (**B and F**), and free BMP-2 (**C and G**) or an empty defect without free BMP-2 (**D and H**) at magnification $\times$ 1 (**A–D**) and 2 (**E–H**). The water content of hydrogels used was 97.8 wt% and the BMP-2 dose was 17 μg/site.

2A and E). On the other hand, no bone formation was radiographically observed in the bone defects applied with empty gelatin hydrogels (Fig. 2B and F) and free BMP-2 (Fig. 2C and G); the appearance was similar to that of empty defect without free BMP-2 (Fig. 2D and H).

Figure 3 shows histological sections of ulna defects 6 weeks after application with the gelatin hydrogel incorporating BMP-2. When applied with the gelatin hydrogel incorporating BMP-2, the bone defect was histologically occupied by newly regenerated bone tissue (Fig. 3A). On the other hand, no bone regeneration was detected at the defect applied with the empty gelatin hydrogel (Fig. 3B) or free BMP-2 (Fig. 3C) and no application (Fig. 3D), while remarkable ingrowth of soft connective tissue into the defect was observed.

Figure 4 shows the time profiles of BMD values of the ulna defect of rabbits after application with the gelatin hydrogel incorporating BMP-2. The BMD value of the bone defect applied with the gelatin hydrogel incorporating BMP-2 was significantly higher than that of the empty gelatin hydrogel, free BMP-2, and empty defect without free BMP-2 after 4 and 6 weeks of application.

Effect of water content of gelatin hydrogels incorporating BMP-2 on bone regeneration

Figure 5 shows soft x-ray photographs of the bone defects 6 weeks after application with gelatin hydrogels incorporating BMP-2. Irrespective of the hydrogel type, bone regeneration at the defect was radiographically detected although the extent of radiopaque area for the hydrogel with a water content of 97.8 wt% (Fig. 5B and F) was larger than that with the higher (Fig. 5C and G) and lower (Fig. 5A and E) water contents. On the other hand, no bone formation was radiographically observed at the bone defect applied with free BMP-2 (Fig. 5D and H).

Figure 6 shows the BMD values of the ulna defects 6 weeks after application with gelatin hydrogels incorporating BMP-2. It was clear that the gelatin hydrogel with a water content of 97.8 wt% (Fig. 6B) enhanced the BMD value of the ulna defect to a significantly higher extent than did the hydrogels with water contents of 93.8 (Fig. 6A) and 99.7 wt% (Fig. 6C). Free BMP-2 did not enhance the BMD value of the bone defect (Fig. 6D).

DISCUSSION

The present study demonstrates that the bone regeneration at the segmental bone defect of rabbit ulna induced by gelatin hydrogels incorporating BMP-2 was greatly influenced by the water content of hydrogels. The water content of hydrogels could be easily changed by altering the concentration of gelatin and glutaraldehyde in crosslinking reaction (Table 1). Our previous study has

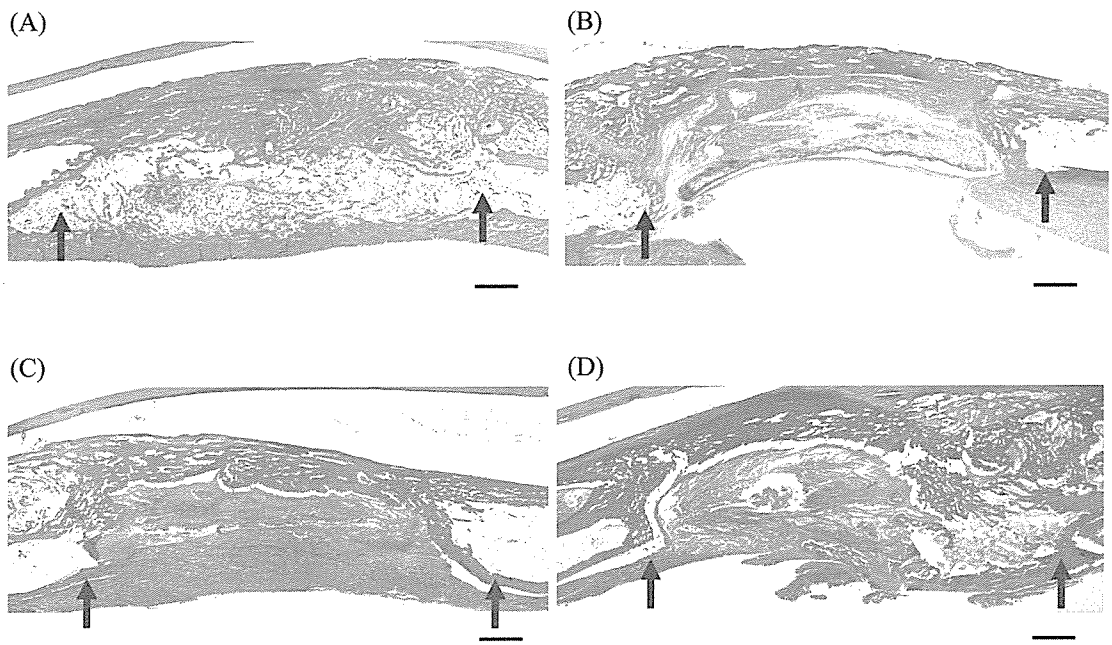

FIG. 3. Histological sections of ulna defects 6 weeks after application with a gelatin hydrogel incorporating BMP-2 (**A**), an empty gelatin hydrogel (**B**), and free BMP-2 (**C**) or an empty defect without free BMP-2 (**D**). The water content of the hydrogels used was 97.8 wt% and the BMP-2 dose was 17 μg/site. Arrows indicate the edge of the defect. Every bar corresponds to 2 mm. (Color images are available online at www.liebertonline.com/ten).

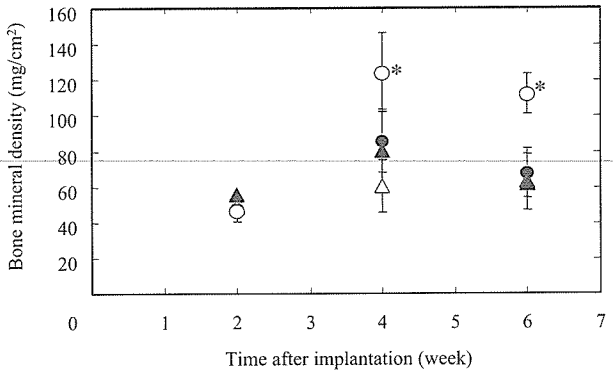

FIG. 4. The time course of BMD at the ulna defect of rabbits after application with gelatin hydrogels incorporating BMP-2 (○), empty gelatin hydrogels (●), and free BMP-2 (△) or empty defect without free BMP-2 (▲). The water content of the hydrogels used was 97.8 wt% and the BMP-2 dose was 17 μg/site. *p <0.05; significance against the BMD value of other groups at the corresponding time.

demonstrated that gelatin hydrogels enabled the release of BMP-2 at the implanted site for extended time periods, and the *in vivo* release profile of BMP-2 was controllable by the water content of hydrogels.[15] The release period of BMP-2 became longer with a decrease in the water content of hydrogel carriers. When the *in vivo* time profile of hydrogel degradation was compared with that of BMP-2 retention, a good correlation between the two profiles was observed.[15] These findings suggest that the BMP-2 release is governed by the degradation of the hydrogel carriers. Furthermore, our previous research revealed that significantly higher ectopic bone formation was induced by the BMP-2-incorporated gelatin hydrogel with a water content of 97.8 wt% than with the lower or higher water contents. This phenomenon suggests that a certain release period of BMP-2 is possibly necessary for local enhancement of the bone induction activity.

In this study, we selected a rabbit model of 20 mm long ulna bone defects as a stable critical-sized defect, according to the method of Hollinger *et al.*[6,8,9] The 20 mm length ulna bone resection did not impair function for the experimental animal. Hollinger and colleagues reported that no bone formation was found both for the empty defect without free BMP-2 and the absorbable collagen carrier (Helistat) without BMP-2 incorporation at the critical-sized (20 mm length) defect of rabbit ulna bone. Similarly to the findings in Hollinger's study, no bone regeneration was detected for the empty defect without free BMP-2 (Figs. 2H and 3D) from the viewpoint of the radiographic and histological observations. Free BMP-2 treatment did not induce bone regeneration at the defect (Figs. 2G and 3C). The hydrogel itself had no ability for bone regeneration because the BMP-2 free, empty

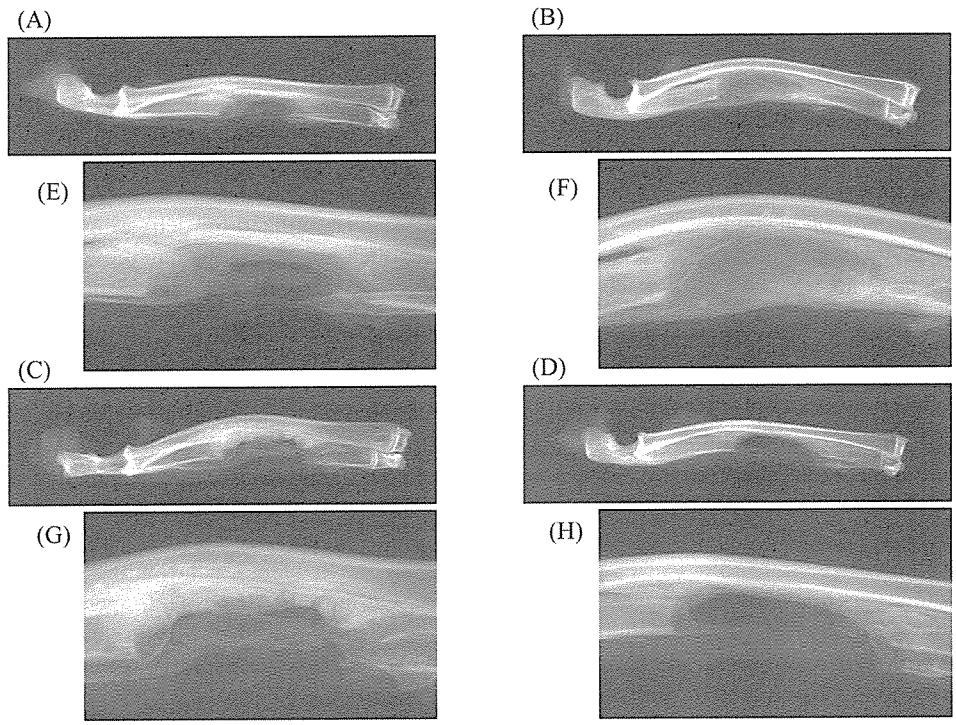

FIG. 5. Radiographic appearance of ulna defects 6 weeks after application with gelatin hydrogels incorporating BMP-2 (**A–C, E–G**) and free BMP-2 (**D and H**) at the magnification × 1 (**A–D**) and 2 (**E–H**). The water content of hydrogels used was 93.8 (**A and E**), 97.8 (**B and F**), and 99.7 wt% (**C and G**), respectively. The BMP-2 dose was 17 μg/site.

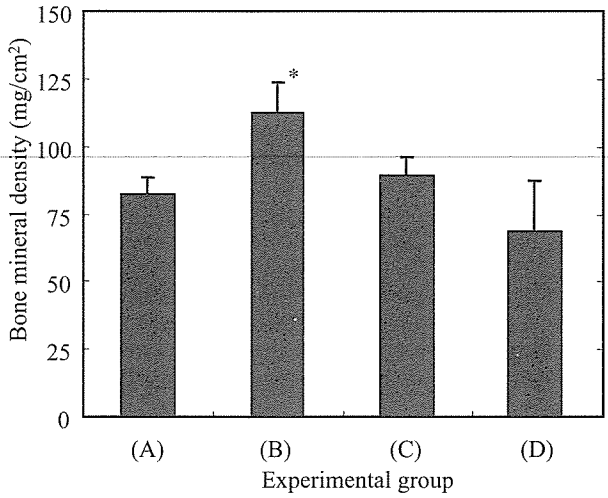

FIG. 6. Influence of the water content of gelatin hydrogels on the BMD at the ulna defect of rabbits 6 weeks after application with gelatin hydrogels incorporating BMP-2 (**A–C**) and free BMP-2 (**D**). The water content of hydrogels used was 93.8 (**A**), 97.8 (**B**), and 99.7 wt% (**C**), respectively. The BMP-2 dose was 17 μg/site. *$p < 0.05$; significance against the BMD value of other groups at the corresponding time.

gelatin hydrogel showed similar appearance to that of empty defect without free BMP-2 (Figs. 2F and 3B). These appearances strongly indicate that the size of the defect used to assess bone regeneration should be large enough to avoid spontaneous healing. The application of materials without any osteoinduction activity into the critical-sized defect results in the formation of fibrous connective tissue rather than bone formation (Fig. 3B–D).

The osteoinduction activity of BMP-2 released from gelatin hydrogels incorporating BMP-2 was evaluated in terms of bone regeneration at the ulna bone defect. The time course of BMD values at the critical-sized defect clearly showed that the BMD value was significantly higher than that of the empty gelatin hydrogel, free BMP-2, and no application 4 and 6 weeks after application of gelatin hydrogels incorporating BMP-2. No enhancement of the BMD value was found for other experimental groups (Fig. 4). Furthermore, Hollinger *et al.* reported that the collagen sponges incorporating BMP-2 promoted a significant increase in the percentage of radiopacity from 2 to 4 weeks, but thereafter no difference with time was observed.[6] Indeed, their histological examinations revealed that numerous bony trabeculae were observed at 4 weeks, and the trabeculae were consolidating and cortices were reforming by 8 weeks after implantation. However, it is impossible to directly compare the present data with Hollinger's because their dose of BMP-2 was 35 μg, which is larger than that used in our study. As shown in Figure 3A, cortices were observed 6 weeks after the application of the gelatin hydrogel incorporating BMP-2 even at the lower BMP-2 dose (17 μg) that we used. Taken together, it is apparent that the period to evaluate the osteoinduction activity should be long enough to fill the critical-sized defect with newly regenerated bone.

The activity of gelatin hydrogels incorporating BMP-2 to induce bone regeneration greatly depended on their water content (Figs. 5 and 6). It is likely that the fast-degraded hydrogel contributed neither to prolonged retention of BMP-2 nor to protection of the defect from the ingrowth of fibrous tissue. When the rate of hydrogel degradation is too slow compared with that of bone regeneration at the ulna defect, the hydrogel remaining in the defect may physically impair bone regeneration, even though long-term release of BMP-2 is achievable. Similar to this appearance, we have demonstrated that gelatin hydrogels with a slow degradability physically impaired bone regeneration induced by gelatin hydrogels incorporating TGF-β1.[13] Interestingly, this dependency of the osteoinduction activity for gelatin hydrogels incorporating BMP-2 is highly correlated to that observed in our previous ectopic bone induction experiments, which revealed that the osteoinduction activity of the hydrogel with a water content of 97.8 wt% was significantly higher than that with the higher and lower water contents.[15] These findings indicate that a balance in the time profile between the BMP-2 retention and the bone formation is essential for the bone regeneration induced by gelatin hydrogels incorporating BMP-2.

Various doses of BMPs and delivery systems, such as collagen, bioceramics, lactide-glycolide co-polymers, and ethylene glycol-lactic acid co-polymer, were employed to induce bone regeneration at a bone defect.[4,16] Among them, a commercially available collagen sponge has been investigated as a promising carrier to reduce the BMP-2 dose, utilized for delivering BMP-2 in the clinical application.[3,5] Indeed our previous study also indicated that the collagen sponge incorporating BMP-2 showed enhanced osteoinduction activity at the subcutaneous tissue around the implanted site, while it could achieve the *in vivo* BMP-2 release for 2 weeks. However, upon comparing the level of osteoinduction activity, the gelatin hydrogel with the profile of BMP-2 release for 4 weeks was superior to the collagen sponge.[15] Although there are several possible reasons for the enhanced osteoinduction activity, the time period of BMP-2 release may be one key factor contributing to the gelatin superiority. On the other hand, Hollinger *et al.* compared the property as a carrier of BMP-2 release for bone regeneration between the collagen sponge and the poly (D,L-lactide) sponge. Irrespective of the carrier type, the same dose of BMP-2 (35 μg) was required to promote nearly identical bone healing in an unilateral rabbit radius model.[6] Although it is impossible to directly compare the present data with the Hollinger's because of their larger