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A Novel Approach to Therapeutic Angiogenesis for Patients With Critical Limb Ischemia by Sustained Release of Basic Fibroblast Growth Factor Using Biodegradable Gelatin Hydrogel

— An Initial Report of the Phase I-IIa Study —

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Background Limb ischemia remains a challenge. To overcome shortcomings or limitations of gene therapy or cell transplantation, a sustained release system of basic fibroblast growth factor (bFGF) using biodegradable gelatin hydrogel has been developed.

Methods and Results A phase I-IIa study was performed, in which 7 patients had critical limb ischemia. They were intramuscularly injected with 200μg of bFGF-incorporated gelatin hydrogel microspheres into the gastrocnemius of the ischemic limb. End-points were safety and feasibility of treatment after 4 and 24 weeks. One patient was excluded from the study for social reasons, but only after symptomatic improvements. In the evaluation of the other 6 patients, significant improvements were observed in the distance walked in 6 min (295±42 m vs 491±85 m for pretreatment vs after 24 weeks, p=0.023) and in transcutaneous oxygen pressure (53.5±5.2 mmHg vs 65.5±4.0 mmHg, p=0.03). The rest pain scale also improved (3.5±0.2 vs 1.0±0.6, p=0.022). The ankle-brachial pressure index improved at 4 weeks but not at 24 weeks. Among 5 patients who had a non-healing foot ulcer, the ulcer was completely healed in 3 patients, reduced in 1, and there was no change in 1 patient at 24 weeks. The blood levels of bFGF were undetected or within the normal level in all patients.

Conclusions The sustained release of bFGF from gelatin hydrogel might be simple, safe, and effective to achieve therapeutic angiogenesis because it did not need genetic materials or collection of implanted cells, and because it did not have any general effects, which was supported by there being no elevation of the bFGF serum level. (*Circ J* 2007; **71**: 1181–1186)

Key Words: Angiogenesis; Basic fibroblast growth factor; Limb ischemia

Gene therapy or cell transplantation might have shown encouraging results in various clinical studies in the cardiovascular field![1–8] However, there are still unignorable concerns for effectiveness, immune or inflammatory responses of genetic materials;[9–11] and invasiveness, which stems from the collection of implanted cells such as general anesthesia or granulocyte-stimulating factor (G-CSF) administration.[6–8,12–14]

As a novel approach, we have developed a drug delivery system of potent growth factors such as basic fibroblast growth factor (bFGF), using biodegradable acidic gelatin hydrogel![15,16] We have demonstrated the effectiveness of bFGF protein released from gelatin hydrogel in various animal models (ie, either non-diabetic or diabetic) for acute myocardial infarction, prevascularization for cardiomyocyte transplantation to the ischemic heart, limb ischemia, and bone regeneration of the sternum![17–24] One of the most important advantages of the system is that it uses biodegradable gelatin hydrogel instead of genetic materials as a sustained release carrier for angiogenic growth factors.

Based on the results in animals, we started a clinical trial to test the safety and feasibility of the sustained release system of bFGF from gelatin hydrogel in patients with critical limb ischemia, who had no option of medical or surgical treatment.

Methods

Study Population

Patients qualified to participate in the study if they had chronic limb ischemia [atherosclerosis obliterans (ASO) or thromboangiitis obliterans (Buerger's disease)], including

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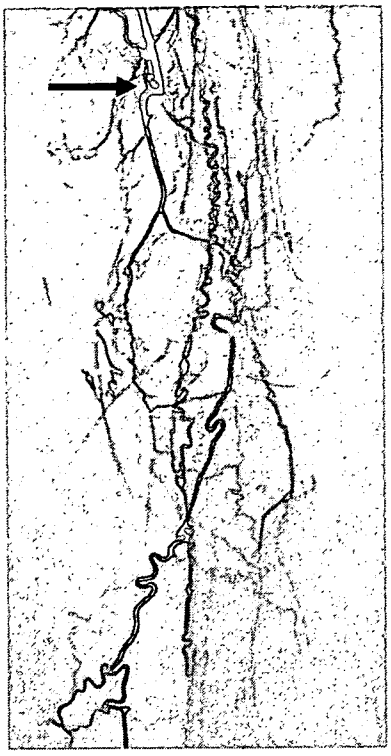

Fig 1. Representative digital subtraction angiography of "no-option" patient (Case 4). The popliteal artery was totally occluded (black arrow) and there were no infrapopliteral main arteries enhanced. Tortuous, corkscrew collateral vessels around the occlusion were observed.

rest pain, non-healing ischemic ulcers, or both, and if they were not candidates for catheter or surgical revascularization (no expandable/graftable infrapopliteal artery). Fig 1 shows the representative digital subtraction angiography of a "no-option" patient. The diagnosis of Buerger's disease was based on the criteria proposed by Olin:[25] (1) onset before age 45 years; (2) current (recent) history of tobacco use; (3) the presence of distal-extremity ischemia (infrapopliteal or infrabrachial) indicated by claudication, rest pain, ischemic ulcers, or gangrene; (4) exclusion of autoimmune or connective tissue diseases, hypercoagulable states, and diabetes mellitus; (5) exclusion of a proximal source of emboli by echocardiography and arteriography; and (6) consistent arteriographic findings in the clinically involved and non-involved limbs.

We excluded patients who showed allergy to gelatin hydrogel. Other exclusion criteria were: proliferative retinopathy, and evidence of a malignant disorder, which required treatment. We obtained written informed consent from all patients. The Ethics committee of Kyoto University approved the study protocol (protocol #524). Patient enrollment began in February 2005.

Preparation of bFGF-Incorporated Gelatin Hydrogel Microspheres

Human recombinant bFGF with an isoelectric point of 9.6 was purchased from Kaken Pharmaceutical Co (Tokyo, Japan). A gelatin sample with an isoelectric point of 5.0 was isolated from the bovine bone through the alkaline process (Nitta Gelatin Co, Osaka, Japan). Gelatin hydrogel microspheres were prepared in an aseptic room as previously described[15,16] Briefly, gelatin hydrogels were prepared through the glutaraldehyde cross-linking of gelatin in an aqueous solution. The resulting hydrogels were soaked in an aqueous solution of glycine for 3 h to block free aldehyde groups in the hydrogels; they were then washed with double distilled water. Gelatin hydrogels were pulverized by using a homogenizer. The homogenates were passed through sieves with different mesh sizes. The microspheres with a diameter ranging from 50 to 100 μm were collected and freeze-dried. After sterilization of the microspheres, we confirmed that there were no residual glutaraldehyde and bacterial contamination in the mircospheres. To incorporate bFGF into gelatin microspheres, an aqueous solution of bFGF (200 μg) was applied to freeze-dried microspheres (100 mg); they were then left at an ambient temperature for 1 h. The microspheres slowly released bFGF for approximately 3 weeks.

Study Design

The bFGF-incorporated gelatin hydrogel microspheres were injected into the gastrocnemius of the unilateral ischemic limb (single administration), and its safety and feasibility were evaluated (ie, the phase I-IIa study). We used the dose of bFGF (200 μg) in view of safety standards according to our previous animal studies[17–24] and other clinical reports[2–5] We did not have a control group in this study because that was only required in the phase I trial and, more importantly, the patients who participated were ill (ie, they were not candidates for conventional treatments). Oral medications such as vasodilators or antiplatelet drugs remained unchanged during the study period. No intravenous drugs such as prostaglandins were used during the study period. Patients were followed up to 24 weeks after the treatment.

End-Points

The primary end-point was the safety of the treatment, as evaluated by the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) Ver 3.0. The secondary end-point was feasibility of the treatment, as defined by the improvements in rest pain, the distance walked in 6 min (m)[26,27] the ankle-brachial pressure index (ABI), transcutaneous oxygen pressure (TcO_2, mmHg), laser Doppler perfusion image (LDPI) analyzer[7,22–24] (Moor Instruments, Devon, UK) (the mean value of the blood perfusion in the back of the ischemic foot: relative unit), thermography (mean temperature of the ischemic foot: °C), and the status of ulcer-healing. We measured the TcO_2 as follows: after cleansing the measurement site with ethanol, we applied the probe, and heated the skin surface to 43.5°C. When a steady-state temperature was achieved, a value expressed in mmHg was recorded. The measurement was performed when patients were breathing room air in a supine position.

The improvements were evaluated by the changes from baseline to week 4 and 24. Rest pain was scaled as previously reported[7] The blood level of bFGF was measured before treatment, and 1, 2, 7 and 28 days after the treatment.

Procedure

The bFGF-incorporated gelatin hydrogel microspheres were dissolved into 40 ml of saline and intramuscularly injected into each injection site (40 sites), with a 3×3 cm grid and by using a 23-gauge needle under spinal anesthesia.

Table 1 Results of the Study

Case	Age/Sex	Diagnosis	DM	HD	Past history	ABI	6 min	Pain***	TcO_2	LDPI	Therm	Foot ulcer
1	24/M	Buerger	No	No	Bypass (occluded)	+15%	+87%	0	+80%	+105%	+1.1°C	Healed
2	64/M	ASO	Yes	Yes	BMCT	−12%	−20%	+4	−10%	+1%	+0.9°C	No change**
3	40/M	Buerger	No	No	Bypass (occluded)	+9%	+26%	+1	+10%	+71%	−0.6°C	Healed
4	33/M	Buerger	No	No	Splenomegaly	+50%	+124%	0	+19%	+18%	−0.3°C	Healed
5	53/M	Buerger	No	No	No	−10%	+69%	0	+15%	+43%	+1°C	(−)
(6)*	62/F	ASO	No	No	No	(−)	(−)	(−)	(−)	(−)	(−)	(Reduced at 4 weeks)
7	69/M	ASO	Yes	Yes	PTA (repeated)	+28%	+83%	+1	+56%	+64%	+1.2°C	Reduced

Values of ABI, 6 min, TcO_2, and LDPI are expressed as % increase (decrease) from the baseline.
**Case 6 was excluded from this study because of social reasons 4 weeks after the treatment.*
***Superficial femoral artery showed new stenosis during the study period.*
****Rest pain scale: +4, severe pain unresolved with non-steroidal anti-inflammatory drugs (NSAID); +3, moderate pain NSAID necessary; +2, slight pain NSAID unnecessary; +1, very slight pain; 0, completely resolved.*
DM, diabetes mellitus; HD, hemodialysis; ABI, ankle-brachial pressure index; 6 min, the distance walked in 6 min; Pain, rest pain scale (see above); TcO_2, transcutaneous oxygen pressure; LDPI, laser Doppler perfusion image; Therm, thermography; ASO, atherosclerosis obliterans; BMCT, bone marrow cell transplantation; PTA, percutaneous transluminal angioplasty.

Table 2 Changes in Parameters of Limb Ischemia (Mean±SD)

	Pretreatment	4 weeks	24 weeks
ABI	0.62±0.12	0.73±0.14*	0.68±0.11
6-min walk (m)	295±42	448±81*	491±85*
Rest pain scale	3.5±0.2	1.3±0.4*	1.0±0.6*
TcO_2 (mmHg)	53.5±5.2	66.5±5.0*	65.5±4.0*
LDPI (relative unit)	436±66	520±80*	614±61*
Thermography (°C)	27.2±0.54	27.9±0.44*	27.4±0.48

**p<0.05 vs pretreatment.*
Four and 24 weeks, 4 and 24 weeks after the treatment.
Abbreviations see in Table 1.

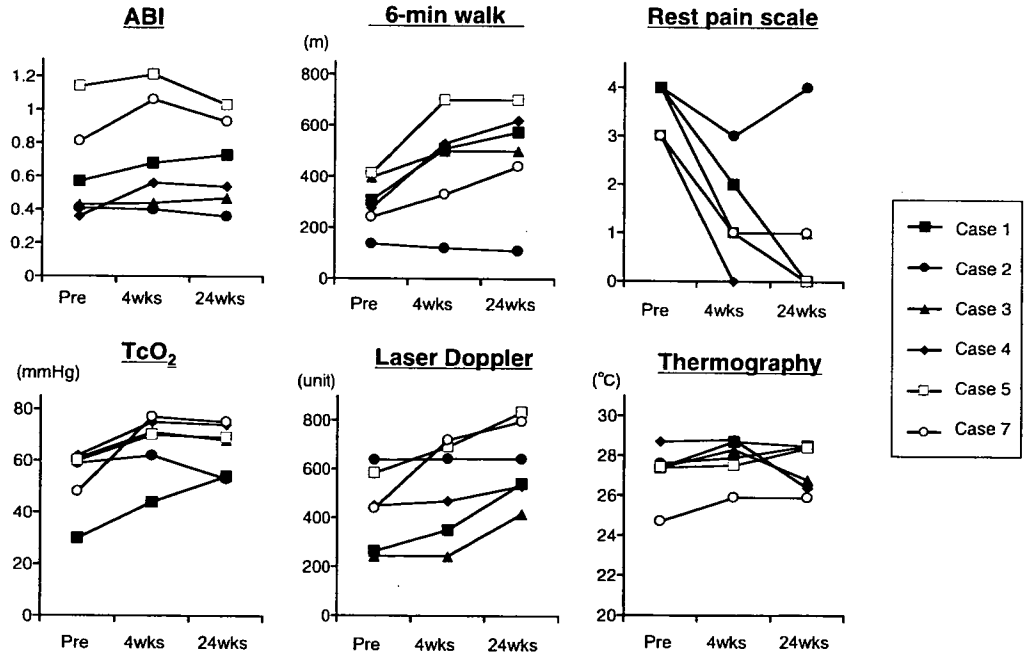

Fig 2. Changes in parameters of limb ischemia in each patient. ABI, ankle-brachial pressure index; 6-min walk, distance walked in 6 min; TcO_2, transcutaneous oxygen pressure; wks, weeks.

Statistical Analysis

All values are expressed as mean±SD. Changes in variables from baseline to week 4 or week 24 were analyzed with the Wilcoxon t-test. All statistical analyses were performed with Statview software (SAS Institute Inc NC, USA). A p value <0.05 was considered to be significant.

Results

Seven patients were entered into the study (49.3±17.2

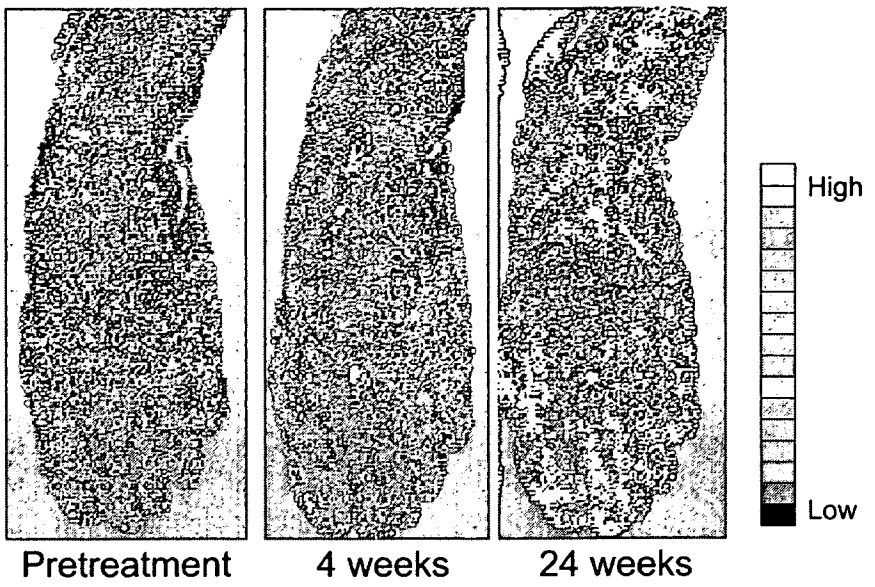

Fig 3. Laser Doppler perfusion image analysis in Case 4.

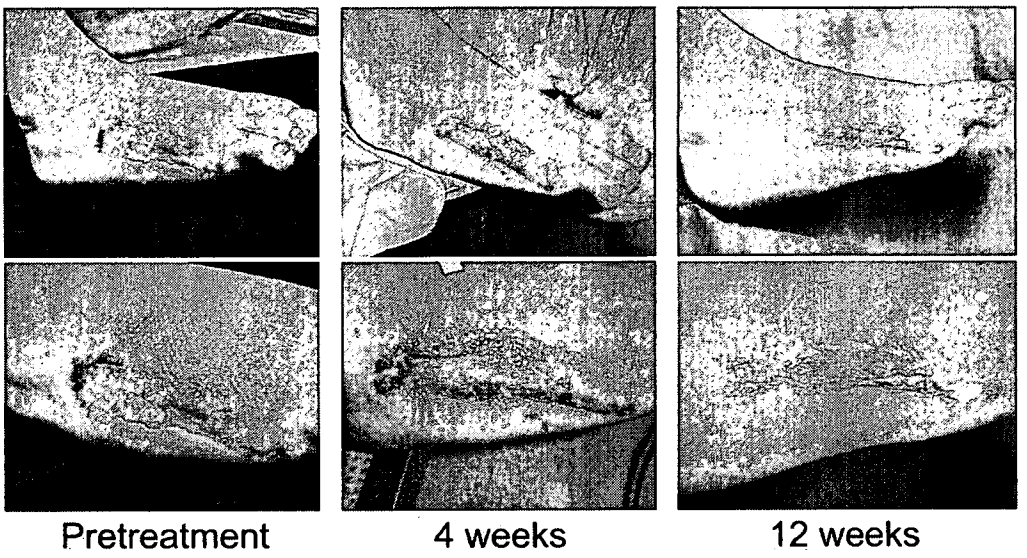

Fig 4. Complete healing of a foot ulcer in Case 1. Pretreatment, non-healed ulcer (90×30 mm) with ischemic skin; 4 weeks, reduced ulcer with reddish skin; 12 weeks, a completely healed ulcer.

years old, 6 males) (Table 1). Among them, 4 patients (Case 1, 3, 4, and 5) were diagnosed as having Buerger's disease and the other 3 had ASO. Cases 2 and 7 were on chronic hemodialysis. Case 4 had splenomegaly of an unknown origin, and was referred to our institute because he could not have G-CSF for peripheral blood mononuclear cells transplantation for fear of splenic rupture[28,29]

Primary End-Point

There were no deaths or events that were against the NCI CTCAE Ver 3.0 during the whole study period. The treatment did not induce focal inflammation or edema at the injected site. In blood analysis, WBC and CRP transiently elevated, but did not sustain, and normalized within 2 weeks in all patients except 1 patient whose foot ulcer did not heal at 24 weeks (Case 2). Blood levels of bFGF were undetected or within the normal value in all patients.

Secondary End-Point (Tables 1,2; Fig 2)

Subjective Parameters The distance walked in 6 min (295±42 m, pretreatment) increased both at 4 weeks (448±81 m, p=0.023) and 24 weeks (491±85 m, p=0.023). Similarly, the rest pain scale (3.5±0.2, pretreatment) improved both at 4 weeks (1.3±0.42, p=0.015) and 24 weeks (1.0±0.6, p=0.022). Three patients (Cases 1, 4, and 5) became free from rest pain completely.

Objective Parameters ABI (0.62±0.12, pretreatment) improved at 4 weeks (0.73±0.14, p=0.024), but not at 24 weeks (0.68±0.11). However, at 24 weeks, 4 patients showed an increase in ABI from 9% to 50% from the baseline. TcO_2 (53.5±5.2 mmHg) increased both at 4 weeks (66.5±5.0 mmHg, p=0.015) and 24 weeks (65.5±4.0 mmHg, p=0.03). LDPI (436±66 relative unit) also increased both at 4 weeks (520±80 relative unit, p=0.024) and 24 weeks (614±61 relative unit, p=0.015) (Fig 3).

Five of the 6 patients had a non-healing foot ulcer; the

ulcers were completely healed in 3 patients, reduced in 1, and no change in 1. Fig 4 shows the drastic improvement of a non-healed foot ulcer in Case 1. One patient (Case 2) did not show healing of the ulcer progressed stenosis of the superficial femoral artery during the study period.

Discussion

We have shown for the first time that the sustained release of bFGF from gelatin hydrogel microspheres effectively increased blood flow in the ischemic limbs, as assessed by substantial increases in ABI, TcO_2, LDPI, and skin temperature. The treatment also significantly improved the distance walked in 6 min and rest pain (complete regression in half of the patients). Ischemic ulcers were completely or partially improved except in 1 case that showed stenosis of the superficial femoral artery during the study period. In addition, the sustained release of bFGF from gelatin hydrogel did not induce focal inflammation at the injected site. Thus, we believe that the method is promising.

The gene transfer of angiogenic growth factors might have shown good results and safety in phase I-IIa clinical trials, although there are still concerns about the unpredictable duration and level of gene expression, or immune or inflammatory responses of genetic materials[1-4,9-11] In addition, autologous bone marrow cell transplantation needs the aspiration of cells under general anesthesia[6-8] Peripheral mononuclear cell transplantation needs the systemic administration of G-CSF; they might induce serious complications such as myocardial infarction, particularly in patients with systemic atherosclesosis[12,13] or splenic rupture, although it has been reported to be rare[28,29] The sustained release of bFGF from gelatin hydrogel does not require gene therapy, general anesthesia, or G-CSF and therefore might solve these problems.

As a carrier biomaterial, gelatin hydrogel might be suitable for clinical use in terms of easy processability and the versatile controlled release of various growth factors[15,16] Gelatin hydrogel is easily processed to microsphere, sheet, or disk. Microspheres are easily dispersed in the water and can be injected into various organs, while sheets could be placed on the heart, bone, and other tissues[17-24] Furthermore, by changing the cross-linking extent, each growth factor could be released at a desirable rate and duration for tissue regeneration[15,16] They enable a sustained release by a single administration across various fields of regenerative medicine.

We used a sustained release of bFGF because bFGF shows not only potential for angiogenesis and arteriogenesis, but also synergistic effects with other angiogenic agents such as VEGF[30] HGF[24,31] or PDGF-BB[32] In addition, we have shown that a combination of bFGF and sarpogrelate, a serotonin blocker[22] or heparin[23] enhanced collateral vessel flow effectively. Therefore, bFGF might induce more mature vessels and promote more collateral vessel development than other angiogenic agents, which is important to improve long-term results. This advantage is prominent particularly in high-risk patients who have severe diabetes mellitus and hypercholesterolemia, or who are on chronic hemodialysis.

Although this is an initial report of the project of sustained release of bFGF from biodegradable gelatin hydrogel microspheres for patients with severe limb ischemia, the results are promising in its effectiveness to increase blood flow and relieve signs and symptoms. Moreover, the serum level of the bFGF did not increase at any time after the treatment, which suggests no systemic effects of bFGF such as hypotension or proteinuria[2,5] If no systemic effects are confirmed, the method will be a purely local treatment that is very safe and suitable for patients with carcinoma or proliferative retinopathy or cerebral/cardiac arterial disease[9,10]

Cases 5 and 7 showed a high ABI in spite of their severe ischemic symptoms. In Case 5, an ABI measurement of the dorsalis pedis artery was high, however, a pulse of the posterior tibial artery was undetectable. The toe-brachial pressure index might be more reliable than the ABI to the patient[33] In contrast, a high ABI in Case 7 might be caused by the non-compressible leg arteries with severe atherosclerosis, which is often observed in patients with long-standing diabetes mellitus, in elderly patients, and patients who require hemodialysis for end-staged-renal disease[27] Therefore, we should have had used toe-brachial pressure index instead of ABI.

There are some limitations in this report/study. First, number of the patients is low becaise this is the first/preliminary report. In addition, the follow-up period was short for the same reason. The fate of the limb circulation late after the treatment should be clarified. Second, because of the phase I (-IIa) nature of the study and because the study deals with "no other option" patients, we did not include a control group who received just a placebo. To confirm the benefit of the method in more detail, a prospective and randomized study will be necessary, perhaps by using patients who are less ill.

In summary, therapeutic angiogenesis by the sustained release of bFGF by using gelatin hydrogel is so far safe and promising. Further investigation is warranted.

Co-Investigators

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◆EXPERIMENTAL INVESTIGATION

Hydrogel-Mediated Release of Basic Fibroblast Growth Factor From a Stent-Graft Accelerates Biological Fixation With the Aortic Wall in a Porcine Model

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Purpose: To evaluate the local reaction of the aortic wall induced by basic fibroblast growth factor (bFGF) released from a gelatin hydrogel coated on the outer surface of a stent-graft for the purpose of biological fixation.

Methods: A total of 18 nitinol-based, polyester-covered stent-grafts were implanted in 6 porcine aortas for 1 month. The implanted stent-grafts were divided into 3 groups: the control group (uncoated), the hydrogel group (coated with hydrogel containing water), and the bFGF group (coated with hydrogel containing bFGF). After stent-graft implantation, the results of intravascular ultrasound (IVUS) and qualitative and quantitative microscopic examinations were compared among the groups.

Results: In the bFGF group, a thin white lamellar tissue was observed on IVUS images. Significantly more new intimal tissue formation was observed in all the bFGF group animals than in the other 2 groups, and alpha smooth muscle (SM) actin–positive cells (αSMCs) were detected in this new tissue. The αSMCs within the fabric of tightly woven grafts were significantly more abundant in the bFGF group than in the other groups.

Conclusion: The local controlled release of bFGF from the stent-graft significantly accelerated the proliferation of new intimal tissue between the aorta and the stent-graft and within the graft materials. These findings suggest that a graft can be fixed biologically to the aortic wall, which may contribute to the shrinkage of aneurysms following stent-grafting.

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Key words: Biological fixation, stent-graft, basic fibroblast growth factor, controlled release, hydrogel, aortic wall

Endovascular stent-grafting for aortic aneurysms has proven to be extremely effective in treating aortic aneurysms,[1] especially in poor surgical candidates.[2,3] One of the most important advantages of stent-grafting compared to open surgery is its relatively low invasiveness, resulting in extremely low periprocedural morbidity and mortality.[4,5] On the other hand, because of the simple mechanical compression of the relatively thin artificial graft to the aortic wall with an internal metallic stent, the graft never tightly contacts the aortic wall. This is especially true in the case of an aortic wall rendered irregular due to arteriosclerosis, with variously sized spaces remaining between the graft and the

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wall. It is speculated that these spaces could be one of the causes of endoleak, endotension, and stent-graft migration, which are the major reasons for progressive enlargement and rupture of aortic aneurysms following stent-grafting.[6–8] Sealing of these spaces is considered to be essential in preventing migration and to obtain complete aneurysm exclusion with long-term clinical success.

The concept of biological fixation between the aortic wall and stent-graft has attracted attention of late.[9,10] Marty et al.[9] enhanced biological fixation by causing extensive granulation tissue invasion into the pores of polyurethane-covered endoprostheses, but healing was inadequate in stent-grafts made with polyester, which has been the main type of graft material used clinically.

In recent years, the application of various genes or growth factors has been investigated in the field of regeneration medicine and tissue engineering. Among them, basic fibroblast growth factor (bFGF) had shown its effectiveness and has been applied clinically for accelerated wound healing.[11,12] Conklin et al.[13] also reported that bFGF increased the proliferation rate of both endothelial cells and endothelial progenitor cells in their experimental study.[13] In addition, Tabata et al.[14] developed a biodegradable gelatin hydrogel as a vehicle for controlled release of bFGF; they confirmed that biologically active bFGF was released to surrounding tissue as a result of in vivo degradation of the acidic gelatin hydrogel. Using this gelatin hydrogel, we postulated that these new techniques might have a potential for achieving biological fixation between a stent-graft and the aortic wall. Hence, we created a new stent-graft that could gradually release bFGF from gelatin hydrogels coated on the outer surface of a stent-graft. We studied the local reaction caused by controlled release of bFGF and explored its potential for biological fixation between the aortic wall and stent-graft.

METHODS

Study Design

The biological reactions evoked between the stent-graft and aortic wall following endovascular stent-grafting in a pig were compared in 3 groups: the bFGF group, in which a polyester graft was coated with gelatin hydrogel crosslinked with a bFGF solution; the hydrogel group, in which the graft was coated with gelatin hydrogel containing water; and the control group consisting of a non-coated graft. Eighteen stent-grafts (6 in each group) were implanted in the abdominal aortas of 6 pigs. The observation period was 1 month.

Animals

All animals were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals in Kanazawa University. The protocol was approved by our institution's Animal Care Committee. Six Landrace-Large White-Duroc pigs weighing a mean 39.3±3.0 kg were boarded and given oral anticoagulation with aspirin (81 mg/d) and ticlopidine (100 mg/d) from 3 days before the procedure to the end of the observation period.

Stent-Graft

The frame was a self-expanding 25-mm-long Matsui-Kitamura stent,[15] which is made from a single 0.3-mm-diameter superelastic nitinol wire (Memoalloy; Tokin Inc., Tokyo, Japan) having a transformation temperature <0°C. The fabric cover was a seamless woven polyester cylindrical graft measuring 15 mm long with a porosity of 250 mL/min/cm^2. The outer diameters of the stent-grafts were 10% to 20% larger than the aorta measured by preoperative aortography.

Twelve grafts were coated with gelatin hydrogel.[16] Gelatin samples with an isoelectric point of 5.0 (molecular weight 99000; Nitta Gelatine Co., Osaka, Japan) were dissolved in double-distilled water with a consistency of 5% wt/wt at 40°C so that these gelatin hydrogels would dissolve over 1 week. The aqueous gelatin solution was chemically crosslinked with glutaraldehyde (target water content 95%) to prepare the hydrogels. Before coating, the graft materials were charged for 1 minute with 100 V for ionization, and then the graft materials were covered with the hydrogels. The closslinking reaction was

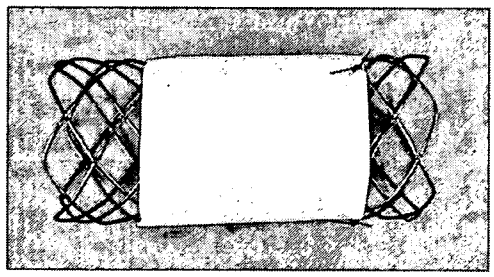

Figure 1 ◆ Stent-graft: MK stent and polyester fabric.

allowed to proceed for 24 hours at 4°C, and the crosslinked hydrogel-coated grafts were immersed in a 50-mM aqueous glycine solution at room temperature for 1 hour to block residual aldehyde groups. The grafts were rinsed twice with double-distilled water and then freeze-dried to preserve them for extended periods of time.

To prepare the stent-grafts, the hydrogel-coated grafts were slid over the stents, attached with 5-0 polypropylene sutures, and sterilized with ethylene oxide gas (Fig. 1). Using aseptic techniques, 6 stent-grafts were impregnated with bFGF using human recombinant bFGF (Trafermin Fiblast spray; Kaken Pharmaceutical Co., Tokyo, Japan), which is commercially available for skin ulcers and burns. The freeze-dried hydrogel-coated stent-grafts were soaked for 1 hour at 37°C in a solution of 1-mg/mL bFGF in distilled water. The other 6 hydrogel-coated stent-grafts were soaked in distilled water for 1 hour at 37°C. The 6 control stent-grafts were soaked in distilled water for 1 hour at 37°C.

To examine how much hydrogel was lost mechanically during the stent-graft delivery procedure, the bFGF was radiolabeled with iodine 125 (^{125}I) using the chloramine-T radioiodination process. Ten microliters of an aqueous bFGF solution (10 mg/mL) was added into 190 μL of a buffer containing 0.5M potassium phosphate buffer (KPB) and 0.5M potassium chloride (pH 7.5). Five microliters of a sodium ^{125}I solution and 100 μL of 0.05M KPB solution containing 0.02 mg chloramine-T were added to the bFGF solution. After agitation at room temperature for 2 minutes, 100 μL of 0.01M phosphate-buffered saline (pH 7.4) containing 0.4 mg of sodium metabisulfite was added to stop the radioiodination process. The resulting mixture was passed through a PD-10 desalting column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) to remove uncoupled ^{125}I molecules.

The radioactivity of a bFGF-impregnated graft was measured with a gamma-counter before and after the stent-graft was pushed through the delivery sheath in vivo. After testing 3 samples, the residual concentration of bFGF after stent-graft delivery was 91.3%, 94.9%, and 92.1%, respectively. Based on these values, the quantities of bFGF initially adherent to the stent-grafts after deployment were estimated as ~110, 114, and 111 μg, respectively. In this study, it was not possible to measure bFGF released in vivo; however, the degradation of bFGF- impregnated gelatin hydrogels was confirmed previously in the subcutis of mice,[14] and it is assumed that hydrogel degradation and bFGF release to the surrounding tissue would be similar.

Stent-Graft Procedure

Animals were placed under general anesthesia after premedication with azaperone (4 mg/kg), atropine sulfate (0.05 mg/kg), and ketamine hydrochloride (15 mg/kg). Anesthesia was induced with a nitrous oxide (2 L), oxygen (2 L), and 5% sevoflurane mixture by inhalation. An endotracheal tube was placed to maintain anesthesia. Pancuronium bromide (0.1 mg/kg) was administrated intravenously for muscle relaxation. Monitoring consisted of electrocardiography, invasive blood pressure measurements, and percutaneous recording of oxygen saturation. During the procedure, all pigs were heparinized to maintain an activated coagulation time between 250 and 350 seconds.

The left femoral artery was exposed, and a 7-F sheath (Terumo, Tokyo, Japan) was inserted by direct puncture. A 4-F straight catheter with calibrated markers (Medikit, Tokyo, Japan) was inserted. Aortography (Infunux-Celeve CC; Toshiba Medical Systems, Tochigi, Japan) and intravascular ultrasound (IVUS) (Intraimaging System TU-C200; Terumo) were performed to measure the aortic diameter at several points and select the deployment positions. After surgical exposure of the right common femoral artery,

a 14-F short sheath (11 cm; Medikit) was inserted, and a 0.035-inch stiff guidewire (Terumo) was passed to the thoracic aorta under fluoroscopic guidance. A 12-F long sheath (80 cm; Medikit) was inserted carefully at the point where the stent-graft would be placed to avoid injury to the distal aorta, and the stent-graft was inserted into the 12-F long sheath over the guidewire. After each stent-graft was deployed, it was dilated using an XXL Large Balloon Catheter (Boston Scientific, Natick, MA, USA). Three stent-grafts were placed in each animal: the control device was placed at the uppermost site, the hydrogel-only stent-graft in the middle, and the bFGF-impregnated device at the lowermost site. After stent-graft placement was complete, aortography and IVUS were performed again to check the patency of the aorta. The access sites were sutured surgically.

Specimen Retrieval and Histological Analysis

After 1 month, aortography and IVUS were performed. The pigs were euthanized and their abdominal aortas retrieved; the specimens were preserved in 20% neutral buffered formalin for 1 week. After careful elimination of the stent wire so as not to injure the surrounding tissue, the specimens were embedded in paraffin. The middle portion of the graft was taken for examination in the axial plane, and the proximal and distal ends were taken for examination in the longitudinal plane. Sections were cut into thin slices and stained with hematoxylin and eosin, elastica van Gieson (EVG), and antibodies to alpha smooth muscle (α-SM) actin.

The specimens were assessed for the thickness of the neointima covering the inside of the graft as determined by IVUS, neointimal ratio (percentage of the neointimal area along the total vascular lumen in the axial plane) and neointimal thickness by EVG staining, and α-SM actin–positive cells within the graft material on the axial section in each group. The area and thickness of the neointima were manually traced on the digitalized axial and longitudinal EVG-stained slides and analyzed using the National Institutes of Health Image Analysis Software (Image J, for Macintosh; http://rsb.info.nih.gov/ij/). The intimal thickness measurements on both IVUS and the histological specimens were the mean values of 3 discrete points in the longitudinal plane.

Statistical Analysis

All data are expressed as means ± standard deviation. Upon detection of significant increases by an analysis of variance, post-hoc pairwise comparisons were conducted using Tukey's test, with the level of statistical significance taken as $p<0.05$. Statistical analyses were performed using SPSS software (SPSS, Chicago, IL, USA).

RESULTS

All endovascular procedures were successful without any complications. All of the pigs survived the observation period uneventfully. At follow-up, all stent-grafts were patent on aortography, and no stent-graft migration was observed. Angiographically, mild stenosis was seen in 5 of 6 stent-grafts in the bFGF group (Fig. 2). In these cases, thin echogenic lamellar tissue was observed on IVUS (Fig. 3). The thickness of the echogenic tissue was 20.8±51.0 μm in the control group, 314±298 μm in the hydrogel group, and 593±490 μm in the bFGF group ($p=0.024$ versus control).

Macroscopic Findings

On gross examination, the adventitial aspect of the specimen showed slight bulging in all groups. On the luminal side, all of the specimens were completely covered with a glistening white-tinged layer, which continued from the cranial to the caudal portion of the stent-graft and showed complete incorporation into the aortic wall.

Histological Findings and Analysis

In all specimens, the medial layer became thinner, lost its normal wavy disposition, and sometimes showed atrophic changes (Fig. 4). The stent struts and grafts were completely covered by the new tissue (neointima). The neointima, which developed in the space

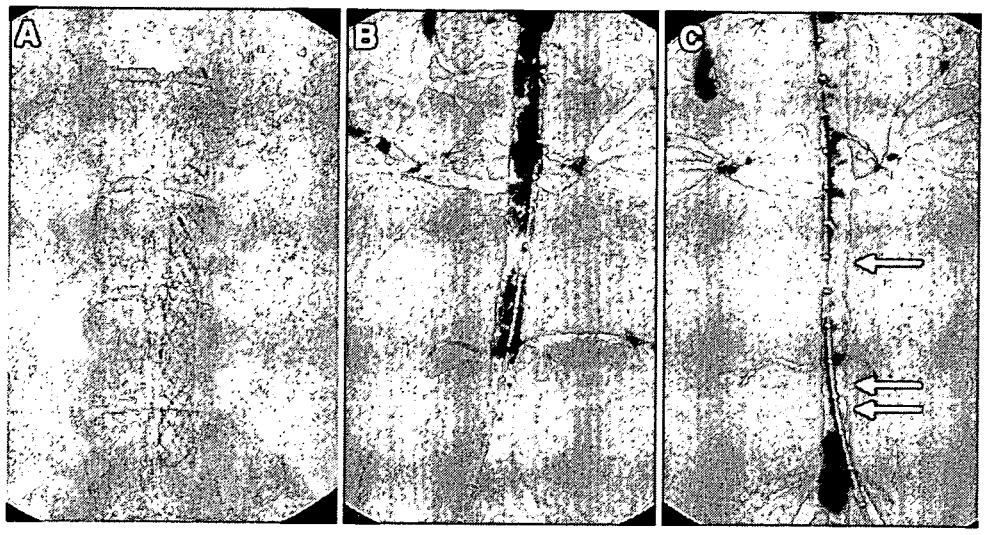

Figure 2 ◆ **(A)** Post deployment fluoroscopy of the stent-grafts (from the top: control, hydrogel-coated, and hydrogel with bFGF–coated). Digital subtraction angiography just after stent-grafting **(B)** and 1 month later **(C)** showed slightly stenotic changes at the level of the hydrogel-coated stent-graft (arrow) and bFGF-impregnated stent-graft (double arrow).

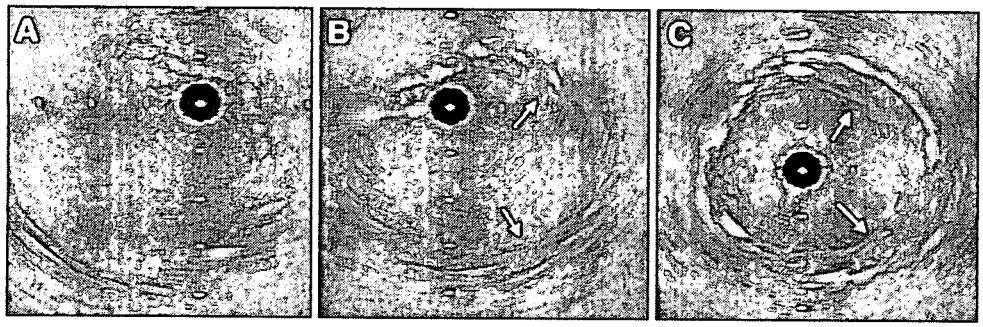

Figure 3 ◆ **(A)** IVUS at 1 month in the control group showed no intimal tissue. **(B)** In the hydrogel group, slight white tissue (arrows) can observed, while in the bFGF group **(C)**, thin white lamellar tissue (arrows) was obvious.

Figure 4 ◆ Histological cross section (elastica van Gieson stain, original magnification ×40) in the control group **(A)**, the hydrogel group **(B)**, and the bFGF group **(C)**. All the polyester grafts were covered by neointimal tissue (*). In the bFGF group, a large amount of new tissue both inside and outside the graft was observed. G: graft material, A: aortic wall, L: lumen.

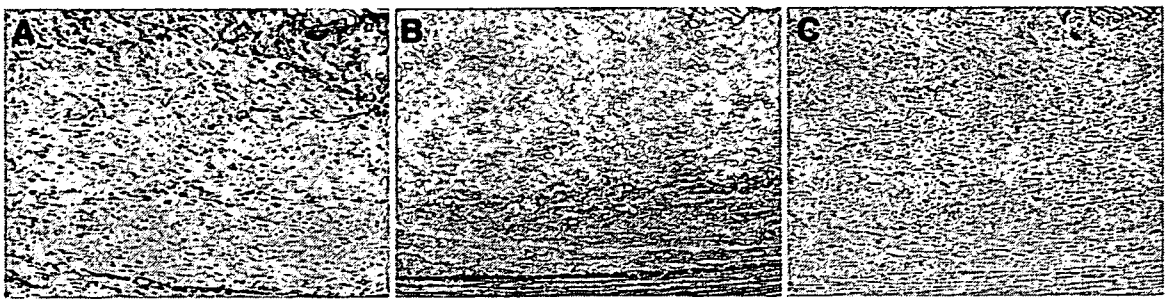

Figure 5 ◆ Histological cross sections of the neointimal tissue between the aortic wall and graft material in the bFGF group [hematoxylin-eosin (**A**), elastica van Gieson (**B**), α-SM actin stain (**C**); original magnification ×200]. The neointima consisted of fibrous tissue, inflammatory cells, collagen, and some elastin. These new tissues had large quantities of α-SM actin–positive cells.

between the aortic media and the graft, contained fibrous tissue, inflammatory cells, collagen, and a little elastin. In contrast, the inner spaces of the graft (luminal side) were occupied by more loose fibrous tissue, collagen, and elastin (Fig. 5). The neointima contained numerous α-SM actin–positive cells on both sides of the graft. These findings were observed in all groups. No capillary ingrowth was seen in any group.

Although the grafts were close-woven, new cells were observed infiltrating the spaces between the fibers of the graft, and the α-SM actin–positive cells were more abundant (Fig. 6), whereas fewer α-SM actin–positive cells were observed in the other groups. The total numbers of α-SM actin–positive cells within the graft materials per axial section were 99.5±35.4 in the control group, 133.5±34.3 in the hydrogel group, and 248.0±55.1 in the bFGF group. There were significant differences between the control and bFGF groups ($p<0.001$) and the hydrogel and bFGF groups ($p=0.001$).

The neointimal ratio was 9.3%±3.4% in the control group, 12.6%±4.2% in the hydrogel group, and 21.3±7.5% in the bFGF group. The differences in these ratios between the hydrogel and bFGF groups ($p=0.02$) and between the control and bFGF groups ($p=0.004$) were statistically significant. The intimal thickness was 11.3±7.7 μm in the control group, 19.7±10.9 μm in the hydrogel group, and 38.0±23.7 μm in the bFGF group ($p=0.026$ versus control).

DISCUSSION

Endovascular stent-grafting for aortic diseases is widely performed, but endoleaks

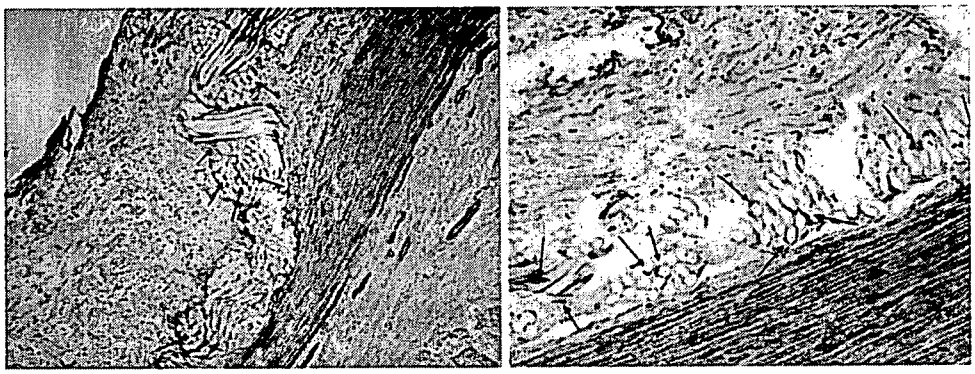

Figure 6 ◆ Histological cross sections in the bFGF group (α-SM actin staining, magnification ×100). New cells were observed between the fibers of the graft, and some α-SM actin–positive cells were also seen in the bFGF group (arrows).

and endotension are critically important problems of this procedure.[6–8] Since the goal of stent-grafting is not only prevention of aneurysmal growth but also shrinkage of the aneurysm, biological fixation of the graft to the aortic wall at the neck is important. However, polyester graft material, which is most widely used in aortic stent-grafts, does not fix to the vessel wall and does not heal adequately. Malina et al.[17] have shown that healing provides poor fixation of Dacron stent-grafts in humans, and fixation relies on the mechanical properties of the stent-grafts, which are exposed to high, pulsatile blood pressure. Unless there is firm perigraft healing between the graft and the aortic wall, biological fixation seems unlikely.

bFGF, a naturally occurring substance in human beings, was originally characterized in vitro as a growth factor for fibroblasts and capillary endothelial cells. In vivo, this growth factor plays an important role in the proliferation of a variety of cells, including mesodermal and neuroectodermal cells. In blood vessels, it has been demonstrated to stimulate the proliferation and migration of endothelial cells, SMCs, and fibroblasts.[18] However, the simple application of bFGF in a solution exerts no biological activities because of its short half-life in vivo, so the controlled release of bFGF is essential. Tabata et al.[14] developed a biodegradable gelatin hydrogel carrier for this purpose. Using this system, they confirmed that biologically active bFGF was released to the surrounding tissue gradually as a result of in vivo hydrogel degradation, whose rate is dependent on its water content.[14,16] Hence, it is possible to change the degradation time by adjusting the water content. In this study, we chose a 1-week degradation time because we wanted to see the reaction after bFGF release within a limited observation period.

In this study, we tested the hypothesis that the controlled release of bFGF from a stent-graft would accelerate aortic healing (including intimal hyperplasia), resulting in biological fixation with the aortic wall compared with a stent-graft without bFGF incorporation. To that purpose, we developed a new type of polyester-based stent-graft coated with hydrogels that allow controlled release of ~100 μg of bFGF over 1 week. All the materials used for this new device have been applied clinically.

Our results revealed that bFGF significantly accelerated the proliferation of new intimal tissue. However, accelerated proliferation was also seen in hydrogel-coated stent-grafts without bFGF, but the increase was minimal and not significantly different compared to the control group. We speculate that this could be due to the potential biocompatibility of the gelatin hydrogel, which itself could serve as a scaffold for cell proliferation. Therefore, the accelerated neointima formation seen in this study was mainly due to bFGF release and partially due to the biological reaction of hydrogel itself. In vivo, these cell proliferations presented as mild stenosis of the aorta. When applied to a small artery, this could be a concern, but it would not be clinically relevant in the large human aorta.

Since this was a preliminary study, the amount of bFGF employed was the maximal dose we could impregnate and the degradation time was short so as to estimate the initial reaction of the surrounding tissue. More investigations would be needed when the technique is applied to human beings.

Another important finding of the histological evaluation was the existence of α-SM actin–positive cells, probably SMCs or myofibroblasts, not only in new intimal tissue but also within the fabric of the tightly woven polyester graft. In cases of in-stent restenosis, Kearney et al.[19] reported that new tissue is composed of α-SM actin–positive cells with phenotypic characteristics of "activated" SMCs surrounded by a loose, light-staining extracellular matrix. The role of SMCs is to maintain the strength and elasticity of the aortic wall by producing elastin, collagen, and other matrix proteins.[20] In this study, the existence of α-smooth muscle–positive cells within the graft might represent the process of cell and matrix proliferation through the graft material.

According to these observations, we postulate that hyperplastic neointima occupying the space between the aortic wall and graft infiltrates into the spaces between the graft fibers, connecting with the neointima on the luminal side of the graft to achieve biological fixation of the stent-graft. This process can be

strongly enhanced by bFGF, which could make the biological fixation tighter and more durable, probably due to expected development of α-SM actin–positive cells.

van der Bas et al.[21] were the first to attempt to combine endovascular aortic aneurysm repair and bFGF. They showed that Dacron prostheses impregnated with collagen, heparin, and bFGF induced graft healing in vitro[21] and in a pig model[22] compared to non-impregnated stent-grafts. There are several differences between their work and our study, such as the porosity of the graft, the drug carrier, and the method of coating. In spite of these differences, we obtained similar results, demonstrating that stent-graft impregnation with bFGF was clearly beneficial for biological fixation with the aortic wall.

Limitations

First, the aortas used in this study had no atherosclerosis. Second, the observation period was relatively short. We surmised that increasingly more cell proliferation would occur as the observation period was prolonged, but we considered that 1 month was long enough to check initial reactions and predict possible changes of the aortic wall.

Third, we inserted 3 stent-grafts per pig to reduce the experimental period and costs; this design feature might have influenced the results. However, we deployed the stent-graft incorporated with bFGF in the most downstream portion of the aorta to avoid any secondary effect caused by released bFGF.

Fourth, there is a possibility that some injuries to the distal aorta might have occurred during implantation of the stent-grafts and thus have influenced to some degree the healing of the device, especially the more distally placed bFGF stent-grafts. However, because there was no difficulty or friction during insertion of the sheath catheter, it was considered to be unlikely that the distal aorta was injured.

Lastly, the real-time measurement of released bFGF in vivo was impossible technically in our study, but we assumed that hydrogel degradation and bFGF release to the surrounding aortic wall might be similar to previous studies.[14]

Conclusion

The local, controlled release of bFGF from the hydrogel coating on the surface of a polyester stent-graft significantly accelerated the proliferation of new intimal tissue on the aorta and within the graft in a porcine model. These findings suggest that the graft surface and aortic wall can be fixed biologically. It is highly likely that the tight attachment of a stent-graft and aortic neck may reduce minor type I endoleak or endotension. To evaluate the healing between the bFGF-coated stent-graft and an aortic aneurysm, further study using an aortic aneurysm model is required.

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Skull Bone Regeneration in Nonhuman Primates by Controlled Release of Bone Morphogenetic Protein-2 from a Biodegradable Hydrogel

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ABSTRACT

The objective of this study was to investigate the feasibility of biodegradable gelatin hydrogels as the controlled-release carrier of bone morphogenetic protein-2 (BMP-2) to enhance bone regeneration at a skull defect of nonhuman primates. Hydrogels with 3 different water contents were prepared through glutaraldehyde crosslinking of gelatin with an isoelectric point of 9.0 under varied reaction conditions. A critical-sized defect (6 mm in diameter) was prepared at the skull bone of skeletally mature cynomolgus monkeys, and gelatin hydrogels incorporating various doses of BMP-2 were applied to the defects. When the bone regeneration was evaluated by soft radiography and bone mineral density (BMD) examinations, the gelatin hydrogel incorporating BMP-2 exhibited significantly higher osteoinduction activity than did an insoluble bone matrix that incorporated BMP-2 (one of the best osteoinduction systems), although the activity depended on the water content of hydrogels. BMD enhancement was highest for the gelatin hydrogel that had a water content of 97.8 wt% among all types of hydrogels. Moreover, the gelatin hydrogel enabled BMP-2 to induce the bone regeneration in nonhuman primates even at low doses. We conclude that the controlled release of BMP-2 for a certain time period was essential to inducing the osteoinductive potential of BMP-2.

INTRODUCTION

BONE MORPHOGENETIC PROTEIN (BMP) has been expected as a therapeutic protein to induce regeneration repairing of bone injures and defects[1] since it has induced significant bone regeneration both orthotopically and ectopically in the body.[2] However, preclinical studies show that physiologically high doses of BMP are required to achieve bone formation for nonhuman primates, which is quite different from the requirements in rodents.[3] One possible reason for this species-dependent dose issue is the immature technology used to administer BMP *in vivo*. On the other hand, recombinant human BMP-2 and BMP-7 (osteogenic protein-1) have already been applied as regeneration therapy of human bone at a defect too large to be repaired only through natural self-healing.[4] However, efficient bone regeneration cannot always be expected because of the short half-life of BMP administered in the body. Therefore, it is necessary to develop an administration carrier of BMP for the localized release at the site applied over the time period required. The drug delivery system, such as controlled-release technology, will resolve the administration issue and reduce the adverse effects caused by high doses and repeated regimens.

BMP has been combined with various biodegradable carriers, such as collagen,[5,6] β-tricalcium phosphate,[7] and lactide-glycolide copolymers,[8,9] for controlled release. However, few

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studies have evaluated *in vivo* BMP release for bone regeneration. Recently, we designed biodegradable hydrogels of gelatin for the *in vivo* controlled release of BMP-2. This hydrogel system enabled BMP-2 to be retained at the implanted site for extended periods and consequently enhanced its ability to induce bone regeneration; this is in marked contrast to results seen with the solution form of BMP-2.[10] The controlled release of BMP-2 induced bone regeneration at the defect of rabbit ulna, whereas the BMP-2 solution was ineffective.[11]

The objective of this study is to evaluate the feasibility of the gelatin hydrogel as a controlled-release carrier of BMP-2 to enhance bone regeneration at a bone defect of monkey skulls. Hydrogels with different levels of biodegradability were prepared by changing the concentration of gelatin and glutaraldehyde in the hydrogel preparation. We examined the effect of the hydrogels' biodegradability and BMP dose on the promotion of bone regeneration; we compared the regeneration effect seen with insoluble bone matrix (IBM) that incorporated BMP-2, which previous studies have found to be the best choice.[12,13]

MATERIALS AND METHODS

Materials

A gelatin sample with an isoelectric point of 9.0 and human recombinant BMP-2 were supplied by Nitta Gelatin Co. (Osaka, Japan) and Astellas Pharma Inc. (Tokyo, Japan), respectively. IBM[12] (particle size, 320–620 μm) was supplied by Yoshinori Kuboki, Professor Emeritus, Department of Oral Health Science, Graduate School of Dental Medicine, Hokkaido University (Hokkaido, Japan). Glutaraldehyde, glycine, and other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) and were used without further purification.

Preparation of gelatin hydrogels and IBM that incorporated BMP-2

Hydrogels were prepared through the chemical crosslinking of aqueous gelatin solution with glutaraldehyde, according to the method described elsewhere.[10] Briefly, an aqueous gelatin solution mixed with glutaraldehyde was cast into a polypropylene dish (138×138 mm^2; BIO-BIK, Osaka, Japan) at various concentrations of glutaraldehyde and gelatin (Table 1), followed by the crosslinking reaction at 4°C for 12 h. The crosslinked hydrogel prepared was punched out to obtain the disks of 6 mm in diameter for the following *in vivo* experiment. The hydrogel disks 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, the hydrogel disks were freeze-dried and sterilized with ethylene oxide gas. The hydrogel weight was measured before and after complete drying at 70°C under vacuum conditions, and the water content (the wt% of water in the wet hydrogel to the hydrogel) was calculated from the 2 weights.

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

To prepare the gelatin hydrogel that incorporated BMP-2 for the *in vivo* experiment, 20 μL of phosphate-buffered saline (PBS; pH, 7.5) containing various amounts of BMP-2 (5, 20, and 200 μg) was dropped onto a freeze-dried gelatin hydrogel disk; the disks were left overnight at 4°C. Similarly, 20 μL of PBS containing 5 μg of BMP-2 was dropped onto IBM and was left overnight at 4°C. As controls of these samples, BMP-2–free, empty gelatin hydrogel and IBM were obtained by dropping 20 μL of BMP-2–free PBS onto a freeze-dried gelatin hydrogel and IBM, respectively.

Surgical procedure to evaluate bone regeneration at site of monkey skull defects

The skull defect model of monkeys was prepared according to a method described elsewhere.[14] All procedures adhered to the institutional guidelines on animal experimentation. After the intramuscular injection of xylazine hydrochloride (1.17 mg/kg body weight), the monkeys (*Macaca fascicularis*; male; weight, 4–6 kg; age, 4–5 years) were further anesthetized by the intramuscular injection of ketamine hydrochloride (2.3 mg/kg). In addition, local analgesia was induced by subcutaneous administration of 1% lidocaine solution into the subcutis of the right temple (10 mL/head). The scalp and underlying temporal muscle were cut, and the temporoparietal region of the skull was exposed. After incision of the pericranium, 5 defects (each 6 mm in diameter) in each monkey's parietal bone were prepared by using a microdrill (without injuring the underlying dura mater), assisted by use of a surgical microscope; the distance between the neighboring defects was 6 mm. We attempted to create the defects at the same anatomic site in each skull, defined by the temporal and occipital crests and the temporoparietal suture. A preliminary experiment revealed that a 6 mm skull defect did not naturally close even after 6 months without any applications. In addition, it is difficult to prepare 5 defects with a larger diameter at the monkey parietal bone. Thus, we decided to use 6 mm as defect diameter in this study. As shown in Table 2, we applied the following to the skull bone defects: 6 mm gelatin hydrogel disks that incorporated BMP-2 (5 μg/site) and contained various water contents (93.8 and 99.7 wt%), gelatin hydrogel that incorporated BMP-2 (5, 50, 200 μg/site) and contained a water content of 97.8 wt%, and IBM that incorporated BMP-2

TABLE 2. THE OVERALL EXPERIMENT DESIGN FOR *IN VIVO* EVALUATION OF THE SKULL BONE REGENERATION IN MONKEYS AND RABBITS

Animal species	*Experiment*	*Application*	*BMP-2 dose*	*Evaluation*	*Implantation period*	*Number of animals*
Monkey	Skull bone regeneration in several scaffolds	PBS Solution	0 μg/site	X-ray measurement; Histological evaluation; Bone mineral density measurement	12 weeks	6
		Empty gelatin hydrogel [1]	0 μg/site			
		Gelatin hydrogels [2] incorporated BMP-2	5 μg/site			
		IBM	5 μg/site			
	Influence of the BMP-2-dose on the skull bone regeneration	Free BMP-2	200 μg/site	Bone mineral density measurement		
		Gelatin hydrogel [1] incorporated BMP-2	0, 5, 50, 200 μg/site			
Rabbit	Skull bone regeneration in several scaffolds	PBS solution	0 μg/site	Bone mineral density measurement	8 weeks	11
		Free BMP-2	5 μg/site			
		Empty gelatin hydrogel	0 μg/site			
		Gelatin hydrogels [2] incorporated BMP-2	5 μg/site			
		IBM	5 μg/site			

[1] Water content: 97.8 wt%
[2] Water content: 93.8, 97.8, and 99.7 wt %

(5 μg/site). As controls, the BMP-2–free empty gelatin hydrogel, 20 μL of free BMP-2 solution (200 μg/site), and PBS solution were applied to the skull bone defect. After treatment, the pericranium and skin were carefully sutured with a 4-0 nylon monofilament to secure the samples. For each experimental group, 3 different defects were randomly selected from 3 different monkeys.

Skull bones were measured radiographically at 100 V and 10 mA for 2.75 sec under anesthetized conditions 1, 2, 3, 4, 6, 8, 10, and 12 weeks after application. At 12 weeks after the procedure, all the animals were sacrificed by overdose administration of anesthetic agents. The skull bone that featured the 5 defects was removed and used for all evaluations, including soft radiography, dual-energy x-ray absorptiometry (DEXA), and histologic observations. Over the 12-week period, no monkeys showed extraordinary behavior or lost weight.

Surgical procedure to evaluate bone regeneration at rabbit skull defects

In vivo bone regeneration was also evaluated for bone defects in rabbit skulls according to a surgical procedure reported elsewhere.[15] All the procedures adhered to Kyoto University's institutional guidelines on animal experimentation. As for the monkey model, the head skin of rabbit (New Zealand white; male; weight, 3.5 kg) was cut to expose the skull bone under anesthetization. After pericranium incision, bilateral skull defects 6 mm in diameter were carefully prepared by a microdrill with the aid of an operating microscope so as not to injure the underlying dura mater. As shown in Table 2, the gelatin hydrogel that incorporated BMP-2 (5 μg/site) was applied to the skull defect; the BMP-2–free empty gelatin hydrogel and 20 μL of free BMP-2 solution (5 μg/site) were employed as controls. Then, the pericranium and skin were carefully sutured with a 4-0 nylon monofilament. For each experimental group, 3 different defects of 3 different rabbits were used; the right or left defect was selected randomly. The animals were sacrificed 8 weeks after the procedure, and the skull bone was removed (together with the defect) and fixed with 10 vol% aqueous neutral formalin solution for histologic observation.

Assessment of bone regeneration

Bone regeneration at the skull defect was assessed by soft radiography, DEXA, and histologic examinations. The bone defect was radiographically observed by soft radiography (Hitex-100, Hitachi, Tokyo, Japan) at 54 kVP and 2.5 mA for 20 sec. The bone mineral density (BMD) of each bone defect was measured at the $5 \times 5\ mm^2$ region of interest by using DEXA with a bone mineral analyzer (DSC 600EX-III, Aloka Co., Tokyo, Japan). This instrument was calibrated with a phantom of known mineral content according to the manufacture's instruction. Each scan was obtained at a speed of 20 mm/sec, and scanning length was

1 mm. Similarly, the BMD value of the intact bone that surrounded the defects was measured as a control.

Bone specimens were placed into 10% neutral phosphate-buffered formalin solution, decalcified with 10% formic acid, and processed for the paraffin embedding. The 3 sections, 3 μm thick, from the center of bone specimens were prepared and stained with hematoxylin and eosin to view by light microscopy (AX-80T, Olympus, Tokyo, Japan).

Statistical analysis

All the data were statistically analyzed by using Fisher's least significant difference test for multiple comparisons, and statistical significance was indicated by a P value less than 0.05. The experimental results were expressed as the mean ± the standard deviation.

RESULTS

Radiographic examination of monkey skull defects

Figure 1 shows the soft radiographs of monkey skull defects 12 weeks after application of different gelatin hydrogels and the IBM that incorporated BMP-2. Bone regeneration was detected for the gelatin hydrogel that incorporated BMP-2 and had a water content of 97.8 wt%, regardless of BMP-2 concentrations (Fig. 1C, 1F, and 1G). On the other hand, little bone formation was radiographically observed at the bone defect applied with PBS solution (Fig. 1A), gelatin hydrogels that incorporated BMP-2 and had water content of 93.8 and 99.7 wt% (Fig. 1B and 1D), empty gelatin hydrogel (Fig. 1E), and the IBM that incorporated BMP-2 (Fig. 1H).

Histologic evaluation

Figure 2 shows the histologic sections of monkey skull defects 12 weeks after application of different gelatin hydrogels and the IBM that incorporated BMP-2. When the empty gelatin hydrogel with a water content of 97.8 wt% was applied to the skull defect, bone regeneration was insignificant and soft connective tissues had infiltrated into the defect (Fig. 2E). In contrast, new bone regeneration was found in the skull defect to which the BMP-2–incorporated gelatin hydrogel with a water content of 97.8 wt% was applied (Fig. 2C, 2F, and 2G), and the defect was completely closed by the newly formed bone tissue. BMP-2–incorporated gelatin hydrogels with water contents of 93.8 and 99.7 wt% at a low (5 μg/site) BMP-2 dose did not induce bone regeneration. The gelatin hydrogel with a water content of 93.8 wt% did not degrade and remained at the skull defect 12 weeks after application.

Mineral deposition at the sites of bone defects of monkey and rabbit skulls

Table 3 summarizes BMD at the skull defect of monkeys 12 weeks after application of different gelatin hydrogels and the IBM that incorporated BMP-2. The BMP-2–incorporated gelatin hydrogel with a water content of 97.8 wt% enhanced the BMD of the skull defect to a significantly higher extent than did PBS application, although the BMD was lower than that of the intact bone. The BMD depended on the types of hydrogels and was significantly higher for the hydrogel with a water content of 97.8 wt% than for the hydrogel with 99.7 wt% water content. On the other hand, BMD was not significantly enhanced with BMP-2–incorporated gelatin hydrogels with water contents of 93.8 and 99.7 wt% or with the BMP-2–

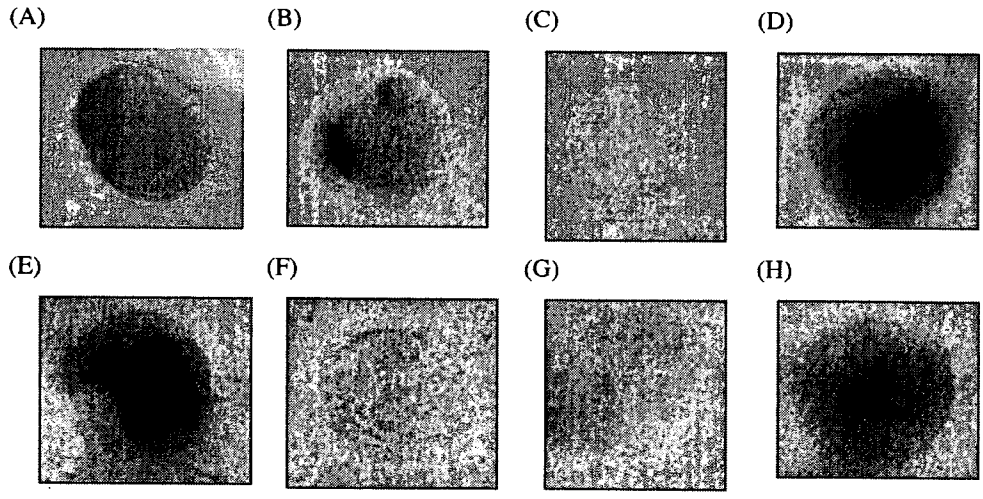

FIG. 1. Radiographic images of bone defect of monkey skulls 12 weeks after application with phosphate-buffered saline solution (**A**), gelatin hydrogels that incorporate bone morphogenetic protein-2 (BMP-2) with various water contents (**B–G**) and insoluble bone matrix that incorporated BMP-2 (**H**). The water content of hydrogels was 93.8 wt% (**B**), 97.8 wt % (**C** and **E–G**), or 99.7 wt% (**D**). The BMP-2 doses were 0 μg/site (**E**), 5 μg/site (**B–D** and **H**), 50 μg/site (**F**), and 200 μg/site (**G**).

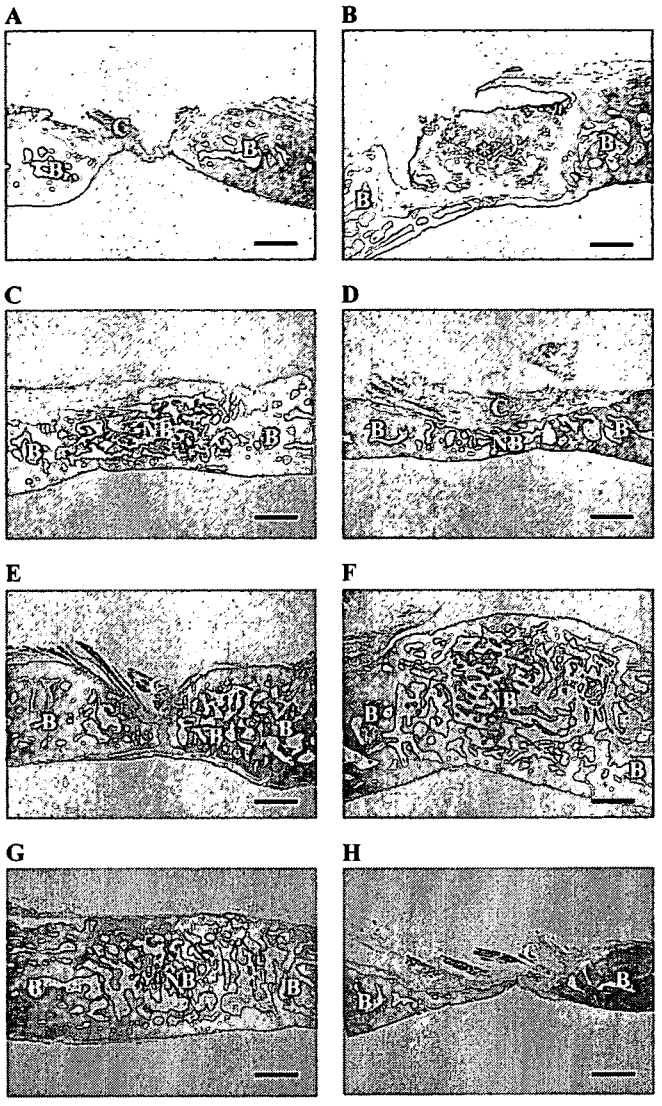

FIG. 2. Histologic cross-sections of bone defect of monkey skulls 12 weeks after application with phosphate-buffered saline solution (A), gelatin hydrogels that incorporated bone morphogenetic protein-2 (BMP-2) with various water contents (B–G) and insoluble bone matrix that incorporated BMP-2 (H). The water content of hydrogels was 93.8 wt% (B), 97.8 wt% (C and E–G), or 99.7 wt% (D). The BMP-2 doses were 0 μg/site (E), 5 μg/site (B–D and H), 50 μg/site (F), and 200 μg/site (G). B, bone; C, connective tissue; NB, new bone. An asterisk indicates the gelatin hydrogel that incorporated BMP-2 remaining in the defect. The bar length is 1 mm. Color images available online at www.liebertpub.com/ten.

incorporated IBM. The BMP-2–free empty hydrogel did not contribute to any increase in the BMD of the skull defect.

Figure 3 shows the influence of the BMP-2-dose on the bone regeneration induced by BMP-2–incorporated gelatin hydrogels with a water content of 97.8 wt%. The BMD at the skull defect applied with gelatin hydrogels that incorporated BMP-2 increased with the increasing BMP dose. The gelatin hydrogel that incorporated 200 μg of BMP-2 (water content, 97.8 wt%) enhanced the BMD to a significantly higher extent than did the BMP-2–free gelatin hydrogel, and BMD was similar to that of the intact bone. Free BMP-2 did not enhance BMD at the bone defect, and tended to be lower than that applied with the gelatin hydrogel that incorporated 200 μg of BMP-2.

TABLE 3. BONE MINERAL DENSITY AT MONKEY SKULL DEFECTS 12 WEEKS AFTER APPLICATION

Application	*Hydrogel water content (wt%)*	*BMD (mg/cm²)*
PBS		80.1 ± 7.8
Empty gelatin hydrogel	97.8	128.9 ± 14.8
Gelatin hydrogel incorporating BMP-2	93.8	104.9 ± 6.3
	97.8	188.8 ± 14.6*†
	99.7	70.0 ± 22.2
IBM		99.2 ± 4.8

The BMP-2 dose was 5 μg/site.

The BMD of intact monkey skulls was 216.4 ± 11.4 mg/cm².

*$p < 0.05$, significant against the BMD value at the skull bone defect after application of BMP-2-free PBS.

†$p < 0.05$, significant against the BMD value at the skull bone defect after application of gelatin hydrogels incorporation BMP-2 with a water content of 99.7 wt%

Table 4 summarizes BMD at the skull defect of rabbits 8 weeks after application with different gelatin hydrogels and

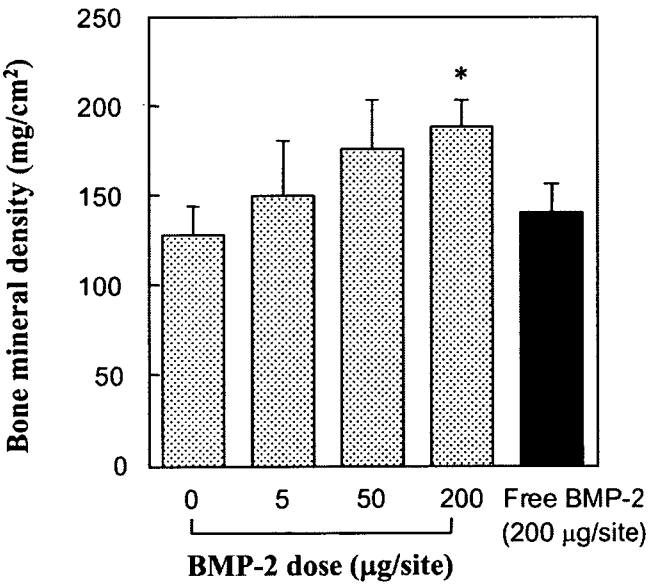

FIG. 3. Effect of bone morphogenetic protein-2 (BMP-2) dose on the bone mineral density at the bone defects of monkey skulls 12 weeks after application of free BMP-2 (black bar) and gelatin hydrogels that incorporated BMP-2 (gray bars). The water content of hydrogel was 97.8 wt%. *$P < 0.05$ compared with bone mineral density at the skull defect applied with BMP-2–free gelatin hydrogel. The experimental results were expressed as the mean of the standard deviation.