dose (35 μg), our hydrogel release system enabled BMP-2 to enhance bone regeneration even at the lower dose (17 μg). The BMP-2 dose necessary to promote bone regeneration may be reduced by a good design of release profile of BMP-2.

Many preclinical studies reveal that BMP at physiologically high doses is required to achieve bone formation in non-human primates, which is different from the case of rodents.[4] One of the possible reasons for this species-dependent dose issue could be the immature technology to modify the *in vivo* retention of BMP. Considering the present results, we can say with fair certainty that the gelatin hydrogel is a promising carrier used to deliver BMP-2. This hydrogel contributes to reduce the dose of BMP-2 suitable for successful bone regeneration and to diminish the side effects elicited by excessive administration of BMP-2 in the body.

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In Situ Regeneration of Adipose Tissue in Rat Fat Pad by Combining a Collagen Scaffold with Gelatin Microspheres Containing Basic Fibroblast Growth Factor

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ABSTRACT

This study is an investigation to evaluate *in situ* adipose tissue regeneration in fat pads. Gelatin microspheres with different water contents were prepared for the controlled release of basic fibroblast growth factor (bFGF). After a collagen sponge scaffold was incorporated by the microspheres containing 0, 0.01, 0.1, 1, and 10 μg of bFGF with or without syngeneic rat preadipocytes (1×10^5 cells/site) into a defect of rat fat pad, adipogenesis at the implanted site of scaffold was evaluated histologically. *In situ* formation of adipose tissue accompanied with angiogenesis was observed in the scaffold implanted with the microspheres containing 1.0 μg of bFGF, although the extent was less at the lower and higher bFGF doses. The *in situ* formation induced by the microspheres containing bFGF was significantly higher than that induced by free bFGF of the same dose. Adipogenesis was enhanced with time after implantation up to 4 weeks and thereafter leveled off. Such *in situ* adipogenesis was reproducibly induced by implantation of collagen scaffold incorporating gelatin microspheres containing 1 μg of bFGF, whereas addition of rat syngeneic preadipocytes did not promote the adipogenesis. The degradation of microspheres and the consequent FGF release became faster with an increase in the water content of gelatin microspheres. Less *in situ* formation of adipose tissue was observed at the lower water content of microspheres, which showed longer-term bFGF release. We conclude that combination of scaffold collagen with an appropriate controlled release of bFGF was essential to achieve the *in situ* formation of adipose tissue even without preadipocytes.

INTRODUCTION

BREAST CANCER is exemplified as one of the most common tumors for women in the western world. Mastectomy results in the loss of one or both breasts, which has often caused mental problems for patients. Therefore, reconstruction trials of female breasts have been performed and reported.[1–5] Autografting of fat pad has a long history in plastic and reconstructive surgeries for augmentation of soft tissues.[6] It was reported that autologous adipose tissues, such as the fat graft of a few millimeters in size and semiliquid, were transplanted to depressed regions or scars in the breast.[3,7] Despite the enthusiasm for such free-fat autografting, however, researchers have been disappointed by the progressive absorption over time of grafted tissue.[8–12] Microscopic examination of

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free-fat autografts removed demonstrated the necrosis of adipocytes and occupation of graft tissue by the host. In addition, the transplanted fat cells hardly proliferated. On the other hand, artificial implants, such as silicone or saline prosthesis, have been clinically used. However, all of the procedures have substantial disadvantages and morbidity.[5]

Recently, tissue engineering has been gaining favor as a newly emerging biomedical technology to repair or regenerate a body defect by combining cells of high proliferation and differentiation potential with an artificial matrix of cells scaffold and growth factor.[13] This tissue engineering technology is also applicable for regeneration of fat tissue, and some trials of adipose tissue engineering have been reported.[14–17]

Preadipocytes, which are defined as a precursor of adipocytes present between mature adipocytes in adipose tissue, were expected as one cell source potential for soft-tissue engineering.[9] The proliferative activity of preadipocytes is high, whereas matured adipocytes lose their capacity to divide.[18] The adipocyte precursor cells have been isolated from the stroma of adult adipose tissue, while their differentiation in culture has been investigated in the last two decades.[19] This has made it possible to isolate the precursor cells from the processed lipoaspirate from patients, and the cells differentiate into osteogenic, adipogenic, chondrogenic, and neurogenic lineages, which is advantageous for tissue engineering.[17,20]

There are two possible strategies based on tissue engineering to induce adipogenesis. The first strategy is to make use of cells that have the potential for proliferation and differentiation to form adipose tissue. The cells are brought into a body site where formation of adipose tissues is expected. For example, it is reported that a cell line of preadipocytes induced formation of fat tissue at the site of subcutaneous injection in nude mice.[19] Patrick *et al.* have succeeded in forming adipose tissue in a porous scaffold of poly(lactic-co-glycolic acid) pre-seeded with preadipocytes autologously isolated after subcutaneous implantation into rats.[15,21] Adipose tissue engineering by use of collagen scaffold combined with human preadipocytes has been reported.[22,23] This is the first report of adipogenesis by use of human cells, which experimentally confirmed the possibility of fat tissue engineering for human trial. If originally existing preadipocytes are used for *in situ* adipogenesis, it will be more convenient because it takes much time to isolate and proliferate the cells. Thus, the second strategy is to induce *in vivo* formation of adipose tissue based on preadipocytes originally existing in the body. If it is possible to provide a local environment suitable for the proliferation and differentiation of such cells, formation of adipose tissue will be expected without exogenous transplantation of cells necessary for adipogenesis. It has been demonstrated that adipogenesis in the mouse subcutis could be achieved only by injection of simple mixtures of basic fibroblast growth factor (bFGF) and an extract of basement membrane extract (Matrigel®, Becton Dickinson Labware, Bedford, MA).[24] Matrigel enabled bFGF to promote the angiogenic response,[25] which may be essential for generation and maintenance of adipose tissue. It has also been reported that adipogenesis in the rat subcutis could be achieved by injection of styrenated gelatin microspheres incorporating bFGF, insulin, and insulin-like growth factor.[26] We have demonstrated that controlled release from gelatin hydrogels enabled bFGF to significantly enhance the angiogenic effect *in vivo*.[27–31] After subcutaneous implantation of Matrigel combined with this bFGF release system into the mouse back, significantly higher adipogenesis at the implanted site was observed than that of the mixed Matrigel and free bFGF.[32,33] These findings experimentally confirmed that it was possible to induce adipogenesis even by preadipocytes originally present if a local environmental field suitable to tissue regeneration in the body is provided. However, because the Matrigel scaffold is a mouse-derived material, the human application is practically impossible. Thus, we have tried to create the regeneration environment by combining a sponge of type I collagen and the controlled release system of bFGF. After implantation of human preadipocytes into the back of nude mice, adipose tissue was formed at the co-implanted site of collagen sponge incorporating gelatin microspheres containing bFGF within 6 weeks postoperatively. Subcutaneous implantation of a collagen scaffold incorporating the release system of bFGF without human preadipocytes did not induce adipogenesis in the back of nude mice.[34]

Considering the regeneration of fat tissue after mastectomy, defect to be regenerated is adipose tissue. Thus, it is necessary to prepare an animal model in which a defect is surrounded with inherent adipose tissue. The objective of this study is to examine regeneration of adipose tissue in a new defect model that is close to the environment of the breast defect after mastectomy. A collagen sponge scaffold was incorporated by gelatin microspheres containing bFGF for controlled release and implanted into the defect of a rat fat pad to evaluate the *in situ* formation of adipose tissue in the sponge. Adipogenesis was evaluated by determining the percentage of adipose tissue newly formed in histological sections. We also examined the effect of the bFGF dose and the release profile of bFGF from gelatin microspheres, and the effect of preadipocytes on the formation of adipose tissue as well as the time course of adipogenesis.

MATERIALS AND METHODS

Materials

An aqueous solution of type I collagen, prepared from porcine tendon with pepsin treatment (3 mg/mL, pH 3.0)

in HCl was kindly supplied by Nitta Gelatin Inc., Osaka, Japan. A polypropylene (PP) mesh with an aperture of 200 μm (Fig. 1) was purchased from FLON Industry Inc. An aqueous solution of human recombinant bFGF (10 mg/mL) was kindly supplied by Kaken Pharmaceutical Co., Ltd., Tokyo, Japan. A gelatin sample with an isoelectric point (IEP) of 5.0 (Nitta Gelatin Inc., Osaka, Japan) was prepared through an alkaline process of type I collagen obtained from bovine bone. α-Minimal essential medium and fetal calf serum (FCS) were purchased from Nissui Pharmaceutical Co., Ltd., and ICN Biomedicals, Inc., USA, respectively. Double-distilled and deionized water (DDW) was prepared with a Milli-Q water filter system (MILLI-Q SP UF: Millipore Co., MA, USA). Other chemicals were purchased from Wako Pure Chemical Industries Ltd., Kyoto, Japan and used without further purification.

Fabrication of collagen-PP scaffold

For preparation of collagen sponges, briefly, 0.1 mL of collagen solution was poured into a polystyrene mold (96 well; COSTAR®, Corning Inc., NY, USA). The collagen solution was frozen at –80°C and freeze-dried to obtain a collagen sponge. The freeze-dried sponge was punched out to obtain collagen sponge discs (5.5 mm in diameter, 3.0 mm thickness). The sponge discs were dehydrothermally cross-linked at 140°C for 12 h under vacuum condition (0.1 torr), followed by chemical cross-linking in 0.2 wt% of glutaraldehyde aqueous solution (4°C, 12 h). The resulting sponge discs were immersed in 50 mM glycine aqueous solution at 37°C for 1 h to block the residual aldehyde groups of glutaraldehyde. Then, the collagen sponge discs were rinsed three times with DDW at 37°C and freeze-dried. The PP mesh was immersed in acetone for 1 h to remove oils and fats, and rinsed five times with DDW for 10 min. The collagen sponge disc was surrounded by the PP mesh. Because the mesh is not biodegradable and biocompatible, it is possible to recognize the boundary between the collagen sponge and the surrounding original tissue even after sponge degradation *in vivo*. The cross-linked collagen-PP scaffolds were sterilized with ethylene oxide gas at 40°C for the following *in vivo* experiments (Fig. 1).

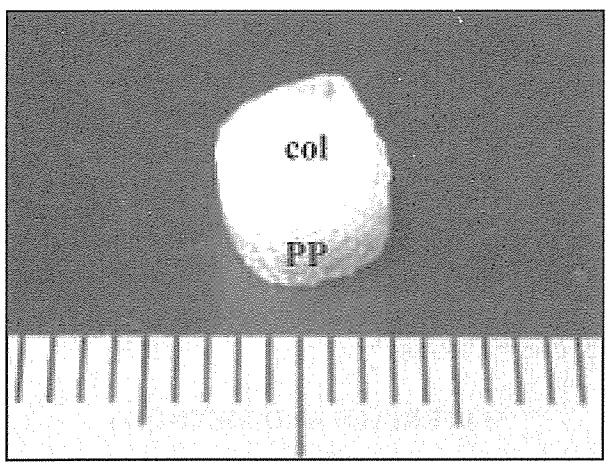

FIG. 1. The surface of a collagen sponge disc (col) was surrounded by a polypropylene mesh (PP) with an aperture of 200 μm to prepare the collagen-PP scaffold.

Preparation of gelatin microspheres containing bFGF

Gelatin microspheres were prepared by chemical cross-linking of gelatin in a water-in-oil emulsion state.[35] An aqueous solution of 10 wt% gelatin (10 mL) was prepared at 40°C, while an impeller stirring at 420 rpm was used for 10 min to yield a water-in-oil emulsion. The emulsion temperature was decreased to 4°C for the natural gelation of gelatin solution. The resulting micro-spheres were washed three times with cold acetone, collected by centrifugation (5000 rpm, 4°C, 5 min), fractionated in size by sieves with apertures of 70 and 100 μm, and air-dried at 4°C. The non-cross-linked and dried gelatin microspheres (20 mg) were placed in 20 mL of 0.1 wt% Tween 80 aqueous solution containing 25, 50, 100, and 500 μL of 25 wt% glutaraldehyde solution and stirred at 4°C for 24 h to allow the gelatin to cross-link. After washing by centrifugation with DDW, the microspheres were agitated in 20 mL of 100 mM aqueous glycine solution at room temperature to block the residual aldehyde groups of glutaraldehyde. The resulting microspheres were washed with DDW by centrifugation and freeze-dried. The water contents of gelatin microspheres prepared were 99, 98, 95, and 90 vol% when 25, 50, 100, and 500 μL of glutaraldehyde solution were added to cross-linking reaction solution. The microsphere diameter was measured by viewing at least 100 microspheres with a light microscope and was found to range from 60 to 130 μm in phosphate-buffered saline solution (PBS, pH 7.4) swelling.

The original bFGF solution was diluted with DDW to adjust the bFGF concentrations to 0.5, 5, 50, and 500 μg/mL. The aqueous solution of bFGF (20 μL) was dropped onto 1 mg of freeze-dried gelatin microspheres, followed by incubation at 25°C for 1 h for impregnation of bFGF into the microspheres. The bFGF solution was completely absorbed into the microspheres through the impregnation process because the solution volume was much less than that theoretically required for the equilibrated swelling of microspheres. As a control, 20 μL of PBS was dropped onto 1 mg of freeze-dried gelatin microspheres to prepare bFGF-free, empty gelatin microspheres. A series of studies[27–31] indicated that bFGF was released from the gelatin hydrogel microspheres of release carrier not by simple diffusion, but by the water-solubilization of bFGF accompanied by hydrogel degra-

dation. In this release system, the time period of growth factor release was not influenced by the hydrogel shape, but was controlled only by changing that of hydrogel degradation.[30,31]

Isolation and culture of rat preadipocytes

Preadipocytes were primarily isolated from the fat pad of syngeneic Wistar rats (Shimizu Laboratory Supply, Japan). The fat pad was washed with PBS to carefully remove blood cells, then minced and digested by 520 U/mL collagenase (Nitta Gelatin Inc., Osaka, Japan) in a water bath at 37°C for 60 min with shaking. The digested fat pad was suspended in Medium 199 containing 10 vol% FCS, followed by centrifugation (200 × g, 5 min, 4°C) to collect the supernatant. After washing twice with the medium, the cells obtained were cultured in a cell-culture flask (75 cm^2, Corning 430720, 1×10^3 cells/cm^2) in the medium containing 0.1 μg/mL of bFGF at 37°C and 5% CO_2-95% air atmosphere pressure. The cells were expanded by subculturing two times and subjected to *in vivo* experiments. The cell morphology was fibroblast-like. When cultured in the presence of 50 nM of insulin, 100 nM of dexamethasone, 10 μg/mL of transferrin, and 200 pM of triiodothyronine for 14 days, the cells accumulated fat droplets inside. This suggested that the isolated cells had an inherent nature to differentiate into matured adipocytes.

In vivo *experiments*

The *in vivo* experiments are listed in Table 1. Groups I and V were performed to evaluate the effect of the bFGF concentration, Group I (1.0) was used to evaluate the effect of the implantation time period, Groups I (1.0), II, III, and V were used to evaluate the effect of preadipocytes presence, and Groups IV and VI were to evaluate the bFGF release profile on the *in situ* formation of adipose tissue in the collagen-PP scaffold.

Female Wistar rats (110–140 g; Shimizu Laboratory Supply, Japan) were used. Anesthesia was achieved with a mixture of ketamine hydrochloride (40 mg/kg intraperitoneally) and xylazine (8 mg/kg intraperitoneally) with additional doses given intraperitoneally as necessary during the experiments. All the surgical procedures were done under clean conditions using steam-sterilized instruments with the surgeon masked and using laboratory coat and sterile gloves. Under anesthesia, the area around the incision was shaved and a 0.5% iodine solution was applied. In each rat, an approximately 4-cm incision was made over the skin obliquely to the median line at 10 mm cephalad to the fat pad of the rat inguinal region. The skin above the fat pad was gently reflected by the forceps to implant the collagen-PP scaffold. Then an approximately 6 mm incision was made in the fat pad, and the incision was opened in a circle. The collagen-PP scaffold incorporating gelatin microspheres containing different amounts of bFGF with or without rat preadipocytes was carefully implanted into the circle of the fat pad of the rat as the side of the collagen-PP scaffold was surrounded by the original adipose tissue. Also, the upper and lower surfaces of the collagen-PP scaffold faced the skin and loose areolar tissue and abdominal wall musculature. The PP mesh of collagen-PP scaffold was fixed to the original adipose tissue of rats with nonabsorbable 5–0 sutures (Johnson & Johnson Co., New Brunswick, NJ, USA). After implantation of the collagen-PP scaffold, the skin was closed with 5–0 sutures (Johnson & Johnson Co.). All the animal experiments were performed according to the Guidelines of Animal Experiment of Kyoto University (1985). Each experimental group was composed of four rats. Two, 4, and 6 weeks after implantation, the rats were sacrificed by an overdose injection of anesthetic, and the scaffold, including the native adipose tissue, was carefully taken off. The specimen was fixed with 10% neutralized formalin solu-

TABLE 1. EXPERIMENTAL GROUPS

Group code	*bFGF (μg/site)*	*Water content of gelatin microspheres (vol%)*	*Preadipocytes (×10^5 cells/site)*
I (0.01)	0.01	98	0
I (0.1)	0.1	98	0
I (1.0)	1.0	98	0
I (10)	10	98	0
II	1.0	98	1
III	0	98	1
IV (90)	1.0	90	0
IV (95)	1.0	95	0
IV (98)	1.0	98	0
IV (99)	1.0	99	0
V (PBS)	0	98	0
VI (free bFGF)	1.0	—	0

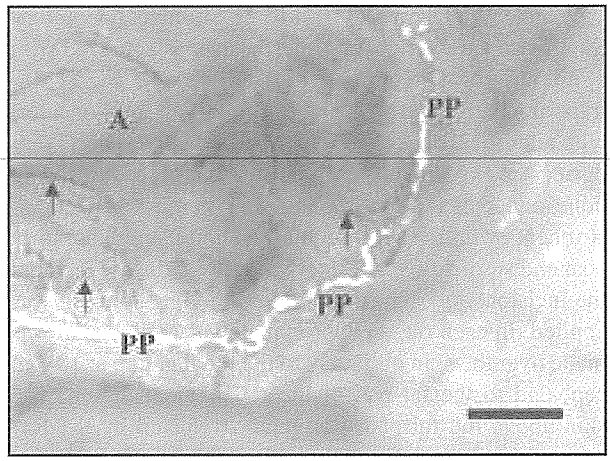

FIG. 2. Tissue appearance of collagen-PP scaffold incorporating gelatin microspheres containing 1 μg of bFGF 4 weeks after implantation into the defect of rat fat pads. PP: polypropylene mesh. A: original adipose tissue. Arrows indicate blood vessels induced. Scale bar = 1 mm.

tion, embedded in paraffin, and sectioned (5 μm in thickness) at the portion of the implanted site as centrally as possible, followed by staining with hematoxylin and eosin (HE). Microphotographs of cross-sections from four different scaffolds were taken at a similar magnification to histologically evaluate the formation of adipose tissue. The percent of newly formed adipose tissue adipocytes was analyzed by measuring the area of matured adipocytes in the PP mesh using a computer program of Image-Pro Plus 3.01 (Media-Cybernetics, Silver Spring, MD).

Statistical analysis

All the results were statistically analyzed by the unpaired Student's t test and $p < 0.05$ was considered to be statistically significant. Data were expressed as the mean $\pm$ the standard deviation of the mean (SD).

RESULTS

Tissue appearance of collagen-PP scaffold implanted

Figure 2 shows the tissue appearance of collagen-PP scaffold immediately and 4 weeks after implantation into the defect of fat pad. Angiogenesis was observed on the surface of collagen scaffold. The shape of scaffold was kept constant from the beginning to 4 weeks.

Influence of bFGF dose on the in situ formation of adipose tissue

Figure 3 shows the histological sections of rat fat pad 4 weeks after implantation of collagen-PP scaffold incorporating gelatin microspheres containing different amounts of bFGF. Apparently, co-implantation of microspheres containing bFGF formed adipose tissue in the

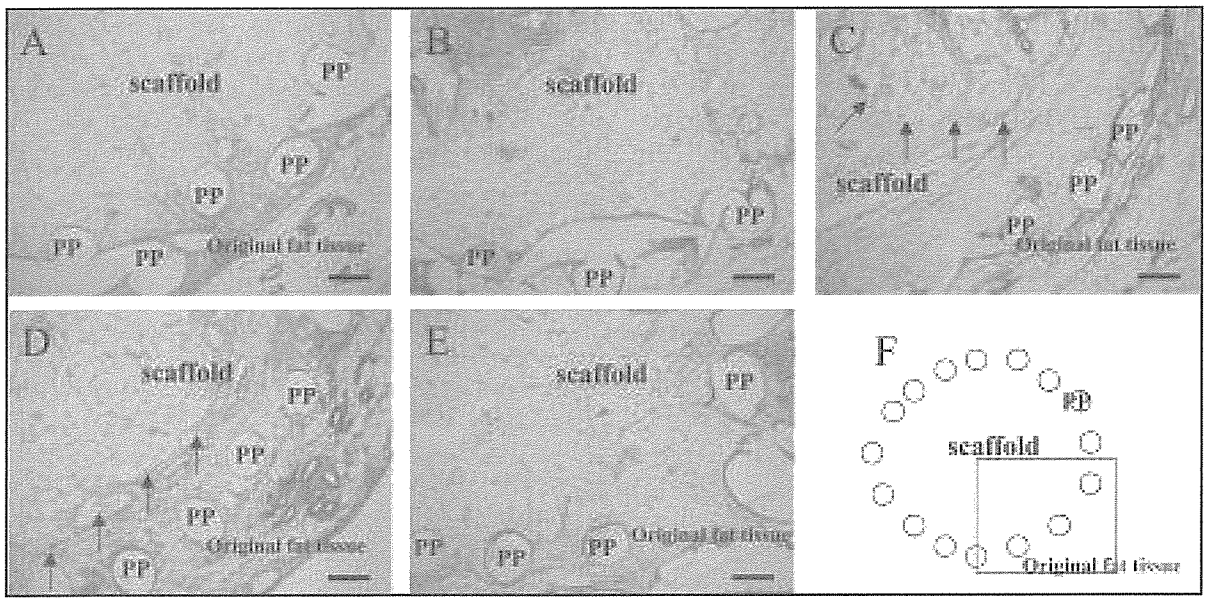

FIG. 3. Histological sections of the implanted site of collagen-PP scaffold incorporating gelatin microspheres containing 0 (A), 0.01 (B), 0.1 (C), 1.0 (D), and 10 μg of bFGF (E) 6 weeks postoperatively. PP: polypropylene mesh. Arrows indicate adipose tissue newly reconstructed. (F) Schematic illustration of the area of histological section photographed. Scale bar = 200 μm. (Magnification: $\times$100, HE staining)

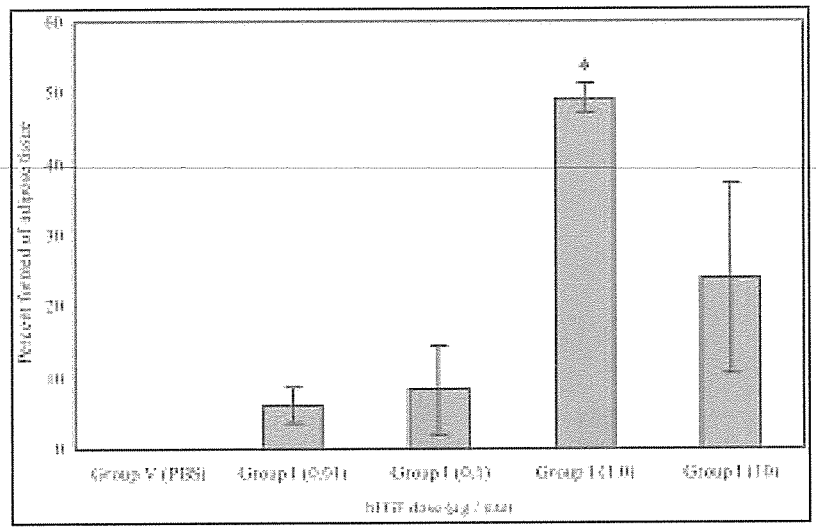

FIG. 4. Effect of the bFGF dose on the percentage of adipose tissue newly formed 4 weeks after implantation of collagen-PP scaffolds incorporating gelatin microspheres containing different amounts of bFGF. $*p < 0.05$; significant against the percentage of adipose tissue formed at the scaffold incorporating microspheres containing bFGF at other amounts.

scaffold surrounded by rat adipose tissue. When the bFGF dose was 1.0 μg, gelatin microspheres containing bFGF significantly induced formation of adipose tissue. Less formation of adipose tissue was observed at bFGF doses of 0.01, 0.1, and 10 μg. Especially, 10 μg of bFGF induced an inflammatory reaction in the collagen scaffold implanted site. No formation of adipose tissue was observed at the collagen scaffold implanted site together with PBS or bFGF-free, empty microspheres. Figure 4 shows the bFGF dose dependence of *in situ* adipogenesis. Adipogenesis was assessed by determining the area percentage of adipose tissue to the total area within the PP mesh on the histological sections 4 weeks after co-implantation of collagen scaffold and gelatin micro-spheres containing bFGF. When gelatin microspheres containing 1.0 μg of bFGF were used, the percent formed of adipose tissue was significantly higher than that of other gelatin microspheres with or without bFGF incorporation.

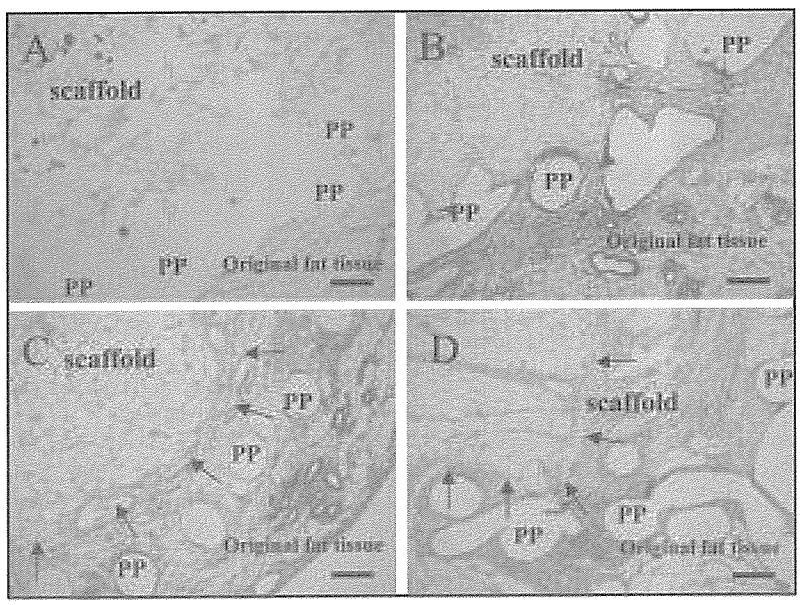

FIG. 5. Histological sections of the implanted site of collagen-PP scaffold incorporating gelatin microspheres containing 1 μg of bFGF 0 (**A**), 2 (**B**), 4 (**C**), and 6 weeks postoperatively (**D**). PP: polypropylene mesh. Arrows indicate adipose tissue newly reconstructed. Scale bar = 200 μm. (Magnification: $\times$100, HE staining)

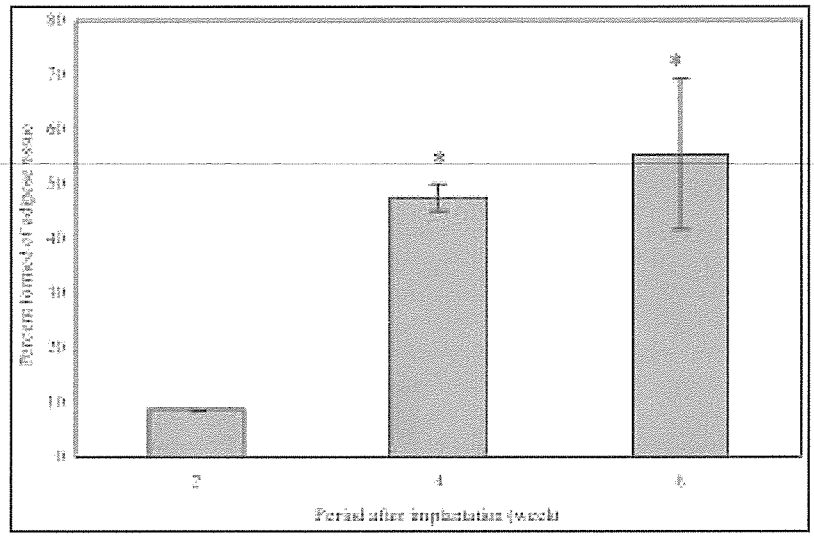

FIG. 6. Time course of percentage of adipose tissue newly formed after implantation of collagen-PP scaffold incorporating gelatin microspheres containing 1.0 μg of bFGF. *$p < 0.05$; significant against the percentage of adipose tissue newly formed 2 weeks after implantation.

Time course of adipose tissue formation after implantation of collagen scaffold incorporating gelatin microspheres containing bFGF

Figure 5 shows the histological sections at the implanted site of collagen-PP scaffold incorporating gelatin microspheres containing 1 μg of bFGF at different time intervals after implantation. Mature adipocytes accumulating lipid inside were observed in the scaffold 4 weeks after implantation of collagen-PP scaffold combined with gelatin microspheres containing bFGF. However, the histological site of newly formed adipose tissue was different from that of the original adipose tissue. Figure 6 shows the time profile of area percentage of adipose tissue newly formed to the total area within the PP mesh on histologic sections after implantation of collagen-PP

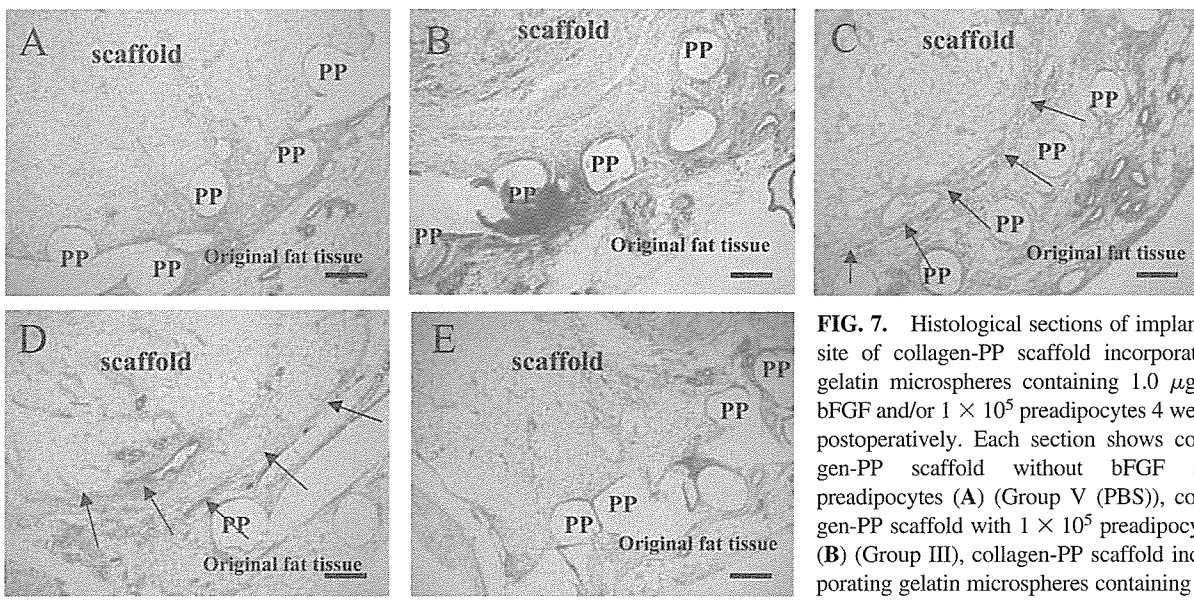

FIG. 7. Histological sections of implanted site of collagen-PP scaffold incorporating gelatin microspheres containing 1.0 μg of bFGF and/or 1×10^5 preadipocytes 4 weeks postoperatively. Each section shows collagen-PP scaffold without bFGF and preadipocytes (**A**) (Group V (PBS)), collagen-PP scaffold with 1×10^5 preadipocytes (**B**) (Group III), collagen-PP scaffold incorporating gelatin microspheres containing 1.0 μg of bFGF (**C**) (Group I (1.0)), collagen-PP scaffold incorporating gelatin microspheres containing 1.0 μg of bFGF and 1×10^5 preadipocytes (**D**) (Group II), and collagen-PP scaffold mixed with 1.0 μg of free bFGF (**E**) (Group VI). Arrows indicate adipose tissue newly reconstructed. Scale bar = 200 μm. (Magnification: ×100, HE staining)

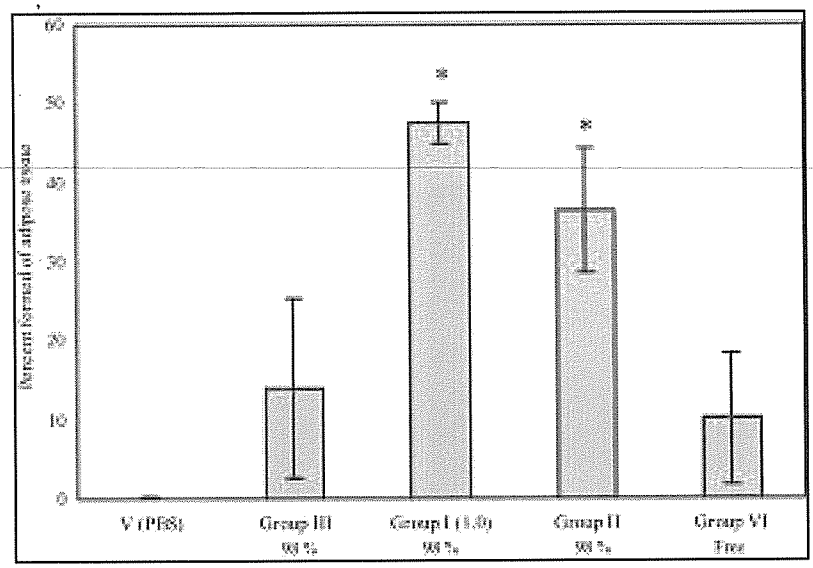

FIG. 8. Effect of the rat syngeneic preadipocytes on the percentage of adipose tissue newly formed after implantation of collagen-PP scaffold with or without incorporation of gelatin microspheres containing 1.0 μg of bFGF. $*p < 0.05$; significant against the percentage of adipose tissue formed at the scaffold incorporating preadipocytes without gelatin microspheres containing 1.0 μg of bFGF (Group III).

scaffold incorporating bFGF contained in gelatin microspheres with implantation time. The adipose tissue was formed with time to attain a certain significant level 4 weeks after implantation, and the formed level was retained until 6 weeks. There were few adipocytes in the area close to the PP mesh surrounding the collagen scaffold while inflammation cells were observed between adipose tissue newly formed and the PP mesh (Fig. 5D).

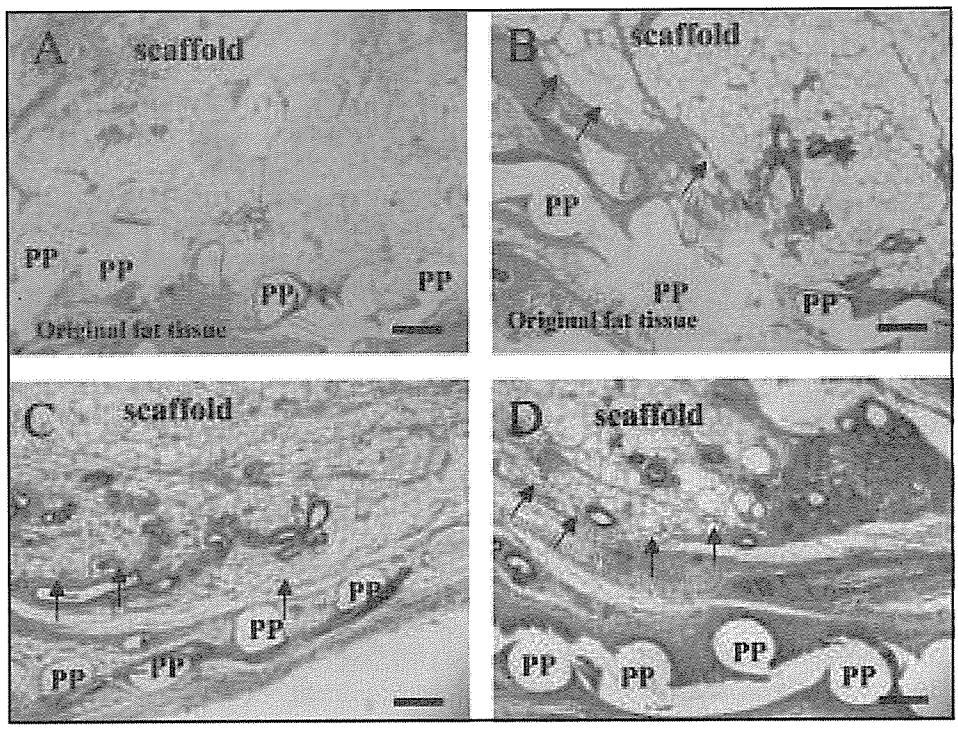

FIG. 9. Histological sections of the implanted site of collagen-PP scaffold incorporating gelatin microspheres containing 1.0 μg of bFGF at water contents of 90 (**A**), 95 (**B**), 98 (**C**), and 99 vol% (**D**) 4 weeks postoperative. Arrows indicate adipose tissue newly reconstructed. Scale bar = 200 μm. (Magnification: $\times$100, HE staining)

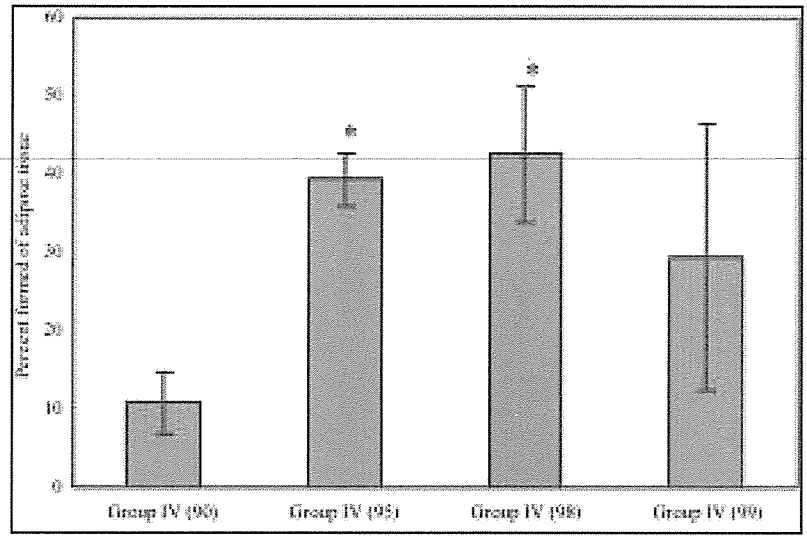

FIG. 10. Effect of the water content of gelatin microspheres containing 1 μg of bFGF on the percentage of adipose tissue newly formed 4 weeks after implantation of collagen-PP scaffolds. $*p < 0.05$; significant against the percentage of adipose tissue newly formed at the scaffold containing gelatin microspheres incorporating 1.0 μg of bFGF at a water content of 90 vol% (Group IV (90)).

Influence of preadipocytes addition on the in situ *formation of adipose tissue*

Figure 7 shows the histological sections of scaffold implanted 4 weeks after implantation. Matured adipocytes accumulating lipid were observed in the scaffold 4 weeks after implantation of collagen-PP scaffold incorporating gelatin microspheres containing bFGF and syngeneic rat preadipocytes. Irrespective of preadipocytes presence, a similar level of adipogenesis was observed when gelatin microspheres containing bFGF were used. However, the collagen-PP scaffold incorporating rat preadipocytes alone was much less effective, whereas no formation of adipose tissue was observed in the scaffold alone. Figure 8 shows the percent area of adipose tissue newly formed in the rat fat pad 4 weeks after implantation. The area of adipose tissue newly formed from the implantation of collagen-PP scaffold incorporating gelatin microspheres incorporating bFGF was larger compared with that of collagen-PP scaffold incorporating preadipocytes. Adipose tissue was not detected in the control group of collagen-PP scaffold without the controlled release of bFGF and preadipocytes.

Influence of bFGF release profile on the in situ *formation of adipose tissue*

Figure 9 shows the histological sections of rat fat pad 4 weeks after implantation of collagen-PP scaffold incorporating gelatin microspheres containing 1.0 μg of bFGF with different water contents. Many cells were observed in the HE histological section after implantation of collagen-PP scaffold incorporating gelatin microspheres containing bFGF with a water content of 90 vol%, but mature adipocytes accumulating lipids were not observed. On the other hand, mature adipocytes accumulating lipids were observed in the scaffold incorporating gelatin microspheres containing 1 μg of bFGF with water contents of 95 and 98 vol%. Figure 10 shows dependence of the bFGF release profile on the *in situ* adipogenesis. Adipogenesis was assessed by determining the area percentage of adipose tissue to the total area within the PP mesh on the histological sections 4 weeks after co-implantation of collagen scaffold and gelatin microspheres containing 1 μg of bFGF with different water contents (90, 95, 98, and 99 vol%). When gelatin microspheres with a water content of 95 or 98 vol% were used as the release carrier of bFGF, the amount of adipose tissue newly formed was significantly higher than that of gelatin microspheres containing the same dose of bFGF with a water content of 90 vol%.

DISCUSSION

We have designed an animal model to evaluate the regeneration of adipose tissue in the defect of fat pad and demonstrated five major findings for *in situ* adipose tissue regeneration. These include the necessity of controlled release of bFGF for adipose tissue regeneration, an optimal concentration of bFGF, an optimal time period of bFGF release, unnecessity of syngeneic preadipocytes, and feasible scaffold of type I collagen.

The defect model of fat pad was prepared to properly simulate the tissue environment of breast after partial mastectomy. That is, the defect is surrounded with natural adipose tissue. Some tissue-engineering trials have

been reported on adipose tissue engineering in mouse or rat subcutis.[21,24,26,32–34,36–38] However, if adipose tissue engineering is applied to surgical reconstruction of a mammal defect, the defect surgically prepared is mostly surrounded by natural adipose tissue. In this study, adipogenesis in the adipose tissue is investigated to compare with that in the ectopic site.

Controlled release of bFGF was a key technology for adipose tissue engineering. This is because the collagen sponge combined with free bFGF resulted in poor adipogenesis compared with the scaffold combined with gelatin microspheres containing bFGF (Figs. 7 and 8). The present study clearly indicated that an environment suitable to induce adipogenesis could be created by combination of a collagen scaffold with the bFGF release system. There will be several reasons to be considered for the bFGF effect on adipogenesis. It is possible that the controlled release of bFGF induced angiogenesis, resulting in efficient proliferation and maturation of adipose precursor cells migrated into the advance angiogenesis-induced scaffold because of good oxygen and nutrients supply to the cells. Indeed, the previous studies about adipogenesis with Matrigel indicated that angiogenesis induced by bFGF release could generate a good environment for tissue regeneration.[32,33] bFGF itself acts on the preadipocytes to accelerate their proliferation,[39] or other growth factors that are provided by the bFGF-induced vasculature enables the cells to proliferate.[40] It is conceivable that the collagen scaffold plus the preadipocytes without the bFGF release system did not induce angiogenesis enough to maintain the survival of cells transplanted, resulting in no formation of fat tissue. The collagen scaffold does not function as the carrier for bFGF release,[41] which will cause poor angiogenesis in the collagen scaffold combined with preadipocytes and free bFGF. As a result, it is possible that the scaffold-cells-free bFGF combination results in poor adipogenesis compared with the scaffold-cells-released bFGF. The direct effect of bFGF on preadipocytes is still controversial. It has been reported that bFGF has no adipogenic effect on human precursor cells[42] and shows an inhibitory effect on the adipogenesis of rat preadipocytes.[43] On the other hand, sheep preadipocytes are reported to differentiate in a culture medium containing bFGF.[39] In addition, it has been demonstrated that bFGF exhibits an accelerating effect on the adipogenesis of human precursor cells[44] or an enhancing adipogenic effect on rat precursor cells.[45] In the research studies, bFGF is applied in the solution form, which is different from the application form of bFGF in this study. Although the adipogenesis effect of bFGF is not clear at the moment, it is conceivable that the controlled release of bFGF increases the number of preadipocytes and the rate of adipocyte differentiation, resulting in totally enhanced adipogenesis.

The adipogenic effect of bFGF should be considered. The bFGF dose dependence indicated that there was an optimal concentration range of bFGF for *in situ* adipogenesis (Figs. 3 and 4). It was reported that the amount of newly formed adipose tissue increased with an increase in the concentration of bFGF, which was immobilized in Matrigel.[24] On the other hand, there was an optimal dose of bFGF controlled release from gelatin microspheres for adipose tissue formation, and there was an optimal dose of bFGF immobilized in gelatin microspheres for capillary density at 2 weeks after implantation.[26] The optimal dose of bFGF from gelatin microspheres was the same with our previous report.[34] Probably, a low dose of bFGF is not enough to exert its angiogenetic or adipogenetic effect even though the bioactive bFGF is released from the gelatin microspheres. On the other hand, when the bFGF dose is too high, in addition to the two effects of bFGF, fibrous tissues into the collagen scaffold would become pronounced. A high dose of bFGF caused an inflammatory response at the implanted collagen scaffold. It may be that inflammation at the scaffold is too severe to induce tissue regeneration.

Cellularity in the collagen scaffold incorporating the controlled release of bFGF and/or preadipocytes changed with time after implantation. There were many cells in the scaffold 2 weeks after implantation, but the majority were fibroblast-like cells and there were few matured adipocytes. The period of bFGF release from the gelatin microspheres with a water content of 98 vol% was about 2 weeks, whereas the increase in the percent of newly formed adipose tissue was observed between 2 and 4 weeks (Figs. 5 and 6). This time lag may be explained based on the biological action of bFGF. bFGF mainly acts on preadipocytes to accelerate their proliferation.[46] However, it has little influence on their differentiation. As a result, it is possible that the bFGF release would enable preadipocytes to infiltrate into the scaffold and to increase their number. Thereafter, it needs a few weeks to infiltrate into the scaffold and subsequently differentiate the increased preadipocytes into matured fat cells *in vivo*. The percentage of newly formed adipose tissue was almost similar between 4 and 6 weeks of implantation. The maximum percent of newly formed adipose tissue was about 50%. It was noted that the center area of the scaffold and the area close to the polypropylene mesh performed poorly. It was suggested that preadipocytes were infiltrated from the original adipose tissue by the controlled release of bFGF, but there were few adipocytes close to the PP mesh in some cases, and inflammatory cells were observed. We think that because some collagen-PP scaffolds had a small gap between the collagen sponge and PP mesh, preadipocytes could not attach to the collagen scaffold, and infiltrated focal inflammatory cells remained there. Although the histological site of newly formed adipose tissue seemed to be different from that of the original adipose tissue in terms of the size of

matured adipocyte, it might be considered that it was still not enough blood supply in this size of scaffold. bFGF ionically complexed with the gelatin (IEP = 5) constituting the microspheres and is released during *in vivo* degradation of the gelatin microspheres.[31] The retention period depended on the water content of the gelatin microspheres, which directly affects their degradability.[30] The ability of bFGF-incorporated microspheres to induce *in situ* adipose tissue regeneration depended on their water content (Figs. 9 and 10). It was possible that the slow degrading gelatin microsphere contributed to prolonged retention of bFGF. When the rate of microsphere degradation was too slow compared with that of adipose tissue regeneration at the skull defect in the fat pad, it was thought that induced rat preadipocytes derived from around original fat tissue were not fully differentiated to mature adipocytes. On the other hand, free bFGF and VI (99%) did not significantly enhance the percent area of newly formed adipose tissue at the rat skull model after 4 weeks implantation. This indicated that controlled release of bFGF was very important for *in situ* adipose tissue regeneration. It was indicated that a balance in the time profile between the bFGF release and the *in situ* adipose tissue regeneration was essential for the adipose tissue regeneration induced by bFGF incorporated into gelatin microspheres in the collagen-PP scaffold.

The present study clearly indicated that *in situ* adipose regeneration was not necessary to add preadipocytes (Figs. 7 and 8). The Matrigel plus bFGF without preadipocytes induced adipogenesis in the subcutis,[24,32,33] whereas no adipogenesis by the combination of bFGF and a collagen I sponge was observed in the mouse subcutis.[34] In this study, adipogenesis by the combination of bFGF and a collagen I sponge was observed in the rat fat pad. The difference of adipogenesis can be explained in terms of different environment. It is conceivable that Matrigel gives preadipocytes infiltrated a better environment for their differentiation. This is because it contained some growth factors and extracellular matrix (ECM) components suitable for *de novo* adipogenesis. Preadipocytes were unnecessary in our study because many preadipocytes are present in the original adipose tissue. It is possible that preadipocytes were recruited into the scaffold by the controlled release of bFGF. It has been recognized in recent cell biology that preadipocytes are multipotent for differentiation.[47] Preadipocytes are committed or determined to differentiate into fat cells, and the cell differentiation can be promoted depending on the microenvironment.[19,37] It is well recognized that the number of adipocytes and their precursor cells are only less than half that of the total cells present in the adipose tissue and the remaining cells are vascular-related cells, such as various blood cells, endothelial cells, and pericytes.[17] After 4 weeks implantation of the collagen-PP scaffold incorporating gelatin microspheres containing 1 μg bFGF and syngeneic rat preadipocytes into rat femoral muscle, not muscle tissue but adipose tissue was observed in the histological section (data not shown). This indicated that rat syngeneic preadipocytes differentiated easily into adipocytes. It was considered, however, that addition of preadipocytes was not affected well because there were many preadipocytes around the collagen-PP scaffold.

The scaffold is one of the important factors for adipose tissue engineering. Implantable materials used for adipose tissue engineering have predominantly been porous biodegradable polymer foams.[14,48] For instance, poly(L-lactic co-glycolic) acid scaffolds preseeded with preadipocytes have demonstrated adipose tissue formation.[21] However, synthetic polymer foams will probably not be the optimal choice for many applications as they are too rigid and would be uncomfortable for patients. A more appropriate choice may be a biodegradable collagen sponge reinforced by poly(glycolic acid) fiber.[49] Nonbiodegradable scaffolds have also been investigated. For instance, Kral and Crandall recently demonstrated that the attachment and proliferation of preadipocytes on fluorotex monofilament-expanded polytetrafluoroethylene scaffolds coated with various extracellular matrices.[50] Matrigel is an essential material to induce *de novo* adipogenesis.[24,32,33] However, because the Matrigel scaffold is a mouse tumor-derived material, it is practically impossible to apply it to humans. Moreover, in the case of gel-type scaffold, the degradation of gel is necessary to allow cells to infiltrate into the scaffold because the pore size of gel is not large enough for cell infiltration.[51] On the other hand, for the sponge-type scaffold, it is likely that cells readily infiltrates into the scaffold *in vivo* without degradation of scaffold.[23] The optimal scaffold for breast tissue engineering remains unclear. Modification with adhesive bioactive substances will give the scaffold new biological functions. However, this strategy is complicated by the fact that the constitution and distribution of the ECMs further varies adipocyte differentiation.[52] Although an optimal matrix for preadipocyte is not yet known, type I collagen is a material widely used in tissue regeneration and a porous collagen matrix supports cellular ingrowth and new matrix synthesis. Indeed, preadipocytes readily adhere to laminin-1 as compared to other ECMs, but preadipocytes also adhere to type I collagen.[53] In this reconstructed model of adipose tissue, we used a type I collagen scaffold. When large enough numbers of original preadipocytes exist, a scaffold of type I collagen incorporating controlled release of bFGF has induced infiltration of cells from original fat tissue, their growth, and adipogenesis.

In situ adipose tissue regeneration was achieved by implantation of a collagen scaffold incorporating the controlled release system of bFGF without preadipocytes into the defect of adipose tissue in the rat fat pad.

Presently, adipose tissue regeneration is being investigated at the defect radioirradiated.

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Periurethral Injection of Sustained Release Basic Fibroblast Growth Factor Improves Sphincteric Contractility of the Rat Urethra Denervated by Botulinum-A Toxin

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Purpose: We evaluated the effects of sustained release basic fibroblast growth factor injection in rat urethra denervated by botulinum-A toxin (Wako Life Science, Osaka, Japan).

Materials and Methods: A total of 30 female Sprague-Dawley rats underwent periurethral injection of 10 U botulinum-A toxin to induce chemical denervation of the urethral sphincter. Leak point pressure in the waking state was determined and a significant decrease in leak point pressure vs that in control rats was confirmed (mean ± SD 58.7 ± 6.2 vs 120.7 ± 13.0 cm H_2O, $p < 0.0001$). Two weeks later 0, 50 and 200 μg basic fibroblast growth factor incorporating 200 μl gelatin hydrogels in 10 rats each were injected into the urethral sphincter, enabling sustained release of basic fibroblast growth factor for 2 weeks. Four weeks later injection leak point pressure measurement and histological evaluation of the urethra were performed.

Results: Leak point pressure in rats with 50 and 200 μg basic fibroblast growth factor injection was significantly higher than in rats with the 0 μg injection (82.7 ± 9.0 vs 95.1 ± 6.2 and 119.3 ± 8.1 cm H_2O, $p = 0.0021$ and <0.0001, respectively). Maximum cross-sectional area of the urethral smooth muscle layer in the 50 and 200 μg groups significantly increased compared with that in the urethra in the 0 μ group, which was considered 100% (114.1% ± 15.8% and 132.5% ± 13.4%, $p = 0.029$ and <0.0001, respectively). Similarly the cross-sectional area of the striated sphincter in the 50 and 200 μg groups was greater than the 100% in the 0 μg group (112.3% ± 15.6% and 124.3% ± 14.1%, $p = 0.069$ and 0.0007, respectively). Vascular density in the urethral peri-atrophic zone in the 50 and 200 μg groups was significantly higher than in the 0 μg group ($p = 0.027$ and <0.0001, respectively).

Conclusions: Sustained release basic fibroblast growth factor injection in the chemically denervated urethral sphincter facilitates regeneration of the urethral muscles and improves sphincteric contractility. Endoscopic periurethral injection of basic fibroblast growth factor incorporating gelatin hydrogels may be an attractive therapy for stress urinary incontinence.

Key Words: urethra; urinary incontinence, stress; fibroblast growth factor 2; botulinum toxin type A; rats, Sprague-Dawley

Stress urinary incontinence is the complaint of involuntary urine leakage during effort, exertion, sneezing or coughing. It is the most common type of urinary incontinence in women.[1] Although many conservative treatments for SUI are available, surgical therapy seems to be the best option for achieving long-term continence. However, surgery for SUI, such as the urethral sling procedure, sometimes cause postoperative voiding difficulty and a limited cure rate in SUI cases due to intrinsic sphincteric deficiency.[2] If a periurethral injection method could achieve good durability and safety, this could potentially be a more optimal minimally invasive treatment for SUI.

Recent reports show that regenerating human and animal tissues such as bone and myocardium can be enhanced by sustained release of bFGF.[3–5] bFGF promotes angiogenesis and proliferation of mesenchymal cells, including those of the skeleton and smooth muscle.[6] For successful application of growth factors some effective delivery system is required.[5,7] Recent studies show that collagen and its denatured derivative, gelatin, are highly versatile carriers for various growth factors, including bFGF, and they achieve successful application of growth factors.[8,9]

We investigated the potential usefulness of sustained release bFGF injection in the urethral sphincter muscles using gelatin hydrogel for SUI treatment. We evaluated its physiological and histological effects in female rat urethra denervated by botulinum-A toxin injection.

MATERIALS AND METHODS

Animals and Study Design

A total of 30 female Sprague-Dawley rats weighing 300 to 330 gm underwent periurethral injection of 10 U botulinum-A toxin to induce chemical denervation of the urethral sphincter. Two weeks later the rats were divided into 3 groups of 10 each, and 0, 50 and 200 μg bFGF incorporating 200 μl gelatin hydrogel were injected periurethrally. LPP

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Study received Tokyo University Animal Experiment Committee approval.

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measurement and histological evaluation of the urethra were performed 4 weeks after bFGF injection. The experiment was approved by Tokyo University Animal Experiment Committee and animal treatment was done in accordance with National Institutes of Health animal care guidelines.

Incompetent Urethra Induced by Botulinum-A Toxin Injection

Each rat was anesthetized with 75 mg/kg ketamine and 15 mg/kg xylazine intramuscularly, and placed supine with the lower legs abducted. An insulin syringe with a 28 gauge needle was used to inject botulinum-A toxin. Physiological saline containing botulinum-A toxin (5 U/100 μl) was injected periurethrally at the mid urethra, which was located at the level of the pubis symphysis. A total of 10 U/200 μl botulinum-A toxin was injected into each urethra.

Preparation and Injection of bFGF Incorporating Gelatin Hydrogels

Human recombinant bFGF was used in the current study. A gelatin sample was isolated from bovine bone. bFGF microspheres were prepared as described previously.[5,10] Briefly, gelatin microspheres were prepared through glutaraldehyde cross-linking of gelatin. The microspheres were washed with acetone at 4C, recovered by centrifugation and freeze dried. bFGF was immersed in the microspheres for 1 hour before use. Gelatin hydrogel has proved to be a highly versatile carrier for bFGF that enables sustained release of bFGF for 2 weeks in vivo.[5,8,9]

Two weeks after botulinum-A toxin injection 0, 50 and 200 μg bFGF incorporating 200 μl gelatin hydrogel were injected with the original syringe with an 18 gauge needle into the mid urethra of 10 rats in each of the 3 groups in the same manner used for botulinum-A toxin injection.

LPP Testing

Four weeks after bFGF injection LPP was determined. The bladder was exposed through a lower midline abdominal incision using anesthesia. A polyethylene catheter with a 0.95 mm external diameter (Unique Medical, Tokyo, Japan) was inserted into the bladder dome. The catheter was tunneled subcutaneously, let out on the back of the neck and surgically secured. LPP measurement was performed 2 days later with the rat in the waking state. A rat in the prone position was put in a transparent plastic cage, which enabled us to observe leakage from the urethral meatus. LPP in the waking state was determined using a modified method of the intravesical pressure clamp model reported by Lee et al.[11] Briefly, intravesical pressure was clamped by connecting a saline reservoir to the bladder catheter using pressure tubing. The reservoir was mounted on a metered vertical pole for controlled height adjustment. Intravesical pressure was increased from 0 cm H_2O upward until indigo stained saline was seen leaking from the urethral meatus. The pressure at which leakage occurred was defined as LPP. An average of 3 consecutive LPPs was used as a data point in each rat. To avoid measuring intravesical pressure at voiding infusing saline volume was maintained at less than 50% of bladder capacity in each rat, which was measured on cystometry 30 minutes before LPP measurement. For cystometry the intravesical catheter was connected using a 3-way stopcock to a pressure transducer and microinjection pump. Room temperature saline was infused at a rate of 0.1 ml per minute to elicit a bladder contraction and voiding. The average of 3 consecutive cystometric results was used as a data point in each animal.

Histological Evaluation

After LPP measurements the rats were sacrificed. The bladder and urethra were removed, fixed in 10% formalin solution and embedded in paraffin. Tissue sections (6 μm) were stained with hematoxylin and eosin or Masson's trichrome stain. Quantitative morphometric analysis of the urethra was performed using a light microscope at 40× magnification. The maximum cross-sectional areas of the smooth muscle layer and striated sphincter were determined using a computerized digital morphometric analysis system (Adobe® Photoshop® 4.0J and NIH Image 1.61). To evaluate the effect of neovascularization of bFGF the number of vessels per unit area (200 × 200 μm^2) in the urethral peri-atrophic zone was counted in 5 randomly chosen fields per slide per animal by 2 blinded pathologists.[5] The average number of the vessels was used to assess vascular density for statistical analysis.

Statistical Analysis

All LPP and histological data are presented as the mean ± SD. Student's unpaired 2-tailed t test was used for comparisons of parameters between the groups of rats with $p < 0.05$ considered statistically significant.

RESULTS

In a preliminary study we evaluated the physiological and histological effects of botulinum-A toxin injection in the mid urethra using the methodology described. We identified 10 U botulinum-A toxin as an optimum dose for chemical denervation of the rat urethra based on data obtained from periurethral injections of 200 μl saline containing 2.5, 5, 10 and 20 U botulinum-A toxin in female Sprague-Dawley rats (data not shown). Subsequently 5 female rats underwent periurethral injection of saline containing botulinum-A toxin (10 U/200 μl). As a control, 5 rats underwent injections with 200 μl saline alone. At 2 weeks an intravesical catheter was inserted in the bladder and LPP was measured. A significant decrease in LPP in rats in the botulinum-A toxin injection group compared with that in control rats was confirmed (see table). Histological evaluation revealed remarkable shrinkage of the smooth muscle layer and striated sphincter in rats in the botulinum-A toxin group (see table and fig. 1).

Figure 2 shows representative LPP results in rats with and without bFGF injection. Six weeks after botulinum-A

Effects 2 weeks after periurethral injection of 10 U botulinum-A toxin in rats

	Mean Control ± SD	Mean Botulinum-A ± SD	p Value
LPP (cm H_2O)	120.7 ± 13.0	58.7 ± 6.2	<0.0001
% Cross-sectional area:			
Smooth muscle layer	100	57.1 ± 10.5	0.0001
Striated sphincter	100	67.0 ± 6.8	0.0016

Five rats per group.

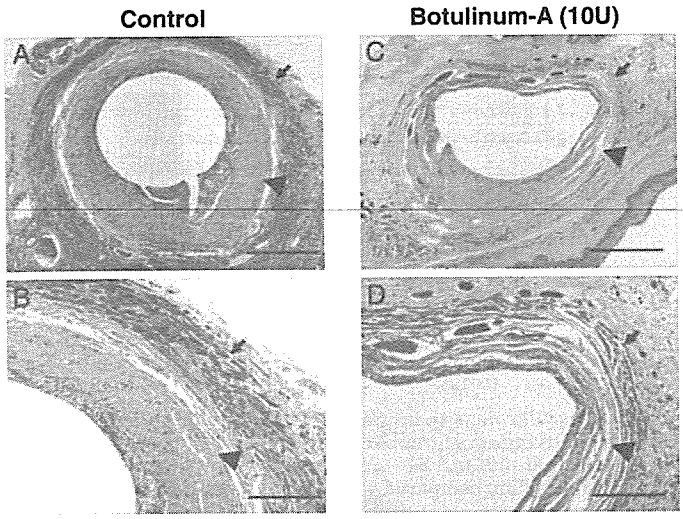

FIG. 1. Histological findings in rat urethra 2 weeks after saline (*A* and *B*) and botulinum-A toxin (*C* and *D*) injection. Remarkable shrinkage of striated sphincter (arrow) and smooth muscle layer (arrowhead) in rat with 10 U botulinum-A toxin injection was observed compared with that in control rat urethra. Masson's trichrome stain. Scale bar indicates 500 (*A* and *C*) and 200 μm (*B* and *D*).

toxin injection mean LPP in a rat without bFGF injection seemed to recover spontaneously (86.0 cm H_2O). However, LPP in a rat with 200 μg bFGF injection was improved to 121.0 cm H_2O. Figure 3 shows the results of LPP measurement. LPP in rats with 50 and 200 μg bFGF injection was significantly higher than in rats without bFGF injection (82.7 ± 9.0 vs 95.1 ± 6.2 and 119.3 ± 8.1 cm H_2O, p = 0.0021 and <0.0001, respectively). Figure 4 shows representative urethral histological findings in the rats 4 weeks after bFGF injection. The smooth muscle layer and striated sphincteric components were remarkably recovered from shrinkage due to botulinum-A toxin injection. Quantitative morphometric analysis of the urethra revealed that the maximum cross-sectional area of the urethral smooth muscle layer with 50 and 200 μg bFGF injection significantly increased compared with that in rats without bFGF injection, which was considered 100% (114.1% ± 15.8% and 132.5% ± 13.4%, p = 0.029 and <0.0001, respectively, fig. 5). Similarly striated sphincter cross-sectional area in rats with 50 and 200 μg bFGF injection was greater than in 0 μg bFGF injected rats, which

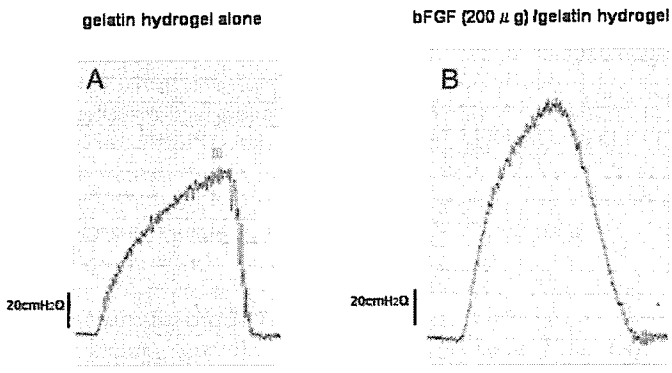

FIG. 2. Representative LPP in rats 4 weeks after injection of 0 and 200 μg bFGF incorporating gelatin hydrogels. Pressure at which leakage occurred was defined as LPP, corresponding to point where pressure decreased in traces.

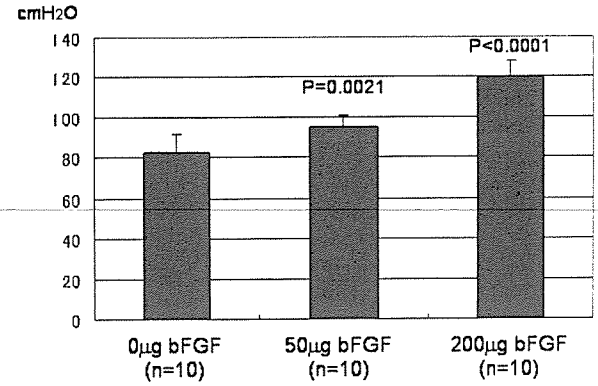

FIG. 3. LPP 4 weeks after injection of 0, 50 and 200 μg bFGF incorporating gelatin hydrogels was significantly higher in rats with 50 and 200 μg injection vs that in rats without injection (82.7 ± 9.0 vs 95.1 ± 6.2 and 119.3 ± 8.1 cm H_2O, respectively).

was considered 100% (112.3% ± 15.6% and 124.3% ± 14.1%, p = 0.069 and 0.0007, respectively, fig. 5). Figure 6 shows that bFGF injection induced marked angiogenesis in the urethral peri-atrophic zone. In contrast, no such histological changes were observed in rats without bFGF injection. Vascular density in the urethral peri-atrophic zone in the 50 and 200 μg bFGF groups was significantly higher than in the 0 μg bFGF group (p = 0.027 and <0.0001, respectively, fig. 7).

DISCUSSION

The current study demonstrates that periurethral injection of sustained release bFGF facilitates regeneration of the urethral muscles and improves sphincteric contractility. We used periurethral injection of botulinum-A toxin to establish an animal model of the impaired urethra. Previous studies have shown that pudendal nerve transection and electrocauterization of tissues lateral to the mid urethra successfully produce models of the impaired urethral sphincter.[11,12] Bot-

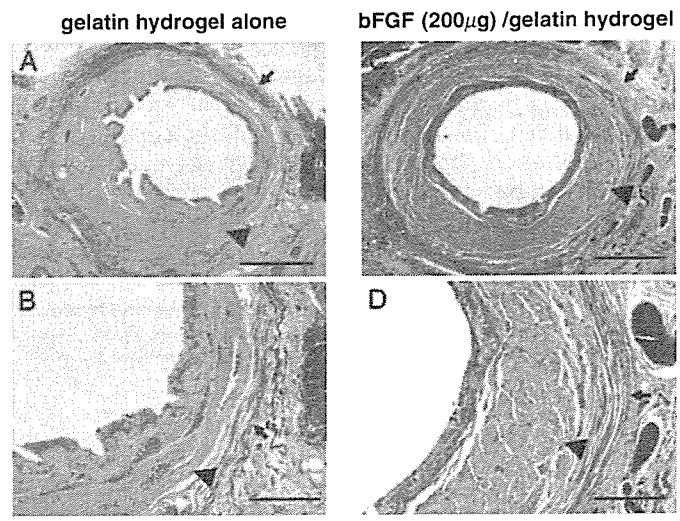

FIG. 4. Histological findings in rat urethra 4 weeks after injection of gelatin hydrogel alone (*A* and *B*) and bFGF incorporating gelatin hydrogel (*C* and *D*). In rat with 200 μg bFGF injection striated sphincteric (arrow) and smooth muscle (arrowhead) components remarkably recovered from shrinkage due to botulinum-A toxin injection. Masson's trichrome stain. Scale bar indicates 500 (*A* and *C*) and 200 μm (*B* and *D*).

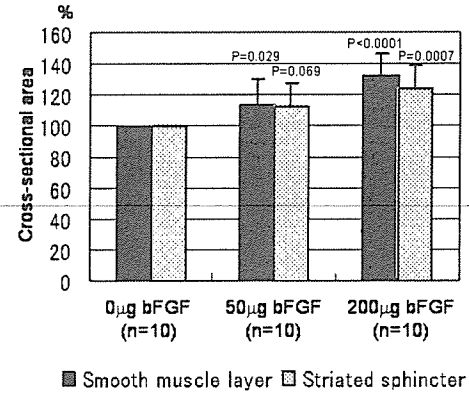

FIG. 5. Quantitative morphometric analysis of urethra after 0, 50 and 200 μg bFGF injection revealed that maximum cross-sectional areas of smooth muscle layer urethra in rats with 50 and 200 μg injection significantly increased vs that in rats without injection, considered 100%. Similarly striated sphincter cross-sectional in 50 and 200 μg groups was greater than in rats without injection, considered 100%.

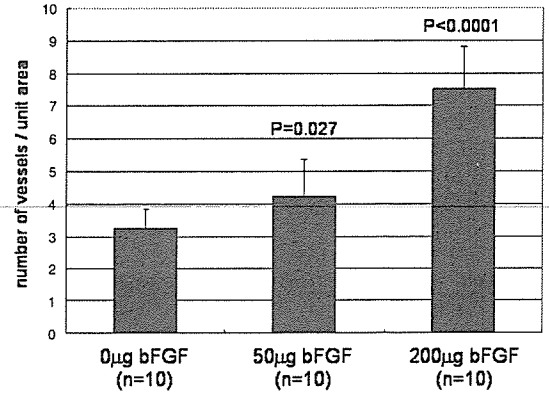

FIG. 7. Vascular density in urethral peri-atrophic area in 0, 50 and 200 μg bFGF injection groups. Number of vessels per unit area (200 $\times$ 200 μm^2) in 50 and 200 μg injection groups increased in dose dependent fashion compared with that in 0 μg injection group.

ulinum-A toxin injection in the urethra has also been reported to be effective for detrusor-sphincter dyssynergia in patients with spinal injury.[13] Chemical denervation of botulinum-A toxin induced a significant decrease in LPP, and remarkable shrinkage of the smooth muscle layer and striated sphincter, as shown. Periurethral botulinum-A toxin injection does not require an abdominal incision and it is a feasible method for inducing the impaired urethral sphincter.

bFGF has been reported to promote angiogenesis and proliferation of mesenchymal cells, including skeletal and smooth muscles.[3–6] For example, some studies of rats with myocardial infarction demonstrated that bFGF administration increased collateral vessels, enhanced cardiac function and decreased infarction size.[5,7,14] However, in other studies bFGF administration following the development of myocardial infarction exerted no additional effects on collateral growth and functional recovery.[15] In general the biological effects of growth factors in their free form are limited because the half-time in vivo is too short. The biological half-time of bFGF was reported to be less than 50 minutes.[16] Recent studies showed that collagen and its denatured derivative, gelatin, are highly versatile carriers for exogenous growth factors, including bFGF.[3–5,8,9] Biologically active bFGF was released as a result of in vivo degradation of the gelatin hydrogel. When gelatin hydrogel incorporating ^{125}I labeled bFGF was implanted into the back subcutis of mice,

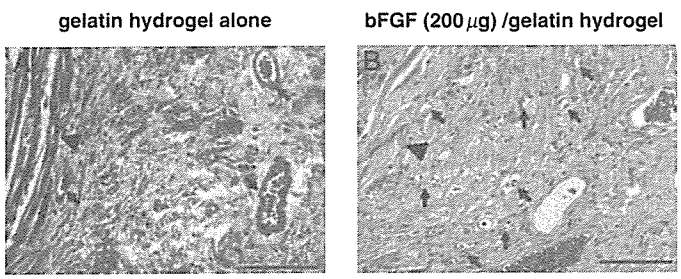

FIG. 6. Neovascularization in urethral sphincter peri-atrophic area in rats with 0 (*A*) and 200 μg (*B*) bFGF injection 4 weeks after treatment. Increased number of vessels (arrows) was observed close to sphincter (arrowhead) in rat with 200 μg injection. In contrast, no significant changes were observed in rat with 0 μg injection.

bFGF radioactivity remaining decreased with time and the retention period was prolonged with a decrease in hydrogel water content.[9] The release profile can be controlled by changing the hydrogel water content.[8,9] The study of rats with myocardial infarction done by our collaborators revealed that there was 15-fold more remaining bFGF in the heart 72 hours after intramyocardial administration of bFGF microspheres compared with that after intramyocardial injection of free bFGF in solution.[17] The hydrogel system used in the current study has been confirmed to permit controlled release of bFGF in vivo for 2 weeks.[5,8,9] Our results clearly demonstrate that bFGF incorporating gelatin hydrogels facilitates neovascularization and regeneration of smooth and striated urethral muscles, and improves urethral function. Taken together these findings suggest that the bFGF incorporating a gelatin hydrogel system can maintain enough bFGF concentration in the sphincteric region for a certain period to achieve sufficient effectiveness.

Recently the injection of allogenic/autologous MDCs in the urethra demonstrated potential usefulness for the treatment of SUI due to intrinsic sphincteric deficiency. First steps toward this new therapy have been reported.[11,12] In rodent models the investigators noted that intraurethral injection of allogenic immortal myoblasts developed from skeletal muscle biopsies facilitates sphincter regeneration. They also observed significant improvement in LPP in rats with MDC injection, which was comparable to that in control rats. The current study similarly demonstrated that LPP in rats with 200 μg bFGF injection was 119.3 $\pm$ 8.1 cm H_2O, comparable to the 120.7 $\pm$ 13.0 cm H_2O in control rats. Strasser et al recently developed transurethral ultrasound guided implantation of monoclonally cultured myoblasts and fibroblasts in pigs, and noted that injected cells survived at least 10 weeks and myoblasts formed new myofibers.[18,19] Furthermore, they performed a clinical study of ultrasound guided transurethral injection of autologous muscle stem cells and reported promising results.[20] In 59 patients, including men with incontinence after prostatectomy, small skeletal muscle biopsies were taken from the upper arm under local anesthesia. The fibroblasts were mixed with a small amount of collagen as carrier material and injected into the urethral submucosa to treat mucosal atrophy. The myoblasts were directly injected into the rhabdosphincter to reconstruct the muscle. Incontinence was improved in all

cases with a cure rate of 86% after stem cell injection, although long-term efficacy remains to be determined.[20]

In the current study sustained release bFGF injection showed significant efficacy for improving LPP in the animal model, which was comparable to that of MDC injection. We believe that our periurethral bFGF injection system will be a feasible and less invasive method when its usefulness is confirmed in well designed clinical trials in the future. Combined injection of MDCs with sustained release bFGF may also be an attractive alternative for SUI treatment.

CONCLUSIONS

Our results demonstrate that sustained release bFGF injection significantly improves the sphincteric contractility of the chemically denervated rat urethra. Endoscopic periurethral injection of bFGF incorporating gelatin hydrogels may be an attractive therapy for SUI due to an impaired sphincter.

ACKNOWLEDGMENTS

Human recombinant bFGF was provided by Kaken Pharmaceutical Co., Ltd., Tokyo, Japan.

Abbreviations and Acronyms

bFGF	=	basic fibroblast growth factor
LPP	=	leak point pressure
MDC	=	muscle derived cell
SUI	=	stress urinary incontinence

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Circ J 2006; **70:** 1190–1194

Combined Treatment of Sustained-Release Basic Fibroblast Growth Factor and Sarpogrelate Enhances Collateral Blood Flow Effectively in Rabbit Hindlimb Ischemia

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Background The effectiveness of sustained-release basic fibroblast growth factor (bFGF) in potentiating arteriogenesis and angiogenesis was evaluated, as well as determining whether chronic oral administration of sarpogrelate, a serotonin blocker, would further increase collateral blood flow in the rabbit hindlimb following surgical induction of ischemia by femoral artery extraction.

Methods and Results Two weeks after femoral artery removal, the rabbits were assigned to 1 of 4 experimental groups and treated for 4 weeks: group A, no treatment; group B, supplemented with diet containing sarpogrelate; group C, single intramuscular injection of sustained-release form of bFGF microspheres; group D: combined treatment with sustained-release bFGF and sarpogrelate. Endpoint measurements performed at 6 weeks found that the ischemic hindlimb blood flow was significantly improved in the rabbits that received sustained-release bFGF, with a further significant improvement in those with the additional administration of sarpogrelate. Angiographic assessment revealed augmented density of collateral vessels in the medial thigh region in the rabbits given the combined treatment.

Conclusions The findings demonstrate that sustained-release bFGF stimulated the development of collateral vessels, and additional administration of sarpogrelate produced a further improvement in hindlimb blood flow in the rabbit hindlimb ischemia model. (*Circ J* 2006; **70:** 1190–1194)

Key Words: Basic FGF; Collateral circulation; Hindlimb ischemia; Sarpogrelate; Serotonin blocker

Acute or chronic interruption in blood flow in peripheral arterial disease (PAD) results in inadequate tissue perfusion, cellular damage and loss of function. With the current rise in the geriatric population worldwide, the prevalence of PAD is now increasing. The best therapeutic strategies are interventional or surgical procedures to either reopen or to bypass the occlusion to reestablish blood flow to the ischemic region. One alternative approach to treating PAD is to promote the inherent ability to develop collateral vessels that improve and maintain blood flow to the ischemic area.

Augmentation of collateral blood flow is achieved by the growth and dilation of the preexisting collateral vessels. Collateral growth encompasses the proliferation of capillaries in the ischemic area (angiogenesis) and the maturation of preexisting collateral vessels (arteriogenesis), with the latter being more important in terms of increasing the collateral blood flow![1–5]

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Therapeutic angiogenesis with growth factors has been introduced for the treatment of PAD[6,7] but although local delivery of basic fibroblast growth factor (bFGF) augmented collateral circulation in a rabbit hindlimb ischemia model[8] the limitation of the use of recombinant bFGF lies in its low accumulation in the ischemic tissue and its rapid inactivation in vivo. Gelatin hydrogel microspheres incorporating bFGF have been developed for sustained-release[9] and we recently demonstrated the effectiveness of a sustained-release bFGF system in various pathological conditions, including hindlimb ischemia![10–12]

The dilation of collateral vessels is achieved by administration of a serotonin blocker, sarpogrelate![3,14] because newly developed collateral vessels are constricted by platelet activation or endothelial dysfunction![15,16]

Thus, the purpose of the present study was to evaluate the effectiveness of sustained-release bFGF for potentiating arteriogenesis and angiogenesis, and to determine whether chronic oral administration of sarpogrelate further increases the collateral blood flow in a rabbit hindlimb ischemia model.

Methods

Animal Model

Forty-four adult Japanese White rabbits (male, 3–4kg) were used for the completion of this study. All procedures related to the use of animals were approved by the Institu-