Enhanced cartilage regeneration by slow-release of basic fibroblast growth factor impregnated in gelatin microspheres

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Abstract

A cartilage graft is used for various reconstructions in the plastic surgery field. Auricular and costal cartilages are generally harvested, and the amount of collectable cartilage is often limited. The optimal utilization of collected cartilage to maximize the effect of cartilage graft has always been attempted resulting in unfavorable donor deformity with pain when harvest volume is large. Tissue reconstruction with minimizing donor site sacrifice is the ideal method. In this study, we introduced a bFGF slow-release system on a cartilage disk and investigated the possibility of quantitatively growing cartilage in a large animal model.

Key words: cartilage regeneration, bFGF, slow-release system

Introduction

The function and morphology of cartilage tissue vary depending on the region. Cartilage is histologically classified into the following 3 types: 1 hyaline cartilage, 2 elastic cartilage, and 3 fibrous cartilage. Articular, alar, and costal cartilages are classified as hyaline cartilage, auricular and epiglottic cartilages are classified as elastic cartilage, and intervertebral discs and the meniscus are classified as fibrous cartilage. It is widely known that regeneration capacity of the cartilage is very limited, and once deformation occurs, the restoration of cartilage is very difficult regardless of the cause such as congenital, traumatic, aging-related, and inflammatory deformations.

Cartilage graft has been frequently applied to improve the external surface morphology of the face in various congenital and acquired diseases. Costal and auricular cartilages were mainly selected for the cartilage donor site. Auricular cartilage shows superior elasticity and workability, but the amount of collectable cartilage is very small. Costal cartilage is advantageous in that it has mechanical strength and the amount that can be collected is large. However, in children, thoracic deformity after cartilage harvest and serious complication such as pneumothorax is frequently induced. If a method to quantitatively expand a small amount of collected cartilage tissue into a large volume is developed, it would provide an ideal cartilage material for reconstruction with minimal donor sacrifice. Unfortunately, no such study has been reported.

bFGF (Fiblast®, Kaken Pharmaceutical Co., Ltd., Tokyo) promotes cell proliferation and angiogenesis, and it is widely used for treating skin ulcers and acute open wounds in the clinical setting. Because of the short half-life, single administration is not sufficient to maintain tissue effective concentration and the pharmacological effect. In this study, we prepared slow-release bFGF using gelatin microspheres and applied it to a cartilage disk from auricle, which subcutaneously implanted as a autologous graft in a large animal (canine) model. The effect of the bFGF

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slow-release system on a grafted auricular cartilage disk was objectively evaluated and the possibility of quantitatively growing cartilage was investigated.

Materials and Methods

All animal procedures were conducted in accordance with regulatory standards of the Kinki University Animal Experiment Committee. A total of 9 female beagles aged 6-8 weeks, Hamaguchi Laboratory Animals, Hyogo) were used in the study. The animals were housed in individual cages maintained at 23°C and 50% humidity under the standard 12 hour-light/dark cycles. They were fed about 300 g of solid food (CD55α, CLEA Japan, Tokyo) once daily, and were given free access to drinking water. All surgical procedures were performed under general anaesthesia. After the animals were fasted for 12 hours, xylazine (Selactar®, 0.15 mL/kg, Bayer Japan, Tokyo) was injected intramuscularly in the gluteal region for induction anaesthesia, and pentobarbital (Somnopentil®, 0.4 mL/kg, Kyoritsu Seiyaku, Tokyo) was injected intravenously for general anaesthesia. The depth of general anaesthesia was monitored by the disappearance of eyelash reflex, and additional amount of pentobarbital was injected when needed. The auricles were cut, and auricular cartilage was harvested by removing the skin, cutaneous tissue, muscle, and perichondrium from the auricles.

Preparation of the bFGF-slow release system: To prepare the bFGF-slow release system, gelatin microspheres were manufactured as bFGF carriers[1]. First, 0.2 mL of 10% gelatin solution (pI=5, bovine bone gelatin, Nitta Gelatin, Osaka) was added to 5 mL of olive oil. The mixture was left to stand at 40°C for 1 hour, stirred, and cooled to 4°C to make particles. Excess olive oil was washed with 1.5 mL acetone. The solution was centrifuged at 4°C and 5,000 rpm for 5 minutes, washed 3 times with 4°C acetone, and was dried in a 4°C refrigerator for 1 hour to collect precipitating gelatin particles. Next, the gelatin particles were cross-linked by adding 1 mL of 0.1% polyoxyethylene sorbitan monooleate and 5 μL of 25% glutaraldehyde to 1 mg of gelatin particles, and stirring at 4°C for 24 hours. The gelatin particle suspension was centrifuged for 5 minutes at 5,000 rpm, and glycine solution was added to the pelleted gelatin particles. The solution was stirred for 1 hour at room temperature, and was washed three times with distilled water by centrifugation. Ultrapure water was added to the gelatin particles, and the solution was filtered using strainers with pore sizes of 70 and 30 μm to collect particles of equal sizes. Gelatin particles with diameters 30-70 μm were collected and snap-frozen in liquid nitrogen. After freeze-drying, the gelatin microspheres (approximately 10 μm in diameter) were sterilized by ethylene oxide gas. To incorporate bFGF into the gelatin microspheres, 100 μg of bFGF (Trafermin, Kaken Pharmaceutical, Tokyo) was dissolved in 60 μL of Ca^{2+} and Mg^{2+}-free PBS, and was added to 10 mg of gelatin microspheres. The solution was left to stand at 4°C for 24 hours.

Effect of bFGF slow-release system on canine auricular cartilage: The harvested auricular cartilage tissues were cut into circular cartilage disks (5-mm diameter) by a dermal punch. The following experimental groups were prepared: bFGF slow-release group (n=6) in which gelatin microspheres containing bFGF were applied onto the surfaces of the cartilage disks, and the control (n=6) in which only gelatin microspheres were applied. Cartilage disks were implanted as follows. First, dogs were shaved from the head to the back of the neck under general anesthesia. A 5-cm incision was made in the parietal area, and then the scalp flap was elevated above the periosteum. After observing the superficial and deep temporoparietal fascia, cartilage disks of experimental group and the control were autologously implanted (one implant on each side) between the two fascial layers.[2] The wound was closed using a 5-0 suture (Sigma, Tokyo) (Fig.1). The specimen was harvested at 5, 10, and 20 weeks after the implantation, the diameter was measured using a caliper, and evaluated histologically. The thickness of the specimen was measured from the histological sections, and the volume of the specimen was calculated based on the area and thickness.

Histological and immunohistochemical study: All in vivo specimens were collected after autologous implantation and fixed in 10% formalin and embedded in paraffin. The paraffin blocks were sectioned into 3 μm slices using a microtome (LEICA SM2000R), and then stained with hematoxyline eosin and safranin O.[1] The specimens were also stained for SOX5 and type II collagen.[3] Tissue fragments were deparaffinised,

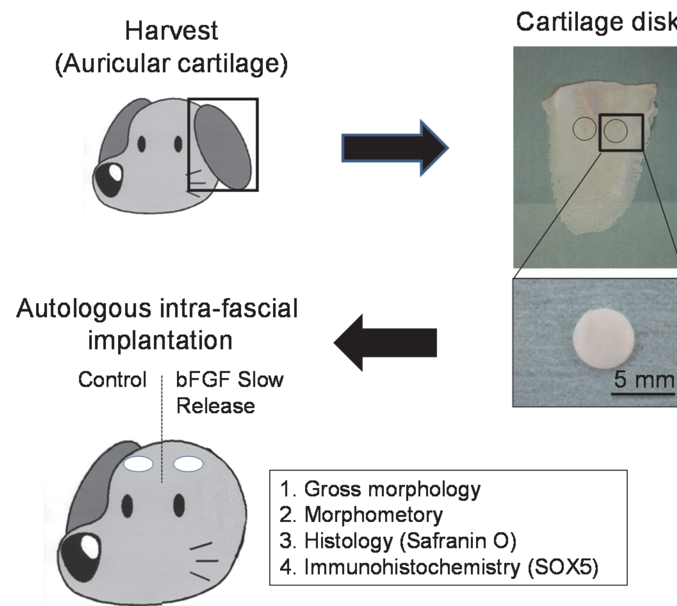

Figure 1 Protocol for experiment

treated with 1x Target Retrieval Solution (Dako, Kyoto), and heated to 100°C using a microwave for 5 minutes three times for antigen retrieval. The samples were then washed with 1x PBS (8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 136 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4), and endogenous peroxidase was inactivated by adding 0.3% H_2O_2 in methanol. Next, the samples were treated with blocking solution (1% bovine serum albumin (BSA), 600 mM NaCl, 50 mM Tris-HCl) for 30 minutes, and endogenous biotin and avitin were inactivated by adding Biotin-Blocking System (Dako, Kyoto, Japan). The samples were washed with PBS (same as above), and were incubated with 4 µg/mL of rabbit anti-dog SOX5 IgG antibody (Aviva Systems Biology, San Diego, CA) or normal rabbit IgG antibody (negative control, Dako, Kyoto) overnight at 4°C. After incubation with one of the primary antibodies, the samples were washed with 1x PBS, incubated with anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA), and washed again with 1x PBS. The secondary antibody was labeled with peroxidase (Vectastain ABC/HRP kit, Vector Laboratories, Burlingame, CA), and stained with diaminobenzidine (DAB) solution for colorimetric assessment.

Statistical analysis: The student's t-test was used to compare two means, and the one-way analysis of variance (ANOVA) and Holm's post-hoc test were used to compare more than three means. All statistical analyses were performed using R Environment (R Project), and $p < 0.05$ was considered to be statistically significant.

Results

The effect of the bFGF slow-release system on the implanted autologous cartilage disks was evaluated histologically after intra-fascial implantation. Gross morphology showed that all implanted disks appeared white and shiny. The autologous cartilage disks in the bFGF slow-release group demonstrated significant increase in the diameter and thickness at 5 weeks. Over the course of implantation, diameter remained the same level while the thickness remarkably decreased (Fig. 2). At 20 weeks after implantation, cartilage disks became approximately two times larger in area, being 4 times larger in volume when compared with those of the control (Fig. 3). The diameter and thickness of the implanted cartilage disks did not show significant changes in the control.

Histological evaluation of the cartilage disks in the bFGF slow-release group revealed thickening of the disk which was positively stained with safranin O at 5 weeks. In addition, there was a marked increase in cell density in the peripherary of the cartilage disks (Fig. 4). As these findings suggested increased cell proliferation-related events in the bFGF slow-release group, the specimens were subsequently stained for SOX5, a

transcription factor that controls the differentiation of mesenchymal cells into chondrocyte lineage. While there were only a few SOX5-positive ($SOX5^+$) cells in the center of the cartilage disk in the control, many $SOX5^+$ cells were found around the cartilage disks in the bFGF slow-release group at 5 weeks (Fig.5). There were few SOX5-positive ($SOX5^+$) cells in both groups at 20 weeks after implantation. These findings suggest that chondrocyte proliferation and chondrogenesis associated with up-regulated SOX5 expression was induced by the bFGF slow-release system.

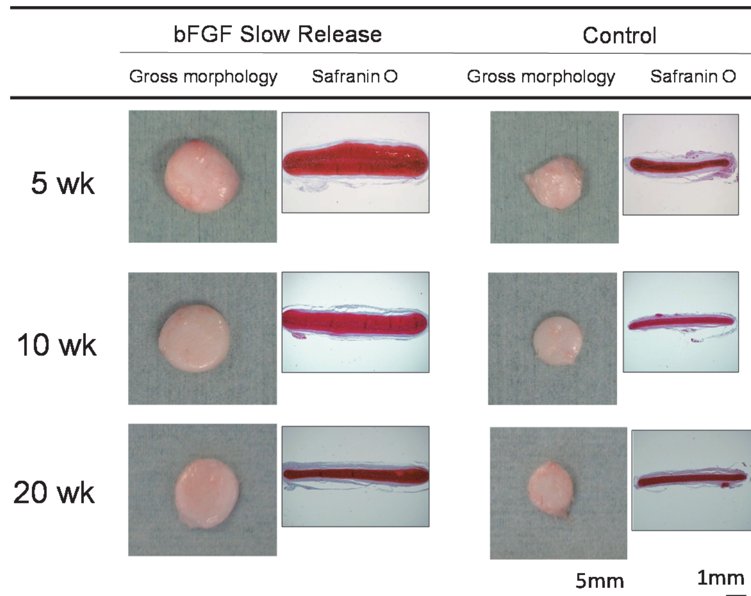

Figure 2 Gross morphology and histology (safranin O) of the cartilage disks following autologous implantation (n=6, representative images are shown)

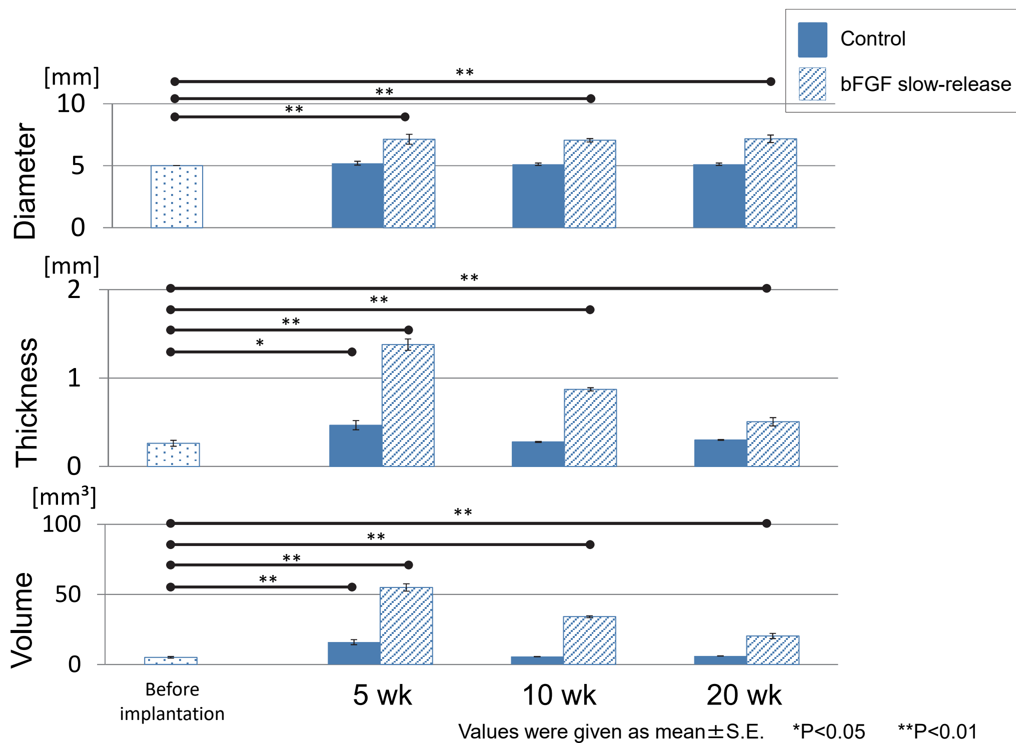

Figure 3 Changes of diameter, thickness, and volume of the implanted cartilage disks

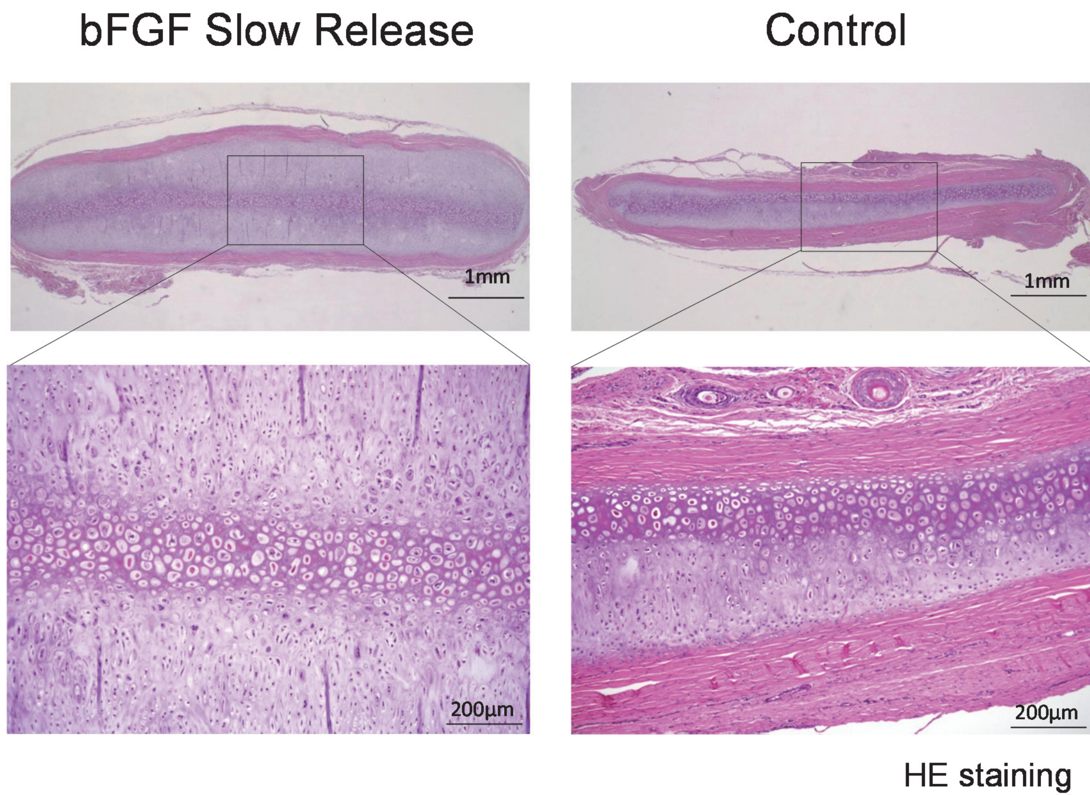

Figure 4 Histology of the cartilage disk at 5 weeks after implantation (n=6, representative images are shown)

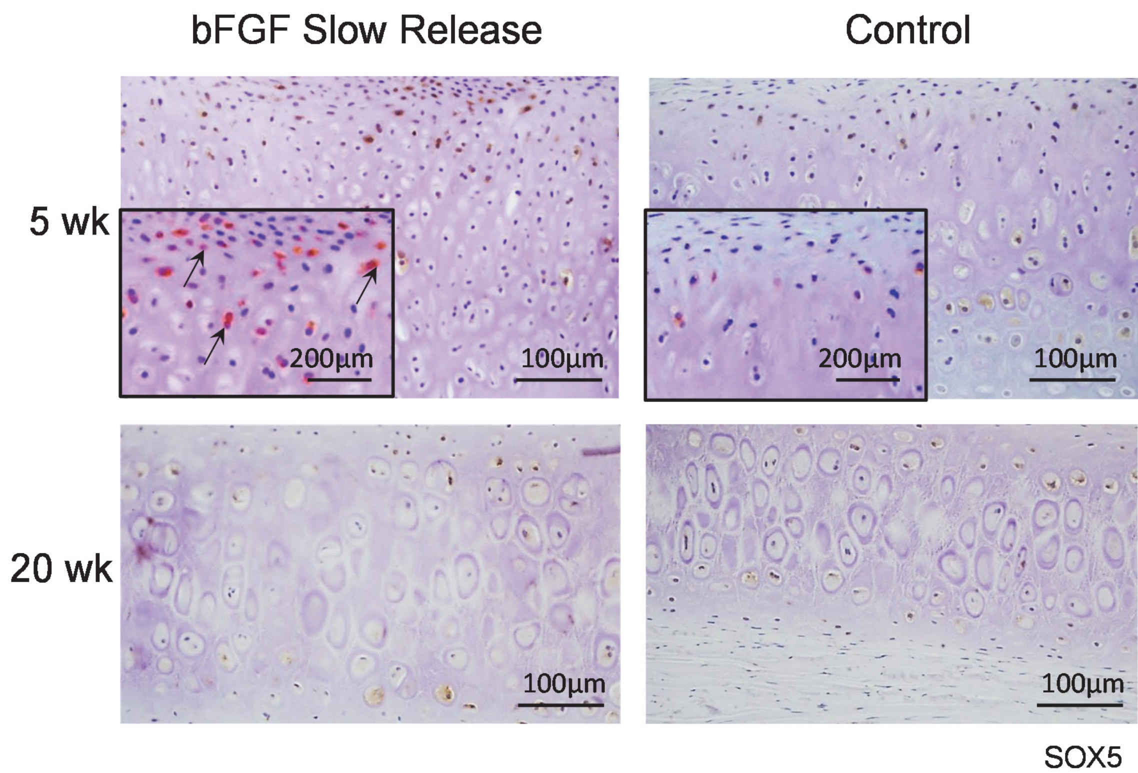

Figure 5 Immunohisotochemical study of the cartilage disks on SOX5 expression (n=6, representative images are shown)

Discussion

bFGF induces cell proliferation and angiogenesis in various cell types.[4] It inhibits the synthesis of type X collagen at the final stage of chondrocyte proliferation.[5] A previous study demonstrated that a single administration of bFGF does not have sufficient pharmacological effects *in vivo*.[6] The bFGF slow-release system using gelatin microspheres was introduced to stably supply bFGF for a prolonged period in vivo. bFGF with an isoelectric point of 9.5 and acidic gelatin with an isoelectric point of 5.0 electrostatically interact to form a compound. The slow-release of bFGF is generated by the gradual degradation of gelatin, and the duration of such slow-release is proportionate to the degradation rate of the gelatin. Gelatin is superior in terms of safety with regard to the infectious genetic materials such as virus vectors and plasmids. In addition, based on previous basic study results, the slow-release system is advantageous in that it acts locally and in the capacity to control the amount of slowly released growth factor.[2.] The system enables the slow-release of bFGF for approximately 14 days *in vivo*.[7]

In a small joint cartilaginous defect, the diameter of which is smaller than 3 mm can be repaired spontaneously. But the repairing mechanism of the cartilaginous tissue was not found in the defect greater than 5 mm. In this experiment, 5-mm diameter of the circular cartilage disk was selected based upon such previous information on the size of the defect in cartilaginous tissue repair.

In cartilage tissue, bFGF is known to be less transported as the chondrocytes are surrounded by the extracellular matrix which works as a barrier for bFGF diffusion.[8] In order to evaluate the effect of bFGF on chondrocytes in cartilage tissue, we applied the bFGF slow-release system with gelatin microspheres onto the cartilage disks prior to autologous implantation (Fig. 1). The result revealed that the implanted cartilage disks were drastically enlarged (Fig. 4), and the volume of the disk was significantly increased (Fig. 2 and 3). These results suggest that the bFGF slow-release system promotes the proliferation and chondrogenesis within the cartilage disks. The long-term effects of the bFGF slow-release system remain to be elucidated.

A recent study suggested that SOX family of transcription factors plays an important role in controlling differentiation of mesenchymal stem cells into chondrocytes.[3] Specifically, SOX9 regulates mesenchymal cells and promotes their aggregation and differentiation into chondrocytic lineage, while SOX5 and SOX6 determines the differentiation of prechondrocytes into chondroblasts.[9] In the present study, the distribution of Sox5-positive (SOX+) cells at 5 weeks after implantation was consistent with the histologically hypertrophied region in the bFGF slow-release group, suggesting that b-FGF induced the quantitative expansion of the cartilage disk through cell proliferation and differentiation, and its basic mechanism involved the proliferation of chondroblasts.

Blood vessels and nerves are absent in cartilage and the tissue viability depends on the nutritional diffusion from the surrounding tissues. The bFGF slow-release system are reported to introduce angiogenesis maximally at day 4 after its subcutaneous administration and the capacity to maintain angiogenesis decreases over time [6]. In this study, cartilage disks were quantitatively expanded due to the influence of the bFGF slow-release system. Subsequently, nutritional diffusion decreased with a qualitative decline of the surrounding angionegesis and immature mesenchymal stem cells thereafter, resulting in a loss of thickness but maintenance of the diameter. In this experiment, histological findings around the autologous cartilage graft were not provided. Further study on the effect of bFGF around the graft is needed.

From this study, the bFGF slow-release system is considered to be a simple method capable of expanding a cartilage disk quantitatively. The system may become a useful tissue-increasing method to quantitatively expand a small amount of cartilage tissue in order to fill large defects. The establishment of an effective surgical procedure involving minimal sacrifice in the cartilage harvest using this new tissue-increasing method is under way.

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