学位論文

Prefabrication of Vascularized Allogenic Bone Graft in a Rat by implanting a Flow-through Vascular Pedicle and Basic Fibroblast Growth Factor containing Hydroxyapatite/Collagen Composite

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Prefabrication of Vascularized Allogenic Bone Graft in a Rat by Implanting a Flow-Through Vascular Pedicle and Basic Fibroblast Growth Factor Containing Hydroxyapatite/Collagen Composite

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Abstract

Background Basic fibroblast growth factor (bFGF) is known to stimulate bone formation and angiogenesis. Hydroxyapatite/collagen composite (HAp/Col) is also known to have very strong bone conductive activity. In this study, prefabrication of vascularized allogenic bone (allo-bone) graft was attempted in recipients by implanting vascular bundles from recipients into the transplanted allo-bone graft. Furthermore, the effect of bFGF-containing HAp/Col on the prefabricated vascularized allo-bone graft was investigated.

Methods In this study, 32 Sprague-Dawley rats were used as donors, and bone grafts were collected from their femora. Thirty-two Wistar rats (recipients) were divided into four groups, and the allo-bone grafts were transplanted into the thigh region. In the experimental groups, one or both of the flow-through saphenous vascular bundles and 100-μg bFGF-containing HAp/Col were implanted into the medullary cavity of the allo-bone grafts. In the control group, neither was implanted. These rats were sacrificed at 4 weeks after transplantation, and bone formation, angiogenesis, and bone resorption in the transplanted allo-bone grafts were evaluated histologically and genetically.

Results Bone formation and angiogenesis in the transplanted allo-bone graft were effectively stimulated by implanting vascular bundles or bFGF-containing HAp/Col on both histological and genetic evaluations compared with the control group. The most significant stimulation was observed in the group in which both were implanted. Bone resorption was not stimulated in any group.

Conclusion By implanting a flow-through vascular bundle and bFGF-containing HAp/Col, an ideal vascularized allo-bone graft that had high bone formative and angiogenetic activities and did not stimulate bone resorptive activity was prefabricated.

Keywords
- allo-bone graft
- bFGF
- hydroxyapatite/collagen

Autologous bone (auto-bone) grafts have usually been used for reconstruction of bone defects caused by severely comminuted bone fractures or resection of malignant bone tumors. Furthermore, allogenic bone (allo-bone) grafts have also been used when the volume of auto-bone grafts was insufficient to reconstruct the bone defects, or to compensate for the disadvantages of auto-bone grafts, such as the risk of donor-site morbidity.[1–3]

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However, nonvascularized auto-bone grafts or allo-bone grafts are not suitable for treating massive segmental bone defects of more than 6 cm in size[4] because the graft bones are often resorbed before revascularization is achieved.[1] In such cases, vascularized auto-bone grafts or massive nonvascularized allo-bone grafts with intramedullary vascularized auto-bone graft are usually selected.[5–7]

Earlier and more secure bone union is achieved using vascularized auto-bone grafts than with nonvascularized auto-bone grafts. However, the size of graft bone depends on the donor site, and the size of graft bone is usually somehow limited.[8]

On the other hand, combined grafts (allo-bone graft shell plus vascularized allo-bone graft) can fill a bigger bone defect, but they sometimes require more time for bone union.[9] Both methods cannot avoid the problem of sacrificing normal tissue.

In our previous study, we attempted to prefabricate vascularized allo-bone grafts in rat recipients. In this model, the saphenous vascular bundles of the recipient rats were implanted into transplanted donor bones. In addition, bone morphogenetic protein (BMP) was administered to stimulate bone formation in the transplanted bone. Indeed, BMP stimulated bone formation strongly, but we found that BMP also stimulated bone resorption in the transplanted bone. Consequently, antibone resorptive agents, such as bisphosphonates, were needed to prevent the bone resorption caused by BMP. However, bisphosphonates were found to delay the union between the transplanted bone and the recipient bone.[10]

Basic fibroblast growth factor (bFGF) is known to stimulate bone formation and angiogenesis and does not stimulate bone resorption.[11,12] Therefore, we hypothesized that we could stimulate bone formation and angiogenesis in the prefabricated vascularized allo-bone graft without stimulating bone resorption by administration of bFGF into the allo-bone graft.

However, the half-life of bFGF in the body is generally too short to exert its full biological activity effectively when injected directly into the bone defect without using any carrier. It has been shown that when bFGF is ionically complexed with acid gelatin, it is stored and released slowly as the hydrogel degrades. As a result, angiogenesis is significantly enhanced, and the level of bone regeneration is increased in comparison to free bFGF.[13] Artificial bone and atelocollagen have also been reported as useful carriers of bFGF.[13–20]

Recently, use of a porous hydroxyapatite/collagen composite (HAp/Col) as artificial bone has started. HAp/Col, which is composed of nanoscale crystals of hydroxyapatite and type 1 atelocollagen derived from porcine skin, was developed.[21] The nanostructure of the composite resembles that of natural bone, and the composite exerts high osteoconductivity and is resorbed by osteoclasts when implanted in bone defects.[21,22] For this reason, we decided to use HAp/Col composite material (ReFit, HOYA Technosurgical Co., Tokyo, Japan) in this study as a carrier of bFGF. ReFit was a $10 \times 10 \times 10\ mm^3$ cube and was constructed with 80% HAp and 20% Col. This proportion of HAp and Col is very similar to that found in natural bone.[23]

In this study, vascularized allo-bone graft was prefabricated in the recipient rat by implanting a flow-through vascular bundle from the recipient rat into transplanted nonvascularized allo-bone graft. Furthermore, the bFGF-containing HAp/Col composite was implanted into the medullary cavity of the transplanted allo-bone graft. Using this experimental model, whether bFGF-containing HAp/Col composite stimulates bone formation and angiogenesis and does not stimulate bone resorption in the prefabricated vascularized allo-bone grafts was investigated histologically and genetically.

Materials and Methods

Animals

In this study, 32 female 9-week-old Sprague-Dawley rats were used as donors and 32 male 9-week-old Wistar rats were used as recipients. All rats were purchased from Japan SLC (Hamamatsu, Japan). During the experimental period, the animals were housed in cages (floor area: 988 cm^2; height: 18 cm) and allowed ad libitum access to water and pelleted commercial rodent diet (Oriental Yeast Co., Tokyo, Japan). All in vivo studies were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006) and approved by the Institutional Animal Care and Use Committee of our institution.

Preparation of Allo-Bone Graft

Graft bone (length: 10 mm) was collected from the midshaft of the femora of donor rats, and slits were made on the bones. These bone samples were heat-sterilized using a Telos Lobator SD-2 Bonebank System (Telos GmbH, Marburg, Germany). During this sterilization process, the collected bone achieved a temperature of 82.5°C for 15 minutes. The sterilized bones were then preserved at −80°C.

Preparation of bFGF-Containing Artificial Bone

A spongiform porous HAp/Col (Refit) was used as artificial bone. It was cut into rectangular blocks ($2 \times 2.5 \times 10$ mm) for implanting into the medullary cavity of allo-bone grafts. In addition, 100 μg of bFGF (Fiblast, Kaken Pharmaceutical, Tokyo, Japan) were mixed with 40 μL of distilled water to make bFGF solution (2.5 μg/μL).

Then, to make bFGF-containing HAp/Col composite blocks, 40 μL of bFGF solution were added into each block so that each block contained 100 μg of bFGF.

In Vitro Analysis of bFGF Release Kinetics

A total of 42 HAp/Col composite blocks containing 100 μg of bFGF were individually placed in 10 mL of phosphate buffered saline (pH 7.4) containing 0.5% bovine serum albumin and incubated at 37°C for 1, 6, 24,48, 72, 96, and 120 hours (six were used for each time point). At each time point, media samples were collected and immediately frozen for storage at −80°C until analysis.

The amounts of bFGF in the media samples were quantified using an enzyme-linked immunosorbent assay kit (Quantikine Immunoassay kit DFB50, R&D Systems,

Table 1 Classification of the groups in this study

Group	Treatment
Control (group C)	Nonvascularized allo-bone
Group V	Vascularized allo-bone
Group F	Nonvascularized allo-bone with bFGF-containing HAp/Col
Group VF	Vascularized allo-bone with bFGF-containing HAp/Col

Abbreviations: allo-bone, allogenic bone; bFGF, basic fibroblast growth factor; HAp/Col, hydroxyapatite/collagen composite.

Minneapolis, MN) according to the manufacturer's instructions. The percentage of released bFGF at each time point was determined as the ratio of the amount of bFGF in 10 mL of the sample, which was calculated by reference to the standard curve, relative to the original amount (100 µg) in the bFGF-containing HAp/Col composite. This experiment was performed according to the method of Ishii et al.[24]

Experimental Design and Surgical Procedure

In this study, animals were divided into a control group ($n = 8$; nonvascularized allo-bone) and three experimental groups ($n = 8$ each) (►**Table 1**).

The surgical procedure for transplantation was as follows: recipient rats were anesthetized by intraperitoneal injection of pentobarbital sodium (Somnopentyl, Kyouritsu Seiyaku, Tokyo, Japan) at a dose of 5 mg/100 g body weight. A 3-cm incision was then made in the medial aspect of the thigh, and the saphenous artery and vein were exposed and separated from surrounding tissues. The prepared nonvascularized allo-bone graft was then transplanted into the thigh region of the recipient rat (►**Fig. 1**), and the saphenous vascular bundle was implanted into the medullary cavity of the transplanted bone in a flow-through manner.

These surgical procedures were performed under an operating microscope. In the C and F groups, the allo-bone graft was transplanted without implantation of the saphenous vascular bundle. In the F and VF groups, the medullary cavity of the transplanted bone was filled with bFGF-containing HAp/Col.

These surgical procedures were performed on both sides.

Harvest of Prefabricated Vascularized Allo-Bone Grafts

All animals were sacrificed at 4 weeks after transplantation. These animals received subcutaneous administration of fluorescent-labeled calcein (1 mg/100 g; Wako Pure Chemical Industries Ltd., Osaka, Japan) 2 days before sacrifice. After sacrifice, the transplanted bones were collected from the recipients. The bone samples of the right side were used for histological evaluation. On the other hand, total ribonucleic acid (RNA) was extracted from the bone samples of the left side.

Histological Preparation and Contact Microradiographs

Bone samples of the right side were divided into two fragments at the center of the bones by cutting with a band saw. The proximal sides were fixed in 10% buffered formalin and then embedded in methyl methacrylate (MMA Embedding Medium, Merck, Darmstadt, Germany). These embedded bone samples were ground to a thickness of 100 µm, and digital contact microradiographs (100 kVp, 2 µA, 5 minutes; µFX-l000; Fujifilm, Tokyo, Japan) were taken for evaluation of new bone formation. The bone samples were then ground to 30 µm for microscopic evaluation. Distal sides were fixed in 10% cold buffered formalin for 3 days. These bones were then decalcified in 10% ethylenediaminetetraacetic acid at 4°C for 4 weeks and embedded in JB-4 glycol methacrylate (JB-4 Embedding Medium, Polysciences, Warrington, PA). Sections with a thickness of 5 µm were cut with a microtome and stained with hematoxylin and eosin (H&E) stain to demonstrate angiogenesis or with tartrate-resistant acid phosphatase (TRAP) stain to demonstrate osteoclasts. TRAP stain was made using 5 mg of naphthol AS-MX phosphate

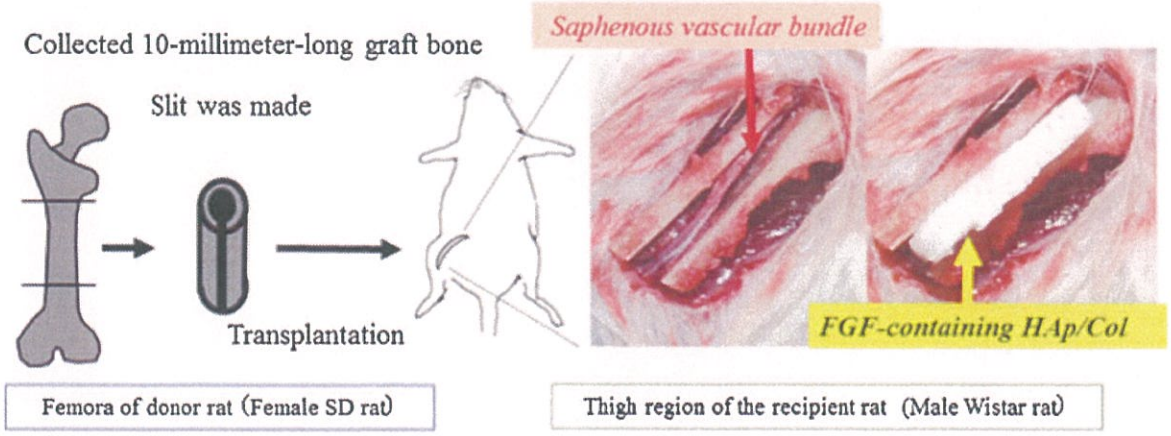

Fig. 1 Allo-bone graft is transplanted into the thigh region of the recipient rat, and the saphenous vascular bundle is implanted into the medullary cavity of the transplanted bone in a flow-through manner. The medullary cavity of the transplanted bone is filled with basic fibroblast growth factor containing hydroxyapatite/collagen composite.

(Sigma-Aldrich), 0.25 mL of N-N-dimethylformamide, 25 mL of sodium acetate (0.2 M), 115 mg of sodium tartrate, and 30 mg of Fast Red AL Salt (Sigma-Aldrich, St. Louis, MO) in 50 mL of TRAP buffer.

Histological Analysis

Histological analyses were performed with an all-in-one fluorescence microscope (FSX100, Olympus Corporation, Tokyo, Japan) and histomorphometric software (cell Sens Dimension Desktop 1.14, Olympus Corporation, Tokyo, Japan).

Undecalcified Specimens

Undecalcified specimens were used for evaluation of calcein uptake by the mineralized bone. The percentage of fluorescent calcein-labeled bone surfaces (%LS: length of labeled bone surface/total length of bone surface × 100) was used as a bone formation parameter.

Decalcified Specimens

Patency of the implanted saphenous vascular bundle and restructured small vessels in the transplanted allo-bone graft was confirmed using H&E-stained specimens.

Angiogenesis was evaluated by counting the numbers and measuring the total area of the newly formed vessels. The newly formed vessel number (N.NV: total newly formed vessel number/total area of bone marrow) and the percentage of newly formed vessel area (%NV: newly formed vessel area/total area of bone marrow × 100) were used as angiogenesis parameters.

Decalcified specimens were also used for evaluation of bone resorption. Osteoclasts were counted on the endosteal and periosteal surfaces of allo-bone grafts using TRAP-stained sections. The percentage of osteoclast surface (% OcS: length of bone surface covered with osteoclasts/total length of bone surface × 100) and osteoclast number (N.Oc: total osteoclast number/total length of bone surface) were used as bone resorption parameters.

Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from the collected bone of the left side using Isogen (Nippon Gene, Tokyo, Japan). The RNA was then reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using High Capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, CA) for reverse transcription polymerase chain reaction (RT-PCR). Quantitative PCR was performed on an Eco Real-Time PCR System (Illumina Inc., San Diego, CA) using Power SYBR-Green PCR Master Mix (Applied Biosystems). The primers (none morphogenetic protein-2 [BMP-2], osteocalcin, type-1 collagen, bone alkaline phosphatase [BAP], vascular endothelial growth factor [VEGF], receptor-activator of nuclear kappa B ligand [RANKL]) for quantitative PCR were synthesized and validated by Hokkaido System Science Co., Ltd. (Hokkaido, Japan). BMP-2, osteocalcin, type-1 collagen, and BAP are gene products related to bone formation. VEGF is a gene product related to angiogenesis. RANKL is gene product related to bone resorption.

Statistical Analysis

Statistical computation of data was performed using GraphPad Prism (ver. 5.04 for Windows, GraphPad Software, San Diego, CA). Differences among treatment groups were tested using one-way analysis of variance. If significant differences were found, differences between means for the two groups were tested using Fisher's protected least significant difference test. Values of $p < 0.05$ were considered significant.

Results

Time Course of bFGF Release from bFGF-Containing Artificial Bone

The mean release ratio of bFGF from the bFGF-containing artificial bone increased over time: 76.0% ± 0.2% after 1 hour, 89.0% ± 0.5% after 6 hours, 90.8% ± 0.1% after 24 hours, 92.3% ± 0.5% after 48 hours, 91.8% ± 0.2% after 72 hours, 91.8% ± 0.2% after 96 hours, and 92.2% ± 0.3% after 120 hours. At each interval up to 48 hours, there was a significant increase in the release of bFGF. Beyond 48 hours, the amount of bFGF in the media samples did not increase significantly (►**Fig. 2**).

Revascularization of Transplanted Allo-Bone Grafts

At 4 weeks after allo-bone graft transplantation, bleeding was observed from all prefabricated vascularized allo-bone grafts when they were collected (V and VF groups).

On histological examination, blood flow in the saphenous vascular bundles was maintained, and restructured small vessels were also observed in the bone marrow area in these groups, especially in the VF group (►**Fig. 3**).

The N.NV values of the control, F, V, and VF groups were 0.0000273 ± 0.00000021, 0.0000627 ± 0.00000018, 0.0002245 ± 0.00000060, and 0.0004935 ± 0.00000082, respectively. The N.NV values of the F group were higher than those of the control group. On the other hand, the N.NV values of the bones of the VF group were the most significantly increased (►**Fig. 4A**). The %NV values of the control,

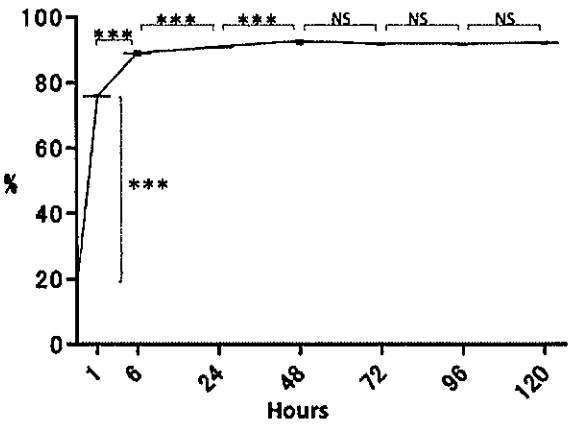

Fig. 2 Graph showing the time course of basic fibroblast growth factor (bFGF) release. Data are expressed as means ± standard deviation of the percentage of released bFGF in six samples for each time point (one-way analysis of variance test) ***$p < 0.001$; NS, not significant.

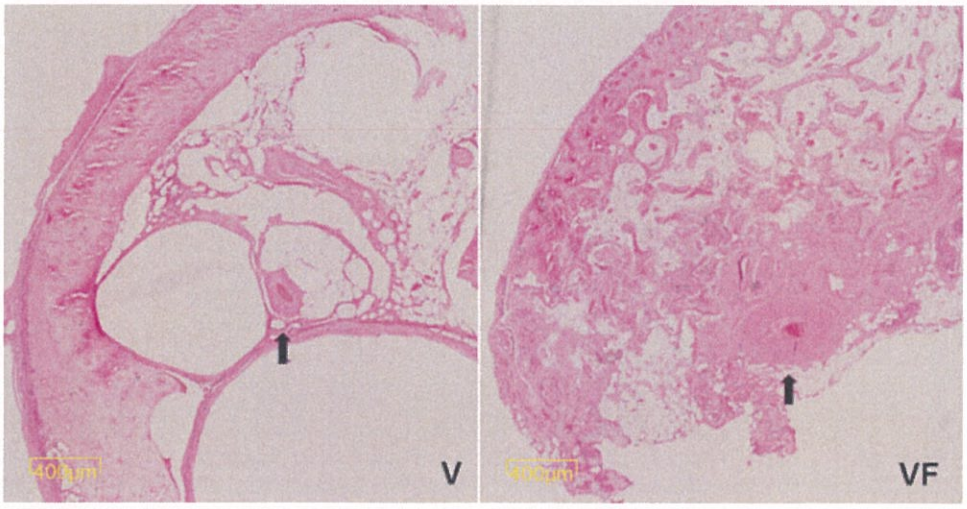

Fig. 3 Decalcified specimens stained with hematoxylin and eosin. In the V and VF groups, blood flow in the saphenous vascular bundles is maintained.

F, V, and VF groups were 0.12 ± 0.0018%, 0.61% ± 0.0012%, 0.79 ± 0.0023%, and 5.40 ± 0.0364%, respectively. The %NV values of the V and F groups were not significantly higher than in the control group. On the other hand, the %NV values of the VF group were significantly higher than in the other groups (►**Fig. 4B**).

Contact Microradiographs

Contact microradiograph showed increased bone formation in the V and F groups, with the most significant increases in the VF group. In the C group, no increase of bone formation was observed (►**Fig. 5**).

Histological Findings (Bone Formation)

The %LS values of the control, F, V, and VF groups were 4.8 ± 4.1%, 14.3 ± 6.0%, 16.5 ± 4.1%, and 40.1 ± 9.3%, respectively. The %LS values of the bones of the F and V groups were increased compared with the control group. On the other hand, the %LS values of the bones of the VF group were the most significantly increased (►**Fig. 6**).

Histological Evaluation of Decalcified Specimens (Bone Resorption)

The %OcS values of the bones of the control, F, V, and the VF groups were 6.1 ± 3.2%, 5.2 ± 1.9%, 5.3 ± 1.4%, and 3.8 ± 2.3%, respectively. The %OcS values of the bones did not increase in any of the groups.

The N.Oc values of the control, F, V, and VF groups were 0.00103 ± 0.00052, 0.00083 ± 000035, 0.00126 ± 0.00020, and 0.00041 ± 0.00018, respectively. The N.Oc values of the bones also did not increase in any of the groups (►**Fig. 7**).

Genetic Analysis

Quantitative Reverse Transcription-Polymerase Chain Reaction (Relative Messenger RNA Expression)

The expression levels of genes related to bone formation (BMP-2, osteocalcin, type-1 collagen, BAP) were slightly increased in the F and V groups in comparison to the control group. On the other hand, the expression levels of these genes were significantly increased in the VF group. Similar

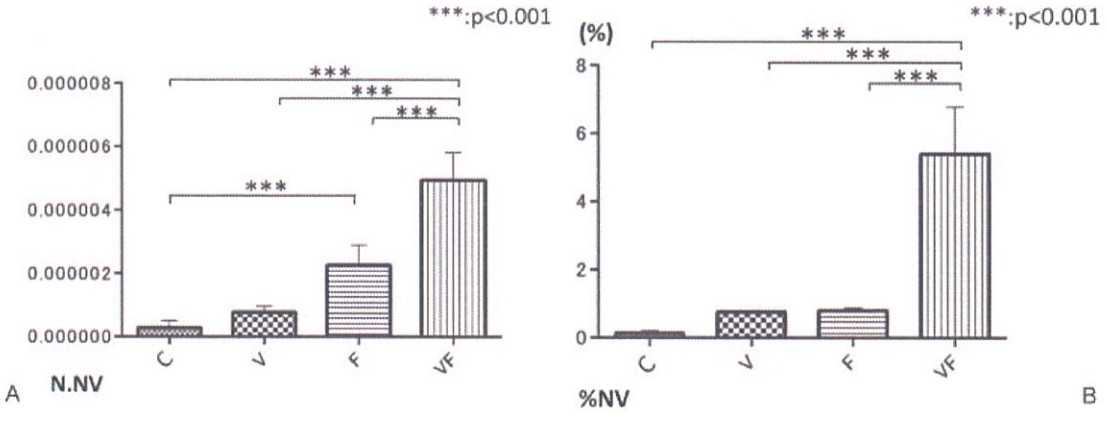

Fig. 4 Decalcified specimens stained with hematoxylin and eosin. Angiogenesis in different groups is evaluated as the newly formed vessel number (N.NV: total newly formed vessel number/total area of bone marrow); (A) and the percentage of newly formed vessel area (%NV: newly formed vessel area/total area of bone marrow × 100); (B). ***$p < 0.001$.

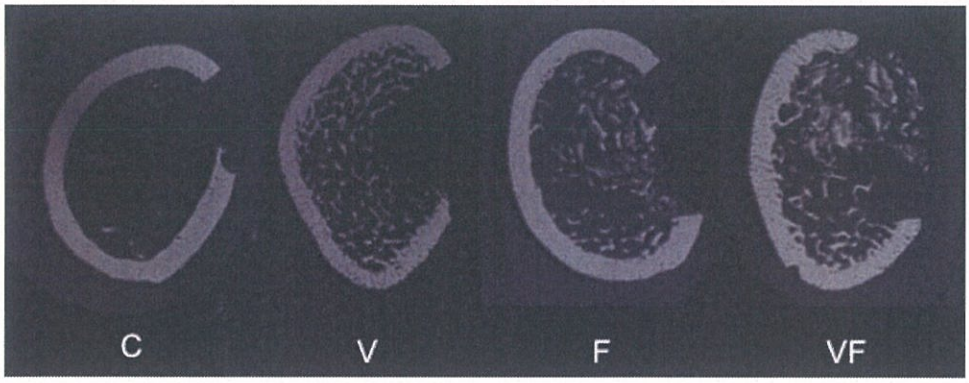

Fig. 5 Contact microradiographs. Increased bone formation is observed in the V and F groups, and the most significant increase is seen in the VF group.

observations were made regarding the expression levels of the angiogenesis-related gene (VGEF). The expression levels of the gene related to bone resorption (RANKL) did not increase in any of the groups (►**Fig. 8**).

Discussion

The limitation of the length of vascularized auto-bone grafts is from 14 to 24 cm, even if it is a vascularized fibula graft.[25] The use of a vascularized fibula graft is valuable in the treatment of long bone defects. Yet, stress fractures are frequently observed during the first postoperative year when a sole fibula is used for such a long bone defect because the vascularized bone graft is still weak and unable to bear full weight.[26]

To compensate for the weakness of the vascularized long fibular graft, a combined vascularized fibula graft and allo-bone graft[5,27] was performed to combine the advantages provided by the mechanical endurance of a massive allo-bone graft and the biological properties of the vascularized

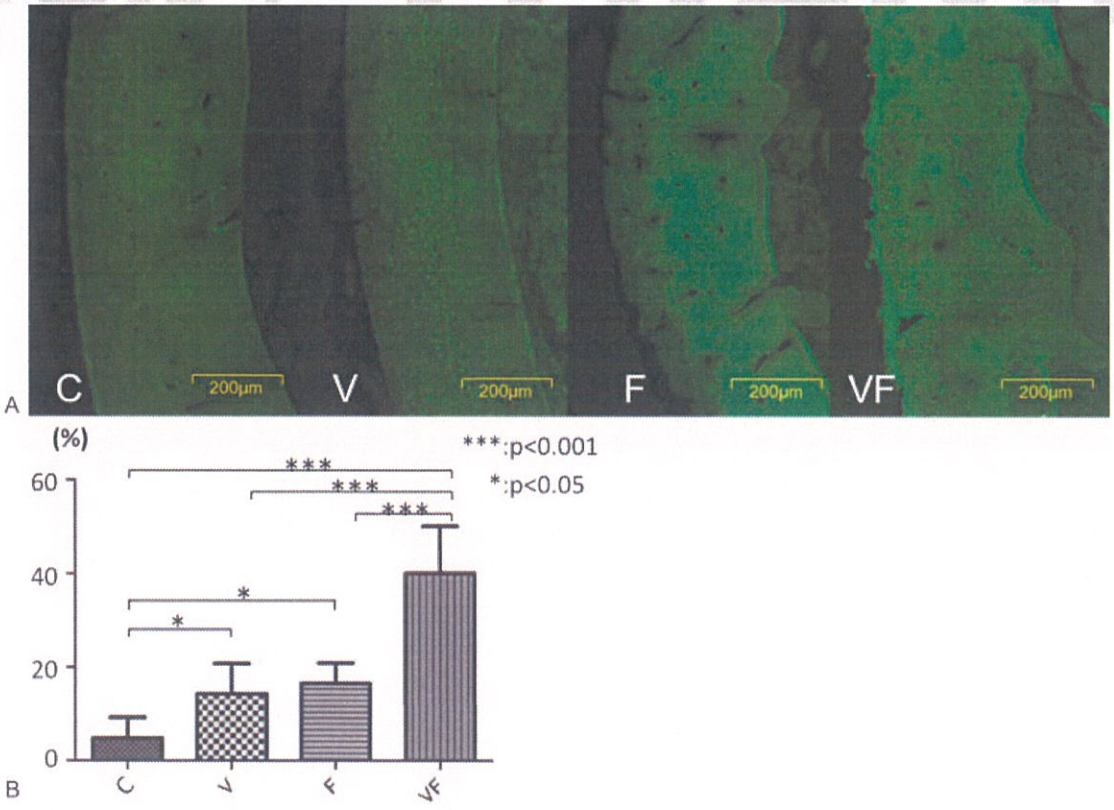

Fig. 6 Histological findings. **(A)** Undecalcified specimens under fluorescent microscopy. **(B)** New bone formation is evaluated as the percent of labeled bone surface.

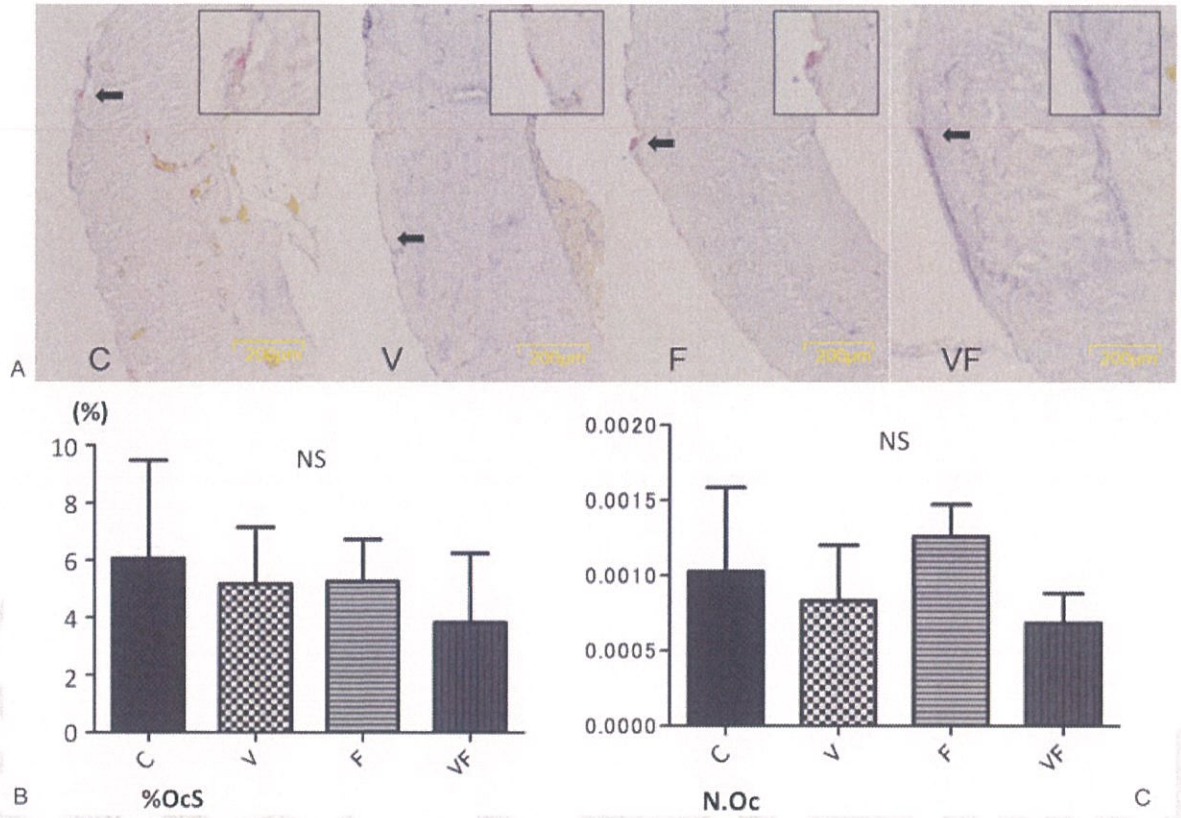

Fig. 7 Histological findings. (**A**) Decalcified specimens stained with tartrate-resistant acid phosphate stain. Bone resorption in different groups is evaluated as the percent of osteoclast surface (%OcS: length of bone surface covered with osteoclasts/total length of bone surface × 100; (**B**) and osteoclast number (N.Oc: total osteoclast number/total length of bone surface); (**C**). NS, Not significant.

fibula graft. The allo-bone graft provides adequate bone stock and early stability, while the vascularized fibula graft facilitates host-allo-bone graft union. However, even when the combined graft was performed, it was difficult to allow early weight-bearing. Another way to reinforce the graft strength was a double-barrel vascularized fibular graft. However, the limitation of the graft length was only 12 cm because the maximum length of fibular graft that we can harvest is 24 cm.[25]

Moreover, the 24-cm-long harvest of fibula can induce other complications at the donor site, such as ankle joint instability. In any case, these methods have a major problem due to the loss of normal bone tissues.

Therefore, we planned to prefabricate a vascularized allo-bone graft in the recipient by introducing the recipient vessels into the transplanted allo-bone graft.

Furthermore, we effectively used cytokines to stimulate revascularization and bone formation in the prefabricated vascularized allo-bone graft.

There are several cytokines that stimulate revascularization and bone formation, such as BMP and bFGF.[10–12] In our previous study, we used BMP to stimulate revascularization and bone formation. BMP was administered into transplanted allo-bone graft, and it indeed stimulated revascularization and bone formation significantly. However, it also stimulated bone resorption.[10]

Basic-FGF is known to stimulate angiogenesis and bone formation and not to stimulate bone resorption.[11,12,14] Thus, in this study, bFGF was used as a stimulator for angiogenesis and bone formation. However, free-FGFs are readily degradable in vivo, leading to loss of biological activity and functions.[28–30] Edelman et al reported that when bFGF is delivered without stabilization, bFGF diffuses rapidly, undergoes proteolysis, and consequently loses bioactivity under normal physiological conditions.[31]

To obtain satisfactory performance, FGFs are adsorbed onto or encapsulated within various scaffolds to secure biological activity in a sustained and controllable manner. Reported examples of such scaffolds include alpha tricalcium phosphate,[20] glycosaminoglucan hydrogel,[29] atelocollagen,[17] and HAp/col.[32]

The reasons to use HAp/Col to make bFGF controlled release in this study were that HAp/Col has good absorbency because of its spongiform nature and it is easy to deal with because of its softness.

There are some reports about bone tissue reconstruction using bFGF, in that bFGF can promote osteogenesis and angiogenesis more effectively by letting itself become controlled release.[11–15]

Kawaguchi et al reported that their preliminary experiments using rat and rabbit models demonstrated that the local half-life of the injected 125I-labeled FGF-2 in the gelatin

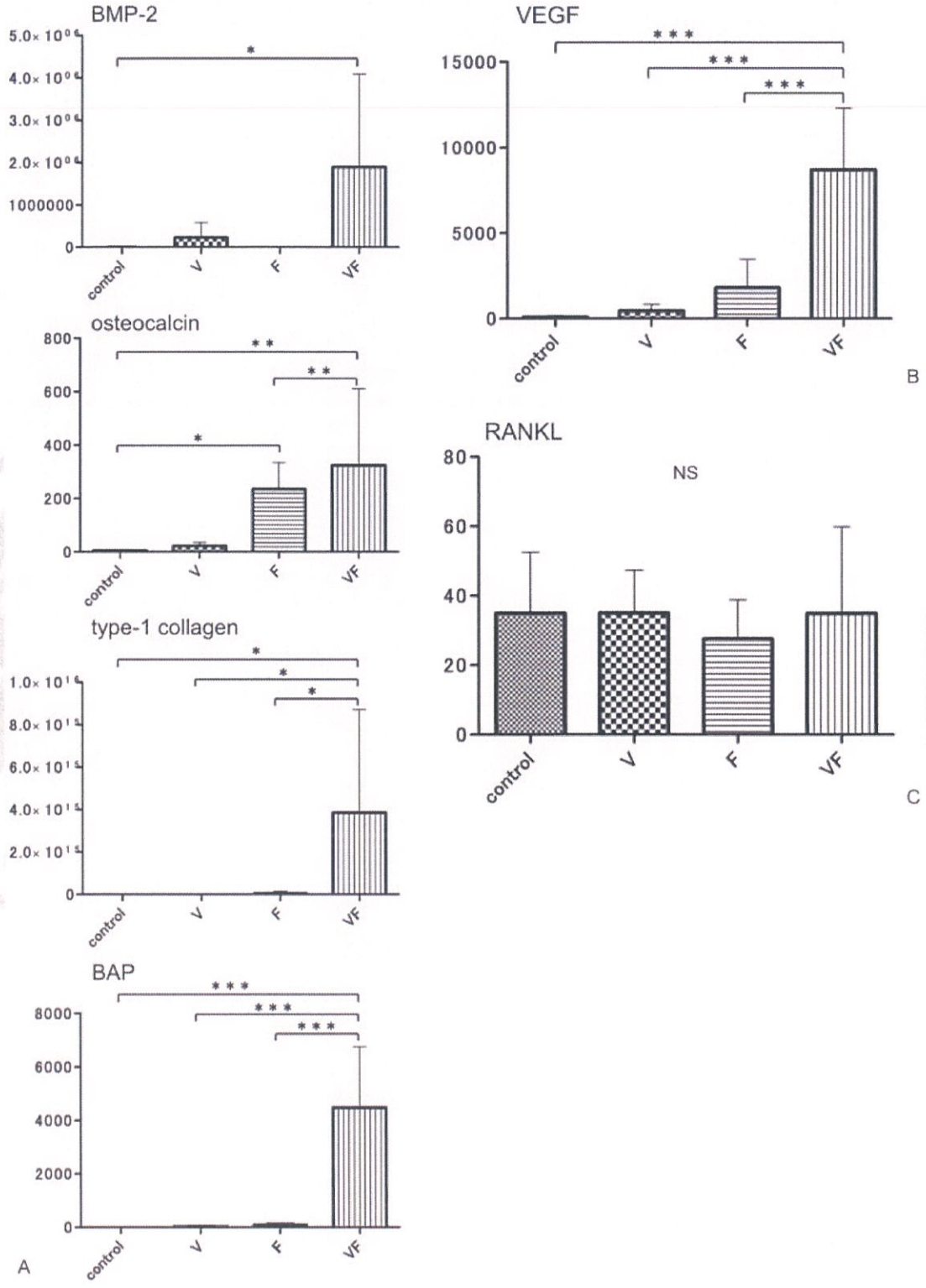

Fig. 8 Relative messenger ribonucleic acid expression of genes related to **(A)** bone formation, **(B)** angiogenesis, and **(C)** bone resorption. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, not significant.

hydrogel was 1 to 2 days, but the percentages of 125I-labeled FGF-2 remaining localized were approximately 20, 3, and 1% at 1, 2, and 3 weeks, respectively.[11] Kimura et al reported that one possible way to artificially enhance in vivo angiogenesis is to allow bFGF to be released in a controlled manner over an extended period of time.[14] Song et al reported that degradation of gelatin matrices containing growth factors with lower water content or higher cross-linking density is slow, resulting in slow and sustained release of growth factors over longer periods of time.[15]

However, there have been no reports using allo-bone grafts filled with bFGF-containing artificial bone.

Controlled-release of bFGF from HAp/Col was confirmed in this study, and HAp/Col is also known to have osseous conduction ability. For these reasons, we thought that HAp/Col might be a good functional scaffold.

There are some reports suggesting that several factors are necessary for bone regeneration. These factors include a functional scaffold, a supply of the cellular ingredients that are required to make osseous tissues, and growth factor.[14,33] In this study, the HAp/Col and allo-bone grafts were used as functional scaffolds, the flow-through vascular bundles was implanted to supply the cellular ingredients, and bFGF was used as the growth factor.

On histological examination, restructured small vessels were observed in the bone marrow area with the vascular bundle and bFGF-containing HAp/Col. Furthermore, in the genetic analysis, the expression levels of the angiogenesis-related gene (VEGF) were higher in the allo-bone graft in which both the vascular bundle and bFGF-containing HAp/Col were added compared with the bone in which only the vascular bundle or bFGF-containing HAp/Col was added.

The results of the present histological and genetic analyses also suggest that bone formation in the allo-bone graft in which both the vascular bundle and bFGF-containing HAp/Col were added, was significantly stimulated compared with that in the allo-bone graft in which only the vascular bundle or bFGF-containing HAp/Col was added.

On PCR analysis, the expressions of the bone formation and angiogenesis parameters were higher in the VF group than in the F group. The reason why the F and VF groups had such different expression patterns on PCR may be as follows. Although there are scaffold and growth factors (bFGF) in the transplanted allo-bone of the F group, the cellular ingredient supply was insufficient because of the lack of the vascular bundle. On the other hand, there was a substantial cellular ingredient supply from the vascular bundle in the VF group, and the supplied rich cellular ingredients were stimulated by bFGF and produced large amounts of bone formative and angiogenetic factors such as BMP-2, VEGF, and so on.

Bone resorption in the allo-bone graft was not stimulated by adding the vascular bundle and bFGF-containing HAp/Col. These facts suggest that by adding vascular bundle and bFGF-containing HAp/Col, we can prefabricate an ideal allo-bone graft that has significant angiogenesis and osteogenesis activities and does not have excessive bone resorptive activity.

The limitation of this study is that it only showed that angiogenesis and osteogenesis in the transplanted allo-bone graft were stimulated by implanting the vascular bundle of the recipient and bFGF-containing HAp/Col, but whether the prefabricated vascularized allo-bone graft has sufficient quality to reconstruct a massive bone defect was not demonstrated. To assess the implanted allo-bone's own quality, it is important to evaluate bone formation, bone resorption, and angiogenesis within the transplanted allo-bone independently. Thus, this study was performed before doing an osteosynthesis study. If an osteosynthesis experiment were performed first, it would not be possible to extract the RNA only from prefabricated vascularized allo-bone. Indeed, to demonstrate clinical efficacy, it is necessary to demonstrate increased bone union rates, but this is the next issue that needs to be addressed. Since this was a limitation of this study, an osteosynthesis study is now being planned.

Conclusion

Angiogenesis and osteogenesis in allo-bone graft in a rat recipient were promoted by adding both the vascular bundle and bFGF-containing HAp/Col. Furthermore, bone resorption of the allo-bone graft was not promoted by this method. Thus, this method is considered to be an ideal way to prefabricate vascularized allo-bone graft.

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