学位論文

Effects of VEGF on Prefabricated Vascularized Bone Allografts in Rats

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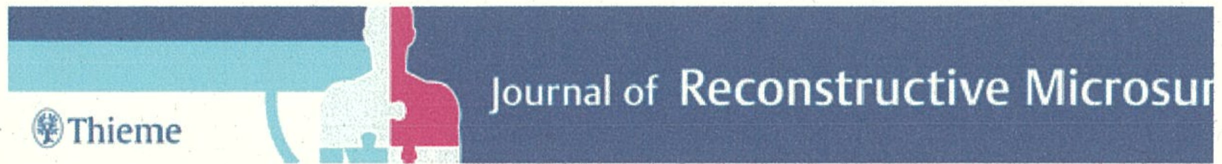

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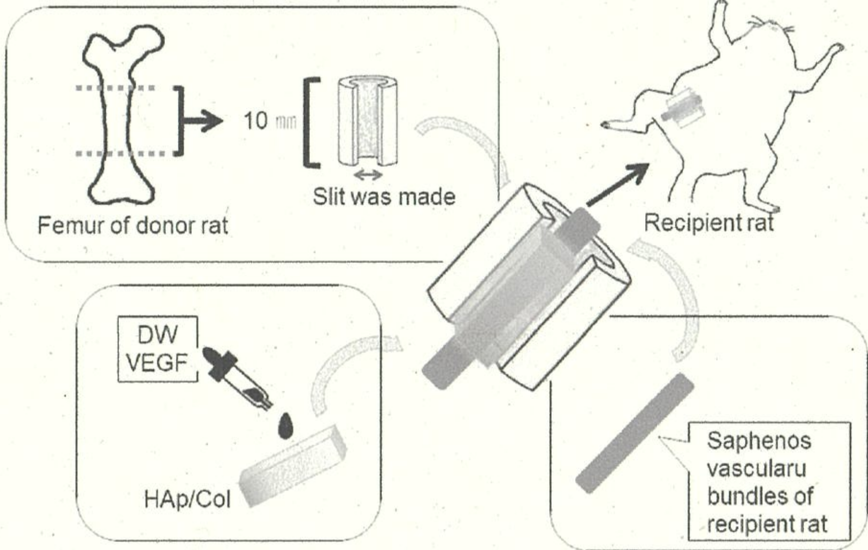

Fig. 1 Surgical procedure used in the present study. Graft bones were collected from the femur of the donor rats. Transplant surgery was performed on the recipient's thigh, and a saphenous vein was passed through the slit in the transplanted bone cavity, followed by HAp/Col containing reagents.

93x59mm (300 x 300 DPI)

Group	Treatment
Group 1 (C group)	Non-vascularized bone allograft
Group 2 (V group)	Vascularized bone allograft
Group 3 (HAp group)	Non-vascularized bone allograft with HAp/Col
Group 4 (HAp +V group)	Vascularized bone allograft with HAp/Col
Group 5 (VEGF+V group)	Vascularized bone allograft with VEGF-containing HAp/Col

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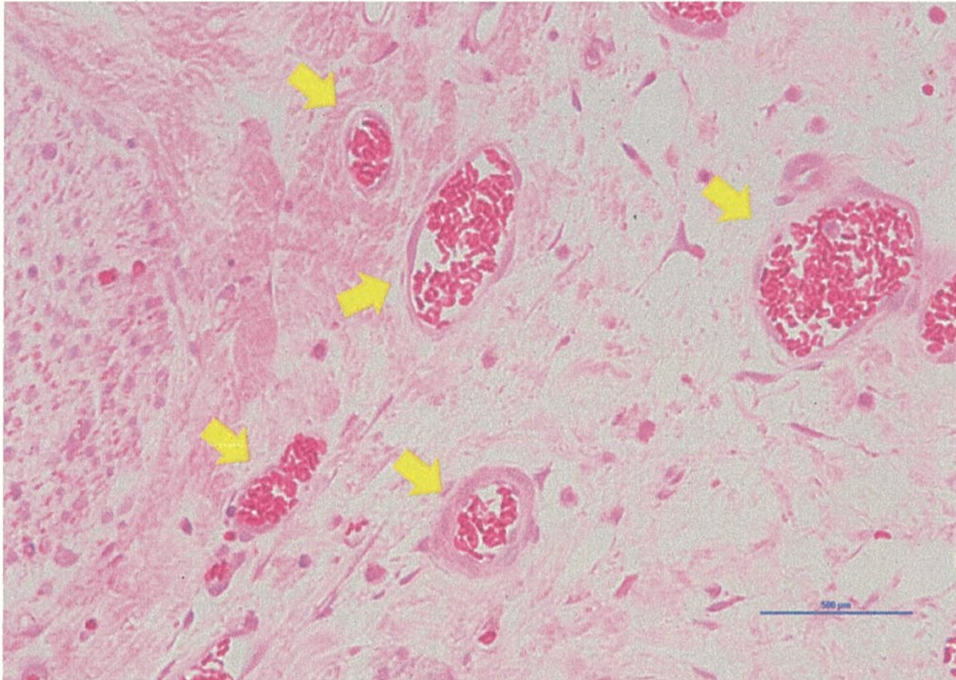

Fig. 2 The luminal structures containing red blood cells were counted as blood vessels (arrow).

71x50mm (300 x 300 DPI)

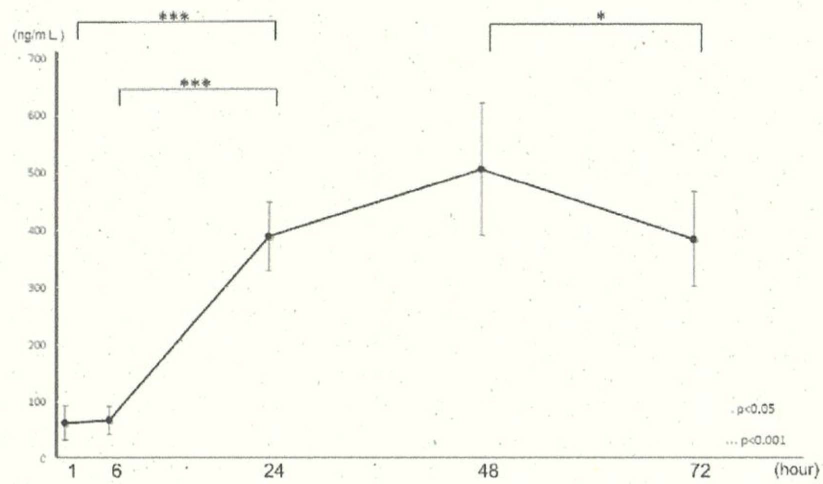

Fig. 3 Time course of vascular endothelial growth factor release. Data are expressed as the mean ± standard deviation of the VEGF concentration at six time points.

88x63mm (300 x 300 DPI)

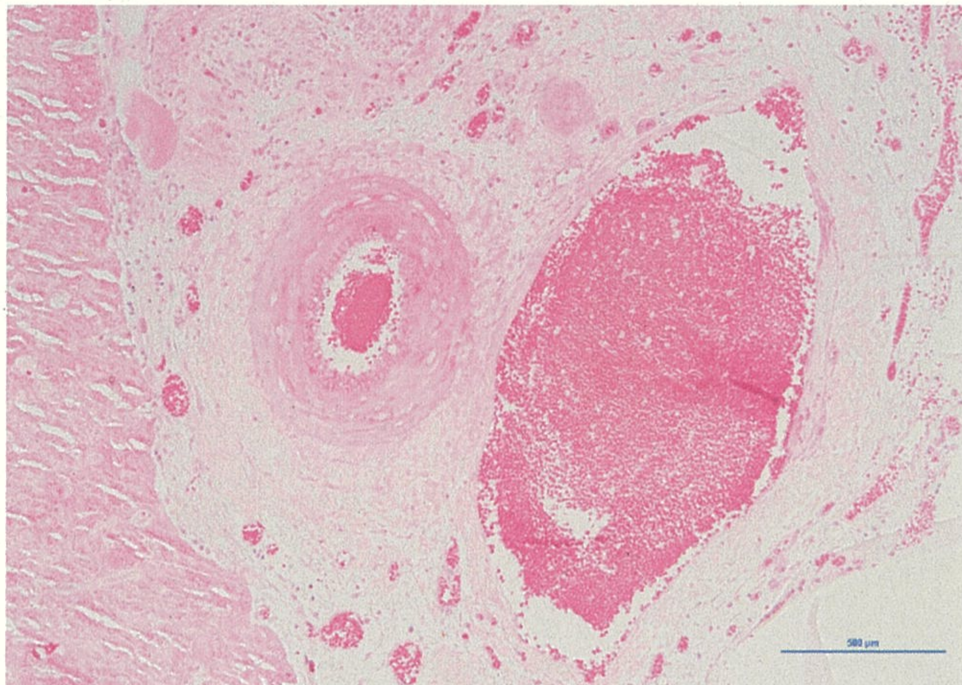

Fig. 4 The vascular bundle grafted through slits into the graft bone. The patency of the implanted vascular bundle and the formation of small vessels around the vascular bundles were confirmed histologically.

84x60mm (300 x 300 DPI)

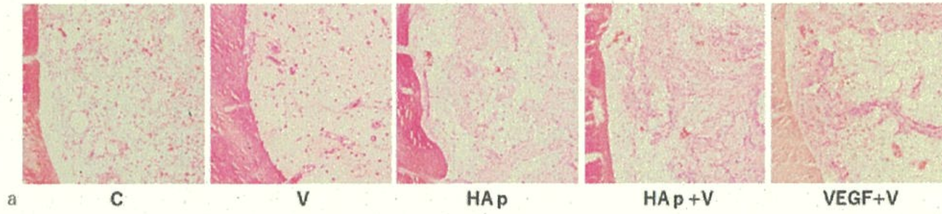

Fig. 5 Evaluation of angiogenesis. a) Decalcified specimen stained with hematoxylin and eosin (100×). The tubular structures with red blood cells were recognized as newly formed blood vessels

107x24mm (300 x 300 DPI)

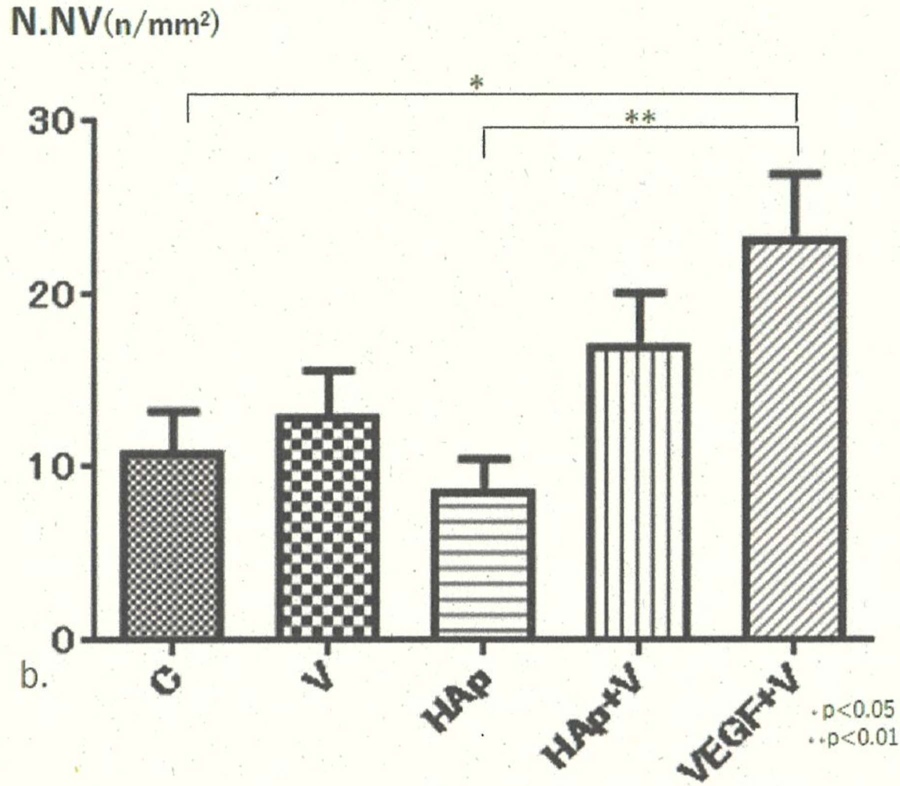

Fig.5 b) The number of newly formed blood vessels in the specimens were counted

53x46mm (300 x 300 DPI)

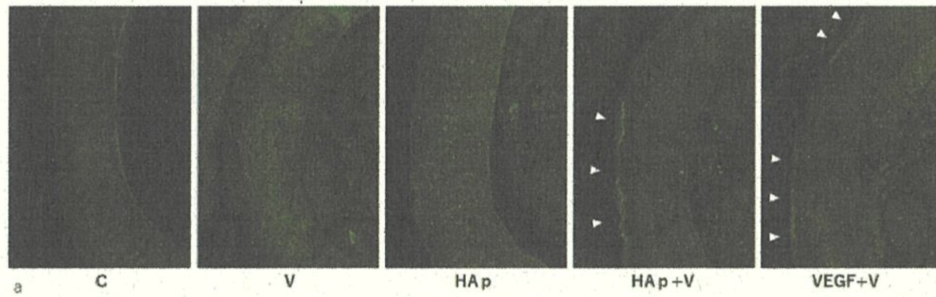

Fig. 6 Evaluation of bone formation. a) Undecalcified specimen. Fluorescence-labeled bone surfaces were recognized as newly formed bone (100×)

120x39mm (300 x 300 DPI)

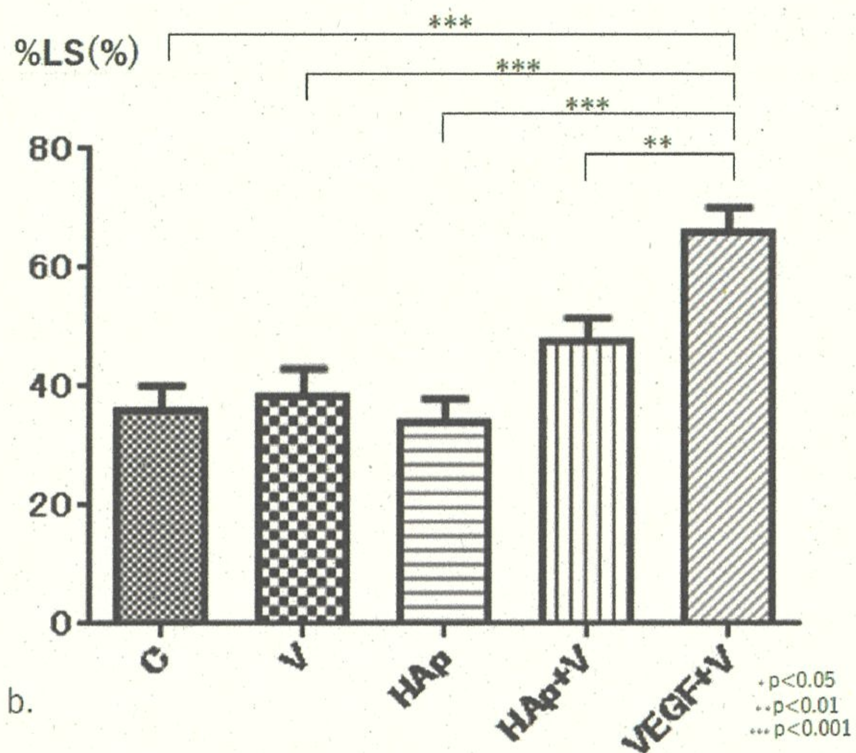

Fig. 6 b) Bone formation was evaluated as the percent of labeled bone surface.

57x48mm (300 x 300 DPI)

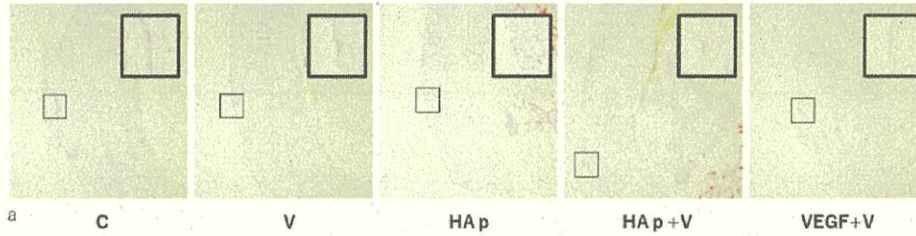

Fig. 7 Evaluation of bone resorption. a) Decalcified specimens stained with tartrate-resistant acid phosphatase (100×). The red stained cells were recognized as osteoclasts

109x28mm (300 x 300 DPI)

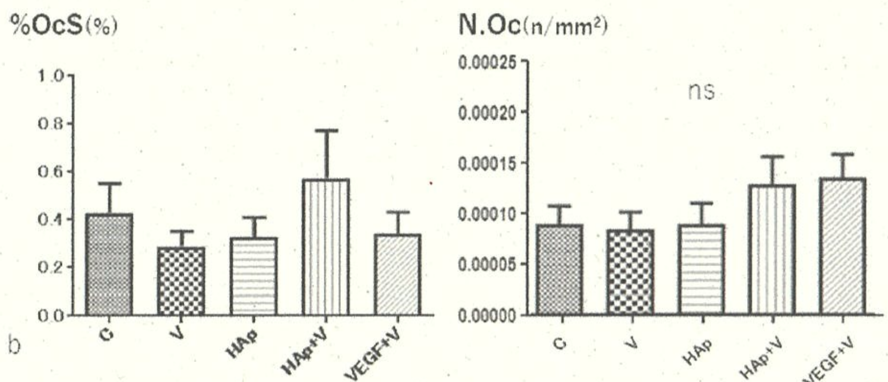

Fig. 7b) Bone resorption was evaluated as the percent of bone surface covered with osteoclasts (%OcS) and the number of osteoclasts on the bone surface (N.Oc).

72x34mm (300 x 300 DPI)

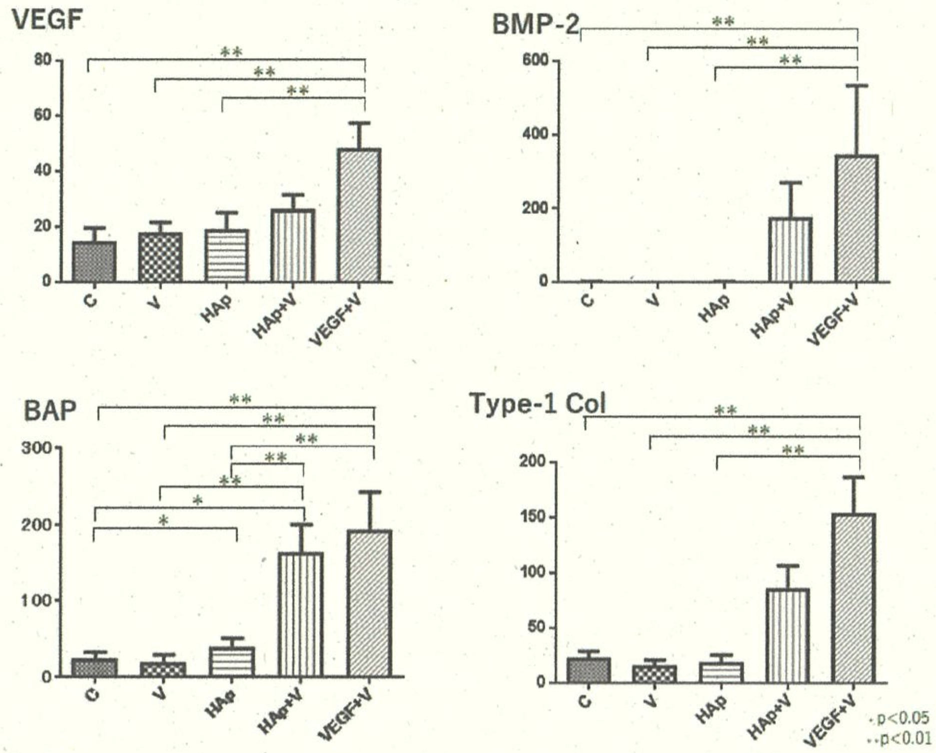

Fig. 8 Expression levels of genes related to angiogenesis and bone formation. VEGF is related to angiogenesis, and BMP-2, BAP, and Type-1 Col are related to bone formation.

73x61mm (300 x 300 DPI)

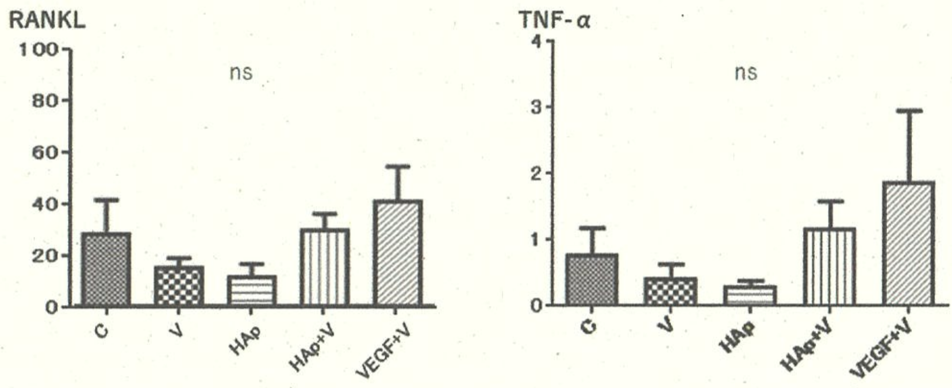

Fig. 9 Expression levels of genes related to bone resorption.

88x41mm (300 x 300 DPI)

Introduction

Massive bone defects after wide resection of malignant bone tumors or a serious injury are difficult to treat and reconstruct in terms of function. Non-vascularized autologous (auto) or allogenic (allo) bone grafts are commonly used to treat bone defects, but neither are suitable for treating large defects (> 6 cm).[1] Existing methods for treating such massive bone defects include vascularized bone autografts,[2–4] cadaveric bone allograft combined with vascularized bone autografts, and prosthetic devices. However, vascularized bone autografts require the scarification of healthy bone tissue, are limited by size. Cadaveric bone allografts combined with vascularized bone autografts are ideal in terms of size and durability, but this treatment also requires scarification. Prosthetic devices are problematic in terms of durability. To solve these problems, we attempted to establish a new method to prefabricate vascularized bone allografts without vascularized bone autograft in recipient rats.

In our previous study, which prefabricated a vascularized bone allograft in recipient rats,[5] the saphenous veins of recipient rats were implanted into non-vascularized bone allografts to establish vascularization. Bone morphogenetic protein (BMP) was then administered into the transplanted bone allograft to stimulate bone formation. The results showed that bone formation was significantly stimulated. However, we also

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observed that bone resorption was significantly stimulated by BMP in the bone allografts, thereby requiring the administration of anti-bone resorptive agents such as bisphosphonate.[6,7] To solve this problem, in the present study, instead of BMP, we used vascular endothelial growth factor (VEGF) because it is known to promote bone formation by promoting angiogenesis, but not to promote bone resorption.[8] In addition, we used a hydroxyapatite/collagen composite (HAp/Col). HAp/Col is an artificial bone made of nanoscale crystals of hydroxyapatite and type-1 atelocollagen that has been shown to have strong osteoconductive activity[9] as a carrier of VEGF.

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The purpose of this study was to prefabricate vascularized bone allografts in recipient rats by implanting vascular bundles with blood flow-through and HAp/Col containing VEGF inside the medulla of the transplanted bone allograft. After prefabrication, histologic and genetic evaluations were conducted in regard to bone formation and resorption.

Materials and Methods

Animals

Sprague Dawley rats (n=50) were used as donors and Wistar rats (n=50) as recipients. All rats were 9 weeks old and purchased from Japan SLC (Hamamatsu, Japan). The

rats were reared in a cage (width: 240 mm, length: 355 mm, height: 200 mm) and allowed to drink water and eat pelleted commercial food freely (Oriental Yeast Co., Tokyo, Japan). This in vivo study was approved by the Institutional Animal Care and Use Committee of our institution (No. 1665) and carried out in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006).

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Preparation of VEGF-containing Artificial Bone

At 2 h before surgery, distilled water (DW) or 10 μg of VEGF (mixed with DW, 1 μg/μL, Pepro Tech, Rocky Hill, NJ, USA) was added into a spongiform porous HAp/Col composite (ReFit; HOYA Technosurgical Co., Tokyo, Japan) (10 mm × 2 mm × 2 mm) and kept at room temperature.

Preparation of Bone Allografts

The graft bones from donor rats were taken from the mid-shaft of the femoral bone and adjusted to 10 mm in length. Next, slits were made for the implantation of flow-through vascular bundles. A Telos Lobator SD-2 Bonebank System was used for heat sterilization (Telos GmbH, Marburg, Germany). The graft bones were heated to 82.5

°C for 15 min using the lobater before being stored in a freezer at –80°C.

Surgical Procedure

Bone graft surgery was performed subcutaneously on the recipient's thigh, and a saphenous vein was passed through the slit in the transplanted bone cavity, followed by HAp/Col containing reagents. Various combinations of HAp/Col and vascularized/non-vascularized were applied (Fig. 1). Next, 10 rats each were categorized into the following five groups: Group 1 was the C group (control group; non-vascularized, transplantation of bone allograft only, no HAp/Col); Group 2 was the V group (vascularized only, no HAp/Col); Group 3 was the HAp group (non-vascularized, used HAp/Col containing DW); Group 4 was the HAp+V group (vascularized, used HAp/Col with DW); and Group 5 was the VEGF+V group (vascularized, used HAp/Col containing VEGF) (Table 1). All surgeries were performed under anesthesia by intraperitoneal injection using pentobarbital sodium (Somnopentyl®; Kyouritsu Seiyaku, Tokyo, Japan) as an anesthetic. The dose used was 5 mg/100 g body weight.

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In Vitro Analysis of VEGF Release Kinetics

HAp/Col composite blocks (10 mm × 2 mm × 2 mm) containing 10 μL of VEGF were placed in a six-well plate and filled with phosphate-buffered saline containing 0.5% bovine serum (10 mL) albumin. Then, HAp/Col composite material was incubated at 37 °C. Six samples were taken at 1, 6, 24, 48, and 72 h, respectively, and concentration of VEGF was measured at each of the five time points. Media samples were collected at each time point and frozen at –80 °C until analysis. The concentration of VEGF precipitated from the HAp/Col composite material was measured with the Quantikine® ELISA Rat VEGF Immunoassay, which is an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA). This experimental model was based on Ishii et al.[10]

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Harvesting of Vascularized Prefabricated Bone Allografts from Recipient Thighs

All graft bones were collected at 4 weeks after transplantation. Two days before sacrificing, bone fluorescent labeling was carried out by subcutaneous injection of 1 mg/100 g calcein (Wako Pure Chemical Industries Ltd., Osaka, Japan). These bone allografts were then subjected to histological and genetic evaluations.

Histological Evaluation

The collected bone allografts were divided into two fragments using a band saw (BS-300CP; Meiwafosis, Tokyo, Japan). One of the fragments was fixed in 10% buffered formalin, and methyl methacrylate (MMA Embedding Medium; Merck, Darmstadt, Germany) was used for the embedding substrate. The processed bone was sliced into 100-μm thick sections and then ground into 30-μm thick sections for the microscopic evaluation of bone formation. Other fragments were fixed in 10% cold-buffered formalin for 3 days, and then decalcified in 10% ethylenediaminetetraacetic acid at 4 °C for 4 weeks. For embedding, glycolic acid methacrylate (JB-4 embedding medium; Polyscience, Warrington, PA) was used. The embedded bone was sliced into 5-μm sections and subjected to hematoxylin and eosin (H&E) and tartrate-resistant acid phosphatase (TRAP) staining.

An FSX100 microscope (Olympus Corp., Tokyo, Japan) was used to carry out the observations, and Olympus Corporation software (Cell Sens Dimension Desktop 1.14) was used to carry out the measurements.

Decalcified Specimens

The evaluation of angiogenesis and bone resorption was carried out using decalcified

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specimens. Using the specimens stained with H&E, the newly formed vessel (N.NV: number newly formed vessel number / total area of bone marrow) was evaluated. A luminal structure with red blood cells in the lumen was counted as a vessel (Fig. 2). In addition, using the specimens stained with TRAP, as parameters of bone resorption, the percentage of osteoclast surface (%OcS: length of bone surface covered with osteoclasts / total length of one surface × 100) and osteoclast number recovered (N.Oc: total osteoclast number / total length of bone surface) were used.

Undecalcified Specimens

Calcein uptake by the mineralized bone was evaluated using undecalcified specimens. As a parameter of bone formation, we used the percentage of labeled bone surface (%LS: length of labeled bone surface / total length of bone surface × 100).

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Genetic Evaluation

Total RNA was obtained from the transplanted bone allografts, which consisted of bone tissue, bone marrow tissue, and vascular bundles. Next, the collected RNA was reverse-transcribed to complementary deoxyribonucleic acid (cDNA) via reverse transcription quantitative polymerase chain reaction (RT-qPCR). These procedures

used ISOGEN (Nippon Gene, Tokyo, Japan), High Capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, CA, USA), Power SYBR-Green PCR Master Mix (Applied Biosystems), and the ECO Real-Time PCR System (Illumina Inc., San Diego, CA, USA). Data were normalized by comparison with a template standard curve of dilution using the ECO Real-Time PCR System Software v3.0. The relative mRNA expression of one gene influencing angiogenesis (VEGF), three genes influencing bone formation (bone morphogenetic protein-2 [BMP-2], bone alkaline phosphatase [BAP], and type-1 Col), and two genes influencing bone resorption (receptor activator of nuclear factor-κB ligand [RANKL] and tumor necrosis factor-α [TNF-α]) were selected. These primers for RT-qPCR were synthesized by Hokkaido System Science Co., Ltd. (Hokkaido, Japan).

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Statistical Analysis

All data in this study were analyzed using GraphPad Prism (ver. 5; GraphPad Software, San Diego, CA, USA). Statistical processing was performed using one-way factorial analysis of variance and Tukey's multiple comparison tests, with $p < 0.05$ set as the level of significance.

Results

Sustained VEGF Release from HAp/Col

The concentration of VEGF in the media sample increased significantly until 24 h, and then showed an increasing tendency until 48 h. Beyond 48 h, no increase in the concentration of VEGF was observed (Fig.3).

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Evaluation of Revascularization and Angiogenesis

At the time of bone collection, bleeding from all transplanted bone allografts and vascular bundles in which vascular bundles had been implanted (the V, HAp+V, and VEGF+V groups) was observed. Moreover, the patency of the implanted vascular bundles and the formation of small vessels around the vascular bundles were confirmed histologically in these three groups (Fig. 4). In the evaluation of angiogenesis using H&E-stained decalcified specimens, N.NV was significantly higher in the VEGF+V than in the C and HAp groups, and N.NV tended to be higher in the HAp+V compared with the C and HAp groups (Fig. 5).

Evaluation of Bone Formation (Undecalcified Specimen)

%LS was significantly higher in the VEGF+V than in the C, V, HAp, and HAp+V

groups. %LS tended to be higher in the HAp+V compared with the C, V, and HAp+V groups, but this difference was not significant (Fig. 6).

Evaluation of Bone Resorption (Decalcified Specimen)

Regarding %OcS, no statistical differences were found between any groups, and no increases in N.Oc were observed (Fig. 7).

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Genetic Analysis

The expression level of VEGF, which is an angiogenesis-related gene, as shown in Figure 8, was significantly higher in the VEGF+V than in the C, V, and HAp groups, and tended to be higher than that in the HAp+V group.

Regarding the bone formation-related genes, the expression level of BMP-2 was significantly higher in the VEGF+V than in the C, V, and HAp groups, and tended to be higher in the HAp+V compared with the C, V, and HAp groups. The expression levels of BAP were significantly higher in the HAp+V and VEGF+V groups than in the C, V, and HAp groups. The expression level of type-1 Col was significantly higher in the VEGF+V than in the C, V, and HAp groups, and tended to be higher in the HAp+V compared with the C, V, and HAp groups.

No increase in the expression levels of the bone resorption-related genes (RANKL, TNF-α) was seenin any of the groups (Fig. 9).

Discussion

Several surgical methods are currently used for reconstructing large bone defects. The transplantation of non-vascularized bone allografts with vascularized bone autografts, such as vascularized fibular grafts, is an effective method.[2,3] However, this procedure requires the scarification of healthy bone tissue. Vascularized bone allografts could therefore be a useful alternative. Although some authors have reported success in vascularized bone allotransplantation using animal models, to preserve the transplanted bone allografts, those studies involved the administration of immunosuppressants, which can cause severe side effects.[4]

The method introduced in the present study prefabricates vascularized bone allografts without the sacrifice of healthy bone tissue and eliminated the need for immunosuppressants. These features are a major advantage of our method.

On the other hand, to promote the use of prefabricated vascularized bone allografts as a therapeutic option, angiogenesis and bone formation need to be accelerated within the transplanted bone. To achieve this, we added osteogenesis-stimulating agents such

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as BMP into the prefabricated vascularized bone allografts.

In our previous experiment, BMP was found to have a strong potential to stimulate bone formation in prefabricated vascularized bone allografts. However, BMP is also known to stimulate osteoclastogenesis and osteoclast function,[6,7] and in fact, bone resorption in the transplanted bone was strongly stimulated, enough to require an attempt to use basic fibroblast growth factor (bFGF) instead of BMP because bFGF has a stimulating effect on bone formation, but unlike BMP, does not have a strong stimulating effect on bone resorption.[11] The results of that study were satisfactory, but because of the very short in vivo half-life of bFGF, a scaffold was needed for controlled release.[11]

This time, we considered the function of blood flow on bone regeneration, and used VEGF because it is known to stimulate angiogenesis strongly.[8,12] As angiogenesis is a very important factor for bone formation,[9,13] we thought that VEGF would also stimulate bone formation through its angiogenetic effect. In fact, the osteogenic effects of VEGF have been well reported.[14–16] On the other hand, similar to bFGF, the half-life of VEGF within tissues is very short.[17] Therefore, we considered that a scaffold would be needed to obtain satisfactory performance in terms of VEGF.

In our previous experiment using bFGF, we used HAp/Col as a scaffold, and it was

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confirmed that bFGF demonstrated sufficient osteogenetic performance and was released from HAp/col for 48 h.[11] Moreover, HAp/Col is known to have good osteoconductive activity, and to be replaced in the bone tissue of the recipient.[18,19] These characteristics of HAp/Col could also offer great advantages as a scaffold for bFGF.

Regarding the scaffold for VEGF, alginate hydrogels,[20] gelatin,[21] chitosan,[22] and nanodiamond-based injectable hydrogels[23] have been used. In this study, we used HAp/Col as a scaffold for VEGF because we had already confirmed that HAp/Col was a useful scaffold for bFGF. As a matter of fact, in the present study, VEGF was released from HAp/Col for 48 h, which suggests that HAp/col also could be a useful scaffold for VEGF.

As mentioned above, VEGF is known to stimulate angiogenesis. In the present study, although the number of newborn blood vessels was not significantly increased in the transplanted bone allograft after the implantation of HAp/Col or the vascular bundles, it was significantly increased by the addition of VEGF in the presence of HAp/Col and the vascular bundles. This fact suggests that even if the transplanted bone allografts are non-vascularized, significant revascularization is induced if VEGF is added in the presence of both a good scaffold and vascular bundles.

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Regarding bone formation, VEGF is also known to stimulate osteoblast function directly.[14] In the present study, based on genetic evaluations, the expression levels of BMP-2, BAP, and Type-1 Col mRNA were significantly stimulated by the addition of VEGF, as was the bone labeling volume in histological evaluations. We speculated that this stimulated osteogenesis was induced by not only the stimulating effect of VEGF on angiogenesis, but also the direct effect on osteoblast function. It is also known that osteoblasts release VEGF.[24] In this study, the stimulated expression of VEGF was also observed in the prefabricated vascularized bone allografts with VEGF-containing HAp/Col at the time of bone collection. We thought that this stimulated expression of VEGF was derived from osteoblasts that proliferated after the addition of VEGF, and that the stimulated production of VEGF induced new angiogenesis and bone formation. Thus, the angiogenesis caused by the addition of VEGF, the osteoblast proliferation caused by the increased angiogenesis and the direct effect of VEGF, and the production of VEGF caused by proliferated osteoblasts may induce a positive spiral.

On the other hand, the stimulating effect of VEGF on osteoclast differentiation and proliferation was not observed either genetically or histologically. Regarding the effect of VEGF on bone resorption, VEGF has been reported to stimulate osteoclast differentiation and function in several studies.[25,26] However, Enoki et al.[27] reported that

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osteoclast differentiation was suppressed when macrophages were cultured with vascular endothelial cells. It was therefore considered that VEGF actually stimulated osteoclast differentiation and function immediately after the bone allograft transplantation. However, angiogenesis was also stimulated simultaneously by VEGF, and the proliferated vascular endothelial cells may have suppressed osteoclast differentiation. As a result, the stimulating effect of VEGF on osteoclastogenesis was thought to be countered, and thus, no stimulated bone resorption was observed at the time of transplanted bone collection.

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A limitation of the present study was that although the stimulating effect of VEGF on bone formation and angiogenesis was confirmed in the vascularized prefabricated bone allografts, whether large bone defects can be effectively reconstructed using this technique remains unclear. However, the results of this study suggest that prefabricated vascularized bone allografts with the addition of VEGF-containing HAp/Col may have considerable potential to reconstruct large bone defects. Further experiments are needed to confirm this hypothesis.

Conclusion

VEGF-containing HAp/Col effectively stimulated angiogenesis and bone formation in

prefabricated vascularized bone allografts, but did not stimulate bone resorption. The method introduced in the present study may therefore become a useful tool for the treatment of massive bone defects after wide resection of malignant bone tumors or serious injuries. Currently, it is possible to preserve different allogeneic bones collected from various parts of the body. Therefore, in clinical practice, it should be possible to select and transplant a bone graft that matches the size and shape of the bone defect. In addition, peripheral vascular bundles, which have previously been used in vascularized flaps, can be selected from around the bone defect and introduced into the bone graft. We believe that this method has merit because it can be completed without sacrificing major blood vessels.

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Introduction

Massive bone defects after wide resection of malignant bone tumors or a serious injury are difficult to treat and reconstruct in terms of function. Non-vascularized autologous (auto) or allogenic (allo) bone grafts are commonly used to treat bone defects, but neither are suitable for treating large defects (> 6 cm).[1] Existing methods for treating such massive bone defects include vascularized bone autografts,[2–4] cadaveric bone allograft combined with vascularized bone autografts, and prosthetic devices. However, vascularized bone autografts require the scarification of healthy bone tissue, are limited by size. Cadaveric bone allografts combined with vascularized bone autografts are ideal in terms of size and durability, but this treatment also requires scarification. Prosthetic devices are problematic in terms of durability. To solve these problems, we attempted to establish a new method to prefabricate vascularized bone allografts without vascularized bone autograft in recipient rats.

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The purpose of this study was to prefabricate vascularized bone allografts in recipient rats by implanting vascular bundles with blood flow-through and HAp/Col containing VEGF inside the medulla of the transplanted bone allograft. After prefabrication, histologic and genetic evaluations were conducted in regard to bone formation and resorption.

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Materials and Methods

Animals

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°C for 15 min using the lobater before being stored in a freezer at −80°C.

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Histological Evaluation

The collected bone allografts were divided into two fragments using a band saw (BS-300CP; Meiwafosis, Tokyo, Japan). One of the fragments was fixed in 10% buffered formalin, and methyl methacrylate (MMA Embedding Medium; Merck, Darmstadt, Germany) was used for the embedding substrate. The processed bone was sliced into 100-μm thick sections and then ground into 30-μm thick sections for the microscopic evaluation of bone formation. Other fragments were fixed in 10% cold-buffered formalin for 3 days, and then decalcified in 10% ethylenediaminetetraacetic acid at 4 °C for 4 weeks. For embedding, glycolic acid methacrylate (JB-4 embedding medium; Polyscience, Warrington, PA) was used. The embedded bone was sliced into 5-μm sections and subjected to hematoxylin and eosin (H&E) and tartrate-resistant acid phosphatase (TRAP) staining.

An FSX100 microscope (Olympus Corp., Tokyo, Japan) was used to carry out the observations, and Olympus Corporation software (Cell Sens Dimension Desktop 1.14) was used to carry out the measurements.

Decalcified Specimens

The evaluation of angiogenesis and bone resorption was carried out using decalcified

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specimens. Using the specimens stained with H&E, the newly formed vessel (N.NV: number newly formed vessel number / total area of bone marrow) was evaluated. A luminal structure with red blood cells in the lumen was counted as a vessel (Fig. 2). In addition, using the specimens stained with TRAP, as parameters of bone resorption, the percentage of osteoclast surface (%OcS: length of bone surface covered with osteoclasts / total length of one surface × 100) and osteoclast number recovered (N.Oc: total osteoclast number / total length of bone surface) were used.

Undecalcified Specimens

Calcein uptake by the mineralized bone was evaluated using undecalcified specimens. As a parameter of bone formation, we used the percentage of labeled bone surface (%LS: length of labeled bone surface / total length of bone surface × 100).

Genetic Evaluation

Total RNA was obtained from the transplanted bone allografts, which consisted of bone tissue, bone marrow tissue, and vascular bundles. Next, the collected RNA was reverse-transcribed to complementary deoxyribonucleic acid (cDNA) via reverse transcription quantitative polymerase chain reaction (RT-qPCR). These procedures

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used ISOGEN (Nippon Gene, Tokyo, Japan), High Capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, CA, USA), Power SYBR-Green PCR Master Mix (Applied Biosystems), and the ECO Real-Time PCR System (Illumina Inc., San Diego, CA, USA). Data were normalized by comparison with a template standard curve of dilution using the ECO Real-Time PCR System Software v3.0. The relative mRNA expression of one gene influencing angiogenesis (VEGF), three genes influencing bone formation (bone morphogenetic protein-2 [BMP-2], bone alkaline phosphatase [BAP], and type-1 Col), and two genes influencing bone resorption (receptor activator of nuclear factor-κB ligand [RANKL] and tumor necrosis factor-α [TNF-α]) were selected. These primers for RT-qPCR were synthesized by Hokkaido System Science Co., Ltd. (Hokkaido, Japan).

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Statistical Analysis

All data in this study were analyzed using GraphPad Prism (ver. 5; GraphPad Software, San Diego, CA, USA). Statistical processing was performed using one-way factorial analysis of variance and Tukey's multiple comparison tests, with $p < 0.05$ set as the level of significance.

Results

Sustained VEGF Release from HAp/Col

The concentration of VEGF in the media sample increased significantly until 24 h, and then showed an increasing tendency until 48 h. Beyond 48 h, no increase in the concentration of VEGF was observed (Fig.3).

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Evaluation of Revascularization and Angiogenesis

At the time of bone collection, bleeding from all transplanted bone allografts and vascular bundles in which vascular bundles had been implanted (the V, HAp+V, and VEGF+V groups) was observed. Moreover, the patency of the implanted vascular bundles and the formation of small vessels around the vascular bundles were confirmed histologically in these three groups (Fig. 4). In the evaluation of angiogenesis using H&E-stained decalcified specimens, N.NV was significantly higher in the VEGF+V than in the C and HAp groups, and N.NV tended to be higher in the HAp+V compared with the C and HAp groups (Fig. 5).

Evaluation of Bone Formation (Undecalcified Specimen)

%LS was significantly higher in the VEGF+V than in the C, V, HAp, and HAp+V

groups. %LS tended to be higher in the HAp+V compared with the C, V, and HAp+V groups, but this difference was not significant (Fig. 6).

Evaluation of Bone Resorption (Decalcified Specimen)

Regarding %OcS, no statistical differences were found between any groups, and no increases in N.Oc were observed (Fig. 7).

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Genetic Analysis

The expression level of VEGF, which is an angiogenesis-related gene, as shown in Figure 8, was significantly higher in the VEGF+V than in the C, V, and HAp groups, and tended to be higher than that in the HAp+V group.

Regarding the bone formation-related genes, the expression level of BMP-2 was significantly higher in the VEGF+V than in the C, V, and HAp groups, and tended to be higher in the HAp+V compared with the C, V, and HAp groups. The expression levels of BAP were significantly higher in the HAp+V and VEGF+V groups than in the C, V, and HAp groups. The expression level of type-1 Col was significantly higher in the VEGF+V than in the C, V, and HAp groups, and tended to be higher in the HAp+V compared with the C, V, and HAp groups.

No increase in the expression levels of the bone resorption-related genes (RANKL, TNF-α) was seenin any of the groups (Fig. 9).

Discussion

Several surgical methods are currently used for reconstructing large bone defects. The transplantation of non-vascularized bone allografts with vascularized bone autografts, such as vascularized fibular grafts, is an effective method.[2,3] However, this procedure requires the scarification of healthy bone tissue. Vascularized bone allografts could therefore be a useful alternative. Although some authors have reported success in vascularized bone allotransplantation using animal models, to preserve the transplanted bone allografts, those studies involved the administration of immunosuppressants, which can cause severe side effects.[4]

The method introduced in the present study prefabricates vascularized bone allografts without the sacrifice of healthy bone tissue and eliminated the need for immunosuppressants. These features are a major advantage of our method.

On the other hand, to promote the use of prefabricated vascularized bone allografts as a therapeutic option, angiogenesis and bone formation need to be accelerated within the transplanted bone. To achieve this, we added osteogenesis-stimulating agents such

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as BMP into the prefabricated vascularized bone allografts.

In our previous experiment, BMP was found to have a strong potential to stimulate bone formation in prefabricated vascularized bone allografts. However, BMP is also known to stimulate osteoclastogenesis and osteoclast function,[6,7] and in fact, bone resorption in the transplanted bone was strongly stimulated, enough to require an attempt to use basic fibroblast growth factor (bFGF) instead of BMP because bFGF has a stimulating effect on bone formation, but unlike BMP, does not have a strong stimulating effect on bone resorption.[11] The results of that study were satisfactory, but because of the very short in vivo half-life of bFGF, a scaffold was needed for controlled release.[11]

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This time, we considered the function of blood flow on bone regeneration, and used VEGF because it is known to stimulate angiogenesis strongly.[8,12] As angiogenesis is a very important factor for bone formation,[9,13] we thought that VEGF would also stimulate bone formation through its angiogenetic effect. In fact, the osteogenic effects of VEGF have been well reported.[14–16] On the other hand, similar to bFGF, the half-life of VEGF within tissues is very short.[17] Therefore, we considered that a scaffold would be needed to obtain satisfactory performance in terms of VEGF.

In our previous experiment using bFGF, we used HAp/Col as a scaffold, and it was

confirmed that bFGF demonstrated sufficient osteogenetic performance and was released from HAp/col for 48 h.[11] Moreover, HAp/Col is known to have good osteoconductive activity, and to be replaced in the bone tissue of the recipient.[18,19] These characteristics of HAp/Col could also offer great advantages as a scaffold for bFGF.

Regarding the scaffold for VEGF, alginate hydrogels,[20] gelatin,[21] chitosan,[22] and nanodiamond-based injectable hydrogels[23] have been used. In this study, we used HAp/Col as a scaffold for VEGF because we had already confirmed that HAp/Col was a useful scaffold for bFGF. As a matter of fact, in the present study, VEGF was released from HAp/Col for 48 h, which suggests that HAp/col also could be a useful scaffold for VEGF.

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As mentioned above, VEGF is known to stimulate angiogenesis. In the present study, although the number of newborn blood vessels was not significantly increased in the transplanted bone allograft after the implantation of HAp/Col or the vascular bundles, it was significantly increased by the addition of VEGF in the presence of HAp/Col and the vascular bundles. This fact suggests that even if the transplanted bone allografts are non-vascularized, significant revascularization is induced if VEGF is added in the presence of both a good scaffold and vascular bundles.

Regarding bone formation, VEGF is also known to stimulate osteoblast function directly.[14] In the present study, based on genetic evaluations, the expression levels of BMP-2, BAP, and Type-1 Col mRNA were significantly stimulated by the addition of VEGF, as was the bone labeling volume in histological evaluations. We speculated that this stimulated osteogenesis was induced by not only the stimulating effect of VEGF on angiogenesis, but also the direct effect on osteoblast function. It is also known that osteoblasts release VEGF.[24] In this study, the stimulated expression of VEGF was also observed in the prefabricated vascularized bone allografts with VEGF-containing HAp/Col at the time of bone collection. We thought that this stimulated expression of VEGF was derived from osteoblasts that proliferated after the addition of VEGF, and that the stimulated production of VEGF induced new angiogenesis and bone formation. Thus, the angiogenesis caused by the addition of VEGF, the osteoblast proliferation caused by the increased angiogenesis and the direct effect of VEGF, and the production of VEGF caused by proliferated osteoblasts may induce a positive spiral.

On the other hand, the stimulating effect of VEGF on osteoclast differentiation and proliferation was not observed either genetically or histologically. Regarding the effect of VEGF on bone resorption, VEGF has been reported to stimulate osteoclast differentiation and function in several studies.[25,26] However, Enoki et al.[27] reported that

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osteoclast differentiation was suppressed when macrophages were cultured with vascular endothelial cells. It was therefore considered that VEGF actually stimulated osteoclast differentiation and function immediately after the bone allograft transplantation. However, angiogenesis was also stimulated simultaneously by VEGF, and the proliferated vascular endothelial cells may have suppressed osteoclast differentiation. As a result, the stimulating effect of VEGF on osteoclastogenesis was thought to be countered, and thus, no stimulated bone resorption was observed at the time of transplanted bone collection.

A limitation of the present study was that although the stimulating effect of VEGF on bone formation and angiogenesis was confirmed in the vascularized prefabricated bone allografts, whether large bone defects can be effectively reconstructed using this technique remains unclear. However, the results of this study suggest that prefabricated vascularized bone allografts with the addition of VEGF-containing HAp/Col may have considerable potential to reconstruct large bone defects. Further experiments are needed to confirm this hypothesis.

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Conclusion

VEGF-containing HAp/Col effectively stimulated angiogenesis and bone formation in

prefabricated vascularized bone allografts, but did not stimulate bone resorption. The method introduced in the present study may therefore become a useful tool for the treatment of massive bone defects after wide resection of malignant bone tumors or serious injuries. Currently, it is possible to preserve different allogeneic bones collected from various parts of the body. Therefore, in clinical practice, it should be possible to select and transplant a bone graft that matches the size and shape of the bone defect. In addition, peripheral vascular bundles, which have previously been used in vascularized flaps, can be selected from around the bone defect and introduced into the bone graft. We believe that this method has merit because it can be completed without sacrificing major blood vessels.

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