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ORIGINAL ARTICLE

Ectopic Bone Induction by BMP-loaded Collagen Scaffold and Bone Marrow Stromal Cell Sheet

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SYNOPSIS

We combined the use of bone morphogenetic protein-2(BMP-2)-loaded collagen scaffold and cultured bone marrow stromal cell (BMSC) sheet in order for developing new bone tissue engineering.

Rat BMSC sheets were generated in the presence of dexamethasone, β -glycerophosphate and ascorbic acid. Four types of the grafts were implanted in the intermuscular region of rat femurs: control group, collagen sponge; cell group, BMSC sheet in collagen sponge; BMP group, BMP-2-loaded collagen sponge; and BMP-cell group, BMSC sheet in BMP-2-loaded collagen sponge. Four weeks after surgery, histological and histomorphometric analysis were performed.

The BMP-cell group demonstrated abundant new bone formation compared to all the other groups ($p<0.05$) and marked resorption of the implanted collagen when compared to the control and cell groups ($p<0.05$).

The combination of BMP-2-loaded collagen sponge and BMSC sheet appeared to successfully promote an osteoinduction.

Key words: bone tissue engineering, bone marrow stromal cell, BMP-2, cell sheet, collagen sponge scaffold

INTRODUCTION

Bone inductive therapies have been developed using tissue engineering with three essential factors, *i.e.*, osteogenic cells, osteoinductive growth factor and a scaffold for bone-forming cells. Bone marrow stromal cells (BMSCs) include multipotent mesenchymal stem cells. BMSCs could differentiate into several cell-types including osteoblasts^{1, 2}, and implantation of BMSCs with various biological scaffolds enables to induce a new bone *in vivo*³⁻⁵. Grafting with cal-

cium alginate to alveolar bone defects in dogs accelerated bone regeneration⁶. Bruder *et al.*⁷ reported that the combined implantation with hydroxyapatite/ β -tricalcium phosphate ceramics and bone marrow-derived mesenchymal stem cells facilitated bone formation: They insisted that a ceramic scaffold carrying cell source could be primarily biocompatible and be able to provide mechanical stiffness compared to the ceramics scaffold *per se*.

A biological scaffold may play an

important role in osteogenic differentiation, angiogenesis and space-preservation for cell repopulation and extracellular matrix reconstruction. In previous studies, several types of scaffolds for bone tissue engineering were utilized: collagen^{8, 9}, gelatin¹⁰, calcium phosphate^{4, 11}, hydroxyapatite^{3, 12}, and synthetic polymer^{5, 13}. Type I collagen is a major component of the extracellular matrix of connective tissue and bone, and therefore, it appears to be a biocompatible scaffold for bone cells. A mixture of fibrillar atelocollagen (FC) and heat-denatured atelocollagen (HAC) sponge (weight ratio, 9:1) have been developed for artificial skin^{14, 15}. FC provides mechanical stiffness to the sponge, while HAC possesses high tissue compatibility. FC-HAC sponge transplanted subcutaneously in rat promoted cell infiltration. Kato *et al.*¹⁶ have demonstrated osteoblastic proliferation, high expression of osteocalcin and resultant new bone in the surgically-operated defects of the mandible, which have been filled with FC-HAC, while FC implantation allowed a less amount of regenerated bone. Therefore, they conjectured that FC-HAC sponge serve as an excellent scaffold for successful bone augmentation. Shimoji *et al.*¹⁷ demonstrated the accelerated bone augmentation by FC-HAC sponge implantation in the perforation area of rat femur, and speculated that FC-HAC served as a scaffold for bone marrow cells. Taken together, FC-HAC sponge appears to be a successful scaffold for osteogenic differentiation, as well as for proliferation and differentiation of BMSCs.

Bone morphogenetic protein-2 (BMP-2) plays a critical role in osteoblastic cell differentiation. BMP-2 stimulates osteoblastic differentiation not only from osteoprogenitor cells¹⁸, but also from non-osteogenic cell lines, such as fibroblasts¹⁹, myoblasts²⁰ and

preadipocytes²¹. Yamagiwa *et al.*²² found that BMSCs could differentiate into osteoblasts by BMP-2. Furthermore, many animal studies^{12, 23} have demonstrated ectopic bone formation *in vivo* by means of BMP-2.

In this study, we propose a new technique for bone tissue engineering, by means of the combined application with cultured BMSCs and BMP-2-loaded FC-HAC sponge. We have performed histological and histomorphometric evaluation on the regenerated bone, assessed the overall efficacy of the approach, and closely investigated the behavior of bone cells.

MATERIALS AND METHODS

The experimental protocol followed institutional animal use and care regulations of Hokkaido University (Animal Research Committee of Hokkaido University, Approval No.08-0104).

Bone marrow cell preparation

Bone marrow stromal cells were obtained from femoral shafts of 9 Fisher 344 male rats (6 weeks old, weight 100-120 g) after euthanasia using an overdose of diethyl ether (Wako, Osaka, Japan). The femur bone was excised aseptically, and the ends of the bone were removed. The bone marrow was flushed out using 5 ml of culture medium (GIBCO[®] MEM α + GlutaMAX, Invitrogen, San Diego, CA, USA) through an 18-gauge needle. The released cells were plated in standard medium: culture medium containing 10% FBS (GIBCO[®] Fetal Bovine Serum Qualified, Invitrogen, San Diego, CA, USA) and antibiotics (GIBCO[®] 100 U/ml penicillin and 100 μ g/ml streptomycin, Invitrogen, San Diego, CA, USA). The cells were kept in an incubator at 37°C in a humidified atmosphere containing 95% air and 5% CO₂.

When the cells reached confluency, BMSCs were subcultured using

conventional techniques employing trypsin-ethylenediamine tetraacetic acid (GIBCO® Trypsin-0.5% EDTA, Invitrogen, San Diego, CA, USA).

Preparation of culture-expanded BMSC sheets

The cells released at passage 3 were seeded at 5×10^5 cells per culture flask (Iwaki Tissue Culture Flask 25 mm², Asahi Glass, Tokyo, Japan). When the cells reached confluency, the culture medium was changed to osteogenic medium: standard medium containing 50 µg/ml ascorbic acid (L (+) - Ascorbic Acid, Wako, Osaka, Japan), 10 mM β-glycerophosphate (Glycerol 2-Phosphate Disodium Salt *n*-Hydrate, Wako, Osaka, Japan) and 100 nM dexamethasone (Wako, Osaka, Japan).

Subsequently, 7×10^5 cells at passage 3 were added to one flask on day 3, 5, and 7, respectively. On day 10, layered cells were detached from the substratum, forming cell sheets by a scraper and trypsin-EDTA treatment (Fig. 1-A). These BMSC sheets were used for the following examinations.

Cytochemical and histological examination of BMSC sheets

ALP staining

BMSCs at passage 3 before layering culture and BMSC sheets were prepared for alkaline phosphatase (ALP) staining. Cells were fixed in 10% buffered formalin and rinsed with phosphate-buffered saline. Then, ALP staining was performed using New Fuchsin Substrate System (Dako, Carpinteria, CA, USA). The fixed cells were soaked and incubated for 10 minutes in Substrate-Chromogen Reagent, which was prepared by mixing 2.5 mol/L tris buffer concentrate (pH 8.8), distilled water, alkaline phosphatase substrate concentrate (pH 8.2), 0.5% new fuchsin solution, and activating agent. Subsequently,

the stained cells were examined using light microscopy.

Morphologic Analysis

BMSC sheets were prepared for morphologic analysis. BMSC sheets were fixed in 10% buffered formalin and embedded in paraffin. Six-micrometer-thick sections were prepared, stained with hematoxylin and eosin (HE), and examined using light microscopy.

Preparation of collagen sponge

Collagen sponge was provided by Olympus Terumo Biomaterials Corp. (Tokyo, Japan), which had been prepared as described previously¹⁵. Atelocollagen (Koken, Tokyo, Japan) in a dilute HCl solution (0.3%; pH 3) was neutralized by adding concentrated phosphate buffer in NaCl to a final concentration of 0.1% collagen, 30 mM Na₂HPO₄ and 0.1 M NaCl. This collagen solution was incubated at 37°C for 4 hours. The resulting fibrous precipitate was referred to as FC. HAC was prepared from atelocollagen in a dilute HCl solution by heating at 60°C for 30 minutes.

A composite of FC (0.3%) and HAC (1%) was prepared by mixing the two at a ratio of 9:1 (w/w), respectively. This composite material was adjusted to a final concentration of 4% and made in a form of a sponge by lyophilization at -30°C. This sponge was dehydrothermally cross-linked at 110°C for 2 hours and used as an FC-HAC sponge. In this study, 34 pieces of FC-HAC sponge of size 4×4×4 mm were used.

BMP-2 construct

Recombinant human BMP-2 (98% purity) was donated by Astellas Pharma (Tokyo, Japan). The BMP-2 was diluted with culture medium (GIBCO® MEMα + GlutaMAX, Invitrogen, USA) to produce a stock solution at 100 µg/mL.

Surgical procedure

Seventeen F344 male rats (10 weeks old, weight 190-210 g) were given general anesthesia with inhalation of diethyl ether and intraperitoneal injections of 0.6 ml/kg sodium pentobarbital (Somnophenyl, Kyoritsu Seiyaku, Tokyo, Japan), and local injection of 2% lidocaine hydrochloride with 1:80,000 epinephrine (Xylocaine Cartridge for Dental Use, Dentsply-Sankin K.K., Tokyo, Japan).

All animals received implants bilaterally at femoral sites. The femoral skin was incised, muscle exposed and implants placed in intermuscular sites of biceps femoris muscle 15 mm from the knee joint (Fig. 1-C). The recipient sites were randomly assigned to four groups and received each implant as described below. **Control group:** FC-HAC sponge soaked with 30 μ l of culture medium. **Cell group:** FC-HAC sponge with BMSC sheet; an FC-HAC sponge was soaked with 30 μ l of culture medium and incised by a scalpel. Subsequently, the BMSC sheet was inserted approximately at the center of the sponge through an incision (Fig. 1-B). **BMP group:** FC-HAC sponge soaked with 30 μ l of BMP solution. **BMP-cell group:** BMP-loaded FC-HAC sponge with BMSC sheet; FC-HAC sponge was soaked with 30 μ l of BMP solution and made an incision. Subsequently, the BMSC sheet was inserted approximately at the center of sponge through an incision.

The skin was closed with nylon sutures (Softretch 4-0, GC, Tokyo, Japan) and tetracycline hydrochloride ointment (Achromycin Ointment, POLA Pharma, Tokyo, Japan) was applied to the wound.

Histological procedure

Four weeks postsurgery, the animals were euthanized using an overdose of diethyl ether. Implants were excised with

surrounding tissues, fixed in 10% buffered formalin, decalcified in 10% EDTA (pH 7.0), and embedded in paraffin according to standard procedures. Six-micrometer-thick sections were prepared and stained with hematoxylin and eosin (HE). The stained sections were examined using light microscopy.

Histomorphometric analysis

Five of the stained sections were selected for histomorphometric measurements; one was approximately in the central area of the implant and the other four were at 600 and 1200 μ m from either side of the central area, respectively. The areas of newly formed bone and residual sponge were measured by a software package (ImageJ 1.41, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Means and standard deviations of newly formed bone area and residual sponge area were calculated for each group. Statistical analysis was performed using one-way ANOVA/Games Howell post-test for newly formed bone area and Kruskal-Wallis test/Bonferroni's modification of Mann-Whitney *U* test for residual sponge area. *P*-values <0.05 were considered statistically significant. All the statistical procedures were performed using a software package (SPSS 11.0, SPSS Japan, Tokyo, Japan).

RESULTS

Cytochemical and histological observations of BMSC sheets

Although BMSCs did not show ALP-positivity (Fig. 2-A), the BMSC sheet came to be positive for ALP (Fig. 2-B and C). The BMSC sheet with approximately 50 μ m thickness contained highly increased cell population and abundant extracellular matrices (Fig. 2-D).

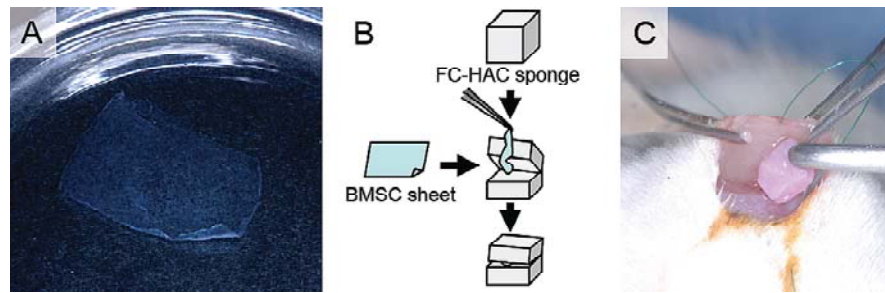


Figure 1. Implantation of the FC-HAC sponge with BMSC sheet. A) BMSC sheet. B) BMSC sheet was inserted approximately at the center of the FC-HAC sponge incised by a scalpel. C) Rats received implants at intermuscular sites.

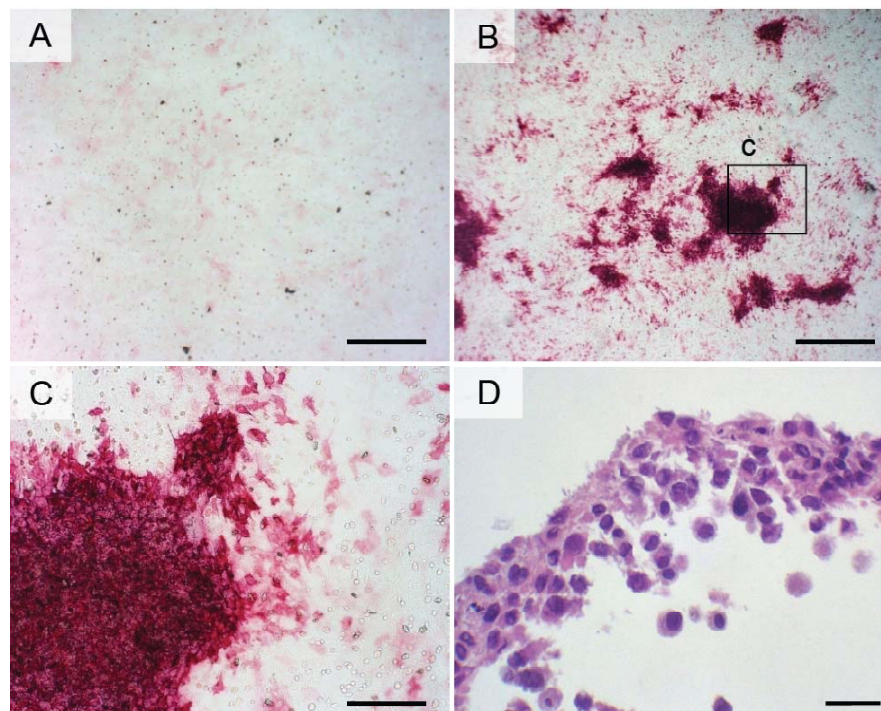


Figure 2. Cytochemical and histological findings of BMSC sheets. A) ALP staining of BMSCs before layering culture. Cells were not positive for ALP staining. B) ALP staining of BMSC sheet. Cells were positive for ALP staining. C) Higher magnification of the framed area (c) in B. D) HE staining of BMSC sheet. Scale bars: A and B = 1 mm; C = 200 µm; D = 20 µm.

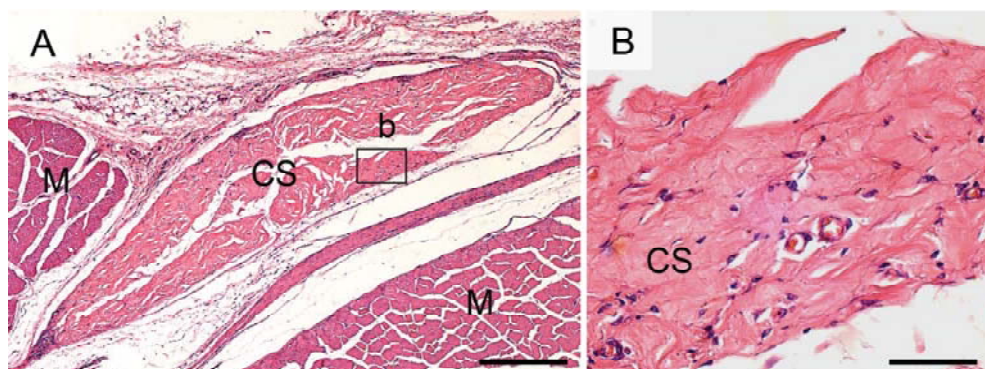


Figure 3. Histological findings in the control group. A) Residual FC-HAC sponge was displayed at the intermuscular site. B) Higher magnification of the framed area (b) in A. Fibroblast-like cells infiltrated into the FC-HAC sponge. M, muscle; CS, FC-HAC sponge; HE staining; scale bars: A = 500 µm; B = 50 µm.

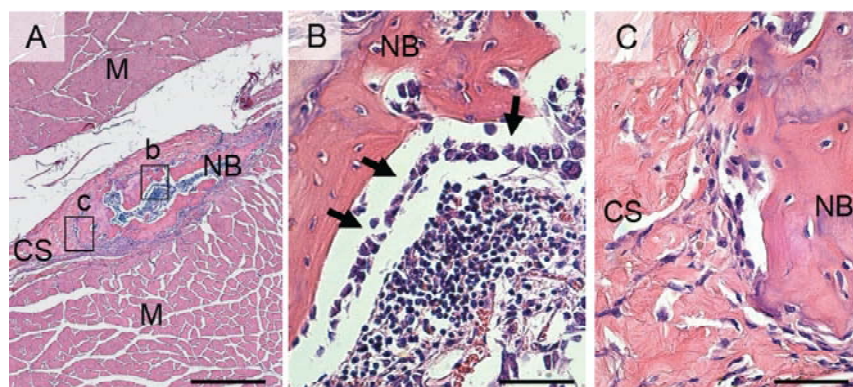


Figure 4. Histological findings in the cell group. A) Newly formed bone was observed at the intermuscular site. B) Higher magnification of the framed area (b) in A. Developed trabecular bone demonstrated localized many osteoblasts (arrow) and a few osteoclasts, and embedded osteocytes. Bone marrow included haematopoietic cells, adipocytes and blood vessels. C) Higher magnification of the framed area (c) in A. FC-HAC sponge including several fibroblastic cells remained in the periphery of newly formed bone. M, muscle; CS, NB, new bone; FC-HAC sponge; HE staining; scale bars: A = 500 μ m; B and C = 50 μ m.

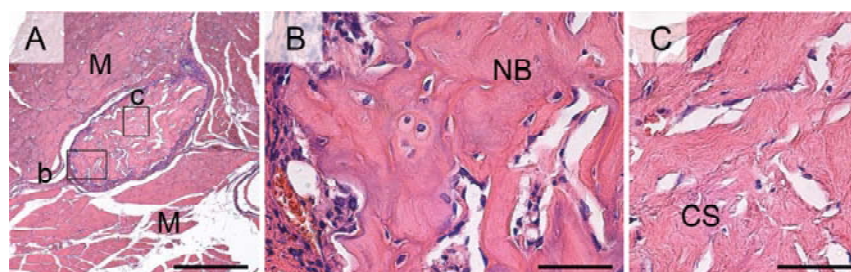


Figure 5. Histological findings in the BMP group. A) Ectopic bone induction was observed at the intermuscular site. B) Higher magnification of the framed area (b) in A. Newly formed bone included few osteocytes, osteoblasts, and little marrow tissue. C) Higher magnification of the framed area (c) in A. Residual FC-HAC sponge was partly discernible. M, muscle; NB, new bone; CS, FC-HAC sponge; HE staining; scale bars: A = 500 μ m; B and C = 50 μ m.

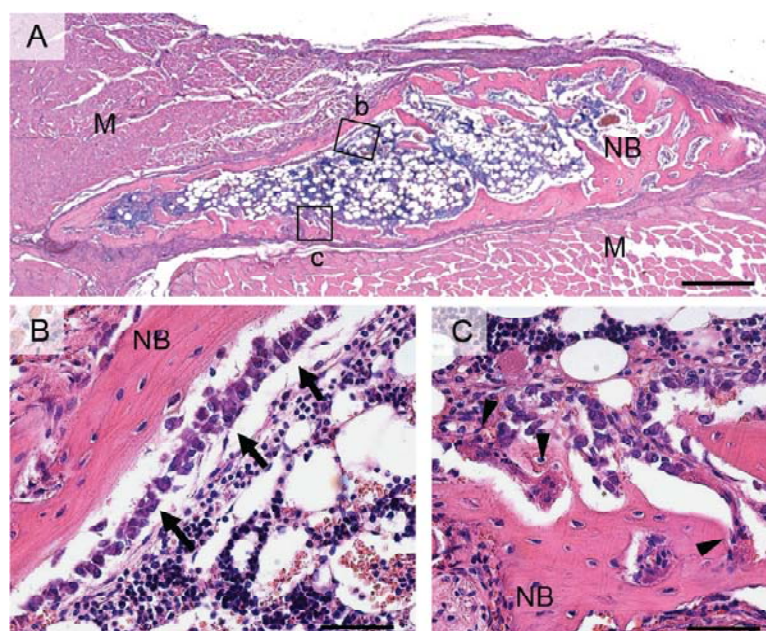


Figure 6. Histological findings in the BMP-cell group. A) A large amount of newly formed bone appeared at the intermuscular site. B) Higher magnification of the framed area (b) in A. Many osteoblastic cells (arrow) had covered the surfaces of trabeculae. Bone marrow included haematopoietic cells, adipocytes and blood vessels. C) Higher magnification of the framed area (c) in A. Osteoclastic cells (arrow heads) surrounded trabecular bones. M, muscle; NB, new bone; HE staining; scale bars: A = 500 μ m; B and C = 50 μ m.

Histological observations

Control group: Newly formed bone was not evident, and instead, all recipient sites were filled with connective tissue. Residual FC-HAC sponge was noticeable in seven of eight specimens (Fig. 3-A). Few inflammatory cells were seen, and fibroblast-like cells had infiltrated into the FC-HAC sponge (Fig. 3-B). **Cell group:** Five of eight specimens demonstrated new bone with developed trabecular bones (Fig. 4-A) which localized many osteoblasts and a few osteoclasts, and embedded osteocytes. Bone marrow included haematopoietic cells, adipocytes and blood vessels. Trabeculae of regenerated bone revealed many cement lines indicating active bone remodeling (Fig. 4-B). In four specimens, FC-HAC sponges including several fibroblastic cells were observed in the periphery of newly formed bone (Fig. 4-C). Inflammatory cells were rarely seen in both new bone and the remnants of FC-HAC sponge. **BMP group:** Six of eight specimens exhibited new bone (Fig. 5-A). However, the newly formed bone included few osteocytes, osteoblasts and little marrow tissue (Fig. 5-B). Residual FC-HAC sponge was partly discernible (Fig. 5-C). **BMP-cell group:** A large amount of newly formed bone appeared in all

specimens (10/10 sites) (Fig. 6-A). Newly formed trabeculae had numerous cement lines implying accelerated bone remodeling, and many osteoblastic cells had covered the surfaces of trabeculae. Bone marrow included haematopoietic cells, adipocytes and blood vessels (Fig. 6-B). Many osteoclasts surrounded trabecular bones: some were localized on the trabeculae, while others were separated but close to them (Fig. 6-C). FC-HAC sponge rarely remained.

Histomorphometric analysis

Histomorphometric parameter on the new bone area was $0 \pm 0 \text{ mm}^2$ (control group), $0.049 \pm 0.062 \text{ mm}^2$ (cell group), $0.237 \pm 0.290 \text{ mm}^2$ (BMP group) and $2.045 \pm 1.329 \text{ mm}^2$ (BMP-cell group): Bone formation in the BMP-cell group was significantly greater than all the other groups (Fig. 7-A).

The index of residual sponge area in the control group, cell group, BMP group and BMP-cell groups was $0.633 \pm 0.576 \text{ mm}^2$, $0.128 \pm 0.190 \text{ mm}^2$, $0.053 \pm 0.150 \text{ mm}^2$, and $0.005 \pm 0.016 \text{ mm}^2$, respectively. Grafted collagen had been markedly-reduced in the BMP-cell group compared to the control and cell groups. FC-HAC sponge broadly remained in the control group, compared to the BMP group (Fig. 7-B).

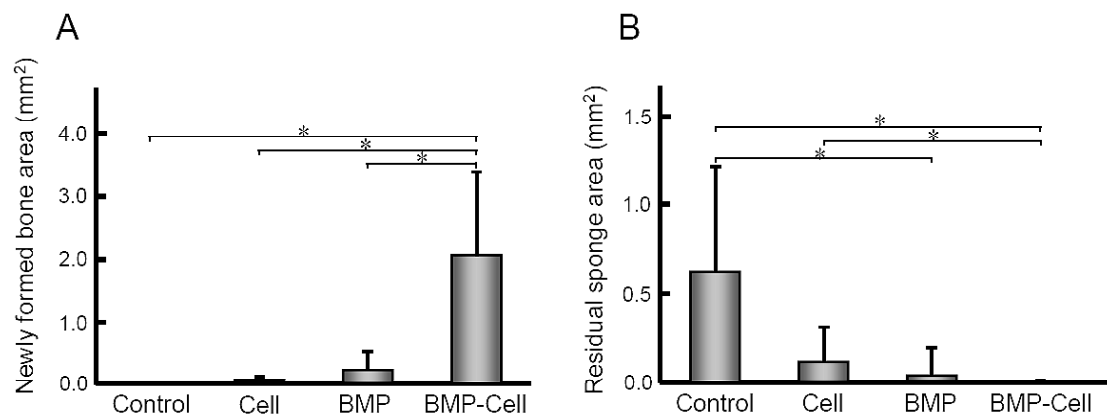


Figure 7. Histomorphometric analysis. A) Newly formed bone area, B) Residual sponge area observed for all groups. The results are expressed as mean $\pm$ standard deviation (SD). * ($p < 0.05$). Statistical differences in each group were analyzed using one-way ANOVA/Games Howell post-test for A, and Kruskal-Wallis test/Bonferroni's modification of Mann-Whitney U test for B.

DISCUSSION

For implantation of BMSCs to ectopic sites, a cell-sheet system was selected in this study. In regenerative therapy, it is well known that viable cell transplantation can promote tissue regeneration; various cell suspensions were seeded onto a biological scaffold and then implanted immediately^{3, 7}. Cell sheet engineering was recently developed as a novel method for cell transplantation^{24, 25}. Cell sheets are advantageous as a large number of cells can be easily transplanted into a recipient site and cell-to-cell junctional protein and extracellular matrix like collagen are retained in the sheet structure. Furthermore, Cell sheets are able to rapidly adhere to surrounding tissues²⁴. Therefore, it seems likely that, in this study, BMSC sheets take advantage for tissue regeneration, due to numerous cell transplantation and rapid adhesion to collagen sponges.

The histological findings of this study demonstrated ectopic bone formation by the implantation of BMSC sheets in the cell group. The BMSCs might contain multipotent mesenchymal stem cells, which could differentiate into several cell types including osteoblasts^{1, 2}. Many studies using BMSCs with various biological scaffolds showed new bone formation in the transplantation site³⁻⁵. In this study, BMSCs cultured in osteoinductive medium increased ALP activity. Many studies have reported that BMSCs expressed ALP and osteoblast-related proteins in the presence of β -glycerophosphate and dexamethasone^{1, 2, 26, 27}. Therefore, BMSCs might differentiate into osteoblastic cells by cultivation in medium added these chemicals. On the other hand, Miyamoto et al.²⁸ demonstrated that BMSCs, when cultured in type I collagen gel, revealed an intense ALP-activity and gene expressions for osteoblastic differentiation including bone sialoprotein,

osteocalcin, osteopontin and type I collagen $\alpha 1$ chain, which therefore indicate a pivotal role of collagen during osteoblastic differentiation of BMSCs. Mizuno et al.²⁹ also revealed in a mouse study that the subcutaneous implantation of BMSCs, which had been cultured in type I collagen gel, caused ectopic bone formation. Therefore, it seemed likely that osteoblastic differentiation of BMSCs was directly stimulated by the type I collagen in the FC-HAC sponge.

Abundant bone formation was seen in the BMP-cell group compared to other groups. BMP-2 has the ability to transform pluripotent stem cells into osteoprogenitor cells¹⁹ and induce osteogenesis^{12, 23}. Implantation of BMP-2 with collagen material induced ectopic bone formation in animal studies^{8, 9}. BMP-2 loaded into FC-HAC sponge promoted BMSCs proliferation and differentiation into osteoblasts, suggesting stimulated bone formation. Therefore, the biological event for osteogenesis in the BMP-cell group might be affected by the combined application of BMP and BMSCs implantation. Osteoblastic MC3T3-E1 cells show chemotactic reaction toward culture medium containing BMP-2³⁰. From this evidence, BMP might stimulate proliferation, differentiation and migration of mesenchymal stem cells in intermuscular sites in the BMP and the BMP-cell groups.

In the BMP group, newly formed bone had little bone marrow and blood vessels. Many studies have demonstrated that bone marrow cells can differentiate into vascular endothelial cells^{31, 32}. Fujimura et al.³³ reported the relationship between osteoinductive activity of BMP-2 and blood flow. Considering these reports, less vascularization in the BMP group might cause less bone formation than the BMP-cell group. On the other hand, hard tissue resorption by osteoclasts participate in hard tissue formation and maturation in a coupling

phenomenon during ectopic bone formation with BMP application^{34, 35}. In the cell and the BMP-cell groups, many osteoclastic cells appeared in the periphery of newly formed bone and trabeculae had numerous cement lines. Therefore, the bone construction of the BMP group might be poorer than that of the cell and the BMP-cell groups due to low bone remodeling activity.

In the present study, we applied FC-HAC sponge as a regenerative scaffold. The control group noted significant residual FC-HAC sponge and no inflammatory cell infiltration. Thus, as been shown by previous studies, FC-HAC sponge is a biocompatible material^{14, 15}. Residual collagen was observed at the periphery of newly formed bone in the cell group, whereas little was seen in the BMP-cell group. It was considered that BMP-loaded FC-HAC sponge might allow not only proliferation of implanted sheet-forming cells but also migration of undifferentiated mesenchymal cells around the implanted FC-HAC sponge in the early stage. Consequently, the early exchange of space between degrading collagen and newly formed bone may be performed.

CONCLUSION

Implantation of BMP-loaded FC-HAC sponge with BMSC sheet stimulated ectopic bone induction. It is reasonable to expect that combination of BMP-loaded FC-HAC sponge and BMSC sheet may provide a more effective tissue engineering approach for bone reconstruction.

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