Bone regeneration with osteogenic matrix cell sheet and tricalcium phosphate: An experimental study in sheep

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AIM
To determine the effects of a cell sheet created from sheep bone marrow and tricalcium phosphate (TCP) on osteogenesis.

METHODS
Bone marrow cells were harvested from a sheep and cultured in a minimal essential medium (MEM) containing ascorbic acid phosphate (AscP) and dexamethasone (Dex). After 2 wk, the formed osteogenic matrix cell sheet was lifted from the culture dish using a scraper. Additionally, harvested bone marrow cells were cultured in MEM only as a negative control group, and in MEM with AscP, Dex, and β-glycerophosphate as a positive control group. For in vitro evaluation, we measured the alkaline phosphatase (ALP) activity and osteocalcin (OC) content in the media of the cultured cells from each group. For in vivo analysis, we prepared an experimental group comprising TCP scaffolds wrapped with the osteogenic matrix cell sheets and a control group consisting of the TCP scaffold only. The constructs were implanted into sheep with a dehiscence created in the lateral femoral condyle of the femur.
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implanted subcutaneously into athymic rats and the cell donor sheep, and bone formation was confirmed by histology after 4 wk.

RESULTS

In the in vitro part, the mean ALP activity was 0.39 ± 0.03 mg/well in the negative control group, 0.67 ± 0.04 mg/well in the sheet group, and 0.65 ± 0.07 mg/well in the positive control group. The mean OC levels were 1.46 ± 0.33 ng/well in the negative control group, 3.92 ± 0.16 ng/well in the sheet group, and 4.4 ± 0.47 ng/well in the positive control group, respectively. The ALP activity and OC levels were significantly higher in the cell sheet and positive control groups than in the negative control group (P < 0.05). There was no significant difference in ALP activity or OC levels between the cell sheet group and the positive control group (P > 0.05). TCP constructs wrapped with cell sheets prior to implantation showed bone formation, in contrast to TCP scaffolds alone, which exhibited poor bone formation when implanted, in the subcutaneous layer both in athymic rats and in the sheep.

CONCLUSION

This technique for preparing highly osteoinductive TCP may promote regeneration in large bone defects.

Key words: Cell sheet; Osteogenesis; Sheep; Bone marrow; Mesenchymal stromal cell

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Core tip: An osteogenic matrix cell sheet derived from sheep bone marrow enhances osteogenic differentiation. We found that the osteogenic matrix cell sheets on tricalcium phosphate discs efficiently promotes bone formation.

Kira T, Akahane M, Omokawa S, Shimizu T, Kawate K, Onishi T, Tanaka Y. Bone regeneration with osteogenic matrix cell sheet and tricalcium phosphate: An experimental study in sheep. World J Orthop 2017; 8(10): 754-760. Available from: URL: http://www.wjgnet.com/2218-5836/full/v8/i10/754.htm DOI: http://dx.doi.org/10.5312/wjo.v8.i10.754

INTRODUCTION

Massive bone defects that result from trauma or tumor resection, osteomyelitis, or osteonecrosis require bone grafting and represent a great burden in clinical practice. Although an autologous bone graft transferred as either vascularized or non-vascularized tissue remains the gold standard to treat bone defects, the graft procedure is associated with complications at donor sites[1,2]. Allografts carry high risks of infections, immunological rejection, and poor rate of bone-healing[3]. Although artificial bone material possesses some osteoinductive and osteoconductive activities, its osteogenic potential is limited[4].

Bone marrow mesenchymal stromal/stem cells (BMSCs) are capable of differentiating into osteoblasts, chondrocytes, or adipocytes in vitro and are widely applied in bone tissue engineering[5]. They are preferably combined with scaffolds to prevent the BMSCs from flowing out of the target site[6].

We previously developed a new technique of BMSC transplantation using osteogenic matrix cell sheets (OMCSs) derived from rat BMSCs to induce osteogenesis[7]. Because these OMCSs do not require a scaffold and maintain intercellular networks with the extracellular matrix that they produce, these sheets can be used in various graft sites in animal models[8,9]. Furthermore, these OMCSs produce growth factors, such as bone morphogenetic protein and vascular endothelial growth factor. Therefore the OMCSs represent an ideal candidate for promoting new bone formation. However, no studies have investigated in vivo osteogenesis of OMCSs in a large animal model.

This study aimed to investigate whether OMCSs could promote in vivo osteogenesis in a sheep model. The sheep is a frequently used model for orthopedic research for several reasons: The bone size is large enough to allow complex orthopedic procedures to be performed and for medical devices and biomaterials to be tested; the lifespan of the animal is short enough for age-related studies in diseases such as osteoarthritis and osteoporosis to be performed; and bone remodeling in sheep is comparable to that in humans[10,11].

MATERIALS AND METHODS

BMSC preparation

BMSCs were obtained from the humeral head of a 2-year-old male Corriedale sheep (40.0 kg body weight; Japan Lamb, Hiroshima, Japan) by bone marrow aspiration under general anesthesia with intravenous atipamezole (20 µg/kg IV, ZENOAQ, Fukushima, Japan) and induction with intravenous ketamine (2 mg/kg IV, Daiichi Sankyo Propharma, Tokyo, Japan). The aspirated cells were collected in two 75-cm² culture flasks (Falcon; BD Biosciences, Franklin Lakes, NJ, United States) containing 15 mL of regular medium comprising minimal essential medium (Nacalai Tesque, Kyoto, Japan) supplemented with 15% fetal bovine serum (Gibco Life Technologies, Carlsbad, CA, United States) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin; Nacalai Tesque). Cells were cultured in a humidified atmosphere of 95% air and 5% carbon dioxide at 37 °C. After reaching confluence (at approximately day 14), the primary cultured cells were released from the culture substratum using trypsin-EDTA (Nacalai Tesque).

OMCS preparation and cell culture

To create OMCSs, the cells released from the primary culture were seeded at 2 × 10⁵ cells/cm² in culture dishes
for subculture in regular medium containing 10 nmol/L dexamethasone (Dex, Sigma, St. Louis, MO, United States) and 0.28 mmol/L L-ascorbic acid phosphate magnesium salt n-hydrate (AscP, Wako Pure Chemical Industries, Kyoto, Japan) until they reached confluence (at approximately day 14). After two rinses with PBS (Gibco), the cell sheet was lifted using a scraper. The cell sheet was easily detached from the culture dish by gentle scraping in PBS, starting from the periphery of the sheet (Figure 1A). As positive and negative controls for osteoblastic differentiation, respectively, released cells were also seeded at the same cell density and cultured in osteoinductive medium (containing 10 nmol/L Dex, 0.28 mmol/L AscP, and 10 mmol/L β-glycerophosphate) or in regular medium (without Dex, AscP or β-glycerophosphate) until they reached confluence.

**In vitro study**

**Alkaline phosphatase activity measurement:** Alkaline phosphatase (ALP) activity was measured in cells cultured in 12-well plates (Falcon), as reported previously [12]. For each condition, six wells were evaluated. ALP activity is represented as the amount of p-nitrophenol released after 30 min of incubation at 37 °C. The measurements were repeated twice.

**Osteocalcin measurement:** The Osteocalcin (OC) content of the culture medium was measured by an ELISA developed in a previous study [13]. Briefly, conditioned medium was collected at day 12 and an aliquot (100 µL) of 1:10 diluted medium was analyzed. The OC measurements evaluated four wells for each group, and the measurements were repeated twice.

**In vivo study**

**Construction of tricalcium phosphate scaffold wrapped with a OMCS:** Sterilized porous beta tricalcium phosphate (TCP) ceramics (Superpore; discs: 5 mm in diameter and 2 mm thick; 60% porosity) were purchased from Pentax (Tokyo, Japan). Constructs with OMCSs were prepared by wrapping the OMCS around the TCP immediately previous to transplantation (Figure 1B and C). Construct implantation in the subcutaneous layer of athymic rats and the cell donor sheep: TCP constructs were implanted into the subcutaneous layer on the backs of athymic 7-wk-old male F344/Ncid-mu/nu rats (CLEA Japan Inc., Tokyo, Japan) as described previously [7,14]. Additionally, constructs were implanted subcutaneously into the abdomen of the cell donor sheep under the general anesthesia conditions described above. We also prepared control groups in which TCP discs were implanted subcutaneously into athymic rats and at a different site in the sheep. For each group, six constructs were implanted into one recipient rat to produce the subcutaneous implantation model, and all constructs were implanted into the cell donor sheep. After 4 wk, the implanted constructs were harvested and bone formation was histologically evaluated. The harvested discs were fixed in buffered formalin (Wako Pure Chemical Industries). Each disc was embedded in paraffin after decalcification, cut through the middle of the disk, and then stained with hematoxylin and eosin for histological evaluation.

**Animal care and use statement**

The care and handling of the rats and the sheep used in this study was approved by the animal care committee of our institute and met the standards of the National Institutes of Health.

**Statistical analysis**

The ALP activity values and OC levels are presented as mean and SD. One-way analysis of variance with post-hoc multiple comparisons using Tukey’s test was conducted to determine statistical significance. Values of P < 0.05 were considered statistically significant.

**RESULTS**

**In vitro study**

In the in vitro study, the mean ALP activity was 0.39 ± 0.03 mg/well in the negative control group, 0.67 ± 0.04 mg/well in the sheet group, and 0.65 ± 0.07 mg/well in the positive control group (Figure 2). The mean OC levels were 1.46 ± 0.33 ng/well in the negative control group, 3.92 ±
0.16 ng/well in the sheet group, and 4.4 ± 0.47 ng/well in the positive control group, respectively. The ALP activity and OC levels were significantly higher in the cell sheet and positive control groups than in the negative control group (\( P < 0.05 \)). There was no significant difference in ALP activity or OC levels between the cell sheet group and the positive control group (\( P > 0.05 \)).

**In vivo study**

Figures 3 and 4 show representative histological sections of constructs subcutaneously implanted at 4 wk into athymic rats and the cell donor sheep, respectively. The low-magnification images show higher levels of bone formation in the TCP-cell sheet construct sections than in sections of TCP discs without the cell sheet, in
DISCUSSION

Our study demonstrates that we can successfully use large animal models to derive and culture OMCS, and that TCP constructs wrapped with sheep OMCSs lead to bone formation after implantation. This is important, as sheep bone healing is more comparable to that of humans than rodents used in previous models.

Even though BMSCs alone have osteogenic potential, their implantation as a cell suspensions leads to an uneven distribution and weak adhesion of cells to the bone surface, potentially leading to cell defluvium from the target site\(^{15}\). In contrast, cell sheets bear intact cell-cell junctions and an extracellular matrix, which provide mechanical support to the cell and thereby maintain the viability of the cells at the transplanted site\(^{16}\). Furthermore, prior culture with medium that includes agents that potentiate bone formation, such as Dex and AscP, allows for already pre-determined cells to be implanted\(^{17}\), which have a better chance of laying down additional matrix and integrating with the transplant site than cells that are still actively engaged in proliferation.

BMSCs including osteoblasts and osteoprogenitor cells are bone-forming cells that express various osteoblastic markers such as OC, and exhibit ALP activity\(^{18,19}\). ALP is considered a relevant biochemical marker in osteoblast differentiation. The activity and correct localization of ALP are necessary for bone development and differentiation\(^{20,21}\). OC is considered a late marker of osteogenic differentiation and its expression at high levels indicates maturation and terminal differentiation of osteoblasts\(^{22}\). Others have suggested that Dex may inhibit OC expression through direct binding of the Dex-activated glucocorticoid receptor to negative glucocorticoid response elements in the OC promoter\(^{23}\). However, we observed enhanced OC expression in OMCSs compared with the negative control group\(^{23}\). The effect of Dex is dependent on its concentration and on the stage of cellular differentiation. The Dex concentration used in this study (10 nmol/L) is considered an appropriate concentration for the differentiation of BMSCs committed to the osteogenic lineage. The ALP activity and OC levels observed in the OMCS suggest that the osteogenic differentiation ability of the BMSCs was enhanced by AscP and Dex.

OMCSs can be easily detached from the plastic dish and transplanted subcutaneously with or without a scaffold, and has osteogenic potential to form new bone tissue. The TCP scaffold also has osteoinductive properties in bone; however, minimal osteogenesis was observed inside the TCP without the cell sheet in athymic rats, presumably because of the small number of osteoprogenitor cells available for integration into the disc in the subcutaneous layer. Conversely, substantial amounts of new bone was found inside the TCP that had

Figure 4  Hematoxylin and eosin-stained sections at 4 wk after implantation into the subcutaneous layer of the donor sheep. A low-magnification image of a β-tricalcium phosphate (TCP) disc without the cell sheet shows poor bone formation (A and B) whereas relatively high level of bone formation is visible in and around TCP wrapped with an osteogenic matrix cell sheet cultured in minimum essential medium containing ascorbic acid and dexamethasone (C and D). Asterisks indicate bone tissue.
been wrapped with an OMCS. We suggest that these transplanted sheep cells from the OMCSs migrated into the TCP disc, proliferated, differentiated into osteoblasts, and mineralized, and thus induced bone formation.

Results similar to those for athymic rats were obtained for implantation in the cell donor sheep. We have previously investigated cell sheets derived from rodent bone marrow and reported their efficacy for osteogenesis, angiogenesis and reconstructive surgery\cite{9,24,26}. We found that severe fracture and nonunion could be united by the implantation of OMCSs\cite{25}, and that OMCSs can enhance early bone tunnel healing in a tendon graft model\cite{8}. Furthermore, we show that a vascularized tissue-engineered bone scaffold composed of OMCSs wrapped around vascular bundles within a TCP mediates abundant vascularization and osteogenesis\cite{25}. Although these studies reported that OMCS transplantation is useful for bone reconstruction, large animal studies are required to further support the potential of OMSC transplantation for clinical application. Guo et al\cite{26} created cell sheets using canine cells, and showed that cell sheets exhibited normal activity and a preserved extracellular matrix and multilayer cell structure, and displayed osteogenic induction. They used AscP (vitamin C) to create cell sheets from bone marrow mesenchymal stem cells, whereas we used a combination of AscP and Dex to create our cell sheets. Culturing cell in the medium containing AscP and Dex can stimulate osteogenic differentiation and complete cell sheet formation; therefore, using induction medium containing AscP and Dex may be more suitable for creating cell sheets for application in bone reconstruction surgery\cite{17}.

We chose a sheep model because sheep have bone properties similar to those of humans, with similar bone turnover and remodeling activities\cite{27,28,29}. Furthermore, their size allows for the simultaneous implantation of many TCP constructs\cite{26,27,28}, and the subcutaneous implantation approach used in this study permitted the implantation of multiple TCP constructs in one sheep. Thus, the results of these in vivo studies suggest that human OMCSs can induce high levels of osteogenesis in TCP.

There were a few limitations in our study. First, we only used 2-mm-thick TCP. In future studies, we aim to use thicker TCP discs to assess their osteogenic ability when transplanted with OMCSs. Second, the experimental period in the present study was relatively short. Therefore, a longer follow-up study is required to investigate whether the implanted TCP remains in the sheep or is broken down and resorbed by the newly formed tissue. Third, we did not confirm whether the bone that formed in the TCP construct was derived from host cells or donor cells. Finally, we need to verify osteoinductive and osteoconductive ability of human OMCSs. Concerning these points, further study will be necessary.

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**ACKNOWLEDGMENTS**

We would like to thank to Ms Kunda F and Ms Matsumura M (Nara Medical University) for their technical assistance.
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**P-Reviewer:** Emara KM, Searfi S  **S-Editor:** Ji FF  **L-Editor:** A  **E-Editor:** Lu YJ
