The Significance of Performing Osteogenic Differentiation in Human Bone Tissue-Derived Mesenchymal Stromal Cells

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SYNOPSIS

An important element of treatment for Cleft lip/cleft palate is the attainment of normal occlusion. We recultured human bone tissue-derived mesenchymal stromal cells (hBT-MSCs) in vitro following cryopreservation for ≥10 years, divided the cells into osteogenic differentiation and nondifferentiation MSC groups, and compared the cells in the two groups. In vitro expression of osteoblast markers was then measured. Furthermore, we created a hybrid-type bone substitute and transplanted it into the skulls of 8-week-old male nude rats. Eight weeks later, the skulls were examined by Micro CT. We then performed microscopic analysis of grafts after hematoxylin and eosin (HE) staining and calculated the surface ratio. Immunohistochemical staining with an anti-human osteocalcin antibody was also performed and NEO STEM™ expression was confirmed by fluorescence microscopy. With regard to osteoblast marker expression, alkaline phosphatase and osteonex were significantly elevated in the osteogenic differentiation MSC group (P < 0.05). Micro CT revealed osteogenesis in the hybrid-type bone substitute grafts. HE staining clearly indicated new bone formation within porous hydroxyapatite in the hybrid-type bone substitute grafts. Immunohistochemical staining indicated anti-human osteocalcin antibody expression and NEO STEM™ expression in the sites exhibiting new bone formation. The volume of bone formation was also significantly higher in the osteogenic differentiation MSC group than in the nondifferentiation MSC group (P < 0.05). We observed excellent osteogenic capability of hBT-MSCs following cryopreservation for ≥10 years, and we believe that our hybrid-type bone substitute can be adapted for future clinical application.

Key words: mesenchymal stem cells, hybrid-type bone substitute, osteogenic differentiation, cryopreservation

INTRODUCTION

Cleft lip/cleft palate (CL/CP) is a congenital abnormality that occurs in approximately 1 in every 500 Japanese infants. Affected patients require multiple surgical procedures with different objectives such as gain-of-function and cosmetic improvement. An important element of treatment for CL/CP is the attainment of normal occlusion. This requires bone formation in the defects of the alveolar cleft along with proper tooth
eruption. Currently, grafting of autologous iliac crest cancellous bone to the alveolar cleft region is the treatment of choice for bone formation in bone defects\textsuperscript{1,2}.

The ideal surgical period for this secondary bone grafting is between 5 and 8 years of age, when the anterior teeth are replaced by permanent teeth. However, more than one bone graft procedure may be needed for successful treatment, and multiple procedures may be required if the collection of enough cancellous bone is difficult because of the patient’s young age or if the bone graft gets absorbed. We are involved in the research and development of bone substitutes using tissue engineering to decrease the level of surgical invasion in such CL/CP cases. Research on bone-graft substitutes generally uses mesenchymal stem cells acquired via bone marrow puncture. In contrast, our basic research uses MSCs\textsuperscript{3,4} acquired from the primary culture of the surplus iliac bone tissue obtained for secondary bone grafting. We have previously established a separation / proliferation / cryopreservation method for collecting bone-derived MSCs from bone tissue\textsuperscript{5}. We have also used tissue engineering to develop a hybrid-type bone substitute using bone-derived MSCs following long-term cryopreservation\textsuperscript{6}.

With the aim of clinical application of this bone substitute, we have previously reported basic research on the osteogenic capability of hBT-MSCs when subcutaneously implanted in the dorsal regions of animals\textsuperscript{7}. Here, we investigated the amount of bone formation that could be expected with hBT-MSCs in bone tissue areas suitable for clinical application. First, we analyzed the expression of osteoblast markers to examine the \textit{in vitro} bone-forming potential of these hBT-MSCs. Next, we used an \textit{in vivo} experiment to examine whether osteogenic differentiation is necessary as a pretreatment before transplanting these cells into bone graft areas.

**MATERIALS AND METHODS**

This study was approved by our ethics committee and individual consent was obtained in each cases. All animal experiments were conducted within the guidelines for animal experimentation established by the Center for Genetic Studies of Integrated Biological Functions (Kitasato University School of Medicine, no.2012-068). We used MSCs that had undergone long-term cryopreservation at \(-80^\circ\text{C}\). The cells had originally been obtained after primary culture of the surplus iliac bone tissue collected during secondary bone grafting performed in the Department of Plastic and Cosmetic Surgery at the Kitasato University School of Medicine. Eleven samples were obtained from 6 male and 5 female patients aged 5–18 years (mean age: 8.9 years). No subjects had any infections or diseases of note.

1. **Cryopreservation of primary culture and hBT-MSCs**

Collected bone cells were seeded into 25-cm\(^2\) flasks (Sumitomo Bakelite Co, Tokyo, Japan) in a basal medium of alpha-minimum essential medium (\(\alpha\)-MEM; Life Technologies Corporation, Carlsbad, CA) in which 10% fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO) and antibiotics (100 U/mL penicillin, 100 \(\mu\)g/mL streptomycin) were added. Cells were cultured in 5% CO\(_2\) at 37\(^\circ\text{C}\), with the medium being changed twice every week. Once the cells became subconfluent, they were transferred into 75 cm\(^2\) flasks, suspended in Cell-banker\textsuperscript{™} (Nippon Zenyaku Kogyo Co., Ltd. Fukushima, Japan), which contains blood serum, and were cryopreserved at \(-80^\circ\text{C}\).
2. Reculture and osteogenic differentiation
The cryopreserved hBT-MSCs were thawed at room temperature, placed into the basal medium described above with the addition of 1-ng/mL b-FGF (R and D Systems, Inc., Minneapolis, MN), and cultured in 5% CO₂ at 37°C. One week later, cells were confirmed to be subconfluent, and some of these cells were then subcultured under the same conditions to continue proliferation. These cells were labeled as the nondifferentiation MSC group. Osteogenic differentiation was induced in the remainder of the cells by adding 10⁻⁷ mol/L dexamethasone (Sigma-Aldrich), 50 µmol/L L-ascorbic acid (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 10-mmol/L β-glycerophosphate (Calbiochem, Merck, Whitehouse station, NJ). These cells were labeled as the osteogenic differentiation MSC group and were cultured for 1 additional week, with the medium being changed twice every week. NEO-STEM™ (Bite-rials Co., Ltd.) at a concentration of 0.2 mg/mL was added to the culture as an osteoblast marker 48 hours before transplantation.

3. In vitro evaluation of osteoblast marker expression
Markers for assessing osteoblastic activity of the cells were chosen based on the report by Ogata et al. Thus, using real-time reverse transcriptase-polymerase chain reaction (RT-PCR), we measured the expression of runt-related gene 2 (Runx2), ALP, osteocalcin, and osterix in the nondifferentiation MSC group and the osteogenic differentiation MSC group. Total RNA was extracted from cultures using

Figure 2  Schematic diagram of the experiment
HA, hydroxyapatite; real-time RT-PCR, real-time reverse transcriptase–polymerase chain reaction.
the RNeasy® Mini kit (Qiagen, Valencia, CA) and reverse-transcribed into cDNA as per the manufacturer’s instructions. PCR primer sequences were designed using the Primer 3 software program from Gen Bank. Primer sequences are listed in Table 1. All 11 specimens were analyzed and statistical significance was assessed using the unpaired Student’s t-test (*P < 0.05, ** P < 0.01).

4. Seeding of hBT-MSCs in HA
The present study used 5mm diameter, 2mm thick HA discs with 85% porosity (Ca_{10}[PO_{4}]_{6}[OH]_{2}; Pentax, HOYA, Tokyo, Japan) as a scaffold. The nondifferentiated MSCs and osteogenic differentiated MSCs grown from cryopreserved hBT-MSCs were seeded into HA at 1 × 10^5 cells / 30 µL, placed into 6 well cell culture plates (Sumilon, Sumitomo Bakelite), and incubated for 24 h in 5% CO_2 at 37°C. These samples were then referred to as hybrid-type bone substitute.

5. Transplantation into nude rats
We created 9 samples of hybrid-type bone substitute and transplanted them into 8 week-old male nude rats (F344/Njcl-run/run, Clea Japan, Tokyo, Japan) under anesthesia, which was induced by intramuscular injections of a mixture of 0.5 mg/kg of midazolam, 0.5 mg/kg of vetrophage, and 0.3 mg/kg of medetomidine. Mid-sagittal skin incisions extending up to the level of the periosteum were made. After elevating the periosteum, 5mm holes were made in the left and right sides of the skull with a trepan having an external diameter of 5mm. Areas close to the sutures of the skull were avoided. Care was taken to ensure that the holes only removed the layers of the skull bone without damaging the dura mater. Our hybrid-type bone substitute was then grafted, with the side on which cells were seeded positioned next to the dura mater. Rats in whom the skull holes were made without grafting (skull-loss model) were used as controls. Eight weeks after the transplant procedure, a trepan with an external diameter of 11 mm was used to extract the bone substitute samples along with normal bone.

6. CT imaging
The extracted samples were imaged with Micro CT (inspeXio SMX-90CT; Shimadzu Co., Tokyo, Japan) to analyze bone density.

7. Histological and immunohistochemical assessments
The extracted samples were fixed with 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries Ltd, Japan), decalcified for 24 hours with K-CX (Falma CO, Ltd, Japan), and cleaned before paraffin embedding. The middle section of each sample was set as the sagittal section and slices of approximately 4μm thickness were

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<th>Table 1 Gene specific primers used in present study</th>
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Runx2: Runt-related gene 2
ALP: Alkaline phosphatase
made. These slices were then microscopically observed after HE staining. To confirm that the bone tissue formed was derived from the engrafted human cells, we performed immunohistochemical staining with the anti-human osteocalcin antibody.

In addition, according to a report by Takeda et al., we observed the specimens using fluorescence microscopy to confirm NEO STEM™ expression. Bone formation volume was investigated using Image J version 1.36b (National Institutes of Health, USA http://rsb.info.nih.gov/ij/) analytical software to calculate the surface ratio using microscopic observations of HE stained specimens.

RESULTS

1. Osteoblast marker expression according to real-time RT-PCR

Expressions of ALP and osterix were significantly higher in the osteogenic differentiation MSC group than the non-differentiation MSC group ($P < 0.05$) (Figure 3). Although the expressions of runX2 and osteocalcin were also higher.

![Figure 3](image_url) Osteoblast marker expression according to real-time RT-PCR
Significant differences were noted for ALP and osterix expression but not for Runx2 or osteocalcin (*$P < 0.05$, **$P < 0.01$)

![Figure 4](image_url) Micro CT images
A. Skull-loss model
B. Hybrid-type bone substitute grafting
Figure 1 Grafting of hybrid-type bone substitute into nude rats.
A. Skull hole creation
B. Hybrid-type bone substitute grafting

Figure 5 Histological and immunohistochemical staining
A. HE staining, low magnification: Clear new bone formation in HA pores of the hybrid-type bone substitute.
B. Immunohistochemical staining, low magnification: Anti-human osteocalcin antibody
C. Immunohistochemical staining, low magnification: NEO STEM™
Cells positive for anti-human osteocalcin NEO STEM™ expression were observed in bone formation regions, confirming formation of new bone from human-derived cells.

Figure 6 Comparison of histological staining and immunohistochemical staining
A. HE staining of osteogenic differentiation cells
B. Anti-human osteocalcin antibody in osteogenic differentiation cells
C. HE staining of nondifferentiation cells
D. Anti-human osteocalcin antibody in nondifferentiation cells
Bone tissue in HA pores indicated that rat-derived bone tissue and human-derived bone tissue were working together to fill the bone defects. The area of human-derived bone tissue formation was extensive in the osteogenic differentiation group.
in the osteogenic differentiation MSC group than in the nondifferentiation MSC group, no statistically significant difference was apparent.

2. Results of Micro CT
No bone formation was noted in the skull-loss model. Bone formation was observed in the hybrid-type bone substitute graft areas.

3. Histological assessments and immunohistochemical assessments
HE staining did not indicate any new bone formation in the control group, but new bone formation was clearly observed within the HA pores of the hybrid-type bone substitute. Immunohistochemical staining revealed cells positive NEO STEM™ expression and anti-human osteocalcin antibody in the areas of new bone formation. These results confirmed that new bone was formed and that it had originated from the human-derived cells. Bone tissue within HA pores indicated that rat-derived osteoblasts and human-derived osteoblasts were working in unison to heal the bone defects. The area of human-derived bone tissue formation was extensive in the osteogenic differentiation group. Bone formation volume was also significantly higher in the osteogenic differentiation MSC group than in the nondifferentiation MSC group \((P < 0.05)\).

DISCUSSION
Embryonic stem cells (ES cells) and MSCs are well-known cell sources for tissue engineering research\(^{11-14}\). In particular, in bone substitute research, MSCs from bone marrow aspirates acquired by bone marrow puncture are a common cell source. However, MSCs form just 0.01% of the bone marrow aspirate collected. In order to obtain the amount of cells required for regenerative medicine, cells need to be cultured and osteogenic differentiation factors need to be added to pluripotent MSCs to force them to differentiate into osteoblasts. We focused our research on using outgrowth MSCs from primary cultures of surplus bone tissue collected during secondary bone grafting and that removed during jaw deformity surgery as a cell source. While we have previously reported bone formation by cells after short term cryopreservation, the present study investigated the potential of cells that had undergone cryopreservation for \(\geq 10\) years.

Our examination of in vitro expression of osteoblast markers found that scores for each marker increase even
without the addition of factors to promote osteogenic differentiation, confirming that even these cells, which were ≥ 10 years old, were similar to preosteoblasts with osteoblastic properties. In the osteogenic differentiation group, significantly higher expressions of ALP and osterix were observed. The lack of a statistically significant increase in osteocalcin expression may have been due to the short induction period of 7 days, making it difficult to observe rises in osteocalcin because this marker tends to increase later. These results are consistent with the findings of studies on the bone formation process and suggest that bone-derived MSCs that have been cryopreserved for ≥ 10 years undergo the same normal differentiation process. These results also show that bone-derived MSCs retain high bone-forming potential even after being preserved for ≥ 10 years and therefore could constitute an extremely useful cell source for bone tissue engineering.

We created a hybrid-type bone substitute for use in grafts and compared bone formation in bone defects. It has been reported that 5 mm diameter, 2 mm thick discs of HA (Ca_{10}[PO_4]_6[OH]_2; Pentax, HOYA, Japan) with porosity of 85% are useful as a scaffold. This type of HA was therefore used to incorporate the cells in this study. Holes of 5 mm were created in the skulls of 8 week old nude rats and the bone substitute was grafted onto these regions. A defect size of 5 mm has been reported as a non-healing model and we did not observe any bone formation in our control group. New bone formation from the human-derived bone cells was observed in all areas onto which the hybrid-type bone substitute was grafted. In a previous study, we grafted the same hybrid-type bone substitute subcutaneously onto the dorsal aspects of mice and only observed bone formation in the bone induction group. However, in the present study, we found that bone formation was possible even with nondifferentiation induction when implanted in the skeletal induction group suggested that induction of osteogenic differentiation in bone tissue-derived MSCs before grafting made it more effective as a hybrid-type bone substitute. Our results also suggest that nondifferentiated MSCs could be used to graft areas within existing bone tissue such as the alveolar cleft region or bone cysts. It is clear that artificial induction of graft cells should be kept to a minimum to ensure safety and that both nondifferentiated cells and differentiated cells could be used to create the hybrid-type bone substitute according to the state of the bone defect undergoing grafting.

We used safe autologous tissue as a cell source under strict ethical guidelines and were able to confirm excellent bone formation capability in bone tissue-derived MSCs that had undergone long-term preservation for ≥ 10 years. We therefore conclude that this bone substitute could be expanded for use in clinical applications.

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REFERENCES


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