Bone marrow stromal cells (MSCs) have multi-lineage differentiation capabilities and are focused on as a cell source for various cell therapies. To facilitate the availability of MSCs, cryopreservation technique is one of the critical factors for the cell therapies. In this study, effects of cryopreservation on capabilities of MSCs derived from a nonhuman primate were tested, aimed at a clinical application for tissue-engineered bone reconstruction.

Effects of cryopreservation on the MSCs' adhesion rate, proliferation, and osteogenic differentiation in vitro were compared with non-cryopreserved MSCs. Bone formation capabilities were also tested using an extraskeletal bone induction model. The bone formation induced by the combination of cryopreserved MSCs and an artificial bone scaffold was confirmed in all cases. The amount of bone formation at each case was irregular, but the results were suggested the possibility of cryopreserved MSCs on clinical use.

Key words: MSCs, cryopreservation, bone reconstruction

Introduction

In the field of orthopaedic and oral surgery, various methods have been investigated for reconstructing large bone defects, using autograft, allograft, and artificial bone substitutes. Whereas autologous bone graft is the most successful method to reconstruct bone defects and is routinely practiced at present, it is impossible to eradicate complications such as donor site morbidity, risks for infection, neurovascular injury, and further, the amount of available graft is limited. Allograft is also useful but its osteogenic capability is inferior to that of autograft, and it is accompanied with risks for infectious disease, including some that are unidentified. Some artificial bone substitutes have been developed and though they are free from the abovementioned complications with autograft or allograft, their osteogenic abilities are limited. For these reasons, effective alternatives to autograft have been required.

Bone marrow-derived stromal cells (MSCs), which are cultured from bone marrow, aspirate comparatively easily. MSCs have multi-lineage differentiation capability, including osteogenic lineage. Focusing on their
osteogenic capability, MSCs have been studied as a cell source which provides artificial bone with osteointegraftivity, and recently, there have been some clinical trials of bone reconstruction using combinations of artificial bone and MSCs.\(^{10-12}\) Furthermore, MSCs have been also used for treatment of congenital skeletal disorders such as osteogenesis imperfecta,\(^{13,14}\) and used with peripheral hematopoietic stem cell transplantation for treatment of myelosuppression induced by leukodystrophy or chemotherapies for cancers utilizing their supportive effects on hematopoietic stem cells.\(^{15-16}\) To achieve desirable clinical treatment using MSCs, it is essential to use cells with optimal conditions. However, the state of MSCs changes during the culture period,\(^{17,18}\) and the optimal cell condition does not always correspond to the time of the therapies. Therefore, a cryopreservation technique which does not diminish their ability would expand the usefulness and the effectiveness of the treatments using MSCs.

In this study, aimed at bone reconstruction using MSCs, we tested the effects of cryopreservation on MSCs of a nonhuman primate using a commercially available freezing reagent by in vitro analysis and also in vivo bone formation analysis.

### Materials and methods

All animal experiments are conducted according to guidelines of the Tokyo Medical and Dental University for the care and use of laboratory animals.

### Experimental Animals

Cynomolgus monkeys (male, 4-5kg body weight, n=5) were used for each experiment in vitro analysis and in vivo analysis.

### Cell Culture

To obtain monkey MSCs, bone marrow (3–5 ml) was aspirated from greater trochanters of femurs of cynomolgus monkeys under general anesthesia using a bone biopsy needle (Jamshidi Bone Marrow Biopsy/Aspiration Needle, Cardinal Health, USA). Each bone marrow was seeded into T-75 culture flasks (Becton, Dickinson and Company, USA) with Dulbecco’s Modified Eagle’s medium (DMEM; Sigma-Aldrich Co, St. Louis, USA) containing 10% fetal bovine serum (Sigma-Aldrich Co), 1% Antibiotic-antimycotic (10,000 units/ml penicillin G sodium, 10,000 \(\mu\)g /ml streptomycin sulfate, and 25 \(\mu\)g /ml amphotericine B; Invitrogen Co, NY, USA), and 10\(^{-7}\)M dexamethasone (Sigma-Aldrich Co) and cultured at 37°C in a humidified atmosphere with 5% CO\(_2\).

24 hours later, the medium was exchanged for fresh DMEM medium, and the non-adherent cells were removed. Subsequent exchanges of the DMEM medium were performed two times per week.

When the flasks became nearly confluent at 9–12 days of the culture, the attached cells were washed with PBS, and released by treatment with 0.25% trypsin / 0.01 mol EDTA (Invitrogen). The cells were resuspended in the medium and plated at 4000 cells/cm\(^2\) into T-75 flasks. When the cells reached a near-confluent state, the DMEM medium was added with supplements for osteogenic introduction: 10\(^{-7}\) M dexamethasone, 10 mM β-glycerophosphate (Sigma-Aldrich Co.) and 50 mg/ml ascorbic acid phosphate (Wako, Japan).

The osteogenic induction was maintained for 4 days, and then the cells were released by 0.25% trypsin / 0.01mol EDTA and divided into two groups; one was a cryopreserved group and the other was a non-cryopreserved group used as a control. The cells of both groups obtained from each monkey were used for the following in vitro and in vivo analyses, though the cryopreserved group was analyzed after a cryopreservation procedure.

### Cryopreservation of Cells

The cells in the cryopreserved group were collected in a 50ml centrifugation tube (Becton, Dickinson and Company) and centrifuged at 1000g for 3min at 4 °C.

Next the cells were suspended in a reagent for cell cryopreservation (Cell Banker, serum free type, Nippon Zenyaku Kogyo Company, Japan) at a concentration of 10\(^6\)/ml, and the cell suspension was distributed into cryo-tubes. The tubes were directly placed in a deep freezer (−80 °C) to rapidly freeze the cell suspension, stored for 24 hours, and then transferred into a deep freezer (−150 °C).

After 10 days cryopreservation, the tubes were taken out of the deep freezer and quickly thawed in a water bath (37 °C). The thawed cell suspension was transferred into a 50 ml centrifugation tube with an adequate volume of the DMEM medium containing 10% fetal bovine serum and 1% Antibiotic-antimycotic and centrifuged at 1000g for 3min at 4 °C. The cells were then resuspended in the medium for the use in the following experiments.

### Cell adhesion analysis

The cells from each monkey of two groups were suspended in the DMEM medium containing 10% fetal...
bovine serum and 1% Antibiotic-antimycotic and plated onto 10cm dishes at 4000 cells/cm², and incubated for 12h in a culture chamber. After the incubation, the plates were rinsed with PBS to wash away the nonadherent cells, and the adherent cells were detached with trypsin-EDTA; they were counted with a hemocytometer.

Cell proliferation analysis
The cells of each group were seeded on a 12-well culture plate (Becton, Dickinson and Company) at 2000 cells/cm² and cultured in the medium containing 10% fetal bovine serum and 1% Antibiotic-antimycotic.

For the cell proliferation analysis, the total DNA content of each sample was measured. At 4, 8 and 12 days of the assay, the cells attached on each well were lysed with 500 μl of 0.2% Triton-X100 (Sigma-Aldrich Co) and sonicated using Vibra-Cell (SONICS, USA) on ice. DNA content was measured using a PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer’s instruction. The sonicated samples or the standards (35 μl) were mixed with a working solution of the PicoGreen reagent (35 μl) in a 96-well plate and incubated for three minutes at room temperature. Fluorescence intensity of each well was measured with a spectrofluorometer (Mx 3000p, Stratagene, La Jolla, CA, USA) at emission and excitation wavelengths of 516 and 492 nm, respectively.

Analysis for alkaline phosphatase activity
To measure alkaline phosphatase activity, the cells from each group were plated on a 12-well culture plate at a density of 5000 cells/cm². They were cultured in an osteogenic medium containing 10% fetal bovine serum, 1% Antibiotic-antimycotic and supplements for osteogenic introduction: 10⁻⁷M dexamethasone, 10mM β-glycerophosphate (Sigma-Aldrich Co) and 50 mg/ml ascorbic acid phosphate (Wako, Osaka, Japan). At 4, 8 and 12 days of the osteogenic induction, the cells were lysed with 500 μl 0.2% Triton-X100 at room temperature and sonicated on ice.

The samples (10 μl) from each tube or standards were mixed with 100 μl substrate buffer (10mM disodium p-nitrophenylphosphate hexahydrate, 0.056M 2-amino-2-methyl-1, 3-propanediol, and 1mM MgCl₂, Wako) and incubated for 30 minutes at 37 °C in each of a 96-well plate. After the incubation, absorbance at 405nm was measured using a microplate reader (Micro Plate Reader model550, BIORAD, USA ). Total ALP enzyme activity value of each well was determined using a standard curve. ALP enzyme activity values were normalized by the total DNA content of each sample measured by the abovementioned method.

Implantation
In each monkey, bone formation capabilities of the both groups were tested using an autologous extraskeletal transplantation model. The cells of each group were suspended in the culture medium at a concentration of 2 x 10⁶ /ml. Then the cell suspension was introduced into three porous β-tricalcium phosphate (β-TCP) blocks (5 x 5 x 5mm, porosity: 75%, pore size: 100-200 μm, Osferion , Olympus Biomaterial, Tokyo, Japan) for each group with negative pressure using the airtight container. After the introduction, the blocks were incubated at 37°C in a humidified atmosphere with 5% CO₂ for 3h for cell attachment on the blocks, and then the blocks were used for the following implantation.

General anesthesia was induced with intramuscular administration of ketamine (5mg/kg) and maintained with inhalational anesthesia of isoflurane(1.0%-2.0%). After intubation, one monkey was placed in a prone position, shaved the back and draped in the usual sterile manner. Three incisions were made on the right side of the monkey’s back, and three muscle pouches were created, and the implants with the control cells were implanted into them. Ten days after the implantation of the control group, the same procedures were performed on the left side of the monkey’s back for the cryopreserved group. The implanted blocks were harvested at five weeks after each implantation.5 monkeys (case1 to 5) in all were treated as the same way on each experimental time.

Histological examinations
The harvested implants were fixed in 10% neutral buffered formalin and decalcified with K-CX solution (Falma, Tokyo, Japan). From five evenly divided sections of the samples embedded in paraffin, slices with 5 μm in thickness were prepared and stained with hematoxylin and eosin and then observed using an optical microscope. Bone formation areas , where lamellar bone structures and osteocytes were recognized, were selected using image processing software (Adobe Photoshop 7.0) and measured using image analyzing software (Scion image). Then average value of the ratio of bone formation areas to whole section of the implant on the 5 sections from 1 block was calculated and defined as average bone formation occupancy of that block. (n=3)
Statistical analysis

Statistical analyses were performed using Wilcoxon signed-rank test, with $\alpha = 0.05$, and two-way repeated measures ANOVA.

In this study, 80% of the mean of the control group was used for the index which is assumable that serious clinical problems would not be occurred by using the cryopreserved cells. Cell therapies are profitable but it is difficult to prepare for cells on good timing and cryopreserved cells will useful in such clinical occasion. The index is the valuation basis considering the negative effect by cryopreserving.

Results

Effects of cryopreservation on cell adhesion

The bone formation occupancy
\[= \left( \frac{\text{Bone area}}{\text{Sectional area}} \right) \times 100 \]

Fig. 1. The cube in Fig. 1 is the outline of the extracted sample block. The broken line shows the location of the sample slice which was used for histological examinations. The bone formation occupancy was calculated with software (Scion image). The average value on 5 sections was used as a substitute for the bone formation data of 1 block.

Fig. 2. Sample slice from implanted block stained with hematoxylin eosin. a: osteoblast cells  b: lamellar bone c: $\beta$-tricalcium phosphate. Scale bar=200 $\mu$m
The effect of the cryopreservation procedure on MSCs’ attachment was examined by counting the attached cells on culture dishes 12 hours after the plating. The average rate of attached cells of the cryopreserved group was 79.2%, whereas that of the control was 87.7%. Figure 3 shows the average cell-adhesion rate of two groups. From this result, we observed that the cell adhesion capability was with limited influence of cryopreservation and consequently the cell plating densities of the following analyses were not corrected. (Fig. 3)

**Effects of cryopreservation on cell proliferation**

To assess whether the cryopreservation affects the proliferation ability of the cells, the total DNA contents were quantified at 4, 8 and 12 days of the culture. As shown in Figure 4, there were not statistically significant differences between the groups at any point by two-way repeated measures ANOVA.

Each value at this assay of cryopreserved group was all over 80% of the mean of the control group. From these results, cryopreservation of the MSCs is considered not to affect their proliferation capability after thawing. (Fig. 4)

**Effects of cryopreservation on cells alkaline phosphatase activity in vitro**

To evaluate the influences of the cryopreservation, the control cells and the thawed cryopreserved cells were cultured in the osteogenic induction medium, and their ALP activity was measured at 4, 8 and 12 days of the osteogenic induction. Through the experimental period, the mean of the ALP activities of the cryopreserved group tended to be slightly higher than those of the control. (Fig. 5). The value at each case was uneven, and was all over 80% of the mean of the control group.

**Effects of cryopreservation on ectopic bone formation**

Comparisons of the bone formation capabilities were made at five weeks after the implantation. All samples were retrieved without surgical complications such as infection and disruption of the implants. Bone formation was observed in all samples of both of the groups (Fig. 6). From this data, the bone formation occupancy from each animal varied widely, from 0.9% to 14.55%, but there are no statistical difference with the use of Wilcoxon signed-rank test. ($\alpha=0.05$)

Each value at this assay of cryopreserved group was over 80% of the mean of the control group except for the case 2. In the case 2, the value of cryopreserved group was 38% of the control group.

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**Fig. 3.** Cell adhesion rate, calculated as $\frac{(\text{detatched cells number})}{(\text{plated cells number})} \times 100$. The adherent cells were counted with a hemocytometer. The error bar indicate SD. (n=5)

**Fig. 4.** Cell proliferation analysis. Total DNA content of each sample was measured. The error bar indicate SD. (n=5)

**Fig. 5.** Alkaline phosphatase activity, total ALP enzyme activity value of each sample was determined by standard curve and normalized by the total DNA content of each sample. The error bar indicate SD. (n=5)
Discussion

For clinical usage of MSCs to reconstruct bone defects, it is critical to prepare the cells with satisfactory condition and at proper stage of the culture. However, since capabilities of MSCs vary individually in proliferation and differentiation rates, preparation of optimal cells just at the day of the surgery is accompanied by some difficulties. Therefore, cryopreservation of MSCs would be a critical technique if capabilities of the cells were retained after the cryopreservation procedure which includes freezing, storage, and thawing processes. Cryopreservation of tissue and cells has been studied since the 1700s and cryopreservation technique is now routinely used to stock spermatozoa, oocytes, embryos, and many types of cells from animals and humans. As for MSCs, studies on cryopreserving technique using not only animals but human-derived MSCs have been reported. Bruder et al. reported that proliferation and osteogenic capabilities of cryopreserved human MSCs with 24 hours storage in liquid nitrogen were almost equal to those of the non-cryopreserved cells. Hirose et al. stated that cryopreserved human MSCs retained ability to generate mineralized matrix in vitro; their colleague also performed more extensive studies using long-term stored MSCs and reported that expression of cell surface markers and mineralizing capability of cryopreserved cells were not different from those of the noncryopreserved cells. However, all these reports were from in vitro studies, so the effects of cryopreservation of MSCs on bone formation capability were still elusive. Yoshikawa et al. demonstrated the bone formation capacity of rat-derived cryopreserved MSCs using an extraskeletal bone formation model of a syngenic rat, though they did not compare to the model of non-treated cells. However, it is also reported that there were differences between MSCs derived from human and rat in response to bone morphogenetic protein 2, which is potent osteogenic growth factor. Therefore, selection of an animal species to be used in a study is important, especially if the study is aimed at a clinical application. There have been some reports about bone formation capability about composites of nonhuman primate derived MSCs and porous materials including b-TCP. However, most of these studies used transplantation models into skeletal sites, and therefore the osteoinductivity of the implants could not be assessed definitively. The experimental model used in this study was a bone formation model at an extraskeletal site, which can clearly demonstrate osteoinductivity of transplanted materials.

In this study, we compared capabilities of cryopreserved MSCs and noncryopreserved MSCs derived from a nonhuman primate, in vitro and in vivo. Both the cell groups showed excellent cell adhesion rate, proliferation capability, and differentiation capacity into osteogenic lineage in vitro. There was some difference between the groups, but it was subtle and not indicated low ability of cryopreserved cells. In the quantitative bone formation analysis, all the MSCs in both the groups demonstrated extraskeletal bone formation, and though each bone formation ratio varied widely, there were no statistical differences between the groups. We think the dispersion may be due to the condition of the recipient sites, because the animal had received stresses from the implantation surgery of the control group only 10 days before. To clearly elucidate the effects of cryopreservation on bone formation ability, another study plan may be required; the result indicates, at least, that 10 days’ cryopreservation does not necessarily diminish bone formation capability of MSCs derived from nonhuman primates.

Conclusion

In conclusion, we confirmed the bone formation by cryopreserved MSCs derived from non-human primate in vivo. There were some differences between the capabilities of cryopreserved and those of noncryopreserved MSCs in vitro and in vivo. Especially in bone formation analysis, the cryopreserved MSCs demonstrated equivalent bone formation capability to that of the noncryopreserved cells. Cryopreservation would
expand the availabilities of various clinical treatments using MSCs in the near future.

References


