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Bone Formation by Cryopreserved Human Bone Marrow-derived Mesenchymal Stem Cells *In Vitro* -Preliminary results using human marrow cells from two cases-

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Cryopreserved human bone marrow-derived mesenchymal stem cells from two donors were thawed and seeded on culture surfaces. The cells retained high cell viability immediately after thawing. When the cells were cultured in the presence or absence of dexamethasone, the cells proliferated to reach confluency at culture day 7. The dexamethasone-treated cells formed abundant mineralized nodules at the extracellular regions after culture day 14, while the cells without dexamethasone never showed bone formation. The calcein uptake into the extracellular matrices was detected in the dexamethasone-treated cells with culture periods but not in the non-treated cells. These results suggest that human bone marrow-derived mesenchymal stem cells maintain bone-forming ability after cryopreservation.

Key words: Bone marrow, Mesenchymal stem cell, Cryopreservation, Mineralization, Tissue engineering

1. INTRODUCTION

With the rapid progress in tissue engineering, cultured cells will find various utilities in construction of regenerative tissues. Effective usage of cultured cells has been developed for the fabrication of cell-based tissue engineered devices and living cells with differentiated functions could be combined with various scaffolds including polymer and ceramics. As cryopreservation technology of cultured cells has progressed [1, 2], cryopreserved cells have potentially important implications for the clinical applications due to the limitation of supplied cells. We have previously reported that primary cultures of mesenchymal stem cells derived from bone marrow cells could differentiate into osteoblasts by the treatment of dexamethasone to form bone matrices with abundant minerals on ceramic surfaces [3, 4, 5, 6]. Recently, we have newly developed the monitoring system of mineralization processes by cultured cells using calcium binding fluorescent dyes [7, 8]. The calcein uptake was also quantified *in situ* by using an image analyzer throughout the duration of culture periods. The exogenously added calcein never affected bone formation by mesenchymal stem cells. Furthermore, the uptake of calcein well correlated with the calcium content of the mineralized bone matrix, resulting that this would be convenient and reliable method for monitoring *in vitro* mineralization by cultured cells.

In this report, we examined the calcein uptake into extracellular regions of cryopreserved/thawed human bone marrow-derived mesenchymal stem cells (hMSCs) from two donors to assess the *in vitro* bone-forming capability.

Experimental procedures

Primary culture and cryopreservation of hMSCs:
Human bone marrows were obtained from two donors with informed consent (donor age 71

and 55 years old). Three milliliters of bone marrow was harvested from the ilium of each donor by needle aspiration and centrifuged at 900 rpm for 5 min. After the supernatant with fat layer was discarded, the remaining supernatant (buffy coat and red blood cell layer) was added into Falcon T-75 flasks containing 15 mL of Minimum Essential Medium-α (MEM-α, Invitrogen) with 15% human serum or fetal bovine serum (FBS) and antibiotics and incubated under a 5% CO_2 atmosphere at 37°C for 10 days to 2 weeks until confluence. The confluent cells were released from Falcon T-75 flasks using 0.1% trypsin/ 0.05% EDTA. After trypsinization, the cell suspension was centrifuged at 900 rpm for 5 min. Precipitated cells were suspended in Cell Banker (Juji Field) at a cell density of $5X10^5$ cells/mL and cryopreserved at −150°C. In this report, we used the cells stored for 36 months for the donor of 71 years old and 12 months for that of 55 years old at −150°C.

Cell viability assay:
Cell viability was assessed with the LIVE/DEAD Viability assay kit (Molecular Probes) based on a simultaneous determination of living and dead cells with two probes, calcein-bis [(acetyloxy) methyl] ester (calcein-AM) for intracellular esterase activity and ethidium homodiner-1 (EthD-1) for plasma membrane integrity [9]. Cryopreserved hMSCs were thawed in MEM-α containing 15% FBS and the working solution (2 mM of calcein-AM and 5mM of EthD-1 in PBS) was added directly to the cell suspension. After a 15-min incubation at 37°C, the stained cells were observed under a fluorescent microscope (Model IX70, Olympus).

Culture of Cryopreserved/thawed hMSCs:
Cryopreserved hMSCs were thawed in MEM-α supplemented with 15% FBS. Subcultures at a cell density of $2X10^4$ cells/well in Falcon 24-well culture dishes were performed with 1 mL of MEM-α supplemented with 15% FBS, 10 mM β-glycerophosphate (Calbiochem), 0.28 mM ascorbic acid 2-phosphate (Wako Pure Chemical), and antibiotics with or without 100 nM dexamethasone under a 5% CO_2 atmosphere at 37°C for 25 days. The culture medium was renewed three times a week.

Quantitative fluorescence analysis of calcein uptake in cryopreserved/thawed hMSCs:
To enable the assay of the calcein uptake, calcein (Dojindo) at a final concentration of 1 μg/mL was added to subcultures of hMSCs. A single 24-well dish was analyzed at each time point during the subsequent culture period (culture day 4 through day 25). For the assay, the cells were washed twice with Calcium-free phosphate-buffered saline (PBS, Invitrogen), after which 1 mL of the culture medium was added. The fluorescence of the incorporated calcein was visualized and quantified by using an image analyzer (Typhoon 8600, Amersham Pharmacia Biotech) (526 nm short-pass filter) and was also observed by using a fluorescent microscope (Model IX70, Olympus). After the assay, the cell culture was continued to culture in the presence of calcein.

Results and Discussion

Osteogenic capability of cryopreserved/thawed hMSCs in culture.

Cell viability immediately after thawing was assessed with the LIVE/DEAD Viability assay kit. Living cells are distinguished by the presence of ubiquitous intracellular activity, determined by the enzymatic conversion of the no fluorescent cell-permeant calcein-AM to the intensely fluorescent calcein. The viable cells were found to be 82% and 86% of the total cells from two donors by cell counting under fluorescent microscopy. These results show that even after the cryopreservation of hMSCs, cell viability is well retained.

Cryopreserved/thawed hMSCs were then subcultured in the presence or absence of dexamethasone. Under these conditions, the dexamethasone induces the undifferentiated mesenchymal cells into osteoblasts that produce a mineralized matrix about 14 days after the start of the subculture as previously reported by our group. In order to check whether the cryopreserved hMSCs could have proliferative and osteogenic potential, we observed bone-forming ability of the cells after thawing by culturing the cells with or without dexamethasone for long culture periods. Phase-contrast microscopy demonstrated that

the cells actively proliferated to reach confluency after 7 days in culture. The cells of two cases cultured in the presence of dexamethasone showed abundant mineral deposition around the cells to form mineralized nodular aggregates comparable to primary cultured hMSCs. In contrast, non-treated cells did not exhibit bone-forming ability (Fig. 1).

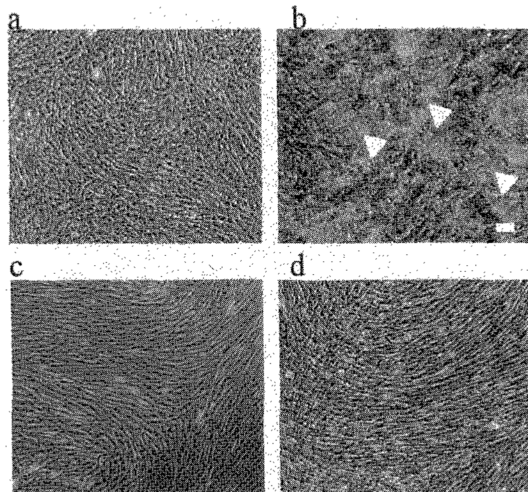

Fig. 1. Phase-contrast microscopy of cryopreserved/thawed hMSCs in culture. Cryopreserved hMSCs (donor age; 71 years) were thawed and cultured in the presence (a, b) or absence (c, d) of dexamethasone. The cells were observed under a phase-contrast microscope at culture day 7 (a, c) and day 21 (b, d). Arrowheads indicate bone mineral nodules present in extracellular regions of the cells. Scale bar represents 50 μm.

Recently, we have reported a novel quantitative method for mineralization by cultured cells utilizing a Calcium-binding fluorescent dye, calcein [7, 8]. We have combined hMSCs with bioceramics scaffolds to fabricate tissue-engineered bone tissues. With the rapid advancement in tissue engineering, *in situ* analysis methods for cellular functions are required for monitoring the functions in time dependent manner. Mineralization of cultured osteoblasts has been observed with Alizarin Red S staining. However, it is impossible to monitor further mineralization of cultured osteoblasts after the Alizarin Red S staining. In order to overcome the shortcoming, we developed the direct fluorescent quantitative measurement of the mineralization by cultured osteoblasts using fluorescent dyes of calcein. Calcein was found to be specifically incorporated and deposited into extracellular bone matrices evidenced by the co-staining with Alizarin Red S. Advanced characteristics of the method included the monitoring of the mineralization of the same specimens of cultured cells in a time dependent manner due to continuous cultivation without fixation processes of cell layers. To evaluate the degree of mineralization by cryopreserved/thawed hMSCs, we added calcein to the culture medium and observed the cells with a fluorescent microscope and then quantified the fluorescent intensity of deposited calcein. Fluorescent microscopy demonstrated that the cells treated with dexamethasone could have the ability of mineralization and calcein uptake (Fig. 2 a and b). By contrast, the cultures without the dexamethasone did not display matrix formation and the calcein uptake was negligible (Fig. 2 c and d). Measurements to quantify the amount of calcein deposited into mineralized matrices of the cells were performed using the image analyzer. Increase in fluorescent intensity as a result of the calcein uptake was clearly determined only in the cells treated with dexamethasone as culture days proceeded.

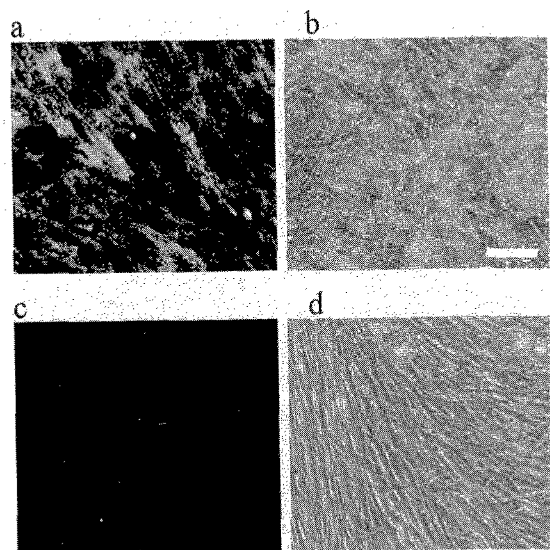

Fig. 2. Incorporation of calcein into

extracellular matrices of cryopreserved/thawed hMSCs in culture.
Cryopreserved hMSCs (donor age; 71 years) were thawed and cultured in the presence (a, b) or absence (c, d) of dexamethasone with addition of calcein. At culture day 21, the cells were observed with a fluorescent microscope. Calcein-uptake into extracellular regions actively occurred in the dexamethasone-treated cells (a), while did not occur in the non-treated cells at all (c). Phase-contrast micrographs of (b) and (d) show the same microscopical regions of (a) and (c), respectively. Scale bar represents 50 μm.

Taken together, the results from two cases of old age clearly showed that cryopreserved /thawed hMSCs maintained the dexamethasone-dependent osteogenic potential. Furthermore, the cells were morphologically, phenotypically and functionally comparable with primary hMSCs. The facts that hMSCs could possess the differentiated activity into osteoblasts even after cryopreservation mean the clinical significance because one of the main issues we should address to realize regenerative medicine using living cells is to secure sufficient cells with high differentiated potential. We are now doing extensive research whether cryopreserved hMSCs have universal capability for the proliferation and differentiation regardless of donor ages. We believe that cryopreserved hMSCs could become a promising candidate of cell sources for fabrication of regenerative bone tissues.

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