Yonsei Medical Journal
Vol. 45, Suppl., pp. 61 - 67, 2004

Original Article

# Clinical Application of Marrow Mesenchymal Stem Cells for Hard Tissue Repair

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Human marrow mesenchymal stem cells were cultured in a medium containing glycerophosphate, ascorbic acid, and dexamethasone (Dex) on alumina ceramic discs and on tissue culture polystyrene (TCPS) dishes. Cell proliferation followed by osteogenic differentiation was observed to be equal on both culture substrata. The differentiation resulted in the appearance of bone-forming osteoblasts, which fabricated mineralized matrices on these substrata. Stem cells kept at 4℃ for 24 h outside a $CO_2$ incubator maintained a viability level of more than 90%. The regenerative cultured bone outside the incubator also maintained high alkaline phosphatase activity for several hours. These results verified that cultured bone fabricated at a cell processing center can be transported to distant hospitals for use in hard tissue repair. To date, the tissue engineered cultured bone formed on alumina ceramics in this environment have already been used in clinical situations, such as total ceramic ankle replacements.

**Key Words:** Bone marrow, mesenchymal stem cells, bone formation, alumina ceramics

## INTRODUCTION

It is well known that mesenchymal stem cells (MSCs) reside in bone marrow and that the MSCs can differentiate into osteochondrogenic lineage.[1-3] The differentiation can be confirmed by *in vivo* implantation[4-6] and *in vitro* culture of MSCs.[7-10] We proposed a new technical concept for hard tissue repair based on the osteogenic differentiation capability of MSCs.[11] The cascade of the concept consists of culture expansion of the number of MSCs from the patient's fresh marrow, differentiation of the MSCs into osteoblasts, then fabrication of bone matrix on the various substrata. The regenerative cultured bone exhibits an immediate new bone-forming capability after *in vivo* implantation.[12] This new bone-forming capability can be attributed to the implanted cultured osteoblasts as well as to the bone matrix, which contains cytokines and mineralized crystals. Since January 2002, we initiated the use of this concept in clinical applications[13,14] and have transported cultured bone from our center (Tissue Engineering Research Center) to two hospitals, Nara Medical University Hospital and Sinshu University Hospital (distances of 40 km and 200 km from our center, respectively). Because of the long distances, transportation of the tissue engineered biomaterials requires that the cellular functions of the cultured bone should be maintained at high levels for a long time. Another important issue is the monitoring of a mineralized matrix produced by cultured cells on culture substrata, especially on a ceramic surface to be used in orthopedic surgery. Here, we report a newly developed method of monitoring matrix formation by culturing patient MSCs on alumina ceramics. We also assayed the cellular functions such as viability of the cultured MSCs and the osteogenic parameters of the cultured bone after prolonged periods

Received January 30, 2004

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outside a $CO_2$ incubator.

## MATERIALS AND METHODS

### Materials (ceramics)

Alumina ceramic disks (30-mm diameter, 1.5-mm thick) were kindly provided by Kyocera Co., Ltd. (Kyoto, Japan). The disks were transparent single-crystal alumina disks that were manufactured using the Czochralski crystal growth technique. Before using the disks as cell culture substrates, we subjected them to autoclave sterilization (120℃, 20 min).

### Marrow cell preparation and culture

After obtaining the informed consent of eight donors 25 to 70 years old, their bone marrow cells were obtained from the iliac crest by needle aspiration. Three mL of the marrow aspirates were immediately combined with 3 mL of phosphate-buffered saline minus $Ca^{2+}$ and $Mg^{2+}$ (PBS) containing 30 IU of heparin. After centrifugation, the plasma and fat layers were removed from the supernatant. The remaining nucleated cells, together with the red blood cells, were poured into two T-75 flasks (Coster Co., Cambridge, MA) containing 15 mL of medium to give the primary culture. The medium (maintenance medium) consisted of Eagle's alpha minimal essential medium (a-MEM; Nacalai Tesque, Inc., Kyoto, Japan) containing 15% fetal bovine serum (JRH Biosciences, Lenexa, KS) and antibiotics (100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 $\mu$g/mL amphotericin B; Sigma Chemical Co., St. Louis, MO, USA). The primary cultures were maintained under a 5% $CO_2$ atmosphere at 37℃. The maintenance culture medium was renewed three times a week. As part of the medium change, floating cells (red blood cells and hematopoietic cells) were removed so that the adherent cells became almost confluent about ten days after the creation of the primary culture. The adherent cells are known to contain cells of mesenchymal types.[15] The nearly confluent cells were released from the substrates using 0.05% trypsin 0.53-mM EDTA. The cells were centrifuged to concentrate at a cell density of $5\times10^5$ cells/mL and suspended in a storage solution (Cell Banker, Juji Field, Inc.). The Cell Banker is a ready-to-use storage solution containing dimethyl sulfoxide (DMSO). As a simplified process of program freezing, the MSCs in the storage solution were stored at 4℃ for 10 min, -30℃ for 1 h, and -80℃ for 2-3 days. Finally, the MSCs were stored at -152℃ until use.

After the frozen MSCs were thawed, the cells were seeded into control Falcon tissue culture polystyrene (TCPS) dishes, and onto alumina disks placed in six-well TCPS dishes at a cell density of 5,000 cells/$cm^2$. The samples were then subcultured for two weeks with 2 mL of the maintenance medium supplemented with 10 mM $\beta$-glycerophosphate (Merck KGaA, Darmstadt, Germany). To induce osteogenic differentiation, 20.5 ug/mL L-ascorbic acid 2-phosphate magnesium salt n-hydrate and 100 nM dexamethasone (Dex) (Wako Pure Chemical Industries Ltd., Osaka, Japan) were added to the medium.

### Visualization of mineralized matrix by fluorescence emission during subculture

During the subculture periods, to enable the detection of the mineralized extracellular matrix of the cultured cells, 1 $\mu$g/mL of calcein (Dojindo Laboratories, Kumamoto, Japan) was added to the culture wells whenever the culture medium was renewed. Prior to the assay, the subcultured cell layers were washed twice with PBS, after which 2 mL of maintenance medium was added. The fluorescence of the calcein in the mineralized extracellular matrix of the cultured cells was visualized and observed by using a fluorescence microscope (IX 70, OLYMPUS Co., Ltd., Japan).[16] The fluorescence of the incorporated calcein was also visualized and quantified by using an image analyzer (Typhoon 8600, Molecular Dynamics Inc., CA, USA) (526 nm short-pass filter). This assay was done for the same culture wells at each time point during the subsequent culture periods.[17]

### Measurement of ALP activity of the 14-day subcultured cells

The cell layers were washed twice with PBS and collected by scraping with 1 mL of 10-mM Tris-

buffer (pH 7.4, 1-mM EDTA, 100-mM NaCl). The cell suspension was then sonicated and centrifuged at 13,000 g for 1 min at 4℃. An aliquot (20 $\mu$L) of the supernate was assayed for ALP activity using a p-nitrophenyl phosphate substrate (Zymed Laboratories Inc., CA, USA).[8,10] The activity of the cell layer / well was represented by p-nitrophenol, which was released after incubation for 30 min at 37℃.

### Cell viability assay

MSC viability was analyzed using a Nucleo Counter (ChemoMetec A/S, Allerød, Denmark), which is intended for counting cells, employing a fluorescence microscope adapted to a relatively low optical magnification. After immediate thawing of the frozen MSCs, $500 \times 10^3$ of the cells were subcultured into two T75 flasks for about 1 week to reach near confluence. The cells were trypsinized as described above, suspended in 5 ml of MEM and centrifuged at 400 × g for 5 min. After centrifugation, the cell pellet was suspended in 1.5 ml of saline and stored for a period from 0 (immediately used) to 24 h at 4℃. Then, 40 ul of the cell suspension was diluted 5 times with saline and was analyzed on the NucleoCounter with cartridges before and after treatment with a lysis buffer, giving an estimate of non-viable and total cells, respectively. The cell count system was based on propidium iodide (PI) staining.

## RESULTS AND DISCUSSION

The first step of our approach involves the proliferation of mesenchymal stem cells (MSCs) from fresh bone marrow. As described in the Methods and Materials section, we usually took 3 mL of aspirated marrow cells from the donor iliac bone and placed them into two T-75 flasks. From this 3 mL of fresh bone marrow, we were able to harvest about $10 \times 10^6$ adherent cells after about 10 days of primary culture. The adherent cells were negative for hematopoietic markers (CD14 and CD34) but positive for antigens present in mesenchymal cells (CD29 and CD44). These results indicate that the cultured cells derived from bone marrow were mesenchymal types.[15]

The MSCs were subcultured in the presence and absence of Dex. Under these conditions, Dex induces the undifferentiated MSCs into osteoblasts that produce mineralized matrices on a variety of materials about 14 days after the subculture is started, as we previously reported.[8,10] The *in vitro* fabricated mineralized matrices together with the appearance of osteoblasts can be designated as "regenerative cultured bone".[15] As shown in Fig. 1A, the culture with Dex after 14 days showed mineralization, which was confirmed by calcein

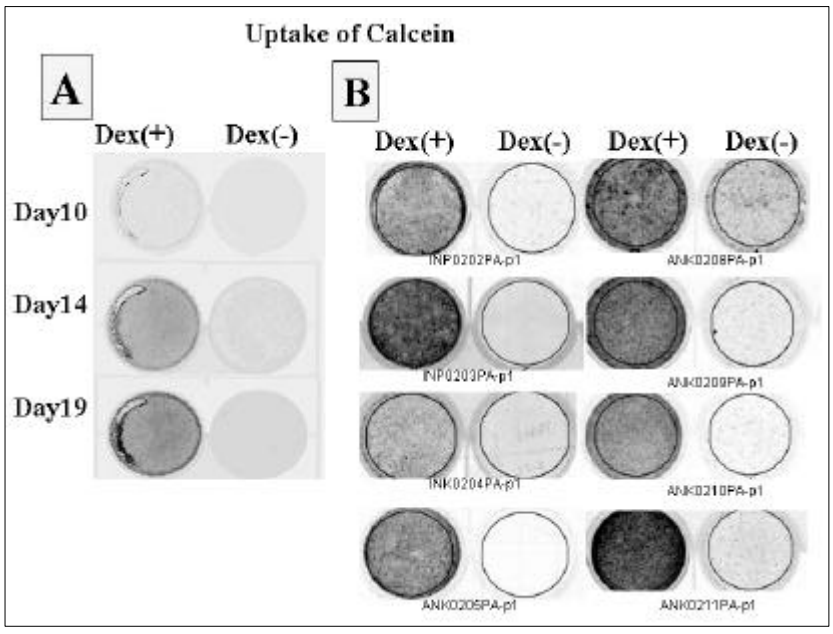


**Fig. 1.** Mineralization of MSCs on culture dish. A: Progress of fluorescence uptake with time. MSCs were cultured in the presence dexamethasone (Dex +) or absence (Dex-) of Dex for 10 to 19 days in 12 well plates. Fluorescent dye of calcein was supplemented in the culture medium. After washing with PBS, the culture dish was visualized using an image analyzer. Figures shown were derived from identical culture wells for Dex+ and Dex-, respectively. Black dots indicate the deposition of calcium shown by fluorescence uptake. B: Fluorescence uptake of different MSCs. Eight MSCs derived from the marrow of different patients were cultured for 14 days. Details are described in the Materials and Methods section.

uptake using an image analyzer. The amount of uptake represented by fluorescence intensity was parallel with calcium deposition (data not shown). Thus, the intensity can be used for detecting the volume of mineralized matrix in cultured bone. Significantly, the intensity of the entire culture area can be visualized. All of the culture with Dex showed extensive mineralization but the culture without Dex showed only a trace of mineralization. However, due to the individual osteogenic capacity of MSCs from the donor marrow of different patients, a broad variety of intensities can be seen after 2 weeks of subculturing with Dex (Fig. 1B).

During the two-week subculture period, the proliferation and differentiation of the MSCs on the alumina disks, as well as on the control culture dishes, were compared by phase contrast microscopy. The tissue culture polystyrene (TCPS) dishes are fabricated from crystal-grade polystyrene by a vacuum-gas plasma process. The TCPS surfaces exhibit a slightly hydrophilic nature since the treatment introduces carboxyl and hydroxyl groups into their surfaces. Under typical culture conditions using TCPS dishes, different types of cultured cells can adhere and proliferate on the surfaces, although the cells exhibit little attachment to bare polystyrene surfaces because of their high hydrophobicity. For this reason, TCPS dishes are commonly utilized for maintaining cultured cells. As shown in Fig. 2, the cells cultured with Dex showed good cell adhesion and proliferated to reach confluence at day 7 on both the alumina and TCPS substrates. At day 14 of the subculture, the Dex(+) cells cultured on both substrates formed mineralization detected by phase contrast microscopy and fluorescence microscopy, which was used to monitor fluorescence emission of deposited calcein in mineralized areas. Thus, attachment, proliferation, and mineralization of MSCs occurred in much the same way on the alumina surface as well as on the culture dish surface (Fig. 2). Our recent findings of biochemical assay of the cultured MSCs on alumina ceramics also confirmed this evidence.[18] These findings strongly support our approach using regenerative cultured bone fabricated on alumina total joint prosthesis to prevent the loosening of joint replacements, as described later.

All the data indicate that MSCs can show

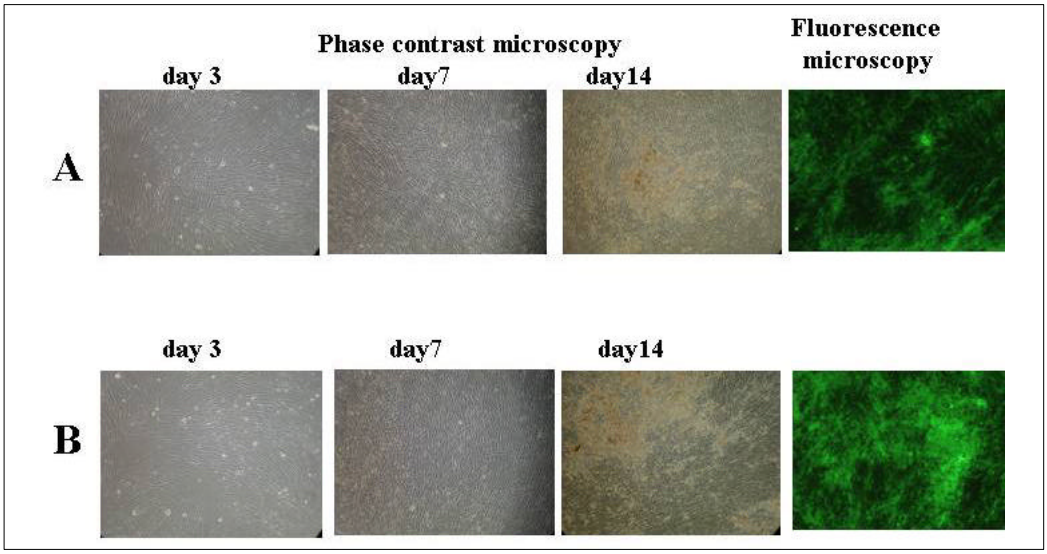


**Fig. 2.** Phase contrast and fluorescence micrographs of MSCs cultured on alumina disks and control culture dishes. MSCs were subcultured on single-crystal alumina disks (A) and control tissue culture polystyrene dishes (B) for two weeks in the presence of (Dex). Phase-contrast microscopy was used to compare the cells cultured on the alumina disks with those cultured in the culture dishes. On both the alumina and culture dishes, the cells exhibited good attachment and early proliferation at day 3, reached confluence at day 7, and showed nodular aggregates indicating mineralization at day 14. In contrast, no mineralization was observed in the cells cultured without Dex, even after 14 days of subculture (data not shown). Mineralization was also clearly detected by fluorescence microscopy (original magnification: 100X). Details are described in the Materials and Methods section.

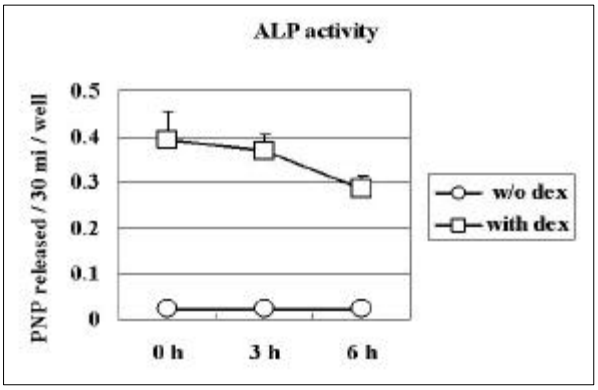


**Fig. 3.** ALP activity of cultured bone. MSCs of a 61-year-old male patient were subcultured with (□) or without (O) dexamethasone. After two weeks, the culture dishes were taken from the $CO_2$ incubator and allowed to culture at 4℃ for several h. The ALP activity of the cultured cell layers were then measured using the method described in the Materials and Methods section. The data represents the mean ± SD (standard deviation) of the four samples.

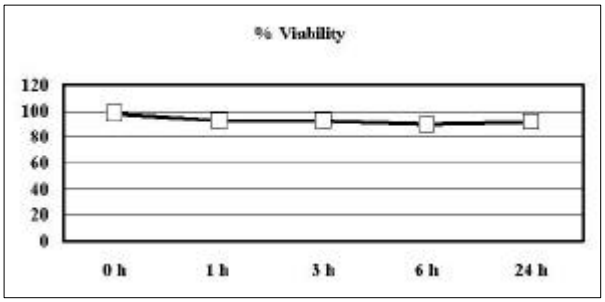


**Fig. 4.** Viability of MSCs. MSCs of a 70-year-old patient cultured about 1 week were taken from the $CO_2$ incubator and stored in saline for 0 to 24 h at 4℃. The viability of the cells was then assayed as described in the Materials and Methods section.

osteogenic differentiation together with bone matrix formation when cultured in the presence of Dex. Therefore, formed regenerative cultured bone can be used for osseous reconstruction. However, in considering transportations of the cultured bone, an important question is whether the cultured bone maintains its functions outside a $CO_2$ incubator. If the cultured bone can continue to function, the next question is how long. To answer these questions, we took the 2-week cultured (with Dex) bone on the tissue culture plates from the $CO_2$ incubator, and allowed the culture to continue at 4℃ for several hours. As shown in Fig. 3, high ALP activity could be seen at 3 h and the activity was comparable to that at 0 h. Although some decrease in activity was seen at 6 h, the activity was still significantly higher than that of the culture without Dex. Therefore, regenerative cultured bone is durable cultured tissue retaining its osteogenic function, and the tissue can be transported for a long distance without major loss of this function.

We utilized the cultured bone derived from MSCs for the purpose of osseous reconstruction. The MSCs without further osteogenic differentiation could be used for bone reconstruction[19] and also for cartilage reconstruction.[20] Due to these reports, we also checked the viability and osteogenic capacity of MSCs settled outside a $CO_2$ incubator. The subconfluent cultured MSCs were trypsinized and stored in saline at 4℃ for 0 to 24 h. The MSCs stored in saline were then used for the viability assay. As shown in Fig. 4, a viability level of more than 90% was detected in the MSCs stored in saline for 24 h. Interestingly, further culturing of the MSCs in the presence of Dex resulted in osteogenic differentiation; the differentiation capability was not seriously affected by the storage time (data not shown).

All of these results show that human MSCs can show osteogenic differentiation on alumina ceramics comparable to that on "gold standard" culture dishes and the MSCs as well as the differentiated cells (osteoblasts) have durable viability and osteogenic function. Consequently, we harvested and cultured about 10 ml of fresh marrow cells from patients to increase the number of MSCs followed by osteogenic differentiation on alumina ceramic prosthesis. The regenerative cultured bone on the prosthesis was transported to the university hospital where total joint replacement surgery using the tissue-engineered prosthesis was performed (Fig. 5). The treatments can be expected to result in early bone fixation, thus avoiding the loosening of implants. We also fabricated the cultured bone on artificial bone such as porous hydroxyapatite and tricalcium phosphate ceramics. They were used for osseous reconstruction after bone tumor excision surgery. We have already treated more than 30 patients using regenerative cultured bone and none of the patients reported any serious complications. Although long-term follow-ups are needed to

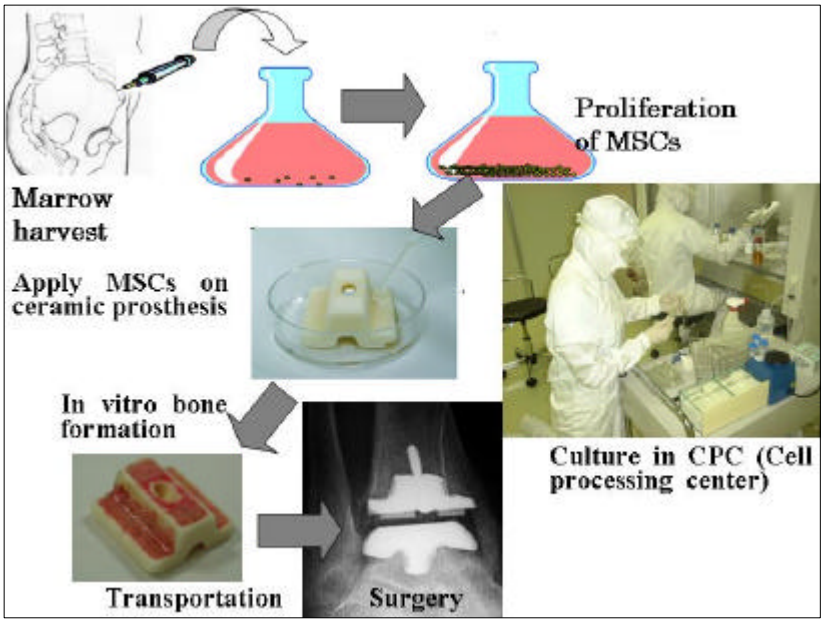


**Fig. 5.** Schematic presentation of clinical application of MSCs for osteoarthritic joints. After proliferation of MSCs from the fresh marrow of a patient, the cells were applied to the surface of alumina ceramics and subcultured for 2 weeks in an osteogenic medium. The lower left figure shows the ALP staining after the subculture using the MSCs from a 65-year-old patient. The tissue-engineered alumina ceramics were then used for joint replacement surgery at the hospital. The entire culturing procedure was done at the Cell Processing Center (CPC) of the Tissue Engineering Research Center.

confirm the efficacy of the treatment, short-term follow-ups have shown excellent results. Thus, the tissue-engineered approach using cultured bone is leading to exciting new dimensions of cell-based therapy for hard tissue reconstruction.

## ACKNOWLEDGMENTS

This work was undertaken by the Three-Dimensional Tissue Module Project, METI (A Millennium Project) and partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan. We wish to express our gratitude to Kyocera Co., Ltd. (Kyoto, Japan) for supplying the alumina ceramic disks used in this study.

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