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TECHNOLOGY DEVELOPMENT

Bioreactor Expansion of Human Adult Bone Marrow-Derived Mesenchymal Stem Cells

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ABSTRACT

Supplementation of mesenchymal stem cells (MSCs) during hematopoietic stem cell (HSC) transplantation alleviates complications such as graft-versus-host disease, leading to a speedy recovery of hematopoiesis. To meet this clinical demand, a fast MSC expansion method is required. In the present study, we examined the feasibility of using a rotary bioreactor system to expand MSCs from isolated bone marrow mononuclear cells. The cells were cultured in a rotary bioreactor with Myelocult medium containing a combination of supplementary factors, including stem cell factor and interleukin-3 and -6. After 8 days of culture, total cell numbers, Stro-1$^+$CD44$^+$CD34$^-$ MSCs, and CD34$^+$CD44$^+$Stro-1$^-$ HSCs were increased 9-, 29-, and 8-fold, respectively. Colony-forming efficiency-fibroblast per day of the bioreactor-treated cells was 1.44-fold higher than that of the cells without bioreactor treatment. The bioreactor-expanded MSCs showed expression of primitive MSC markers endoglin (SH2) and vimentin, whereas markers associated with lineage differentiation, including osteocalcin (osteogenesis), type II collagen (chondrogenesis), and C/EBP-α (CCAAT/enhancer-binding protein-α) (adipogenesis), were not detected. Upon induction, the bioreactor-expanded MSCs were able to differentiate into osteoblasts, chondrocytes, and adipocytes. We conclude that the rotary bioreactor with the modified Myelocult medium reported in this study may be used to rapidly expand MSCs. STEM CELLS *2006;24:2052–2059*

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is routinely performed after chemo/radiotherapy to rescue the hematopoiesis of patients with cancer. Despite a significant improvement of the quality of life, most patients inevitably suffer a short period of severe neutropenia and thrombocytopenia after HSCT. New transplantation strategies are therefore needed to reduce these complications. Mesenchymal stem cells (MSCs) have been shown to support hematopoiesis in vitro [1]. In fetal sheep and nonobese diabetic/severe combined immunodeficient mice, cotransplantation of MSCs with hematopoietic stem cells (HSCs) accelerated hematopoiesis reconstitution [2–4]. In human trials, the supplementation of autologous MSCs during HSCT after myeloablative chemotherapy has also been performed with encouraging outcomes [5]. MSC supplementation may therefore offer a solution to minimize HSCT-associated complications. In addition, it has been revealed that MSCs are immunoprivileged and transplantable across allogeneic barriers [6]. Allogeneic MSC supplementation [7, 8] may provide clinical benefit in a broader context.

The static amplification of MSCs is a time-consuming procedure and prone to contamination [1]. The increasing clinical applications appeal for an alternative to rapidly expand MSCs [9]. In previous studies, MSCs have been cultured alongside HSCs in spinner flasks without losing their multiple mesenchymal-lineage differentiation potentials [10, 11]. Such a mixed culture system is mutually beneficial for both MSC and HSC growth because the suspension system mimics the in vivo bone marrow microenvironment/niche containing various cytokines, chemokines, and growth factors [12]. The three-dimensional rotary bioreactor is considered to be the next generation of spinner flask with the unique environment of minimal shear stress and microgravity, which is particularly suitable for the mammalian cell survival. The large-scale expansion of HSCs has been previously achieved using the bioreactor system [13].

The aim of the present study was to examine the feasibility of expanding MSCs from isolated bone marrow mononuclear cells (MNCs) in a rotary bioreactor system. After 8 days of culture in a bioreactor with modified Myelocult medium (StemCell Technologies Inc., London, http://www.stemcell.com), the numbers of Stro-1$^+$CD34$^+$CD44$^+$ MSCs, CD34$^+$Stro-1$^-$CD44$^+$ HSCs, and total cells increased by 29-, 8-, and 9-fold, respectively. The bioreactor-expanded MSCs expressed primi-

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tive mesenchymal cell markers, maintained a high level of colony-forming efficiency-fibroblast per day (CFE-F/day), and were capable of differentiating into chondrocytes, osteoblasts, and adipocytes upon appropriate inductions. The results confirmed that the rapid expansion of (primitive) MSCs together with HSCs can be achieved using the rotary bioreactor system.

Materials and Methods

Bone Marrow Cell Preparation

After consent was obtained, bone marrow was collected under general anesthesia through aspiration from the iliac crests of patients undergoing total hip replacement (ages 46–81, $n = 6$). The aspirates were aseptically transferred into sterile heparinized phosphate-buffered saline (PBS) (Sigma, Dorset, U.K., http://www.sigmaaldrich.com) and processed within 1–3 hours. For isolation of bone marrow MNCs, the whole marrow washouts were layered onto Lymphoprep (1.077 g/ml; Nycomed, Roskilde, Denmark, http://www.nycomed.com) and centrifuged at 1,840 rpm for 30 minutes at room temperature. After centrifugation, the MNCs that remained in the buffy coat layer were collected.

MSCs in Static Culture

Freshly isolated MNCs were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, and 2 mM L-glutamine (Invitrogen Corporation, Paisley, U.K., http://www.invitrogen.com) and seeded into T75 flasks at a density of 1×10^5 cells per cm^2. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. The first feeding was at day 7 and twice weekly thereafter until confluence.

MSCs in Bioreactor Culture

Freshly harvested MNCs were inoculated into two 50-ml RCCS bioreactors (Synthecon, Inc., Houston, TX, http://www.synthecon.com) at a concentration of 1×10^6 cells per milliliter. Two different medium preparations were used. Medium one is Myelocult medium, supplemented with 50 ng/ml stem cell factor (SCF), 10 ng/ml interleukin (IL)-3 and IL-6 (PeproTech EC Ltd., London, http://www.peprotechec.com), and freshly dissolved 10^{-6} M hydrocortisone (StemCell Technologies Inc.). This is hereafter referred to as modified Myelocult medium. Medium two (control) is DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, and 2 mM L-glutamine and hereafter is referred to as DMEM. The rotation speed used for the bioreactor was 20 rpm. The cells were incubated at 37°C for 8 days in a humidified atmosphere with 5% CO_2. The first feeding was at day 3 with 20% medium exchange without retention of cells. In addition, the growth factor supplements were reduced to 10 ng/ml of SCF and 2 ng/ml of IL-3 and IL-6 from the first medium exchange. The feeding was performed daily afterward. The limiting dilution feeding (LDF) protocol used here is based on the assumption that the changes of total cell and progenitor cell numbers measured can be extended to those removed during the feeding procedure [10]. The cells removed each time were used either for total cell counts (during the first 2 days of culture, cells were counted by randomly removing 50 μl from the filling port) or for flow cytometric analysis.

Tri-Color Flow Cytometry

All antibodies (Abs) were purchased from DakoCytomation Ltd. (Cambridgeshire, U.K., http://www.dako.co.uk) unless otherwise specified. Sample cells from day-1, -4, and -8 bioreactor culture were first treated with ammonium chloride solution to lyse any potential contaminated red blood cells prior to flow cytometric analysis. The remaining cells were washed in cold PBS buffer containing 5% human AB serum (Sigma). Separate aliquots of 1×10^5 cells in a final volume of 50 μl were used for each binding for 30 minutes at 4°C in the dark. The Abs used were 10 μl of monoclonal mouse anti-human Stro-1 (extracted from hybridoma provided by Dr. P.J. Simmons, Matthew Roberts Laboratory, Adelaide, South Australia, Australia), 2 μl of phycoerythrin (PE)-conjugated anti-human HCAM/CD44 (DF1485; Insight Biotechnology Limited, Wembley, U.K., http://www.insightbio.com), 2 μl of R-phycoerythrin-cyanin 5 (RPE-Cy5)-conjugated anti-human CD34 (Clone BIRMA-K3), 2 μl of secondary fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse Abs, and equal volume of isotype-matched FITC-, PE-, and RPE-Cy5-conjugated negative controls. Finally, all the cells were resuspended in 1 ml of 1% paraformaldehyde (Sigma) and kept in the dark at 4°C. The analysis was performed on a Partec Cytoflow Space flow cytometer (Partec GmbH, Münster, Germany, http://www.partec.com) equipped with the Partec FloMax software.

CFE-F Assay

CFE-F assay was used to examine the functional property of modified Myelocult bioreactor-expanded MSCs. Both freshly harvested MNCs (control) and bioreactor output cells were resuspended in DMEM and seeded into six-well plates (NUNC A/S, Roskilde, Denmark, http://www.nuncbrand.com) at a density of 1×10^5 cells per cm^2. A third group was also included with MNCs suspended in the modified Myelocult medium to examine the effect of this medium on static culture. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2, and the first feeding was 1 week later and twice weekly thereafter until colonies appeared. The cells were then fixed with 95% ethanol and stained with hematoxylin. The colonies were counted manually under a light box and presented as CFE-F = colony counts per 10^6 MNCs. Because the colony-forming time was slightly different among groups (e.g., colonies appeared sooner in bioreactor output cells than in the static control), the CFE-F data were further corrected for time differences and presented as CFE-F/day.

Western Blot

Total proteins of both bioreactor-expanded and static control MSCs were extracted using standard methods and quantified using a BCA (bicinchoninic acid) protein assay kit (Pierce Technology, Perbio Science UK Ltd., Cramlington, U.K., http://www.piercenet.com). The procedure was carried out as previously described [14]. Briefly, nitrocellulose membranes were incubated with the following mouse monoclonal anti-human Abs: endoglin (SH-2), vimentin (DakoCytomation Ltd.), collagen type II, CCAAT/enhancer-binding protein-α (C/EBP-α)

(Insight Biotechnology Limited), osteocalcin (OC) (Abcam, Cambridge, U.K., http://www.abcam.com), and β-actin (Sigma) overnight at 4°C, followed by immune detection with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Igs) (DakoCytomation Ltd.). The antigen-Ab complexes were visualized with western blotting luminal reagent (Insight Biotechnology Limited).

Tri-Lineage Differentiation of the Bioreactor-Expanded MSCs

The reagents were all purchased from Sigma unless otherwise specified. Because the induction procedure is routinely performed on monolayer or pellet cells, the bioreactor output cells were allowed to attach prior to tri-lineage inductions. The passage-one MSCs were plated onto Petri dishes or LAB-TEK chamber slides (NUNC A/S) at a density of 2×10^4 cells per cm^2. For chondrogenic induction, additional aliquots of 1×10^6 cells were pelleted down and cultured in 15-ml polypropylene tubes (Iwaki brand; Scitech Division, Asahi Glass Co. Ltd., Chiba, Japan, http://www.agc.co.jp/english) alongside the chamber slide cultures. The chondrogenic induction medium consisted of serum-free DMEM supplemented with 0.1 μM dexamethasone (Decadron; Merck & Co., Inc., Whitehouse Station, NJ, http://www.merck.com), 0.2 mM ascorbic acid-2 phosphate, 1 mM sodium pyruvate, and 1:100 diluted ITS+ Premix (BD Biosciences, Oxford, U.K., http://www.bdbiosciences.com). Transforming growth factor-β-1 (PeproTech) was freshly added at a final concentration of 10 ng/ml each time. The medium was changed every other day. After 28 days, the cells cultured in chamber slides were stained for collagen type II, and pellet cells were embedded in paraffin for alcian blue staining or processed for transmission electron microscopy (TEM). For osteogenesis, 0.1 μM dexamethasone, 0.2 mM ascorbic acid-2 phosphate, and 10 mM β-glycerophosphate were added to the DMEM containing 10% FBS. The medium was changed twice weekly. After 18 days, the cells were subject to Von Kossa and alkaline phosphatase (ALP) staining and immunocytochemistry (ICC) examinations for OC, type I collagen, vimentin, and CD105 expressions. The adipogenic supplements consisted of 10^{-6} M dexamethasone, 0.50 mM methylisobutylxantine, and 50 uM indomethacin. The medium was changed twice a week, and after 12 days of culture, oil red O staining was performed to detect the accumulated lipid vacuoles in the cells.

ICC

A standard three-step indirect ICC method was used. In brief, after fixation in 95% ethanol, the cells were treated with 10% goat serum and incubated with primary monoclonal mouse anti-human collagen type II (1:200) and OC (1: 200), biotinylated secondary polyclonal goat anti-mouse Igs (1:100; DakoCytomation Ltd.), and ALP-streptavidin (1:100; Vector Laboratories, Ltd., Peterborough, U.K., http://www.vectorlabs.com). The positive staining was visualized with a Fast Red substrate pack (ZYMED Laboratories Inc., Cambridge, U.K., http://www.zymed.com), and the cells were counterstained with hematoxylin (Sigma). Negative controls were obtained by omitting the primary Abs.

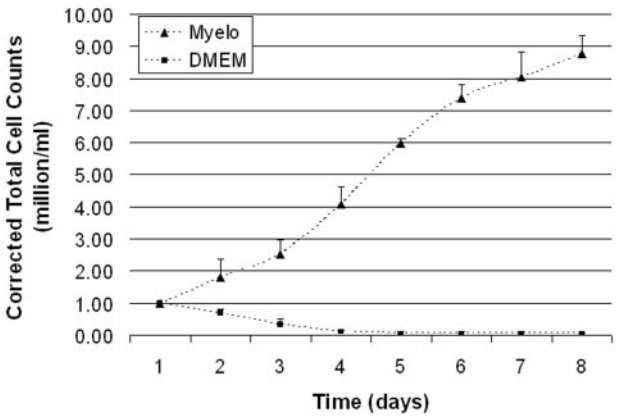


Figure 1. Total cell counts as a function of time in the bioreactor with two different media. Freshly isolated human bone marrow mononuclear cells were inoculated into the bioreactor with either modified Myelocult medium (▲) or DMEM (■) at a density of 1×10^6 cells per milliliter. Sample cells removed from the culture were counted manually throughout the culture period. By the end of 8 days, the total cell expansion upon input was 8.93 ± 0.41-fold and 0.11 ± 0.03-fold for the modified Myelocult bioreactor culture and DMEM bioreactor culture, respectively. The data are corrected for the dilution effect, and each time point represents the mean of six independent experiments, with error bars representing SD. Abbreviations: DMEM, Dulbecco's modified Eagle's medium; Myelo, Myelocult medium.

TEM

To determine whether the cells underwent chondrogenic differentiation, the pellets were fixed for TEM, washed in 0.1 M sodium cacodylate buffer, postfixed in 0.1 M osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated through graded ethanol solutions, and embedded in epoxy resin (Araldite; Sil-Mid Limited, Coleshill, West Midlands, U.K., http://www.silmid.com). Semithin sections were stained with 1% toluidine blue (Sigma) to select appropriate fields; ultrathin sections were cut with diamond knives, placed on mesh hexagonal copper (3.5 mm) grids, contrasted with uranyl acetate and lead citrate, and examined with a JEM-100CX II electron microscope (JEOL Ltd., Tokyo, http://www.jeol.com.au).

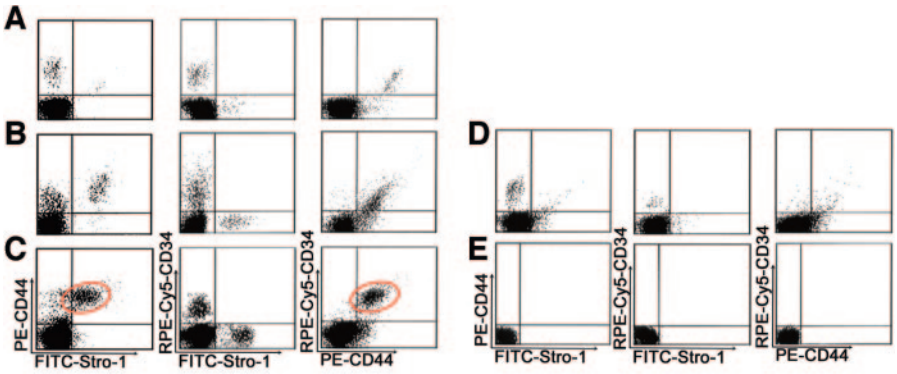
Statistics

The means of different populations were compared by analysis of variance using software SPSS version 13.0 (SPSS Inc., Chicago, http://www.spss.com), and $p < .05$ was considered statistically significant. The data are presented as the mean ± SD of the pooled six independent experiments.

RESULTS

Cell Expansion Kinetics

After inoculation, the initial proliferation was slow with $2.55 \pm 0.42 \times 10^6$ cells per milliliter recorded at day 3 (Fig. 1). After the first medium exchange, the average total cell count reached $4.07 \pm 0.44 \times 10^6$ cells per milliliter at day 4, and the expansion rate remained high for the following 2 days. The expansion speed gradually slowed down at days 7 and 8 as the growth curve began to level off. By the end of culture, the cell density increased from the initial 1×10^6 cells per milliliter to $8.93 \pm 0.41 \times 10^6$ cells per milliliter, and the average net expansion



F

Days	Myelocult (×100%)		DMEM (×100%)	
	Stro-1$^+$44$^+$ MSCs	34$^+$44$^+$HSCs	Stro-1$^+$44$^+$ MSCs	34$^+$44$^+$ HSCs
1	0.47	3.64	0.47	3.64
4	2.31	5.25	0.30	1.40
8	13.7	30.00	0.21	0.79
net expansion (fold)	29.15	8.24	0.45	0.22

Figure 2. Phenotypic analysis of cells cultured in the bioreactor with two different media. Tri-color flow cytometry examinations using FITC anti-Stro-1, PE anti-CD44, and RPE-Cy5 anti-CD34 antibodies were performed on the cells from the bioreactor with modified Myelocult and DMEM. **(A):** Data from both culture systems at day 1. **(B):** Data from cells cultured in the bioreactor with modified Myelocult medium at day 4. **(C):** Data from cells cultured in the bioreactor with modified Myelocult medium at day 8. Circles indicate the two stem cell subpopulations. **(D):** Data from cells cultured in the bioreactor with DMEM at day 4. **(E):** Data from cells cultured in the bioreactor with DMEM at day 8. Each panel is divided into four quadrants. **(F):** The percentage of cells in the destination quadrant(s). MSCs are defined as Stro-1$^+$ CD44$^+$CD34$^-$, and HSCs are CD34$^+$CD44$^+$Stro-1$^-$. Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; HSC, hematopoietic stem cell; MSC, mesenchymal stem cell; PE, phycoerythrin; RPE-Cy5, R-phycoerythrin-cyanin 5.

was approximately ninefold over input. However, when cells were treated in the bioreactor with DMEM, total cell counts declined rapidly, and cells were barely detectable by the end of the 8-day experimental period. The cell counts decreased from 1×10^6 cells per milliliter at day 1 to $0.11 \pm 0.03 \times 10^6$ cells milliliter ml at day 4 (Fig. 1). By the end of day 8, few cells still existed and the average net expansion was only 0.06-fold.

Expansion of MSCs in Bioreactor with the Modified Myelocult Medium

Tri-color flow cytometry was used to detect whether two stem cell subpopulations, MSCs and HSCs, were simultaneously expanded in bioreactor culture. The subpopulations were defined by Stro-1$^+$CD44$^+$CD34$^-$ for MSCs and CD34$^+$CD44$^+$Stro-1$^-$ for HSCs. There was no overlapping between Stro-1 and CD34, as shown by the two-parameter histogram consisting of FITC-Stro-1 on the *x*-axis and RPE-Cy5-CD34 on the *y*-axis. The data showed that both MSCs and HSCs increased significantly ($p < .05$) after 8 days of culture in the bioreactors with the modified Myelocult medium (Fig. 2). Upon inoculation, the MNC populations contained an average of 0.47% MSCs and 3.64% HSCs (Fig. 2A). At day 4, the MSC subpopulation increased to 2.31% and HSCs to 5.25% (Fig. 2B). By the end of the 8-day culture, the percentage reached 13.7% for MSCs and 30% for HSCs (Fig. 2C). The net expansion of the MSC and HSC percentages within the MNCs was 29- and 8-fold, respectively (Fig. 2F). In addition, the CD44 expression was also increased through the culture period. At both day 4 and day 8, the PE-fluorescence densities of CD44 were higher than that of day 1, especially for MSCs (Fig. 2B and 2C). In the control group with DMEM, the two subpopulations were not able to expand as seen by continuous loss of cells at all the time points tested. At day 4, the MSC subpopulation reduced to 0.30% and HSCs to 1.40% (Fig. 2D); by the end of culture, both subpopulations were barely detectable, with only 0.21% of MSCs and 0.79% of HSCs recorded (Fig. 2E). The net expansion in control DMEM after culture was 0.45-fold for MSCs and 0.22-fold for HSCs (Fig. 2F).

Comparison of CFE-F/Day of MSCs Obtained from Various Culture Conditions

CFE-F/day of the output cells after bioreactor culture with modified Myelocult medium was 1.44-fold over that of the static culture with DMEM (2.3 ± 0.2 vs. 1.6 ± 0.4, $p < .05$; Table 1). The CFE-F/day for the cells in the static culture with modified Myelocult was, however, decreased 40% compared with that of the static culture with DMEM (1.0 ± 0.2 vs. 1.6 ± 0.4). The average colony-forming time was 12 days for bioreactor-expanded MSCs with the modified Myelocult medium and 16 days for either static culture with the modified Myelocult medium or DMEM. At day 12, healthier and bigger colonies were seen in the cultures of bioreactor-expanded MSCs with the modified Myelocult medium (Fig. 3A), whereas in the static cultures with DMEM the colonies appeared smaller and immature (Fig. 3B).

Protein Expression of the Bioreactor-Expanded MSCs

Western blot showed that after probing with specific Abs, bioreactor-expanded MSCs were positive for both primitive mes-

Table 1. Comparison of CFE-F/day among the MNCs with different treatments

MNC culture condition	Mean culture time (days)	CFE-F/day						
		Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Mean ± SD
Bioreactor[a] culture	12	2.4	2.1	2.5	2.2	2.1	2.3	2.3 ± 0.2[c]
Static culture[a]	16	1.0	0.8	1.4	0.9	1.1	1.0	1.0 ± 0.2[c]
Static culture[b] (control)	16	1.6	1.1	2.1	2.0	1.6	1.2	1.6 ± 0.4

[a] Modified Myelocult medium.
[b] Dulbecco's modified Eagle's medium.
[c] $p < .05$ analysis of variance.
Abbreviations: CFE-F/day, colony-forming efficiency-fibroblast per day; MNC, mononuclear cell.

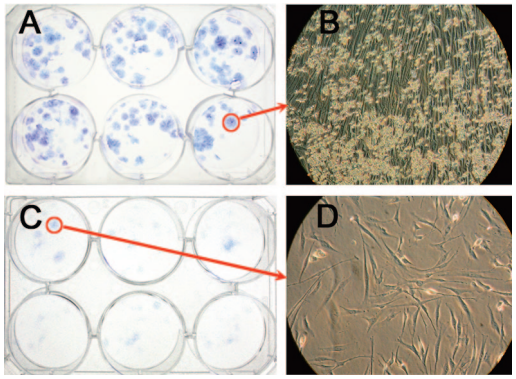


Figure 3. Comparison of colony appearances in colony-forming efficiency-fibroblast (CFE-F) assay. **(A):** The output cells after 8 days of bioreactor culture with the modified Myelocult medium were subjected to the standard CFE-F assay procedure. **(B):** The output cells after 8 days of bioreactor culture with the donor-matched freshly isolated bone marrow mononuclear cells (MNCs) were subjected to the standard CFE-F assay procedure. After 12 days, the bioreactor output cells showed densely arranged colonies with typical fibroblastic-like cells and numerous hematopoietic stem cells in the culture, whereas the MNCs formed only sparsely aligned colonies. Magnification: ×100.

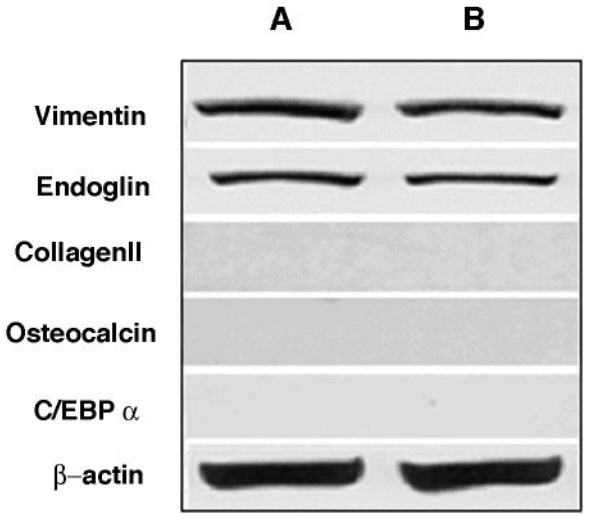


Figure 4. Comparison of the protein expressions on mesenchymal stem cells (MSCs). **(A):** Bioreactor-expanded MSCs. **(B):** Donor-matched static-expanded MSCs (control). Western blot examinations showed that the two sets of MSCs exhibited a similar protein expression pattern. Both groups expressed primitive mesenchymal markers vimentin and endoglin but not the differentiation markers type II collagen (chondrogenesis), osteocalcin (osteogenisis), and CCAAT/enhancer-binding protein-α (C/EBP-α) (adipogenesis). The data are typical of six independent experiments.

enchymal progenitor cell markers vimentin and endoglin (SH2), whereas the lineage-specific markers type II collagen (chondrogenesis), OC (osteogenesis), and C/EBP-α (adipogenesis) were not detected (Fig. 4, lane A). The expression pattern of the above markers was similar in the MSCs cultured in static control (Fig. 4, lane B).

Multilineage Differentiation Potentials of the Bioreactor-Expanded MSCs

After chondrogenic induction for 28 days, the bioreactor-expanded MSCs were positive for type II collagen immunostaining (Fig. 5A); paraffin-embedded cell pellets stained with alcian blue showed that chondrogenically differentiated cells were encapsulated in the chondrocytic lacunae (Fig. 5B, inset); TEM examinations of the cell pellets demonstrated typical features of hyaline cartilage, with randomly oriented (collagen) fibrils and (proteoglycan) granules (Fig. 5B). When treated with osteogenic medium over time, the cells underwent a morphological transformation from long, thin, and smooth-edged cells to more extended polygonal-shape cells. At approximately day 13, the cells were positive for both ALP (Fig. 5D) and OC (Fig. 5E), and at approximately day 18, the extracellular matrix mineralization was confirmed by Von Kossa staining (Fig. 5C). After treatment with adipogenic medium for an average of 12 days (from 7 to 17 days), the cells showed a reduction in nuclear size and the accumulated lipid vacuoles within and around the cells were positive for the oil red O staining (Fig. 5F).

Discussion

In this study, we investigated the feasibility of expanding MSCs in a rotary bioreactor with the modified Myelocult medium. After 8 days of bioreactor treatment, the percentage of MSCs among the inoculated MNCs increased up to 29-fold. The expanded MSCs maintained their stemness as proven by both phenotypic analysis and functional assays.

It is well known that microgravity conditions promote cell growth as seen in outer space. Colvin et al. were the first to introduce the concept that microgravity conditions promoted stem cell expansion [15]. From then on, numerous publications have provided evidence that microgravity positively regulates cell growth. The bioreactor used here was invented by NASA to simulate tissue culture condition in outer space and is considered to be the next generation of spinner flasks. It offers the unique environment of microgravity and minimal shear stress, which enables cells to grow much as they would in vivo. The mammalian cells are

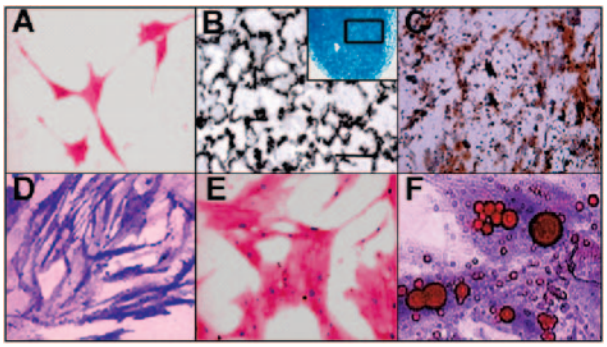


Figure 5. Multilineage differentiations of the bioreactor-expanded mesenchymal stem cells. **(A):** After 28 days of chondrogenic induction, the cells were positive for collagen type II. **(B):** Transmission electron microscopy showed cell pellets with a fibrillar network typical of hyaline cartilage, containing randomly distributed granules. Inset shows the macromorphology of a cartilage cell pellet on an alcian blue-stained paraffin section (internal bar: 2 μm). **(C):** After 18 days of osteogenic induction, the mineralization was confirmed by Von Kossa staining in the culture. **(D):** Cells were positive for alkaline phosphatase. **(E):** Cells were also positive for osteocalcin. **(F):** For adipogenic induction, the lipid vacuoles accumulated in the differentiated cells were stained by oil red O. Magnifications: ×400 **(A, D–F)**, ×11,800 **(B)**, ×200 **(C)**.

particularly shear-sensitive and cannot survive at higher agitation rates. In this sense, the bioreactor is superior to the spinner flask because the latter employs continuous agitation and may disrupt the well-being and further expansion of the stem cells.

In the bioreactor system, three important parameters affecting cell survival are feeding regime, shear force effect, and culture medium with supplements. The LDF protocol used in the present study has certain advantages. First, as the culture progresses, rapid stem cell expansion may lead to the depletion of stimulatory cytokines and the production of suppressive factors in the culture system, compromising the further expansion of primitive stem cells [16, 17]. Medium exchange by LDF allows the dilution of inhibitory factors and retention of stimulatory ones at the same time. It has been proven that, both in bioreactor and static cultures, daily feeding with dilution of cells (without retention of the cells in the discarded medium) will improve the expansion of hematopoietic progenitors as well as total cell counts compared with feeding every other day with total cell retention [18–20]. Second, to achieve the optimal cell expansion in vitro, the interchange of metabolic waste and nutrients in the bioreactor culture system should be comparable with that occurring in vivo. A daily exchange of 20% of the total medium in the suspension culture system mimics the plasma perfusion rate in bone marrow, which is approximately 0.1 ml/ml marrow per minute [21]. Current results confirm that 20% dilution feeding is suitable for rapid expansion of primitive MSCs (Figs. 4 and 5).

The cytokine cocktail used in the present study is a combination of SCF, IL-3, and IL-6. The concentration of the cytokines used in the cocktail was determined in our preliminary experiments by screening the effects on MSC proliferation by various concentrations of SCF (1, 5, 10, 50, and 100 ng/ml) and IL-3 and IL-6 (1, 5, 10, 25, and 50 ng/ml). SCF, the ligand for the tyrosine kinase receptor c-kit, is an early-acting cytokine for hematopoiesis. It maintains and preserves long-term hematopoietic progenitors per se compared with the synchronized effect that many other cytokines need. Tong et al. have shown that the administration of SCF increased the absolute number of $CD34^+$ cells and primitive myeloid progenitor cells after chemotherapy in patients with breast cancer [22]. IL-3 is a controversial cytokine; reports are conflicting in regard to its ability to inhibit or stimulate stem cell expansion. It has been demonstrated that IL-3 abrogated the expansion and self-renewal of primitive stem cells in a concentration-dependent manner [23]. In both human and murine models, IL-3 showed inhibitory effects on HSC reconstitution [1, 24]. On the other hand, IL-3 also promotes HSC proliferation at a high level of quality [25]. In the current study, IL-3 exhibited positive effects on the proliferation and preservation of both MSCs and HSCs, which may be partially explained by the finding of a significant increase of $CD45^-CD123^+$ (IL-3-α receptor) cells with osteogenic potential after suspension culture [11]. Unlike in previous studies [10, 11], IL-6 was also added into the cocktail after a screening test showed that IL-6 alone stimulated MSC expansion. It has been suggested that IL-6 improves the transplantation outcome of human stem cells and stimulates hematopoiesis (especially thrombopoiesis) [22, 26]. Interestingly, it has been demonstrated that IL-6 has an antitumor effect [27]. The presence of IL-6 may help to eliminate the possible contamination of malignant cells in bone marrow harvests [28]. This may further strengthen the purging effect of the bioreactor during clinical practice [29].

Upon inoculation, we used a boost dose for the cytokine cocktail and switched to a lower concentration after the first medium exchange. Our preliminary data showed that the cell expansion was more rapid when the boost dose with higher concentrations of cytokine was used. The boost dose may help the primitive stem cells move from the quiescent state into the cell proliferation cycle, thus shortening the culture period. However, continuous addition of high doses of cytokines in HSC culture promotes rapid cell proliferation as well as cell differentiation [30]. Therefore, a combination of high (upon inoculation) and low (from first feeding) doses of cytokine supplements was used in the present study to ensure the expansion and preservation of primitive stem cell subpopulations.

Apart from the added exogenous cytokines, the endogenous factors produced by MSCs and HSCs in the suspension configuration also help to prevent the apoptosis and differentiation of the progenitor cells. As within the bone marrow cavities, MSCs and HSCs are always in close contact and support each other through the release of various growth factors, cytokines, and chemokines. In HSC culture, a stromal feeding layer is required for the growth of long-term culture-initiating HSCs by providing cytokines and growth factors such as leukemia inhibitory factor (LIF), Flt-3 ligand (FL), and granulocyte-macrophage colony-stimulating factor [31, 32]. LIF is constitutively expressed by bone marrow stroma cells and plays several roles in preventing differentiation of HSCs [20]; FL is capable of recruiting HSCs into the cell proliferation cycle and promoting cell survival through inhibition of apoptosis [33]. The understanding of the complex cytokine profiles in the rotary bioreactor suspension culture system will provide insights for ex vivo stem cell manipulation.

In the present study, the rotary speed of the bioreactor was 20 rpm, which was determined after different tests using 10, 15, 20, and 23 rpm. We found that 20 rpm was the optimal speed to prevent cell aggregation during the culture period in the biore-

actor. We also found that cells started forming aggregates after 8 days in the rotary bioreactor. It has been postulated that microgravity conditions favor high-quality stem cell expansion for only a short period [15]. As culture progressed, extra efforts such as filtering the aggregates using 70-μm cell strainers and repeated pipetting had to be adopted to separate the cells; this was not practical after day 13, because the cells in the bioreactor became heavily aggregated. Because the total cell counts reached a peak at approximately day 8 and started declining afterward, the duration of 8 days was chosen as an average time for keeping the cells in the rotary bioreactor. In addition, the serological factors in the culture system have been reported to accelerate the differentiation of stem cells and may have a negative effect on retention of progenitor cells [34]. Even in serum-free conditions, the early-stage long-term repopulating HSCs were lost after 9 days of culture despite a further increase of the later-stage colony-forming cells and $CD34^+$ cells [35]. Based on above observations, the slow-down of cell proliferation after approximately 8 days in the current bioreactor suggests the exhaustion or arrest of the early progenitor cells (Fig. 1). In clinical applications, the potential side effects related to serum supplementation have to be avoided. Baksh et al. optimized their culture configuration by using 10-fold-higher cytokine supplements in the absence of serum [10, 11]. The quality of MSC output after such a culture remained high. Efforts to define an alternative serum-free culture condition for MSC bioreactor expansion are under way.

In static culture, a small fraction of MSCs are actively engaged in the proliferation stage of the cell cycle ($S+G_2+M$), whereas nearly 80% MSCs are in the quiescent state (G_0) [36–38]. The average colony-forming time for the current bioreactor outputs was 12 days compared with 16 days for the non-treated control cells (Table 1), implying that the bioreactor treatment has increased the proliferation ability via promoting quiescent progenitors into the cell cycle and, consequently, shortened the time needed for colony formation upon plating.

CONCLUSION

The rotary bioreactor with the modified Myelocult medium reported in this study may be used to rapidly expand primitive MSCs.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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