Xeno-free proliferation of human bone marrow mesenchymal stem cells

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Abstract The proliferation of human bone marrow mesenchymal stem cells (MSCs) employing xeno-free materials not containing fetal calf serum (FCS) and porcine trypsin was investigated for the regenerative medicine of cartilage using MSCs. Four sequential subcultivations of MSCs using a medium containing 10% FCS and recombinant trypsin (TrypLESelect™) resulted in cell growth comparable to that with porcine trypsin. There was no apparent difference in the cell growth and morphology between two kinds of MSC stored in liquid nitrogen using 10% FCS plus DMSO or serum-free TC protector™. MSCs were isolated from human bone marrow cells, stored in liquid nitrogen, and sequentially subcultivated four times employing conventional materials that included FCS, porcine trypsin, and DMSO, or xeno-free materials that included serum-free medium (MesenCult-XF™),

TC protector™ and TrypLESelect™. Cells in the culture using the xeno-free materials maintained typical fibroblast-like morphology and grew more rapidly than the cells in the culture using the conventional materials, while the cell surface markers of MSCs (CD90 and CD166) were well maintained in both cultures. Chondrogenic pellet cultures were carried out using these subcultivated cells and a medium containing TGFβ3 and IGF1. The pellet culture using cells grown with the xeno-free materials showed an apparently higher gene expression of aggrecan, a chondrocyte marker, than the pellet culture using cells grown with the conventional materials. Consequently, MSCs that are isolated, stored, and grown using the xeno-free materials including the serum-free medium (MesenCult-XF™), TC protector™, and recombinant trypsin (TrypLESelect™) might be applicable for regenerative medicine of cartilage.

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Introduction

Mesenchymal stem cells (MSCs) in adult bone marrow have been shown to give rise to multiple mesodermal tissue types, including bone and cartilage (Caplan 1991), tendon (Caplan et al. 1993), muscle (Wakitani et al. 1995), fat (Dennis and Caplan 1996),

and marrow stromal cells (Majumdar et al. 1995, 1998). MSCs have an adherent, fibroblast-like morphology, and the surface antigens were characterized as CD45[−], which is a representative marker of hematopoietic cells, and CD90⁺ and CD166⁺, which were reported to be markers of MSCs (Arai et al. 2002; Bruder et al. 1998; Stute et al. 2004; Wexler et al. 2003). These stem cells are isolated from bone marrow aspirates and, because of their multilineage potential, present exciting opportunities for cell-based therapeutic applications. In fact, therapeutic modalities have been described for the use of MSCs in cartilage (Wakitani et al. 1994), bone (Bruder et al. 1998), tendon (Young et al. 1998), and bone marrow stroma (Lazarus and Caplan 1995) regeneration.

MSCs should be expanded *in vitro* after isolation from bone marrow in order to be utilized for regenerative medicine, because the number of MSCs isolated from one adult bone marrow aspirate is too small (i.e., 2,000 cells, Bruder et al. 1997) to effect tissue regeneration. It was reported that human bone marrow MSCs could be passaged in a medium containing fetal calf serum (FCS) up to the 38th population doubling level (Δ PDL) while still maintaining their osteogenic potential (Bruder et al. 1997).

It is very important in a cell cultivation process aimed at clinical transplantation to remove completely FCS and other animal-derived materials such as trypsin used for cell harvesting from the culture used, because calf serum and other animal-derived materials may be contaminated with agents causing diseases such as BSE. Moreover, FCS was reported to stimulate the differentiation of MSCs during proliferation culture, which may lead to an unstable culture process (Yokoyama et al. 2008).

The cell-growth-stimulating activity of fish serum for MSCs is yet unclear, although the use of fish serum has been investigated (Fujiwara et al. 2007, 2009, 2010). The *in vitro* cell-growth-stimulating activity of adult human serum was much lower than that of fetal serum. On the other hand, fibroblast growth factor was reported to stimulate several kinds of primary cell including MSCs (Tsutsumi et al. 2001). Thus, the combined use of adult human serum with basic fibroblast growth factor (FGF2) was reported to be effective for removing FCS from the growth medium for MSCs (Takagi et al. 2003). However, the quality of adult human serum is unstable and varies between donors.

Although many researchers have investigated the use of serum-free media containing substitutes such as hormones (Barnes and Sato 1980; Shipley and Ham 1983; Clark et al. 1981) including MesenCult-XFTM medium (Karen et al. 2004) and their quality might be more stable than those of adult human serum and fish serum, the differentiation capacity of MSCs that are grown using them is yet unclear.

FCS is required not only for cell proliferation but also to minimize cell damage during cell storage in liquid nitrogen. Recently, xeno-free products such as TC protectorTM and recombinant trypsin (TrypLESelectTM) have been supplied to minimize cell damage during cell storage in liquid nitrogen and to recover adherent cells, respectively.

However, there was no report on a xeno-free system for MSC proliferation culture combining serum-free medium, TC protectorTM, and TrypLESelectTM, in which not only cell proliferation but also cell quality, such as surface marker and differentiation activity, were investigated.

In this study, the influences of the isolation, storage, and subcultivation of human bone marrow MSCs employing xeno-free materials without FCS and porcine trypsin on the phenotypes of surface antigens and chondrogenic capacity were investigated for the regenerative medicine of cartilage using MSCs.

Materials and methods

Cell preparation and subcultivation

Bone marrow was obtained from human donors (Donor A, 69 years old, male; donor B, 17 years old, male; donor C, 72 years old female). All the subjects enrolled in this research gave their informed consent, which was approved by our institutional committee on human research, as was the study protocol. Approximately 10 ml of unfractionated bone marrow was obtained by routine iliac crest aspiration. The nucleated cell (NC) concentration was determined with Turk's solution and a hemocytometer. Two methods with or without FCS were employed to prepare and subcultivate adherent cells containing human MSCs. In the former conventional method, the bone marrow aspirate (donors A, B, and C) was diluted with growth medium, plated in a dish (21 cm², Corning, Tokyo) to a concentration of 6.0×10^5 NCs/cm² and cultured at 37 °C in a

humidified atmosphere containing 5% CO₂ for 16 days, during which time the medium was changed on days 1, 2, 9. On day 16, the cells were detached using trypsin–EDTA (Sigma, St. Louis, MO, USA) and inoculated to the original dishes. On day 18, the cells were detached again, counted by the trypan blue dye-exclusion method, suspended in the growth medium supplemented with 10% DMSO, and stored in liquid nitrogen. After thawing, the cells were inoculated to a dish (55 cm², Corning, Tokyo) at a density of 0.15×10^4 cells/cm² and medium change was performed every week thereafter. The cells were detached with trypsin and subcultivated further when the culture reached near confluence. DMEM-LG (Gibco, NY, USA) containing 10% serum, 2,500 U/l penicillin, and 2.5 mg/l streptomycin was employed as the growth medium.

In the latter xeno-free method, MesenCult-XF Attachment Substrate (Stem Cell Co., Palo Alto, CA, USA) was diluted 28 times with PBS and the dish was coated with 1.75 ml of the diluted solution overnight before inoculation. The bone marrow aspirate (donors B and C) was diluted with the growth medium, plated in the coated dish to a concentration of 6.0×10^5 NCs/cm² and cultured at 37 °C in a humidified atmosphere containing 5% CO₂ for 13 days, during which time half of the medium was changed on day 10. On day 13, the cells were detached using TrypLESelect™ (Invitrogen Co., Carlsbad, CA, USA) following the manufacturer's instructions, counted by the trypan blue dye-exclusion method, suspended with TC protector, and stored in liquid nitrogen. After thawing, the cells were inoculated onto the coated dish (55 cm², Corning, Tokyo) at a cell density of 0.15×10^4 cells/cm² and medium change was performed every week thereafter. The cells were detached with TrypLESelect™ and subcultivated further when the culture reached near confluence. MesenCult-XF (Stem Cells) containing MesenCult-XF Supplement (Stem Cell) and L-glutamine (Stem Cells) was employed as the growth medium. The increase in ΔPDL was calculated from the fold increase in cell concentration during subcultivation.

All culture experiments were performed in triplicate and the mean is shown.

Chondrogenic differentiation

High-density pellet cell cultures were initiated by centrifugation (500×g for 5 min) of 2.5×10^5 cells

suspended in 0.5 ml of the differentiation medium in 15-ml conical tubes. The pellet was incubated for 3 weeks at 37 °C in 5% CO₂, during which time the medium was changed every 3 days. The pellet was hydrolyzed as previously reported to obtain suspended cells for analysis (Matsuda et al. 2005).

The differentiation medium was DMEM-HG supplemented with 10 ml/l ITS-Premix™ (BD Bioscience, Franklin Lakes, NJ USA), 2,500 U/l penicillin, 2.5 mg/l streptomycin, 50 µg/ml L-ascorbic acid 2-phosphate (Wako Pure Chemicals, Osaka), 100 µg/ml sodium pyruvate (Wako), 40 µg/ml proline (ICN Biomedicals, Costa Mesa, California, USA), 39 ng/ml dexamethasone (ICN Biomedicals), 10 ng/ml TGF-beta3 (Peprotech, Rocky Hill, NJ, USA) and 100 ng/ml IGF-I (Peprotech).

RNA preparation and RT-PCR analysis

Total RNA was extracted from the cells using the RNeasy minikit (Qiagen, Victoria, Australia). DNase-treated RNA was used to produce cDNA using Omniscript and Sensiscript RT kits (Qiagen) and the Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). PCR was performed with cDNA using a HotStar Tag Master Mix kit (Qiagen) and an ABI PRISM 7700 system (Applied Biosystems) using the primers (Sense 5'-AGTCCTCAAG CCTCTGTACTCA-3', Antisense 5'-GCAGTTGAT TCTGATTCACGTTTC-3'), probe (5'-ATGCTTCC ATCCAGCTTCTCCGG-3') for aggrecan, and actin as the standard (NM 001101; Applied Biosystems).

The cDNA prepared with RNA isolated from primary human chondrocytes in the articular cartilage was employed as a positive control for PCR analysis. The aggrecan expression level was determined using Eq. 1.

$$\text{Aggrecan expression level (\%)} = \frac{\frac{\text{Amount of aggrecan (cultured cell)}}{\text{Amount of actin (cultured cell)}}}{\frac{\text{Amount of aggrecan (control)}}{\text{Amount of actin (control)}}} \times 100 \quad (1)$$

Flow cytometry analysis

Cells harvested by trypsinization were stained with mouse IgG anti-human CD90 (Chemicon, San Diego, CA, USA) and fluorescein isothiocyanate

(FITC)-conjugated goat anti-murine IgG (Chemicon). Thereafter, the cells were stained with phycoerythrin (PE)-conjugated anti-human CD166 antibodies (Beckman Coulter, Miami, FL, USA) and analyzed using a flow cytometer (EPICS XL; Beckman Coulter) equipped with an argon laser (488 nm).

Results

Subcultivations using recombinant trypsin

In order to study the usefulness of recombinant trypsin, MSCs isolated from bone marrow (donor A) and stored in liquid nitrogen using 10% FCS-containing medium with DMSO were subcultivated 3 times using 10% FCS-containing medium and trypsin (porcine trypsin or recombinant TrypLESelect). Two ways were employed to inoculate cells detached from the culture dish using the recombinant trypsin to the next culture. In one way, the cell suspension containing the recombinant trypsin was directly transferred to the next culture. In the other way, after the culture supernatant was removed by centrifugation (1,000 rpm, 5 min), the harvested cells were suspended in fresh medium and then transferred to the next culture.

The average increase in PDL is shown and the standard deviations are not shown because they were too small (Fig. 1). The cells grew monotonically in all the cultures. There was no significant difference in PDL at day 40 between cultures using porcine trypsin or recombinant trypsin with centrifugation, while the PDL of the cells cultivated using recombinant trypsin without centrifugation was markedly lower than that of the culture using porcine trypsin. There was no apparent difference in cell morphology between the cells at day 40 in these three types of culture, and they contained some polygonal cells besides fibroblast-like cells (Fig. 2a, b, c).

Cell stock using TC protector

In order to study the usefulness of FCS-free cell stock reagent (TC protector), MSCs isolated from bone marrow (donor A) using 10% FCS-containing medium were stored in liquid nitrogen using 10% FCS medium containing DMSO or TC protector. These cells were thawed using 10% FCS-containing medium and cultivated in a dish for 14 days.

Fig. 1 Comparison of subcultivations using porcine trypsin and TrypLESelect. Cells that were isolated from donor A using 10% serum-containing medium and stored using 10% serum-containing medium and DMSO were incubated using 10% FCS medium and subcultivated 3 times using porcine trypsin (circle) or TrypLESelect (square, triangle). Centrifugation for the removal of TrypLESelect at the subcultivation was performed in the culture indicated by the square symbol. The average of triplicated cultures is shown

There was no marked difference between the two storage methods in terms of recovery and viability of the thawed cells (Table 1). The initial cell adhesion yield and mean generation time of the cells stored using TC protector were almost the same as those of the cells stored using 10% FCS medium containing DMSO. There was also no apparent difference between the two methods for cell stock in terms of cell morphology after 14 days of growth (Fig. 2d, e).

Subcultivation of adherent cells in bone marrow using xeno-free materials

In order to study the usefulness of completely xeno-free materials combining recombinant trypsin (TrypLESelect), FCS-free cell stock reagent (TC protector), and serum-free medium (MesenCult XF), bone marrows of new donors B and C were used, because adherent cells in the bone marrow of donor A had been isolated using FCS-containing medium. MSCs were isolated from bone marrow (donors B and C), stored in liquid nitrogen, and subcultivated using conventional materials (FCS-containing medium, porcine trypsin, and cell stock reagent containing FCS and DMSO) or the xeno-free materials

Fig. 2 Microscopic observation of cells in culture. Cells at the end of subcultivations shown in Fig. 1 using porcine trypsin (a) or TrypLESelect with (b) or without (c) centrifugation. Cells at the end of culture inoculated with the cells stored using FCS-containing medium and DMSO (d) or TC protectorTM (e) shown

in Table 1. Cells derived from donor B subcultivated as shown in Fig. 3 using the conventional (f, h) or xeno-free materials (g, i) at passage numbers 1 (f, g) and 4 (h, i). A scale bar in the right bottom is for all photos

Table 1 Comparison of cell stock reagents

	Cell stock reagents	
	FBS + DMSO	TC protector
Stored		
Cell number (10^5 cells)	10	10
Recovered		
Cell number (10^5 cells)	7.3	7.8
Viability (%)	78.6	79.6
0 Day		
Cell density (10^3 cells/cm ²)	1.5	1.5
1 day		
Cell density (10^3 cells/cm ²)	0.68 ± 0.10	0.71 ± 0.08
Viability (%)	96.3 ± 0.5	96.4 ± 0.4
14 days		
Cell density (10^3 cells/cm ²)	7.02 ± 0.78	7.27 ± 0.96
Viability (%)	94.4 ± 1.8	95.0 ± 1.0
Mean generation time (h)	91.9	91.5

(serum-free medium (MesenCult XF), recombinant trypsin (TrypLESelect), and serum-free cell stock reagent (TC protector)).

Cell number was determined at the time of stock and subcultivations, and the increase in Δ PDL was calculated on the assumption that the content of adherent cells among bone marrow nucleated cells (NCs) was 0.1% (Fig. 3). Both cells derived from donors B and C grew apparently faster in the culture with the xeno-free materials than in the culture with the conventional materials. Passage number of cells was defined as one (P1) at the cell stock and increased as subcultivation progressed (P1-P4 for cells from donor B, P1-P5 for cells from donor C). Cells in the culture with the xeno-free materials showed a thinner morphology than those in the culture with the conventional materials, while the cells maintained a typical fibroblast-like morphology throughout the whole period of culture in both cultures (Fig. 2f–i).

Characteristics of cells subcultivated with xeno-free materials

Cell surface antigens and chondrogenic activity of the passaged cells shown in Fig. 3 were analyzed. Because these analyses required a large amount of cells, the cells in each passage were pooled before analysis. Surface antigen was analyzed for the pooled cells ($n = 1$) only in later passage of subcultivations of cells from donors B and C (Table 2). The pellet culture was performed using the pooled cells in triplicate during the subcultivation of cells from only donor B ($n = 3$) and the aggrecan gene expression level was determined before and after the pellet culture (Table 3).

The percentage of CD90-positive cells was maintained at around 90% or higher in each passage during both subcultivations using the conventional and xeno-free materials (Table 2). The percentage of CD166-positive cells was almost 100% in all the passages determined.

Pellets were successfully obtained by the pellet culture employing these passaged cells from the bone marrow of donor B. The aggrecan expression level of the cells was apparently higher after the pellet culture than before the pellet culture, while the cells before the pellet culture showed almost no aggrecan expression

Fig. 3 Subcultivation of adherent cells in bone marrow. Cells in bone marrow (donor B *square*; donor C *circle*) were cultivated, stored in liquid nitrogen, and further subcultivated using the conventional (*open symbols*) or xeno-free materials (*closed symbols*). Cell number was determined at the time of stock and subcultivation, and the increase in population doubling level (Δ PDL) was calculated on the assumption that the content of adherent cells among bone marrow nucleated cells was 0.1%

Table 2 Analysis of cell surface markers of subcultivated cells

	Passage	Δ PDL	CD90 (%)	CD166 (%)
Donor B				
Conventional	P4	12.9	97.4	99.8
Xeno-free	P3	17.9	88.3	99.9
	P4	22.1	93.4	100.0
Donor C				
Conventional	P3	12.1	98.4	99.8
	P4	14.6	97.4	99.8
	P5	17.0	98.7	99.8
Xeno-free	P3	19.9	92.3	99.9
	P4	23.3	88.5	99.9
	P5	26.5	95.4	100.0

(Table 3). The aggrecan expression level of the cells passaged with the xeno-free materials was markedly higher than that of the cells passaged with the conventional materials.

Discussion

Remaining recombinant trypsin may not be deactivated by serum components and may inhibit cell growth thereafter, because the cell growth in the subcultivation without the removal of recombinant trypsin by centrifugation was apparently slower than that in other subcultivations using porcine trypsin or recombinant trypsin with centrifugation (Fig. 1). Thus, centrifugation was performed at the subcultivation using recombinant trypsin in later experiments.

The centrifugation and re-suspension of cells may decrease the concentration of FCS carried-over to approximately 1/50. So, the FCS concentration in the cell stock might be at most 0.2% and it may not affect the cell growth. The difference in the kinds of trypsin and cell stock reagent had no apparent effect on the cell growth rate (Fig. 1, Table 1). Thus, the reason for the apparently faster cell growth in the subcultivations using the xeno-free materials than with the conventional materials might be the difference in the growth media of 10% FCS-containing DMEM and serum-free MesenCult-XF (Fig. 3). The composition of MesenCult-XF should be clarified when this xeno-free culture system is applied in clinical use.

Table 3 Chondrogenic capacity of subcultivated cells

	Passage	Δ PDL	Aggrecan expression (%)	
			Before	After induction
Conventional	P3	9.6	0.42	13.4 $\pm$ 7.6
	P4	12.9	0.16	8.8 $\pm$ 2.6
Xeno-free	P2	14.3	0.03	48.4 $\pm$ 5.7
	P3	17.9	0.06	75.1 $\pm$ 17.1
	P4	22.1	0.03	63.0 $\pm$ 13.4

Pellet cultures ($n = 3$) were performed using the subcultivated cells derived from donor B. Aggrecan gene expression was determined before ($n = 1$) and after the pellet culture

A sufficient amount of cells could not be harvested at smaller passage numbers and their surface markers were not analyzed. Almost all and approximately 90% of the cells during both subcultivations using the conventional and xeno-free materials expressed CD166 and CD90, respectively (Table 2). There was almost no aggrecan expression in the cells during the subcultivations of the cells from donor B (Table 3). Thus, almost all the cells were MSCs even when using the xeno-free materials.

The amount of bone marrow aspirate from donor C was small and the number of cells was insufficient for the pellet culture. The aggrecan expression level markedly increased during the pellet cultures and the cells subcultivated using the xeno-free materials reached a higher level than the cells subcultivated with the conventional materials (Table 3). While some cell populations bearing higher chondrogenic capacity could be selected during the subcultivations with the xeno-free materials, the mechanism is not clear. Anyway, the subcultivations using the xeno-free materials maintained higher chondrogenic capacity than those using the conventional materials.

Cartilage (5 cm in length, 2 cm in width, 4 mm in thickness, cell density of 5×10^6 cells/ml) contains 2×10^7 chondrocytes. Generally, 10 ml of bone marrow aspirate contains approximately 1×10^9 NCs and 1×10^5 MSCs if the content of MSCs among nucleated cells is 0.01% (Pittenger et al. 1999). Consequently, MSCs should be expanded at least 200-fold for regenerative medicine; the fold increase in cell number corresponds to the increase observed after a Δ PDL of 8 ($2^8 = 256$). However, this should be the minimum requirement, because there might be some loss of cells during transplantation operation and several pieces of cartilage-like constructs should be

transplanted in some patients. In this study, MSCs could be expanded up to 22.1 Δ PDL, which might be a sufficient expansion in cell number for clinical use.

Conclusions

In conclusion, MSCs in bone marrow aspirate could be isolated, stored, and expanded to a sufficient number for clinical use through subcultivations using xeno-free materials maintaining the surface markers of MSCs and chondrogenic capacity. Thus, this xeno-free proliferation system for bone marrow MSCs might be applicable to the regenerative medicine of cartilage.

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