***In vitro* proliferation of human bone marrow mesenchymal stem cells employing donor serum and basic fibroblast growth factor**

Mutsumi Takagi^{1,*}, Tetsuya Nakamura¹, Chikayoski Matsuda¹,
Takako Hattori², Shigeyuki Wakitani³ and Toshiomi Yoshida¹

¹International Center for Biotechnology, Osaka University, 2-1 Yamada-oka, Suita, Osaka 565-0871, Japan;

²Department of Orthopedic Surgery, Osaka-Minami National Hospital, 2-1 Kidohigashi-machi, Kawachinagano, Osaka 586-8521, Japan; ³Orthopedic Surgery, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan (*Author for correspondence, current address: Division of Molecular

Chemistry, Graduate School of Engineering, Hokkaido University, Sapporo, 060-8628, Japan, fax: +81-11-706-6567, E-mail: takagi-m@eng.hokudai.ac.jp)

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Abstract

A method for the *in vitro* proliferation of human bone marrow mesenchymal stem cells (MSCs) employing a medium not containing fetal calf serum (FCS) was developed for a regenerative medicine of cartilage using MSCs. Without using density-gradient centrifugation, the bone marrow aspirate was poured into a dish (6.0×10^5 nucleated cells/cm²) with DMEM medium containing 10% serum (FCS or donor serum) and basic fibroblast growth factor, and incubated at 37 °C under a 5% CO₂ atmosphere. The density of adhesive cells incubated with the medium containing human serum and basic fibroblast growth factor (10 ng/ml) almost reached confluence at 19d and was 1.4–2.7 times that in the medium containing only FCS. The density of cells incubated with the medium containing only human serum was 0.1–0.6 times that in the medium containing only FCS. The content of CD45[−] CD105⁺ cells among the cells harvested after a 19-d incubation in the medium containing human serum and basic fibroblast growth factor was higher than 90%. This high content and chondrogenic activity, which was confirmed by pellet cultivation and staining with Safranin O, were maintained even after further subcultivation in the medium to 17 population doubling levels. Consequently, this method might be applicable to *in vitro* proliferation of MSCs for the regeneration of cartilage.

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). MSCs have an adherent, fibroblast-like morphology, and the surface antigens

were characterized as CD45[−], which is a representative marker of hematopoietic cells, and CD105⁺, which is the TGF- β receptor endoglin (Barry et al. 1999; Pittenger et al. 1999). 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 medicines, because the number of MSCs isolated from one adult bone marrow aspirate is too small (e.g., 2000 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 (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 FCS completely from the medium used, because calf serum is not only animal-derived but may be contaminated with agents causing diseases such as BSE. Although many researchers have investigated the use of serum-free media containing expensive substitutes such as hormones, these media were applicable mainly to the proliferation of cell lines and not primary cells. (Barnes and Sato 1980; Clark et al. 1981; Shipley and Ham 1983) 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 cells including MSCs. (Tsutsumi et al. 2001)

Industrialization of regenerative medicine would require automatic cell processing, because human labor for highly aseptic cultivation operations is extremely costly. However, most of the reported techniques for the isolation of MSCs from bone marrow aspirates employ cell separation by density-gradient centrifugation. This is too complicated and delicate to be carried out automatically, while other operations during cultivation such as medium changing and washing are suitable for automation.

In this study, the *in vitro* proliferation of MSCs isolated from bone marrow aspirates without density-gradient centrifugation employing medium containing donor serum and basic fibroblast growth factor (FGF2) was investigated. The influence of the subcultivation of MSCs in the medium on the phenotypes of the surface antigens and the chondrogenic capacity was also investigated.

Materials and methods

Cell preparation and culture methods

Bone marrow was obtained from human donors (Table 1). All 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. Two methods with or without density-gradient centrifugation were employed to prepare adherent cells containing human MSCs. In the former method, the bone marrow aspirate was diluted with the separation medium (DMEM-LG (Gibco, NY, USA) supplemented with 10% FCS (Gibco)) to give a nucleated cell (NC) concentration of 1.0×10^7 cells/ml and was then fractionated by Ficoll (Amersham) density-gradient centrifugation at 13,000g (2000 rpm) for 20 min. The NC concentration was determined with Turk's solution and a hemocytometer. The mononuclear cell (MNC)-enriched fraction was collected, rinsed with the growth medium described below, plated in a dish (21 cm², Corning, Tokyo) at 6.0×10^5 NCs/cm² and cultured at 37 °C in a humidified atmosphere containing 5% CO₂ for 19d, during which time the medium was changed on days 9 and 16. In the latter method, the bone marrow aspirate was diluted with the growth medium, plated in the dish to a concentration of 6.0×10^5 NCs/cm² and cultured at 37 °C in a humidified atmosphere containing 5% CO₂ for 19d, during which time the medium was

Table 1. Human MSCs donor profile

Donor	Donor age	Gender	Cell concentration (10 ⁷ /ml)*
A	65	F	2.00
B	66	F	1.30
C	76	F	3.70
D	24	M	0.83
E	46	F	1.60
F	56	F	2.60
G	73	F	2.20
H	22	F	2.92
I	22	F	2.09
J	25	M	1.00
K	23	F	1.59
L	28	M	0.99
M	26	F	1.11

*Nucleated cell concentration in each bone marrow aspirate.

changed on days 1, 2, 9 and 16. On day 19, the cells were detached using trypsin-EDTA (Sigma, St. Louis, MO, USA), counted by the trypan blue dye-exclusion method and subcultivated at a cell density of $0.6\text{--}1 \times 10^4$ cells/cm². Adherent cell density was determined using the nuclei staining method (Sanford et al. 1950) 1d after inoculation. When the culture reached near confluence, the cells were subcultivated further.

The growth medium was composed of DMEM-LG (Gibco, NY, USA), 10% serum, 2500 U/l penicillin, 2.5-mg/l streptomycin and FGF2 (0–10 ng/ml, Peprotech). FCS (Gibco) or human serum, which was obtained from the same donor as that of the bone marrow after informed consent, was employed. All culture experiments were performed, in duplicate and the mean was shown.

Chondrogenic differentiation

High-density pellet cell cultures were initiated by centrifugation (500g 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 2 weeks at 37 °C in 5% CO₂, during which time the medium was changed at week 1. The pellet was then rinsed twice with PBS, fixed in 20% formalin, dehydrated through a graded series of ethanol, infiltrated with isoamyl alcohol, and embedded in paraffin. Sections of 3-μm thickness were cut through the center of the pellets and were stained with 1% Safranin O in 1% sodium borate.

The differentiation medium consisted of DMEM-HG (Gibco), 2500 U/l penicillin, 2.5 mg/l streptomycin, 10 ng/ml transforming growth factor-β1 (TGF-β1, Gibco), 39 ng/ml dexamethasone (ICN Biomedicals), 50 μg/ml 1-ascorbic acid 2-phosphate (Wako Pure Chemicals, Osaka), 100 μg/ml sodium pyruvate (Wako), 40 μg/ml proline (Wako), 6.25 μg/ml bovine insulin (Wako), 6.25 μg/ml transferrin (Wako), 6.25 μg/ml selenous acid. (Wako), 5.33 μg/ml linoleic acid (Wako), and 1.25 mg/ml bovine serum albumin (Wako).

Flow cytometry analysis

Cells were stained with mouse IgG anti-human CD105 (Beckman Coulter) and phycoerythrin (PE)-conjugated anti-murine IgG (Beckman

Coulter). Then, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-human CD45 antibodies (Beckman Coulter), and analyzed using a flow cytometer (EPICS XL, Beckman Coulter) equipped with an argon laser (488 nm).

Results

Adherent cell preparation without density-gradient separation

Nucleated cells from various donors with or without density-gradient separation were inoculated at the same density (6.0×10^5 NCs/cm²) and incubated for 19d. There was no apparent difference in the density of adherent cells harvested at 19d between previous method with density-gradient separation ($1.8\text{--}2.5 \times 10^4$ cells/cm²) and new method without the separation ($1.3\text{--}3.4 \times 10^4$ cells/cm²) (Table 2). Almost all adherent cells prepared using both methods were fibroblast-like (data not shown).

Effect of FGF2 plus human serum on adherent cell preparation

Nucleated cells from two donors, without being subjected to density-gradient separation, were inoculated into the medium containing human serum (10%) and FGF2 (0–10 ng/ml), and incubated for 19d. Ten nanograms per milliliter of FGF2 gave a 2.1–4.0-fold higher density of adhered cells after 19d than the culture without FGF2 addition, while lower concentrations of FGF2 had almost no effect (Figure 1).

Table 2. Cell proliferation with and without density-gradient centrifugation

Donor	Density-gradient centrifugation	Cell density (10 ⁴ /cm ²)*
A	+	2.50
B	+	1.80
C	–	2.44
D	–	2.60
E	–	2.10
F	–	3.40
G	–	1.30

*Cell density was measured after incubation for 19 days.

Figure 1. Effect of FGF2 concentration in the medium containing human serum on adherent cell density prepared from bone marrow aspirates. Bone marrow aspirates from donors A and B were incubated for 19d in the medium supplemented with human serum (10%) and FGF2 (0, 0.1, 1, 10 ng/ml).

Nucleated cells from two donors, without being subjected to density-gradient separation, were inoculated into media containing FCS (10%), human serum (10%), and human serum (10%) plus FGF2 (10 ng/ml), respectively. The adherent cell density at 19d in the culture supplemented with human serum plus FGF2 was 1.4–2.7 times that in the medium containing only FCS, while the cell density in the medium containing only human serum was 0.1–0.6 times that in the medium containing FCS (Figure 2). Microscopic observation of adherent cells in the 19-d culture of bone marrow cells from donor D in the medium containing human serum plus FGF2 showed subconfluent and fibroblast-like morphology (data not shown).

Subcultivation of adherent cells in the medium containing human serum plus FGF2

Adherent cells propagated from bone marrow aspirates were further subcultivated several times

Figure 2. Comparison of stimulating activities for adherent cell growth between medium containing human serum and FCS. Bone marrow aspirates from donors C and D were incubated for 19d in the medium supplemented with FCS (10%) or human serum (HS) (10%) plus FGF2 (0, 10 ng/ml).

in the medium containing human serum (10%) plus FGF2 (10 ng/ml). Although the adherent cell density 1d after inoculation was slightly lower than the inoculum cell density, adherent cells grew thereafter during each subcultivation (Figure 3). Average generation times during subcultivations were in the range of 64.1–118.4 h.

Characteristics of adherent cells cultured in the medium containing human serum plus FGF2

Cell surface antigens and chondrogenesis activity were analyzed for nucleated cells in the bone marrow aspirate, adherent cells propagated from bone marrow aspirate in the medium containing human serum plus FGF2, and adherent cells following subcultivation. Although the percentages of CD45⁻ CD105⁺ cells among cells in the bone marrow aspirates were less than 0.1%, approximately 90% of adherent cells prepared from bone marrow were negative for CD45 and positive for CD105 (Table 3). The PDL of these prepared adherent cells was calculated on the assumption that the content of adherent cells in bone marrow nucleated cells was 0.1%. The percentage of

Figure 3. Subcultivation of adherent cells prepared from bone marrow. Adherent cells prepared from bone marrow aspirates of donors A and B and cultured in the medium supplemented with human serum (10%) and FGF2 (10 ng/ml) were further subcultivated in the same medium. Average generation time (g h) was calculated using the final cell concentration and that on d1 during each subcultivation.

Table 3. Cell surface antigen profile

Donor	PDL*(-)	CD45 ⁻ CD105 ⁺ (%)
L	0	< 0.1
	8.3	89.6
	17.2	86.1
M	0	< 0.1
	6.8	92.2

*Population doubling level (PDL) was calculated on the assumption that the content of adherent cells in bone marrow NCs was 0.1%.

CD45⁻ CD105⁺ cells was maintained close to 90% even after subcultivation (17.2 PDL).

Pellet cultures were performed employing these cells. However, pellets of nucleated cells in bone marrow aspirates could not be obtained. Pellets were successfully obtained by employing adherent cells prepared from bone marrow aspirates and further subcultivated using the medium containing human serum plus FGF2. These pellets were positively stained with Safranin O (Figure 4).

Discussion

Adherent cell preparation without density-gradient separation could provide a cell yield from bone marrow aspirates comparable with that obtained using density-gradient separation (Table 2).

Figure 4. Microscopic observation of a section of pellet culture. Pellet cultivations were performed for 2 weeks employing adherent cells at each PDL, and the sections were stained with Safranin O.

Automation of cell processing, including MSC preparation from bone marrow, would be important for the industrialization of regenerative medicine, because manual aseptical operation is extremely costly. This method of preparing adherent cells with a high content of MSCs without density-gradient separation should have an advantage over the conventional method using density-gradient separation because this type of separation requires delicate operation and cannot easily be automated.

The density of adherent cells harvested from a bone marrow aspirate after a 19-*d* incubation in the medium containing only human serum (10%) was only 3.4–9.5% that obtained using the medium containing human serum plus FGF2 (10 ng/ml) (Figure 2). The average generation times for these incubations were calculated to be 312 and 76 h, respectively, on the assumption that the content of adherent cells in the inoculum aspirate was 0.1%. For example, it took approximately 400 h to increase the number of adherent cells in the bone marrow aspirate of donor H to 2×10^7 cells employing the medium containing human serum and FGF2 (data not shown) and this would be prolonged to 1600 h without FGF2. Thus, the acceleration of MSC proliferation by FGF2 would, be advantageous for clinical applications.

If only the addition of FGF2 without donor serum addition could grow cells with high growth rate maintaining cell differentiation capacity, it might be much advantageous for patients. However, no cultivation experiment was performed, in which cells were incubated with FGF2 addition without any serum. Based on the previous results about the development of serum-free medium mainly for cell lines, we considered that only one growth factor could not play the all role of FCS.

The growth stimulating activity of the medium containing human serum and FGF2 on MSCs was superior to that of the medium containing FCS (Figure 2). In addition, the continuous growth of MSCs using the medium containing human serum and FGF2 was stable during a series of subcultivations (Figure 3).

During many researches previously performed about the effect of serum addition on mammalian cell growth, marked variation of growth stimulating activity between the lot of FCS was shown and usual concentration of FCS addition to medium was set to 10% because 10% was enough for

almost all of cell lines and FCS lots. There might be also some variation in growth stimulating activity of donor serum for MSCs between donors even if the concentration of 10% was employed. However, the addition of FGF2 may compensate the variation.

There were no marked difference in yield of MSCs between donors C and D (Figure 2) and in differentiation between donor L and M (Figure 4). These observation preliminary suggested that proliferation method of MSCs employing donor serum and FGF2 could be applicable independent from donor age and gender.

It was reported that 1×10^7 nucleated cells following the density-gradient separation of a bone marrow aspirate contained 2200 MSCs (Bruder et al. 1997) and the content of MSCs among the nucleated cells was approximately 0.2%. The contents of SH-2-positive (CD105⁺) and CD45⁻ cells among adherent cells prepared from bone marrow aspirates after density-gradient separation were reported to be 98 and 99%, respectively, (Majumdar et al. 1998) The contents of CD105⁺ CD45⁻ cells among nucleated cells in the bone marrow aspirate and adherent cells harvested after incubation of bone marrow cells in the medium containing human serum, and FGF2 were less than 0.1% and 89–92%, respectively (Table 3). These were similar to other reported values.

Because the nucleated cells in bone marrow aspirates contained less than 0.1% CD105⁺ CD45⁻ cells and could not form a solid pellet, this indicates that the content of MSCs among the nucleated cells in bone marrow aspirates is very low. On the other hand, adherent cells prepared by a 19-*d* incubation of bone marrow nucleated cells in the medium containing human serum and FGF2 contained approximately 90% CD105⁺ CD45⁻ cells and could form a solid pellet which stained well with Safranin O (Figure 4). This indicated that MSCs exhibiting the CD105⁺ CD45⁻ surface antigen and chondrogenic capacity were selectively expanded by incubation in the medium.

The PDL of adherent cells after preparation was calculated on the assumption that the content of adherent cells in bone marrow nucleated cells was 0.1%. Adherent cells harvested after several subcultivations employing the bone marrow aspirate and serum from donor L contained 86.1% CD105⁺ CD45⁻ cells (Table 3) and could form a

solid pellet that stained well with Safranin O (Figure 4). The PDL of these MSCs was calculated to be 17.2 (Table 3). Thus, MSCs in the bone marrow aspirate could selectively proliferate up to a PDL of 17.2 in the absence of density-gradient separation when the medium containing human serum and FGF2 was used.

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 chondrocyte cells. Generally, 10 ml of bone marrow aspirate contains approximately 1×10^9 nucleated cells 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, which fold increase in cell number corresponds to the increase observed after a PDL of 8 ($2^8 = 256$). However, this should be the minimum requirement, because there might be some loss of cells due to transplantation operation and several pieces of chondrocyte should be transplanted in some case. In this study, MSCs could be expanded up to 17.2 PDL, which, meant more 500 ($2^9 = 512$) times increase (total $2^{17} \times =$ approximately $130,000 \times$) and this technique might satisfy MSCs cell number for one patient (donor).

The amount of serum required also should be considered for clinical application. Initially, 1×10^9 NCs containing 1×10^5 MSCs will be inoculated to a dish (55 cm^2 , 10 ml) at the density of 6×10^5 NCs/ cm^2 . Medium will be changed four times during 19d of incubation. This process needs 1500 ml of medium. ($10 \text{ ml/dish} \times 30 \text{ dishes} \times 5 = 1500 \text{ ml}$). In order to expand more MSCs depending on the clinical necessity, for example more eight times, MSCs harvested (2.5×10^7 MSCs) should be further cultivated three more passages if one passage could expand them two times. MSCs (2.5×10^7 MSCs) will be inoculated to 50 dishes with the inoculum cell density of 1.0×10^4 MSCs/ cm^2 . Three passages should need 3500 ml medium ($50 + 100 + 200 = 350 \text{ dishes}$). Consequently, total amount of medium and donor serum required to expand MSCs $2^{(8+3)}$ times are 5000 and 500 ml, respectively. This amount of serum may be got from the donor separately before and during the culture which takes approximately 40 days.

In conclusion, the MSCs in the bone marrow aspirate could be expanded to a PDL of 17.2 when the medium containing human serum and FGF2

was used even in the absence of density-gradient centrifugation separation and FCS.

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