The Efficiency of Percoll and Ficoll Density Gradient Media in the Isolation of Marrow Derived Human Mesenchymal Stem Cells with Osteogenic Potential

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Background: Percoll and Ficoll density gradient media are widely used in the isolation of human mesenchymal stem cells (hMSCs) for research. However, the efficacy of the two methods in the isolation of hMSCs and their differentiation potentiality have not been evaluated. In this study, the ability of the two methods to isolate MSCs from human bone marrow was compared.

Methods: Colony forming unit-fibroblasts (CFU-Fs) are considered colonies of stem and progenitor cells. Thus it was proposed that MSCs be represented by CFU-Fs. Therefore, we compared the relative efficiencies of the two media, Ficoll and Percoll, in isolating colony forming unit-fibroblasts with alkaline phosphatase activity (CFU-F/ALP$^+$) and the percentages of CD166$^+$/CD34$^-$, CD90$^+$/CD34$^-$, SH3$^+$/CD34$^-$ and CD105$^+$/CD34$^-$ cells in all nucleated cells from bone marrow (BM).

Results: A significantly higher number of nucleated cells could be isolated with Ficoll than with Percoll. The percentages of cells which were CD166$^+$/CD34$^-$, CD90$^+$/CD34$^-$, SH3$^+$/CD34$^-$ and CD105$^+$/CD34$^-$ were significantly higher for the Ficoll group. The colony-forming efficiency from Ficoll isolates (119 $\pm$ 69 CFU-F/ALP$^+$ per dish) was also higher than that from Percoll isolates (46 $\pm$ 35 CFU-F/ALP$^+$ per dish) ($p < 0.01$). However, the average colony size, percentage of CFU-Fs with ALP$^+$ and the differentiation abilities of CFU-Fs were not significantly different between groups.

Conclusions: Ficoll and Percoll are both suitable but the Ficoll methodology is superior to that of Percoll in the preparation of hMSCs.

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Key words: mesenchymal stem cells, Ficoll, Percoll, CFU-Fs

Research interest in mesenchymal stem cells (MSCs) has been increasing because of the potential application of stem cells to human health problems. Under the induction of specific culture medium, MSCs are capable of multilineage differentiation into mesoderm-type cells, e.g., osteoblasts.[1] adipocytes,[2] and chondrocytes,[3] that can possibly be used for autogenous tissue grafting.[4] This approach is described as MSC-based tissue engineering and offers the possibility of a renewable source

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of cellular therapy.[5]

The attempt to use stem cells from a patient's own body for treatment or research requires that stem cells first be isolated and then grown in culture until a sufficient quantity is obtained for further application. Technological challenges remain before these techniques can be incorporated into clinical practice. In particular, MSCs are present in only minute quantities, existing at an estimated frequency of about 1 in 100,000 bone marrow (BM) cells.[6] Several different methods have been described for isolating MSCs, including the use of immuno-magnetic beads,[7-9] density gradient separation[10-14] and direct BM plating.[15,16]

Because of a lack of availability of unique antigens to human MSCs (hMSCs) and the limited popularity of flow cytometry, FACScan sorting is not as widely used as gradient media in MSC isolation. Most MSC populations have been isolated via methodology similar to that originally used by Friedenstein and popularized by Caplan which utilizes the physical property of plastic adherence.[5,17-19] The method of direct BM plating is commonly used for cells from rats,[15,16] mice[20-22] and rabbits,[23,24] whose available BM volume is limited. Density gradient centrifugation is the most widely used method for isolating MSCs from human BM.[10-14] Macrophages, endothelial cells, lymphocytes, and smooth muscle cells also adhere to plastic and contaminate early BM preparations.[10,25] Only fibroblast-like spindle-shaped cells proliferate and form colonies. Colony forming unit-fibroblasts (CFU-Fs) are clearly indicative of cells capable of forming colonies and are representative of the more highly proliferative cells in these cultures.[26] The other types of cells which adhere to the dishes do not proliferate, perhaps because culture conditions are sub-optimal.[26] It has been accepted that under proper experimental conditions, BM CFU-Fs are able to differentiate into bone, cartilage, adipocyte, fibrous tissues and hematopoietic supporting tissues in vitro and in vivo.[5,8,10,18,19,27] CFU-Fs with alkaline phosphatetase activity (CFU-F/ALP$^+$) are thought to have osteogenic potential and are, thus, proposed to be osteoprogenitors.[26]

BM-MSCs are the progenitors of osteoblasts, chondroblasts, adipocytes and several other cell types.[5,10,28] The CFU-Fs, derived from MSCs by culture expansion in vitro, have surface markers positive for CD44, CD54, CD73, CD90, CD105, CD166, SH3 and many other surface proteins, and negative for hematopoietic CD14, CD34, and CD45.[10,17,29] Correlations between formation of bone (in an in vivo osteogenesis assay) and the expression of CD105, CD166 and CD90 have been reported in BM mononuclear cells and mesenchymal progenitor cells from the synovial membrane.[30,31]

Percoll and Ficoll are the density gradient media which are most commonly used for the isolation of human MSCs (hMSCs) from BM.[10-14] Percoll which consists of a suspension of colloidal silica particles (diameter 15-30 nm), has been widely used for separating cells, organelles, viruses, and other subcellular particles[32-34] and is usually used as a first step to enrich cell populations before other finer resolution. Ficoll, a polymer of sucrose with a high synthetic molecular weight, has been traditionally used for separating lymphocytes from other formed elements in blood.[27,35] It has been used in clinical practice for several decades, and its efficiency in separating lymphocytes and its safety in clinical usage are well recognized.[27,35] Both of these gradient media have been applied in the separation of MSCs from BM.[10-14] However, to our knowledge, the relative isolation efficiencies of these two gradient media have not been documented.

In this study, mononuclear cells were isolated from BM with these two different density gradient media, Percoll and Ficoll, and the efficiencies of separation of cells (CD166$^+$/CD34$^-$, CD90$^+$/CD34$^-$, SH3$^+$/CD34$^-$ and CD105$^+$/CD34$^-$) in BM nucleated cells from other unwanted cells by the two media were compared. Furthermore, using the percentile of number and size of CFU-F/ALP$^+$, the proliferation and differentiation capacities of CFU-Fs were further investigated.

METHODS

Bone marrow aspiration

BM aspirates were taken, with informed consent, from the iliac crest of nine adult donors during an autogeneous bone graft-harvesting procedure (Table 1), under a protocol approved by an institutional review board. Ten milliliters of BM aspirates were separated into equal volumes (5 ml), placed into two glass tubes and immediately sent to the laboratory to undergo further isolation procedures.

Table 1. Patient Characteristics

Subject	Age/Gender	Donor site	Surgical procedure	Other information
1	72/F	Posterior iliac crest	Lumbar spine posterolateral fusion	Diabetes hypertension
2	63/M	Posterior iliac crest	Lumbar spine posterolateral fusion	Hypertension
3	54/M	Posterior iliac crest	Lumbar spine posterolateral fusion	Smoker
4	36/M	Anterior iliac crest	Osteosynthesis of femoral non-union	
5	76/F	Posterior iliac crest	Lumbar spine posterolateral fusion	Diabetes hypertension
6	58/F	Posterior iliac crest	Lumbar spine posterolateral fusion	
7	44/F	Anterior iliac crest	Osteosynthesis of tibial non-union	
8	82/M	Posterior iliac crest	Lumbar spine posterolateral fusion	Hypertension, coronary artery disease
9	74/F	Posterior iliac crest	Lumbar spine posterolateral fusion	Chronic renal insufficiency, hypertension

Isolation of bone marrow nucleated cells

Percoll

The procedures for isolating CFU-Fs from BM were followed according to previously published methods.[17] A 5 ml aliquot of BM aspirate, diluted with an equal volume of the control medium [DMEM-LG with 10% fetal bovine serum (FBS)], was enriched for CFU-Fs by density gradient centrifugation over a Percoll cushion (density 1.077 g/cm^3, Amersham Biosciences, U.S.A.) by centrifugation at 1100 *g* for 30 min. The cells at the medium-Percoll interface were collected, counted, and plated on 100-mm culture dishes at a density of 10^7 cells per dish. The dishes were incubated at 37°C in a humidified 5% carbon dioxide environment.[17]

On the day 4 of culture, the non-adherent cells were removed by replacing the culture medium. The medium in three of the dishes (passage 0) was replaced with osteogenic induction medium (DMEM-LG with 10% FBS, 0.1 μM dexamethasone, 0.05 mM ascorbic acid-2-phosphate, and 2 mM β-glycerophosphate),[11] and these cultures were maintained in this medium for 12 days. The other cultures were maintained in control medium until the cells were near confluence and their differentiation ability was further analyzed.

Ficoll

A 5 ml aliquot of BM aspirate, diluted with an equal volume of the control medium, was layered onto the Ficoll (density 1.077g/cm^3, Amersham Biosciences) and centrifuged at 1100 *g* for 30 min. The cells at the medium-Ficoll interface were then collected and counted. The cells were processed under the same conditions as described above for the Percoll group.

Surface markers of BM cells

The BM cells were analyzed for surface markers by flow cytometry. In brief, immediately after enrichment by density gradient centrifugation, the BM cells were stained for 20 min with an empirically determined amount of CD34, CD166, CD90, SH3, and CD105 antibody (all antibodies were purchased from Becton-Dickinson, Franklin Lakes, New Jersy, U.S.A.) at a concentration of 5×10^5 cells/ml. The labeled cells were analyzed on a FACScan (Becton-Dickinson) by collecting 10,000 events with the Cell Quest software program (Becton-Dickinson). In addition, BM cells were analyzed for surface markers as described above after both density gradient enrichment and culture in control medium when the cells were nearly confluent.

Cytochemical staining of CFU-Fs

After being cultured in osteogenic induction medium for 12 days, cells were fixed with cold 10% neutral-buffered formalin (30 min at 4°C) and then assayed for alkaline phosphatase (ALP) activity.[36] Briefly, the substrate solution was prepared by dissolving 8 mg of naphthol AS-TR phosphate in 0.3 ml of N,N'-dimethylformamide, while a separate solution of fast blue BB was prepared by dissolving 24 mg in 30 ml of 100 mM Tris (pH 9.6). The above

solutions were mixed and then 10 mg of $MgCl_2$ was added and dissolved, and the pH was adjusted to 9.0 with 1N HCl. The cells were incubated with fresh substrate at 37°C for 30 min, then rinsed extensively with distilled H_2O and photographed. After being assayed for ALP activity on day 12, CFU-F colonies with 50 or more cells, the accepted value for defining a colony,[19] were visually scored as positive (blue stain, CFU-F/ALP$^+$). ALP-positive colonies were counted.

Cell proliferative capacity

The size of the CFU-Fs reflect cell growth;[7] therefore, the size and number of colonies were recognized as the cell proliferation potential. The size and number of colonies were determined by image analysis of each plate with Imagine-Pro Version 4.5 (Media Cybernetic, Silver Spring, MD, U.S.A.).

Osteogenic differentiation of CFU-Fs

For in vitro osteogenic assay, cells (passage one) were plated at a density of 3×10^3 cells/cm^2 in 6-well culture dishes and osteogenic differentiation was induced in osteogenic induction medium. The matrix calcium content and ALP activity of CFU-Fs from different gradient media were quantified to evaluate the capacity for osteogenesis.

On days 4, 8, 12 and 16, the ALP activity was evaluated by exposing the triplicate cultures to 1 ml of ALP substrate buffer (50 mM glycine, 1 mM $MgCl_2$, pH 10.5) that contained 2.5 mM *p*-nitrophenyl phosphate. Then, the optical density was determined by spectrophotometry at a wavelength of 405 nm. Values were corrected for the number of cells by DNA contents of plated cells.[37]

Mineralization was determined as previously described.[11] On day 28, cells were scraped off the culture dish in 0.6 N HCl, and then the calcium was extracted from the cells by shaking for 6 h to evaluate the matrix calcium content. Values were corrected for the number of cells by DNA contents of plated cells.

For the DNA assay, one mL of 0.1 N NaOH was added per well in the six-well culture dish, and then neutralized with 0.1 N HCl in 5 M NaCl, and 100 mM NaH_2PO_4. This mixture was combined with 1 mL of 0.7 μg/mL Hoechst 33258 (Sigma, St. Louis, MO, U.S.A.) in water. Fluorescence was read at an excitation of 360 nm and emission of 460 nm and compared with a certified calf thymus DNA standard (Sigma). Differences in DNA content were assumed to reflect differences in cell number.

Chondrogenic and adipogenic differentiation of CFU-Fs

The potential of the CFU-Fs to differentiate to lineages of mesenchymal tissues was further confirmed with chondrogenic or adipogenic induction media.

The chondrogenic potential of cells (passage one) was evaluated in an aggregate culture system.[38, 39] In brief, cells were trypsinized, counted, and resuspended in chondrogenic medium [DMEM-HG supplemented with 1% ITS +Premix™ (BD, Franklin Lakes, NJ, U.S.A.), 100 μM ascorbate-2-phosphate, 10^{-7} M dexamethasone, 0.1 mM nonessential amino acids (Gibco, Carlsbad, CA, U.S.A.), 1 mM sodium pyruvate (Gibco), and 10 ng/mL transforming growth factor-β1 (R&D systems, Minneapolis, MN, U.S.A.)] at a density of 1.25×10^6 cells/mL. Aliquots containing 2.5×10^5 cells were placed in polypropylene 96-well plates.[39] On day 21, triplicate aggregates from each group were processed for histologic evaluation or for glycosaminoglycan (GAG) and DNA quantification.

The aggregate GAG content was quantified by a previously described method.[40] Aggregates were digested with papain.[41] Aliquots of 25-μL of the papain-digested extracts were mixed with 250 μL of 0.02% safranin O. The mixtures were filtered through the nitrocellulose membrane with a dot blot apparatus (Bio Rad, Hercules, CA, U.S.A.). The individual dots were cut out from the filter, and incubated in 10% cetylpyridinium chloride. The absorbance of these extracts was read at 536 nm and compared with chondroitin sulfate standards (Seikagaku America, Falmouth, MA, U.S.A.). The aggregate DNA content was measured as described above. To additionally evaluate chondrogenesis in the aggregates, samples were formalin-fixed and paraffin-embedded. Adjacent 7-μM sections were stained with safranin O for proteoglycan deposition.

The adipogenic potential of cells was evaluated by a previously published method.[10] In brief, cells (passage one) were plated at a density of 100,000 cells/well in a 6-well plate and grown to confluence in control medium. Adipogenic differentiation was induced by subjecting confluent monolayers to 3

rounds of adipogenic treatment. Each consisted of 72 hours in adipogenic induction medium (DMEM-HG, 10% FBS, 1 μM dexamethasone, 0.5 mM methylisobutylxanthine, and 10 μg/ml insulin), followed by 72 hours in maintenance medium (DMEM-HG, 10% FBS, and 10 μg/ml insulin). Cells were assayed after an additional week in maintenance medium. For each experiment, at least five microscopic visual fields (200-fold magnification) were counted, and the number of cells that contained lipid vacuoles was calculated.

Cytotoxicity of gradient medium

Tetrazolium assay (MTT) was used to assess the growth-inhibitory or cytotoxic effects of the two gradient media.[6] In brief, cells (passage one) were plated in a 24-well culture dish at 6000 cells per well in a mixture of basal medium with Percoll or Ficoll in serial dilutions and incubated at 37°C in a humidified 5% carbon dioxide atmosphere. After 24 hr of culture, a MTT assay was performed and quantified directly with an enzyme-linked immunosorbent assay (ELISA) plate reader.

Statistical analysis

Data are presented as the mean and standard deviation (SD) of continuous response variables. The paired t-test was used to assess the difference between Ficoll and Percoll in the efficiency of isolation of hMSCs from BM. Before the analysis, the p value was set at 0.01 for each test.

RESULTS

Mononuclear cells isolated from Ficoll density centrifugation showed higher CFU-F forming efficacy than those isolated with Percoll

After enrichment by density gradient centrifugation, the total number of nucleated cells isolated from 5 ml BM aspirate was between 36×10^7 and 6×10^7. The average number of nucleated cells in the Ficoll group was $25.3 \pm 8.9 \times 10^7$, which differed significantly from that in the Percoll group ($13.6 \pm 6.6 \times 10^7$, $p < 0.01$). Thus, Ficoll gradient media allowed recovery of more nucleated cells from the BM than Percoll gradient media.

After 4 days of culture, the cells with adherence capacity were attached onto dishes. Grossly, more cells were attached in the Ficoll groups than in the Percoll groups. The adherent cells were heterogeneous stromal populations including fibroblast-like spindle-shaped cells, oval cells and some with the appearance of giant cells. Small colonies were visible at about one week, and became clearer as the incubation period prolonged.

The mean colony-forming efficiency for the whole donor group was $94 \pm 81/10^7$ mononuclear cells. CFU-F/ALP$^+$ were obtained from all of the donors after the 12-day culture period. The number of CFU-F/ALP$^+$ in the Ficoll group was $119 \pm 69/10^7$ mononuclear cells, which is statistically different from the number in the Percoll groups ($46 \pm 35/10^7$ mononuclear cells, $p < 0.01$) (Fig. 1A). In the Ficoll group, the average colony size was 17.7 ± 10.4 mm while that in the Percoll group was 14.6 ± 11.8 mm ($p = 0.069$).

The percentage of CFU-Fs/ALP$^+$ over colonies were $76 \pm 6\%$ and $77 \pm 9\%$ for Ficoll and Percoll, respectively. These two groups did not differ statistically ($p = 0.71$). Fig. 1B plots a typical CFU-Fs/ALP$^+$ experiment.

CD166$^+$/CD34$^-$, CD90$^+$/CD34$^-$, SH3$^+$/CD34$^-$ and CD105$^+$/CD34$^-$ cells in mononuclear cells isolated by Ficoll and Percoll

After enrichment by the gradient media, freshly isolated cells were analyzed immediately. The percentages of CD166$^+$/CD34$^-$, CD90$^+$/CD34$^-$, SH3$^+$/CD34$^-$ and CD105$^+$/CD34$^-$ nucleated cells in the Ficoll group were $17.04 \pm 3.54\%$, $3.0 \pm 1.3\%$, $10.1 \pm 3.2\%$ and $5.33 \pm 1.67\%$, respectively, which were significantly different from the corresponding results in the Percoll group ($10.79 \pm 2.17\%$, $1.25 \pm 0.74\%$, $6.11 \pm 2.59\%$ and $3.03 \pm 1.37\%$, respectively, $p < 0.01$). Ficoll gradients allowed recovery of more of all these nucleated cells from BM than Percoll gradients (Fig. 2). For enriched BM cells cultured in control medium until near confluence, the percentages of CD166$^+$/CD34$^-$, CD90$^+$/CD34$^-$, SH3$^+$/CD34$^-$, and CD105$^+$/CD34$^-$ cells showed no significant differences between groups (data not shown).

CFU-Fs isolated by Ficoll and Percoll showed similar osteogenic capacity

Cells (passage one) were grown for four weeks in osteogenic induction medium to verify the osteogenic potential of BM nucleated cells isolated

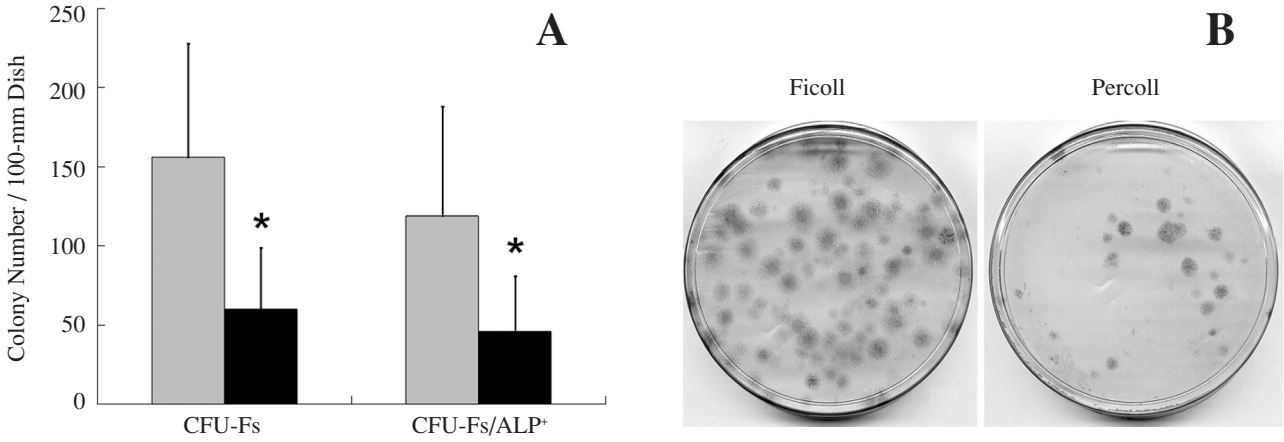

Fig. 1 Production of CFU-Fs from human BM cells. (A) After BM was enriched with Ficoll (gray bar) or Percoll (black bar), 10^7 nucleated BM cells were plated into one 100-mm dish with control medium. On day 4 of culture, the medium was replaced with osteogenic induction medium. After 12 days of culture in osteogenic induction medium, the colonies were counted and stained to determine ALP activity. Data are expressed as mean ± SD for CFU-Fs or CFU-Fs/ALP$^+$ from nine donors. * Significant difference ($p < 0.01$) compared to the Ficoll group. (B) The figure shows the results of a representative experiment of CFU-Fs/ALP$^+$ from nine different samples.

by Ficoll and Percoll. Under the influence of dexamethasone, β-glycerophosphate and ascorbate, the isolated CFU-Fs assumed a less elongated, polygonal appearance and formed aggregates or nodules. Quantitative assays revealed that increases in ALP activity on days 8, 10, and 12, which were then down regulated (Fig. 3C). Cell cultures treated with osteogenic induction medium produced a mineralized extracellular matrix stained with Alizarin Red S in both the Ficoll and Percoll groups (Fig. 3A OS). In untreated cell cultures, there was an absence of these phenotypes (Fig. 3A Control). The osteogenic capacity of CFU-Fs, as shown by the matrix calcium content/mineralization (Fig. 3B) and the ALP activity (Fig. 3C) of the cells did not statistically differ between two groups, when measured either per well (Fig. 3C, center) or normalized to DNA content (Fig. 3C, right).

CFU-Fs isolated by Ficoll and Percoll showed similar chondrogenic and adipogenic capacities

Under the influence of TGF-β1, all CFU-F preparations evaluated in this study were capable of forming a chondrogenic pellet containing large round cells surrounded by abundant extracellular matrix. Safranin-O staining, indicative of the presence of highly negatively charged sulfated GAG, was observed in the central region of the aggregates after 21 days in chondrogenic culture in both groups (Fig. 4A). Cells isolated with different density gradient media did not show a significant difference in capacity for chondrogenic differentiation. Analysis of the DNA content of the aggregates demonstrated no significant differences between the groups in the amount of DNA per aggregate at the time points analyzed (Fig. 4B, left). Meanwhile, the GAG content was similar between the groups both per aggregate (Fig. 4B, center) and when normalized to DNA content (Fig. 4B, right).

In vitro adipogenesis experiments showed that after 25 days of multiple induction treatment, 30% to 50% of the CFU-Fs committed to this lineage, and lipid vacuoles continued to develop over time. Induction was apparent by the accumulation of lipid-rich vacuoles within cells (Fig. 4C). The percentage of cells containing lipid vacuoles was not significantly different between groups (Fig. 4D).

Cytotoxicity of the gradient medium

CFU-Fs (passage one) were cultured with a mixture of control medium and Percoll or Ficoll in serial dilutions to assess the growth-inhibitory or cytotoxic effects of these two gradient media. The CFU-Fs exhibited greater cell death as the ratio of gradient medium increased in both groups. However, the two gradient media showed no significant differ-

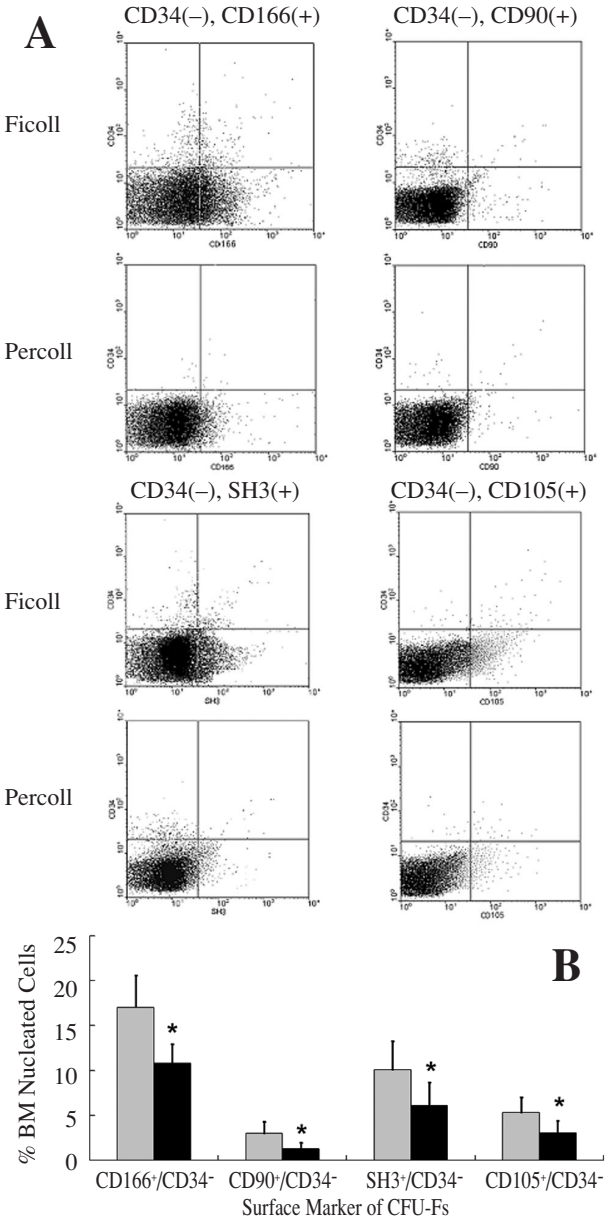

Fig. 2 Flow cytometry of BM cells after gradient enrichment. (A) Immediately after enrichment by density gradient centrifugation, BM cells were stained with antibodies against CD34, CD166, CD90, SH3, and CD105. The figure shows the results of a representative experiment from nine different samples. (B) Results show that after enrichment with gradient media, the number of $CD166^{+}/CD34^{-}$, $CD90^{+}/CD34^{-}$, $SH3^{+}/CD34^{-}$ or $CD105^{+}/CD34^{-}$ cells was significantly higher in the Ficoll group (gray bar) than the Percoll (black bar) group. Data are expressed as mean ± SD for CFU-Fs from nine donors. * Significant difference ($p < 0.01$) compared to the Ficoll group.

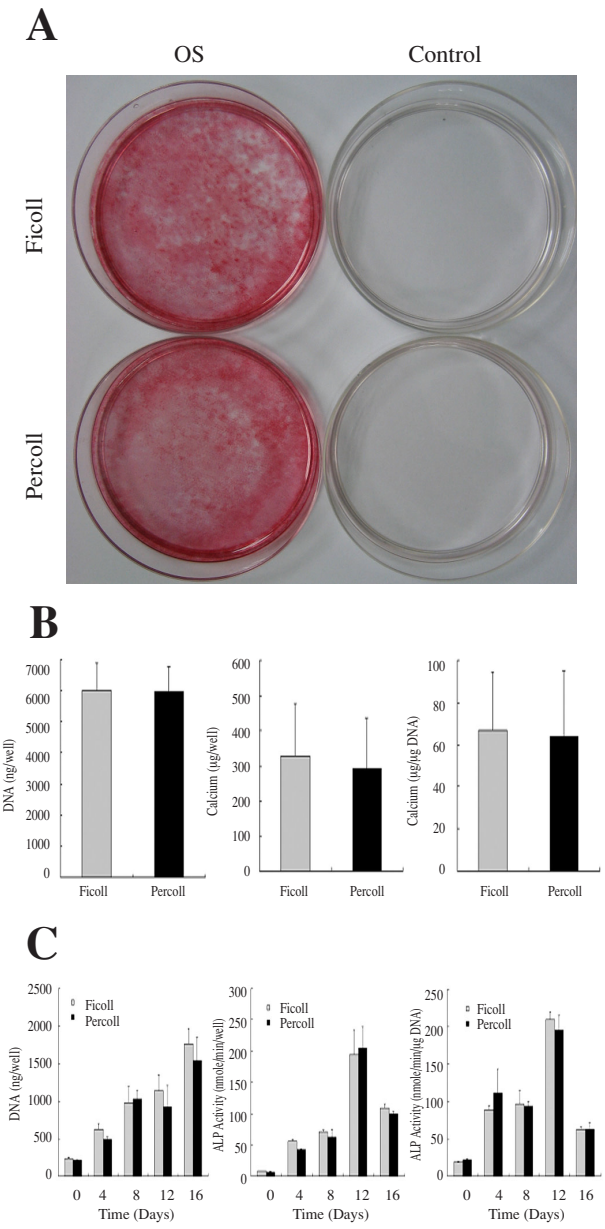

Fig. 3 Osteogenic capacity of CFU-Fs. (A) CFU-Fs, cultured with osteogenic induction medium (OS) or control medium (Control) for 28 days, were stained with Alizarin red S to determine the calcium rich extracellular matrix. The figure shows the results of a representative experiment from nine different samples. (B, C) The osteogenic capacities of CFU-Fs, from the Ficoll (gray bar) or Percoll (black bar) groups, were further confirmed with the quantification assay for calcium content in the extracellular matrix (B) on day 28 and ALP activity (C) on days 0, 4, 8, 12 and 16 with osteogenic induction cultures. Data are expressed as mean ± SD for CFU-Fs from nine donors.

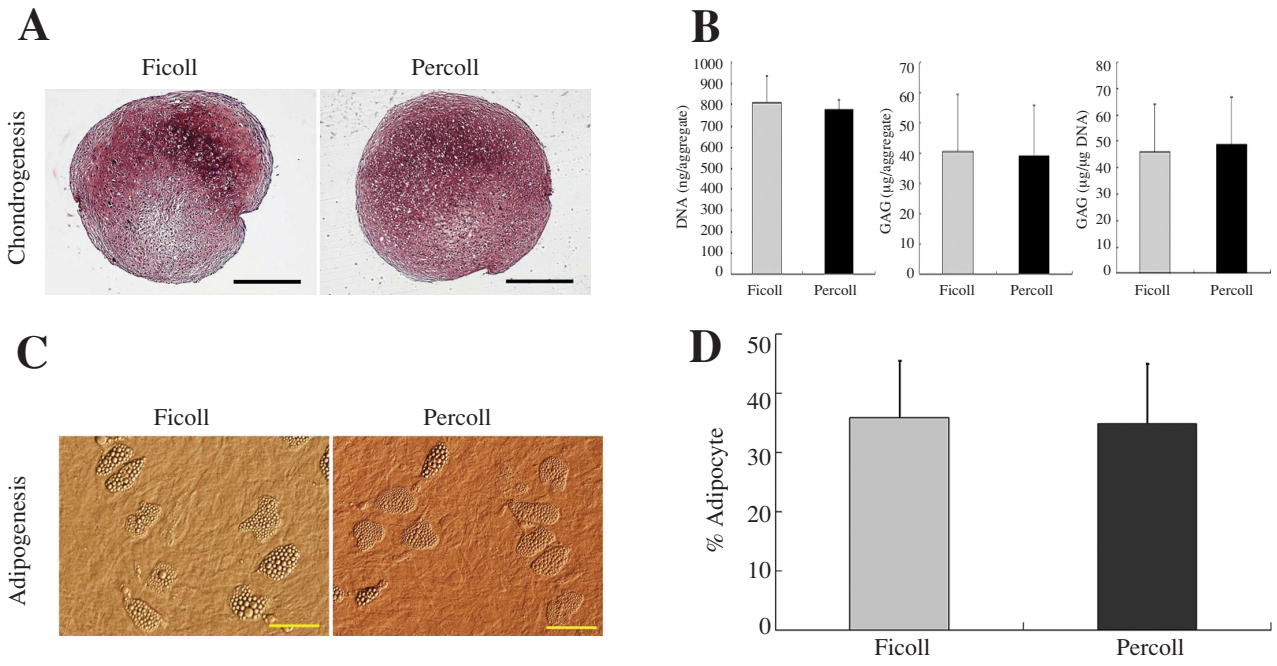

Fig. 4 Chondrogenic and adipogenic capacity of CFU-Fs. (A) Microscopic appearance of safranin-O-stained aggregates made with CFU-Fs isolated with different density gradient media, Ficoll or Percoll, and cultured in chondrogenic induction medium for 21 days. (original magnification, x 40, bar = 500 μm). The figure shows the results of a representative experiment from nine different samples. (B) Glycosaminoglycan content in the aggregates made with CFU-Fs previously isolated with Ficoll (gray bar) or Percoll (black bar) and cultured in chondrogenic induction medium for 21 days. Data are expressed as mean ± SD for CFU-Fs from nine donors. (C) Microscopic appearance of CFU-Fs isolated with Ficoll or Percoll and cultured in 3 rounds of adipogeinc treatment. (original magnification, x 200, bar = 100 μm). The figure shows the results of a representative experiment from nine different samples. (D) The adipogenic capacities of CFU-Fs, from the Ficoll (gray bar) or Percoll (black bar) groups, were further evaluated by counting the percentage of cells containing lipid vacuoles. Data are expressed as mean ± SD for CFU-Fs from nine donors.

ence in their growth-inhibitory or cytotoxic effects as evaluated by MTT assay (data not shown).

DISCUSSION

In the current study, we found that the number of $CD166^+/CD34^-$, $CD90^+/CD34^-$, $CD105^+/CD34^-$ or $SH3^+/CD34^-$ cells was greater after the BM cells were enriched with Ficoll than with Percoll. Meanwhile, the total number of CFU-F/ALP^+ and the colony-forming efficiency were significantly higher in the Ficoll group than in the Percoll group. These findings suggested that Ficoll is able to isolate CFU-Fs with osteogenic potential from BM more efficiently than Percoll. Colonies derived from CFU-F assays are heterogeneous in both size and differentiation potential.[42,43] We do not know of any variation in the differentiation ability between CFUs which are CFU-Fs/ALP^+ or CFU-Fs/ALP^-, or any variation in the isolation efficiency of CFU-Fs with the potential of other mesenchymal lineages. The CFU-Fs (passage one) isolated by these two gradient media have similar proliferation and differentiation abilities, including osteogenesis, chondrogenesis and adipogenesis. Furthermore, the average colony size did not differ between the two groups (data not shown), indicating that the proliferation capacity of CFU-Fs from the same donor was not affected by the isolation medium. Based on the results of the MTT assay (data not shown), the growth-inhibitory and cytotoxic effects of the gradient medium showed no significant differences. The possible reason for these observations is that Percoll and Ficoll gradient centrifugation isolated the same cell fractions from the BM aspirates, but with different enrichment efficacy. Therefore, the characteristics of cells isolated by

either Percoll or Ficoll density centrifugation were both able to form CFUs with similar proliferation activity (colony size), similar differential capacities (osteogenesis, chondrogenesis and adipogenesis) and similar percentile of CFU-Fs/ALP$^+$ over colonies. However, we do not know the exact mechanism causing the difference in isolation efficacy between the two gradient media.

Investigators have reported studies of MSCs using different methods of isolation and expansion, and different approaches to characterize the cells.[8,10,17,21,30] It is increasingly difficult to compare and contrast study outcomes, which hinders progress in the field. There are differences in the surface markers among reported studies, which may be explained by variations in culture methods and/or differentiation stages of the cells.[8,10,17,30] Therefore, minimal criteria for defining cells as MSCs, including a number of surface markers, has recently been suggested by a working group within the International Society for Cytotherapy.[29] MSCs must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules.[44] We used some of the surface markers in the list above, CD34, CD105, CD90, CD166 and SH3, to compare MSCs isolated from the two methods. Correlations between formation of bone (in an in vivo osteogenesis assay) and the expression of CD105, CD166 and CD90 have been reported in BM mononuclear cells and mesenchymal progenitor cells from the synovial membrane.[30,31] The relationships between specific surface markers with distinct steps along the chondrogenic or adipogenic lineage of hMSCs have not been identified completely.[45] We can not exclude the possibility that MSCs isolated by different methods may not be the same in developmental lineages. However, it is apparent that Ficoll is much more efficient than Percoll in isolating MSCs with the same multipotential differentiation of mesoderm cells.

Apart from the isolation efficiency, the complexity of the procedure is another factor that should be considered. Percoll can either be used in continuous or discontinuous multiple density gradients and both procedures must be diluted to the desired density-stock isotonic Percoll before they are used. A high speed centrifuge must be used with this gradient medium. In contrast, Ficoll is ready to use, and centrifugation at moderate speed is sufficient. In short, cell isolation with Ficoll requires less time and fewer steps and uses less costly equipment, such as the high speed centrifuge, than Percoll. Furthermore, Ficoll is commercially available in an endotoxin-free form, but Percoll is not.[34,46] We currently suggest that Ficoll is superior to Percoll for rapid isolation of human MSCs for clinical use potential.

Conclusions

This study revealed that the Percoll and the Ficoll density gradient media are efficient, rapid and economical for the isolation of hMSCs. The hMSCs isolated by two different methods can be induced into osteogenic, adipogenic and chondrogenic lineages. However, the Ficoll methodology gives a higher yield of hMSCs when cultured in vitro.

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使用比重梯度溶液分離人類骨髓間葉幹細胞效率之研究

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背 景： Pecoll 與 Ficoll 是常使用於分離人類間葉幹細胞的兩種比重梯度溶液。至目前爲止，此兩種梯度溶液在分離人類間葉幹細胞的效率上，未曾被探討過。在這個研究中，我們針對這個議題作深入比較。

方 法： 具有形成細胞菌落能力的纖維母細胞 (CFU-Fs) 可視爲是間葉幹細胞的代表。因此，在這個研究中，我們藉由計數 CFU-Fs 的數目、分析 CFU-Fs 中具有鹼性磷酸媒活性的 CFU-Fs (CFU-Fs/ALK$^+$) 的比例、分析這些細胞帶有表面抗原爲 CD166$^+$/CD34$^-$，CD90$^+$/CD34$^-$，SH3$^+$/CD34$^-$ 與 CD105$^+$/CD34$^-$ 的百分比。藉此比較兩種比重梯度溶液 Pecoll 與 Ficoll，從人類骨髓液中分離間葉幹細胞的效率。

結 果： Ficoll 具有從人類骨髓液中分離出較多有核細胞的能力；在這些細胞中，其表面抗原帶有 CD166$^+$/CD34$^-$，CD90$^+$/CD34$^-$，SH3$^+$/CD34$^-$ 與 CD105$^+$/CD34$^-$ 的比例也明顯較高。Ficoll 分離出的有核細胞，形成 CFU-Fs/ALK$^+$ 菌落的數目上，Ficoll 也明顯比較好；但是在菌落的大小與 CFU-Fs 的分化能力上，則沒有明顯差異。

結 論： Ficoll 與 Percoll 均適用於分離人類間葉幹細胞，但是 Ficoll 提供較好的效能。
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