and treated with 100 mM glycine buffer. Then, cells were washed again three times and incubated with biotinylated antibodies against CD45 or CD54 (both 1:200 dilutions; Biolegend) at 4°C overnight. The next day, cells were re-washed three times and incubated with a streptavidin-FITC conjugate (1:800 dilutions; Biolegend) for 20 min at room temperature. After three washes with DPBS, cells were counterstained with Vectashield mounting medium with DAPI (Vector Laboratories Inc., Burlingame, CA, U.S.A.) and observed under a fluorescence microscope (Axio Obserber. Z-1,Carl Zeiss Japan). Digital images were acquired using an AxioCam HRm and AxioVision software (Carl Zeiss Japan).

Cell number analysis and quantitative ALP assay

Cell number analyses and quantitative ALP assays were performed as described elsewhere with modifications (13). Cells from each group were plated into 24-well plates at a density of 2×10^4 cells per well in serum-containing medium. The next day, cells were fed with non-induction medium (serum-containing medium) or osteogenic induction medium (serum-containing medium with 10 nM dexamethasone (Sigma-Aldrich), 100 μM ascorbic acid (Wako Pure Chemical Industries, Ltd.), and 10 mM glycerol 2-phosphate disodium salt hydrate (β-glycerophosphate, Sigma-Aldrich)). Culture medium was replaced with fresh medium twice a week. After one or two weeks of culture, cell number analyses and quantitative ALP assays were performed with a commercially available *p*-nitrophenyl phosphate tablet set (Sigma-Aldrich) and a cell counting kit-8 (WST-8®; Dojindo).

Briefly, 50 μL of WST-8 were added to each well containing 0.5 mL of fresh medium, incubated for 60 minutes, and absorbance was read at 450 nm to assess cell numbers. After WST-8 analysis, each well was washed twice with DPBS and 400 μL of *p*-nitrophenyl phosphate solution was added to each well. After ten minutes of incubation at 37°C, the conversion to *p*-nitrophenol was stopped with 400 μL 3N NaOH and the absorbance of *p*-nitrophenol was measured at 405 nm. ALP activity was expressed as *p*-nitrophenol absorbance (OD; 405 nm) / WST-8 absorbance (OD; 450 nm).

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (real-time qPCR) was conducted to investigate the differences in the expression of osteogenic marker genes. Cells from each group were plated in six well plates at a density of 1×10^5 cells per well in serum-containing medium. The next day, cells were fed with non-induction medium (serum-containing medium) or osteogenic induction medium. Culture medium was replaced with fresh medium twice a week. After one or two weeks of culture, total RNA was extracted with RNeasy® Mini Kit (QIAGEN Science, Maryland, U.S.A.).

First-strand cDNA syntheses were performed with PrimeScript® RT Master Mix (Perfect Real Time) (Takara Bio Inc., Shiga, Japan). Real-time qPCR was conducted using the primers for osteopontin (forward: gatgaaccaagcgtggaaac; reverse: tgaaactcgtggctctgatg), core-binding factor subunit alpha-1 (*Cbfa1*; forward: gccaggttcaacgatctgag; reverse: gaggcggtcagagaacaaac), osteocalcin (forward: agctcaaccccaattgtgac; reverse: agctgtgccgtccatacttt), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; forward: aactcccattcctccacctt; reverse: gagggcctctctcttgctct) that have been described previously (15). The SYBR® *Premix* Ex Taq™ II (Tli RNase H Plus) (Takara Bio Inc.) was used for real-time qPCRs according to the manufacturer's instructions. The reactions were performed with a Thermal Cycler Dice® Real Time System II (Takara Bio Inc.) at 95°C for 30 sec, and then 40 cycles at 95°C for five sec and 60°C for one min. The cycle threshold (Ct) values were calculated by the second derivative maximum method, and the relative quantities were calculated based on a standard curve generated with serial dilutions of cDNA. *GAPDH* was used as an internal control.

***In vitro* mineralization assays**

To investigate differences in the mineralizing potentials of BMSCs, cells from each group were plated in 24 well plates at a density of 5×10^4 cells per well in serum-containing medium. The next day, cells were fed with non-induction medium (serum-containing medium) or osteogenic induction medium. Culture medium was replaced with fresh medium twice a week. After three weeks of culture, cells were fixed with 70% ethanol at -20°C for 60 min, washed, and stained for 15 min with a saturated solution of Alizarin red S (pH: 4.2) (Sigma-Aldrich) as described elsewhere (16).

Statistical analysis

Data are presented as the mean ± standard deviation. Multiple comparisons were performed with one-way ANOVA and protected Fisher's Least Significant Difference test. Differences were considered statistically significant when $P < 0.01$ or $P < 0.05$.

RESULTS

Cell isolation by each method

The same quantities of marrow cells were processed by either Ficoll-Paque® centrifugation (Ficolled cells), or hemolysis buffer (hemolyzed cells), or were left without treatment (untreated cells) (Fig. 1A). After each treatment, the viable cell number was counted using a Countess® Automated Cell Counter (Invitrogen) following the manufacturer's instructions with fixed settings (sensitivity: 2; size gating: 10 - 60 μm; circularity: 85%). The average number of isolated cells was greatest in the untreated fractions, followed by hemolyzed fractions, and lowest in Ficolled fractions (Fig. 1B).

Bone forming ability of non-cultured samples of untreated, hemolyzed, and Ficoll-treated marrow

The fraction of successful ectopic bone transplants was defined as the percentage of transplants containing ectopic bone/ total transplants. Following transplants of untreated, hemolyzed, or Ficolled cells, we assessed successful transplants as 86%, 57%, or 43%, respectively (Fig. 2A). The average bone score (total bone score/ the number of transplants) were 1.14, 0.86, or 0.43, respectively (Fig. 2B). Representative histological photographs are shown in Fig. 2C - E (2C, untreated cells; 2D, hemolyzed cells; 2E, Ficolled cells).

Flow cytometric analysis of cell surface marker expression in cultured BMSCs isolated by each method

Expression profiles of cell surface markers of hematopoietic cells (CD45), mesenchymal cells (CD54 and CD90), and committed osteogenic cells (ALP) were analyzed by flow cytometry. Flowcytometric analyses were performed for three times and representative expression profiles of cultured, non-induced samples from each group are shown in Fig. 3. The percentages of CD45-positive cells in untreated, hemolyzed, and Ficolled cells were 20%, 17%, and 28%, respectively. Thus, there appeared to be no significant differences in the proportions of hematopoietic cells among the groups (Fig. 3).

Expression profiles of CD54 and CD90, both of which are expressed by mesenchymal cells as well as some hematopoietic cells such as monocytes and dendritic-like cells (17), did not show significant differences among the groups (Fig. 3). In contrast, there was a significant difference in the expression of ALP. The percentages of ALP-positive cells in untreated, hemolyzed, and Ficolled cells were 41%, 41%, and 7%, respectively. Thus, the Ficolled cells might contain a lower proportion of committed osteogenic cells than the other isolates (Fig. 3).

Expression of CD45 and CD54 in cultured BMSCs assessed by immunostaining

Fluorescent immunostaining of CD45 and CD54 was performed to confirm the expression of these cell surface markers in cultured BMSCs. In accordance with the results of flow cytometric analyses, approximately 20% of the cells were positive for CD45 (Fig. 4A - D), while 100% of the cells were positive for CD54 (Fig. 5A - D).

Cell proliferation and ALP activities in non-induction medium or osteogenic induction medium.

Differences in cell proliferation and ALP activities when cultured in non-induction medium or osteogenic induction medium were quantitatively analyzed. As shown in Fig. 6A and 6B, all cell groups showed greater cell proliferation in non-induction medium. Although the Ficolled cells showed relatively slower proliferation regardless of the type of medium, no significant differences were observed among the groups at any sampling point. As for ALP activity, a statistically significant difference was observed after 14 days of culture in non-induction medium. The ALP activity of non-induced Ficolled cells was significantly lower than that of the other isolates (Fig. 6C), in accordance with the results of flow cytometric analyses. Notably, the Ficolled group showed the greatest levels of ALP activity when cultured in osteogenic induction medium for 14 days, though the difference did not reach a statistically significant level (Fig. 6D).

Expression of osteogenesis-related genes in non-induction medium or osteogenic induction medium.

Differences in the expression of osteogenesis-related genes (osteopontin, core-binding factor subunit alpha-1 (*Cbfa-1*), and osteocalcin) were analyzed by real-time qPCR. The expression of osteopontin after seven days of culture both in non-induction medium and in osteogenic induction medium was lowest in the Ficolled group, though this group showed the greatest osteopontin expression after 14 days of culture in osteogenic induction medium (Fig. 7A and 7B). While the expression of *Cbfa-1* was also relatively low in the Ficolled group when cultured in non-induction medium (Fig. 7C), this group showed the greatest *Cbfa-1* expression after 14 days of culture in osteogenic induction medium (Fig. 7D), as observed in osteopontin expression (Fig. 7B).

The Ficolled group also showed the greatest osteocalcin expression after 14 days of culture in osteogenic induction medium (Fig. 7F), though the expression of osteocalcin in this group was relatively high even when cultured in non-induction medium (Fig. 7E).

In vitro mineralization ability

Differences in *in vitro* mineralization abilities were analyzed by alizarin red staining. As shown in Fig. 8, all groups showed *in vitro* mineralization ability after 21 days of culture in osteogenic induction medium. The intensities of the alizarin red staining were comparable among the groups.

Discussion

Committed osteogenic cells are usually classified into three types, *i.e.*, osteoprogenitors, pre-osteoblasts, and osteoblasts according to their stage of differentiation. All of these osteogenic cells possess bone-forming ability without osteogenic induction (18, 19). Accordingly, we first investigated differences in bone-forming ability of non-cultured samples of untreated, hemolyzed, and Ficoll-fractionated bone marrow (BM). As shown in Fig. 2A and 2B, following transplantation, the percentages of successful ectopic bone formation and the average bone scores were greatest in the untreated group and lowest in the Ficolled group, suggesting that Ficolled BM contained the lowest number of committed osteogenic cells.

As for the hemolyzed group, the percentage of transplants successfully achieving ectopic bone formation was lower (Fig. 2A) but the average bone score was comparable to the untreated group (Fig. 2B), suggesting that hemolyzed BM contain greater numbers of committed osteogenic cells than the Ficolled BM.

To further investigate differences in committed osteogenic cell populations, cells from each group were cultivated *in vitro* in non-induction medium and analyzed for differences in the percentages of alkaline phosphatase (ALP)-positive cells by flow cytometry. This approach was utilized because cell surface ALP expression as well as bone-forming ability are important characteristics of committed osteogenic cells (20, 21). As shown in Fig. 3, the percentage of ALP-positive cells was significantly lower in Ficolled cells, though no significant differences were observed in the expression profiles of other cell surface markers (CD45, CD54, and CD90) among the groups, indicating that Ficolled cells are characteristically different from other groups in the proportion of committed osteogenic cells. Quantitative ALP assays also provided supportive evidence that Ficolled cells include a lower proportion of committed osteogenic cells than other fractions (Fig. 6C), though cell number and growth were comparable among the groups (Fig. 6A). On the other hand, no significant differences were observed between untreated and hemolyzed fractions in these assays, suggesting that cultured BMSCs from both untreated and hemolyzed BM contain similar proportions of committed osteogenic cells.

Although these data suggest that the proportion of committed osteogenic cells is significantly lower in Ficolled cells, it remains unknown whether the osteogenic capacity of Ficolled cells is actually inferior to that of other fractions, because Ficolled cells are known to contain uncommitted stem cells (22). Therefore, we next investigated the osteogenic ability of each group's BMSCs when cultured in osteogenic induction medium by analyzing the level of ALP activity. As shown in Fig. 6C and 6D, all induced samples showed greater levels of ALP activities than non-induced samples, suggesting that all groups contain uncommitted stem cells which require osteogenic induction to differentiate into that lineage. Interestingly, the ALP activity of the Ficoll-separated fraction was greater than that of other groups after 14 days of culture in osteogenic induction medium, though the difference did not reach a statistically significant level (Fig. 6D). Those results indicate that although Ficolled cells contain a lower proportion of committed osteogenic cells, this fraction shows greater or at least comparable levels of osteogenic ability when cultured in osteogenic induction medium, possibly because the Ficolled group contained a greater proportion of uncommitted stem cells. To support this, Ficolled cells showed the greatest gene expression of osteopontin, *Cbfa-1*, and osteocalcin after 14 days of culture in osteogenic induction medium (Fig. 7B, 7D, 7E), and they were able to form calcified nodules after 21 days of culture in the induction medium like untreated and hemolyzed fractions (Fig. 8). Therefore, Ficolled cells should be induced before use in bone tissue engineering. In contrast, both untreated and hemolyzed groups could be used without osteogenic induction because both these groups contain committed osteogenic cells. However, it might be better to induce them before use in bone tissue engineering, since their osteogenic abilities also depend on uncommitted stem cells.

The hemolysis treatment of BM is useful for the efficient isolation of BMSCs (9, 10). In fact, the cell yield (harvested cell number after primary culture/ days of primary culture/ initially seeded cell number) calculated for untreated, hemolyzed, and Ficolled group was 0.44, 0.52, and 0.13, respectively. However, this study indicated that some of the committed osteogenic cells contained in BM are lost or damaged during the hemolysis treatment, because the percentage of successful ectopic bone formation was lower in the hemolyzed group than the untreated group. Thus, it might be advisable to use adherent culture of untreated BM to isolate BMSCs for use in bone tissue engineering. As for the Ficoll centrifugation technique, this study revealed that the osteogenic cell population obtained by Ficoll fractionation significantly differs from untreated and hemolyzed populations. As Ficolled cells rarely include committed osteogenic cells, this group is less osteogenic than other groups in the absence of induction. However, when cultured in osteogenic induction medium, the Ficolled fraction shows osteogenic ability comparable to the other fractions, possibly because this group contains a greater proportion of uncommitted stem cells. Therefore, the Ficoll technique might be rather suitable for the isolation of multi-potent BMSCs. These findings should be taken into account when applying either Ficoll separation or hemolysis for the isolation of BMSCs, and it is better to select an isolation technique specific for the intended purpose.

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DISCLOSURE OF INTEREST

The authors have no conflict of interests.

REFERENCES

1. Bruder SP, Fink DJ, Caplan AI. Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. J Cell Biochem. 1994; 56: 283-294.

2. Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp Hematol. 2002; 30: 896-904.

3. Ashton BA, Allen TD, Howlett CR, Eaglesom CC, Hattori A, Owen M. Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo. Clin Orthop Relat Res. 1980; 151: 294-307.

4. Aubin JE, Liu F, Malaval L, Gupta AK. Osteoblast and chondroblast differentiation. Bone. 1995; 17: 77S-83S.

5. Aubin JE. Osteoprogenitor cell frequency in rat bone marrow stromal populations: role for heterotypic cell-cell interactions in osteoblast differentiation. J Cell Biochem. 1999; 72: 396-410.

6. Mendes SC, Tibbe JM, Veenhof M, Bakker K, Both S, Platenburg PP, et al. Bone tissue-engineered implants using human bone marrow stromal cells: effect of culture conditions and donor age. Tissue Eng. 2002; 8: 911-920.

7. McCulloch CA, Struguresku M, Hughes F, Melcher AH, Aubin JE. Osteogenic progenitor cells in rat bone marrow stromal populations exhibit self-renewal in culture. Blood. 1991; 77: 1906-1911.

8. Leboy PS, Beresford JN, Devlin C, Owen ME. Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. J Cell Physiol. 1991; 146: 370-378.

9. Horn P, Bork S, Diehlmann A, Walenda T, Eckstein V, Ho AD, et al. Isolation of human mesenchymal stromal cells is more efficient by red blood cell lysis. Cytotherapy. 2008; 10: 676-685.

10. Peterbauer-Scherb A, van Griensven M, Meinl A, Gabriel C, Redl H, Wolbank S. Isolation of pig bone marrow mesenchymal stem cells suitable for one-step procedures in chondrogenic regeneration. J Tissue Eng Regen Med. 2010; 4: 485-490.

11. Wagner W, Ho AD. Mesenchymal stem cell preparations--comparing apples and oranges. Stem Cel. Rev. 2007; 3: 239-248.

12. Agata H, Asahina I, Watanabe N, Ishii Y, Kubo N, Ohshima S,. et al. Characteristic change and loss of in vivo osteogenic abilities of human bone marrow stromal cells during passage. Tissue Eng Part A. 2010; 16: 663-673.

13. Agata H, Watanabe N, Ishii Y, Kubo N, Ohshima S, Yamazaki M, et al. Feasibility and efficacy of bone tissue engineering using human bone marrow stromal cells

cultivated in serum-free conditions. Biochem Biophys Res Commun. 2009; 382: 353-358.

14. Mankani MH, Kuznetsov SA, Avila NA, Kingman A, Robey PG. Bone formation in transplants of human bone marrow stromal cells and hydroxyapatite-tricalcium phosphate: prediction with quantitative CT in mice. Radiology. 2004; 230: 369-376.

15. Kuroda S, Virdi AS, Dai Y, Shott S, Sumner DR. Patterns and localization of gene expression during intramembranous bone regeneration in the rat femoral marrow ablation model. Calcif Tissue Int. 2005; 77:212-225.

16. Agata H, Kagami H, Watanabe N, Ueda M. Effect of ischemic culture conditions on the survival and differentiation of porcine dental pulp-derived cells. Differentiation. 2008; 76:981-993.

17. Chen-Woan M, Delaney CP, Fournier V, Wakizaka Y, Murase N, Fung J, et al. In vitro characterization of rat bone marrow-derived dendritic cells and their precursors. J Leukoc Biol. 1996; 59:196-207.

18. Włodarski KH. Properties and origin of osteoblasts. Clin Orthop Relat Res. 1990; 252: 276-93.

19. Aubin JE. Bone stem cells. J Cell Biochem. 1998; Suppl. 30/31: 73-82.

20. Herbertson A, Aubin JE. Cell sorting enriches osteogenic populations in rat bone marrow stromal cell cultures. Bone. 1997; 21: 491-500.

21. Kotobuki N, Matsushima A, Kato Y, Kubo Y, Hirose M, Ohgushi H. Small interfering RNA of alkaline phosphatase inhibits matrix mineralization. Cell Tissue Res. 2008; 332: 279-288.

22. Polisetti N, Chaitanya VG, Babu PP, Vemuganti GK. Isolation, characterization and differentiation potential of rat bone marrow stromal cells. Neurol India. 2010; 58: 201-208.

FIGURE LEGENDS

Figure 1. Experimental design and the average number of cells isolated from untreated, hemolyzed, or Ficoll-treated bone marrow (BM)

(A) Experimental design of this study. Rat BM was divided into three portions and the suspensions were either hemolyzed, or subjected to Ficoll fractionation, or left without treatment (untreated). Thereafter, cells obtained by each method were analyzed for differences in osteogenic cell populations with or without *in vitro* cultivation. (B) Average yield of cells from untreated, hemolyzed, and Ficolled BM. Significant differences were observed in average isolated cell numbers among the groups. Data are presented as the mean ± standard deviation, calculated from six repeats of independent experiments (n=6). **: $p < 0.01$

Figure 2. Bone forming ability of non-cultured samples

(A) Assessment of successful ectopic bone formation (transplants containing bone/ total transplants) of non-cultured samples, which was calculated from the results of seven independent experiments, was greatest in the untreated group, followed by the hemolyzed group, and lowest in the Ficolled group ($n = 7$). (B) Average bone score (total bone score/ total transplants) was greatest in the hemolyzed group, followed by the untreated group, and lowest in the Ficolled group. Representative histology of the transplants of non-cultured samples from untreated (C), hemolyzed (D), and Ficolled (E) fractions. Transplants of the Ficolled fraction formed lower amounts of new bone than those of other groups. Green scale bar: 100 μm.

Figure 3. Expression profiles of cell surface antigens of BMSCs grown from each fraction.

BMSCs from each fraction were expanded in non-induction medium and were analyzed by flow cytometry to investigate differences in the expression of CD45, CD54, CD90, and ALP. The percentage of ALP-positive cells was significantly lower in the Ficolled fraction, though no significant difference was observed in the expression profiles of other cell surface markers among the isolates. Blue line represents the isotype control.

Figure 4. Fluorescent immunostaining of CD45 in BMSCs grown from untreated BM. Fluorescent immunostaining of CD45 was performed to confirm the results of flow cytometric analyses. Approximately 20% of cells grown from untreated BM were positive for CD45 even at passage one. (A) DAPI, (B) FITC-CD45, (C) phase contrast, (D) merged image of DAPI, FITC-CD45, and phase contrast photographs.

Figure 5. Fluorescent immunostaining of CD54 in BMSCs grown from untreated BM. Fluorescent immunostaining of CD54 was performed to confirm the results of flow cytometric analyses. Cells grown from untreated BM were 100% positive for CD54. (A) DAPI, (B) FITC-CD54, (C) phase contrast, (D) merged image of DAPI and FITC-CD54.

Figure 6. Cell proliferation and ALP activities in non-induction medium or osteogenic induction medium.

BMSCs grown from untreated, hemolyzed of Ficolled BM were cultured in non-induction medium or osteogenic induction medium and analyzed for differences in cell proliferation and ALP activities. (A) Cell proliferation in non-induction medium. All fractions proliferated similarly in non-induction medium and no significant differences were observed in cell number at either seven or 14 day sampling points. (B) Cell proliferation in osteogenic induction medium. All fractions proliferated similarly and no significant differences were observed in cell number at either sampling point, though cell proliferation in osteogenic induction medium was significantly lower than that in non-induction medium. (C) ALP activity in non-induction medium. A statistically significant difference was observed in ALP activities between Ficolled cells and the other fractions after 14 days of culture in non-induction medium. (D) ALP activity in osteogenic induction medium. Although all fractions showed greater ALP activity than in non-induction medium, the Ficolled fraction showed the lowest ALP activity at the seven day sampling point. However, this fraction showed the greatest ALP activity at the 14 day sampling point. Data are presented as the mean ± standard deviation (n = 3). *: $P < 0.05$.

Figure 7. Expression of osteogenesis-related genes in non-induction medium or osteogenic induction medium.

BMSCs grown from untreated, hemolyzed, or Ficolled BM were cultured in non-induction medium or osteogenic induction medium and analyzed for differences in gene expression of osteopontin, *Cbfa-1*, and osteocalcin. (A) The expression of *osteopontin* when cultured in non-induction medium. (B) Osteopontin expression in osteogenic induction medium. Ficolled fraction showed greatest expression after 14 days of culture. (C) The expression of *Cbfa-1* when cultured in non-induction medium. (D) *Cbfa-1* expression in osteogenic induction medium. Ficolled fraction showed greatest expression after 14 days of culture. (E) The expression of osteocalcin when cultured in non-induction medium. (F) Osteocalcin expression in osteogenic induction medium.