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Characteristic differences among osteogenic cell populations of rat bone marrow stromal cells isolated from untreated, hemolyzed or Ficoll-treated marrow

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Abstract

Background aims. Although bone marrow (BM) stromal cells (SC; BMSC) isolated from adherent cultures of untreated BM are known to contain both committed and uncommitted osteogenic cells, it remains unknown whether BMSC isolated either by hemolysis or Ficoll centrifugation also contain both of these populations. *Methods.* Differences in the osteogenic cell populations of rat BMSC isolated from untreated, hemolyzed or Ficoll-treated BM were analyzed by *in vivo* transplantation, flow cytometry, alkaline phosphatase (ALP) assay, real-time polymerase chain reaction (PCR) and alizarin red staining. *Results.* Transplantation of non-cultured samples indicated that the Ficolled BMSC contained the lowest number of committed osteogenic cells. Flow cytometric analysis of cultured, non-induced samples showed that the percentage of ALP-positive cells was significantly lower in Ficolled BMSC. Quantitative ALP assays confirmed that the lowest ALP activity was in the Ficolled BMSC. Hemolyzed BMSC also contained lower numbers of committed osteogenic cells than untreated BMSC, but still more than Ficolled BMSC. Interestingly, the Ficolled BMSC showed the greatest levels of osteogenic ability when cultured in osteogenic induction medium. *Conclusions.* These findings suggest that, although Ficolled BMSC rarely contain committed osteogenic cells, they are able to show comparable or even greater levels of osteogenic ability after induction, possibly because they contain a greater proportion of uncommitted stem cells. In contrast, induction is optional but recommended for both untreated and hemolyzed BMSC before use, because both these groups contain both committed and uncommitted osteogenic cells. These findings are of significant importance when isolating BMSC for use in bone tissue engineering.

Key Words: *bone marrow stromal cells, Ficoll, hemolysis, osteogenic cells, stem cells*

Introduction

Bone marrow (BM) stromal cells (SC; BMSC) isolated from adherent cultures of untreated whole BM (normal BMSC) contain an uncommitted stem cell population called mesenchymal stromal cells that can differentiate into multiple (osteogenic, chondrogenic, adipogenic, myogenic and neurogenic) lineages (1,2). It remains controversial regarding whether the multilineage differentiation ability of normal BMSC is attributed solely to uncommitted stem cells, because the BMSC population is composed of a heterogeneous collection of cells that might include committed progenitors of each lineage (3). Nonetheless, the osteogenic ability at least of normal BMSC depends on both committed and uncommitted cells, as it has long been known that normal BMSC contain committed osteogenic cells such as pre-osteoblasts (4).

Uncommitted stem cells require induction stimuli to promote osteogenic differentiation (5). Thus BMSC are generally cultured in osteogenic induction medium before use in bone tissue engineering. However, the induction process can be omitted or shortened when BMSC include large numbers of committed osteogenic cells, as committed cells spontaneously differentiate into osteoblasts (4,5). Thus the proportion of committed osteogenic cells affects both the protocol design as well as the total cost of bone tissue engineering. In addition, this

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proportion may significantly affect the osteogenic ability of BMSC, as highly osteogenic BMSC are able to form bone without induction (6). Therefore it is important to investigate the proportion of committed osteogenic cells when analyzing the osteogenic ability of BMSC, although few studies have focused on the importance of that population.

In general, BMSC are obtained from adherent cultures of untreated whole BM because they are able to form adherent colonies unlike non-adherent hematopoietic cells (7,8). However, this technique may be inefficient for the isolation of BMSC because untreated BM contains a large proportion of erythrocytes and their presence can interfere with the initial colony-forming ability of BMSC. Both density-gradient centrifugation over Ficoll® (GE Healthcare UK Ltd, Amersham, UK) and hemolysis treatment with ammonium chloride are techniques used for the removal of erythrocytes from BM to enhance BMSC adherence, and BMSC isolated by these techniques have been shown to possess multilineage differentiation abilities (9,10). However, it remains unknown whether these BMSC have the same differentiative potential as BMSC isolated from whole BM, as the cellular composition of BMSC may vary with the isolation techniques (11). Differences in cellular composition may greatly influence the osteogenic characteristics of BMSC, because the osteogenic ability of normal BMSC depends on at least two different cell populations, i.e. committed osteogenic cells and uncommitted stem cells (5). Therefore, it is important to investigate the osteogenic BMSC populations following isolation by either Ficoll fractionation or hemolysis before applying these techniques to bone tissue engineering. In the present study, we isolated BMSC from untreated, hemolyzed or Ficoll-treated rat BM and analyzed their differences in osteogenic cell populations, with a special emphasis on committed osteogenic cell populations.

Methods

BM isolation from Sprague–Dawley rats

Sprague–Dawley (SD) rats (Jcl. SD, male, 6 weeks old) were purchased from Clea Japan Inc. (Tokyo, Japan). All experiments were approved by the Animal Ethics Screening Committee of The Institute of Medical Science, The University of Tokyo (Tokyo, Japan). After intraperitoneal overdose administration of pentobarbital sodium, the femurs and tibiae were carefully dissected from the killed rats. The tips of the bones were cut with scissors to expose the BM. Thereafter, BM was flushed out from each bone by injecting 7.5 mL Hanks' balanced salt solutions (HBSS; Wako Pure Chemical Industries Ltd, Osaka, Japan) with an 18-gauge needle (Terumo, Tokyo, Japan) attached to a 20-mL syringe (Terumo), and collected in a 50-mL centrifuge tube (Becton Dickinson, Pharmingen, Franklin Lakes, NJ, USA). Then the tubes were centrifuged at 440 ***g*** for 5 min at 4°C. After discarding the supernatants, the marrow cells from each rat were resuspended in 10.5 mL Dulbecco's phosphate-buffered saline (DPBS; Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) and divided among three different tubes (each tube containing 3.5 mL marrow cell suspensions).

Isolation of BMSC from untreated, hemolyzed or Ficoll-treated BM

To isolate BMSC, marrow cell suspensions were processed on Ficoll or hemolyzed as described elsewhere (9).

Ficoll-separated cells

For Ficoll treatment, 3.5-mL marrow cell suspensions were diluted with 7.5 mL DPBS and carefully laid over 15 mL Ficoll-Paque® (GE Healthcare UK Ltd). After centrifugation (400 ***g***, 30 min, 20°C), the mononuclear cell fraction was collected and moved to another new tube. Subsequently, cells were washed twice in DPBS. After centrifugation (400 ***g***, 10 min, 4°C), the supernatants were discarded and the cell pellets obtained were suspended in 1 mL physiologic saline (Otsuka Pharmaceutical Co. Ltd, Tokushima, Japan) for transplantation experiments or suspended in 6 mL α-MEM (Minimum Essential Medium Alpha) (Wako Pure Chemical Industries Ltd) supplemented with 10% fetal bovine serum (JRS, Woodland, CA, USA) and antibiotics/antimycotics for cell-culture analyzes. The suspensions were termed 'Ficolled cells'.

Hemolyzed cells

For hemolysis treatment, 3.5-mL marrow cell suspensions were mixed with 10 mL lysis buffer [0.15 M ammonium chloride (NH_4Cl; Sigma-Aldrich, St Louis, MO, USA), 10 mM potassium bicarbonate ($KHCO_3$; Sigma-Aldrich) and 0.1 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetrasodium salt, tetrahydrate (EDTA·4Na; Dojindo Laboratories, Kumatomo, Japan)] and incubated for 10 min at room temperature on a horizontal shaker. Then the cells were centrifuged (400 ***g***, 10 min, 4°C) and washed twice in DPBS. After centrifugation (400 ***g***, 10 min, 4°C), the supernatants were discarded and the resultant cell pellets were suspended in 1 mL physiologic saline for the transplantation experiment, or suspended in 6 mL serum-containing medium for cell-culture analyzes.

Untreated group

As untreated controls, 3.5-mL marrow cell suspensions were washed twice in DPBS. After centrifugation (400 ***g***, 10 min, 4°C), the supernatants were discarded and the resultant cell pellets were suspended in 1 mL physiologic saline for transplantation experiments or suspended in 6 mL serum-containing medium for cell-culture analyzes.

Transplantation of non-cultured cells isolated by different techniques

Committed osteogenic cells spontaneously differentiate into bone-forming osteoblasts (5). Therefore non-cultured samples of untreated, hemolyzed or Ficolled cells were analyzed for their *in vivo* bone-forming abilities to investigate differences in committed osteogenic cells. Samples were transplanted to ectopic sites to avoid the effects of recipient nude mouse-derived osteogenic cells. One milliliter of saline containing each sample was mixed with 50 mg β-tricalcium phosphate (β-TCP) granules (G1 type OSferion®; Olympus Terumo Biomaterials, Tokyo, Japan) in a 14-mL polypropylene tube (Becton Dickinson) and centrifuged (100 ***g***, 5 min, 4°C). After careful removal of the supernatants, 100-μL 10 mg/mL fibrinogen solution (bovine plasma fibrinogen; F8630; Sigma-Aldrich) and 5-μL 100 U/mL thrombin solution (bovine plasma thrombin; T9549; Sigma-Aldrich) were added to each cell–β-TCP mixture to form a fibrin clot. Thereafter each clotted cell mixture was transplanted into the subcutaneous space on the backs of 6-week-old female BALB/CAJcl-nu/nu mice (Clea Japan Inc.) under anesthesia with pentobarbital sodium. Transplants were harvested 4 weeks after the operation.

Histologic assessment of the transplants of non-cultured samples

Harvested samples were fixed in 10% buffered formalin, decalcified in Kalkitox™, neutralized in 5% sodium sulfate solution (all purchased from Wako Pure Chemical Industries Ltd) and embedded in paraffin. Five-micrometer thick sections were prepared from the middle of each transplant and stained with hematoxylin and eosin (H&E).

Microscopic examination of H&E-stained sections was conducted to determine the number of transplants that contained ectopic bone formation. The percentage of successful ectopic bone formation (number of transplants containing ectopic bone/total transplants) was calculated to assess the bone-forming potential of untreated, hemolyzed and Ficolled cells, as described elsewhere (12). To investigate the bone-forming activity, light microscopic images were captured with a digital camera (Carl Zeiss Japan, Tokyo, Japan) and transferred to a computer. Then the percentage of the area containing bone (new bone area/total area) was assessed manually using Image J (Scion Corporation, Frederick, MD, USA), as described elsewhere (13). The extent of bone formation in each transplant was scored on a semi-quantitative scale in a manner similar to that described previously (14). For example, sections prepared from the middle of each transplant were scored on a scale of 0 to 3: a score of 0 corresponded to no bone formation, while a score of 3 corresponded to abundant bone formation (bone area > 10%) (Table I). Thereafter, the average bone score of the transplants (total bone score/total transplants) was calculated for each group.

In vitro *expansion of BMSC isolated from untreated, hemolyzed or Ficoll-treated marrow cell suspensions*

For further investigations, cells isolated from untreated, hemolyzed or Ficoll-treated marrow cell suspensions were resuspended in 6 mL serum-containing medium for culture expansion as described above. Cells were plated in 3-mL/well volumes in 6-well plates (Becton Dickinson) and maintained in a 37°C, 5% CO_2 incubator. On the following day, the culture medium was replaced with fresh medium of the same type. Adherent cells were re-fed with fresh medium on the fourth day. After 1 week of culture, adherent cells were treated with 0.5% trypsin–EDTA (Invitrogen, Carlsbad, CA, USA), and cells detached within 3 min were passaged and replated in 100-mm tissue culture dishes (TPP Techno Plastic Products AG, Trasadingen, Switzerland) at a density of 6.5×10^3 cells/cm^2 for flow cytometric analyses, or replated into multiwell plates (Becton Dickinson) for other analyzes.

Flow cytometric analysis

Differences in cell-surface marker expression were analyzed with a fluorescent-activated cell sorter (FACS) Aria flow cytometer (Becton Dickinson). Fluorescence isothiocyanate (FITC)-conjugated, phycoerythrin (PE)-conjugated, or allophycocyanin (APC)-conjugated antibodies targeted against CD45, CD54, CD90 (all from Biolegend, San Diego, CA,

Table I. Semi-quantitative scale to estimate the extent of bone formation.

	Bone score (Extent of bone formation in transplant)
0	No bone evident
1	Low bone formation (bone area < 5%)
2	Moderate bone formation (5% < bone area < 10%)
3	Abundant bone formation (bone area > 10%)

USA) and a biotinylated antibody against alkaline phosphatase (ALP; BAM1448; R&D Systems Inc., Minneapolis, MN, USA) were used for the analyzes. The biotinylated antibody was detected with a streptavidin–FITC conjugate (Biolegend). Propidium iodide (Dojindo) was used to detect dead cells.

First-passage cells were detached with trypsin–EDTA and 1×10^6 cells were resuspended in 50 μL ice-cold DPBS. Cells were then incubated with individual antibodies for 20 min on ice. Thereafter, cells were washed and incubated with a streptavidin conjugate for 20 min on ice. Finally, the cells were washed, resuspended in 200 μL ice-cold DPBS, stained with propidium iodide, and analyzed. Data analysis was performed using FlowJo software (TreeStar Inc., San Carlos, CA, USA).

Fluorescent immunostaining

Fluorescent immunostaining of CD45 and CD54 was performed to confirm the results of flow cytometric analyzes. First-passage cells were plated in 24-well plates at a density of 5×10^4 cells/well in serum-containing medium, and cultured until 70% confluent. Thereafter, cells were fixed with 4% paraformaldehyde, washed three times with DPBS, and treated with 100 mM glycine buffer. Then the 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 rewashed 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, USA) 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 analyzes and quantitative ALP assays were performed as described elsewhere with modifications (13). First-passage cells from each group were plated into 24-well plates at a density of 2×10^4 cells/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 1 or 2 weeks of culture, cell-number analyzes 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 WST-8 were added to each well containing 0.5 mL fresh medium, incubated for 60 min, 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 *p*-nitrophenyl phosphate solution was added to each well. After 10 min 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 ((Optical Density 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 differences in the expression of osteogenic marker genes. Second-passage cells from each group were plated in 6-well plates at a density of 1×10^5 cells/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 1 or 2 weeks of culture, total RNA was extracted with an RNeasy® Mini Kit (QIAGEN Science, Germantown, MD, USA). First-strand cDNA syntheses were performed with a 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 (Cbfa-1; forward, gccaggttcaacgatctgag; reverse, gaggcggtcagagaacaaac), osteocalcin (forward, agctcaaccccaattgtgac; reverse, agctgtgccgtccatacttt) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward, aactcccattcctccacctt; reverse, gagggcctctctcttgctct), which have been described previously (15). A SYBR® Premix ExTaq™ II (Tli RNase H Plus) (Takara Bio Inc.) was used for real-time qPCR 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 s, and then 40 cycles at 95°C for 5 s and 60°C for 1 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 BMSC, second-passage cells from each group were plated in 24-well plates at a density of 5×10^4 cells/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 3 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) (Figure 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 (Figure 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, the successful transplants were assessed as 86%, 57% and 43%, respectively (Figure 2A). The average bone scores (total bone score/the number of transplants) were 1.14, 0.86 and 0.43, respectively (Figure 2B). Representative histologic photographs are shown in Figure 2C–E (C, untreated cells; D, hemolyzed cells; E, Ficolled cells).

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

Expression profiles of cell-surface markers of hematopoietic cells (CD45), mesenchymal cells

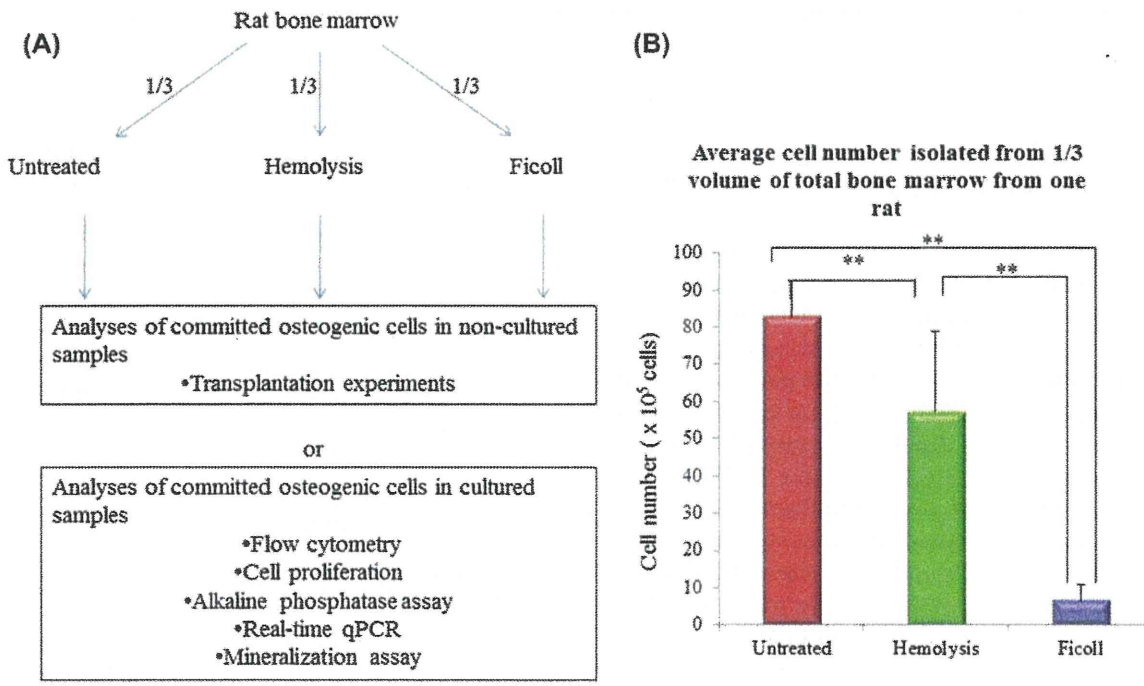

Figure 1. Experimental design and the average number of cells isolated from untreated, hemolyzed or Ficoll-treated BM. (A) Experimental design of this study. Rat BM was divided into three portions and the suspensions were hemolyzed, subjected to Ficoll fractionation or left without treatment (untreated). 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 the average numbers of isolated cells among the groups. Data are presented as the mean ± standard deviation ($n = 6$). $^{**}P < 0.01$.

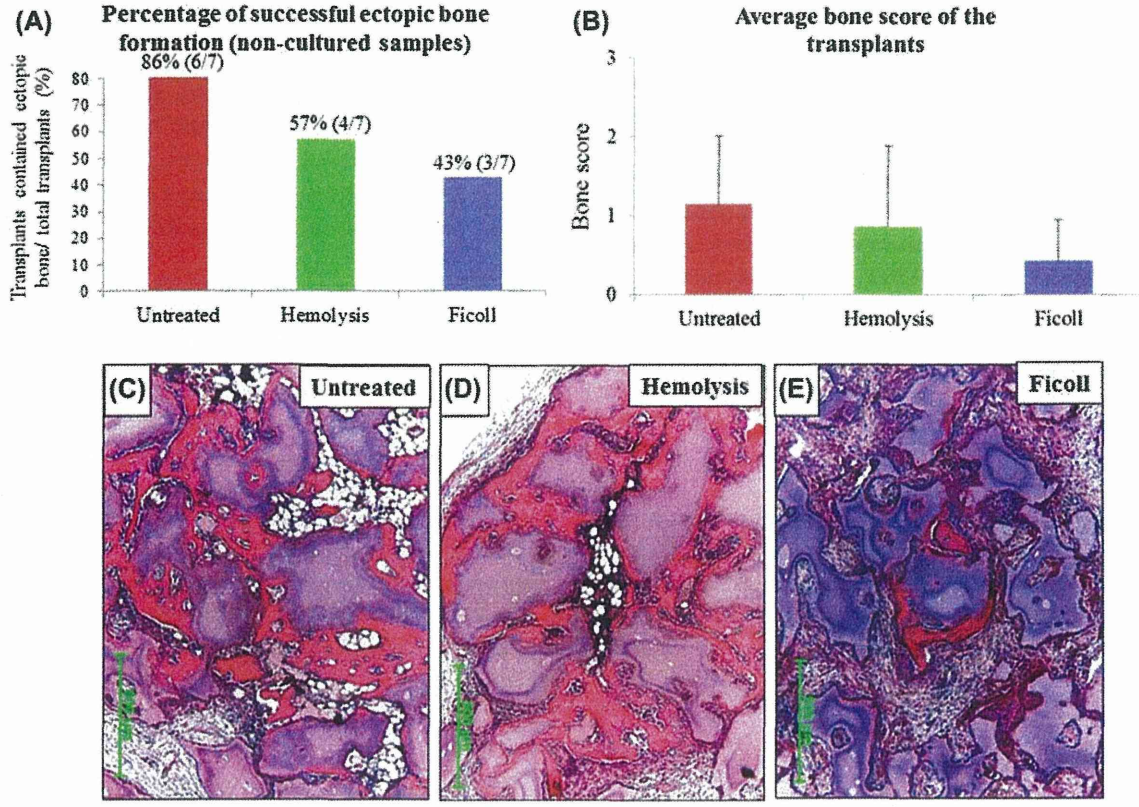

Figure 2. Bone-forming ability of non-cultured samples. (A) Successful ectopic bone formation (transplants containing bone/total transplants) of non-cultured samples, which was calculated from the results of seven independent experiments, was greatest in the untreated group, followed by the hemolyzed group, and lowest in the Ficolled group. (B) The 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 ($n = 7$). 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 the other groups. Green scale bar: 100 μm. color images are available online

(CD54 and CD90) and committed osteogenic cells (ALP) were analyzed by flow cytometry. Flow cytometric analyzes were performed three times and representative expression profiles of cultured, non-induced samples from each group are shown in Figure 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 (Figure 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 (Figure 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 (Figure 3).

Expression of CD45 and CD54 in cultured BMSC assessed by immunostaining

Fluorescent immunostaining of CD45 and CD54 was performed to confirm the expression of these cell-surface markers in cultured BMSC. In accordance with the results of flow cytometric analyzes, approximately 20% of the cells were positive for CD45 (Figure 4A–D), while 100% of the cells were positive for CD54 (Figure 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 analyzed quantitatively. As shown in Figure 6A, B, 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

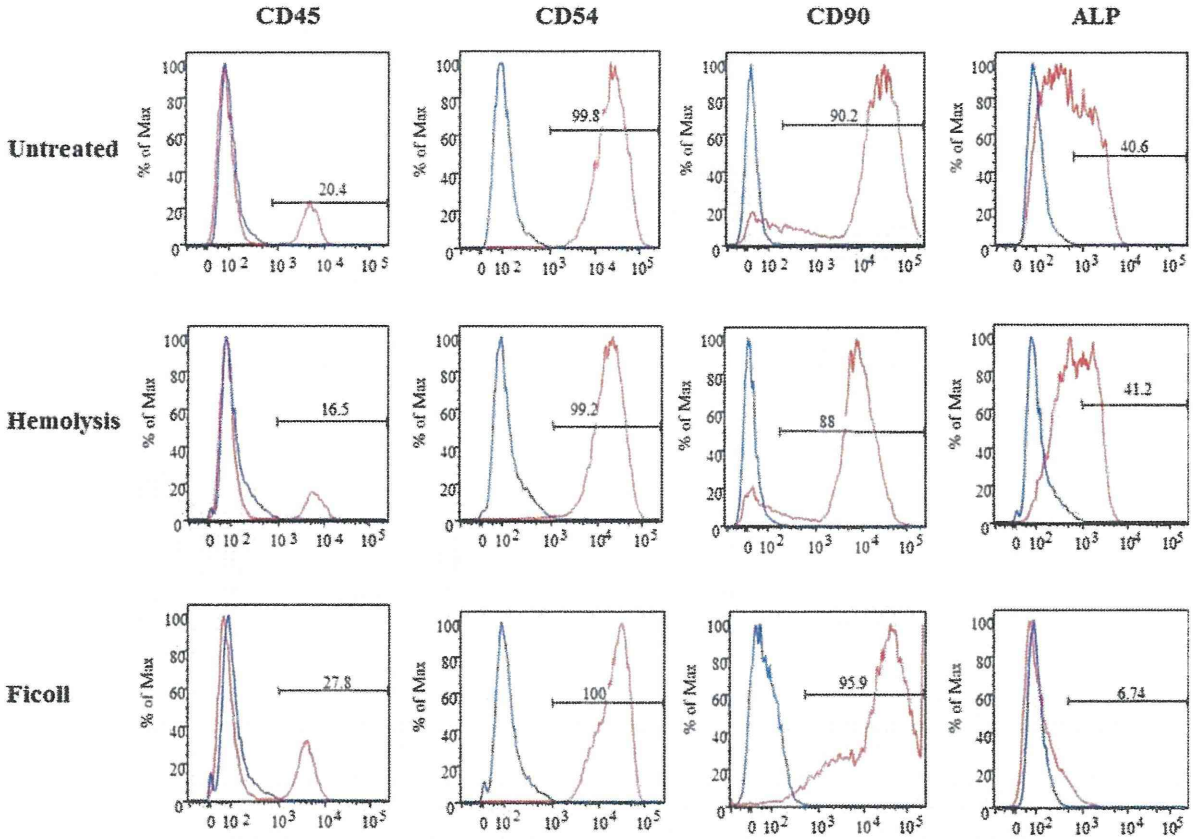

Figure 3. Expression profiles of cell-surface antigens of BMSC grown from each fraction. BMSC from each fraction were expanded in non-induction medium and analyzed by flow cytometry to investigate differences in the expression of CD45, CD54, CD90 and ALP. The percentage of ALP-positive cells was significantly lower in the Ficolled fraction, although no significant difference was observed in the expression profiles of other cell-surface markers among the isolates. Blue line: unstained control cells.

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 (Figure 6C), in accordance with the results of flow cytometric analyzes. Notably, the Ficolled group showed the greatest levels of ALP activity when

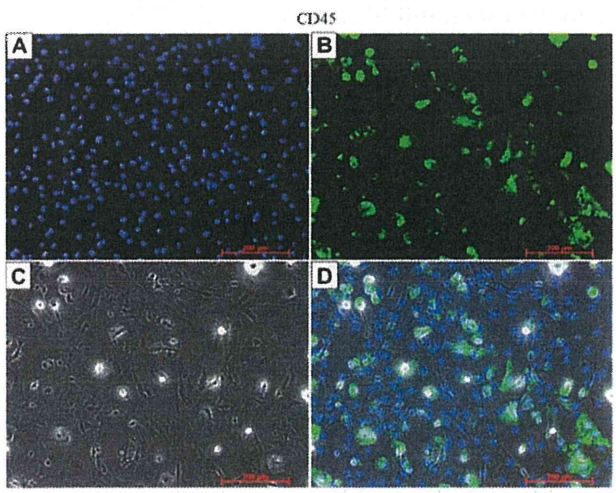

Figure 4. Fluorescent immunostaining of CD45 in BMSC grown from untreated BM. Fluorescent immunostaining of CD45 was performed to confirm the results of flow cytometric analyzes. Approximately 20% of cells grown from untreated BM were positive for CD45, even at passage 1. (A) DAPI, (B) FITC–CD45, (C) phase–contrast, (D) merged image of DAPI, FITC–CD45 and phase–contrast photographs.

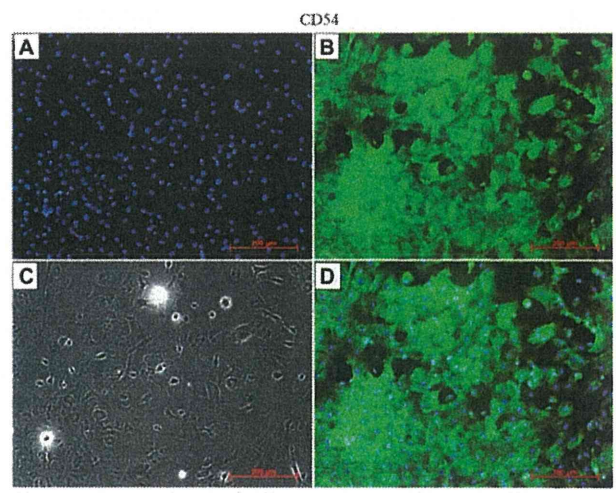

Figure 5. Fluorescent immunostaining of CD54 in BMSC grown from untreated BM. Fluorescent immunostaining of CD54 was performed to confirm the results of flow cytometric analyzes. Cells grown from untreated BM were 100% positive for CD54. (A) DAPI, (B) FITC–CD54, (C) phase–contrast, (D) merged image of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

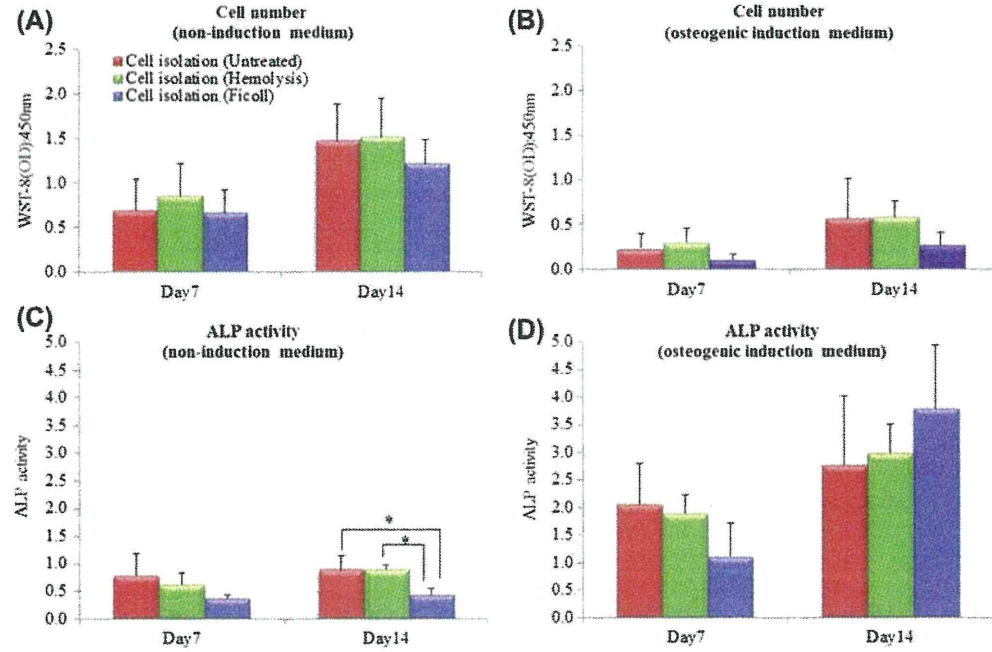

Figure 6. Cell proliferation and ALP activities in non-induction medium or osteogenic induction medium. BMSC grown from untreated, hemolyzed or Ficolled BM were cultured in non-induction medium or osteogenic induction medium and analyzed for differences in cell proliferation and ALP activities. (A) Cell proliferation in non-induction medium. All fractions proliferated similarly in non-induction medium and no significant differences were observed in cell number at either 7- 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, although 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 7-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 ($n=3$). $^{*}P<0.05$.

cultured in osteogenic induction medium for 14 days, although the difference did not reach a statistically significant level (Figure 6D).

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

Differences in the expression of osteogenesis-related genes (osteopontin, Cbfa-1 and osteocalcin) were analyzed by real-time qPCR. The expression of osteopontin after 7 days of culture both in non-induction medium and in osteogenic induction medium was lowest in the Ficolled group, although this group showed the greatest osteopontin expression after 14 days of culture in osteogenic induction medium (Figure 7A,B). While the expression of Cbfa-1 was also relatively low in the Ficolled group when cultured in non-induction medium (Figure 7C), this group showed the greatest Cbfa-1 expression after 14 days of culture in osteogenic induction medium (Figure 7D), as observed in osteopontin expression (Figure 7B). The Ficolled group also showed the greatest osteocalcin expression after 14 days of culture in osteogenic induction medium (Figure 7F), although the expression of osteocalcin in this group was relatively high even when cultured in non-induction medium (Figure 7E).

In vitro *mineralization ability*

Differences in *in vitro* mineralization abilities were analyzed by alizarin red staining. As shown in Figure 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, preosteoblasts 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 the bone-forming ability of non-cultured samples of untreated, hemolyzed and Ficoll-fractionated BM. As shown in Figure 2A,B, following transplantation

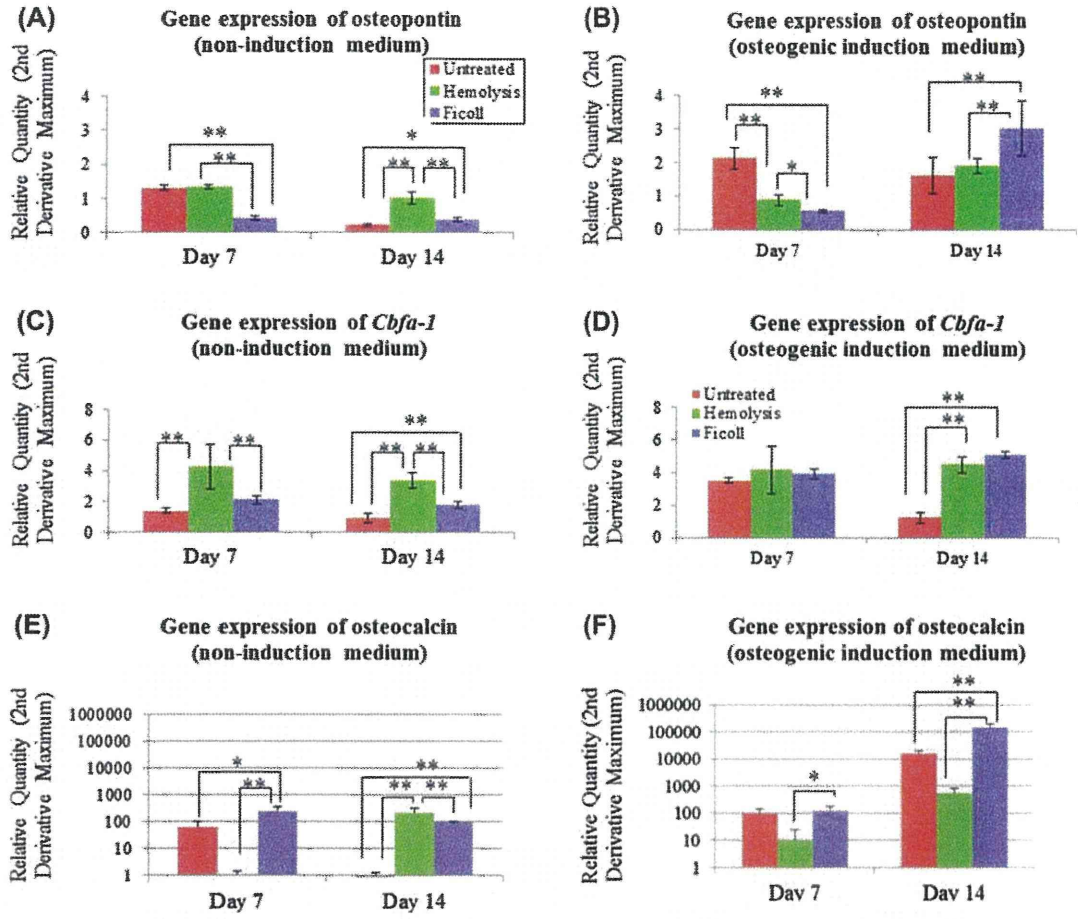

Figure 7. Expression of osteogenesis-related genes in non-induction medium or osteogenic induction medium. BMSC grown from untreated, hemolyzed or Ficolled BM were cultured in non-induction medium or osteogenic induction medium and analyzed for differences in gene expression of osteopontin, Cbfa-1 and osteocalcin. (A) The expression of osteopontin when cultured in non-induction medium. (B) Osteopontin expression in osteogenic induction medium. The Ficolled fraction showed the 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. The Ficolled fraction showed the 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. The Ficolled fraction showed the greatest expression after 14 days of culture. Data are presented as the means ± standard deviation (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$.

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 (Figure 2A) but the average bone score was comparable with the untreated group (Figure 2B), suggesting that hemolyzed BM contain greater numbers of committed osteogenic cells than the Ficolled BM.

To investigate further 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 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 Figure 3, the percentage of ALP-positive cells was significantly lower in Ficolled cells, although 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 were characteristically different from the other groups in the proportion of committed osteogenic cells. Quantitative ALP assays also provided supportive evidence that Ficolled cells included a lower proportion of committed osteogenic cells than the other fractions (Figure 6C), although cell number and growth were comparable among the groups (Figure 6A). On the other hand, no significant differences were observed between untreated and hemolyzed fractions in these assays, suggesting that cultured BMSC from both untreated and hemolyzed BM contained similar proportions of committed osteogenic cells.

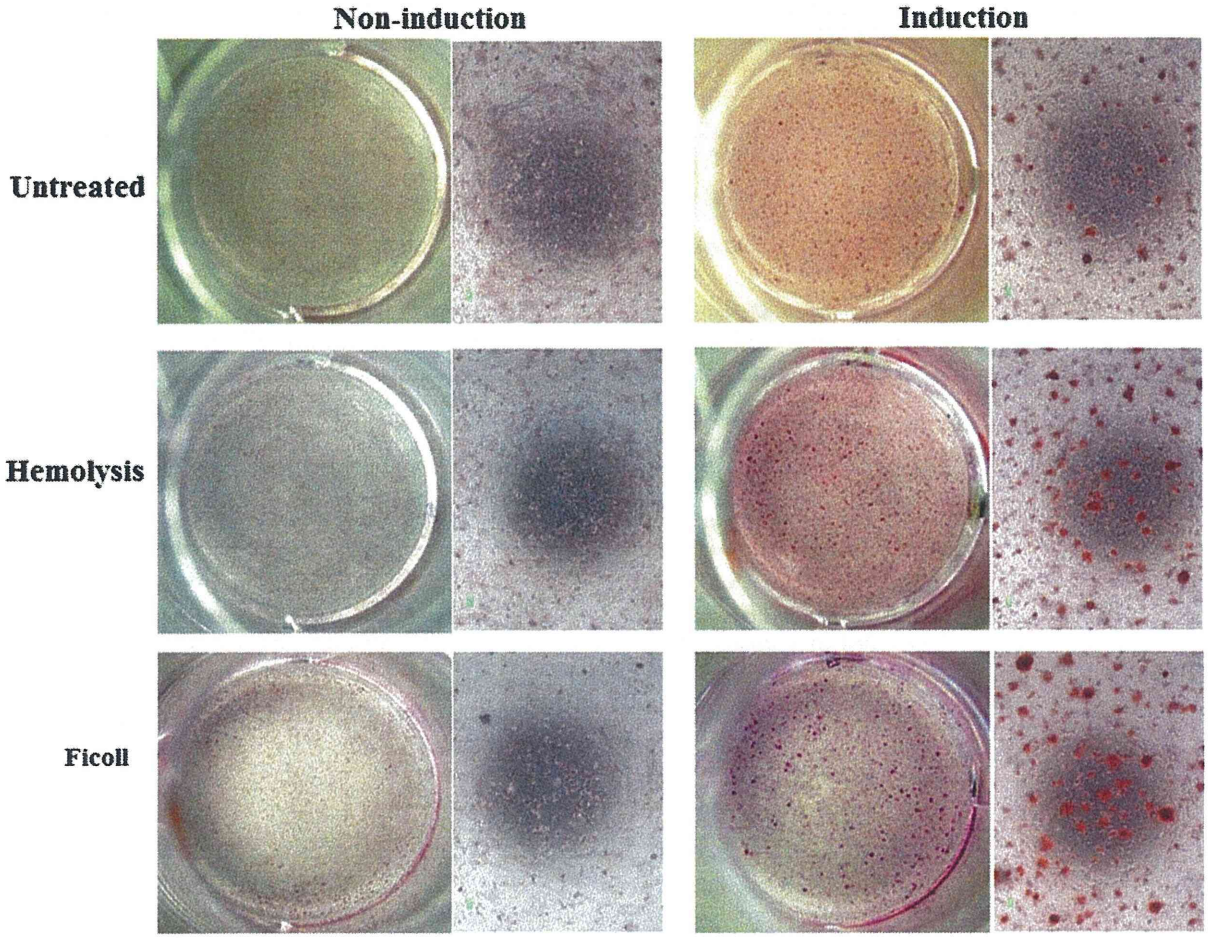

Figure 8. Alizarin red staining after 21 days of culture in non-induction medium or osteogenic induction medium. BMSC grown from untreated, hemolyzed or Ficolled BM were cultured in non-induction medium or osteogenic induction medium for 21 days and analyzed for differences in *in vitro* mineralization ability by alizarin red staining. In non-induction medium, *in vitro* mineralization was not observed in any groups. In contrast, all groups showed comparable levels of calcified nodule formation in osteogenic induction medium.

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 investigated the osteogenic ability of each group's BMSC when cultured in osteogenic induction medium by analyzing the level of ALP activity. As shown in Figure 6C, D, all induced samples showed greater levels of ALP activities than non-induced samples, suggesting that all groups contained uncommitted stem cells that required 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, although the difference did not reach a statistically significant level (Figure 6D). These 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 contains 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 (Figure 7B, D, E), and they were able to form calcified nodules after 21 days of culture in the induction medium, like untreated and hemolyzed fractions (Figure 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, because their osteogenic abilities also depend on uncommitted stem cells.

The hemolysis treatment of BM is useful for the efficient isolation of BMSC (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 groups was 0.44, 0.52 and 0.13, respectively. However, this study indicates 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 BMSC 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 with 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 multipotent BMSC. These findings should be taken into account when applying either Ficoll separation or hemolysis for the isolation of BMSC; it is better to select an isolation technique specific for the intended purpose.

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ORIGINAL ARTICLE

Effect of GDF-5 and BMP-2 on the expression of tendo/ligamentogenesis-related markers in human PDL-derived cells

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OBJECTIVES: The effect of growth differentiation factor 5 and bone morphogenetic protein 2 on human periodontal ligament-derived cells was investigated with special reference to tendo/ligamentogenesis-related markers.

MATERIALS AND METHODS: Effects of each factor were analyzed by quantitative PCR for scleraxis and tenomodulin and by western blotting for scleraxis. After exposure to those factors, STRO-1-positive and STRO-1-negative fractions of human periodontal ligament tissues were isolated with an immunomagnetic cell sorting system, and the expression of scleraxis in each fraction was analyzed by western blotting. Non-separated crude cells were used as a control.

RESULTS: Growth differentiation factor 5 and bone morphogenetic protein 2 did not increase alkaline phosphatase activity in crude periodontal ligament-derived cells. Growth differentiation factor 5, but not bone morphogenetic protein 2, increased the expression of scleraxis in crude, STRO-1-positive and STRO-1-negative periodontal ligament-derived cells. The expression of scleraxis in STRO-1-positive periodontal ligament-derived cells was significantly less compared to that in crude P2 and STRO-1-negative periodontal ligament-derived cells.

CONCLUSION: Growth differentiation factor 5 induced the expression of scleraxis and may enhance tendo/ligamentogenesis in human periodontal ligament-derived cells. The expression of scleraxis was higher in STRO-1-negative fraction, suggesting more differentiated state of the cells.

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Introduction

Periodontal ligament (PDL) is anticipated as a suitable material for periodontal regeneration. But the mechanism of periodontal regeneration is not well known. Recent findings suggest the presence of somatic stem cells in PDL tissue, which are defined as STRO-1 expression (Seo *et al*, 2004). Therefore, it is considered that the STRO-1-positive (STRO-1$^+$) putative stem cells may proliferate and differentiate into both periodontal cells and cementoblasts to regenerate periodontal tissue when it is damaged.

Growth differentiation factor 5 (GDF-5), also known as cartilage-derived morphogenetic protein-1, is a member of the bone morphogenetic protein (BMP) family and the transforming growth factor-β (TGF-β) superfamily. GDF-5 is known as a key regulatory factor of limb skeletal development (Storm *et al*, 1994; Chan *et al*, 1994; Thomas *et al* 1996; Merino *et al*, 1999; Storm and Kingsley, 1999) as well as a regulatory gene of bone, cartilage, and tendon/ligament formation. Although it is known that GDF-5 localizes in periodontal tissue, its physiological roles under normal and diseased conditions remain to be investigated. Our group has demonstrated that GDF-5 regulates differentiation of both dental papilla and follicle during odontogenesis and may co-operatively play crucial roles with other growth factors such as BMP-2 in porcine cells (Sumita *et al*, 2010). Recently, the potential of GDF-5 to regenerate periodontal tissue has been investigated using animal models (Kim *et al*, 2009; Kwon *et al*, 2010;

Lee *et al*, 2010), and the effect of GDF-5 on osteogenic differentiation of human PDL-derived cells was also reported (Nakamura *et al*, 2003). However, available information about its effect on tendo/ligamentogenesis-related markers is still limited. As GDF-5 regulates not only bone and cartilage formation but also tendo/ligamentogenesis during development, it is conceivable to speculate the importance of the role of GDF-5 in the regulation of ligament-related markers in PDL-derived cells.

Bone morphogenetic proteins were originally identified as a molecule to induce ectopic bone formation (Urist, 1965). BMPs also play a variety of roles during development and cell differentiation (Valenzuela *et al*, 1995; Jamali *et al*, 2001; Warren *et al*, 2003). Among the BMPs, BMP-2 is well known as a strong osteogenic inducer and has been clinically used in cases of spinal fusion and alveolar bone regeneration (Kwong and Harris, 2008). Efficacy of recombinant human BMP-2 (rhBMP-2) for periodontal tissue repair has been investigated in animal models. Kinoshita *et al* (1997) reported that rhBMP-2 improved new bone formation, new cementum formation, and connective tissue attachment in circumferential defects created by experimental periodontitis in beagle dogs, and similar findings were also reported using a rat model (King *et al*, 1997). On the other hand, some recent studies have shown the limited effect on cementum formation (Choi *et al*, 2002) and increased ankylosis at the treated sites (Wikesjo *et al*, 2003; Takahashi *et al*, 2005). Currently, the efficacy of BMP-2 on periodontal tissue regeneration remains controversial. Previous studies investigated the effect of BMP-2 on PDL-derived cells and osteoinductive but not proliferation-inducing effects were reported (Kobayashi *et al*, 1999; Zaman *et al*, 1999). However, the effect of BMP-2 on the expression of tendo/ligamentogenesis-related markers has not been reported. Furthermore, those studies used only crude PDL-derived cells, and the effect on isolated putative stem cells was not known.

There are few reports concerning the tendo/ligamentogenesis effect of growth factors on PDL-derived cells. In this study, the effect of BMP-2 and GDF-5 on crude human PDL-derived cells was investigated with special reference to tendo/ligamentogenesis-related markers to regenerate PDL, tendon, or ligament using PDL-derived cells. Furthermore, the effect of those growth factors on either STRO-1$^+$ putative stem cells or STRO-1$^-$ cells was also investigated and compared with crude cells.

Materials and methods

Tissue preparation and cell culture

Periodontal ligament tissues were harvested from extracted teeth (n = 10; average age = 25.8 ± 2.5) of impacted, healthy third molars. The procedure used to harvest the extracted teeth from humans conformed to the tenets of the Declaration of Helsinki, and the experimental protocol was approved by the Ethical Committee at Nagoya University School of Medicine. Informed consent was obtained from each subject prior to donation of the tissue. Each tooth samples was rinsed twice in a phosphate-buffered saline (PBS) solution containing 1000 units ml^{-1} penicillin G sodium, 1 mg ml^{-1} streptomycin sulfate, and 2.5 μg ml^{-1} amphotericin B (Invitrogen, Carlsbad, CA, USA) for 5 min at room temperature. PDL tissue was mechanically removed from the root surface by a scalpel. To avoid contamination by gingival and apical tissue, tissue from the apical and coronal regions of the tooth was discarded. Tissue was digested with 2 mg ml^{-1} collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 1 h in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Thermo Trace Ltd, Melbourne, Australia) and antibiotic–antimycotic solution on a shaker at 37°C. The cell suspension was then centrifuged for 5 min at 440 *g*, and the cell pellet was re-suspended in culture medium (DMEM containing 10% FBS and antibiotic–antimycotic solution). Isolated cells were seeded in a 12-well culture dish. When cells reached 80–90% confluence, they were subcultured (1×10^4 cells in a 10 cm dish) until passage 2.

Treatments with osteogenic induction medium and culture medium with GDF-5 or BMP-2

Osteogenic induction medium consisted of culture medium with 10 nM dexamethasone (Sigma-Aldrich), 100 μM ascorbic acid (Wako, Tokyo, Japan), and 10 mM glycerol 2-phosphate disodium salt hydrate (β-glycerophosphate, Sigma-Aldrich). The optimal concentrations for recombinant mouse GDF-5 (rmGDF-5; R&D Systems, Inc., Minneapolis, MN, USA) and recombinant human BMP-2 (rhBMP-2; R&D Systems, Inc.) treatments were 200 and 100 ng ml^{-1} respectively, based on our preliminary study. First passage PDL-derived cells were seeded at a concentration of 5×10^4 cells per well into 6-well plates. When the cultured cells reached 70% confluence, the cells were subcultured in culture medium (control group), osteogenic induction medium (Dex group), or culture medium with rmGDF-5 (200 ng ml^{-1}, GDF-5 group) or rhBMP-2 (100 ng ml^{-1}, BMP-2 group).

Total cell number and alkaline phosphatase (ALP) activity analyses

Cell proliferation and ALP activity were evaluated at 7 days among control, Dex, BMP-2, and GDF-5 groups. Cell proliferation was measured using WST-8 kit (Wako) according to the manufacturer's protocol. The quantity of pigments was then determined spectrophotometrically. Cells were incubated with medium containing 100 μl ml^{-1} of WST-8 for 1 h. The absorbance was then measured on a spectrophotometer at 450 nm (SmartSpeck™ 3000; BIO-RAD, Tokyo, Japan). ALP activities were measured according to the method of Lowry (1955). An aliquot (5 μl) of the supernatant was added to 1 ml of 50 mM *p*-nitrophenylphosphate containing 1 mM $MgCl_2$ (Sigma-Aldrich) and the mixture were incubated for 6 min at 37°C. 1 ml of 0.2 N NaOH was added to stop the enzymatic reaction, and the absorbance was read at 415 nm with a

spectrophotometer (SmartSpeck™ 3000; BIO-RAD). Each experiment was performed in triplicate for each of three samples.

Reverse transcription-polymerase chain reaction and quantitative PCR

Quantitative PCR (q-PCR) was used to determine the expression of tendo/ligamentogenesis-related genes in human PDL-derived cells cultured with or without rmGDF-5 (200 ng ml^{-1}) and rhBMP-2 (100 ng ml^{-1}) at 1, 3, and 7 days. Total cellular RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis was performed on 2 μg of total RNA by Super Script First-strand Synthesis (Invitrogen) according to the manufacturer's protocol.

Samples were incubated in a Thermal Cycler GP (Takara Bio Inc.) at 95°C/(2 min) for 1 cycle and then 95°C/(60 s), 56°C/(60 s), and 72°C/(60 s) for 20 cycles, with a final 5 min extension at 72°C. After amplification, 10 μl of each product was analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Furthermore, the effect of rmGDF-5 and rhBMP-2 on the expression of scleraxis and tenomodulin (TeM) mRNA in cultured PDL-derived cells was also evaluated by real-time fluorescent quantitative PCR using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster, CA, USA). Reactions were carried out with SYBR Green Master Mix (Applied Biosystems) according to manufacturer's protocol. The PCR consisted of an initial enzyme activation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. PDL-derived cell-specific primer sets used in this experiment were as follows:

Scleraxis
(forward 5′-TGCGAATCGCTGTCTTTC-3′,
reverse 5′-GAGAACACCCAGCCCAAA-3′) (91 bp),

TeM
(forward 5′-TTGAAGACCCACGAAGTAGA-3′,
reverse 5′-ATGACATGGAGCACACTTTC-3′)
(119 bp),

GAPDH
(forward 5′-GCACCGTCAAGGCTGAGAAC-3′,
reverse 5′-ATGGTGGTGAAGACGCCAGT-3′)
(106 bp).

Magnetic activated cell sorting (MACS)

At passage 2, PDL were subjected to immunomagnetic cell sorting using a mini-MACS isolation kit (Miltenyi Biotech, Inc, Bergisch Gladbach, Germany) according to the manufacturer's recommendations. PDL were incubated for 60 min at 4°C with monoclonal anti-human STRO-1 antibody (R&D Systems, Inc.). After incubation, cells were rinsed three times in PBS. Subsequently, the cells were incubated with rat anti-mouse IgM microbeads. After washing with PBS, the labeled cells were filtered through a 70-μm nylon mesh and loaded onto a column surrounded by a magnetic field. STRO-1-positive (STRO-1^+) cells bound to the STRO-1 microbeads were trapped in the column. Both the STRO-1^+ and STRO-1-negative (STRO-1^-) fractions of PDL were collected for western blot analysis.

Western blot analysis

To evaluate the effect of GDF-5 on tendo/ligamentogenesis of PDL-derived cells, western blotting was performed for scleraxis protein. Crude P2 PDL-derived cells were cultured on a 10-cm culture dish until 70% confluent, and washed with PBS twice. They were then cultured for 7 days in serum-free medium with rmGDF-5 (GDF-5-treated group) or without rmGDF-5 (non-treated group), and collected for western blot analysis.

To evaluate scleraxis expression pattern, 7-day-rmGDF-5-treated P2 PDL-derived cells were sorted using MACS, and STRO-1-positive and STRO-1-negative fraction of PDL-derived cells were collected, respectively, for western blot analysis. Cells were homogenized in protein extraction buffer (CytoBuster™; Novagen, Merck KGaA, Darmstadt, Germany) with protease inhibitor cocktail tablets (Complete Mini; Roche, Indianapolis, IN, USA). The protein concentration for the lysate was measured by BCA assay kit (Pierce, Rockford, IL, USA), and the protein was denatured by boiling with SDS and 2-mercaptoethanol solution. An equal concentration of proteins in 7.5–12.5% SDS-polyacrylamide gel was applied to samples to perform electrophoresis. The protein was transferred by iBlot TM Dry Blotting System (Invitrogen). The blotting membrane (nitrocellulose) was blocked with 4% skim milk in PBS at room temperature for 60 min, then immunoblotted using a rabbit polyclonal antibody to scleraxis (abcam; 1:500). Secondary anti-rabbit antibodies conjugated to horseradish peroxidase (Cell Signaling Technology) at a 1:3000 dilution were used for the detection and visualized using a chemoluminescence ECL detection system (Amersham Life Sciences, Arlington Heights, IL, USA). To assess the intensity of bands for these proteins quantitatively, densitometric analysis was performed using ChemiDoc (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with imaging software (Quantity One; Bio-Rad). The intensity level of detected protein bands was divided by the intensity level of β-actin to be standardized. The relative scleraxis expression was defined as the level of expression in each sample divided by that of non-GDF-5-treated cells from the same donor (Figure 3c,d) and crude PDL-derived cells from the same donor (Figure 4b).

Statistical analysis

One-way repeated measures ANOVA were used for western blot analyses to detect any significant difference within each group. When a significant difference was detected, the difference among any selected groups was confirmed using Dunnett's test. Experimental values are presented as mean ± s.d. A P-value of <0.05 was considered to be statistically significant.

Results

Osteogenic differentiation of PDL-derived cells

When crude PDL-derived cells at second passage were treated by osteogenic induction medium, ALP activity increased significantly compared to the other groups. On the other hand, the levels of ALP activity did not change when the cells were treated with either rmGDF-5 or rhBMP-2 (Figure 1).

Effect of GDF-5 and BMP-2 on the expression of tendo/ligamentogenesis-related genes in crude P2 PDL-derived cells

The results from q-PCR showed that rmGDF-5 tended to upregulate the expression of *scleraxis*, although the difference was not significant possibly due to the large variation (Figure 2a). A similar tendency was observed when the expression of *TeM* was analyzed. rmGDF-5 tended to upregulate the expression of *TeM* at all time points and rhBMP-2 tended to upregulate the expression of *TeM* only at day 7, although the differences were not significant (Figure 2b).

Effect of GDF-5 on the expression of scleraxis protein in crude P2 PDL-derived cells and STRO-1$^-$ fraction

The results from western blot analysis showed that both crude P2 PDL-derived cells and STRO-1$^-$ fraction of PDL-derived cells expressed scleraxis protein (Figure 3a,b). There was a significant difference among GDF-5-treated and non-treated crude P2 PDL-derived cells (1.86 ± 0.77, $P < 0.05$) (Figure 3c). In terms of STRO-1$^-$ fraction of PDL, similar tendency was observed, although the difference was not significant (Figure 3d).

Expression pattern of scleraxis protein in GDF-5-treated crude, STRO-1$^+$ and STRO-1$^-$ P2 PDL-derived cells

The results from western blot analysis showed that crude P2, STRO-1$^+$, and STRO-1$^-$ PDL are treated with GDF-5 expressed scleraxis protein (Figure 4a). The relative expression level of scleraxis in STRO-1$^+$ PDL-derived cells was significantly smaller than that in crude cells (0.63 ± 0.17, $P < 0.05$). On the other hand, the relative expression of scleraxis in STRO-1$^-$ PDL-derived cells was almost identical to that in crude cells

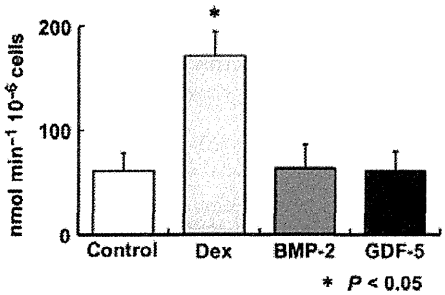

Figure 1 Alkaline phosphatase (ALP) activity of crude PDL-derived cells in passage 2. ALP activity of the Dex group was significantly greater compared with control, BMP-2, and GDF-5 groups. *$P < 0.05$. Values are the mean ± s.d. of five experiments

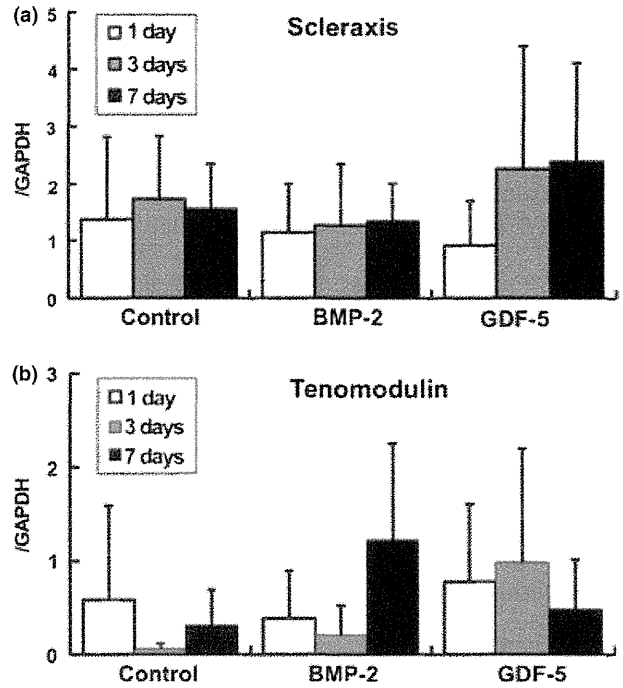

Figure 2 Quantitative RT-PCR analysis for scleraxis (**a**) and tenomodulin (**b**) gene expression of crude PDL-derived cells in passage 2. Cells were cultured with culture medium with or without BMP-2, GDF-5 for 1, 3, and 7 days. There were no significant differences among control, BMP-2, and GDF-5 groups on any time points. Values are the mean ± s.d. of five experiments

(0.91 ± 0.10) and significantly higher than that in STRO-1$^+$ PDL-derived cells ($P < 0.05$) (Figure 4b).

Discussion

PDL-derived cells constantly express both osteogenic/cementogenesis- and tendo/ligamentogenesis-related genes. Although the results from a study using clonal cells demonstrate that single colony-derived PDL cells can express both osteogenic/cementogenesis- and tendo/ligamentogenesis-related genes, crude PDL-derived cells generally consist of heterogeneous multiple colonies (Itaya *et al*, 2009), and the physiology of crude PDL cells may not be identical to that of putative stem cells. A recent study reported that STRO-1 is considered as a marker for mesenchymal stem cells including human PDL stem cells (Seo *et al*, 2004). Although the ratio of STRO-1$^+$ cells in PDL cells varies among research reports, our previous study demonstrated a 33.5% rate of STRO-1$^+$ cells in primary culture, which dropped to 14.7% by the third passage (Itaya *et al*).

The results from osteogenic induction with dexamethasone confirmed that crude PDL-derived cells showed increased ALP activities. However, rmGDF-5 and rhBMP-2 did not affect the ALP activity, which suggest that those factors may not induce bone formation but allow retaining characteristics of ligament cells at the concentration used in this study. Although the results

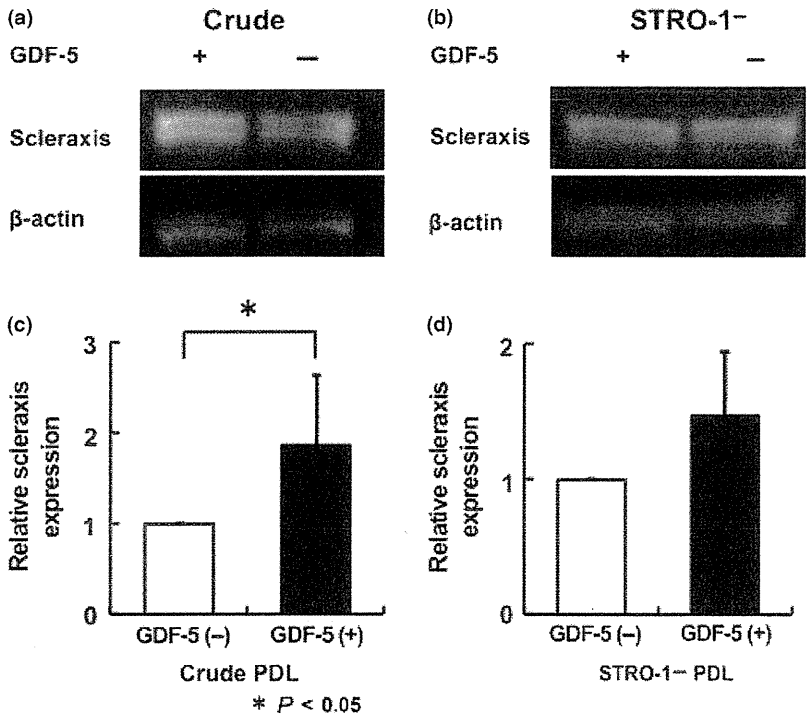

Figure 3 Western blot analyses of scleraxis crude PDL-derived cells (**a**) and STRO-1$^-$ PDL-derived cells (**b**) at passage 2. Expression of scleraxis was detected in all samples. An experiment representative of five similar studies is shown. (**c**) GDF-5-treated crude PDL-derived cells had significantly higher scleraxis expression than the other groups. (**d**) There was a similar tendency to crude PDL-derived cells, but no significant difference in STRO-1$^-$ PDL-derived cells among all groups. Values are the mean ± s.d. of five experiments

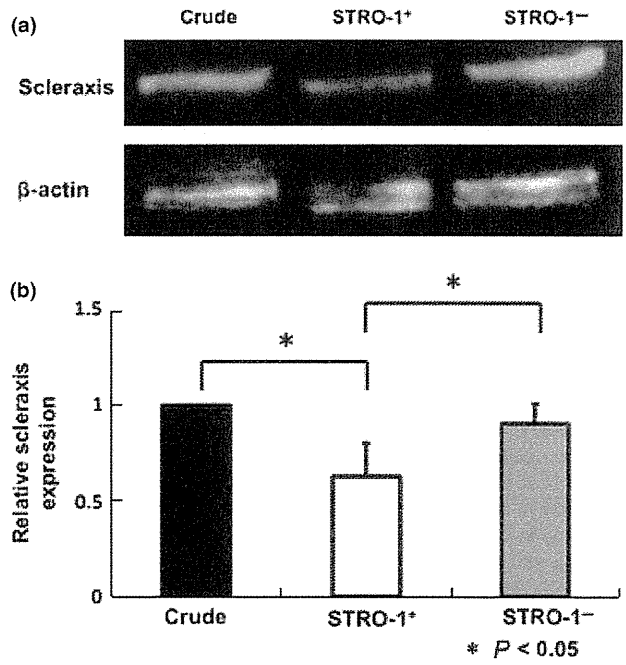

Figure 4 The results from western blot analyses of scleraxis in crude PDL-derived cells, STRO-1$^+$, and STRO-1$^-$ PDL-derived cells at passage 2. All samples were treated with rmGDF-5. (**a**) The expression of scleraxis proteins was detected in all groups for 7 days. An experiment representative of six similar studies is shown. (**b**) Reduced scleraxis expression in STRO-1$^+$ PDL-derived cells was statistically significant compared to crude P2 and STRO-1$^-$ PDL-derived cells. Values are the mean ± s.d. of six experiments

from this study are based on a single dose of rmGDF-5. GDF-5 is known to decrease ALP activity of human PDL-derived cells dose dependently, and the results from this study may be consistent with the previous findings (Nakamura *et al*, 2003). BMP-2 is a strong inducer of osteogenic differentiation in various types of cells. In terms of human PDL-derived cells, BMP-2 may require preconditioning osteogenic induction medium with dexamethasone to induce ALP activities (Hou *et al*, 2007). The result from this study demonstrated that BMP-2 alone was not effective in inducing osteogenic differentiation of crude human PDL-derived cells.

In this study, we focused on the effect of GDF-5 and BMP-2 on the expression of tendo/ligamentogenesis-related genes that is *scleraxis*, *TeM*. Scleraxis is a helix-loop-helix transcription factor that has been shown to be a highly specific marker for tendon and ligament progenitors and differentiating cells (Cserjesi *et al*, 1995; Schweitzer *et al*, 2001; Brent *et al*, 2003, Banos *et al*, 2008). It has been suggested that scleraxis is required for normal tendon differentiation and formation, presumably via regulation of collagen type I promotion (Banos *et al*, 2008). The presence of scleraxis in PDL-derived cells has been reported (Shi *et al*, 2005; Itaya *et al*, 2009). TeM is a member of a new family of type II transmembrane glycoproteins and is predominantly expressed in tendon, ligaments, and eyes (Docheva *et al*, 2005). TeM is known as a late marker of tendon formation, and the presence of TeM in human PDL has been reported (Itaya *et al*, 2009). The results from this study showed that both GDF-5 and BMP-2 affect the differentiation of PDL-derived cells *in vitro*. However, the effect on those differential ligament-makers was not identical and the underlying mechanisms might be complex.

In terms of GDF-5, the induction of scleraxis was demonstrated in crude PDL-derived cells. This finding suggests the potential usefulness of this factor for PDL

regeneration. On the other hand, scleraxis expression was significantly less in GDF-5-treated STRO-1$^+$ fraction. One possibility is that STRO-1$^+$ PDL-derived cells are putative stem cells and not all cells were committed into osteogenic or tendon/ligament lineage yet. Interestingly, the sum of scleraxis expression in STRO-1$^-$ and STRO-1$^+$ PDL-derived cells was less than that of crude cells. The reason for this phenomenon is not clear from this study but it may suggest the important role of cell-to-cell interaction in the maintenance of differentiated status. Cell-to-cell interaction can be achieved via cytokines, growth factors, and also direct contact. Recently, a novel mechanism of cell-to-cell interaction via microvesicles has been reported. Accumulating evidence has shown that stem cells can interact with adjacent and even distant cells with microvesicles, which can transport proteins as well as RNA and affect other cells (reviewed by Camussi *et al*, 2010). Further analyses are required to understand the mechanisms for this differential effect.

The effects of BMP-2 on tendo/ligamentogenesis-related gene expression of PDL-derived cells were heterogeneous. As excessive bone formation around root surface induces ankylosis, the maintenance of PDL is essential for the successful regeneration of periodontal tissue. The usefulness of BMP-2 on periodontal tissue regeneration should be discussed regarding both osteogenesis/cementogenesis and ligamentogenesis around the root surface, which may require further justification.

This study focused on the tendo/ligamentogenesis-related markers and confirmed the effect of those factors not only on crude PDL-derived cells but also on STRO-1$^+$ and STRO-1$^-$ PDL-derived cells. The results from our study showed some potential beneficial effect of GDF-5 on periodontal tissue regeneration. However, the underlying mechanisms appear to be complicated, and the overall benefit of the clinical application of the factor requires further analyses.

Author contributions

Minoru Inoue involved in acquisition, analysis of data; Katsumi Ebisawa involved in research design, drafting & revising the paper; Toshimitsu Itaya and Takayuki Sugito involved in interpretation of data; Aika Yamawaki-Ogata involved in acquisition, analysis of data; Yoshinori Sumita involved in interpretation of data; Ryu Wadagaki involved in acquisition of samples; Yuji Narita involved in research design; Hideki Agata involved in interpretation of data; Hideaki Kagami involved in research design, drafting the paper involved in Minoru Ueda involved in research design.

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ETV6–NTRK3 as a therapeutic target of small molecule inhibitor PKC412

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ABSTRACT

The ETV6–NTRK3 (EN) fusion gene which encodes a chimeric tyrosine kinase was first identified by cloning of the t(12;15)(p13;q25) translocation in congenital fibrosarcoma (CFS). Since then, EN has been also found in congenital mesoblastic nephroma (CMN), secretory breast carcinoma (SBC) and acute myelogenous leukemia (AML). Using IMS-M2 and M0–91 cell lines harboring the EN fusion gene, and Ba/F3 cells stably transfected with EN, we demonstrated that PKC412, also known as midostaurin, is an inhibitor of EN. Inhibition of EN activity by PKC412 suppressed the activity of it downstream molecules leading to inhibition of cell proliferation and induction of apoptosis. Our data for the first time suggested that PKC412 could serve as therapeutic drug for treatment of patients with this fusion.

1. Introduction

The ETV6–NTRK3 (EN) fusion gene which encodes a chimeric tyrosine kinase was first identified by cloning of the t(12;15)(p13;q25) translocation in congenital (or infantile) fibrosarcoma, a mesenchymal malignancy of very young children [1]. Since then, EN has been also found in congenital mesoblastic nephroma [2], secretory breast carcinoma [3] and acute myelogenous leukemia (AML) [4].

ETV6 (also known as *TEL*) is an ETS family transcription factor thought to play a major role in early hematopoiesis and angiogenesis [5,6]. The *ETV6* gene has also been identified as a fusion partner in leukemia-associated chimeric proteins, such as *ETV6-PDGFR* [7], *ETV6-AML1* [8,9], *ETV6-JAK2* [10], *ETV6-ARG* [11], and others [12]. The *NTRK3* gene (also known as *TRKC*) encodes the transmembrane surface receptor for neurotrophin-3 involved in growth, development, and cell survival in the central nervous system [13].

In general, EN fusion transcripts encode the N-terminal pointed (PNT) domain of *ETV6* which is responsible for polymerization [14] fused to the C-terminal protein tyrosine kinase (PTK) domain of NTRK3. This fusion protein is similar in structure to other ETV6 chimeric PTKs [1]. Until now, two types of this fusion gene were found. The first one is detected in the non-hematological maglinancies in which the chimeric transcript encoded exon 1 to exon 5 of the *ETV6* gene fused to nucleotide (nt) 1741 of *NTRK3* gene. The second one is detected in leukemia in which the chimeric transcript encoded exon 1 to exon 4 of *ETV6* gene fused to nt 1741 of *NTRK3* gene.

In general, native *NTRK3* requires an extracellular ligand binding of neurotrophin 3 prior to its dimerization and autophosphorylation [15–19]. However, when fused to ETV6, the extracellular ligand binding domain of NTRK3 is abrogated and ETV6–NTRK3 bypassed this requirement, still keep itself autophosphorylation. Interestingly, in vitro and in vivo experiments have shown that the EN fusion protein has potential transforming activity in several cell lineages including fibroblast [20], hematopoietic cells [21], and breast epithelial cells [3].

The important role of EN in oncogenesis has been well known. However, specific treatment for patients expressing EN has not been achieved. Patients of congenital fibrosarcoma, congenital mesoblastic nephroma and secretory breast carcinoma have been considered as a good prognosis and rarely metastases [22–24]. Unfortunately, patients of leukemia seem to be poor prognosis. Two patients harboring EN have been reported no response to chemotherapy treatment suggesting it as a refractory leukemia [25,26]. Therefore, finding novel therapeutic treatment for leukemia patients harboring EN is extremely necessary.

We hypothesized that inhibition of EN could be an effective therapeutic strategy in treatment of patients harboring EN. In this report, we tested the potential therapeutic utility of the small molecule inhibitor PKC412 as an option for treatment of leukemias associated with EN fusion. PKC412 also known as midostaurin is the broad spectrum inhibitor of serine-threonine/tyrosine-protein kinases including protein kinase C (PKC), vascular endothelial growth factor receptor (VEGFR), fms-like tyrosine kinase (FLT3),

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platelet-derived growth factor receptor (PDGFR) and the stem cell factor, c-KIT [27,28]. Currently, PKC412 is used in phase IIB clinical trials for treatment of acute myeloblastic leukemia (AML) patients with FTL3 mutations [29] with minimal side-effect suggesting the utility of PKC412 for treatment.

2. Materials and methods

2.1. Plasmid construction

The construct of AML type of EN was described somewhere [30].

2.2. Cell lines, culture conditions and transfection

Experiments were conducted using two EN-positive AML cell lines: IMS-M2 and M0–91. IMS-M2 cell line was established from the bone marrow cells taken from a 59-year-old female with AML (FAB-M2), with chromosome abnormalities of 48,XX,add(6)(q27),+8, der(12)t(12;15)(p13;q25)inv(12)(p13;q15),der(15) t(12;15)(p13;q25), +der(15)t(12;15)(p13;q25), ETV6–NTRK3 fusion gene [4,25] and the type A *NPM1* mutation [31]. M0–91, an AML-M0 derived cell line, has recently been identified as a cell line expressing the ETV6–NTRK3 fusion gene by Gu et al. [32]. MOLM-13 and Jurkat cells were used as controls for evaluating PKC412 anti-proliferation effect.

All cell lines were grown in RPMI 1640 medium (Sigma–Aldrich, Japan K.K., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (Nakalai Tesque, Kyoto, Japan) in a humidified incubator of 5% CO_2 at 37 °C. The Ba/F3 cells (ATCC, Manassas, VA, USA), maintained in RPMI 1640 medium supplemented with 10% FBS and 10 ng/ml of recombinant mouse interleukin 3 (IL-3; R&D Systems, Minneapolis, MN) were transfected with AML type of EN construct by lipofectamine (Invitrogen) according to the manufacturer's instructions. Transfected cells were selected in medium containing mouse IL-3 and 1 mg/ml G418 (GIBCO BRL, Gaithersburg, MD, USA) for 2 weeks and subsequently subjected to limiting dilution to isolate single clones.

2.3. Reagents

PKC412, midostaurin was purchased from Sigma–Aldrich Japan (Tokyo, Japan) and dissolved in dimethylsulfoxide (DMSO). Controlled cells were cultured with the same concentration of carrier DMSO as used in the highest dose of reagents. The concentration of DMSO was kept under 0.1% throughout all the experiments to avoid its cytotoxicity.

2.4. Cell proliferation assays

Proliferation was determined by trypan blue dye exclusion test. Cells in suspension were seeded in six-well plates at a density of 1×10^5 cells/ml in the presence of different concentrations of PKC412 for 3 days. In control wells, DMSO instead of PKC412 was added. After the treatment, 10 μl of the cell suspension was mixed with 10 μl of 0.4% trypan blue, and alive cells were counted manually using a hemacytometer. Results were calculated as the percentage of the values measured when cells were grown in the absence of the reagent. All experiments were performed in triplicate.

2.5. Western Blot analysis

The western Blot analysis was described in previous report [33]. Immunoprecipitation (IP) was performed as described previously [34]. Protein samples were electrophoresed through polyacrylamide gel and transferred to Hypond-P membrane (Amersham, Buckinghamshire, UK) by electro-blotting. After washing, the membrane was probed with following antibodies and antibody-binding was detected using enhanced chemiluminescence (ECL) (Amersham). The following antibodies were purchased from Cell Signaling Technology Japan (Tokyo, Japan): Phospho-p44/42 Map kinase (Thr202/Tyr204), phospho-Akt (Ser473), XIAP, Bcl-2, caspase-3, PARP, AKT, p44/42 MAPK, phospho-IKappaBalpha (Ser 32/36), and p-STAT5 (Y694). α-TrkC (C-14), STAT5 (C-17), and NFkB p52 (C-5), survivin (sc-17779), anti-rabbit IgG-HRP (sc-2317), and anti-mouse IgG-HRP (sc-2031) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine 4G10 was from Upstate Biotechnology (Lake Placid, NY, USA). Anti-actin (A2066) was from Sigma–Aldrich.

2.6. Wright-Giemsa staining

For fragmented nuclei and condensed chromatin assessment, cells at a density of 1×10^5 cells/ml were treated with 100 nM PKC412. After 24 h incubation, cells were harvested and fixed onto slides by using a cytospin (Shandon, Shandon Southern Products Ltd., Cheshire, UK). Cells then were stained with Wright-Giemsa solution. Morphology of cells was observed under an inverted microscope.

2.7. DNA fragmentation assay

IMS-M2 cells were treated with or without 100 nM PKC412 for 24 h. Cells then were collected and total genomic DNA (gDNA) was extracted with a standard protocol. For DNA fragmentation assay, 10 μg gDNA of each sample was blotted and electrophoresed on 1.2% agarose gel. DNA fragmentation was observed under UV light.

2.8. Statistical analysis

All data were expressed as the mean ± standard deviation. Statistical analyses were done using Student's *t-test*, in which $p < 0.05$ was the minimum requirement for a statistically significant difference.

3. Results

3.1. PKC412 inhibits EN fusion tyrosine kinase in hematopoietic Ba/F3 cells

We evaluated the transforming property of EN in hematopoietic Ba/F3 cells. EN construct was stably transduced in Ba/F3 cells (Fig. 1A). Stable Ba/F3 cell line was assessed for IL-3 independent growth as a surrogate for transformation (Fig. 1B). EN effectively conferred IL-3 independence to Ba/F3 cells, whereas, Ba/F3 cells transduced with empty vector underwent apoptosis in the absence of IL-3.

To determine whether PKC412 inhibited EN activity, we have used the assay based on the work of Daley and Baltmore [35]. The transfected Ba/F3 cells were treated with or without 100 nM PKC412 for 72 h. Then cell proliferation assay was done to account for the inhibitory effect of reagent. 100 nM PKC412 significantly inhibited the cell proliferation of transfected Ba/F3 cells (Fig. 1C). To confirm whether the cell growth inhibition of PKC412 in transfected Ba/F3 cells is due to the lost of EN phosphorylation, we then checked the phosphorylation of EN in transfected Ba/F3 cells treated with or without 100 nM PKC412 for 8 h. As expected, the phosphorylation of this fusion protein was decreased by PKC412 treatment (Fig. 1D), suggesting that PKC412 inhibited the cell pro-