ATTGTCTCCTCCGCTGCTGC-3′ for bone sialoprotein (J05213, 565 bp), 5′-CTAGGCATCACCTGTGCCATACC-3′ and 5′-CAGTGACCAGTTCATCAGATTCATC-3′ for osteopontin (J04765, 331 bp), and 5′-CCACCGAGACACCATGAGAG-3′ and 5′-CCATAGGGCTGGGAGGTCAG-3′ for osteocalcin (X53698, 419 bp) were used as primers for RT-PCR. Obtained PCR products were separated on 1% agarose gels and stained with ethidium bromide.

FACS analysis

BMSCs at passages 3–4 were harvested with trypsin and EDTA, centrifuged at 1500*g* for 5 minutes, and resuspended at 5×10^6 cells/ml in PBS containing 3% FBS. Aliquots containing 10^5 cells were incubated with individual primary antibodies or control IgG for 30 minutes at room temperature. The cells were washed in PBS containing 3% FBS and incubated with a fluorescent conjugated secondary antibody for 30 minutes at room temperature. Samples were analyzed using a FACSCalibur cytometer (Becton Dickinson), and the data were analyzed using the CELLQUEST software (Becton Dickinson). The following monoclonal antibodies (mAbs) were used: fluorescein isothiocyanate (FITC)-conjugated or R-phycoerythrin (PE)-conjugated antibodies against CD13, CD14, CD29, CD34, CD44, CD49b, CD54, CD56, CD71, CD90, CD105, CD106, CD117, CD124, CD138, CD144, HLA-ABC, HLA-DR, mouse-IgG1, mouse-IgG2a, or mouse IgM (Immunotech Coulter Company); antibodies against CD123, CD140b, CD166, or mouse-IgG3 (Pharmingen); antibodies against Flk-1 (Santa Cruz Biotechnology); antibodies against MT-MMP-1 (Sigma); antibodies against STRO-1 (Genzyme); antibodies against RANKL (R & D Systems), and anti-rabbit-IgG (Chemicon International).

Transplantation of BMSCs into mice

The potential for cells to differentiate into osteoblasts after transplantation into immunodeficient mice was assessed as described.[14,15] Human alveolar BMSCs at passages 3–5 (1.5×10^6 cells) in 1.0 ml of DMEM were mixed with 40 mg β-tricalcium phosphate (β-TCP) powder (OSferion; Olympus Co., Tokyo, Japan). After incubation at 37°C for 90 minutes, the mixture was centrifuged at 1500 rpm for 1 minute, and the supernatant was discarded. The pellet of β-TCP powder with adherent cells was mixed with 15 μl of mouse fibrinogen (3.3 mg/ml solution in PBS) and mouse thrombin (25 U/ml in 2% $CaCl_2$; both from Sigma) to form a fibrin clot. The fibrin clots transplanted into 5-week-old female CB-17 scid/scid (SCID; severe combined immunodeficiency) mice (Nihoncrea, Tokyo, Japan). After anesthetizing by intraperitoneal injection with 10% Nembutal (Dainihon Seiyaku Co., Osaka, Japan) in PBS, five skin incisions were made on the dorsal surface of each mouse, fibrin clots (five per mouse) were transplanted, and incisions were sutured. The transplants were harvested 8 weeks after transplantation.

Immunohistochemical and histomorphometrical analyses of transplanted sample

The transplants were fixed in 4% paraformaldehyde for 1 day, decalcified with 10% formic acid for 3 days, and embedded in paraffin. Four-micrometer-thick sections were prepared in the middle of the transplants, collected on poly-L-lysine-coated slides, and stained using H&E for histochemical examination. To examine human vimentin expression immunohistochemically, endogenous peroxidase was quenched by incubating with 1% H_2O_2 and methanol. The sections were incubated with anti-human vimentin mAb (Dako; 50-fold dilution with Dako Antibody Diluent) for 1 h and treated with Envision (Dako). Color reaction was developed with diaminobenzidine (Dako). To examine osteocalcin expression, the sections were incubated with antihuman osteocalcin mAb (200-fold dilution; Biomedical Technologies, Stoughton, MA, USA) for 1 h, and color was developed with DAKO LSAB +System, HRP (Dako). Bone formation area in three fields (random sampling, 0.41 mm^2/field) in the middle sections—stained with H&E—of the transplants of alveolar BMSC (a9) plus β-TCP powder or β-TCP powder alone was captured by CCD camera (coolpix 4500; Nikon, Tokyo, Japan), and the bone area in the pictures was traced with Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA, USA); the bone area is expressed as the percentage of total area (0.41 mm^2).

RNA extraction from transplanted samples

Total RNA was prepared from the transplants as described.[16] Briefly, extracted samples were snap-frozen in liquid nitrogen and pulverized for 1.5 minutes at 2000 rpm in a mixer mill. A 1-ml aliquot of TriReagent (Sigma) was added directly to the powdered samples and warmed to room temperature. Each sample was transferred to a 1.5-ml microcentrifuge tube and mixed by orbital rotation for 10 minutes at room temperature. After the addition of 0.2 ml of chloroform, samples were vortexed and allowed to sit at room temperature for 15 minutes, and then centrifuged for 20 minutes at 12,000 rpm. The upper aqueous phase was removed and mixed with an equal volume of 70% ethanol. Total RNA was isolated using RNeasy minicolumns and reagents (Qiagen). DNase I (Ambion) treatment was performed to remove genomic DNA from RNA samples according to the manufacturer's protocol.

PCR analysis of implanted samples

First-strand cDNA was synthesized by Omniscript reverse transcriptase (Qiagen; 2 μg total RNA/20-μl reaction volume) using oligo dT primer (Promega). PCR amplification was performed (1 μl cDNA solution/25-μl reaction volume) using oligonucleotide primers corresponding to cDNA sequences for human-specific GAPDH (GenBank Accession no. M33197, 347 bp; sense 5′-CACCAGGTGGTCTCCTCT-3′, antisense 5′-GTACATGACAAGGTGCGG-3′), human-specific osteocalcin (X53698, 315 bp; sense 5′-CATGAGAGCCCTCACA-3′, antisense 5′-AGAGCGACACCCTAGAC-3′), mouse-specific osteocalcin (X04142, 443 bp; sense 5′-AACAGACAAGTCCCACACAG-3′, antisense 5′-GCTGTGACATCCATACTTGC-3′), and human and mouse GAPDH (M33197 and M32599, 268 bp; sense 5′-CACCAGGTGGTCTCCTCT-3′, antisense 5′-GTACATGACAAGGTGCGG-3′) using *Taq* polymerase (Promega). PCR was performed at 94°C/

(45 s), 56°C/(45 s), and 72°C/(60 s) for 35 cycles. After amplification, 10 μl of each reaction was analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

Oligonucleotide primers corresponding to cDNA sequences for the mouse-specific osteocalcin were designed using mouse sequence (X04142). The site selected for mouse-specific osteocalcin has low (<60%) homology to human osteocalcin (X53698). Even if these primers could anneal to human cDNA, the PCR product size would be 419 or 339 bp, which differs from 443 bp of the mouse product. Furthermore, we confirmed that no PCR product was synthesized with these primers and human RNA samples containing osteocalcin mRNA. In the case of human-specific GAPDH (M33197), the antisense primer had no similar homology site to mouse GAPDH (M32599). Thus, the product amplified by these primers corresponds to human GAPDH.

Statistical analysis

Student's *t*-test was used.

RESULTS

Expansion of alveolar BMSCs in culture

We obtained alveolar bone marrow samples from 41 dental patients. BMSCs (fibroblast-like cells) in 29 samples adhered to the culture surface and proliferated in the presence of 10% FBS and bFGF in primary and secondary cultures. No BMSC expansion was observed with the other samples (Table 1). There was no sex difference regarding the expansion of alveolar BMSCs (Fig. 1A), but the BMSC expansion depended on patients' age (Fig. 1B): BMSCs from patients >50 years of age often showed only a few adherent cells in primary cultures, and these did not form colonies within 14 days (data not shown), suggesting an age-related decline in the number of alveolar BMSCs and/or their growth capability. Marrow samples obtained during the course of wisdom tooth extraction or surgery for bone fracture or jaw deformity showed a higher success ratio than did those obtained during surgery for dental implants or dental cyst extraction (Fig. 1C). It remains unknown whether the site of aspiration affects the success ratio (Table 1). The low success ratio for dental implants may be caused by the increased age of the patients (Table 1). Whereas the success ratio increased with an increase in the volume of obtained marrow samples (Fig. 1D), this relationship is questionable because some aspirates contain peripheral blood.

All BMSC lines obtained by ex vivo expansion were maintained at least until passages 3–4 and then stored in liquid nitrogen for differentiation assays; some lines underwent further successive passages for proliferation assays (Fig. 2). Passages were performed when cells were approaching confluence. The cell number was determined at the passage, and the cumulative cell number at the passage is shown in Fig. 2. Alveolar BMSC lines retained their proliferative capacity until passages 8–11. The growth rate during the logarithmic growth phase (1.0 ± 0.2 cell division/day; doubling time, 25 ± 4 h) and proliferative life span (36 ± 10 cell doublings) of alveolar BMSCs were similar to those (0.9 ± 0.1, 28 ± 5, and 33 ± 11, respectively) of iliac BMSCs, although both BMSC lines showed large interindividual variations (Fig. 2).

TABLE 1. ISOLATION OF BMSCs FROM ALVEOLAR BONE

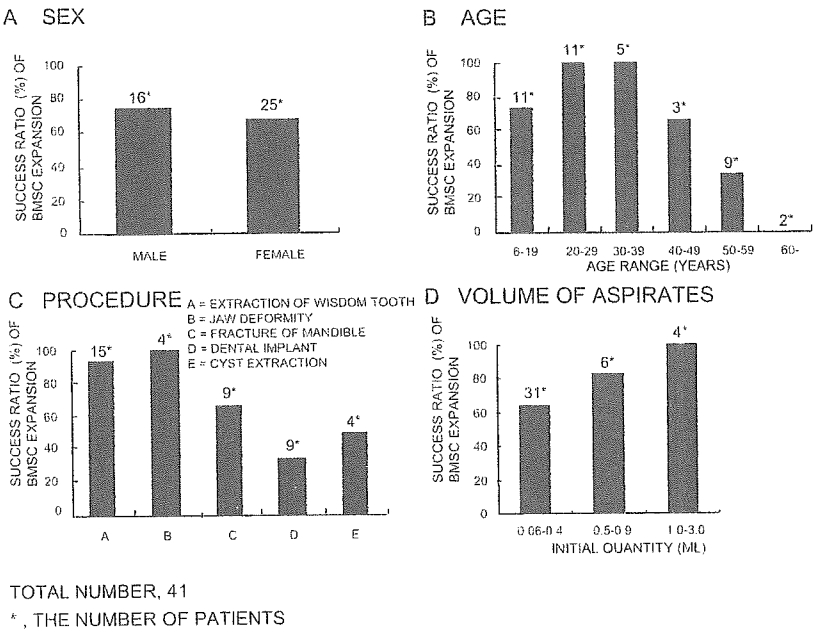
Patients	*Age*	*Sex*	*Procedure*	*Initial quantity (ml)*	*Cell expansion*
a1	25	F	A	0.1	+
a2	29	M	A	0.1	+
a3	31	F	A	0.1	+
a4	24	F	A	0.1	+
a5	25	M	A	0.1	+
a6	19	F	A	0.1	+
a7	35	M	A	0.1	+
a8	19	F	A	0.1	+
a9	22	F	A	0.1	+
a10	14	F	A	0.1	+
a11	19	F	C	0.5	+
a12	20	F	B	3	+
a13	31	F	A	1	+
a14	19	M	B	1	+
a15	22	F	B	0.5	+
a16	25	F	A	0.1	+
a17	58	F	A	0.1	−
a18	59	M	C	0.1	−
a19	53	F	D	0.2	−
a20	53	M	D	0.5	+
a21	53	M	D	0.2	−
a22	17	M	C	0.2	−
a23	52	F	D	0.2	+
a24	12	F	E	0.4	−
a25	40	F	D	0.15	−
a26	41	M	D	0.5	+
a27	41	M	A	0.5	+
a28	57	F	D	0.5	−
a29	18	F	D	0.1	−
a30	53	M	E	0.2	−
a31	66	F	C	0.1	−
a32	71	F	D	0.4	−
a33	21	M	C	1	+
a34	51	M	E	0.2	+
a35	26	M	C	0.4	+
a36	6	F	C	0.1	+
a37	28	F	C	0.1	+
a38	36	M	C	0.2	+
a39	19	F	A	0.3	+
a40	15	F	B	0.15	+
a41	38	M	E	0.06	+

A, extraction of wisdom tooth; B, jaw deformity; C, fracture of mandible; D, dental implant; E, cyst extraction.

Cells in marrow aspirates were seeded and maintained as described in the Materials and Methods section. When marrow samples from 41 patients were examined, the expansion of BMSCs was observed in some cultures, depending on the patients' age.

Osteogenic differentiation of human alveolar BMSCs in vitro

In most cultures of alveolar BMSC lines (a1–a11) maintained in osteogenic conditions for 21–28 days, the mRNA levels of osteopontin, osteocalcin, bone sialoprotein, and ALP (Fig. 3A), as well as the calcification level estimated



TOTAL NUMBER, 41

*, THE NUMBER OF PATIENTS

FIG. 1. Effects of sex, age, disease history, and marrow sample volumes on ex vivo expansion of alveolar BMSCs. Cells in marrow aspirates were seeded and maintained as described in the Materials and Methods section. *The number of patients examined.

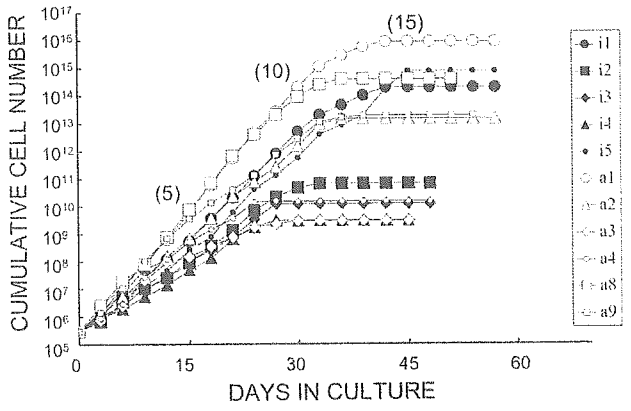


FIG. 2. The growth and proliferative life span of alveolar and iliac BMSCs. Alveolar (a) and iliac BMSC lines (i) obtained from the secondary cultures were seeded and passaged in 10-cm tissue culture dishes at 5000 cells/cm^2. The ages of donors (i1, i2, i3, i4, i5, a1, a2, a3, a4, a8, and a9) were 22, 24, 22, 32, 29, 19, 25, 29, 31, 24, and 19 years, respectively. Passages were performed when cells were approaching confluence. Each point shows the cumulative cell number at the passage. The number of passages is shown in parenthesis.

with alizarin red (Fig. 3B), were higher than those in undifferentiated cultures maintained in medium-A alone. The calcium level in a1–a11 cultures in osteogenic conditions was significantly higher than in undifferentiated cultures (Fig. 3C), but the degree of calcification varied among the lines. Lines a4, a5, and a7 showed the expression of osteopontin and bone sialoprotein at low levels and calcification at high levels on day 28. In contrast, lines a1, a6, and a9 showed expression of osteopontin and bone sialoprotein at moderate or high levels and calcification at low levels on day 28: The latter may represent a delayed calcification process.

Poor chondrogenic and adipogenic potentials of alveolar BMSCs

The differentiation potential of alveolar BMSCs was compared with that of iliac BMSCs using a12–a14 and i1–i3 lines. In the osteogenic conditions, ALP activity and calcium level in alveolar BMSC cultures were similar to those in iliac cultures (Fig. 4A). The chondrogenic potential of alveolar and iliac BMSCs was examined in pellet cultures, because chondrocyte differentiation takes place at high levels in pellet cultures.[17,18] Almost all iliac cells reorganized into a cartilage-like tissue that was stained purple with toluidine blue (Fig. 4B), whereas scarcely any metachromasia was observed with alveolar BMSCs, indicating poor chondrogenesis in the alveolar cell pellets. In the periphery of alveolar cell pellets, a few chondrocytes appeared (Fig. 4D), but the GAG level and ALP activity were lower in alveolar cultures than in iliac cultures (Fig. 4B). Under the adipogenic conditions, most iliac BMSC prosperously accumulated lipid droplets, whereas only a few adipocytes appeared in cultures with alveolar BMSC (Figs. 4C and 4E). Furthermore, alveolar cells showed a lower GAPDH, a marker for adipocytes, than did iliac cells on day 28. Some alveolar BMSC lines showed appreciable adipogenic potential on day 45, and their adipogenic potential was greater than that of fibroblasts (data not shown).

To compare alveolar BMSCs with iliac BMSCs without interindividual variations, we simultaneously isolated alveolar and iliac BMSCs from three beagle dogs. After osteogenic differentiation, ALP and calcium levels in the alveolar BMSC cultures were found to be similar to those in the iliac cultures (Fig. 5). However, under chondrogenic conditions, the alveolar BMSCs synthesized glycosaminoglycan or ALP, and these cells synthesized GAPDH under adipogenic conditions, but only marginally in both cases. In

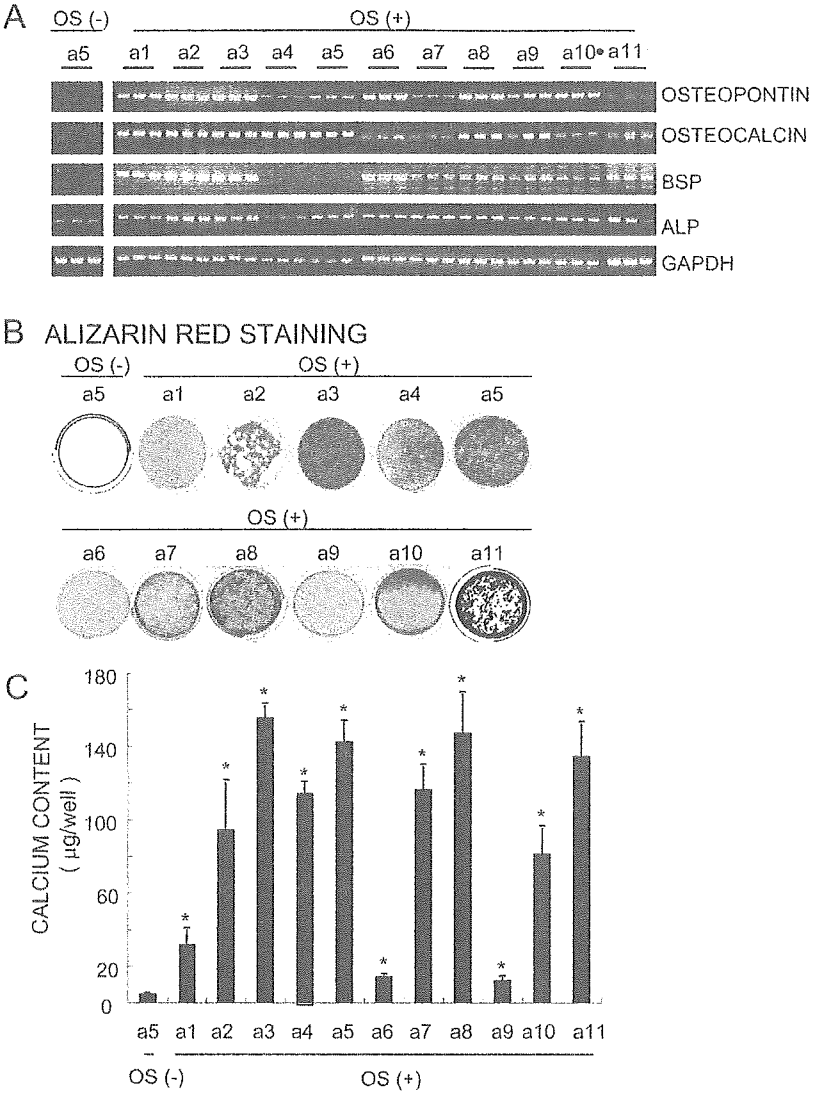


FIG. 3. The osteogenic potential of alveolar BMSCs. Alveolar BMSCs lines (a1–a11) obtained at passage 3 were induced to differentiate into osteoblasts by incubating with the osteogenic medium (OS+) for (B) 21 or (A and C) 28 days. In addition, line a5 was maintained in medium A (OS−) for (B) 21 or (A and C) 28 days. The expressions of osteopontin (OP), osteocalcin (OC), bone sialoprotein (BSP), and ALP mRNA in triplicate cultures was determined by RT-PCR on day 28. (B) Cultures maintained in the osteogenic medium (OS+) or in medium A (OS−) were stained with alizarin red on day 21. (C) The calcium level was determined on day 28. Values are means ± SD for four cultures. $^{*}p < 0.05$.

contrast, the iliac BMSCs underwent either chondrogenic or adipogenic differentiation under these conditions (Fig. 5).

Cell surface antigens

Previous studies have shown that several cell surface antigens, including ALCAM (activated leukocyte cell adhesion molecule), CD29 (integrin β-1), intercellular adhesion molecule (ICAM)-1, platelet-derived growth factor receptor (PDGFR), CD44, CD90, and CD105/SH2, are expressed in BMSCs and/or perichondrium mesenchymal stem cells.[9,19,20] In this study, we examined the expression of 25 cell surface antigens in alveolar and iliac BMSCs by FACS analysis: None of the cell surface antigens examined differed between alveolar and iliac BMSCs (Fig. 6). STRO-1 is a marker for BMSCs.[21] However, alveolar and iliac BMSCs showed STRO-1 expression at a low level (Fig. 6). Previous studies have also shown that STRO-1^{+} cells in undifferentiated human BMSC populations are only 7 ± 6%.[22] Therefore, STRO-1 may be progressively lost with time in these cultures.

Transplantation of alveolar BMSCs

Next we examined whether alveolar BMSCs could differentiate into bone tissue in vivo. Alveolar BMSCs were attached to β-TCP powder and transplanted into SCID mice: 8 weeks after transplantation, new bone formation was observed (Figs. 7B and 7C). In contrast, no bone formation was observed with implants of β-TCP alone (Figs. 7A). Histomorphometrical measurements showed significant differences in bone formation between cell-loaded and unloaded implants: in six transplants (18 fields) with alveolar BMSCs plus β-TCP, bone area was 22 ± 9% compared with 0 ± 0% in four implants (12 fields) with β-TCP alone (Table 2), proving that alveolar BMSCs do indeed enhance initial bone formation.

It was still unknown, however, whether transplanted BMSCs contributed to the bone formation, because host (mouse) cells could have induced bone formation in response to β-TCP. To address this issue, we used mAb against human vimentin that did not cross-react with mouse vimentin. The mAb to human-specific vimentin reacted with osteoblasts and osteocytes around and in new bone

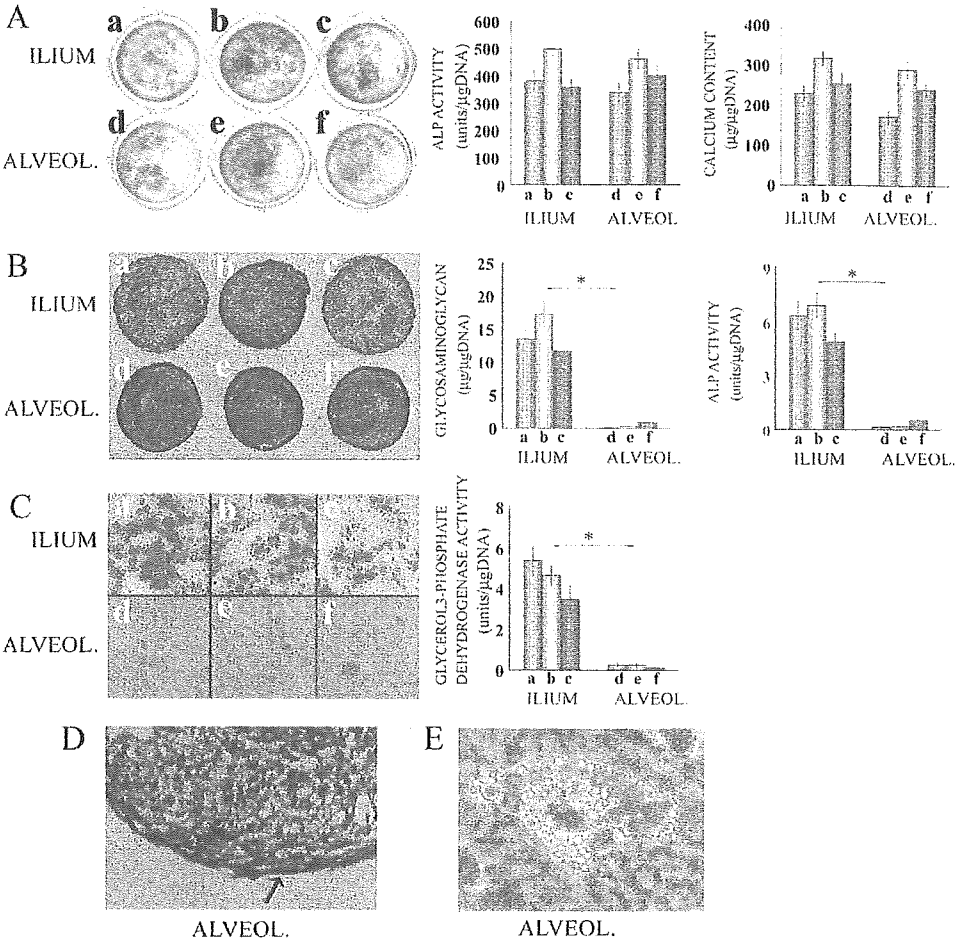


FIG. 4. The differentiation potentials of human alveolar and iliac BMSCs. (A) Human alveolar (a12–a14) and iliac BMSCs (i1–i3) obtained at passage 3 were transferred into the osteogenic medium. The cell layers were stained with alizarin red on day 21. ALP activity and the calcium content of the cell-matrix layers were determined on day 28. (B) Human alveolar and iliac BMSCs obtained at passages 3–4 were incubated in the chondrogenic status in pellet cultures for 28 days and stained with toluidine blue. The levels of GAG and ALP were determined on day 28. In the periphery of the alveolar BMSC pellet, stained with toluidine blue, a few chondrocytes were observed (D). (C) Human alveolar and iliac BMSCs obtained at passage 3 were maintained in the adipogenic status for 28 days. The cells were stained with oil red O. GAPDH activity was determined on day 28. Values are means ± SD for four cultures. $^*p < 0.05$. (E) Magnification of the alveolar BMSC culture maintained in the adipogenic status for 28 days: a few cells became adipocytes in alveolar cultures.

tissues: most osteoblasts and osteocytes, as well as some mesenchymal cells surrounding bone tissue, in the transplants with BMSCs were stained with the human-specific antibody (Figs. 7E and 7F) compared with no human vimentin$^+$ cells in the implants with β-TCP alone (Figs. 7D). Thus, human alveolar BMSCs did in fact reorganize into new bone tissues. Furthermore, osteoblasts, osteocytes, and mesenchymal cells in or near bone tissues in the transplants of alveolar BMSC synthesized osteocalcin (Fig. 8).

To examine whether new bone formation would occur with other alveolar BMSC lines, we examined the expression of human osteocalcin and human GAPDH mRNA, using total RNA isolated from whole transplants. In almost all transplants (11/13) of three alveolar BMSC lines (3/3, 4/6, and 4/4), human osteocalcin and/or human GAPDH mRNA expressions were observed (Fig. 7G), and similar bone formation was observed with human iliac BMSCs (data not shown). In contrast, no human osteocalcin/GAPDH expression was detected in the implants of β-TCP alone, although mouse GAPDH was found in these implants. Unexpectedly, using mouse-specific osteocalcin primers, mouse osteocalcin mRNA was detected in some transplants of human alveolar BMSCs, suggesting that mouse mesenchymal cells partly contributed to bone formation during osteogenic differentiation of human BMSCs. However, no mouse osteocalcin mRNA was detected in the implants of β-TCP alone (Fig. 7G), indicating the absence of bone formation in whole implants of β-TCP alone. These findings indicate that in vivo bone formation in the transplants of alveolar BMSCs was much greater than that in the implant of β-TCP alone.

DISCUSSION

Alveolar BMSCs had the same fibroblastic shape as that reported for BMSCs isolated from the iliac crest, and their

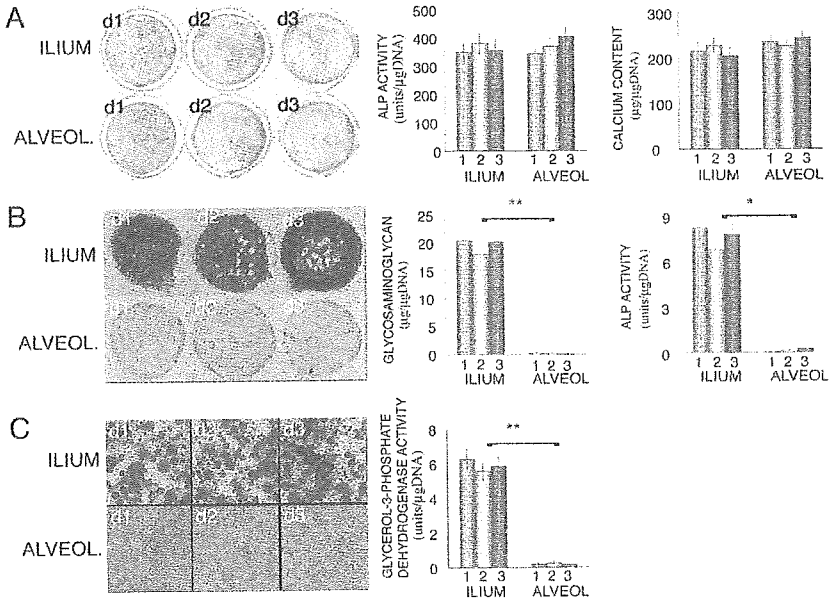


FIG. 5. The differentiation potentials of canine alveolar and iliac BMSCs. (A) Iliac and alveolar BMSCs were isolated from three dogs (d1–d3). Cells obtained from secondary cultures were incubated in the osteogenic status. The cell layers were stained with alizarin red on day 21. ALP activity and the calcium content of the cell-matrix layers were determined on day 28. (B) Iliac and alveolar BMSCs obtained from the secondary cultures were transferred into the chondrogenic medium in pellet cultures for 28 days and stained with toluidine blue. The levels of GAG and ALP activity were determined on day 28. (C) Alveolar and iliac BMSCs obtained from the secondary cultures were maintained in the adipogenic status for 28 days. The cells were stained with oil red O. GAPDH activity was determined. Values are means ± SD for four cultures. $^{*}p < 0.05$; $^{**}p < 0.01$.

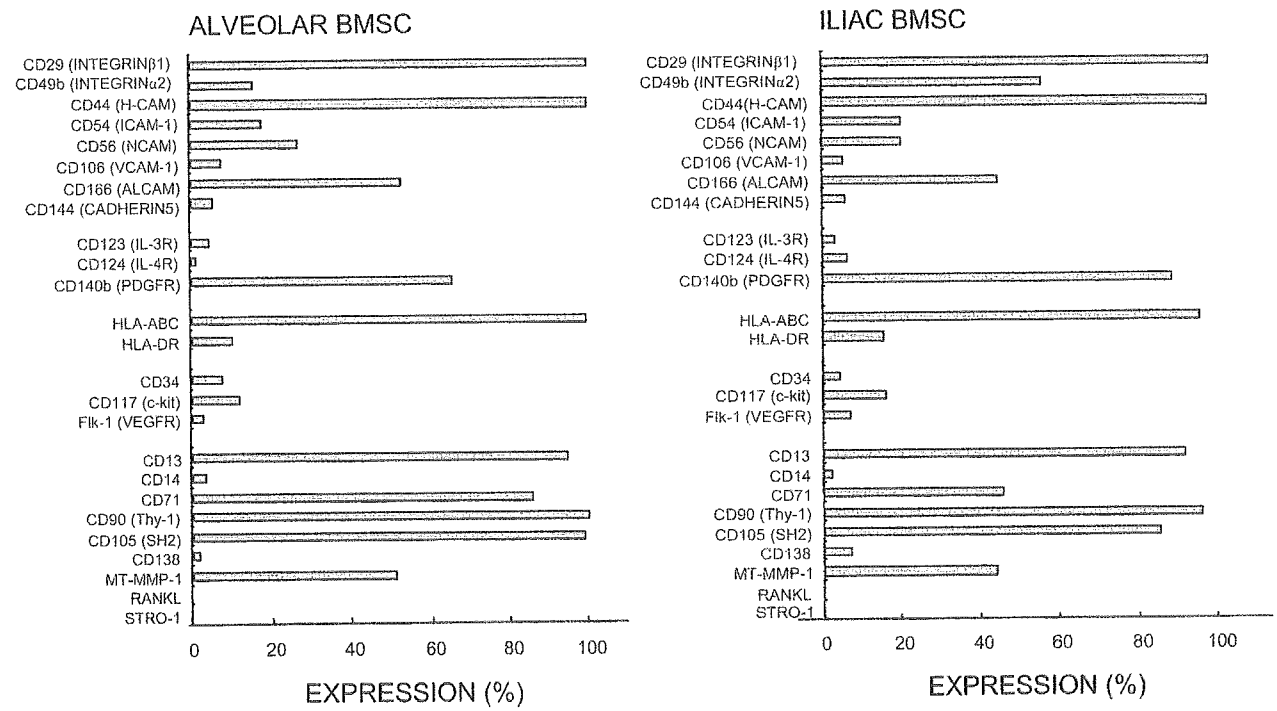


FIG. 6. Cell surface antigen expression by human alveolar and iliac BMSCs. Human alveolar and iliac BMSCs obtained at passage 3 (maintained in OS– medium) reacted with antibodies to cell surface antigens and were analyzed using a FACSCalibur cytometer.

proliferative and osteogenic potentials were similar to those of iliac BMSCs. However, alveolar BMSCs hardly differentiated into chondrocytes, and their adipogenic potential was less than that of iliac BMSCs, at least in the standard differentiation medium: we do not know at present whether other growth factors not included in the medium might enhance the chondrogenic or adipogenic differentiation of these cells. However, we rarely observed cartilage callus formation at the site of a jaw fracture, which may be caused by the poor chondrogenic potential of alveolar BMSCs. The physiological significance of the low adipogenic potential of alveolar BMSCs is not known, but, in any case, we showed here for the first time that topologically different bone marrows contain BMSCs with different features.

Precisely why alveolar and iliac BMSCs have different features remains unknown. BMSCs, like hematopoietic stem cells,[23,24] might have migrated from some bones to others,[20] because it has been suggested that BMSCs mi-

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Molecular markers distinguish bone marrow mesenchymal stem cells from fibroblasts

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Abstract

To characterize mesenchymal stem cells (MSC), we compared gene expression profiles in human bone marrow MSC (11 lines) and human fibroblasts (4 lines) by RT-PCR and real time PCR. Messenger RNA levels of MHC-DR-α, MHC-DR-β, MHC-DR-associated protein CD74, tissue factor pathway inhibitor-2, and neuroserpin were much higher in MSC than in fibroblasts, even in the presence of large interindividual variations. Those of adrenomedullin, apolipoprotein D, C-type lectin superfamily member-2, collagen type XV α1, CUG triplet repeat RNA-binding protein, matrix metalloproteinase-1, protein tyrosine kinase-7, and Sam68-like phosphotyrosine protein/T-STAR were lower in MSC than in fibroblasts. FACS analysis showed that cell surface expression of MHC-DR was also higher in MSC than in fibroblasts. MHC-DR expression decreased after osteogenic differentiation, whereas the expression of adrenomedullin—a potent stimulator of osteoblast activity—along with collagen XV α1 and apolipoprotein D increased after osteogenic differentiation. The marker genes identified in this study should be useful for characterization of MSC both in basic and clinical studies.

Keywords: Bone marrow; Marker genes; Mesenchymal stem cell; Regenerative medicine

Bone marrow mesenchymal stem cells (MSC), which are also called plastic-adherent marrow cells or bone marrow stromal cells, can differentiate into osteoblasts, chondrocytes, adipocytes, tenocytes, and muscle cells in vitro and/or in vivo [1–11]. MSC can easily be isolated from adult bone, and can be expanded with serum ex vivo, so these cells are promising for regenerative medicine: they are already being used for treatment of osteogenesis imperfecta or bone/cartilage defects [7,8]. Nonetheless, MSC have not been fully characterized, and thus it is difficult to examine whether ex vivo expanded MSC population is free of fibroblasts. Bone marrow may contain fibroblasts or can be contaminated by fibroblasts during aspiration, and the fibroblasts—together with MSC—could possibly be expanded in the presence of serum ex vivo. However, the appearance

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of MSC is similar to that of fibroblasts. In addition, we found that several cell surface antigens, previously considered to be MSC markers, were also expressed in fibroblasts at similar levels. In other words, molecular markers for MSC remain unknown. To address this issue, we compared gene expression profiles between human bone marrow MSC and connective tissue fibroblasts—using DNA macroarrays, RT-PCR, and real time PCR. We identified 13 genes differentially expressed between these cells. Since contamination of transplantable cells by fibroblasts may delay regeneration, we used these markers to distinguish MSC from fibroblasts before transplantation at Hiroshima University Hospital.

Materials and methods

MSC and fibroblast cultures. MSC were obtained from iliac crest or alveolar/jaw bone according to a protocol approved by Ethical Authorities at Hiroshima University. In addition, human iliac MSC were purchased from Bio-Whittaker (Walkersville, MD). For isolation of alveolar/jaw bone marrow, we selected patients whose bone marrow sites had been opened during oral surgery, and obtained marrow aspirates using routine syringes and needles [12]. Bone marrow cells including erythrocytes were seeded at a density of 0.1 ml aspirate per 35-mm tissue culture dish (Corning) and maintained in 2 ml DMEM supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin G, and 100 μg/ml streptomycin (medium-A). Three days after seeding, floating cells were removed and the medium was replaced by fresh medium-A. Thereafter, attached cells (plastic-adherent marrow cells) were fed with fresh medium-A supplemented with 1 ng/ml FGF-2. FGF-2 was added every other day. Passages were performed when cells were approaching confluence: the cells were seeded at a density of 5×10^3 cells/cm^2 on 100-mm tissue culture dishes (Corning) and maintained in 10 ml medium-A supplemented with 1 ng/ml FGF-2 [9]. To avoid direct actions of FGF-2 on gene expression, FGF-2 was removed from the culture medium of MSC or fibroblasts 72 h before isolation of RNA. Human skin fibroblasts were purchased from Kurabo (Tokyo, Japan), and human gingival fibroblasts were isolated as described previously [13]. Fibroblasts were also maintained in culture as described above.

Osteogenic differentiation. MSC or fibroblasts at passage fifth to eighth were seeded at 4×10^4 cells per 16-mm well in a 24-well plate, and maintained for 7–28 days in medium-A supplemented with 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 μg/ml ascorbic acid-2-phosphate [1,9].

Microarray analysis and RT-PCR. Total RNA was isolated using TRIZOL reagent (Invitrogen), when the fifth to eighth passage cultures became confluent. Poly(A)$^+$ RNA was purified using Micro poly(A) purist (Ambion). DNA microarray analysis was performed with 0.5 μg poly(A)$^+$ RNA by Kurabo Life Array analysis service (Incyte Genomics; Lot # KL01081).

For RT-PCR, first-strand cDNA was synthesized with 1 μg of total RNA using SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Using the cDNAs as a template, PCR was carried out under the following conditions: denaturation at 94 °C for 30 s and primer extension at 65 °C for 1.5 min in 28 cycles for adrenomedullin; 30 cycles for matrix metalloproteinase-1 (MMP-1), tissue factor pathway inhibitor-2, apolipoprotein D, collagen type XV α1, CUG triplet repeat RNA-binding protein, serine (or cysteine) proteinase inhibitor clade-1 member-1 (neuroserpin), protein tyrosine kinase-7, Sam68-like protein, MHC-DR-α and MHC-DR-β; 33 cycles for C-type lectin superfamily member-2. The sequences of primers are shown in supplementary Table 1. Obtained PCR products were separated on 1% agarose gels,and stained with ethidium bromide.

Real time PCR. With the above cDNAs (1 μg) as a template, real time quantitative RT-PCR analyses were performed using an ABI Prism 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Foster City, CA). Sequences of the primers and probes are shown in supplementary Table 2. The primers and probes for CD73 (4331182-CD74) and GAPDH (4310884E) were purchased from PE Applied Biosystems. The mRNA level relative to that of GAPDH was calculated.

FACS analysis. Cells at passage fifth to eighth were harvested with trypsin and EDTA, centrifuged at 1500*g* for 5 min, and resuspended at 5×10^6 cells/ml in phosphate-buffered saline (PBS) containing 3% fetal bovine serum. Aliquots containing 10^5 cells were incubated with individual primary antibodies or control IgG for 30 min at room temperature. The cells were washed in PBS containing 3% fetal bovine serum and incubated with a fluorescent conjugated secondary antibody for 30 min at room temperature. Samples were analyzed using a FACSCalibur cytometer (Becton Dickinson), and the data were analyzed using CELLQUEST software (Becton Dickinson). The following monoclonal antibodies (mAbs) were used: fluorescein isothiocyanate (FITC)-conjugated or *R*-phycoerythrin (PE)-conjugated antibodies against HLA-DR (MHC-DR), CD13, CD14, CD29, CD34, CD44, CD49b, CD54, CD56, CD71, CD90, CD105, CD106, CD117, CD124, CD138, CD144, MHC-DR, HLA-ABC, mouse-IgG1, mouse-IgG2a or mouse IgM (Immunotech Coulter Company); antibodies against MHC-DR, CD73, CD74, CD123, CD140b, CD166 or mouse-IgG3 (Pharmingen); antibodies against Flk-1 (Santa Cruz Biotechnology); antibodies against MT-MMP-1 (Sigma); antibodies against STRO-1 (Genzyme); antibodies against RANKL (R&D Systems), and anti-rabbit-IgG (Chemicon International).

Statistical analysis. Student's *t* test was used.

Results

Differential expression of candidate marker genes between MSC and fibroblasts

MSC or fibroblast lines were obtained from alveolar/jaw bone, ilium, gums or skin of young adults of similar age (Table 1). Under the osteogenic conditions, alveolar (MSC-1) and iliac MSC (MSC-9) induced matrix calcification—which was stained with alizarin red—on days 21 and 28 (supplementary Fig. 1). All of the other MSC lines—but none of the fibroblast lines—also induced calcification by day 28 (Table 1).

Gene expression profile was compared between MSC-1 and fibroblast-2 using DNA microarrays (9400 genes): many (~100) genes showed different signals (>2-fold) between these cells. To confirm the different expressions, we performed RT-PCR analysis for these genes with seven MSC and four fibroblast lines: many genes showed large interindividual variations or very low expression levels, so the comparison was difficult. Nonetheless, the following genes appeared to be expressed differently between MSC and fibroblasts: the mRNA levels of MMP-1 (Fig. 1A), adrenomedullin, (B), protein tyrosine kinase-7 (C), collagen type XV α1 (D), Sam68-like phosphotyrosine protein/T-STAR (E), C-type lectin superfamily member-2 (F), CUG triplet repeat RNA-binding protein

Table 1
Fibroblast and MSC lines used in this study

Cell lines	Tissues	Age	Sex	OB
Fibroblast-1	Gums	17	M	–
Fibroblast-2	Gums	18	F	–
Fibroblast-3	Skin	29	F	–
Fibroblast-4	Skin	33	F	–
MSC-1	Alveolar/jaw	26	M	+
MSC-2	Alveolar/jaw	24	F	+
MSC-3	Alveolar/jaw	19	F	+
MSC-4	Alveolar/jaw	23	F	+
MSC-5	Alveolar/jaw	24	F	+
MSC-6	Alveolar/jaw	19	F	+
MSC-7	Alveolar/jaw	36	M	+
MSC-8	Ilium	24	F	+
MSC-9	Ilium	22	F	+
MSC-10	Ilium	19	F	+
MSC-11	Ilium	24	M	+

Note. OB, osteogenic differentiation.

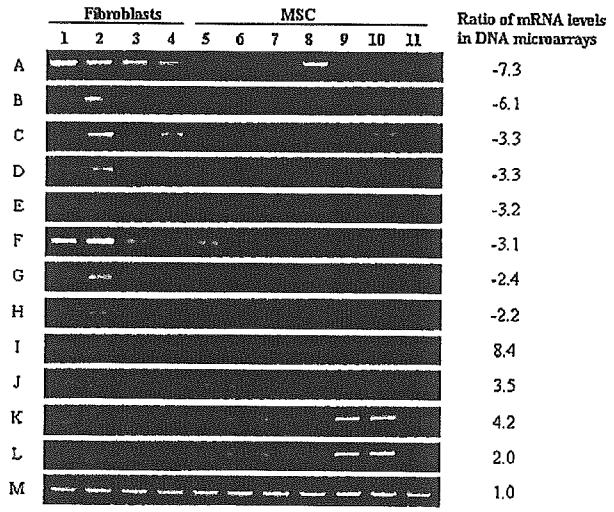

Fig. 1. RT-PCR analysis for genes expressed differently in fibroblasts and MSC. Fibroblast-1, -2, -3, and -4 (lanes 1–4, respectively) and MSC-1, -2, -3, -4, -9, 10, and -11 (lanes 5–11, respectively) were isolated from 11 different donors. The ratio of the mRNA level between MSC and fibroblasts in DNA microarray analysis is shown on the right of the panel. (A) MMP-1; (B) adrenomedullin; (C) protein tyrosine kinase-7; (D) collagen type XV α1; (E) Sam68-like phosphotyrosine protein; (F) C-type lectin superfamily member-2; (G) CUG triplet repeat RNA-binding protein; (H) apolipoprotein D; (I) tissue factor pathway inhibitor-2; (J) neuroserpin; (K) MHC-DR-α; (L) MHC-DR-β; (M) GAPDH.

(G), and apolipoprotein D (H) were lower in MSC than in fibroblasts. In contrast, mRNA levels of tissue factor pathway inhibitor-2 (I), neuroserpin (J), MHC-DR-α (K), and MHC-DR-β (L) were higher in MSC than in fibroblasts. The GAPDH mRNA level in MSC was equal to that in fibroblasts (M).

Real time PCR analysis showed that mRNA levels of these genes, relative to GAPDH, were statistically different ($P < 0.05$) between 11 MSC and 4 fibroblast lines (Fig. 2). Of the 12 genes, MHC-DR-α and -β showed differential expression between iliac and alveolar MSC (see below).

FACS analysis of MHC-DR expression

Since MHC-DR-α and -β mRNA levels were higher in MSC than in fibroblasts, we examined the protein level of MHC-DR by FACS analysis (Fig. 3A): no positive cells were detected with fibroblast lines, whereas MSC lines showed MHC-DR expression at low or moderate levels.

Differential expression of CD74 mRNA between MSC and fibroblasts

Since MHC-DR expression was higher in MSC than in fibroblasts, we compared the expression of MHC-DR-associated protein CD74 in MSC and fibroblasts. The mRNA level of CD74 in iliac MSC was higher than that in fibroblasts, although alveolar/jaw MSC showed CD74 expression at lower levels than did iliac MSC (Fig. 3B).

Changes in marker expressions after osteogenic differentiation

We compared marker gene expressions before and after osteogenic differentiation. The MHC-DR-α and/or -β mRNA levels in five MSC lines decreased after osteogenic differentiation, and the MHC-DR-β mRNA level in alveolar MSC was lower than that in iliac MSC (Fig. 4A). In contrast, apolipoprotein D, adrenomedullin, and collagen type XV α1—which were suppressed both in iliac and alveolar MSC—increased after osteogenic differentiation (Fig. 4B). No changes in mRNA levels of tissue factor pathway inhibitor-2, neuroserpin, C-type lectin superfamily member-2, CUG triplet repeat RNA-binding protein, MMP-1, protein tyrosine kinase-7, or Sam68-like phosphotyrosine protein could be detected after osteogenic differentiation (data not shown). These findings suggest that MHC-DR is a positive marker for undifferentiated MSC, whereas the expression of apolipoprotein D, adrenomedullin, and collagen type XV α1 is temporarily suppressed at the undifferentiated stage.

FACS analysis of cell surface antigens

Some cell surface antigens—in addition to MHC-DR—may be expressed selectively in MSC. SH2 (CD105), SH3 (CD73), ALCAM (activated leukocyte cell adhesion molecule/CD166), CD13, CD29 (integrin β-1), PDGF receptor, CD44 (hyaluronate receptor), and CD90 (Thy-1) were expressed in MSC and/or perichondrium mesenchymal stem cells

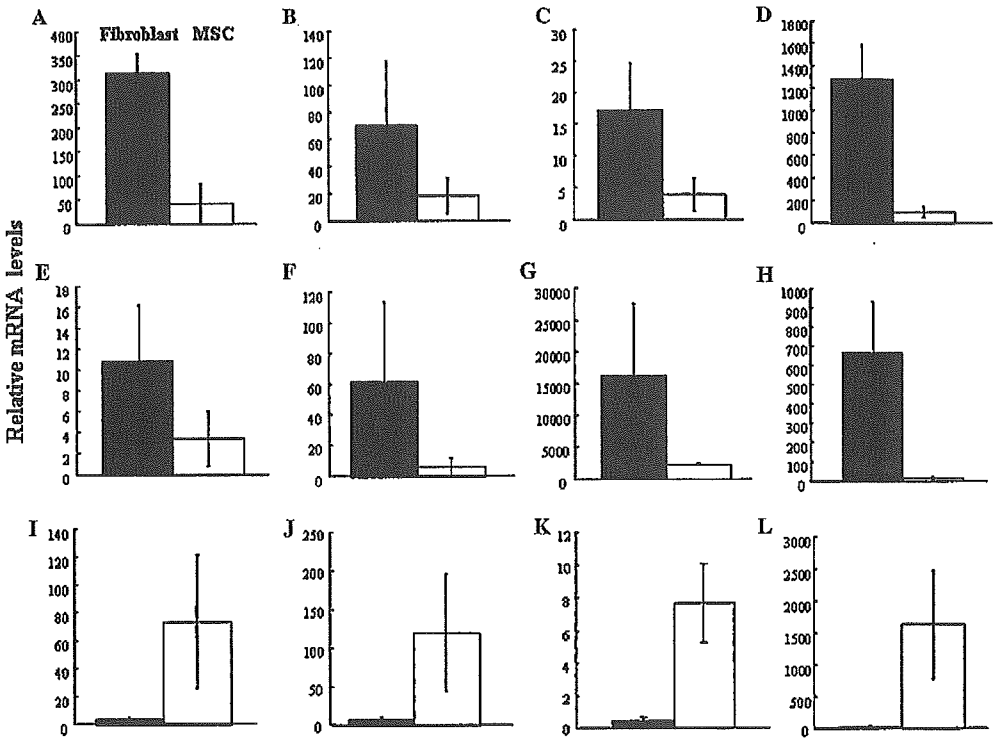

Fig. 2. Real time PCR analysis of candidate genes. Expressions of the candidate genes relative to GAPDH in confluent cultures of fibroblast-1, -2, -3, and -4 (closed columns) and MSC-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, and -11 (open columns) were examined by real time PCR. (A) MMP-1; (B) adrenomedullin; (C) protein tyrosine kinase-7; (D) collagen type XV α1; (E) Sam68-like phosphotyrosine protein; (F) C-type lectin superfamily member-2; (G) CUG triplet repeat RNA-binding protein; (H) apolipoprotein D; (I) tissue factor pathway inhibitor-2; (J) neuroserpin; (K) MHC-DR-α; (L) MHC-DR-β. Values are averages ± SD for 4 or 11 cultures. The mRNA levels of all examined genes were statistically significant between MSC and fibroblast lines ($P < 0.05$).

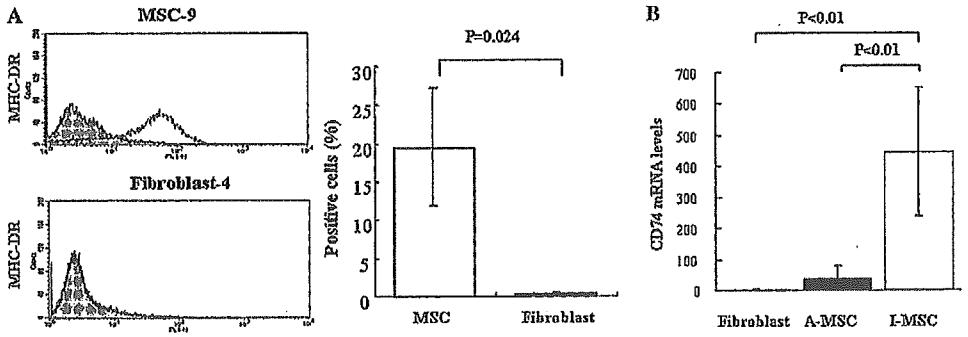

Fig. 3. Expression levels of MHC-DR and CD74 in MSC and fibroblasts. (A) FACS analysis of MHC-DR expression on the cell surface of MSC. MHC-DR expression in fibroblast-2, -3, and -4 (closed columns) and MSC-5, -6, -7, -8, -9, -10, and -11 (open columns) were examined by FACS analysis with anti-MHC-DR antibody (Immunotech Coulter). Values are averages ± SD for three fibroblast or seven MSC lines. (B) The expression of CD74 mRNA in alveolar/jaw MSC (A-MSC), iliac MSC (I-MSC), and fibroblasts. CD74 mRNA expression in fibroblast-1,-2, -3, and -4, alveolar MSC-1, -2, -3, -4, and -5, and iliac MSC-9, 10, and -11 in confluent cultures was examined by real time RT-PCR. Values are averages ± SD for three to five cultures.

[1,14–18]. SH2 and SH3 levels decreased after osteogenic differentiation [14], and ALCAM was expressed selectively in perichondrium mesenchymal cells [15]. However, it is unknown whether these cell surface markers were present or absent in fibroblasts. In this study, we compared the levels of these markers in human MSC and fibroblasts: overall, the surface marker expression levels in MSC in this study were consistent with those reported in the literature [1,14–16,18], but none of the cell surface antigens—including SH2,

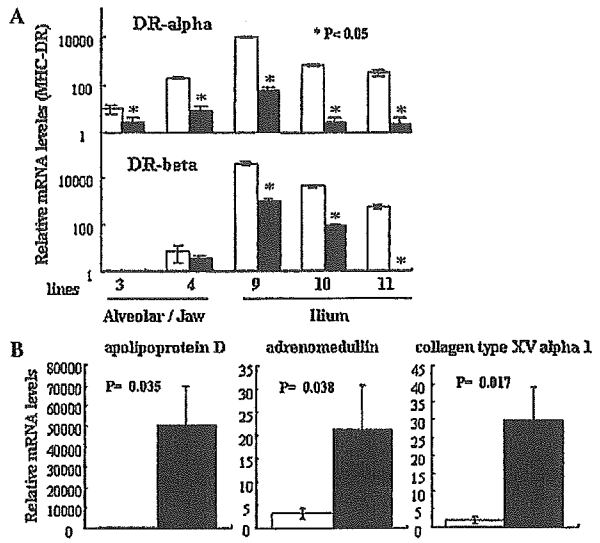

Fig. 4. Effects of osteogenic differentiation on gene expression in MSC. (A) Decrease in MHC-DR mRNA expression after osteogenic differentiation. MSC-3, -4, -9, -10, and -11 were maintained for 28 days in medium-A alone (open column) or in medium-A supplemented with 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 μg/ml ascorbic acid-2-phosphate in the osteogenic status (closed columns). Values are averages ± SD for three cultures. (B) Increases in mRNA levels of marker genes in MSC after osteogenic differentiation. MSC-3, -4, -9, -10, and -11 were maintained for 28 days in medium-A alone (open columns) or in medium-A supplemented with 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 μg/ml ascorbic acid-2-phosphate (closed columns). Values are averages ± SD for five cultures.

SH3, ALCAM, and STRO-1—differed between MSC and fibroblasts (supplementary Fig. 2). The levels of these cell surface markers in iliac MSC or fibroblasts were similar to those in alveolar MSC [12]. STRO-1 has been found in freshly isolated MSC [17], but in this study, MSC showed STRO-1 expression at a low level (supplementary Fig. 2). Previous studies have also shown that STRO-1$^+$ cells in human MSC population are only 7 ± 6% [18], so STRO-1 may be progressively lost with time in these cultures. In any case, our findings suggest that STRO-1 is not essential for the differentiation potential of MSC.

Application of marker genes to regenerative medicine

At Hiroshima University Hospital, we have commenced clinical studies on regenerative medicine for periodontal diseases, using autologous MSC. Before transplantation, we compared the marker gene expressions in patients' MSC (iliac MSC) with those in standard iliac MSC and fibroblast lines (Table 2). In the case of patient-1 (63, male), all examined genes in expanded plastic-adherent cells showed a similar expression pattern to that of standard MSC lines. Fibroblast contamination was unlikely, because MMP-1 and collagen type XV α1 mRNA levels in the marrow cells were only 1% of those in the fibroblastic lines (Table 2). In contrast, MHC-DR-α and -β mRNA levels in the marrow cells were 1000-fold greater than those in the fibroblast lines. Similar results were obtained with cells from patient-2 (39, male), patient-3 (64, female), patient-4 (46, female), patient-5 (25, male), patient-6 (56, female), and patient-7 (22, male) (data not shown). After the quality examination, we were able to transplant these cells with abundant self-confidence.

Table 2
Evaluation of ex vivo expanded MSC population before transplantation using marker genes

Genes	Relative mRNA levels		
	Patient-1	Standard MSC	Standard fibroblasts
MMP-1	28	9 ± 10	1206 ± 1097
Adrenomedullin	3	1 ± 1	15 ± 5
CUG triplet repeat, RNA-binding protein-2	7	6 ± 5	148 ± 107
Collagen type XV α1	1	No signal	475 ± 163
Sam68-like phosphotyrosine protein	No signal	No signal	3 ± 2
C-type lectin, superfamily member-2	3	5 ± 4	231 ± 191
Apolipoprotein D	14	3 ± 2	426 ± 326
Tissue factor pathway inhibitor-2	9	23 ± 17	4 ± 4
MHC-DR-β	2121	563 ± 769	2 ± 1
MHC-DR-α	1561	582 ± 402	2 ± 2

Before transplantation, we examined the expression pattern of marker genes in patient's MSC expanded ex vivo, standard MSC, and standard fibroblasts. The patient's MSC were cultured, and total RNA in confluent cultures at passage 3 was isolated as described in Materials and methods. Gene expression profile in the patient's cells was compared with that in RNA samples of MSC-9, MSC-10, MSC-11, fibroblast-1, fibroblast-2, and fibroblast-3 by real time PCR. Values are averages of duplicate determinations (patient-1) or averages ± SD for three cultures (standard lines).

Discussion

We identified several genes differentially expressed between MSC and fibroblasts, and the differential expression was unrelated to age, sex or culture conditions. We used MSC and fibroblasts from donors of similar age and cultured under similar conditions; sex did not affect the gene expressions (data not shown).

In addition, the difference was not due to in vivo location of the cell, since we used iliac and alveolar MSC, and skin and gingival fibroblasts. Alveolar MSC had potent osteogenic potential in vitro and in vivo, although their chondrogenic or adipogenic potential was less than that of iliac MSC [12]. Alveolar and iliac MSC shared many common marker genes, with a few genes (MHC-DR and CD74) being expressed at different levels.

MSC showed a lower level of MMP-1 and a higher level of its inhibitor—tissue factor pathway inhibitor-2—than did fibroblasts. Tissue factor pathway inhibitor-2 suppresses the activity of the collagenases—MMP-1 and MMP-13—as well as the gelatinases—MMP-2 and MMP-9 [19], so MSC may be less active in collagen-matrix breakdown. On the other hand, reduced expression of neuroserpin may increase activities of tissue-type plasminogen activator [20].

Type XV collagen occurs in basement membrane zones of tissues [21], and type XV collagen-derived endostatin has antiangiogenic actions [22]. Adrenomedullin—a member of the calcitonin family—stimulates osteoblastic activity and bone growth in vivo [23,24]. Accordingly, adrenomedullin mRNA expression increased after osteogenic differentiation of MSC. Protein tyrosine kinase-7 is essential for neural tube closure [25] and is involved in tumor metastasis [26]. Sam68-like phosphotyrosine protein (an Src substrate) is involved in cell proliferation [27,28]. Apolipoprotein D increases platelet-derived growth factor actions and synergistically stimulates migration of vascular smooth muscle cells [29]. Roles of the molecules in MSC remain unclear, but higher or lower levels of these molecules are characteristic of MSC. We are investigating the physiological roles of molecules in MSC or fibroblasts.

We also found that the expression of MHC-DR (class II) was higher in MSC than in fibroblasts, whereas MHC (class I)/HLA-ABC was expressed in MSC and fibroblasts at similar levels (supplementary Fig. 2). The expression level of MHC-DR-associated protein CD74 was also higher in MSC than in fibroblasts. CD74 binds to MHC-DR and this interaction involves the peptide-binding groove of MHC-DR [30]: MHC-DR-α and -β molecules are assembled with CD74 protein in the endoplasmic reticulum [31]. The physiological relevance of MHC-DR and CD74 expression in MSC is unknown, but MSC can modulate immune response [32].

Plastic-adherent marrow cells/MSC are often designated as a colony-forming unit-fibroblastic (CFU-F), and some CFU-F colonies do not show any differentiation potentials. Thus, ex vivo expanded adherent cell populations may contain fibroblast-like cells. In addition, bone marrow samples can be contaminated by connective tissue fibroblasts during surgery or aspiration. Contamination of MSC populations by fibroblasts delays regeneration and could be harmful, and thus we need to confirm the absence of fibroblasts before transplantation. Conventionally we have to incubate transplantable cells in differentiation-induction media for 21–28 days to examine the presence of MSC with multi-lineage differentiation potential, but this does not show the presence of fibroblasts directly. In the present study, we developed a method of distinguishing MSC from fibroblasts promptly before transplantation—using marker genes. This quality examination should be crucial to regenerative medicine with MSC. In addition, identification of marker genes will help us characterize MSC and examine their in vivo location.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.04.118.

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特集 骨代謝疾患の成因と治療～基礎と臨床をつなぐもの～・Therapy

歯周組織の細胞移植療法
骨髄間葉系幹細胞を用いた歯周組織再生療法

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歯周組織の細胞移植療法
骨髄間葉系幹細胞を用いた歯周組織再生療法

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歯周炎で失われた歯周組織を再構築させることは歯周炎治療の最終的な目標である。これまでの基礎研究で，歯周組織欠損部位への骨髄間葉系幹細胞移植が歯周組織再生を促進することを明らかにしてきた。そこで，歯周組織再生治療の全く新しい試みとして，骨髄間葉系幹細胞を利用する方法を考案した。患者の腸骨から骨髄液を採取し，臨床専用の細胞培養室で間葉系幹細胞を分離・増殖させ，医療用アテロコラーゲンゲルとの複合移植体（細胞密度2×10⁷ cells/mL）として歯周組織欠損部へ細胞移植している。現在まで，移植患者7名は，良好な経過をたどっている。今後，さらに基礎・臨床研究を続け，本治療法の効果を検討していく必要がある。

Cell transplantation for periodontal diseases
A novel periodontal tissue regenerative therapy using bone marrow mesenchymal stem cells

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A major goal of periodontal therapy is to reconstruct healthy periodontium destroyed by periodontal diseases. Basic studies have revealed that transplantation of mesenchymal stem cells (MSC) into periodontal defects promotes regeneration of periodontal tissue. We have developed a novel method for periodontal therapy using MSC. Human bone marrow cells are obtained from the iliac crest and expanded in vitro at Cell and Tissue Engineering Center in Hiroshima University Hospital. MSC are, then, isolated and mixed with Atelocollagen at final concentrations of 2×10^7 cells/mL. These MSC in Atelocollagen are transplanted into periodontal osseous defects at the periodontal surgery. The results in all seven patients who received the own MSC transplantation have shown good clinical course. Further basic studies and the continuous clinical trial are needed to prove the effectiveness of the clinical application.

はじめに

歯科領域での外科的治療の特徴として，①術後処置部位を無菌的に保つことが困難，②術後に歯・歯周組織を安静にすることが困難，③患者の審美的要求が強い，などがあげられる。このようなことから，術後できるだけ短期間に障害組織を再生できる治療法の開発は特に重要である。現在までに，人工骨や組織遮断膜，サイトカインを利用し，残存組織の再生能を引き出す再生療法が，歯周炎や顎骨の疾患に対して臨床応用されてきた。しかし，欠損が広範囲に及ぶと，再生に必要とされる十分な数の幹細胞の遊走や増殖が困難なため，量的にも質的にも理想的な組織再生は期待できない。

一方で，細胞を積極的に利用する再生医療は，多様な歯周組織欠損に対応が可能であり，より短期間に組織再生が可能であるという点から歯科領域においても魅力的な医療である。その中で，歯周炎治療では，患者自身の骨髄間葉系幹細胞を移植する方法が，既に臨床研究の段階まできている。本稿では，この「自家間葉系幹細胞移植法を用いた歯周組織再生治療」の基礎研究と臨床研究の概略を紹介する。

歯周組織の構造と歯周炎

歯周組織とは，歯を支えている組織のことであり，歯肉，セメント質（歯根の最表層組織），歯槽骨（顎骨の一部で歯根を覆っている部分の骨），歯周靱帯で構成されている。健常な状態では，歯根はセメント質で覆われており，この表面と歯槽骨の間に線維性結合組織である歯周靱帯が介在することで歯は支持されている。（図1）。従って，歯周靱帯は2つの硬組織に介在する極めて重要な軟組織である。

歯周炎は，口腔内の歯周病原性細菌の感染に対する免疫応答の結果，歯周組織の破壊が生じたものである。すなわち，歯周炎は，歯と歯肉の付着の喪失，セメント質の細菌感染による汚染，歯槽骨の吸収，歯周靱帯の断裂が起こっている状態で，長期間放置すれば症状が増悪し，歯を喪失することとなる。（図1）。

歯周組織再生療法について

歯周炎治療の究極の目標は，歯周炎によって破壊された歯周組織を再生，再構築することである。現在，組織遮断膜やサイトカインを利用して歯周組織を再生させる方法が行われているが，これらの治療法は，“残存する歯周組織由来の幹細胞の再生能力を発揮させる”という戦略である。この戦略には限界があり，歯周組織欠損が広範囲に及ぶと，十分な数の幹細胞の遊走や増殖が困難で，量的にも質的にも理想的な歯周組織再生は期

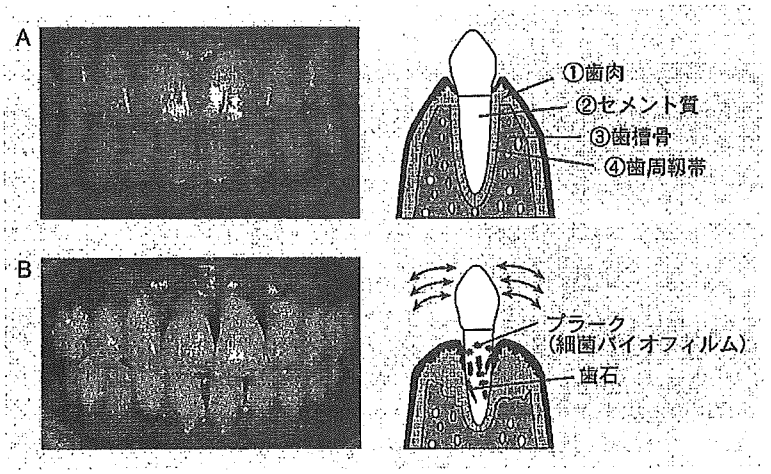


図1　歯周組織の構造

A：歯周組織は① 歯肉，② セメント質，③ 歯槽骨，④ 歯周靭帯から構成されている。

B：歯周炎では，歯と歯肉の付着は壊れ，セメント質の汚染，歯槽骨の吸収，歯周靭帯の断裂が起こっている。

(筆者ら提供)

待できない。多様な歯周組織病変に対応するために，幹細胞を移植して組織再生の量的，質的な問題の解決を図るという新たな歯周組織再生の戦略が必要となっている[1]。

骨髄間葉系幹細胞を利用した歯周組織再生の基礎研究

骨髄間葉系幹細胞は，骨・軟骨・脂肪細胞をはじめ多数の細胞に分化可能なこと[2)~5)]，細胞採取法や培養法が比較的容易なこと，自家細胞の使用で拒絶反応の心配がないことや倫理的問題が回避できるなどのメリットがあり，多くの疾患に対する再生医療の供給源となり得る細胞である。なかでも歯周炎治療の対象となる歯周組織は，歯槽骨，セメント質，歯周靭帯といった複数の異なる組織によって構成されていることから，さまざまな細胞に分化できる間葉系幹細胞は，歯周組織再生に最も適した細胞である。骨髄間葉系幹細胞を移植して歯周組織を再構築する治療法の開発を目指し，まずその基礎研究に取り組んだ。

骨髄間葉系幹細胞の培養に関しては，fibroblast growth factor-2を加えた培地を用いることで，従来の方法よりも10万倍高い効率で増殖させる技術を確立した[5]。この骨髄間葉系幹細胞の分離・増殖法を利用し，ビーグル犬の歯周組織欠損部へ，自家骨髄間葉系幹細胞とアテロコラーゲンゲル(株式会社高研，東京)複合体の移植を行った[6]。

細胞移植後1カ月でアテロコラーゲンゲルのみ移植した群と比較したところ，幹細胞移植群は，顕著な歯周組織の再生が観察され，骨髄間葉系幹細胞移植が歯周組織再生に有効である知見を得た。(図2)。また，green fluorescent protein (GFP)を導入した骨髄間葉系幹細胞を移植すると，再生した歯周組織を構成する細胞がGFP陽性を示し，歯周組織欠損部へ移植した骨髄間葉系幹細胞が，歯周組織構成細胞であるセメント芽細胞，歯周靭帯線維芽細胞，骨芽細胞に分化することで歯周組織再生を促進する可能性が示された。

これらの基礎研究や動物実験の結果をもとに，

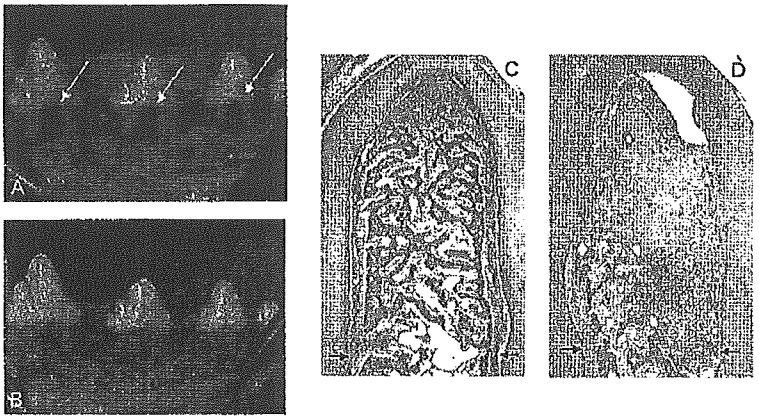


図2　イヌの歯周組織欠損モデルにおける骨髄間葉系幹細胞移植による歯周組織再生

A：イヌの根分岐部に歯周組織欠損を作製（矢印）。
B：細胞複合体を移植した状態。
C, D：術後1カ月の組織像。骨髄間葉系幹細胞移植（C）は，細胞非移植（D）に比べて顕著な歯周組織再生が観察された。矢印は歯周組織欠損の底部。
（文献6より引用）

学内の倫理委員会の承認後，広島大学病院では“自家間葉系幹細胞移植法を用いた歯周組織再生治療”の臨床研究を開始した。（図3）。医科と歯科の診療科で分業体制をとり，学内の基礎講座，関連企業と共同して本治療法の「有効性」と「安全性」を検証している。

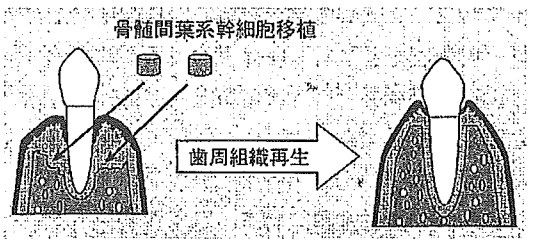


図3　骨髄間葉系幹細胞移植による歯周組織再生

骨髄間葉系幹細胞を歯周組織欠損部に移植し，歯周組織再生を試みる。
（筆者ら作成）

自家間葉系幹細胞を利用した歯周組織再生の臨床研究

臨床研究の流れを図4に示す。インフォームドコンセントを得た後に，選択基準を満たした歯周組織欠損を有する患者に対して，まず約200 mLの末梢血の採血を行い，完全な閉鎖系のバッグを用いて無菌的に約90 mLの血清を分離する。（図5）。これは，一般的な培養で利用する市販のウシ胎児血清では，未知の感染物質が存在する可能性が否定できないため，患者自身の血清を幹細胞の分離・培養に用いるためである。

細胞培養の準備が整うと，骨髄液採取となる。局所麻酔下で，腸骨から骨髄液約15 mL採取するが，外来で採取が可能であり患者は入院の必要はない。採取した骨髄液は，病院内に設置された治療用ヒト細胞専用の細胞培養室に搬送し，患者自己血清を含む培地を使って間葉系幹細胞の分離，培養を行う。

細胞移植手術は，骨髄採取日の3週間後を基本とし，細胞数をコントロールして培養を行っている。血清，細胞培養液，培養細胞に対して，寄生体の感染などに関する安全性検査，移植細胞に関して品質の検証[7]後，細胞密度2×10^7cells/mL

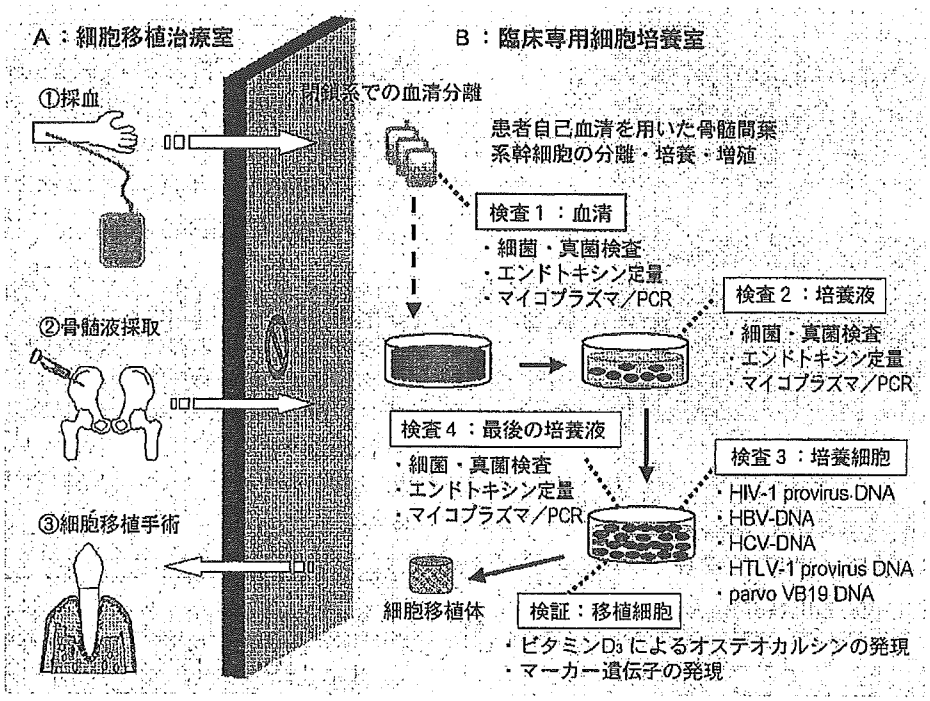


図４　臨床研究の流れ

インフォームドコンセント，スクリーニング検査を経て選択基準を満たした患者は，自己血清分離の採血を受ける（A①）。患者血清を確保した後，骨髄液を採取し（A②），臨床専用細胞培養室（B）で骨髄間葉系幹細胞の培養を約３週間行う。血清，細胞培養液，培養細胞に対しての検査（検査１～４），移植直前の細胞に関しての品質の検証後，細胞移植手術（A③）を行う。

PCR：ポリメラーゼ遺伝子増幅反応，HIV：ヒト免疫不全ウイルス，HBV：B型肝炎ウイルス，HCV：C型肝炎ウイルス，HTLV：ヒトT細胞白血病ウイルス

（筆者ら作成）

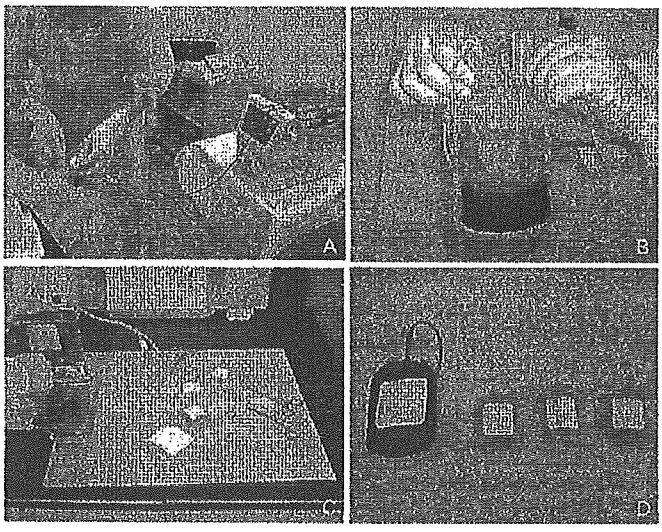
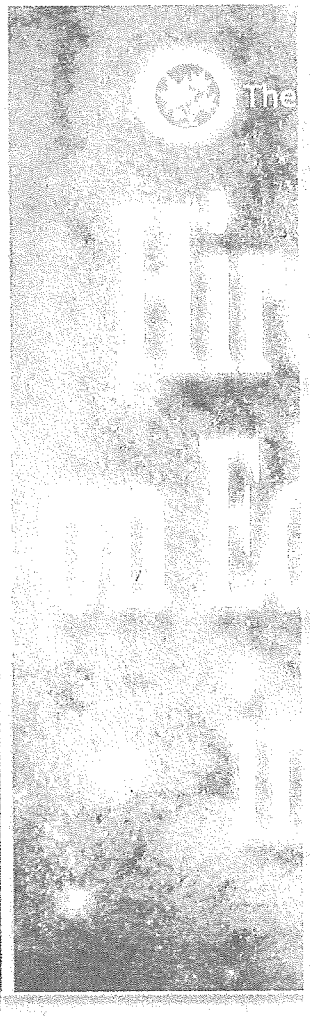
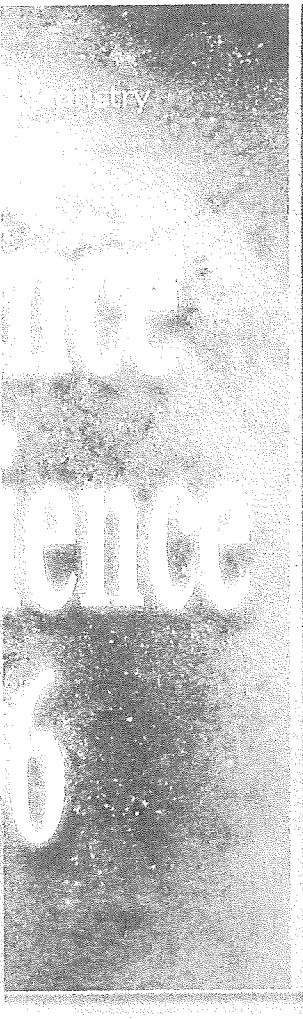


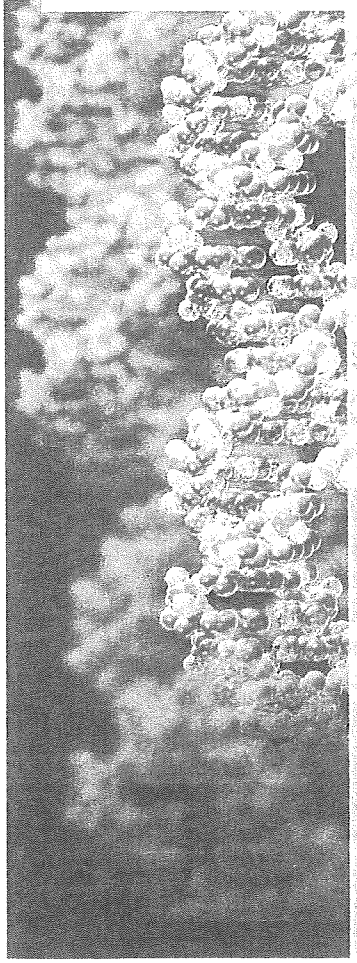
図５　完全閉鎖系バッグによる血清分離システム

専用の血清分離バッグを用い，前腕肘正中静脈から200 mLの採血を行う（A）。血清分離バックを臨床専用細胞培養室へ搬送し，遠心操作によって血清を分離する（B）。その後，血清分離板を使って血清部分だけを連結してある小さなバッグ３つに移し（C），それぞれのチューブをシール，切り離して（D）使用時まで凍結保存する。

（筆者ら提供）







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