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Human Mesenchymal Stem Cells Promote Human Osteoclast Differentiation from CD34$^+$ Bone Marrow Hematopoietic Progenitors*

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ABSTRACT

Interactions between osteoclast progenitors and stromal cells derived from mesenchymal stem cells (MSCs) within the bone marrow are important for osteoclast differentiation. *In vitro* models of osteoclastogenesis are well established in animal species; however, such assays do not necessarily reflect human osteoclastogenesis. We sought to establish a reproducible coculture model of human osteoclastogenesis using highly purified human marrow-derived MSCs (hMSCs) and CD34$^+$ hematopoietic stem cells (HSCs). After 3 weeks, coculture of hMSCs and HSCs resulted in an increase in hematopoietic cell number with formation of multinucleated osteoclast-like cells (Ocls). Coculture of hMSCs with HSCs, transduced with a retroviral vector that expresses enhanced green fluorescent protein, produced enhanced green fluorescent protein$^+$ Ocls, further demonstrating that Ocls arise from HSCs. These Ocls express calcitonin and vitronectin receptors and tartrate-resistant acid phosphatase and possess the ability to resorb bone. Ocl formation in this assay is cell contact dependent and is independent of added exogenous factors. Conditioned medium from the coculture contained high levels of interleukin (IL)-6, IL-11, leukemia inhibitory factor (LIF), and macrophage-colony stimulating factor. IL-6 and LIF were present at low levels in cultures of hMSCs but undetectable in cultures of HSCs alone. These data suggest that coculture with HSCs induce hMSCs to secrete cytokines involved in Ocl formation. Addition of neutralizing anti-IL-6, IL-11, LIF, or macrophage-colony stimulating factor antibodies to the coculture inhibited Ocl formation. hMSCs seem to support Ocl formation as undifferentiated progenitor cells, because treatment of hMSCs with dexamethasone, ascorbic acid, and β-glycerophosphate (to induce osteogenic differentiation) actually inhibited osteoclastogenesis in this coculture model. In conclusion, we have developed a simple and reproducible assay using culture-expanded hMSCs and purified HSCs with which to study the mechanisms of human osteoclastogenesis. (*Endocrinology* **140:** 3736–3743, 1999)

HEMATOPOIETIC progenitors differentiate into osteoclasts through the influence of systemic hormones and factors produced within the bone marrow microenvironment (1). Stromal cells arising from pluripotent mesenchymal stem cells (MSCs) are components of this microenvironment and have been shown to produce extracellular matrix, cytokines, and growth factors that regulate the formation, activity, and survival of osteoclasts *in vitro* (2). The proximity of marrow stromal cells and hematopoietic cells in the marrow suggests that the contact between mesenchymal and hematopoietic cells may signal important intracellular events that, in turn, may regulate the biological activities of these two cell lineages. In support of this idea, several studies, using cells derived from rodent marrow, have demonstrated that cell-to-cell interactions between osteoclast progenitors and stromal cells are mediated through surface molecules such as adhesion molecules and cytokines like cadherin-6/2, macrophage-colony stimulating factor (M-CSF), and TRANCE/RANKL/ODF/OPGL (3–5).

Although *in vitro* models have provided insights into the mechanisms of osteoclastogenesis in rodents (6), such assays do not necessarily reflect human osteoclastogenesis, and several reports have identified mechanistic differences between osteoclastogenesis in animal and human species. For example, PGs stimulate formation of rat osteoclast-like cells (Ocls) (7) but inhibit osteoclastogenesis in cultures of human marrow cells (8). In addition, calcitonin (CT) markedly induces the retraction of rodent osteoclasts (9), whereas this hormone has only moderate effects on human osteoclasts (10). It has been shown that stromal cells may not be essential for human osteoclastogenesis (11), whereas Ocl formation in rodents required stromal cells (6).

Previous studies have revealed several features of human marrow-derived MSCs (hMSCs) and hematopoietic stem cells (HSCs) that are relevant to the present study. hMSCs are pluripotent cells that, under appropriate conditions, can differentiate into osteoblasts, adipocytes, and chondrocytes (12, 13). It has been recently shown: 1) that hMSCs produce numerous hematopoietic cytokines and growth factors that regulate osteoclast differentiation; and 2) that monolayer of hMSCs support long-term cultures initiating cells in long-term cultures of human HSCs (14, 15). In this study, we used hMSCs and HSCs to examine the role of hMSCs in osteoclastogenesis. We found that hMSCs promote HSCs to differentiate into Ocls in the absence of added exogenous growth factors, cytokines, or hormones.

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Materials and Methods

Isolation and osteoblastic differentiation of hMSCs

Human bone marrow aspirates were purchased from Poietic Technologies, Inc. (Gaithersburg, MD), and hMSCs were isolated from fresh

aspirates and culture-expanded as previously described (15). hMSC culture medium consisted of DMEM-low glucose (HyClone Laboratories, Inc., Salt Lake City, UT) supplemented with 10% FBS (Biocell Laboratories, Rancho Dominquez, CA) and 1% antibiotic-antimycotic solution (Life Technologies, Grand Island, NY). When the cultures reached 90% of confluence (~7 days), cells were recovered by the addition of a solution containing 0.05% trypsin-EDTA (Life Technologies) and replated at a density of 5.4×10^3cells per cm^2 flask as passage-1 cells.

Osteogenic differentiation of hMSCs was induced as previously described (13). Briefly, passage-1 hMSCs were treated with an osteogenic supplement (OS) containing 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μM L-ascorbic acid-2-phosphate for 2, 3, 4, 5, 10, or 13 days. At the time of initiation of the osteoclastogenesis coculture, OS-containing medium was replaced with medium without OS, which was used for the duration of the experiments.

Cocultures

Cryopreserved HSCs originated from healthy human bone marrow and were purified at Poietic Technologies, Inc. Bone marrow cell suspensions were centrifuged on Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) to isolate mononuclear cells. HSCs were purified by positive selection using the antibody that recognizes the CD34 cell surface marker (purity > 95%, by flow cytometry) (MiniMACS CD34 isolation kit, Miltenyi Biotec, Auburn, CA). Aliquots of HSCs ($5 \times 10^4/cm^2$) were added to monolayers of hMSCs or OS-treated hMSCs, and the coculture was maintained at 37 C in 95% air-5% CO_2 for up to 3 weeks. Every 3 days, half of the culture medium was carefully removed to minimize loss of nonadherent cells and was replaced with an equal volume of fresh medium.

To assess the effects of 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$] and cytokines on Ocl formation, 1,25$(OH)_2D_3$ (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA); or antibodies to human interleukin (IL)-6, IL-11, leukemia inhibitory factor (LIF), or M-CSF; or control IgG (R&D Systems, Minneapolis, MN) were added to the coculture and maintained through the coculture period for up to 3 weeks.

To determine the role of cell contact in Ocl formation, experiments were performed in which HSCs and hMSCs were cultured in either the same chamber or in chambers separated by 0.45-μm porous membrane (Becton Dickinson and Co., Bedford, MA) for 3 weeks.

Tartrate-resistant acid phosphatase (TRAP) staining

At the end of the culture period, the medium was removed, and the cell layers were fixed by addition of 60% acetone solution in citrate buffer (pH 5.4) for 30 sec, and the cells were washed twice with distilled water and air dried. TRAP-positive ($TRAP^+$) cells were detected using a commercial TRAP staining kit (Sigma Chemical Co., St. Louis, MO). $TRAP^+$ multinucleated cells (three or more nuclei, $TRAP^+$MNCs) were counted manually using a light microscope (16). In some experiments, the cells were counterstained after TRAP staining with 4′,6-diamidino-2-phenylindole (Molecular Probes, Inc., Eugene, OR) to visualize the nuclei.

Immunocytochemistry

For immunocytochemical analysis of vitronectin receptor (VNR), cocultures were fixed by the addition of a solution of 3.7% (wt/vol) paraformaldehyde in PBS for 30 min. The fixed-cells were then incubated sequentially with 1.5% solution of horse serum (30 min) and a 1:50 dilution of antihuman VNR monoclonal antibody. The anti-VNR antibody (kindly provided by Dr. Michael Horton, The Rayne Institute, London, UK) was diluted in PBS, containing 0.15% horse serum, for 30 min. Cells were then subsequently incubated for 30 min with glucose oxidase-conjugated horse antimouse secondary antibody, which is contained in the Vectastain ABC-GO kit (Vector Laboratories, Inc., Burlingame, CA). All incubations were performed at room temperature and were followed by three individual washes with PBS.

RNA preparation and RT-PCR analysis

Total RNA was extracted from hMSC cultures or HSCs and hMSC coculture using the High Pure RNA Isolation kit (Boehringer Mannheim, Indianapolis, IN). RNA yield was determined by absorbance at 260 nm, and PCR was performed for 30 cycles on single-strand complementary DNA prepared from total RNA (1 μg) using a GeneAmp RT-PCR kit (Perkin-Elmer Corp., Foster City, CA). The following conditions were used for PCR: denature at 95°C for 20 sec, anneal at 55°C for 20 sec, polymerize at 72°C for 30 sec, and elongate at 72°C for 10 min. The upstream and downstream primers, respectively, were designed as follows: TRAP: 5′- CGATCACAATCTGCAGTACC-3′ and 5′- ACCCAGTGAGTCTTCAGTCC-3′, PCR product size = 150 bp; CT receptor (CTR): 5′-TTTCCAGGGCTTCTTTGTT-3′ and 5′- CTTGGTTGTTGGCTGGTTC -3′, PCR product size = 205 bp. The CTR primers were designed based on the conserved region of this receptor family. PCR products were separated by electrophoresis through a 1% agarose gel and visualized by staining the gel with ethidium bromide.

Pit formation assay

Bone resorbing activity of cells formed in the cocultures was assessed using smoothened elephant tusk slices prepared from discarded elephant tusks obtained and processed by Dr. E. Hunziker (Berne, Switzerland) or artificial bone, Osteologic discs (Millenium Biologix Inc., Ontario, Canada). Tusk slices ($4 \times 4 \times 0.1$ mm) were sterilized with absolute ethanol, air-dried, and washed several times with sterile PBS solution. hMSCs ($3 \times 10^3/cm^2$) were plated on each slice or disc and cultured for 1 week in hMSC medium. HSCs (5×10^4 cells/cm^2) were added to each well, and the cocultures were maintained for 3 weeks, as described above. At the end of the culture period, cells were stained for TRAP activity to visualize Ocls. Cells on tusk slices were subsequently incubated with a 0.1 M NaOH solution, ultrasonicated for 2 min, rinsed with water to remove Ocls from the slices, and placed in a 1% aqueous toluidine blue solution containing 1% sodium borate for 5 min (17). Photomicrographs of resorption pits were taken using a light microscope. Cells on bone analog were subsequently incubated with an aqueous 6% NaOCl/5.2% NaCl solution for 5 min and rinsed with water to remove Ocls from the discs. The discs were mounted on aluminum stubs and sputter coated with gold palladium. They were viewed on AMRAY scanning electron microscope model 1810 (AMRAY, Inc., Bedford, MA) operating at 20 kV. Images were recorded on polaroid type 55 film (Polaroid Corp., Cambridge, MA).

Transduction of $CD34^+$ cells

To demonstrate that Ocls are derived from HSCs, we cocultured hMSCs with HSCs transduced with a retroviral vector expressing enhanced green fluorescent protein (EGFP). The construction of this retroviral vector (MGIN) expressing EGFP used in this study was reported previously (18). Amphotropic retroviral supernatants were produced in a human 293T cell-based packaging line (Phoenix) provided by Dr. G. Nolan (Stanford University, Stanford, CA), similar to the method previously described (18). HSCs were transduced as follows: freshly thawed retroviral supernatants were mixed with the cell suspension 1:1 (vol/vol) in a 15-ml polypropylene tube, then the following reagents were added: 8 μg/ml polybrene (Sigma Chemical Co., St Louis, MO); Flk2/Flt3 ligand and stem cell factor (100 ng/ml, each) and IL-3, IL-6, TPO, and G-CSF (10 ng/ml, each) (PeproTech Inc., Rocky Hill, NJ). The transduction mixture was centrifuged at $1800 \times g$ at 32–35 C for 4 h. Pelleted cells were washed once with the medium and cultured for 24 h in Roswell Park Memorial Institute (RPMI) medium containing 10% FCS and the cytokines listed above. After 24 h, the transduction step was repeated. Forty-eight hours later, cells (2×10^5 cells) were analyzed for EGFP expression by flow cytometry, to determine the percent of cells that express EGFP. As a control for these experiments, aliquots of cells were transduced with a retroviral vector that did not encode EGFP sequence.

Enzyme-linked immunosorbent assay analysis of cytokines

hMSCs or OS-treated hMSCs (7 day-treatment with OS) were cocultured with HSCs ($5 \times 10^4/cm^2$). Two days later, conditioned medium was collected and analyzed for the presence of IL-1α, IL-6, IL-11, granulocyte/macrophage-CSF (GM-CSF), M-CSF, and LIF using the Quantikine kit (R&D Systems).

Statistical analysis

All data were analyzed by a paired *t* test. Samples were run in triplicate, and data represent the mean ± SE. Each experiment was repeated at least twice.

Results

Roles of hMSCs on the formation of Ocls

The first goal of this study was to establish the role of culture-expanded hMSCs on human Ocl formation. We developed a coculture system of hMSCs and $CD34^+$ cells (HSCs) in the absence of added hormones, cytokines, and growth factors to show that hMSCs promote differentiation of HSCs into $TRAP^+$ multinucleated cells ($TRAP^+$ MNCs, ≥3 nuclei) (Fig. 1A). After 3 weeks, this coculture system yielded approximately 0.12–2 $TRAP^+$ MNCs/10^3 HSCs. In contrast, a human kidney cell line (293T cells) or human skin fibroblasts (SK1087 cells) failed to support the formation of $TRAP^+$ MNCs (Fig. 1, B and C). No $TRAP^+$ MNCs were formed when HSCs were cultured for 3 weeks without hMSCs (Fig. 1D). In the absence of hMSCs, the majority of HSCs degenerated within 2 weeks of culture; however, most of the surviving cells were $TRAP^+$. These data suggest that hMSCs supply factors required for the growth and differentiation of the Ocl precursors.

Because the expression of TRAP is not exclusive to osteoclasts (19), we determined that $TRAP^+$ MNCs also express the VNR (Fig. 2, A and B), shown to be expressed by Ocls. Approximately 60% of total MNCs were stained strongly with the anti-VNR antibody (Fig. 2B, *black arrow*). Approximately 20% of HSCs that survived in culture in the absence of hMSCs stained positive with the anti-VNR antibody (data not shown). RT-PCR analysis of RNA from the coculture showed that messenger RNA (mRNA)-encoding TRAP or CTR, another marker of the osteoclasts, were expressed in the coculture, but not in hMSCs cultured in the absence of HSCs (Fig. 2C).

Bone resorbing activity of the cells formed in the coculture was first assessed by performing a pit formation assay on artificial bone analogs composed of a film of calcium phosphate on glass. We found that cells formed in coculture produced both small and large resorption pits (Fig. 3A). No resorption pits were observed on analogs cultured in the presence of HSCs alone (Fig. 3B). Similarly, HSCs and hMSC cocultures (Fig. 3C), but not cultures of HSCs alone (Fig. 3D), produced resorption pits on slices of elephant tusk dentine.

To further demonstrate the origin of $TRAP^+$ MNCs formed in the coculture and to determine whether gene transfer into HSCs can affect the ability of these cells to undergo Ocl differentiation, hMSCs were cocultured with HSCs transduced with a retrovirus expressing EGFP [30% of HSCs were $EGFP^+$, as determined by flow cytometry analysis (data not shown)]. After 3 weeks, $EGFP^+/TRAP^+$ MNCs were identified in the coculture (Fig. 4). Taken together, the data show that hMSCs support the formation of Ocls from HSCs.

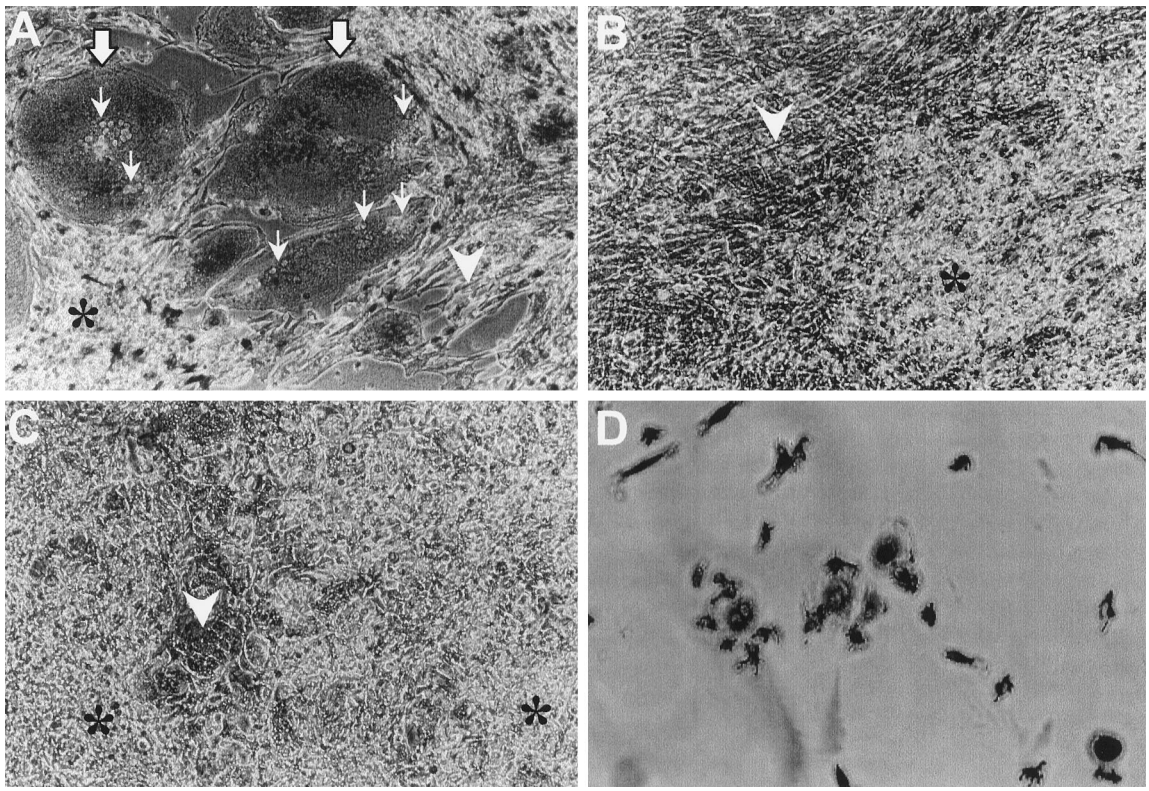

FIG. 1. TRAP staining of 3-week cocultures of $CD34^+$ cells (HSCs) and hMSCs (A), SK1087 fibroblasts (B), 293 cells (C), or HSCs alone (D). Many multinucleated $TRAP^+$ cells (*large arrows*) are seen in coculture of HSCs with hMSCs, compared with skin SK1087 fibroblasts or 293 cells. Small arrows and *asterisks* indicate clusters of nuclei and aggregates of hematopoietic cells, respectively. hMSCs and SK203 fibroblasts, and 293 cells are seen as a layer of spindle- and polygonal-shaped cells, respectively (*arrowhead*).

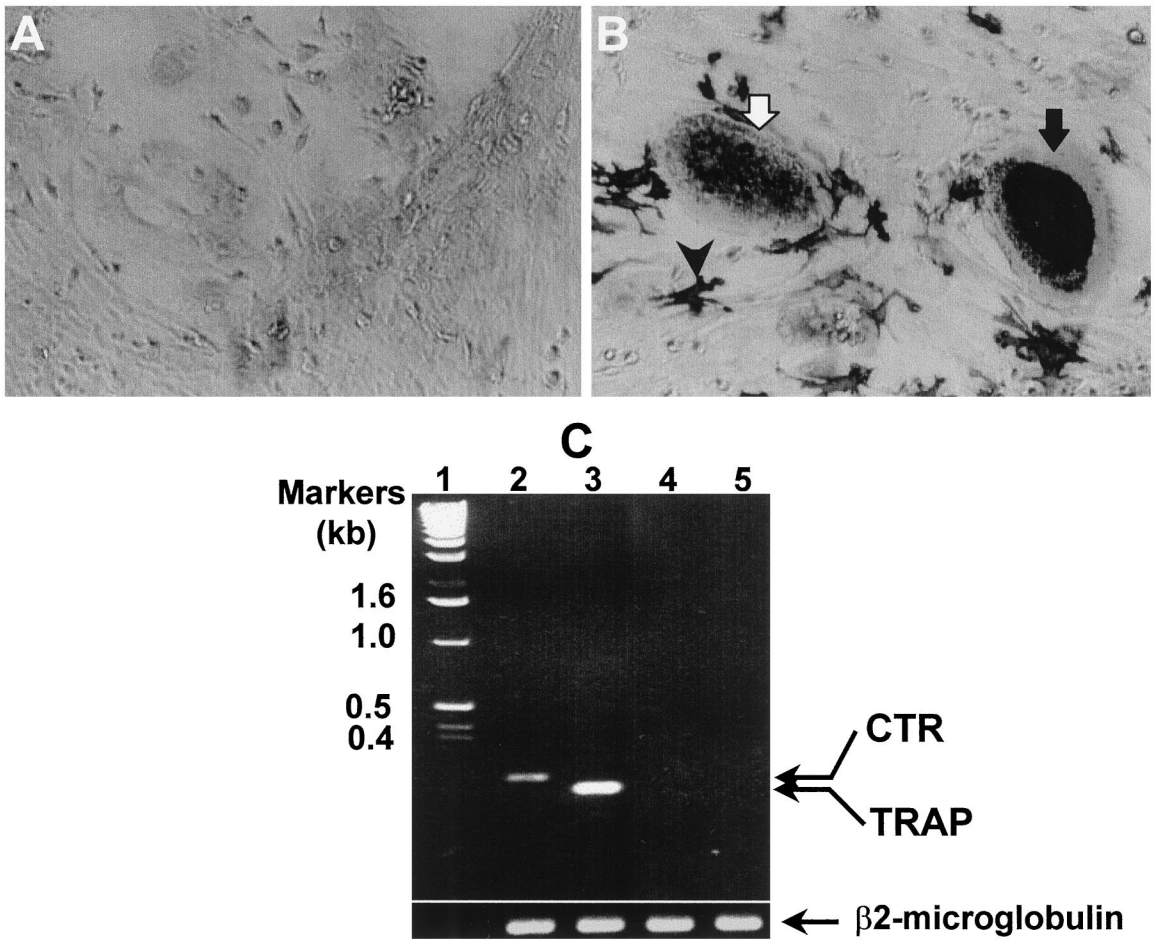

FIG. 2. Immunocytochemical analysis of HSCs and hMSC coculture (A and B): After 3 weeks, coculture was stained with mouse IgG (A) or anti-VNR antibody (B). Large multinucleated cells (*black arrow*) and mononucleated cells (*arrowhead*) were specifically stained by anti-VNR antibody, as compared with the control antibody; hMSCs were not stained with this antibody. Whereas many multinucleated cells showed high levels of staining, a portion of these cells stained slightly with the anti-VNR antibody (*white arrow*). RT-PCR analysis of RNA, prepared from HSCs and hMSC coculture (lanes 2 and 3) or cultures of hMSCs alone (lanes 4 and 5) (C): Data show the expression of both CTR mRNA and TRAP mRNA in cocultures of hMSCs and HSCs. Lane 1, 1-kb DNA ladder; *bottom*, control amplification of β2-microglobulin mRNA.

Effects of cell contact on Ocl formation

To determine whether cell contact is important in hMSC-based Ocl formation, HSCs and hMSCs were cocultured in either the same chamber or in chambers separated by a 0.45-μm membrane. Separation of the hMSCs and HSCs reduced Ocl formation by 75%, suggesting that cell contact is important, but not absolutely required, for Ocl formation (Fig. 5).

Effects of differentiated hMSCs on Ocl formation

Rodent models of Ocl formation have been used to show that mature osteoblasts can support the formation of Ocls (see Ref. 29). To analyze the role of OS-treated hMSCs on Ocl formation, we cocultured HSCs with hMSCs pretreated with OS to induce osteogenic differentiation. hMSCs were cultured in OS medium for various lengths of time (2–13 days) before adding the HSCs. Interestingly, we found that OS-treated hMSCs actually inhibited the formation of Ocls (Fig. 6), whereas dexamethasone alone (which is insufficient in inducing osteogenic differentiation of hMSCs) did not inhibit Ocl formation in this coculture (data not shown). These data suggest that the inhibitory effect of OS-treated hMSCs on Ocl formation is dictated by the differentiated state of hMSCs.

Effects of 1,25(OH)$_2$D$_3$ on Ocl formation

Because 1,25(OH)$_2$D$_3$ is known for its stimulatory effect on Ocl formation, we analyzed the effect of this hormone in this coculture system. We found that Ocl formation was increased if the coculture was performed in the presence of 10^{-9} M, but not 10^{-8} M 1,25(OH)$_2$D$_3$ (Table 1).

Expression and role of cytokines on Ocl formation

hMSCs express numerous growth factors and cytokines, including IL-6, IL-11, M-CSF, stem cell factor, and LIF (14, 15) that stimulate Ocl formation *in vitro*. We found that IL-6, IL-11, LIF, and M-CSF were undetectable in cultures of HSCs alone, whereas the levels of IL-6 and LIF were approximately 10 times higher in HSCs and hMSC coculture than hMSCs alone (data not shown). Coculture of HSCs and hMSCs did not alter the levels of M-CSF (0.76 $\pm$ 0.14 ng/ml *vs.* 0.73 $\pm$

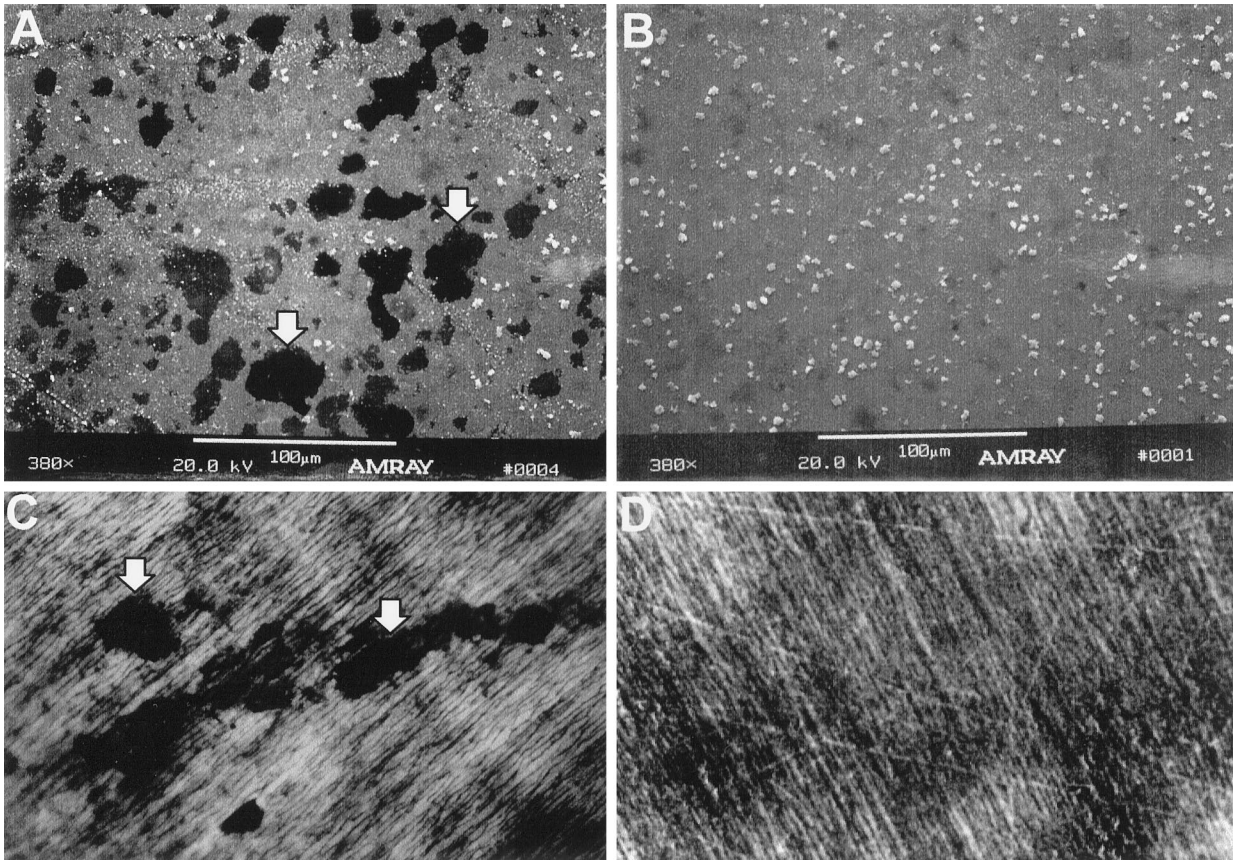

FIG. 3. Formation of resorption pits on bone analog (A) and elephant tusk dentine (C) by HSCs and hMSC cocultures. No pits were formed in culture of HSCs alone (B and D). Note the presence of pits of large areas (*arrow*). A and B, Scanning electron microscope; C and D, light microscope.

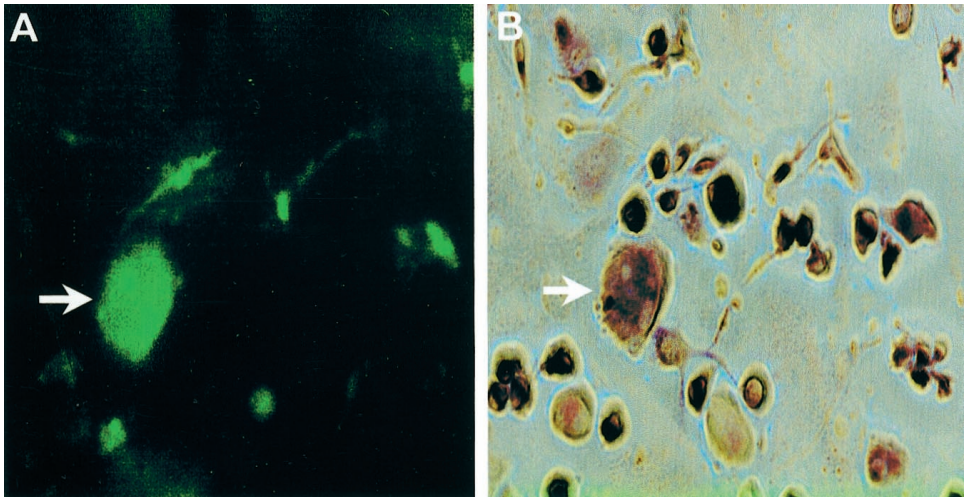

FIG. 4. Formation of Ocls from EGFP-transduced HSCs in coculture with hMSCs. After 3 weeks, cocultures were stained for TRAP, and the photomicrography was taken using fluorescent (A) and light (B) microscopes. Note the presence of an $EGFP^+/TRAP^+$ multinucleated cell (*arrow*) containing 6 nuclei. These nuclei were confimed by 4′,6-diamidino-2-phenylindole staining (data not shown).

0.17 ng/ml) and IL-11 (1.83 ± 0.09 ng/ml *vs.* 1.94 ± 0.15 ng/ml). These data suggest that HSCs induce the production of osteoclastogenic cytokines IL-6 and LIF by hMSCs.

We found that treatment of the hMSCs with OS reduced the levels of IL-6, IL-11, and LIF in the medium (Fig. 7). Because OS-treated hMSCs inhibited the formation of Ocls, we examined the roles of IL-6, IL-11, and LIF in Ocl formation in this coculture. As shown in Table 2, addition of anti- IL-6, IL-11, or LIF antibodies reduced Ocl formation by 75%, compared with untreated cocultures. These data suggest that these cytokines play important roles in Ocl formation mediated by hMSCs.

Discussion

We report that we have developed a coculture of highly purified human CD34+ cells (HSCs) and human MSCs (hMSCs) to demonstrate that hMSCs promote the formation of Ocls that express markers of osteoclasts and resorb bone *in*

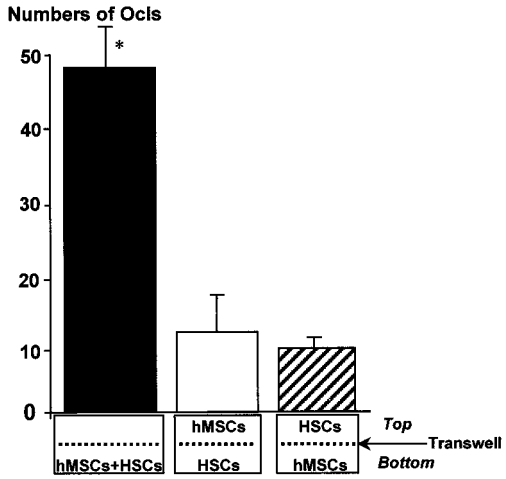

FIG. 5. Role of cell contact in Ocl formation. HSCs and hMSCs were cocultured in the same chamber (■) or separated by means of 0.45-μm filters, as shown in diagram (hMSCs on *top* chamber and HSCs on *bottom* chamber (□); hMSCs on *bottom* and HSCs on *top* (▨). *, $P < 0.01$ *vs.* cocultures separated by filters.

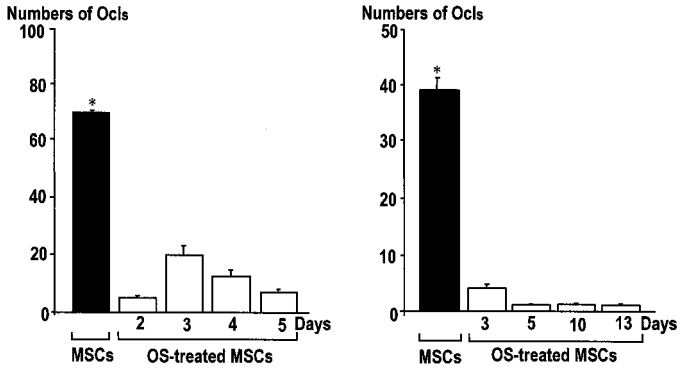

FIG. 6. Inhibition of Ocl formation by OS-treated hMSCs. Osteogenic differentiation of hMSCs was induced by treatment with OS for 2, 3, 4, 5, 10, or 13 days before adding the HSCs. Ocl formation was inhibited in cocultures of HSCs with OS-treated hMSCs (□). *, $P < 0.01$ *vs.* cocultures of OS-treated hMSCs and HSCs.

TABLE 1. Effects of $1,25(OH)_2D_3$ on Ocl formation in HSCs and hMSC coculture

$1,25(OH)_2D_3$	0	10^{-9}M	10^{-8}M
Ocls	17 ± 4	30 ± 5[a]	21 ± 4

After 2 weeks, cocultures were stained for TRAP and the numbers of Ocls (≥3 nuclei) were counted.

[a] $P < 0.05$ *vs.* untreated cultures.

vitro. Coculture of hMSCs with HSCs, which were transduced with a retroviral vector that expresses EGFP, produced $EGFP^+$ Ocls, demonstrating that Ocls arise from these HSCs. Osteoclastogenesis in this coculture is dependent on cell contact and independent of added hormones, cytokines, and growth factors, suggesting that hMSCs supply factors required to induce terminal differentiation of HSCs into Ocls. It is noteworthy that osteoclastogenesis in this coculture system is enhanced by $1,25(OH)_2D_3$, as reported in other models (20, 21). Levels of IL-6 and LIF, well-known regulators of osteoclastogenesis *in vitro* (2) and mediators of bone loss in diseases or caused by estrogen deficiency (22, 23), were elevated in conditioned media from the cocultures of HSCs and hMSCs. Interestingly, HSCs seem to regulate cytokine production by hMSCs, because conditioned media from hMSCs contained low levels of these cytokines. Our data suggest that HSCs induce hMSCs to produce cytokines, although current data cannot rule out the possibility that the induction of these cytokines occurs in both cell lineages. Recently, findings show that HSCs induced IL-6 production by stromal cells and osteoblastic cells (24, 25). The induction of these cytokines within the coculture may be important to the role of hMSCs in osteoclastogenesis, because neutralization of the activities of these cytokines by the addition of specific antibodies significantly inhibited the formation of Ocls.

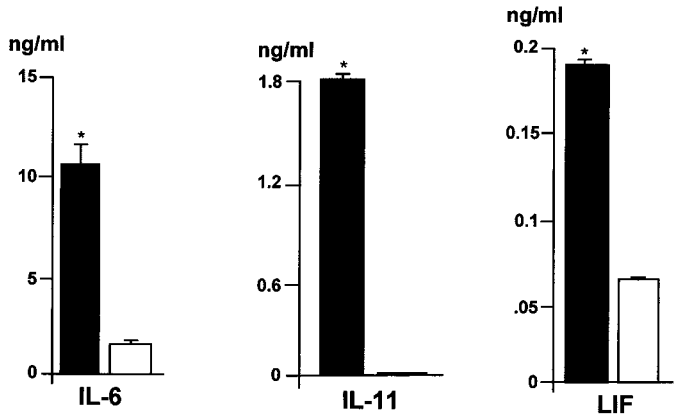

FIG. 7. Measurement of IL-6, IL-11, and LIF in conditioned media in hMSCs and HSC coculture (■) or in OS-treated hMSCs and HSC coculture (□). *, $P < 0.01$ *vs.* cocultures of OS-treated hMSCs and HSCs.

TABLE 2. Effects of anti-IL-6, IL-11, or LIF neutralizing antibodies on Ocl formation in HSCs and hMSC coculture

	0	Control IgG	Antibody to:		
			IL-6	IL-11	LIF
Ocls	21 ± 1	17 ± 5	6 ± 1[a]	4 ± 1[a]	5 ± 2[a]

hMSCs and HSCs were co-cultured for 3 weeks in the presence of nonspecific mouse IgG (control) or anti-IL-6, IL-11, or LIF antibodies (5 μg/ml each). After 3 weeks, cocultures were stained for TRAP and the numbers of Ocls (≥3 nuclei) were counted.

[a] $P < 0.01$ *vs.* untreated cultures. Anti-M-CSF neutralizing antibody (0.5 μg/ml) also significantly inhibited Ocl formation (data not shown).

Ocl formation requires the interactions between Ocl precursors and stromal cells (6) known to secrete cytokines, extracellular matrix molecules, and growth factors. The recent finding that Ocls can be formed from G-CSF-mobilized HSCs peripheral blood only in the presence of IL-1, IL-3, and GM-CSF suggests that stromal cells may not be required for human osteoclastogenesis (11). We have found these cytokines to be less potent than hMSCs in inducing Ocl formation from nonmobilized HSCs (data not shown) and that preventing contact between hMSCs and HSCs reduced Ocl formation. Although mobilization of HSCs by G-CSF may yield cells that can differentiate into Ocls *in vitro* in the absence of stromal cells, our data suggest that human osteoclastogenesis from marrow-derived HSCs is greatly enhanced by physical contact between Ocl precursors and mesenchymal cells, as

previously reported in other models (26, 27). Evidence has accumulated that molecules such as M-CSF (28), cadherin-6/2 (3), and TRANCE/RANKL/ODF/OPGL (4, 5) expressed on the membrane of stromal cells are involved in Ocl formation. Additional studies are required to identify the roles of these molecules in hMSC-mediated Ocl formation.

The relationship between the differentiated state of the mesenchymal cell and its potential to support Ocl differentiation is poorly understood. We have shown that, under defined conditions, hMSCs can differentiate into cells of specific lineages, including osteoblasts, adipocytes, and chondrocytes (12). In this study, we examine the relationship between hMSCs treated with OS and the potential of these cells to support Ocl formation. We found that hMSCs treated with OS for various lengths of time actually inhibit Ocl formation, suggesting that hMSCs regulate osteoclastogenesis as undifferentiated progenitor cells. Although the molecular mechanisms governing the inhibitory effects of OS treatment are not understood, the inhibitory effects may, in part, be caused by the decrease in the levels of IL-6, IL-11, LIF, and M-CSF, which are produced in the coculture, since blocking the activities of the cytokines with neutralizing antibodies inhibits Ocl formation.

Our data showing the inhibitory effects of OS-treated hMSCs on Ocl formation contrast with previous reports in which Ocl formation is promoted by osteoblasts (29). It should be emphasized that, in this study, we have used hMSCs and osteogenic hMSCs of the same marrow donor to demonstrate functional differences between these cell populations. Our data are consistent with the results of studies that demonstrate that Ocl formation is promoted by undifferentiated stromal cell lines (30) and immature osteoblasts (31), and this also occurs in culture of whole bone marrow cells where Ocls are found in close proximity to spindle-shaped stromal cells. The proximity between stromal cells and hematopoietic progenitor cells within the bone marrow cavity, and the recent evidence that ablation of osteocalcin-expressing cells did not prevent osteoclast differentiation (32), support this conclusion. It cannot be ruled out that the stimulatory effects of osteoblastic cells in osteoclastogenesis may result from immature osteoprogenitor cells present in those cell preparations.

It is clear that hMSCs regulate HSC proliferation and differentiation. The ability of hMSCs to maintain long-term cultures initiating cells in long-term bone marrow culture and to support megakaryocytopoiesis from HSCs has been demonstrated (15, 33). In this work, we show that hMSCs promote Ocl differentiation from HSCs. Taken together, the data show that multipotential hMSCs support multilineage differentiation of hematopoietic cells and suggest that our model provides opportunities to understand the mechanisms by which hMSCs regulate human osteoclastogenesis.

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