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

GABRIEL MBALAVIELE, NEELAM JAIswAL, ALICE MENG, LINZHAO CHENG, CHRISTIAN VAN DEN BOS, AND MARK THIEDE
Osiris Therapeutics, Inc., Baltimore, Maryland 21231-3043

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 (OcIs). Coculture of hMSCs with HSCs, transduced with a retroviral vector that expresses enhanced green fluorescent protein, produced enhanced green fluorescent protein+ OcIs, further demonstrating that OcIs arise from HSCs. These OcIs 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)
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 Dominguez, 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 × 10^4 cells per cm² 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 Ficol-Faque (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 × 10^4/cm²) were added to monolayers of hMSCs or OS-treated hMSCs, and the coculture was used for the duration of the experiments.

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.

**Enzyme-linked immunosorbent assay analysis of cytokines**

hMSCs or OS-treated hMSCs (7 day-treatment with OS) were cocultured with HSCs (5 × 10^4 cells/cm²) for 2, 3, 4, 5, 10, or 13 days. The coculture was used for the duration of the experiments.

**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 Cetus, Foster City, CA). The following conditions were used for PCR: denaturation 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'-CGATCACAACTGCTGACCTACG-3' and 5'-ACCGAGTGGAGGTCTTGAGTCCC-3', PCR product size = 150 bp; CTR receptor (CTR): 5'-TTCTCAAGGGCTTCTTTGTTCCAT-3' and 5'-CTTGGTCATTGGCCTGGT-3', PCR product size = 205 bp. The CTR primers were designed based on the conserved region of the receptor family. PCR products were separated by electrophoresis through a 1% agarose gel and visualized by staining the gel with ethidium bromide.

**Transduction of CD34+ cells**

To demonstrate that Ocls are derived from HSCs, we cocultured HSCs with OS-treated HSCs transfected 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 × 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 × 10^6 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.
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 (arrow head).](image)
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)2D3 on Ocl formation

Because 1,25(OH)2D3 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)2D3 (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 ± 0.14 ng/ml vs. 0.73 ±
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...
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)2D3, 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.

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 suggested 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.
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 Ols 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 osteocalin-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 megakaryocytogenesis 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.

Acknowledgment

We are grateful to Don Simonetti and Mark Moorman for flow cytometry analysis of data and to Drs. Michael Horton and Ernst Hunziker for providing us with the 236c antibody to VNR and elephant tusk slices, respectively.
Seventh Annual Gynecology CME Course Announced


As authors of the highly regarded Comprehensive Gynecology textbook, course directors Drs. Arthur L. Herbst and Daniel R. Mishell, Jr. bring their unique expertise to a practical, clinically applicable format. The faculty also includes Drs. Alan Copperman, Sebastian Faro, Steven R. Goldstein, Vicki L. Seltzer, and Sandra R. Valaitis.

The three-day curriculum, developed by the nation’s recognized authorities in the field of gynecology, is structured to provide an annual review of new developments for the physician who attends the female patient, and allows ample opportunity for one-on-one discussion with faculty and colleagues. Topics covered include: primary care concerns, HIV and STDs, infertility, endometrial hyperplasia and carcinoma, vaginitis, hormone replacement, urinary dysfunction, and vaginal sonography.

The Center for Bio-Medical Communication, Inc. (CBC) is accredited by the Accreditation Council for Continuing Medical Education (ACCME) to sponsor continuing medical education for physicians. CBC designates this course for a maximum of 14 hours in Category 1 of the AMA Physicians Recognition Award. AAPA, AAFP, and ACOG credits pending.

As enrollment is limited, early registration is recommended. Early bird tuition (until September 15) is $520; after September 15, tuition is $600. Course organizers have arranged special reduced rates at the Roosevelt Hotel, as well as reduced airfares through a designated travel agent.

For further information, contact: Center for Bio-Medical Communication, Inc., Tel: (201) 342-5300; Fax: (201) 342-7555; E-mail: cmeinfo@cbcbiomed.com.