Role of macrophage-colony stimulating factor and osteoclast differentiation factor in osteoclastogenesis of bone marrow derived stem cells

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Macrophage colony stimulating factor (M-CSF) and osteoclast differentiation factor (ODF) regulate osteoclastogenesis in vivo. Regulation of osteoclast development in vitro by these cytokines has been reported in the present study. Simultaneous addition of ODF and M-CSF during initiation of bone marrow culture inhibited osteoclastogenesis. However, delayed addition of ODF (three days after initiation of the culture) resulted in dramatic increase in phenotypically and functionally mature osteoclasts. Delayed addition of ODF beyond day three decreased osteoclastogenesis. Further, removal of M-CSF as early as day three inhibited ODF-induced osteoclastogenesis. These studies provided evidence for the importance of co-ordinated regulation of osteoclastogenesis by M-CSF and ODF.

Osteoclasts are multinucleated cells derived from the myeloid lineage precursors in bone marrow1. Molecular characterization of osteoclast differentiation and bone resorptive function has been challenging because of several limitations2. Limited availability of osteoclasts in vivo, fragile nature of the cells isolated from bone, difficulty in isolation procedure because of adherence to calcified bone matrix and, lack of availability of osteoclast cell lines warranted an efficient in vitro osteoclast development model with amenability to perform different functional assays. A number of natural and induced mutant mouse models have revealed essential role for macrophage-colony stimulating factor (M-CSF) and osteoclast differentiation factor (ODF) in osteoclastogenesis2,4. M-CSF promotes clonal expansion of hematopoietic progenitors in vitro and in vivo. ODF is expressed by stromal cells, osteoblasts and by activated lymphocytes2,5. It binds to receptor for activation of nuclear factor Kappa B (RANK), a new member of tumor necrosis factor receptor superfamily6,7. Consistent with a requirement for RANK/RANK ligand interaction in osteoclast growth and differentiation, RANK ligand deficiency in mice showed severe osteopetrosis8.

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Materials and Methods

Reagents—Recombinant murine M-CSF, ODF and TNF-α [R&D Systems, Inc (Minneapolis, MN)], gelatin type A, leucocyte acid phosphatase kit, phaloidin mixed isomers and triton X-100 [Sigma Diagnostic (St Louis, MO)], microscope slides [Fisher Scientific (Pittsburgh, PA)] and Crystal/Mount [Biomedia Corporation (Foster City, CA)] were purchased commercially.

Mouse bone marrow culture—Bone marrow cells were flushed from tibiae and femora of FVB/N mice and cultured in DMEM [GIBCO] containing FBS (10%), penicillin-streptomycin and L-Glutamine at 7.5 x 10^5 cells/ml in 24-well plates. Each well was prepared by inserting Thermomax plastic cover slips [Nalge Nunc International (Naperville, IL)] and adding 1 ml of 1% gelatin type A, 2 hr before initiation of
the culture. Cells were fed by replacing the old media with fresh media. M-CSF (50 ng/ml) and ODF (80 ng/ml) were added to the bone marrow cells at different time points as indicated in results section. TNF-α (20 ng/ml) was added with ODF in all the cultures.

Tartrate resistant acid phosphatase (TRAP) staining—Diazotized fast garnet GBS solution (0.2 ml); naphthol AS-BI phosphate solution (0.1 ml); acetate solution (0.4 ml); and tartrate solution (0.2 ml) were mixed with 9 ml of deionized distilled water (ddH2O) prewarmed to 37°C (the TRAP-staining solution). Cells were fixed with 2 ml of fixative solution (25 ml citrate solution, 65 ml acetone, and 8 ml of 37%, formaldehyde) for 30 sec and rinsed thoroughly in ddH2O. One ml of prewarmed (37°C) TRAP solution was added to each well. Plates were incubated in dark for 1 hr at 37°C, rinsed with ddH2O, and counterstained with hematoxylin solution for 2 min. TRAP+ mono, bi, and multinucleated cells were enumerated microscopically (20 fields of 40x). Osteoclast and its precursors appeared with red or maroon granules in the cytoplasm.

Actin ring formation assay—Cells were washed with PBS, fixed for 5 min in 3.7% of formaldehyde in PBS, washed extensively in PBS, dehydrated with acetone, permeabilized with 0.1% triton X-100 in PBS, and then washed again in PBS. Cells were stained with 50 µg/ml of fluorescent phalloidin conjugate solution in PBS for 40 min at room temperature, and then washed with PBS to remove unbound phalloidin conjugate. The actin rings formed by osteoclasts were detected with a fluorescence microscope.

Pit formation assay—BD BioCoat Osteologic bone cell culture System (Bedford, MA) consists of a resorbable artificial bone analog in the form of submicron synthetic calcium phosphate thin films in 24-well discs. At day 9 of bone marrow cell culture, 1 ml of bleach solution (~6% NaOCl, ~5.2% NaCl) was added to each well. The media/bleach mixture was agitated for 5 min and then aspirated. Each disc/well was washed in 2 ml of ddH2O several times. The disc was removed, and washed again in running ddH2O. The discs were air-dried and examined microscopically using phase optics and/or dark fields with 20-100X magnifications. The resorption pits were visualized under transmitted light.

Results

Critical time of addition of ODF for M-CSF dependent osteoclastogenesis from bone marrow cells—ODF is expressed in osteoblasts as membrane bound and secreted forms in bone marrow microenvironment. In vivo administration of ODF increases osteoclastogenesis and bone resorption. Requirement of ODF in osteoclastogenesis is revealed through osteopetrosis in mice deficient in ODF. Soluble ODF circumvents the need for stromal cells and 1,25-dihydroxy vitamin D3 in osteoclastogenesis. Timing of addition of M-CSF along with 1,25-dihydroxy vitamin D3 have been shown to be critical for osteoclast development. Thus simultaneous addition of M-CSF and 1,25-dihydroxy vitamin D3 inhibits osteoclast formation11,12. Since 1,25-dihydroxy vitamin D3 induces production of ODF from the stromal cell lines, we predicted a possible role for timing of addition of ODF in M-CSF dependent osteoclastogenesis. To test this directly, bone marrow cells were cultured in the presence of M-CSF (50 ng/ml) for 9 days in the presence of ODF (80 ng/ml) added at day 0, 3, 5, 6 and 7 after the initiation of the culture. The cells were subsequently maintained in media containing ODF until the end of day 9 culture period and stained with TRAP solution to determine mono, bi, and multinucleated TRAP+ cells. Addition of ODF at day 0, along with M-CSF failed to induce the osteoclast development (Fig. 1). Delaying the addition of ODF until day 3 to M-CSF-induced bone marrow cultures resulted in profoundly increased mono, bi, and multinucleated TRAP+ cells by 130, 7, and 2.3-folds respectively (Fig. 1a,b,c). Further delay in the addition of ODF until day 5, 6 or 7 respectively resulted in reduction of TRAP+ mononucleated cells by 32, 82 and 97%; binucleated TRAP+ cells by 27, 47 and 89% and multinucleated cells by 33, 56 and 93% in comparison to cultures in which ODF was added from day 3-9 (Fig. 1a,b,c).

Effect of removal of M-CSF on ODF-dependent TRAP+ cells in bone marrow cultures—The development of osteopetrosis in mice deficient in ODF or M-CSF established a role for both these cytokines in osteoclast development and maturation in vivo. To determine the optimal time kinetics of M-CSF requirements in vitro differentiation of bone marrow cells to osteoclasts, we tested the effect of M-CSF on ODF dependent OCL differentiation and function. Bone marrow cells cultured in the presence of M-CSF
were cultured in the presence of M-CSF (50 ng/ml added from day 0-9) along with ODF (80 ng/ml added at initiation of the culture). At day 9, the cells were stained with TRAP solution. Mono, bi, and multinucleated cells were counted randomly. The values are presented as number of TRAP positive cells/20 field counted randomly.

ODF (from day 3 until the end) resulted in significant generation of multinucleated osteoclasts, as characteristic TRAP expression was observed. Withdrawal of M-CSF after 3 days induced a decrease in generated mono, bi, and multinucleated TRAP positive cells by 16, 40, and 70%, respectively in cultures in which M-CSF was removed from all 9 days (Fig. 3a-c).

Initiation of ODF and M-CSF for 3 days resulted in osteoclastogenesis. Actin ring formation, was revealed by their ability to resorb artificial bone analog. Thus, at day 9 of the culture, the bone marrow-derived osteoclasts exhibited characteristic prominent resorption pits (Fig. 2d).

Discussion
The results described in this study provide evidence for the importance of co-ordinated regulation of osteoclastogenesis by M-CSF and ODF. While, simultaneous addition of ODF and M-CSF during initiation of bone marrow culture inhibited osteoclastogenesis, delaying the addition of ODF by 3 days after initiation of culture resulted in dramatic increase in phenotypically and functionally competent osteoclast cells. Delayed addition of ODF beyond day 3 decreased osteoclastogenesis. Further removal of M-CSF as early as day 3 inhibited ODF induced osteoclastogenesis. These results showed a requirement for sequential addition of M-CSF and ODF for efficient production of osteoclasts in vitro.

It is likely that M-CSF expands the myeloid precursor pool and permits ODF to act predominantly on the osteoclast precursors to induce them to differentiate into mature osteoclasts. This is consistent with M-CSF to induce RANK, the receptor for ODF in osteo-
clast precursors. Thus, early addition of M-CSF-induced RANK in the osteoclast precursors, which became ideal target for ODF (RANK ligand) added on day 3. The requirement of continuous presence of M-CSF could be attributed to the role of M-CSF in survival and maturation of osteoclasts. This is consistent with earlier reports suggesting that M-CSF stimulate the proliferation and differentiation of osteoclast precursors to mature osteoclasts. Thus, expression of c-fms, the receptor for M-CSF in mature osteoclast, has implicated a role in M-CSF in survival and maturation of osteoclasts.

Our studies demonstrating a requirement for sequential addition of M-CSF and ODF were consistent with the previous observation made with M-CSF and 1,25-dihydroxy vitamin D3, which activates the osteoblasts and promote secretion of ODF. Timing of addition of osteoclast differentiation promoters such as M-CSF and 1,25-dihydroxy vitamin D3 is critical for the osteoclast development. For example, while sequential addition of M-CSF followed by 1,25-dihydroxy vitamin D3 promotes the growth of osteoclasts, simultaneous addition of these two agents inhibits the osteoclast formation. This is consistent with the inhibition of osteoclastogenesis upon simultaneous addition of M-CSF and ODF in the studies described in this report. The current studies emphasized the need for sequential addition of M-CSF and
ODF in bone marrow cultures to generate high number of phenotypically and functionally competent osteoclasts in vitro. This approach allowed generation of increased numbers of functionally mature multinucleated osteoclasts for biochemical and molecular analysis.

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References


