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Osteoclasts Secrete the Chemotactic Cytokine Mim-1

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Osteoclasts are terminally differentiated, multinucleated cells of monocytic origin. In this study, we report that osteoclasts secrete a 35 kD protein and that phorbol myristate acetate treatment stimulates secretion dramatically. Peptide digests of the protein were analyzed by mass spectroscopy. The protein was identified as *myb* induced myeloid protein-1 precursor (mim-1 protein). Mim-1 is expressed specifically by hematopoietic cells and has no known function. It is homologous with the neutrophil chemokine, chondromodulin II, which stimulates proliferation of osteoblasts and chondrocytes. Western analysis showed that osteoclasts secrete mim-1 into culture media. Immunofluorescence studies demonstrated a cytoplasmic and perinuclear distribution of mim-1 in both avian osteoclasts and human osteoclast-like cells. Expression and secretion of a chemokine-like protein by osteoclasts suggests a novel signaling pathway in the bone microenvironment that may be involved in coordinating bone remodeling.

Key Words: **osteoclast; osteoblast; osteoporosis; mim-1; chemokine; bone remodeling.**

Osteoclasts are terminally differentiated cells formed by fusion of precursor cells derived from hematopoietic stem cells (1) circulating in the monocyte fraction (2, 3). Osteoclast differentiation is a complex process that requires M-CSF and is driven primarily by RANKL (ODF, TRANCE) (4, 5). While M-CSF and RANKL, or closely related factors such as TNFα, are essential for differentiation, additional osteoclast-inductive agents or synergistic effectors of RANKL are likely to be important in the development of active, mature osteoclasts (6, 7).

A variety of signaling molecules have been proposed as key elements in signaling between osteoclasts and osteoblasts (8). Osteoclasts respond to numerous factors that are derived from bone or are in the bone microenvironment (9–12). Under conditions of normal bone turnover, bone resorption is followed by new bone synthesis. The mechanisms regulating recruitment of osteoblast precursors into areas of recent resorption are poorly understood, but presumably involve signaling between osteoclasts and osteoblasts (13).

We have identified mim-1 as an osteoclast secreted protein. Mim-1 is expressed by cells of hematopoietic origin (14), and is homologous with the neutrophil chemokine chondromodulin II (LECT2) (15). Immunofluorescence microscopy demonstrates that both mature avian osteoclasts and differentiating human osteoclast-like cells, but not mesenchymal stem cells, express mim-1. Mim-1 secretion by osteoclasts precedes PMA stimulated increases in bone resorption and parallels PMA induced down regulation of PKC isozymes. Secretion of this chemokine-like protein by osteoclasts may be involved in coordinating bone remodeling.

MATERIALS AND METHODS

Osteoclast cultures and bone resorption assays. Avian osteoclasts (egg-laying White Leghorn hens) were isolated as described (16) and L-[^{3}H]-proline-labeled devitalized bone was used as substrate in osteoclast resorption assays (17). Osteoclasts were plated at 2–3 $\times$ 10^3 cells/well on 24 well plates with 100 μg of labeled 20–40 μm ^{3}H labeled bone fragments. To avoid contamination due to fusing macrophages or growth of fibroblast/osteoblast cells bone resorption was

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Abbreviations used: PMA, phorbol myristate acetate; Mim-1, myb-induced myeloid protein-1; PBS, phosphate buffered saline; TRAP, tartrate resistant acid phosphatase; EGTA; ethylene glycol-bis(β-aminoethylether)-*N*,*N*′,*N*′-tetraacetic acid, EDTA, ethylenedinitrilotetraacetic acid; NaF, sodium fluoride; PMSF, phenylmethylsulfonylfluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinylidine difluoride; BSA, bovine serum albumin.

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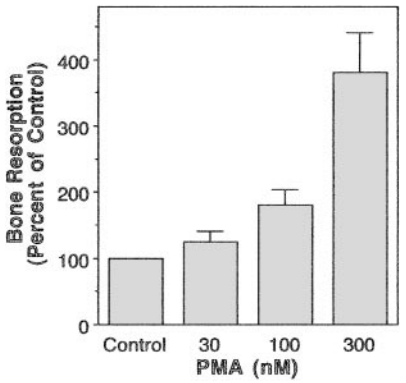


FIG. 1. PMA stimulate osteoclastic bone resorption. Bone resorption assays using 150 μg labeled bone were cultured for 4 days in the presence of increasing concentrations of PMA as described under Materials and Methods. Resorption was quantified by measuring ^{3}H proline released to the media. Data are the mean ± SEM of five separate experiments each performed in quadruplicate.

measured by day 4. Bone degradation was determined by measuring label released to the media. The ^{3}H proline release and pit assays produce comparable results (17, 18).

Human osteoclast-like cell differentiation. Human blood monocytes were isolated by plasma pheresis from healthy volunteers. Blood monocytes (1×10^5 cells/well) were cultured in the presence of 90% confluent MG63 cells in Minimal Essential Media α containing 10% fetal bovine serum, 10^{-7} M dexamethasone, and 10^{-8} M 1, 25 dihydroxyvitamin D_3. TRAP staining was used as a marker for the osteoclast phenotype. In parallel experiments cells were plated at a similar density on 18 mm cover slips and immunostained for mim-1.

Osteoclast lysis and Western analysis. Avian osteoclasts were washed with phosphate buffered saline (PBS) and lysed as previously described (19). Cells were solubilized 1 h with rotation and the Triton insoluble material removed by centrifugation at 15,000*g* for 5 min at 4°C. Lysates (25 μg protein) were resolved on 10% SDS-PAGE. Protein was transferred under standard conditions (20) to PVDF membranes. Mim-1 was detected by Western analysis using a polyclonal antibody to a trpE-mim-1 fusion protein (14) generously provided by Scott Ness, University of New Mexico. Protein concentrations were determined by the Bio-Rad DC assay (Bio Rad, Richmond, CA).

Reduction/alkylation and digestion of protein. Protein bands separated on SDS PAGE gels were reduced, alkylated, and digested in-gel using published procedures (21, 22). Gel pieces were destained, extracted three times for 40 min with 200 mM NH_4HCO_3 in 50% acetonitrile at 30°C, dried by vacuum centrifugation, rehydrated with 10 mM dithiothreitol and reduced for 1 h at 56°C. Proteins were alkylated with 100 mM iodoacetic acid for 30 min, in the dark, at room temperature. The gel pieces were dried again and reswollen with 50 μg/ml trypsin in 100 mM NH_4HCO_3, covered with 200 mM NH_4HCO_3 and incubated overnight at 30°C. The reaction was quenched with 2 μl of 10% trifluoroacetic acid followed by removal of the supernatant. The gel pieces were extracted twice with 100 μl 0.1% trifluoroacetic acid in 60% acetonitrile. The extracts and supernatant were combined, taken to near dryness in a vacuum centrifuge and stored frozen until analyzed.

Microcapillary mass spectrographic analysis. Protein digests were analyzed using a microcapillary HPLC coupled to a Finnigan MAT LCQ Quadrupole Ion Trap Mass Spectrometer. Peptides were resolved using 150 μm (inner diameter) porous polymer monolithic columns (23). Data were generated using the Finnigan triple play data-dependent analysis, in which an ion identified in a full mass range scan is scanned at high resolution to determine its appearance mass and charge state and then fragmented to give a tandem (MS/MS) mass spectrum. Instrument parameters were: 210°C metal capillary, 1.10 kV spray, and 35% relative collision energy. Spectra were collected with two microscans at a 5×10^7 automatic gain control target for full scans, five microscans at 1.5×10^6 automatic gain control target for high resolution scans, and eight microscans at 1×10^7 automatic gain control target for MS/MS scans. MS/MS spectra were searched against the OWL non-redundant protein database (24; Center for Information Technology, NIH, Bethesda MD) as described (25). Results were confirmed by manually comparing observed and predicted fragmentation patterns for the identified peptides.

Immunofluorescence microscopy. Osteoclasts cultured on 18 mm glass cover slips were washed with phosphate buffered saline (PBS), fixed in 3% formaldehyde, permeabilized with 100% methanol for 30 min at −20°C, and rehydrated in 1% BSA in PBS at 23°C for 15 min. Mim-1 polyclonal antibody or nonimmune serum, diluted 1:1000, were incubated on cover slips for 1 h at 23°C. Coverslips were washed four times for 15 min each with PBS. FITC conjugated secondary antibody, 1:1000, was applied to cover slips for 1 h at 23°C, followed by three additional washes. Nuclei were then labeled with Hoescht dye (20 μg/ml), for 1 h.

RESULTS

The phorbol ester PMA stimulates bone resorption 4-fold by isolated osteoclasts with a $K_{0.5}$ between 0.1 and 0.3 μM (Fig. 1). The PMA concentration-dependent increase in bone resorption was paralleled by a decrease in a major 35 kD protein in osteoclast cell lysates (Fig. 2A, arrow). This PMA stimulated decrease in the 35 kD protein was time dependent. The amount of the 35 kD protein was reduced 50% by 6 h of treatment with 0.3 μM PMA (Fig. 2B, arrow). It was clear from these experiments that PMA had minimal effects on other abundant, proteins in osteoclast lysates, sug-

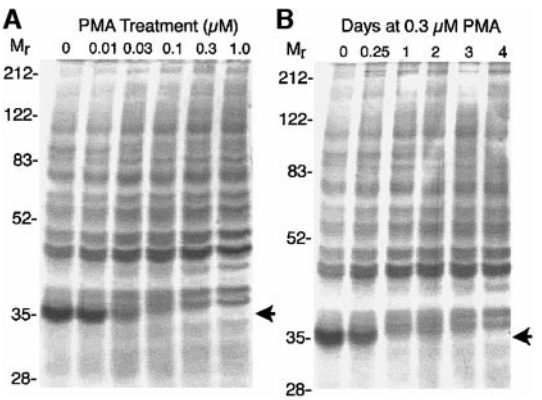
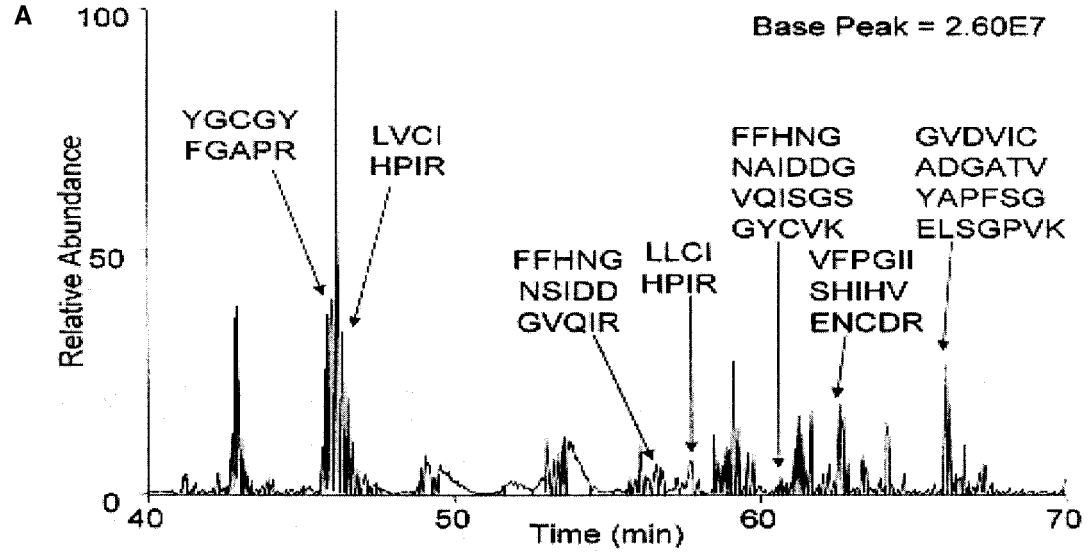


FIG. 2. PMA stimulates concentration- and time-dependent secretion of a 35 kD protein from osteoclast cell lysates. Bone resorption assays were cultured for 4 days in the presence of increasing concentrations of PMA (A) or in the presence of 0.3 μM PMA for the indicated times (B). Osteoclasts were washed and lysed. Cell lysate protein (25 μg) were separated on 10% SDS–PAGE, stained with Coomassie blue. Dried gels were scanned on a UMAX S-12 scanner. Molecular weights (kilodaltons) are indicated on the left. Data are representative of five experiments.



B

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1                mfstkalllaglistalagp w anicagkssn eirtcdrhgc gqysaqrsg- --rphqgvdv lcsagstvya      68 ChondII
1 MPALSLIALL SLVSTAFARQ WEV HPPQQQGRHW AQICSGNPFN RIRGCDRYGC GNYGASR-QG KGEKHKGVDV ICTDGSIVYA      82 Mim-1 N
173                      PPFPQQDAHW AVVCAGNPTN EIRGCDK YGC GYFGAPRRNG KGEKHK GVDV ICADGATVYA     232 Mim-1 C

 69 pftgmivgqe kpyqnknain ngvrisgrgf cvkmfyikpi kykqpikkge klgtllplqk vypgiqshvh iencdssdpt ayl      151 ChondII
 83 PFSGQLSGPI RFFHNGNAID DGVQISGSGY CVKLVCIHPI RYHGQIQKGQ QLGRMLPMQK VFPGIVSHIH VENCDQSDPT HLLRPIPDIS     172 Mim-1 N
233 PFSGELSGPV KFFHNGNAID DGVQIRGSGF CVKLLCIHPI RYNGRISKGQ VLGRMLPMQR VFPGIISHIH VENCDRSDPT SNLERGKGESEMEV 326 Mim-1 C
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FIG. 3. Sequence analysis of 35 kD osteoclast protein. Protein was tryptically digested from gel slices and peptides were resolved by HPLC (A) and sequenced as described under Materials and Methods. The full-length amino sequence of chicken mim-1 (NCBI identifier P08940) is shown (B) and amino acids identified by sequence analysis are illustrated in italics. Some of the peptides had overlapping sequence so that only five peptides are apparent. The full-length sequence of the human mim-1 homolog, chondromodulin II (NCBI identifier AAF 13302), is aligned with mim-1 (lowercase letters; ChondII). Identical amino acids between the repeats in mim-1 (mim-1 N and mim-1 C indicate N and C terminal halves of mim-1) are underlined. Amino acids in chondromodulin II that are identical with one or both repeats in mim-1 are also underlined. Gaps (dashes) were introduced in the chondromodulin II sequence to align the sequence with mim-1.

gesting that the 35 kD protein may have an important regulatory function. Five protein kinase C (PKC) isoenzymes (α, β, δ, ϵ and ζ), representing the three subfamilies of PKC, were also down regulated at least 60% within 8 h by 0.3 μM PMA (26).

The 35 kD protein was one of the most abundant proteins in osteoclast, but its identity was unknown. Therefore, it was isolated and sequenced. Coomassie stained bands were excised from gels post-transfer and tryptically digested (Fig. 3). Tryptic peptides were resolved by HPLC (Fig. 3A). Seven distinct peptides were identified by tandem mass spectrometry. Sequence obtained included 104 of the 326 amino acids of mim-1 (myeloid protein-1), identified previously in neutrophils (14). The amino acids identified by sequence analysis of peptides are illustrated in italics in the full-length sequence of mim-1 (Fig. 3B). One of the peptides identified included the amino acid at position 297, which is the site of a sequence conflict, and was found to be isoleucine rather than tyrosine. No other proteins were identified in the gel band containing mim-1, except a minor contaminant, human keratin. Mim-1 consists of a signal peptide that contains a repeat sequence of approximately 136 amino acids (Fig. 3B). The repeat sequences in mim-1 are aligned with and nearly identical to the human neutrophil chemokine, chondromodulin II (Lect2) (Fig. 3B).

As shown in Fig. 2, osteoclast mim-1 content was PMA responsive. To determine the pattern of mim-1 secretion without stimulation, osteoclasts were cultured in the absence of PMA and mim-1 secretion was determined as a function of time by Western analysis (Fig. 4A). Mim-1 secretion into the media increased throughout the 4-day time course. In separate experiments, osteoclasts were treated with increasing concentrations of PMA, bone resorption was measured, and Western analysis for mim-1 was performed on cell lysates and aliquots of media (Fig. 4B). Western analysis confirmed that the protein secreted by osteoclasts was mim-1. In addition, as bone resorption was stimulated (see Fig. 1), mim-1 decreased in the cell lysates (Fig. 4B, Lysates), and increased in the supernatant (Fig. 4B, Media), a pattern consistent with PMA-dependent secretion.

We tested whether avian and human osteoclast-like cells expressed mim-1 by antibody labeling. This demonstrated that mim-1 is present in both avian osteoclasts and human osteoclast-like cells derived from blood monocytes (Fig. 5). In avian osteoclasts, mim-1 fluorescence was localized in the cytosol and had a

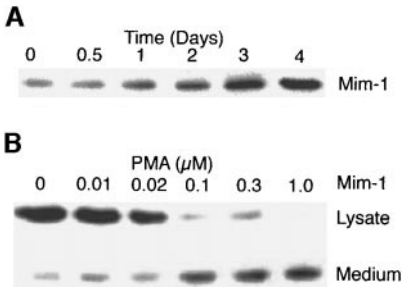

FIG. 4. Western analysis of basal and PMA-stimulated mim-1 secretion by osteoclasts. Osteoclasts were cultured as described above in the absence of PMA and aliquots of media removed at the indicated times (A). In parallel experiments osteoclasts were incubated with the indicated concentrations of PMA for 4 days (B); cells were washed with ice-cold PBS and lysed as described under Materials and Methods. Culture medium was collected from each treatment and boiled in sample buffer. Protein (25 μg) from cell lysates (Lysates) and equal aliquots of the corresponding media (medium) were resolved on 10% SDS-PAGE, transferred to PVDF membrane and the level of mim-1 determined by Western analysis with a polyclonal antibody to mim-1. Data are representative of two separate experiments performed in duplicate.

perinuclear pattern in osteoclasts. The fluorescence intensity decreased in response to PMA treatment (compare Figs. 5A and 5B), in keeping with osteoclast secretion of mim-1. Mim-1 in osteoclasts treated with PMA appeared to be within granules, similar to its distribution in promyelocytes (14). Mim-1 is also present in the human osteoclast precursors (Fig. 5C). In these experiments the plane of focus is at the level of the blood monocytes, which are above the plane of focus of the MG63 cells used in the co-culture system. Consequently, the nuclei of the MG63 cells are out of the plane of focus and appear dark blue rather than bright blue as in the monocytes. Numerous monocytes have begun to fuse as can be seen by the presence of binucleate cells. Background observed with the nonimmune antibody alone was negligible (Fig. 5D).

DISCUSSION

Bone homeostasis is a complex process requiring coordination of the activity of osteoclasts and osteoblasts. A wide variety of molecules are known to be important mediators of cellular signaling between osteoblasts and osteoclasts (7, 12, 27). Many of the known signals are produced by the osteoblasts (28, 29).

We have identified a 35 kD secreted osteoclast protein as mim-1, which was originally cloned from chicken marrow promyelocytes (14). PMA stimulates mim-1 secretion and increased osteoclast activity (Figs. 1 and 2). PMA also increases bone resorption in parallel with increased calmodulin expression and down regulation of all PKC isoenzymes examined (26). Calmodulin antagonists, tamoxifen and trifluoperazine, inhibited PMA-stimulated increases in bone resorption and calmodulin expression but did not effect mim-1 secretion (26).

Mim-1 is very abundant in promyelocytes (14), and the data presented indicate that this is also the case in osteoclasts. The mim-1 homolog, chondromodulin II (Lect2) has chemokine activity (30). It attracts neutrophils (15, 31) and stimulates osteoblast and chondrocyte proliferation (32). Our data indicate that mim-1 is secreted by isolated osteoclasts under basal conditions; it also accumulates in media over a four-day incubation in culture (Fig. 4A). However, mim-1 secretion is substantially increased in response to PMA treatment (Figs. 1 and 2).

The mim-1 sequence consists of two direct repeats of the chondromodulin sequence motif with minor differences. In the repeat sequence only 30 amino acids are non-identical (77% identity) and most of the non-identical sites are conservative substitutions. Chondromodulin II (Lect2) (15, 31) is a 16 kD protein that is expressed primarily in liver, and is a distinct gene product (30).

Previous investigations of mim-1 have focused on transcriptional regulation of mim-1 expression. Northern analysis indicated that bone marrow is the only tissue with detectable expression of mim-1, and that

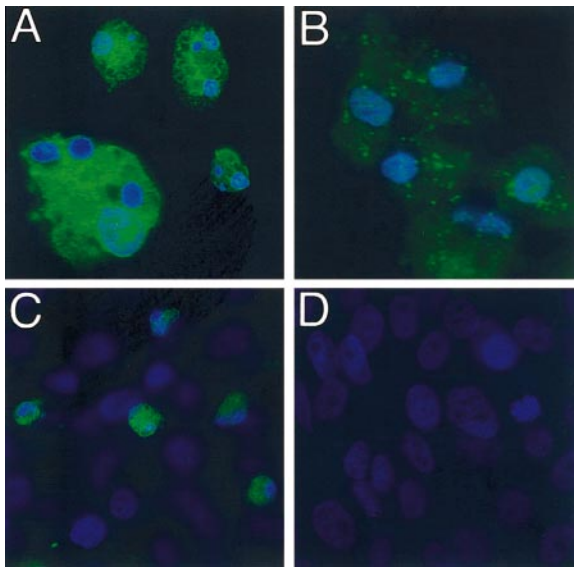

FIG. 5. Immunofluorescence localization of mim-1 in avian osteoclasts and human osteoclast-like cells. Avian osteoclasts were cultured without bone on coverslips as described under Materials and Methods. Human blood monocytes (5×10^4 cells) were cultured for 10 days in the presence of MG63 cells (5×10^5 cells) in the presence of 10^{-8} M 1,25-dihydroxyvitamin D_3, and 25 ng/ml M-CSF. Cells were incubated in the absence (A and C) or presence (B) of 1 μM PMA for 48 h. Avian osteoclasts are illustrated in A and B while human osteoclast-like cells are shown in C and D. Cells were washed with ice-cold PBS, fixed, permeabilized, Hoescht stained for nuclear localization (blue color), and probed with mim-1 (A, B, and C) or nonimmune rabbit serum (D). Data are representative of three separate experiments.

mim-1 was expressed in promyelocytes but was not detected in brain, heart, lung, kidney, liver, muscle, thymus, bursa or spleen of chickens (14). Transcriptional regulation of mim-1 expression is governed by myb. Myb activity, in regulating mim-1 expression, is reported to undergo synergistic activation with C/EBP (33, 34). Transcription of mim-1 is negatively regulated by PU.1, which is induced by M-CSF and is necessary for osteoclast differentiation (35). PU.1 knockouts are osteopetrotic (35), while neutrophils deficient in PU.1 fail to differentiate (36). Myb knockouts are embryonic lethals due to a failure of hematopoiesis (37). There is a negative correlation between mim-1 expression and cell differentiation (14), so that it has been suggested that increasing expression of mim-1 blocks cells from further differentiation.

Mim-1, first identified in promyelocytes, is abundant in osteoclasts (Figs. 1, 2, and 5), which are derived from myeloid precursors. This, together with the fact that mim-1 is secreted preceding a four-fold stimulation of bone resorption with PMA and is homologous with chondromodulin II (Lect2), leads us to propose that mim-1 is an osteoclast-derived chemokine that may be important in bone remodeling.

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