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Proteinase Expression During Differentiation of Human Osteoclasts In Vitro

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Abstract Osteoclasts are macrophage-derived polykaryons that degrade bone in an acidic extracellular space. This differentiation includes expression of proteinases and acid transport proteins, cell fusion, and bone attachment, but the sequence of events is unclear. We studied two proteins expressed at high levels only in the osteoclast, cathepsin K, a thiol proteinase, and tartrate-resistant acid phosphatase (TRAP), and compared this expression with acid transport and bone degradation. Osteoclastic differentiation was studied using human apheresis macrophages cocultured with MG63 osteosarcoma cells, which produce cytokines including RANKL and CSF-1 that mediate efficient osteoclast formation. Immunoreactive cathepsin K appeared at 3–5 days. Cathepsin K activity was seen on bone substrate but not within cells, and cathepsin K increased severalfold during further differentiation and multinucleation from 7 to 14 days. TRAP also appeared at 3–5 d, independently of cell fusion or bone attachment, and TRAP activity reached much higher levels in osteoclasts attached to bone fragments. Two proteinases that occur in the precursor macrophages, cathepsin B, a thiol proteinase related to cathepsin K, and an unrelated lysosomal aspartate proteinase, cathepsin D, were also studied to determine the specificity of the differentiation events. Cathepsin B occurred at all times, but increased two- to threefold in parallel with cathepsin K. Cathepsin D activity did not change with differentiation, and secreted activity was not significant. In situ acid transport measurements showed increased acid accumulation after 7 days either in cells on osteosarcoma matrix or attached to bone, but bone pit activity and maximal acid uptake required 10–14 days. We conclude that TRAP and thiol proteinase expression begin at essentially the same time, and precede cell fusion and bone attachment. However, major increases in acid secretion and proteinases expression continue during cell fusion and bone attachment from 7 to 14 days. J. Cell. Biochem. 78:627–637, 2000.

Key words: ODF; OPGL; TRANCE; cathepsin O2

Macrophages mediate acid degradation of endocytosed material, but degradation of bone by macrophage-derived osteoclasts requires several specialized modifications. Bone is too large to endocytose, it contains large amounts of highly cross-linked collagen, and it buffers acid efficiently. To degrade this substrate, the osteoclast secretes collagenolytic proteinases and uses a cell-surface vacuolarlike H^+-ATPase to drive acid hydrolysis [Blair et al., 1989]. Cellular rearrangements including cell fusion and formation of a tight attachment to bone are also critical for efficient bone degradation [Ross et al., 1993]. Other characteristic osteoclastic products include a tartrate-resistant acid phosphatase (TRAP) [Ek-Rylander et al., 1991], which is not produced in significant quantities on other bone cells, and the vitronectin receptor ($\alpha_v\beta_3$ integrin), which is important in osteoclast attachment [Ross et al., 1993], but not specific to this cell.

Multiple proteinases including metalloproteinases are involved in bone turnover [Blair, 1998]. However, the thiol proteinases, and in particular the recently discovered species cathepsin K (cathepsin O2), are of major importance. Cathepsin K is expressed prominently in mammalian osteoclasts [Tezuka et al., 1994; Inaoka et al., 1995; Li et al., 1995; Bossard et al., 1996; Gelb et al., 1996]. Other acid proteinases, including the aspartate proteinase ca-

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thepsin D and the thiol proteinases cathepsins B and L, are present in osteoclasts, although cathepsin K is the predominant product [Gelb et al., 1996; Drake et al., 1996]. The processing and trafficking of proteinases in bone has not been fully investigated, although mannose-6-phosphate-receptor-dependent transport mechanism, which also functions in the lysosomal trafficking in macrophages, is involved [Baron et al., 1988; Blair et al., 1988]. In the absence of cathepsin K, bone turnover continues, but at a lower rate, and pycnodysostosis, cathepsin K deficiency [Gelb et al., 1996], is nonfatal. Whether expression and distribution of the other acid proteinases [Delaissé et al., 1991; Blair et al., 1993; Goto et al. 1993] change during formation of osteoclasts is not clear.

The relationship of cathepsin K expression to other elements of osteoclast differentiation, including H^+-ATPase expression, TRAP activity, and multinucleation, are not understood. It has been observed that defined cytokine signals, interleukin-1 (IL-1) with tumor necrosis factor α (TNFα [Matayoshi et al., 1996], or the TNF-family protein RANKL (also called ODF, OPGL, TRANCE) alone [Lacey et al., 1998] will induce macrophages primed with CSF-1 to produce osteoclastlike cells. These reports suggest that a single event can induce the specialized proteins characteristic of the osteoclast. However, some aspects of osteoclast differentiation, such as acid secretion, require interaction with the substrate, normally mineralized bone. This substrate interaction, via $\alpha_v\beta_3$ integrin, contributes to cellular regulation through a tyrosine kinase–dependent pathway [Abu-Amer et al., 1997].

We studied the appearance of osteoclast-specific proteins and determined how osteoclastic differentiation affects expression of proteinases also found in macrophages. We used a two-cell in vitro system that models the interaction of preosteoclastic macrophages with the osteoblast, under conditions where both bone-attached and unattached osteoclast-like cells could be observed. Our results indicate that TRAP and cathepsin K appear at similar times, whereas increased acid transport appeared later. The expression of the cathepsin K–related protein, cathepsin B, was increased, but to a lesser extent, and expression continued to increase until 9–14 days. The ability to degrade bone followed the appearance of the characteristic osteoclastic proteins by several days, but correlated well with acid secretion. This suggests that cell fusion and rearrangement to form the mature osteoclast–bone attachment are critical to efficient bone degradation, whereas osteoclast-specific proteins are produced by an earlier step in which coordinated transcription of the major specialized proteins begins.

MATERIALS AND METHODS

Osteoclastic Differentiation

Osteoclast formation was studied in 14-day cultures of human macrophages and osteoblastlike cells. Macrophages were isolated by leukapheresis of healthy volunteer donors using differential centrifugation on a COBE Spectra (COBE BCT, Lakewood, CO), v.4.7 software for mononuclear cell collection, adjusted to 1% red blood cell contamination; 80 ml of leukocyte-enriched cells were collected from 4 to 5 l of acid-citrate dextrose-anticoagulated blood during 90 min. The product was $\sim 10^6$ white cells/ml, with ~70% mononuclear cells and the balance granulocytes, lymphocytes, and red blood cells. The cells were processed without refrigeration, and purified by overnight attachment to tissue culture plastic; >99% of these cells expressed α-naphthyl acetate esterase. Macrophages were used at $\sim 5 \times 10^4/cm^2$. MG63 osteosarcoma cells, passage 44-60 (ATCC, Baltimore, MD), were added at $10^4/cm^2$, and cultures were maintained in Dulbecco's Modified Eagle Medium with 10% HIFCS. At day 1 only, medium was supplemented with 20 ng/ml recombinant human CSF-1 (Genzyme, Cambridge, MA), and devitalized rat bone, 25–40-μm fragments, 50 $\mu g/cm^2$, were added [Blair et al., 1986]. Cell culture media were replaced at 3-day intervals. When stated, removal of MG63 cells used 0.25% trypsin, 10 min at 20°C, and washing three times in phosphate-buffered saline. In macrophage controls, CSF-1 was included in all media to maintain macrophage viability. Each experiment shown used a single macrophage preparation. RANKL and osteoprotegerin expression by MG63 cells was confirmed by polymerase chain reaction. RNA was harvested by phenol/GITC extraction; cDNA was made by random hexamer priming of reverse transcription. Primer pairs were CAGATGGATCCTAATAGAAT and GATCTGGCCAAGAGGAGCAA (GenBank AF01371) for RANKL and ATGAACAAGTTGCT-

GTGCTGCG and GTGTGCCAGCTGTCTGTGTAG (GenBank HSU94332) for osteoprotegerin. These sequences are from the N-terminal portions of the cDNAs, and amplify ~300-bp segments. Thirty cycles of amplification were used. Each cycle was 1 min at 60°C, 1 min at 72°C, and 1 min at 94°C.

Enzyme Assays

Histochemical or colorimetric assays for cathepsins K, B, and D were performed using benzoylcarbonyl-alanyl-glycyl-prolinyl-arginyl- (cathepsin K), benzoylcarbonyl-leucinyl-arginyl-arginyl- (cathepsin B), or benzoylcarbonyl-arginyl-glycyl-phenylalanyl-phenylalanyl-leucyl- (cathepsin D) 4-methoxy-2-naphthylamines (Enzyme System Products, Livermore, CA). Macrophage isolation was verified by α-naphthyl acetate esterase activity [Alvarez et al., 1991]. TRAP was measured using naphthol AS-BI phosphate substrate and fast garnet GBC (2-methyl-4-[(2-methylphenyl)-azo]benzene diazoulum sulfate) coupling, in 0.67 mol/l tartrate, pH 5.6 [Williams et al., 1996], to show phosphatase activity as red. This was used to demonstrate TRAP in cells or after electrophoretic separation in 100 mmol/l citrate, pH 5 in 7% polyacrylamide. The same reaction was used as a colorimetric assay for soluble TRAP activity, measured in 10 μg of cell lysate, but reading the product as net absorbance at 540 nm.

Western Blot Analysis

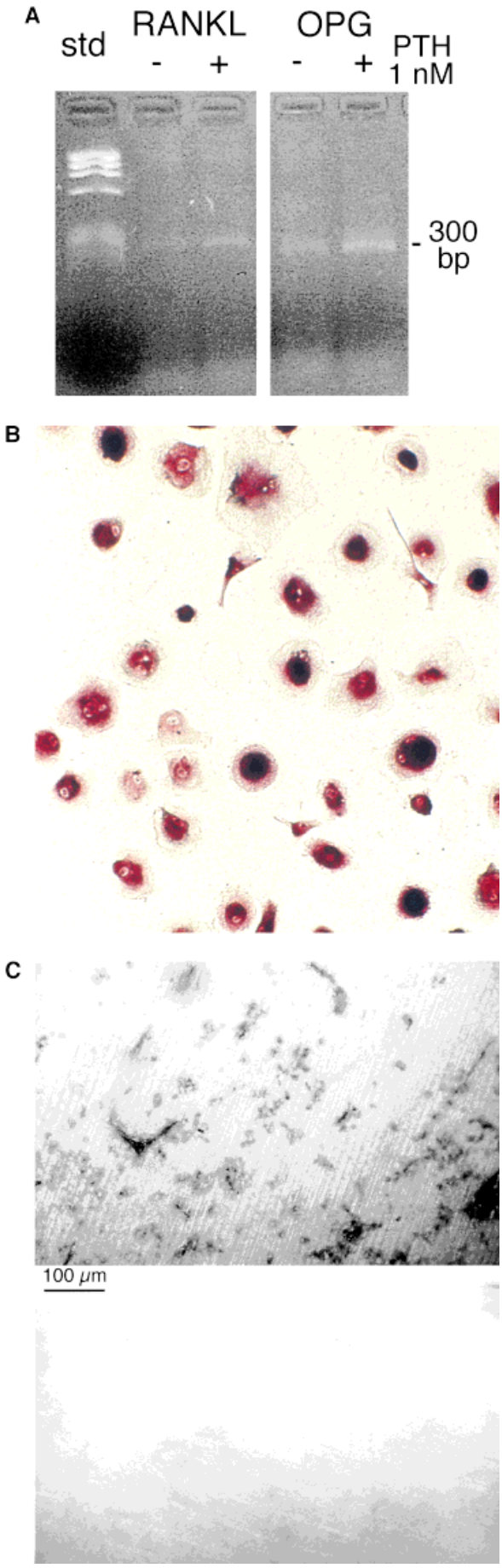
Cathepsins B or K were identified in cell lysates using a monoclonal antibody to cathepsin B (clone CA10, Calbiochem, La Jolla, CA) and polyclonal goat antibody to the C-terminal of cathepsin K (Santa Cruz Biotechnology, Santa Cruz, CA). Western blot analysis used unlabeled antibody reactions and enhanced chemiluminescence detection [Blair et al., 1999]; membranes were blocked in 5% condensed milk overnight in Tris-buffered saline with 0.1% polyoxyethylene sorbitan monolaureate; primary antibodies were used at 1 μg/ml (monoclonal) or 1:1,000 dilution (polyclonal). Antibody reaction steps were 30 min, with three washes between each step. Detection used horseradish peroxidase–coupled rabbit anti-goat or anti-mouse (Amersham, Arlington Heights, IL) and luminol (Amersham) to produce chemiluminescence.

Acid Transport and Bone Degradation Assays

Acid compartments were visualized by the fluorescent weak base method [Baron et al., 1985] using the fluorescent polyamine-pyrrole vital dye lysotracker red DND99 (lysotracker) (Molecular Probes, Eugene, OR). Dye was added at 3 μmol/l to the cultured cells, visualizing fluorescence >520 nm with excitation at 440–460 nm 5 min after addition. Bone degradation was assayed [Williams et al., 1997] by staining pits formed in bone surface with toluidine blue in borate buffers, or by using 20 μg of ^{3}H-labeled substrate measuring label released into supernatant.

RESULTS

Osteoclastic differentiation was studied in vitro in a coculture system using human macrophages and MG-63 human osteosarcoma cells, with 25–40-μm fragments of devitalized bone added so that osteoclastic differentiation on and off substrate could be observed. The properties of the model, and a bone-pitting assay where the culture was performed on a slice of bone, are shown in Figure 1. The in vitro system was similar to the report of Jimi et al. [1996], who achieved human osteoclast differentiation during 3–4 weeks using ST-2 cells [Breuil et al., 1998]. Here, MG63 cells were used; more rapid and repeatable osteoclast development occurred, allowing analysis of osteoclast-specific features and bone degradation within 2 weeks. The MG63 cells were previously shown to express osteoclast-stimulating factors including CSF-1 [Taichman et al., 1996] and m-SCF [Blair et al., 1999] as well as RANKL and its decoy receptor OPG (Fig. 1A). RANKL and OPG were present in MG63 cells in basal medium, and the production of these factors responded to PTH, albeit to a relatively minor degree. This is in keeping with the ability of the osteosarcoma cell lines to induce good osteoclastic development without PTH addition. No cytokines were added to the osteoclast-producing cocultures except for addition of CSF-1, 20 ng/ml, at plating to maintain the macrophages while the MG63 cells stabilized. The appearance of generated osteoclasts after removal of MG63 cells is shown in Figure 1B, demonstrating essentially quantitative production of TRAP-positive cells from the macrophages. Note that essentially all of the trypsin-adherent monocyte-derived cells ex-



pressed TRAP activity at day 14, although ~30% cells were not multinucleated. Bone pitting by these cells at the same culture time is shown in Figure 1C, demonstrating that the osteoclasts formed are functional. Pits were seen in 10-day cultures, but cultures at 7 days showed no measurable pit activity relative to no-cell controls.

Development of TRAP was assayed by histochemical staining (Fig. 2) and after separation of TRAP by nondenaturing gel electrophoresis (Fig. 3). TRAP activity in cells was minimal at day 3, increased rapidly between days 5 and 7, and plateaued by day 10. Activity was present intracellularly but, particularly at later times, the matrix surrounding cells was strongly reactive, in keeping with the major TRAP disposition as a secreted enzyme (Fig. 2). Nondenaturing polyacrylamide electrophoresis isolation of the osteoclast-specific TRAP isoform (Fig. 3) showed similar results, and demonstrated TRAP at day 3 more clearly. At 3 days, when almost all of the cells (>95%) were not polykaryons, but most TRAP-positive cells fused by 10–14 days (Fig. 1B). Gels developed for acid phosphatase but without tartrate showed other nonosteoclast-specific isoforms (not illustrated).

Enzyme activity of cathepsins K, B, and D was measured by fast garnet coupling of naphthylamines, similarly to the TRAP reaction. Attempts at fluorescent coupling using 5-nitrosalicaldehyde, which is suitable for cells that produce modest amounts of thiol proteinases such as chondrocytes and fibroblasts [Van Nooden et al., 1987], produced a copious precipitate in day 5 and later with cathepsin B or K substrates, which precluded analysis in so-

Fig. 1. Properties of the coculture system used to produce human osteoclasts. **A:** Polymerase chain reaction showing that MG63 cells produce the osteoclast-promoting growth factor RANKL and its decoy ligand OPG. Primers and conditions are specified in Materials and Methods. Cells were cultured 72 h with or without 1 nmol/l PTH 1-34 as indicated. **B:** Osteoclasts attached to tissue culture plastic are shown in a tartrate-resistant acid phosphatase (TRAP) stain. This is a 14-day culture, produced without bone, after MG63 cell removal. Essentially all of the cells produce TRAP, but several TRAP-positive cells are mononuclear. **C:** Pits produced by osteoclasts in a coculture on a bone slice. Cells were removed by sonication in detergent and the pitted surface (top panel) was visualized by staining of the exposed collagen in the pits with toluidine blue; control bone is shown in the bottom panel.

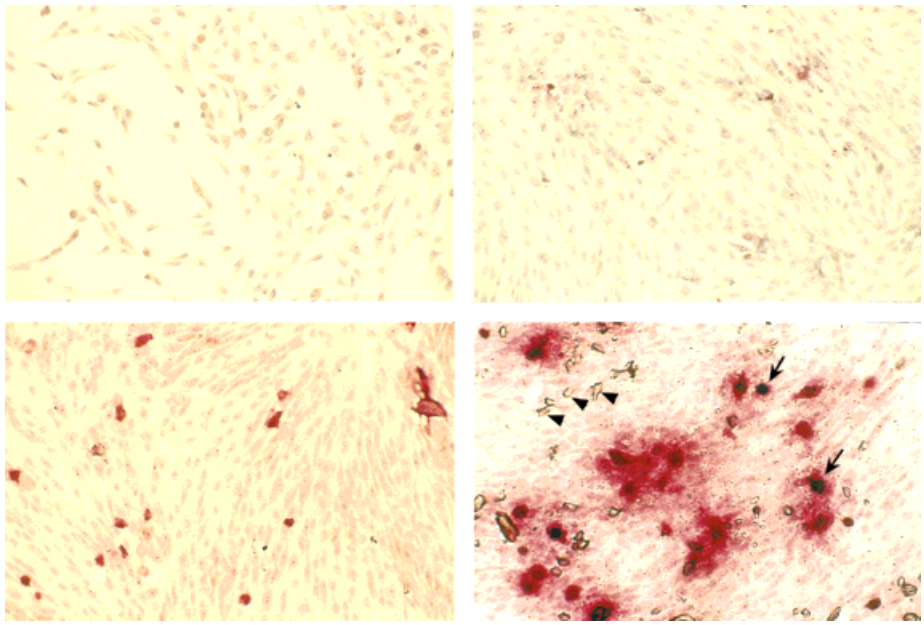


Fig. 2. Tartrate-resistant acid phosphatase (TRAP) activity by histochemical staining during osteoclastic differentiation. In this time-course assay, cocultures of human monocytes with MG63 cells are shown together, and stained for TRAP (red), which is produced only by osteoclasts; the MG63 cells are counterstained with hematoxylin (blue-gray), and photographed in transmitted light. The top left panel shows the appearance of the coculture at 3 days, when TRAP activity is minimal. The top right, bottom left, and bottom right panels show the activity at 5, 7, and 10 days, respectively. The activity is particularly dense on bone fragments (arrows, bottom right panel), which otherwise appear as irregular refractile objects (arrowheads, bottom right panel) when osteoclasts are not attached. The intense reaction at 10 days involves the surrounding MG63 cell-matrix.

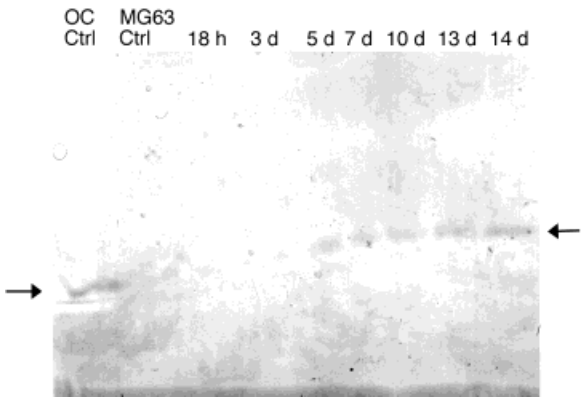


Fig. 3. Tartrate-resistant acid phosphatase (TRAP) enzyme reactivity shown by a colorimetric assay on a nondenaturing gel. Isolation of the tartrate-resistant isoform of acid phosphatase (TRAP) from 1- to 14-day cell lysates. Cell lysates were made using 1% Triton X-100 in citrate buffer at pH 5, separated by electrophoresis on a nondenaturing 7% polyacrylamide gel, and evaluated for a-naphthyl phosphate esterase activity in 0.67 mol/l tartrate. Note that TRAP activity begins to appear at 3 days.

lution or by histochemical staining. Figure 4A summarizes lysate activity, which showed a large increase in cathepsin K activity and a smaller, but distinct, increase in cathepsin B with a similar time course, and at a fraction of the cathepsin K level. The leu-gly-pro-arg (cathepsin K) and ala-arg-arg (cathepsin B) substrates are not absolutely specific, and might be affected by cross-reactivity, so Western blotting was also performed (described below). Cathepsin D activity was essentially invariant, and occurred at still lower levels. The relatively insensitive fast-garnet coupling procedure was also used for histochemical staining (Fig. 4B,C). These reactions showed cathepsin B and K activity at the cell–bone interface, but practically no cathepsin K activity away from bone, and much lower levels of cathepsin B away from bone. Cathepsin D staining gave an intracellular/lysosomal pattern only.

Western blot analysis after removal of MG63 cells (Fig. 5) showed that both cathepsins B and K increased during differentiation. Cathepsin K was seen as early as day 3, but thiol proteinase expression continued to increase after day 7. High-resolution gels showed weak immunoreactive cathepsin K isoforms 2–6 kD above each major species, consistent with variable glycosylation (Fig. 5B). The PI-3-kinase

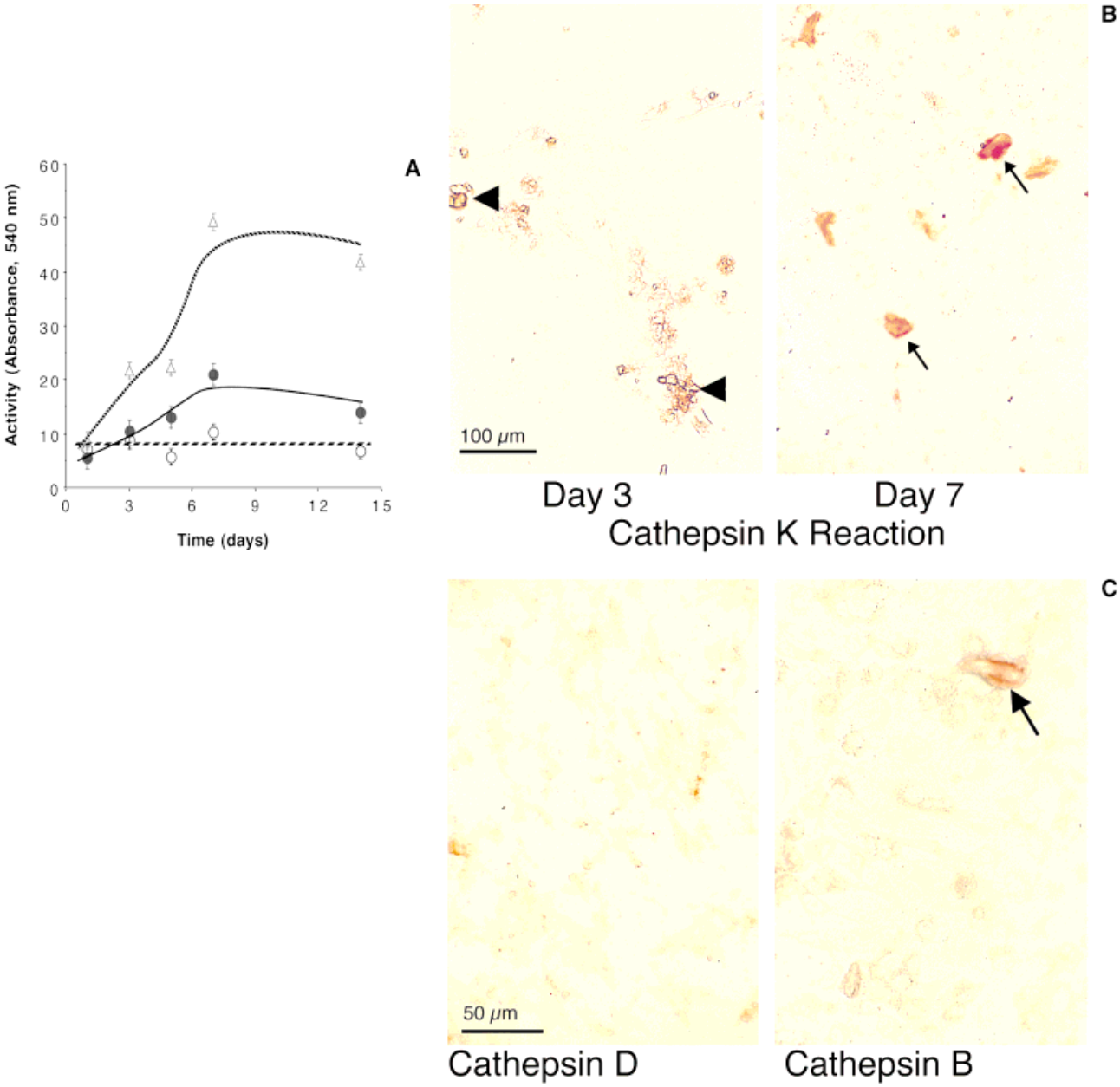


Fig. 4. Activity of proteinases during differentiation. Degradation of benzoylcarbonyl-ala-gly-pro-arg-4-methoxy-2-naphthylamine, benzoylcarbonyl-leu-arg-arg-4-methoxy-2-naphthylamine, and benzoylcarbonyl-arg-gly-phe-phe-leu-4-methoxy-2-naphthylamine were used to measure activity of cathepsins K, B, and D, respectively, using fast garnet reaction to visualize the reaction as red. **A:** Activity of cathepsins in cell lysates. A large increase in cathepsin K activity (triangles), a modest increase in cathepsin B (closed circles), and no change in cathepsin D (open circles) were observed. Results representative of two similar experiments are shown, with duplicate measurements at each time point. **B:** Cathepsin K activity in situ. No activity was observed at 3 days (left panel), but at longer times active enzyme was detectable secreted onto bone particles in the cocultures (right panel). Very little activity was seen within the cells. **C:** Cathepsin D stained coculture cells in a faint lysosomal pattern (left panel). Secreted activity on bone was not detected. Cathepsin B (right panel) was seen faintly in a lysosomal pattern within cells, and was also secreted prominently onto bone particles (arrow).

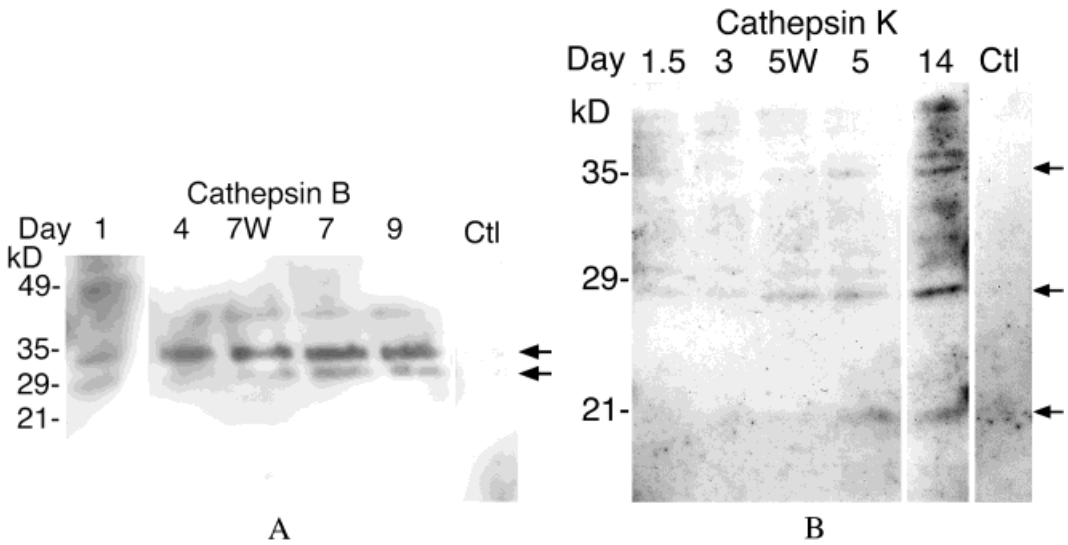


Fig. 5. Western blot analysis of cathepsins B and K as a function of coculture time. Cathepsin B (**A**) was easily detectable in macrophages at plating (day 1) and increased significantly at day 9. Cathepsin K (**B**) was almost undetectable until days 3–5, and increased dramatically from day 5 to day 12. The Western blot method is semiquantitative, but the increase in cathepsin B and a larger increase in cathepsin K are consistent with the activity assays shown in Figure 4A. The cathepsin K isoform of ~20 kD is a known degradation fragment. The PI-3-kinase inhibitor wortmannin (100 nmol/l) did not affect processing of cathepsin B or K (**lanes 7W**) Each panel shows lanes from a single blot; lanes marked **Ctl** were from cultures processed identically to the osteoclast differentiation assays but contained no monocytic precursors, and show the minimal activity attributable to osteoblastic cells or nonspecific reactions. Thiol proteinase blots of osteoclasts show multiple processed/degraded forms of the enzymes; the blots shown are typical results of several experiments (see Results).

inhibitor wortmannin, at 100 nM, did not affect enzyme processing of cathepsins B or K measurably.

Acid secretion was evaluated in situ using the fluorescent weak base lysotracker red DND99 (Fig. 6). At 5 days (top panels), no significant multinucleation was observed and there were no acid lakes at cell–bone interfaces. The lysosomal uptake of mononuclear cells, although distinguishable from that of the MG63 osteosarcoma cells, was of the same order. Results at 7 days were variable. At 10–14 days, polykaryons with greatly increased lysotracker uptake were seen. The acid secretion by osteoclasts forming on bone particles was prominent. Surprisingly, however, the activity of polykaryons not attached to bone was much greater than the lysotracker uptake of mononuclear cells (arrowheads, Fig. 6 lower panels), although this activity was in a vacuolar pattern.

DISCUSSION

The osteoclast expresses proteins made by macrophages, such as lysosomal proteins, vacuolar H^+-ATPase subunits, and the integrin $\alpha_v\beta_3$, many of which retain important roles in the osteoclast [Blair et al., 1989; Ross et al., 1993; Abu-Amer et al., 1997]. Specialized proteins, including TRAP [Ek-Rylander et al., 1991] and cathepsin K [Tezuka et al., 1994; Inaoka et al., 1995; Li et al., 1995; Bossard et al., 1996; Gelb et al., 1996; Drake et al., 1996] are practically undetectable in preosteoclasts, and are induced in a differentiation process that is poorly understood.

Questions include the time course of appearance of specific osteoclastic features, and whether proteins expressed by precursor cells, such as cathepsins B and D, are up- or down-regulated in the process of differentiation. We addressed these questions using in vitro differentiation. Both CSF-1 and RANKL, which support osteoclastic differentiation from marrow monocytes [Lacey et al., 1998], are expressed in the MG63 cell line used to induce the osteoclastic phenotype (Fig. 1). It is possible that stimuli with overlapping specificities, including TNFα and IL-1 [Matayoshi et al., 1996], may be produced by MG63 or by RANKL-stimulated preosteoclastic cells, but these considerations are beyond the scope of the present work.

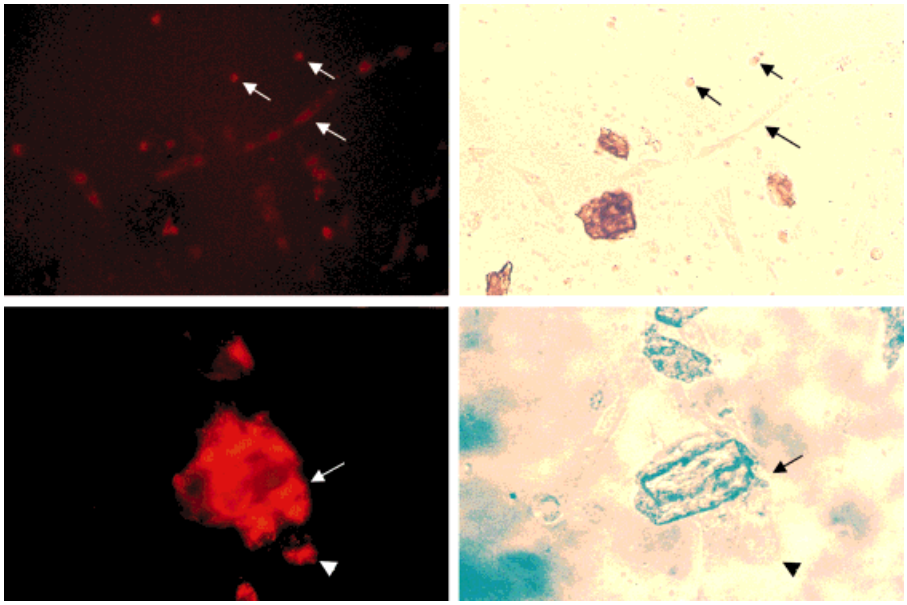


Fig. 6. Evaluation of acid transport with lysotracker. At 5 days (top panels: left, fluorescence; right, phase) spread MG63 cells (bottom arrow) and round macrophages (top arrows) all had fine lysosomal acid uptake patterns, with a slightly greater uptake in the mononuclear cells. Acid secretion on bone (large, angular material) was insignificant. At 14 days, there were prominent acid lakes by osteoclasts on bone (bottom left frame, arrow), and, surprisingly, significant acid uptake in osteoclasts not attached to bone (example indicated by arrowhead). In the cells not attached to bone, uptake was in large vacuoles. Many MG63 cells are present in the frame; these could not be visualized without overexposing the acid compartments on bone. The bone fragments (seen in transmitted light, right panels) are ~50 μM; the bottom pair was photographed at twice the magnification of the top pair.

Osteoclasts secrete the largest quantity of cathepsin K, a highly active acid collagenase and elastase, of any cell type. We detected cathepsin K production at 3–5 days, although it increased in parallel with osteoclastic activity that did not plateau until 10–14 days (Figs. 4 and 5B). Expression of cathepsin K has been studied in a mouse coculture osteoclast development model [Li et al., 1999], with findings consistent with our results. The cathepsin K promoter contains numerous putative control elements, as with other thiol proteinases, in keeping with variable expression in multiple tissues [Gelb et al., 1997; Rood et al., 1997; Li et al., 1999], although it is still not known why so much of it occurs in the osteoclast. Cathepsin K is not unique to the osteoclast, however; it occurs in breast cancer cells [Littlewood-Evans et al., 1997] and smooth muscle [Sukhova et al., 1998].

Cathepsin B is expressed in macrophages and osteoclasts but was not known to be amplified in osteoclasts [Drake et al., 1996]. We found that cathepsin B protein expression and activity increased two- to threefold with osteoclastic differentiation (Figs. 4, 5A). Changes in cathepsin K were much larger; cathepsin K immunoreactive protein and activity were essentially undetectable in macrophages but prominent in osteoclasts, where cathepsin K is the major thiol proteinase. The correlation, albeit imperfect, of cathepsin B and K expression is in keeping with common promoter elements in these closely related thiol proteinases; presumably as-yet-unidentified cathepsin K promoter elements are responsible for the dramatic induction during early osteoclastic differentiation. The aspartate proteinase cathepsin D did not change measurably. This distantly related lysosomal proteinase remained at a basal level. Its activity, unlike cathepsins K and B, was largely in a cellular distribution. This suggests that, in addition to the known role of the mannose-6-phosphate receptor in osteoclast secretion [Baron et al., 1988; Blair et al., 1988], other signals may be involved that allow targeting of specific acid proteinases to intracellular or extracellular locations.

Under the conditions studied, multinucleated cells were commonly seen only after 7 days. Multinucleation was not required for ex-

pression of osteoclastic proteins including TRAP (Figs. 2, 3) and cathepsin K. As with cathepsin K, TRAP is an excellent osteoclast marker but it is not unique to the osteoclast, occurring in some leukemic white blood cells [Janckila et al., 1998]. It has been questioned whether TRAP is a marker of mature osteoclasts, or occurs at intermediate stages of development. Our results suggest that it is indeed not a marker specific to mature osteoclasts, although it appeared at the same time as cathepsin K. Acid transport activity before the onset of multinucleation remained at a basal level similar to the feeder cells. However, its amplification was, surprisingly, not entirely dependent on attachment to mineralized matrix, as prominent vacuolar acidification in unattached cells (Fig. 6).

It is unlikely, based on our findings, that development of osteoclastic markers requires bone attachment, but bone attachment did affect dramatically TRAP or thiol proteinase activity and acid secretion. There was a large difference between enzyme activity on the bone substrate and in nonattached cells for TRAP and either cathepsin B or K, but not cathepsin D. This is in keeping with prominent secretion of active thiol proteinases. A significant amount of cathepsin K was in high-molecular-weight isoforms, suggesting partial processing. Western blot analysis of cathepsin K in tissue typically shows mainly isoforms cleaved into two chains. In addition, we observed minor isoforms 2–6 kD larger than each major protein band, consistent with variable glycosylation.

Bone degradation is dependent on the PI-3-kinase, and processing of cathepsin K in cultured human osteoclasts had been noted to be arrested by 1 μM wortmannin, an inhibitor of this enzyme [Rieman et al., 1998]; a half-inhibitory concentration of 200 nM was reported. We found that 100 nM wortmannin did not change the amount or processing of cathepsins B or K appreciably, and this concentration of wortmannin effectively inhibits the PI-3-kinase [Okada et al., 1994]. Higher concentrations of wortmannin are less specific and modify activity of other pathways including protein kinase C [Natarajan et al., 1997], so we regard the issue of PI-3-kinase involvement in osteoclastic thiol proteinase processing to be unresolved.

To the extent that it was practical to measure expression, we find that TRAP and cathepsin K occur simultaneously at 3–5 days in preosteoclastic cells, whereas major increases in expression of proteinases and acid secretion capacity occurred later, at 10–14 days. Neither multinucleation nor bone attachment appeared to be necessary to trigger TRAP or cathepsin K expression, although efficient bone degradation depends on multinucleation and substrate binding [Blair, 1998]. Our findings are consistent with the hypothesis that a common group of gene-control elements are activated in osteoclast differentiation [Matayoshi et al., 1996; Lacey et al., 1998; Yasuda et al., 1998; Kong et al., 1999], probably involving NF-κB and related transcription control elements dependent on JNK such as the c-Jun transcription factor. c-Jun is involved in osteoclast activation by RANK [Jimi et al., 1999], and is a potent coactivator of PU.1, the m-CSF-dependent transcription factor [Behre et al., 1999]. The genes for cathepsin K, TRAP, and some H^+-ATP subunits transcribed in osteoclasts have been cloned [Lee et al., 1995, 1997; Fleckenstein et al., 1996; Gelb et al., 1997; Rood et al., 1997; Li et al., 1999], and include dozens of potential nuclear factor binding sites, but a connection with c-Jun or NF-κB is not apparent. For example, no NF-κB sites occur near the cathepsin K transcription site [Gelb et al., 1997; Rood et al., 1997; Li et al., 1999], and c-Jun/c-Fos (AP-1) sites are >1 kB upstream in the mouse. Thus, coordinated expression of specific osteoclast products probably involves transcription factors not yet unidentified.

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