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Osteoprotegerin and Osteoprotegerin Ligand Effects on Osteoclast Formation From Human Peripheral Blood Mononuclear Cell Precursors

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Abstract Osteoprotegerin (OPG) and its ligand (OPGL) negatively and positively regulate osteoclastogenesis in the mouse. OPG inhibits osteoclastogenesis by sequestering its ligand, OPGL, the osteoclast differentiation and activation factor. This study demonstrates the effects of soluble muOPGL and huOPG on the developing human osteoclast phenotype, on bone slices, using peripheral blood mononuclear cells (PBMCs), cultured for 2 weeks, without stromal cells. OPGL (2–50 ng/ml), in combination with CSF-1, hydrocortisone (HC), and $1,25(OH)_2D_3$, increases the size of osteoclast-like cells on bone, as defined by the acquisition of osteoclast markers: vitronectin receptor (VR), tartrate-resistant acid phosphatase (TRAP), multinuclearity, and bone resorption. By 14 days, with 20 ng/ml OPGL, the largest cells/10× field have achieved an average diameter of 163 ± 38 μm, but only ~10–20 μm in its absence and the number of osteoclast-like cells/mm^2 bone surface is about 128. By scanning electron microscopy, OPGL-treated (20-ng/ml) cultures contain small osteoclast-like cells on bone with ruffled "apical" surfaces by day 7; by day 15, large osteoclast-like cells are spread over resorption lacunae. At 15 ng/ml OPGL, about 37% of the bone slice area is covered by resorption lacunae. OPG (5–250 ng/ml) antagonizes the effects of OPGL on the morphology of the osteoclast-like cells that form, as well as bone erosion. For cells grown on plastic, Cathepsin K mRNA levels, which are barely detectable at plating, are elevated 7-fold, by 5 days, in the presence, not the absence, of OPGL (20 ng/ml) + CSF-1 (25 ng/ml). Similar findings are observed in experiments performed in the absence of HC and $1,25(OH)_2D_3$, indicating that HC and $1,25(OH)_2D_3$ are not needed for OPGL-induced osteoclast differentiation. In conclusion, this study confirms a pivotal role for OPGL and OPG in the modulation of human osteoclast differentiation and function, suggesting a use for OPG for treating osteoclast-mediated bone disease in humans. J. Cell. Biochem. 72:251–261, 1999.

Key words: bone; resorption; multinucleated cells; vitronectin receptor osteoprotegerin

Osteoprotegerin (OPG) and its ligand (OPGL) regulate osteoclastogenesis in mouse, both in vivo, and in vitro in bone marrow cultures [Simonet et al., 1997; Lacey et al., 1998; Yasuda et al., 1998] and in human peripheral blood mononuclear cell (PBMC) osteoclast-like cell cultures [Matsuzaki et al., 1998]. OPG, a secreted tumor necrosis factor receptor (TNFR)-related protein, functions by sequestering OPGL, a novel tumor necrosis factor (TNF) family member that is a potent osteoclast activation and differentiation factor [Lacey et al., 1998; Yasuda et al., 1998; Matsuzaki et al., 1998], identical to osteoclast differentiation factor (ODF) [Yasuda et al., 1998], TRANCE, or RANKL [Anderson et al., 1997; Wong et al., 1997]. Recombinant soluble OPGL stimulates bone resorption in vivo [Lacey et al., 1998], enhances osteoclast differentiation in mouse bone marrow cultures, activates mature rat osteoclasts, and recognizes a putative cell surface receptor on mouse osteoclast progenitors [Lacey et al., 1998].

It has been known for some time that osteoclast precursors originate from the hematopoietic stem cell [Hagenaars et al., 1989; Sheven et al., 1986; Hattersley and Chambers, 1989; Matayoshi et al., 1996; Kurihara et al., 1989] and that they circulate in the peripheral blood (PB)

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[Walker, 1973; Kahn and Simmons, 1975; Coccia et al., 1980; Walker, 1975]. Only recently have in vitro systems been useful in demonstrating the formation of human osteoclasts from circulating progenitors [Faust et al., in press, Purton et al., 1996; Matayoshi et al., 1996; Fujikawa et al., 1996]. In our hands, homogeneous cultures of small preosteoclast/osteoclast-like cells are generated from PBMC, in the absence of stromal cells, added cytokines, growth factors or steroids. These cultures consist of adherent cells having low nuclearity (mainly mono-, di-, trinuclear, ~10–20 μm in diameter). All other cells die, except for lymphocytes. This occurs within 2–3 weeks when PBMC are cultured at high density in α-MEM + 10% FBS on bone or plastic [Faust et al., in press]. The preosteoclast/osteoclastic cells are tartrate-resistant acid phosphatase positive ($TRAP^+$), cathepsin K-positive (Cath K^+), vitronectin receptor positive (VR^+), calcitonin receptor positive (CTR^+), and exhibit a low level of bone resorption potential.

Although much is known about the development of murine osteoclasts from bone marrow and spleen precursors, very little is known about human osteoclastogenesis. The goal of this study was to characterize the developing human osteoclast phenotype induced by OPGL, in the presence and absence of OPG, using PBMC cultured on bone slices. Soluble muOPGL induced large multinucleated osteoclast-like cell (LMOC) formation (osteoclasts with 100- to 160-μm diameters by 15 days in culture) and function (as assayed by osteoclast-mediated bone resorption, and osteoclast phenotypic markers, VR and TRAP), by 11–16 days of culture. For OPGL-induced LMOCs on bone and plastic, colony-stimulating factor-1 (CSF-1) was required, but not HC or $1,25(OH)_2D_3$. OPGL-induced osteoclast maturation and associated bone resorption progressed over a period of 12–15 days on bone under these culture conditions. OPGL-induced LMOC formation and bone resorption were inhibited by OPG in a dose dependent manner, resulting in small (~10–20 μm in diameter), preosteoclast/osteoclast-like cells of low nuclearity on the bone.

MATERIALS AND METHODS

Reagents

$1,25(OH)_2D_3$ was purchased from Calbiochem (La Jolla, CA). Fetal bovine serum (FBS) was obtained from Cansera (Rexdale, Ontario, Canada). Hydrocortisone (HC) and the TRAP cytochemistry kit (kit number 386) were from Sigma Chemical Co. (St. Louis, MO). CSF-1 was purchased from Genzyme (Boston, MA). Ficoll-Paque (F/H) was from Pharmacia (Piscataway, NJ). Leukapheresis product was obtained from HemaCare Corporation (Sherman Oaks, CA). Heparinized peripheral blood was obtained from volunteer donors. Recombinant CHO huOPG [22–194]-Fc and recombinant *Escherichia coli* muOPGL 158–316 were prepared as described [Simonet et al., 1997; Lacey et al., 1998].

Cell Isolations and Treatments

PBMC were prepared from leukapheresis product and heparinized blood by separation over F/H and resuspended in α-MEM+10% FBS, supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin sulfate and 250 ng/ml fungisone (Bio Whittaker, Walkersville, MD). Both cell sources yielded identical cultures of osteoclasts (Faust et al., in press). Cells (in 0.2 ml of media) were cultured at a concentration of 2.5×10^6 cells/ml in 96-well dishes containing bone slices for 4 h. The slices were then transferred to 24-well plates and fed twice weekly with 1 ml of media supplemented with CSF-1 (25 ng/ml), hydrocortisone (10^{-6} M), $1,25(OH)_2D_3$ (10^{-8} M), and different doses of OPGL (2–50 ng/ml), and OPG (5–500 ng/ml) singly or in various combinations. Osteoclast numbers and sizes were obtained at different doses (2 ng/ml and 20 ng/ml OPGL) by counting, or measuring the diameter, respectively, of multinucleated $TRAP^+$ cells on 10× photomicrographs, taken using a Nikon Microphot-FXA microscope and Nikon DX-DB2 camera. Cell number was expressed as the number of multinucleated cells found on one 10× photomicrograph (n = 2) with a diameter of >10 μm. Cell size was expressed as the average diameter ±SD of the 20 largest cells found on two 10× photomicrographs. In some experiments, ST2 cells (Riken Gene Bank, Tsukuba Science City, Japan) were cultured on top of bone slices in 96-well dishes (5×10^4cells/200 μl) for 24 h before the addition of PBMC.

Staining and Preparation of Cells

Cytocentrifuge preparations of cells dislodged from the plates by gentle scraping and cells remaining on plastic or bone were fixed with 2%

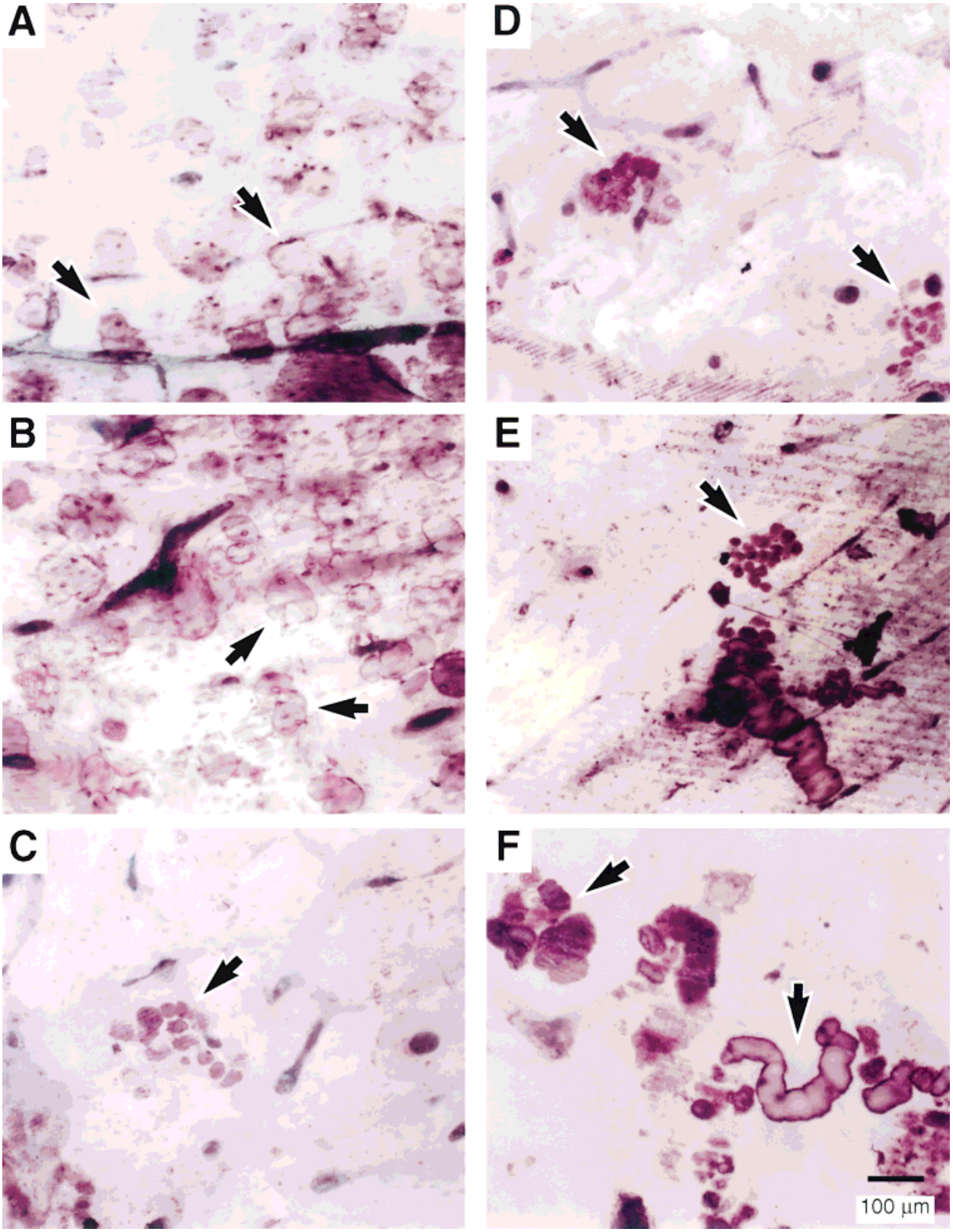

Fig. 1. Osteoprotegerin ligand (OPGL) is a human osteoclast growth and differentiation factor. Peripheral blood mononuclear cells (PBMC) were grown on bone slices as described under Materials and Methods. **A,B:** Toluidine blue-stained bones demonstrating osteoclastic resorption lacunae at 16 and 18 days, respectively, when cells are grown in the presence of OPGL (50 ng/ml) + $1,25(OH)_2D_3$ (10^{-8} M) + hydrocortisone (10^{-6} M) + CSF-1 (25 ng/ml). **C:** Resorption lacunae on bone in α-MEM+10% FBS for 22 days. **D–F:** Resorption lacunae when ST2 stromal cells replace OPGL, at 16, 18, and 21 days in culture, respectively.

paraformaldehyde for 10 min and stained for TRAP activity as previously described (Faust et al., in press). Other cytocentrifuge preparations were fixed with acetone and assayed for vitronectin receptor positive (VR^+) cells using the 23cb antibody as described previously (Faust et al., in press). To visualize cells on the bone with scanning electron microscopy (SEM), bones were washed once with phosphate-buffered saline (PBS), fixed with 1% glutaraldehyde in 0.1 M sodium cacodylic acid (NaCac), pH 7.4 for 2 h, rinsed with 0.1 M NaCac, pH 7.4 (3×), and stored in NaCac, at 4°C.

Osteoclast Resorption Lacunae Assay

Bovine cortical bone slices (6-mm diameter × 0.8 mm) were prepared with a low-speed diamond saw (Buehler, Evanston, IL). Cells were removed from the bone slices, on day 16 of culture, by sonicating (30-s bursts) twice in 2% ammonium hydroxide, twice in distilled water, and twice in acetone. Resorption pits were visualized by staining with 0.1% toluidine blue and light microscopy. The area resorbed on three 20× fields, on each of three bones per condition, was quantitated by image analysis. Some bones were dehydrated in alcohol, sputter coated with 30 nm of gold, using a Hummer VII sputtering system (Anatech Ltd, Alexandria, VA), and then subjected to scanning electron microscopy (model JEOL JSM-5200, Jeol Technics Ltd., Tokyo, Japan).

Cathepsin K RNase Protection Assays

PBMC (1×10^8) were plated on 100 mm dishes in α-MEM+10%FBS, with and without OPGL (20 ng/ml) and CSF-1 (25 ng/ml). On days 0, 5, 9, 12, and 18, five plates per timepoint, the adherent cell layers were washed once and then scraped in PBS. Cells were spun at 460*g* (1,500 rpm) in a Sorvall RT 6000 B centrifuge for 5 min, quick frozen in dry ice/ethanol, and stored at −80°C. RNA was isolated using the Qiagen Mini RNA Kit (Qiagen, Santa Clarita, CA) and checked for integrity by fractionation on 6.6% paraformaldehyde/1% agarose gels. RNase protection assays were performed using the RPA II kit (Ambion, Austin, TX) and 2 µg of total cellular RNA with ^{32}P-labeled RNA probes for Cathepsin K (Gb: U13665, nucleotides 682–1041) and cyclophilin (Ambion). After overnight exposure, the gels were scanned using a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and the signal intensities were analyzed using ImageQuant software. Levels of Cathepsin K mRNA were normalized to cyclophilin mRNA levels.

RESULTS

OPGL Induces Osteoclast Differentiation from Human PBMC Precursors

When PBMCs were cultured on top of bone slices for 16 days in the presence of OPGL (50 ng/ml) + CSF-1 (25 ng/ml) + $1,25(OH)_2D_3$ (D3) (10^{-8} M) + hydrocortisone (HC) (10^{-6} M) (conditions referred to henceforth as "complete media") resorption lacunae formed over the entire bone surface (Fig. 1A). The lacunae increased in size and number by day 18 (Fig 1B). By contrast, cells cultured with α-MEM+10% FBS demonstrated limited resorption by day 22 of culture (Fig. 1C). Four experiments subsequent to this study demonstrated that the combination of CSF-1 and OPGL alone was sufficient for resorption lacunae formation, as omitting D3 and HC alone or together had no effect on osteoclast-like cell formation (see below) and activity. There was no evidence of bone resorption at 16 days when PBMC were cultured in the presence of OPGL (at 50 ng/ml and 100 ng/ml) alone without CSF-1 indicating that OPGL alone was not sufficient for LMOC formation. Resorption lacunae were not observed in the presence of HC+D3+CSF-1, CSF-1, D3, or HC alone (data not shown).

When OPGL was replaced with ST2 stromal cells and treated with HC+D3+CSF-1, small pits were observed at day 16 on a few 20× microsope fields (Fig. 1D). The size and number these pits increased by 18 and 22 days (Fig. 1E

Fig. 2. Scanning electron microscopy (SEM) of osteoprotegerin ligand (OPGL)-induced large multinucleated osteoclast-like cells (LMOC) and resorption lacunae on bone. Peripheral blood mononuclear cells (PBMC) were grown on bone as described in the legend of Fig. 1 for 16 days and either fixed on bone with glutaraldehyde (B–F) and processed for SEM, or removed with ammonium hydroxide and sonicated to visualize resorption lacunae **(A)**. **B:** Cell clusters present on the bone surface. Arrows point to a flattened LMOC cell on bone. **C:** Cell clusters associated with "pseudopods" extending from LMOC over resorption lacunae. **D:** A large resorption lacuna associated with smaller osteoclast-like cells with ruffled "apical" surfaces. **E:** A closer view of a smaller osteoclast-like cell with a ruffled "apical" membrane sitting in a large resorption lacuna. **F:** A closer view of LMOC, demonstrating hair-like projections all over the cell surface and one large pseudopod extending over a resorption lacuna. Resorption lacunae are indicated by arrows in A,C,D,E,F. TRAP-stained cells at day 15 on bone (G), and VR^+ cells at day 11 on bone (H) appear to cover resorption lacunae (arrows point to pits).

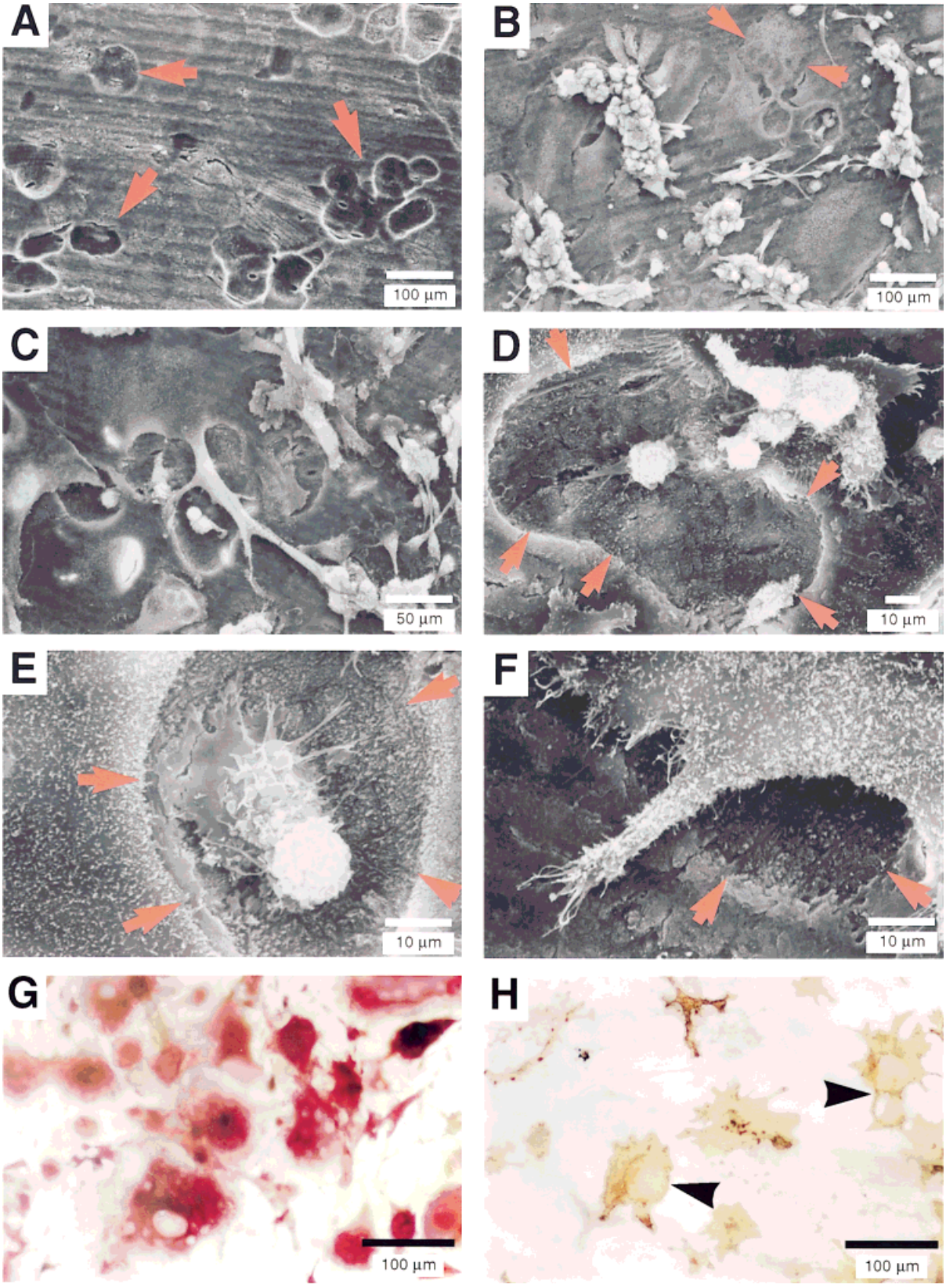

Figure 2.

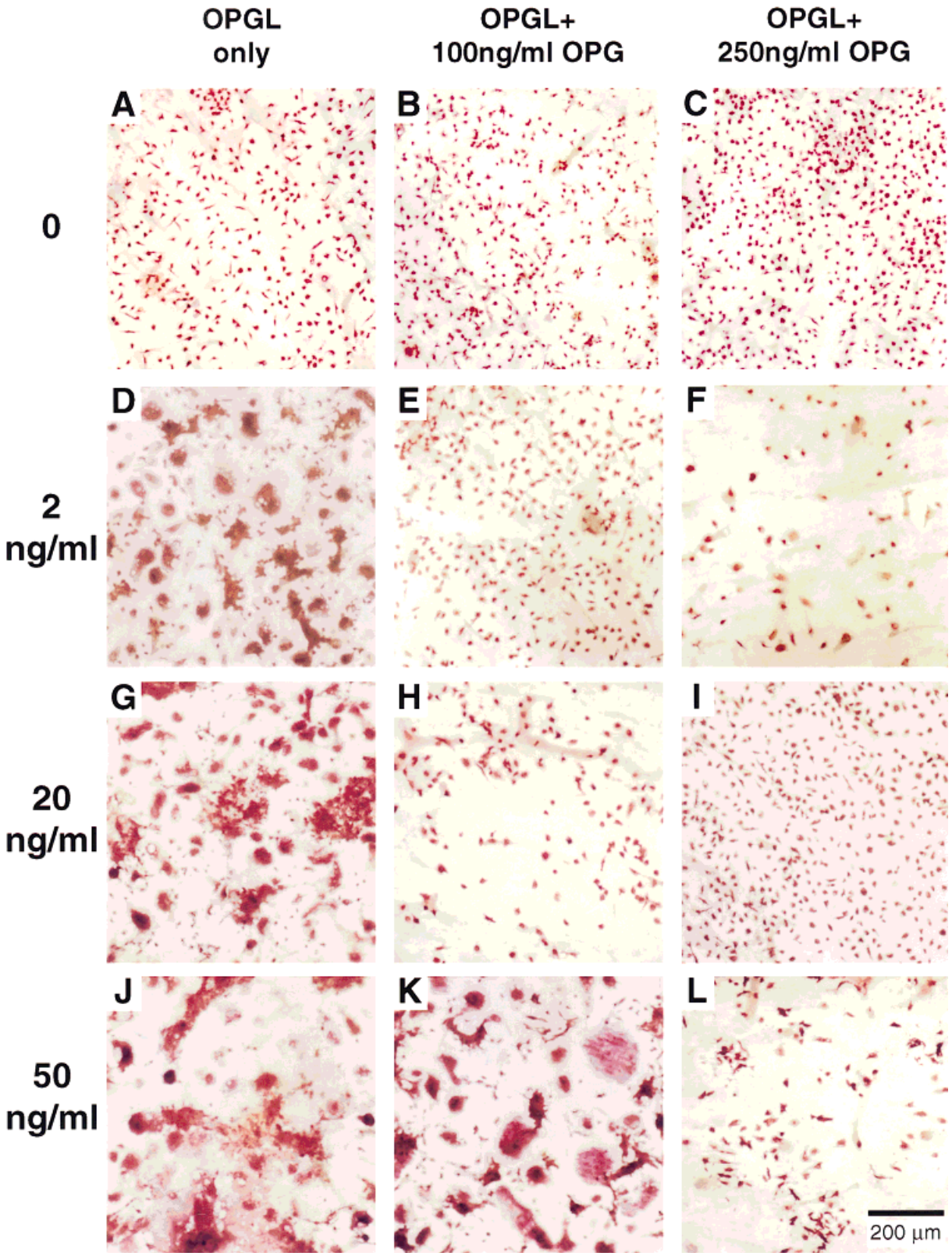

Fig. 3. Osteoprotegerin ligand (OPGL)-induced formation of large multinucleated osteoclast-like cells (LMOC) is inhibited by OPG. Cells were grown as described in the legend to Fig. 1 for 15 days. OPGL induced a dose-dependent increase in osteoclast-like cell size and nuclearity **(A,D,G,J)**. OPG, at 100 and 250 ng/ml, abolished most OPGL-mediated LMOC formation **(E,F,H,I,L)**, except in the case of 50 ng/ml OPGL and 100 ng/ml OPG **(K)**. A: Control panel showing cells cultured in α-MEM+10% FBS demonstrate small $TRAP^+$ preosteoclast/osteoclast-like cells.

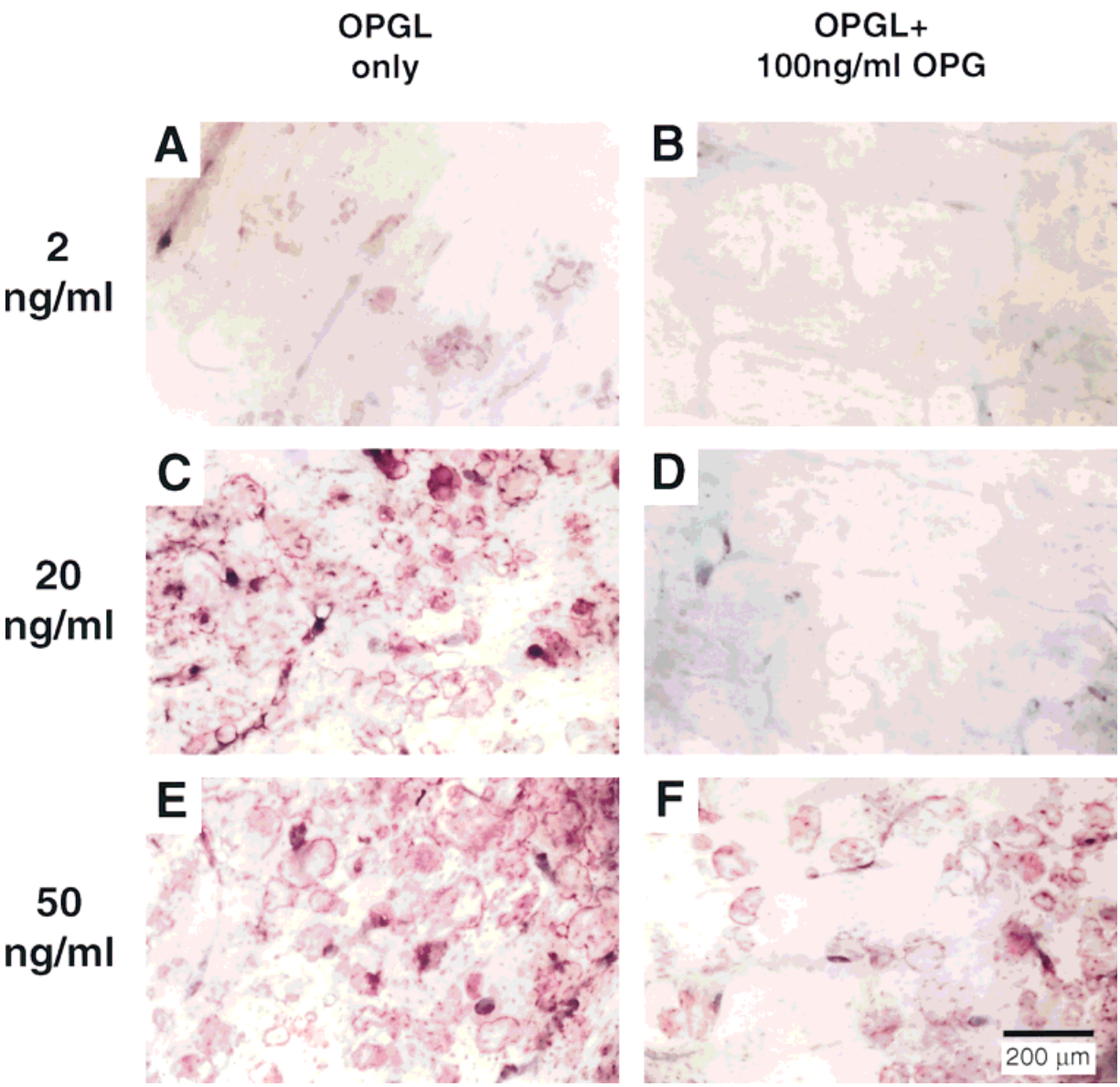

Fig. 4. Osteoprotegerin ligand (OPGL)-induced osteoclastic bone resorption is inhibited by OPG. Cells were removed from an extra set of bones in the experiment from Fig. 3 and stained with toluidine blue to visualize resorption lacunae at day 15. Osteoclast-mediated resorption increased with increasing doses of OPGL **(A,C,E)**; this effect was inhibited by OPG at 100 ng/ml **(B,D)**, except in the case of 50 ng/ml OPGL **(F)**.

and F, respectively) but remained in a few restricted areas on the bone slice (even by 6 weeks of culture). By contrast, OPGL treated cultures generated resorption lacunae that merged with one another to cover the entire bone surface by 21 days (not shown).

Morphology of Human Osteoclast-like Cells Demonstrated by Scanning Electron Microscopy, TRAP Cytochemistry, and VR Immunocytochemistry

Scanning electron microscopy (SEM) of day 16 cultures, grown in complete media, demonstrated the bone surface, before (Fig. 2B–F) and after removal of cells (Fig. 2A). At low magnification the saw marks were covered by flattened osteoclast-like cells spread over the bone surface (Fig. 2B, arrows point to one example). Clusters of mononuclear cells were also seen. At higher magnification, osteoclast-like cells were spread over resorption lacunae (Fig. 2C,F), with collagen fibrils exposed (Fig. 2E). Smaller osteoclast-like cells had extensive ruffled membranes on their "apical" surfaces, extending pseudopods in all directions (Fig. 2E), and

spindle-shaped cells are associated with each other (Fig. 2C). After removal of the cells, resorption lacunae were obvious (Fig. 2A), confirming what was seen using the toluidine blue staining method. In two other experiments, multinucleated osteoclast-like cells were $TRAP^+$, and VR^+ at days 15 and 11, respectively (Fig. 2G,H). On day 11, VR^+ cells appeared to cover resorption lacunae (Fig 2H).

OPGL Stimulates LMOC Formation and Bone Resorption

Larger $TRAP^+$ osteoclast-like cells (Figs. 3A,D,G), associated with larger resorption lacunae (Figs. 4A,C) resulted as OPGL concentrations in complete media were increased from 0, 2, and 20 ng/ml at day 15 of culture. Little difference was observed at 20–50 ng/ml OPGL in cell morphology (Figs. 3G,J) or level of resorption (Figs. 4C,E) at this time. LMOC were intermingled with osteoclast-like cells of low nuclearity, ~10–20 μm in diameter. The average diameters of the largest 20 cells present were 108 ± 19 μm and 163 ± 38 μm, for cells treated with 2 and 20 ng/ml OPGL, respectively, compared with ~10–20 μm in the absence of OPGL. On average there were 104 and 128 LMOC /mm^2 of bone surface, for cultures treated with 2 and 20 ng/ml OPG, respectively.

OPG Inhibits OPGL-Induced Human LMOC Formation and Associated Function

OPG, at 100, 250, and 500 ng/ml (500 ng/ml data not shown), in complete media assayed at day 16, inhibited LMOC and pit formation induced by 2 ng/ml (Figs. 3D–F, 4A,B), and 20 ng/ml OPGL (Figs. 3G–I, 4C,D). Smaller $TRAP^+$ cells, ~10–20 μm in diameter, remained on bone (Fig. 3E,F,H,I). Induction of LMOC formation by OPGL at 50 ng/ml was not inhibited by OPG at 100 ng/ml (Figs. 3K, 4F) but was abolished at higher concentrations of OPG, 250 (Fig. 3L), and 500 ng/ml (not shown). Resorption lacunae were increased by 50 ng/ml OPGL in the presence of 100 ng/ml OPG (Fig. 4F), but OPG at 250 and 500 ng/ml (not shown) inhibited bone resorption induced by this level of OPGL (Fig. 3). For preosteoclast/osteoclast-like cells that arise in cultures of α-MEM+10% FBS (Fig. 3A) [Faust et al., in press], cell morphology appeared the same in the presence (Fig. 3B,C) or absence of different OPG doses, by 16 days. Bone resorption was not detected by this time in any of the cultures.

The effects of OPG (5–100 ng/ml) and OPGL (2.5–15 ng/ml) on bone resorption on day 14 cultures were quantitated by image analysis of bone surfaces and are shown in Figure 5. Resorption increases were observed in the presence of 10 and 15 ng/ml OPGL. No significant decrease in resorption was seen in the presence of 5 ng/ml OPG, but the coaddition of 25 ng/ml OPG abolished the resorption at 10 ng/ml OPGL and reduced the area resorbed with 15 ng/ml OPGL. No resorption was observed in the presence of 50 and 100 ng/ml OPG at any dose of OPGL used in this experiment.

Cathepsin K mRNA Expression

Total cellular RNA, isolated from cells at the time of plating, and at days 5, 9, 12 and 18, treated with and without the combination of OPGL (20 ng/ml) and CSF-1 (25 ng/ml) was analyzed for the expression of Cath K, a lysosomal protease that is an osteoclast expressed gene (Fig. 6). In the presence of OPGL, Cath.K mRNA was expressed earlier (day 5) and transcript levels remained higher than in control cultures throughout the duration of the experiment. This is consistent with enhanced differentiation of LMOC from PBMC induced by OPGL and CSF-1 in these cultures.

DISCUSSION

This study documents OPGL+CSF-1 induced LMOC from PBMC precursors over a 2-week period on bone. Although HC (10^{-6} M) and $1,25(OH)_2D_3$ (10^{-8} M) are included in the experi-

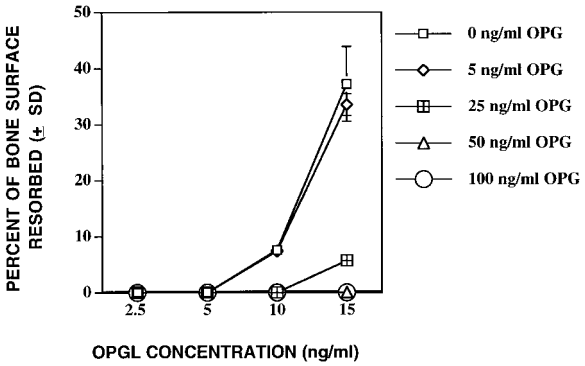

Fig. 5. Quantitation of osteoprotegerin/osteoprotegerin ligand (OPG/OPGL) effects on bone resorption. Cells were grown on bone slices as in the legend of Fig. 1 for 15 days and were treated with 2.5, 5, 10, and 15 ng/ml OPGL in complete media, with and without increasing doses of OPG (5, 25, 50, and 100 ng/ml). Resorption lacunae area was quantitated as described under Materials and Methods.

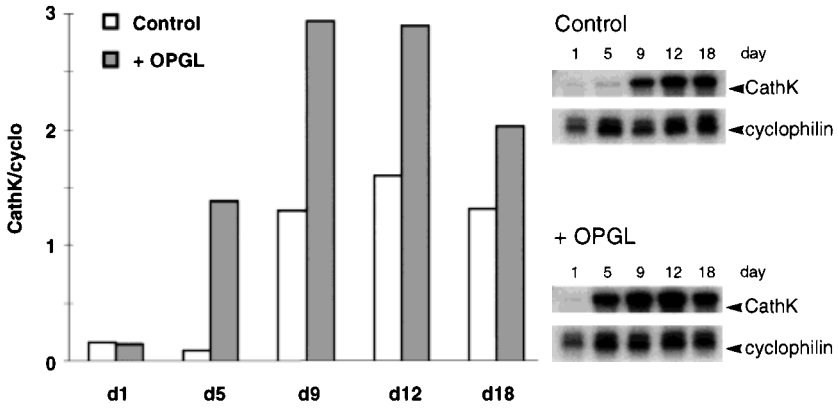

Fig. 6. Cathepsin K mRNA levels. Total cellular RNA was isolated from PBMC cultured on plastic at the time of plating (C1) and at days 5, 9, 12, and 15. Cells were cultured with and without the combination of osteoprotegerin ligand (OPGL) (20 ng/ml) and CSF-1 (25 ng/ml). RNase protection assays were performed with ^{32}P-labeled Cath K and cyclophilin probes. The level of Cath K expression was normalized using cyclophilin expression as a reference as described under Materials and Methods.

ments described, similar results were obtained in the absence of HC and 1,25$(OH)_2$ with OPGL+CSF-1 alone (Shalhoub V, Faust J, unpublished observations). This was also true for cultures on plastic: OPGL+CSF-1 alone induced upregulation of the osteoclast expressed gene Cath K (Fig. 6) along with TRAP$^+$, VR$^+$, and LMOC-like cell formation (not shown). Thus, OPGL replaces stromal cells, vitamin D_3, and glucocorticoids as it does in mouse bone marrow cultures [Lacey et al., 1998], but OPGL cannot bring about LMOC-like cell formation in the absence of CSF-1. Thus, it appears that OPGL fulfills the requirement for the factor that regulates osteoclast maturation [Abe et al., 1986; Yoneda et al., 1993; Hentunen et al., 1994; Fuller et al., 1991; Lee et al., 1991; Horton et al., 1972; Rodan and Martin, 1981] for human cells.

In combination with CSF-1, OPGL dose-dependently increased the size of terminally differentiated osteoclast-like cells on bone and associated resorption. OPG inhibited this effect in a dose-dependent manner confirming findings for mouse [Lacey et al., 1998], and human PBMCs [Matsuzaki et al., 1998]. OPG (0.1–1,000 ng/ml), cannot abolish the formation of preosteoclast-like cells of low nuclearity that form on bone, in control cultures (Shalhoub V, Faust J, unpublished observations and Fig. 3). It is unknown how the formation of these cells is related to the low level of OPG (not shown) and OPGL expressed by cells early in these cultures [Faust et al., in press].

CSF-1 is essential for proliferation and survival of osteoclast lineage cells [Yoshida et al., 1990; Kodama et al., 1991; Felix et al., 1990; Wiktor-Jedrzejczak et al., 1990]. In the presence of CSF-1 (25 ng/ml) alone, pits were not observed by 2 weeks, although a few resorption lacunae were observed at day 37, in one experiment. This is not unexpected because osteoclast-like cells form in culture in the absence of additional OPGL [Faust et al., in press].

By SEM, the osteoclast-like cells on bone were either large and spread out over the bone surface, or, smaller, with ruffled "apical" membranes, facing away from the bone surface (Fig. 2). These small cells, ~10–20 μm in diameter, represent mono-, di-, and trinuclear cells [Faust et al., in press]. At 7 and 11 days, these small cells corresponded to the abundant, small, VR$^+$ preosteoclast/osteoclast-like cells on bone and formed a confluent mat over the bone surface by 11 days (Shalhoub V, Faust J, unpublished observations). By day 15, these cells were less abundant and were beside and within large resorption lacunae (Fig. 2D,E). The "apical" membrane ruffling could represent the result of membrane biochemical changes associated with proliferation/differentiation or bone resorption, or both [Salo et al., 1997; Nesbitt and Horton, 1997]. Whether LMOC formation from PBMCs varies with age, sex, or the presence of skeletal diseases remains to be determined.

In conclusion, this study has reproducibly demonstrated the events leading to the formation of resorbing LMOC, on bone. OPGL, in the presence of CSF-1 induces terminal differentiation of LMOC human cells, in vitro; actions which OPG inhibits. The morphology of osteoclast-like cells on bone, as they develop and resorb, is revealed by light and SEM for the first time. In transgenic overexpressing mice, OPG circulating levels correlate with the severity of osteopetrosis [Simonet et al., 1997] and postnatal OPG-deficient mice develop early-onset osteoporosis and arterial calcification [Bucay et al., 1998]. Coupled with findings in mouse, this study supports similar physiological roles for

OPG and OPGL in the regulation of bone mass in humans.

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