# The Ligand for Osteoprotegerin (OPGL) Directly Activates Mature Osteoclasts

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***Abstract.*** Osteoprotegerin (OPG) and OPG-ligand (OPGL) potently inhibit and stimulate, respectively, osteoclast differentiation (Simonet, W.S., D.L. Lacey, C.R. Dunstan, M. Kelley, M.-S. Chang, R. Luethy, H.Q. Nguyen, S. Wooden, L. Bennett, T. Boone, et al. 1997. *Cell.* 89:309–319; Lacey, D.L., E. Timms, H.-L. Tan, M.J. Kelley, C.R. Dunstan, T. Burgess, R. Elliott, A. Colombero, G. Elliott, S. Scully, et al. 1998. *Cell.* 93: 165–176), but their effects on mature osteoclasts are not well understood. Using primary cultures of rat osteoclasts on bone slices, we find that OPGL causes approximately sevenfold increase in total bone surface erosion. By scanning electron microscopy, OPGL-treated osteoclasts generate more clusters of lacunae on bone suggesting that multiple, spatially associated cycles of resorption have occurred. However, the size of individual resorption events are unchanged by OPGL treatment. Mechanistically, OPGL binds specifically to mature OCs and rapidly (within 30 min) induces actin ring formation; a marked cytoskeletal rearrangement that necessarily precedes bone resorption. Furthermore, we show that antibodies raised against the OPGL receptor, RANK, also induce actin ring formation. OPGL-treated mice exhibit increases in blood ionized $Ca^{++}$ within 1 h after injections, consistent with immediate OC activation in vivo. Finally, we find that OPG blocks OPGL's effects on both actin ring formation and bone resorption. Together, these findings indicate that, in addition to their effects on OC precursors, OPGL and OPG have profound and direct effects on mature OCs and indicate that the OC receptor, RANK, mediates OPGL's effects.

Key words: osteoclast • bone resorption • bone remodeling • osteoporosis • hematopoietic cell growth factors

OSTEOCLASTS (OC)[1] are bone resorbing cells of hematopoietic origin. They arise by the differentiation of OC precursors of the monocyte/macrophage lineage and their function is required not only for the development of the skeleton, but also for mineral homeostasis and normal remodeling of bone in adult animals. The requirement for macrophage colony stimulating factor/colony stimulating factor-1 (MCSF/CSF-1) in the differentiation of OCs is well supported by in vivo and in vitro evidence (Suda et al., 1992). CSF-1 alone, however, is clearly not sufficient to cause differentiation of OCs from hematopoietic progenitors. However, mature, functional OCs will develop from spleen or bone marrow when cocultured with CSF-1-producing osteoblast-like stromal cells in the combined presence of vitamin D3, and dexamethasone (Takahashi et al., 1988). Furthermore, both differentiation and activation of OCs in this coculture system have been shown to require physical contact between the osteoblastic stromal cells and hematopoietic progenitors (Rodan and Martin 1981; Udagawa et al., 1989; Fuller et al., 1991; Suda et al., 1992; Jimi et al., 1996). Based on these results, it has been proposed that a membrane-bound OC factor on stromal cells would stimulate differentiation of OC progenitors as well as activate mature OCs. Although a number of approaches to elucidate the identity of the proposed OC differentiation factor have been taken over many years (Horton, 1972; Abe et al., 1986; Fuller et al., 1991; Lee et al., 1991; Yoneda et al., 1993; Hentunen et al.,

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1. *Abbreviations used in this paper:* MCSF/CSF-1, macrophage colony stimulating factor/colony stimulation factor-1; OC, osteoclasts; OPG/OCIF, osteoprotegerin/osteoclast inhibitory factor; OPGL/ODF OPG, ligand/osteoclast differentiation factor (identical to RANKL and TRANCE); RANK, receptor activator of NF-κB; RANKL, RANK ligand is identical to OPGL/ODF and TRANCE; TNF, tumor necrosis factor; TNFR, TNF receptor; TRANCE, TNF-related activation-induced cytokine is identical to OPGL/ODF; TRAF, TNFR-associated factor; TRAP, tartrate resistant acid phosphatase.

1994), only very recently, and somewhat indirectly, has a clear candidate for such a factor emerged.

Osteoprotegerin (OPG, also called osteoclast inhibition factor, OCIF), a soluble member of the tumor necrosis factor receptor family (TNFR), has profound inhibitory effects on in vitro OC differentiation and bone resorption in vivo (Simonet et al., 1997; Tsuda et al., 1997; Yasuda et al., 1998a). The function of OPG was elucidated by overexpression in transgenic mice, as well as by inhibition of OC differentiation in vitro. OPG transgenic mice developed osteopetrosis with a severity correlated to the level of transgene expression. Finally, OPG knockout mice were recently shown to have severe osteoporosis (Bucay et al., 1998). Taken together, these results provide strong evidence that OPG regulation of OC differentiation is necessary for the maintenance of normal bone density in vivo.

Based on its homology to the TNFR family, Simonet et al. (1997) proposed that OPG might act as a soluble decoy receptor to neutralize a putative OC stimulatory factor on osteoblastic stromal cells, and thus prevent OC differentiation. Direct expression cloning of a transmembrane ligand for OPG (OPGL) has now been accomplished independently by two groups (Lacey et al., 1998; Yasuda et al., 1998b). OPGL (also called osteoclast differentiation factor, ODF), identified using OPG-Fc fusion protein as a probe, is a TNF-related transmembrane cytokine expressed by a number of different cell types, notably including osteoblastic stromal cells (Lacey et al., 1998; Yasuda et al., 1998b). OPGL completely replaces the requirements for stromal cells, vitamin D3 and glucocorticoids during in vitro osteoclastogenesis from spleen or bone marrow progenitors; it binds to a committed OC progenitor and in the combined presence of CSF-l, stimulates the rapid induction of a variety of characteristic OC genes including c-src, tartrate resistant acid phosphatase (TRAP), β-3 integrin (a component of the $\alpha_v\beta_3$ vitronectin receptor), calcitonin receptor and cathepsin K (Lacey et al., 1998; Yasuda et al., 1998b). Furthermore, administration of OPGL to normal mice resulted in a dose-dependent systemic hypercalcemia apparently due to increased OC activity, but not an increase in the number of OCs on the bone surface (Lacey et al., 1998). All of the potent effects of OPGL in vitro and in vivo are completely inhibited by OPG. These data provide evidence that OPGL and OPG are key regulators of osteoclastogenesis.

OPGL is identical to RANKL (receptor activator of NF-κB ligand) and TRANCE (TNF-related activation-induced cytokine); and in vitro evidence suggests that it may play a role in regulating interactions between T cells and dendritic cells (Anderson et al., 1997; Wong et al., 1997). The receptor for RANKL, RANK (receptor activator of NF-κB), was also shown to be involved in $RANK^+$ T cell survival in vitro (Anderson et al., 1997; Wong et al., 1997). In addition, previous data suggested that RANK is also the receptor for OPGL on differentiating (Lacey et al., 1998 and Yasuda et al., 1998b) and mature OCs (Fuller et al., 1998). Hsu et al. (1999) and Nakagawa et al. (1998) have now provided strong evidence that osteoclastogenesis induced by OPGL (RANKL) is directly mediated through RANK on OC precursor cells. Complete support of a dual role for OPGL (RANKL) and RANK in both immune functions and osteoclastogenesis was very recently provided by Kong et al. (1999) who reported on the OPGL knockout mouse. Mice lacking OPGL not only have severe osteopetrosis and defective tooth eruption, but also exhibit defects in T and B cell differentiation and completely lack lymph nodes.

In addition to its role in OC differentiation, we previously reported that OPGL induces a dose-dependent increase in the number of resorption lacunae generated by mature OCs on cortical bone slices (Lacey et al., 1998), suggesting that OPGL also directly activates mature OCs. In the present study we investigate the mechanism by which OPGL increases bone resorption, independent of its role in OC differentiation, in cultures of mature rat OCs and in mice. We present evidence that OPGL acts directly on individual, mature OCs through RANK to rapidly stimulate reorganization of the actin cytoskeleton and induces multiple cycles of bone resorption.

## *Materials and Methods*

### *OC Isolation, Characterization and Cell Culture*

Quantitation of bone resorption by mature OCs was performed essentially as described by Arnett and Dempster (1986; Lacey et al., 1998). In brief, OCs isolated from the long bones of post-natal day 1–3 rat pups were put into suspension by curretting the entire bone in culture media (Hepes-buffered M199 pH 7.0 [GIBCO BRL] containing 10% FBS [Hyclone], penicillin, streptomycin, fungizone and glutamine [GIBCO BRL]). Cells from at least two pups were pooled together for each experiment reported. Large debris were removed by sedimentation at 1 *g* for 1 min and the supernatant was plated onto 4 mm × 4 mm × 400 μm bovine cortical bone slices preequilibrated with $HCO_3$-buffered (1.25 g/liter), M199 in 96-well plates, or directly onto air dried FBS coated glass coverslips. The mature OCs were allowed to attach for 30 min, then most of the more abundant, but less-adherent bone marrow and bone cells were removed by vigorous washing. This procedure generates a sparse culture of cells on the bone slices or coverslips that is enriched for multinucleate, TRAP positive OCs (Fig. 1). Varying numbers of mononuclear cells are also present; (Fig. 6), and although their identity was not established, some of them do express $\alpha_v\beta_3$ and form actin rings (see below) and are presumably immature, mononuclear OCs. The bone slices ($n$ = 4 for each condition) containing mature rat OCs were placed into 24-well dishes with $HCO_3$-buffered M199 control media, or the same media containing test compounds (as indicated) and were incubated for 24 h at 37°C in a humidified, 5% $CO_2$/95% air atmosphere. Coverslips containing OCs were incubated in Hepes-buffered M199 (pH 6.8) in an air incubator at 37°C as indicated below.

### *Bone Resorption Measurements*

Following fixation with 0.25% glutaraldehyde, bone slices were stained for TRAP (Sigma kit 387-A). Mature OCs were defined as highly TRAP positive cells containing three or more nuclei (Fig. 1). The total number of rat OCs on each bone slice was counted (typically 35–50; see Fig. 2 B) using bright field optics on a Nikon Eclipse 800 upright microscope and a 20× objective. After counting, the OCs were removed using 50 mM $NH_4OH$ and brief sonication. The resorption lacunae on the same bone slices were then visualized by toluidine blue staining (Murrills and Dempster, 1990). Individual resorption events were distinguished by a dark border of toluidine blue stain surrounding an excavation. The data presented here record each resorption event separately; often several events are apparent in what is classically called a resorption pit (see Fig. 3 A). The number of resorption events were counted (Lacey et al., 1998) and area measurements were done via a calibrated MetaMorph image analyzer (Leica) coupled to a Nikon Optiphot microscope. Starting at the bottom left hand corner and working up, the entire surface area of each 4 mm × 4 mm bone slice was scanned for resorption lacunae until the entire surface area of each bone slice was examined. When a resorption lacuna came into the viewing screen, the scanning was stopped and the perimeter of each re-

sorption event was traced (see Fig. 3 A). The derived area of each individual measurement was transferred to a spreadsheet. The total number of resorption events per bone slice, total area resorbed, and mean area per event were calculated. The area measurements were sorted by treatment, and the mean area and distribution of event sizes were examined through an appropriate mixed model Analysis Of Variance (ANOVA) complemented with Bonferroni's test when the difference between means was significant at alpha level equals 0.05. The corresponding *P* values are reported in the text. In addition to means comparison, a variance heterogeneity test was performed on the data following the Brown-Forsythe's test (Cochran and Cox, 1957; Brown and Forsythe, 1974). This test was shown to be robust to the underlying distribution. The corresponding *P* values are reported in the text. All the analyses were performed using the Statistical Analysis System software (SAS Institute Inc.) on the UNIX platform. Two additional, independent experiments were performed and similarly analyzed, the results of these are presented in the text.

### *Scanning Electron Microscopy*

Mature rat OCs were isolated and plated on cortical bone slices as described above. Following quantitative analysis of the toluidine blue stained bone slices, they were sonicated in water to remove the stain and any residue. They were dehydrated through a graded ethanol series and left in 100% ethanol overnight. After air drying, the slices were placed in a vacuum desiccator for several hours before being mounted on scanning EM stubs. The mounted slices were sputter coated with 30 nm of gold/palladium. The specimens were examined on a JEOL 5022 scanning EM at 25 KV with a working distance of 20 mm. Resorption lacunae from each slice were identified with scanning EM; representative examples were selected for photography at 750× magnification. For quantitative scoring of the lacunae, the entire surface of two slices per condition, from two separate experiments were analyzed ($n = 4$ slices/condition). A semi-quantitative scale was used to indicate whether a resorbed area contained no exposed collagen fibrils (Ø), a few collagen fibrils (+) or many collagen fibrils (++). In addition, the resorbed areas were scored for whether they contained, 1, 2, 3, or more resorption events within the resorbed area.

### *Antibodies*

Mouse anti–rat β3 was purchased from PharMingen, HRP-linked rabbit anti-FITC was from DAKO, biotinylated goat anti–rabbit and biotinylated horse anti–mouse were from Vector Labs. The rabbit polyclonal anti-RANK antibodies used were generated against a recombinant RANK extracellular domain (residues 31–211) fused to human IgGγ1 Fc (Hsu et al., 1999) essentially as previously described for OPG-Fc (Simonet et al., 1997). The rabbit antiserum was affinity purified on a RANK (31-211)-conjugated Sepharose column to generate the mono-specific anti-RANK–specific antibodies used here.

### *Immunocytochemistry and OPGL Binding to Isolated OCs*

Mature rat OCs were isolated and plated on FBS coated glass coverslips as described above. After a brief incubation in control media (~2 h) the cells were fixed in cold acetone and air dried. The OCs were incubated for 2 h at room temperature with either FITC-labeled OPGL (Lacey et al., 1998) at 20 μg/ml, rabbit anti-RANK at 2.8 μg/ml, or mouse anti–rat β3 at a 1:5 dilution. HRP-linked rabbit anti-FITC, biotinylated goat anti–rabbit or biotinylated horse anti–mouse, respectively, were used to detect the primary reagent. Avidin-Biotinylated enzyme Complex (Vector Labs) was incubated with the biotinylated antibodies, and finally HRP activity was detected using DAB. The coverslips were counterstained with hematoxylin. For competition experiments, a 10-fold excess of unlabeled OPGL (200 μg/ml) was preincubated with FITC-OPGL, or anti-RANK was preincubated with 28 μg/ml of the extracellular domain of RANK both at 4°C overnight. All subsequent incubations were carried out exactly as described above. In addition, an irrelevant mouse IgG incubation served as a general negative control for the staining reagents.

### *Actin Ring Staining and Quantitation*

Mature rat OCs isolated as above were plated onto FBS-coated coverslips in Hepes-buffered M199 media (pH 6.8) containing 10% FBS. After removal of the nonadherent cells, the coverslips were allowed to incubate for ~1 h at 37°C before treatments were initiated. Media were replaced with fresh control or test compounds in Hepes-buffered M199 media (pH 6.8) for the times indicated, then the coverslips were fixed, permeabilized, and stained with a modified version of method 1 in Lakkakorpi and Väänänen (1991). In brief, cells were fixed for 10 min in 3% paraformaldehyde, followed by 0.2% Triton X-100 permeabilization for 10 min. A short blocking step in 5% normal goat serum was included before staining for 1 h at room temperature with 5 units/ml Texas red–labeled phalloidin (Molecular Probes). Coverslips were washed in PBS and then counterstained with DAPI before mounting onto slides using Prolong anti-fade mounting medium (Molecular Probes). The OCs were identified on a Nikon Eclipse 800 upright microscope equipped with epifluorescence optics. OCs were defined as cells containing at least 3 nuclei, and every OC on every coverslip was counted and scored for its type of actin cytoskeletal structure (see Fig. 6 A) by a blinded investigator.

### *OPGL Treatment of Mice*

Male BDF1 mice ($n = 5$) aged 6–8 wk were maintained on normal chow, or were fed low calcium chow (0.02% vs. 0.6% in standard chow) for 48 h before receiving varying doses of OPGL by intravenous injection in a PBS carrier, or PBS alone as control. Orbital blood samples for ionized calcium determination were obtained 1 h after injection from animals anesthetized with inhaled isoflurane. Blood ionized calcium levels were then determined using a Chiron Diagnostics no. 634 blood ionized calcium/pH analyzer. Data (reported as mean ± SEM) were evaluated by ANOVA with Dunnett's post hoc test to allow for multiple comparisons with control, using JMP statistical software (SAS Institute Inc.; Dunnett, 1955).

### *OPG, OPGL, and FITC-OPGL*

The sources and preparation of the protein reagents used in this study have been described in detail in Simonet et al., 1997 and Lacey et al., 1998. In brief, recombinant murine OPGL [158-316] was expressed and purified from *E. coli* (Lacey et al., 1998). FITC-coupled OPGL was prepared using 6-fluorescein-5-(and 6) carboxyamido hexanoic acid succinimidyl ester (Molecular Probes) and murine OPGL [158-316] as described (Lacey et al., 1998). A human OPG [22-201]-hu Fc fusion protein, expressed and purified from Cho cells (Simonet et al., 1997), was used to inhibit the action of OPGL in various experiments.

## *Results*

### *OPGL Stimulates Mature OCs to Resorb Bone*

We previously reported that treatment of mature OCs on bone slices led to the generation of a dose-dependent increase in the number of resorption lacunae (Fig. 7 in Lacey et al., 1998). This increase in resorption events could arise by a number of different mechanisms, for example, OPGL might act directly on the OCs, or indirectly through action on other cells in the culture. Whether direct or indirect, OPGL may act as an OC survival factor, and thus increase the total number of OCs in each experiment. Alternatively, OPGL might activate quiescent OCs such that more of the OCs in the culture are active, or OPGL may activate individual OCs to undergo multiple cycles of resorption during the assay period, or both. To investigate the mode of OPGL action, mature OCs isolated from neonatal rat long bones were plated onto cortical bone slices (Fig. 1) to quantitatively and qualitatively assess the functional consequences of OPGL on bone resorption.

The total area resorbed on each bone slice was quantified by image analysis (see Materials and Methods). We found that OCs treated with OPGL resorb >115,000 $\mu m^2$ (mean of $n = 4$ bone slices) compared with untreated, control OCs which resorbed only ~16,000 $\mu m^2$ of the bone surface area (Fig. 2 A), representing about a sevenfold increase in the total area resorbed per bone slice. This marked increase in the total area resorbed was statistically

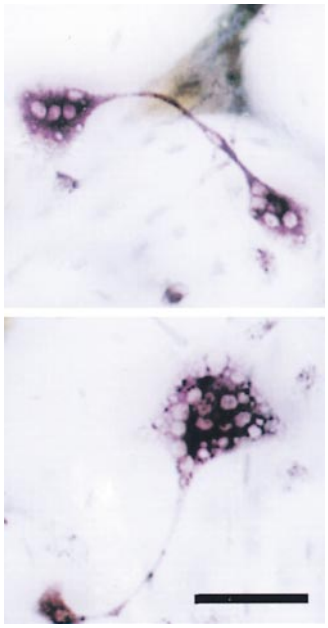

*Figure 1.* Mature rat OCs stain intensely for TRAP. OCs isolated from the long bones of 2-d-old rat pups were plated on cortical bone slices and stained for TRAP (purple). Two representative examples are shown; on average we obtain 30–50 OCs per 4 × 4 mm bone slice, clearly the density of OCs is low in these cultures. Multinuclearity varies from three to ~30 nuclei per OC. Mononuclear cells are visible and some, but not all are TRAP positive. Bar, 50 μm.

significant compared with each of the controls ($P$ = 0.0001) and was completely inhibited by the addition of OPG. However, by itself, OPG treatment of OCs did not significantly alter the total area of bone resorbed compared with the untreated control ($P$ = 0.59; Fig. 2 A). It is noteworthy that both OPG-Fc alone and OPGL plus OPG-Fc treated OCs retain a low level of basal resorption activity. Furthermore, in contrast to the induction of OC differentiation by OPGL (Lacey et al., 1998; Yasuda et al., 1998b), the activation of mature OCs by OPGL occurs in the absence of added CSF-1. In two independent replications of this experiment OPGL caused a similarly significant stimulation of bone resorption. (Experiment 2 showed about a fourfold increase in the total area resorbed/slice and was significant compared with each of the controls, $P$ = 0.0001. Experiment 3 showed about a sevenfold increase and this was significant compared with each of the controls, $P \leq 0.0003$).

### *OPGL Does Not Alter Mature OC Number*

To address the possibility that OPGL was acting as a survival factor or inducing mononuclear OCs precursors to fuse to form multinucleate OCs during the course of the experiment, the total number of TRAP positive multinucleate OCs were counted on each bone slice at the end of the 24-h incubation period. We found that there was no significant difference in the mean total number of OCs on bone slices treated with control media, media containing OPGL, OPG or the combination of OPGL and OPG, ($P$ = 0.093; Fig. 2 B). These results show OPGL does not act by increasing the number of multinucleate OCs in these experiments. Two independent replications of this experiment confirmed that OC numbers do not vary under these different experimental conditions, however, the number of OC per bone slice does vary between different experiments. (Experiment 2, the mean OC number per slice among the four different conditions were not significantly different, $P$ = 0.17; experiment 3, $P$ = 0.20).

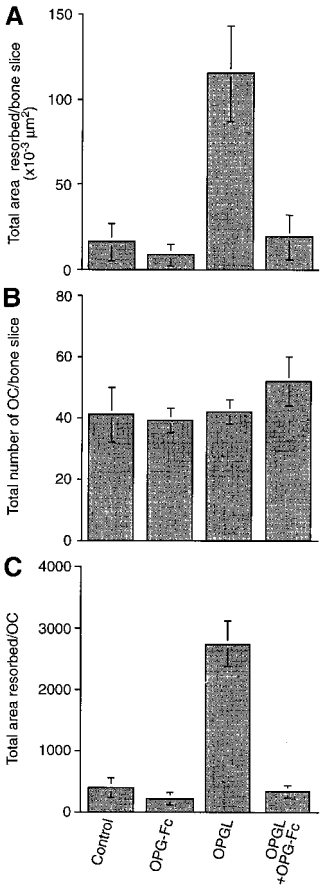


*Figure 2.* Treatment with OPGL increases the total area of bone resorbed without altering OC number. (A) The total area of bone resorbed per bone slice ($n$ = 4) was measured as described in Materials and Methods. Compared with control cultures (untreated), OPGL at 10 ng/ml markedly stimulates osteoclastic bone resorption. A fivefold molar excess of OPG-Fc (130 ng/ml) completely inhibited this stimulation, while OPG-Fc alone (130 ng/ml) has little effect on bone resorption. Data are shown ± 1 SD, compared with the controls, OPGL significantly stimulates the total area of bone resorbed per bone slice ($P$ = 0.0001). (B) OCs containing at least 3 nuclei and exhibiting strong TRAP positive staining from $n$ = 4 bone slices per conditions were counted after 24 h incubation with the indicated treatments (as described above). The data are presented as the average number of OCs per bone slice ± 1 SD, the differences were not statistically significant ($P$ = 0.093). (C) The mean area of bone resorbed per OC is presented for $n$ = 4 slices ± SEM under the same conditions as described above, the differences between OPGL and controls were highly significant ($P$ = 0.0001).

### *OPGL Increases the Area of Bone Resorbed per OC*

To show that the overall effect of OPGL treatment on OCs is to activate either more individual OCs, and/or to activate some individual OC more, we have also expressed the area data as the average of the resorbed area per TRAP positive OC (Fig. 2 C). The data show the striking and significant stimulation of OC resorption by OPGL compared with each of the controls ($P$ = 0.0001). We find that there is about an eightfold increase in the area resorbed per OPGL-treated OCs compared with the control OCs. In two independent replications of this experiment, there was also an approximately eightfold increase in the area resorbed per OPGL-treated OC compared with the controls. (Experiment 2, the area resorbed per OPGL-treated OC was significantly different from each of the controls, $P$ = 0.0001; experiment 3, $P$ = 0.0001.)

### *OPGL Does Not Alter the Area of Individual Resorption Events*

If OPGL induces mononuclear (pre)OCs in the cultures to resorb bone, we would expect to observe an increase in the number of very small resorption events compared with control cultures. On the other hand, if OPGL induces fu-

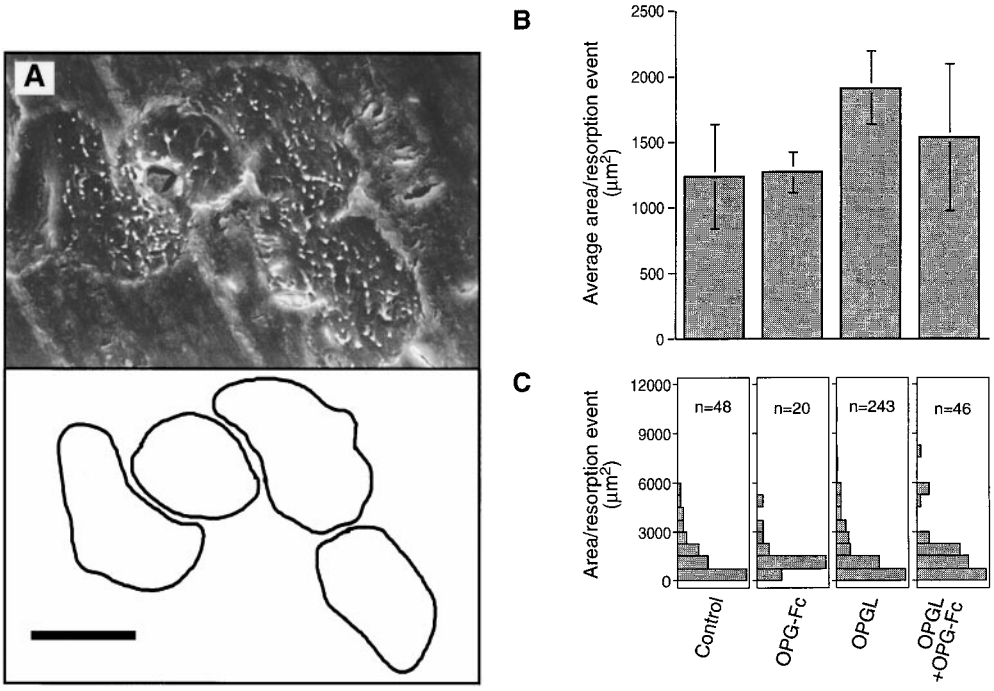


*Figure 3.* OPGL does not change the mean area nor the distribution of sizes of individual resorption events. (A) A scanning EM showing a typical resorption area generated by an OC treated with OPGL (10 ng/ml) is shown in the upper part of A. The drawing in the lower part of A illustrates how individual resorption events are defined by outlining the boundaries of an individual resorption cycle. These are seen as the rim of individual excavations by scanning EM here, or as dark borders on toluidine blue stained bone (not shown). The areas of all of the individual resorption events on bone slices ($n = 4$) per condition were measured as described in Materials and Methods. The scale bar measures 25 μm. (B) The calculated mean area per resorption event ± 1 SD are shown: Control (untreated), OPG-Fc at 130 ng/ml, OPGL at 10 ng/ml, or a combination of OPGL and OPG-Fc at the same concentrations. Differences between the mean areas per resorption event under the 4 conditions shown were not significant ($P = 0.14$). (C) The size distribution of resorption events are displayed in a normalized format to allow the distributions to be visually compared. The number of resorption events differs depending on the different treatments and are shown in each individual panel. Note that the number of resorption events generated by OCs treated with OPG-Fc appears atypically low because only $n = 3$ slices were analyzed, nonetheless, the ratio of resorption events to TRAP positive OCs on these bone slices were not statistically different from control. The results of the statistical analyses indicate that variances (distribution of event sizes) were not significantly different from the controls ($P = 0.135$).

sion of mononuclear cells, many larger resorption events may be generated. To determine whether OPGL treatment altered the area of individual resorption events, we measured the area of each excavation generated by a single round of resorption (Fig. 3 A illustrates on a scanning EM how we define these individual resorption areas). When the resorbed areas were closely related, we were able to identify individual boundaries as darkly stained borders separating individual events or as a prominent rim in scanning EM (Fig. 3 A). As seen in Fig. 3 B, the mean area per resorption event generated under the different experimental conditions is not significantly different ($P = 0.14$). Furthermore, histograms of individual area measurements suggested that the overall distribution of resorption event sizes under the different conditions were quite similar, with most being between 100 and 3,000 $\mu m^2$ (Fig. 3 C). Statistical analyses of the heterogeneity of variances (distribution of sizes) were performed using the Brown-Forsythe's test (Cochran and Cox, 1957; Brown and Forsythe, 1974; see Materials and Methods). The results show that the variance of the event sizes among the four groups was not different ($P = 0.135$) demonstrating that there is no significant increase in the proportion of small or large resorption events. Similar analysis on the mean and variance of resorption event sizes from two additional experiments yielded similar results. (Experiment 2, test of means $P = 0.24$, test of variance $P = 0.37$; experiment 3, test of means $P = 0.58$, test of variance $P = 0.92$.)

### *OPGL Induces Individual Mature OCs to Undergo Multiple Cycles of Resorption*

Scanning electron microscopy (scanning EM) was used to assess if there were any qualitative changes in resorption induced by OPGL. Bone slices were prepared for scanning EM and representative examples of the resorption lacunae observed are shown in Fig. 4. Resorbed areas generated under control conditions, with OPGL and OPG together, or OPG alone (Fig. 4, A, C, or D) were frequently single or double events. In contrast, resorption areas generated by OCs treated with OPGL (Figs. 3 A and 4 B) were frequently connected with 3 to 7 individual resorption events visible within one contiguous resorbed area (Fig. 3 A shows four connected events, while Fig. 4 B shows three connected resorption events). In addition, resorbed areas generated in the presence of OPGL appeared to expose more collagen fibrils regardless of the number of connected events. The generation of physically connected resorption events indicates that single OCs are being induced by OPGL to undergo multiple resorption cycles during the 24-h assay period.

To get a better idea about the magnitude of these quali-

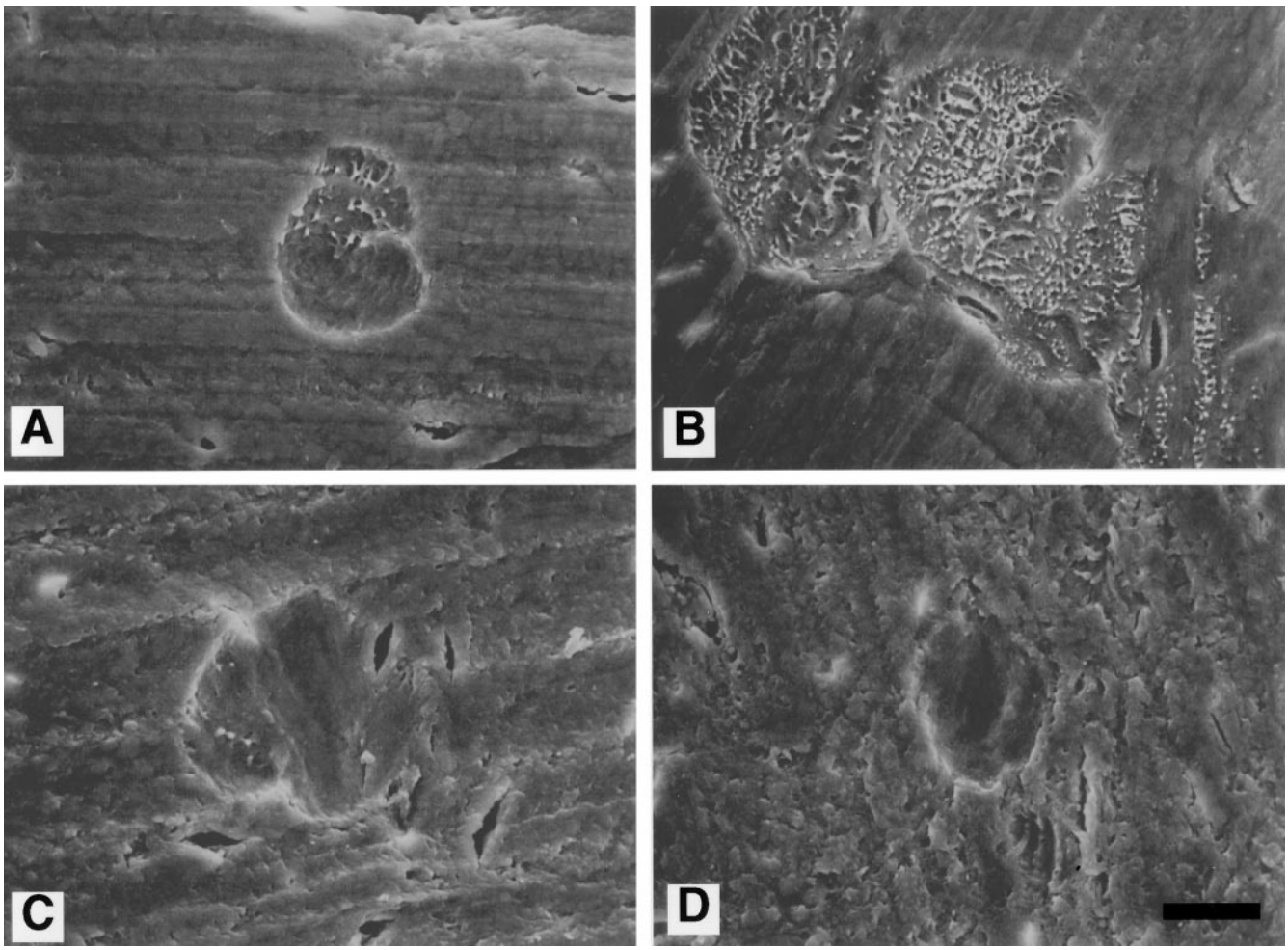


*Figure 4.* Scanning Electron Microscopy reveals that individual OCs are induced by OPGL to undergo multiple cycles of bone resorption. Bone slices from a typical experiment were prepared for, and viewed by scanning EM as described in Materials and Methods. The figure reveals qualitative differences between resorption areas generated under the different conditions shown. (A) Control (untreated). (B) OPGL (10 ng/ml) treatment leads to the generation of numerous connected resorbed areas which expose a network of collagen fibrils. (C) OPGL (10 ng/ml) and OPG-Fc (130 ng/ml) together. (D) OPG-Fc (130 ng/ml) alone. Bar, 25 μm.

tative observations, we have analyzed the appearance of all of the resorbed areas on four bone slices per condition by scanning EM. To score for exposure of collagen fibrils, we used a semi-quantitative scale where (Ø) equals no fibrils exposed (e.g., Fig. 4, C and D), (+) equals a few fibrils exposed (e.g., Fig. 4 A), and (++) equals numerous exposed fibrils (e.g., Figs. 3 A and 4 B). We were able to demonstrate a statistically significance difference (Ø vs. +, ++) between exposure of collagen fibrils by OPGL treated OCs ($n$ = 115 resorbed areas analyzed on four bone slices from two separate experiments) and the untreated control group ($n$ = 31; $P$ = 0.0001) and the OPGL vs. OPGL + OPG-Fc group, ($n$ = 30; $P$ = 0.005).

These same bone slices were also scored for the number of resorption events within each resorbed areas by scanning EM. Compared with each of the controls, OPGL treated OCs generated a statistically significant skew toward multiple-resorption events/resorbed area (the number of resorbed areas with 1 and 2 events were compared with those with 3–7 events). (OPGL vs. control, $P$ = 0.0001; OPGL vs. OPG-Fc, $P$ = 0.0019; and OPGL vs. OPGL + OPG-Fc, $P$ = 0.0044.)

### *Mature OCs Bind OPGL and Express the OPGL Receptor, RANK*

To address whether OPGL acts directly on the mature OCs we asked whether OPGL binds specifically to mature OCs, and if the recently described OPGL receptor, RANK, (Nakagawa et al., 1998; Hsu et al., 1999), is expressed by OCs. A modified, but fully active form of OPGL labeled with FITC (Lacey et al., 1998; see Materials and Methods) was bound to fixed OCs on glass coverslips (Fig. 5) or on bone (data not shown). To amplify the signal from the bound OPGL-FITC, the OCs were incubated with HRP-linked anti-FITC antibody. Essentially all of the multinucleate cells were stained by OPGL-FITC as were some, but not all, of the mononuclear cells (Fig. 5, A and B). This staining was specifically competed by the addition of excess unlabeled OPGL (Fig. 6 C). To determine if the

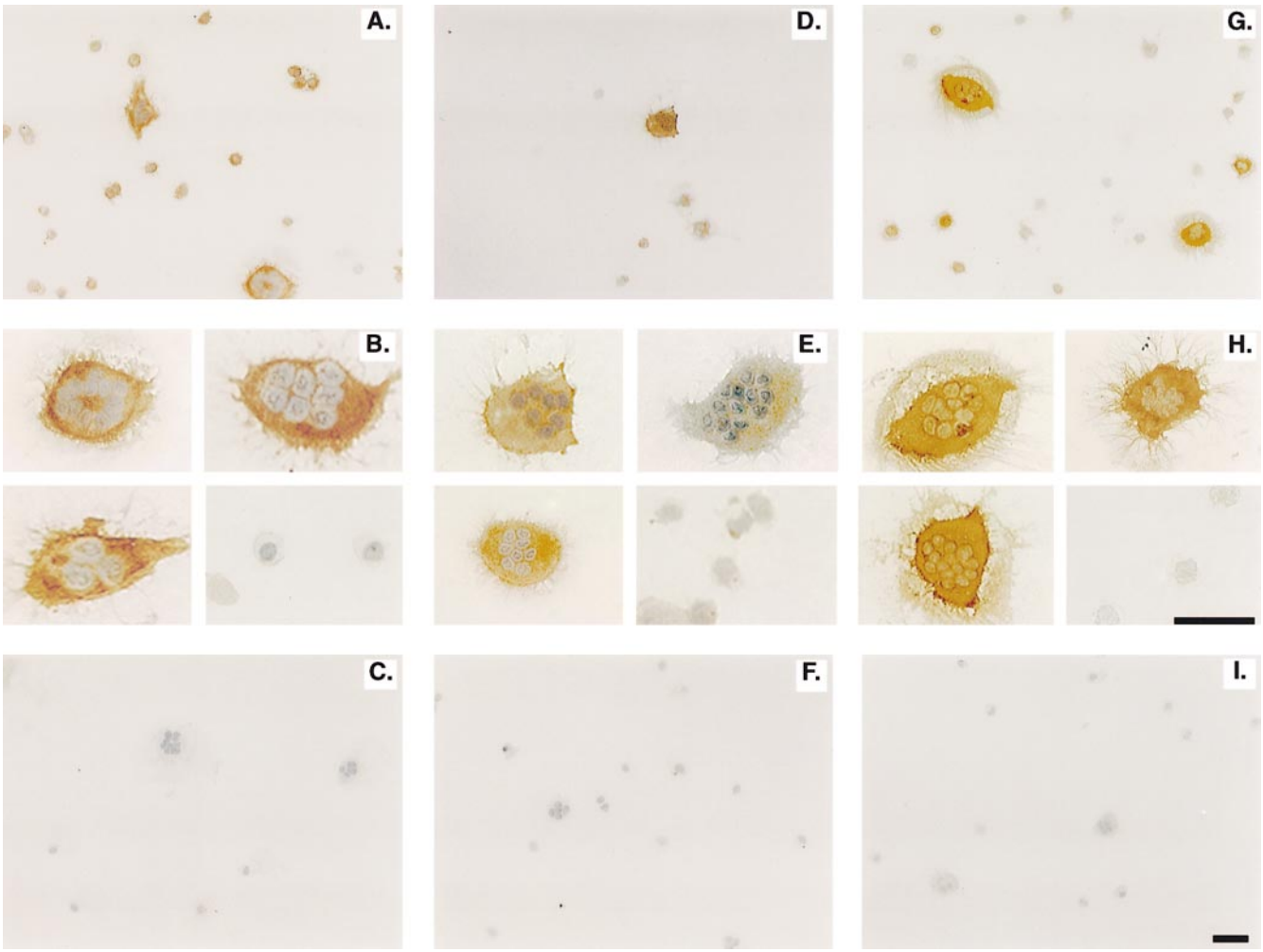


*Figure 5.* Mature osteoclasts specifically bind OPGL and express RANK. Osteoclasts plated on FBS-coated coverslips were stained as described in Materials and Methods. (A) FITC-OPGL binding to multinucleate OCs. (B) Higher magnification of OPGL-binding to multinucleate OCs, as well as some negative mononuclear cells from the same coverslip. (C) Competition of FITC-OPGL with unlabeled OPGL completely blocks binding to OCs and mononuclear cells. (D) Anti-RANK binds to multinucleate OCs. (E) Higher magnification of anti-RANK binding multinucleate OCs, as well as a few negative mononuclear cells from the same coverslip. (F) Competition with soluble RANK completely blocks anti-RANK binding to OCs and mononuclear cells. (G) Anti-$\beta_3$ integrin reacts strongly with multinucleate OCs. (H) Higher magnification of anti-$\beta_3$ integrin stained OCs, as well as some negative mononuclear cells. (I) An irrelevant control mouse antibody does not stain any cells in the culture. A, C, D, F, G, and I are all at the same magnification, while B, E, and H are at a higher magnification. Bars, 50 μm.

OPGL receptor, RANK is expressed on the mature OCs we used a polyclonal antibody directed against the extracellular domain of the receptor (Hsu et al., 1999). Most of the multinucleated OCs, as well as some of the mononuclear cells are positive for RANK (Fig. 5, D and E). To demonstrate the specificity of this antibody, excess antigen was preincubated with the antibody before incubation with the OCs (Fig. 5 F). As a positive control, we stained OCs with antibodies to the well characterized OC cell surface protein, $\alpha_v\beta_3$ integrin (see Rodan and Rodan, 1997) using a $\beta_3$ integrin-specific antibody. Essentially all of the large, multinucleate OCs stained with the $\beta_3$ integrin antibody, as did some of the mononuclear cells (Fig. 5, G and H). In contrast, cells stained with an isotype control antibody were all negative (Fig. 5 I). The staining with anti-RANK is weaker than staining with either OPGL-FITC or anti-$\beta_3$, probably because the OPGL-FITC signal was amplified (see Materials and Methods), and $\beta_3$ is expressed at very high levels on OCs. From these combined data, it appears that OPGL binds directly to mature OCs by binding the OPGL receptor, RANK which is clearly present on the mature multinucleate OCs.

### *OPGL or Anti-RANK Induce the Rapid Reorganization of the OC Cytoskeleton into Actin Rings*

To resorb bone, the OC must become polarized and form a very tight seal with the substratum (bone) before protons and proteases are released into the specialized extracellular resorption compartment. Numerous laboratories have described the specialized actin ring structure that overlies this zone of tight sealing (e.g., Turksen et al., 1988;

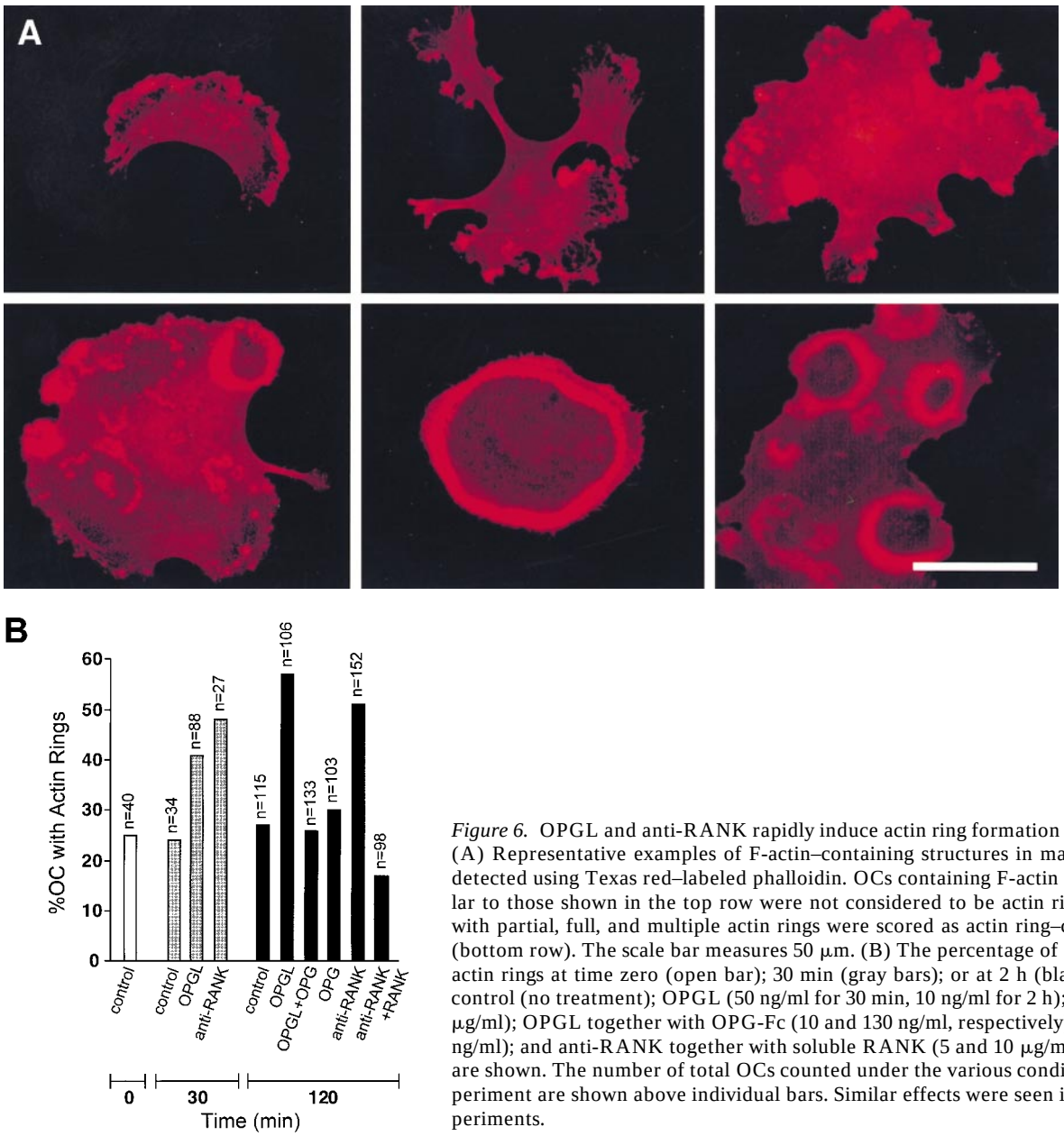


*Figure 6.* OPGL and anti-RANK rapidly induce actin ring formation in mature OCs. (A) Representative examples of F-actin–containing structures in mature OCs were detected using Texas red–labeled phalloidin. OCs containing F-actin structures similar to those shown in the top row were not considered to be actin rings; while OCs with partial, full, and multiple actin rings were scored as actin ring–containing OCs (bottom row). The scale bar measures 50 μm. (B) The percentage of OCs containing actin rings at time zero (open bar); 30 min (gray bars); or at 2 h (black bars) under control (no treatment); OPGL (50 ng/ml for 30 min, 10 ng/ml for 2 h); anti-RANK (5 μg/ml); OPGL together with OPG-Fc (10 and 130 ng/ml, respectively); OPG-Fc (130 ng/ml); and anti-RANK together with soluble RANK (5 and 10 μg/ml), respectively, are shown. The number of total OCs counted under the various conditions in this experiment are shown above individual bars. Similar effects were seen in two other experiments.

Lakkakorpi et al., 1989, 1993; Nakamura et al., 1996; for reviews see, Teti et al., 1991; Murrills et al., 1993; and Väänänen and Horton 1995). (It is also called the clear zone as the actin network in the actin ring appears to exclude organelles from this region.) There is an excellent correlation between actin ring formation and bone resorption (Lakkakorpi and Väänänen, 1991), and it appears that actin ring formation is required for bone resorption (Teti et al., 1991). Given our evidence suggesting that OPGL directly activates mature OCs we were curious to know if OPGL itself, or perhaps our polyclonal antibody to its receptor RANK would stimulate the formation of actin rings as a prerequisite to induction of the first round of bone resorption during the assay.

To quantify actin ring formation in isolated OCs treated under a variety of conditions, we stained fixed OCs for F-actin with Texas red–labeled phalloidin and a blinded investigator tallied the results. Several different types of F-actin containing structures are seen in OCs under all of the experimental conditions, and a few examples are shown in Fig. 6 A. The dot-like structures have been called podosomes, these appear to be short vertical bundles of F-actin which coalesce into larger structures in a time-dependent manner (Fig. 6 A, top panels; Teti et al., 1991; Väänänen and Horton, 1991). The structures we identify as partial-, full-, and multiple-actin rings are illustrated in the bottom panels of Fig. 6 A. The tallied number of these latter structures present under the different conditions were summed and the data are presented as a percentage of the total number of multinucleate OCs (Fig. 6 B). About 25% of OCs in control (untreated) cultures at time zero, 30 min and 2 h contained actin rings. OPGL or anti-RANK antibody treatment for as little as 30 min increased the number of actin ring–containing OCs to ~40–50% of the total.

At 2 h, actin ring formation induced by exposure to OPGL or anti-RANK antibodies increased further, reaching ~50–60%. To demonstrate the specificity of these effects, we used OPG to competitively inhibit OPGL or used OPG alone. Compared with untreated controls, no increase in the number of actin rings was observed under either condition. Finally, when the extracellular domain of RANK was preincubated with the anti-RANK before treatment of the OCs, no stimulation of actin ring formation was observed. As an aside, the mono- and binuclear cells were not included in the analysis shown, as they are not classically defined as (multinucleate) OCs. We did find that the binuclear cells had a similar response to OPGL (2 h control: 29% of the binuclear cells had actin rings; 2 h OPGL-treated: 59% of the binuclear cells had actin rings). It was not possible to unambiguously distinguish mononuclear OCs from other mononuclear cells in the culture, therefore, we could not determine with certainty if OPGL stimulated actin cytoskeletal rearrangements in these cells. Some mononuclear cells clearly contained podosomes and actin ring structures while others contained prominent stress fibers typical of fibroblasts, but these were present in both the control and treated cultures. Taken together, these results show that OPGL, signaling through its receptor, RANK on multinucleate OCs, induces rearrangement of the actin cytoskeleton into actin rings, a structure that is required for OC polarization and the formation of a specialized extracellular bone resorption compartment, and thus OC activity.

### *OPGL Stimulates a Rapid Onset of Hypercalcemia in Mice*

Because OPGL stimulation of isolated OCs induced rearrangement of the cytoskeleton within 30 min, and increased bone resorption in vitro, we examined whether OPGL could rapidly stimulate bone resorption by activating preexisting OCs in vivo. Randomized groups of mice ($n$ = 5), were injected intravenously with OPGL at the concentrations indicated (Fig. 7). After 1 h, the level of ionized calcium in the blood was determined as a measurement of OC activation, and the results are shown in Fig. 7. OPGL dose-dependently increased whole blood ionized calcium levels with significant increases seen at doses of 0.05, 0.1, and 0.5 mg/kg. Since we have previously shown that 3 d treatment of mice with OPGL does not increase the number of OCs at the bone surface these results suggest that within the 1-h treatment time, enough preexisting OCs have become activated by OPGL to produce a measurable increase in ionized calcium in the blood. Maintenance of mice on a low calcium diet for 48 h before dosing with OPGL did not abrogate the significant increase in blood levels of ionized calcium (data not shown), indicating that increases in gut calcium absorption are an unlikely alternate explanation for the increased blood calcium levels.

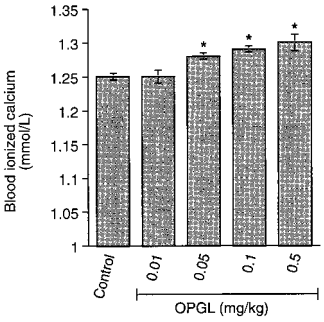


*Figure 7.* OPGL rapidly increases blood ionized calcium levels in mice. Randomized groups of mice ($n$ = 5) were injected intravenously with various concentrations of OPGL or PBS as a control as indicated. At 1 h post-injection, blood samples were obtained, ionized calcium measurements performed (see Materials and Methods), and the data are presented as the mean ± SEM. At OPGL doses of 0.05, 0.1, and 0.5 mg/kg, statistically significant increases in ionized calcium were detected (* indicates significantly different compared with PBS, $P < 0.05$, see Materials and Methods).

## *Discussion*

In this report we investigated the role of the newly described TNF-related protein, OPGL, in OC activation. Previously, Lacey et al. (1998) and Yasuda et al. (1998b) identified OPGL/ODF as the long sought OC differentiation factor. Direct expression cloning was used independently by the two groups to identify OPGL/ODF as the ligand for OPG/OCIF. OPG is expressed only as a soluble form and is now believed to act as a soluble decoy receptor to regulate the action of OPGL on differentiation of OCs. The data presented in the two reports provide strong evidence that OPGL acts directly on a population of OC progenitors, and together with CSF-1 induces terminal differentiation into mature, active OCs. Our data also showed that OPGL activated mature OCs to resorb bone in vitro (Lacey et al., 1998), and recent work supports our previous results (Fuller et al., 1998).

In this report we have shown that OPGL or agonist antibodies to its receptor, RANK, act directly on fully differentiated, mature OCs inducing individual OCs to undergo a rapid rearrangement of their actin cytoskeleton into actin rings and to perform multiple cycles of bone resorption as seen by scanning EM. The data demonstrate that many individual OCs are induced to perform multiple cycles of resorption during the assay period, but we also find that more of the OCs in the cultures appear to be activated as suggested by an increase in the number and density of single, isolated resorption events generated by OPGL treated OCs.

Because PTH has been shown to induce OPGL expression in primary osteoblasts (Yasuda et al., 1998b) and osteoblastic cell lines, (Fuller et al., 1998), it would be interesting to more carefully quantify the relationship between the size and spatial distribution of the resorption events as reported by Murrills et al. (1990). They showed that PTH treatment of rat OC cultures primarily increased the number of resorption foci (defined as resorption lacunae lying within an area of bone covering 1/116th of a bone slice), suggesting that more of the OCs were activated in PTH treated cultures (presumably due to stimulation of OPGL expression by osteoblastic cells present in the cultures). Some of the multiple excavations we observe, especially groups of smaller resorbed areas, may be generated by single large OCs containing multiple actin rings (e.g., see Fig. 6 A, bottom right), or by small OCs performing multiple resorption cycles in a focal region of the bone slice as the result of OPGL activation.

In addition to the quantitative effects of OPGL on OCs, we also found that the resorbed bone surfaces were quite different: while resorbed areas generated by untreated OCs are fairly smooth and usually single, areas resorbed by OPGL activated OCs are frequently multiple connected excavations, which expose numerous collagen fibrils. These continuous excavations appear to be very similar to those described by Chambers et al. (1984) resulting from OC resorption on anorganic bone (hydrazine treated) compared with the intermittent resorption that occurred on whole bone. How these two observations might be related is unknown, but may reflect different OC residence times at the resorption site and/or the probability that the OC will migrate to a new location under the different conditions. It is possible that OC movement and cycle reactivation is mediated by calcium released during each resorption cycle (Malgaroli et al., 1989; Zaidi et al., 1989; for review see Hall and Chambers, 1996).

We found that OPG inhibited the activation of isolated OCs by OPGL in vitro, however basal OC activity was not significantly decreased by OPG alone. However, OPG caused an inhibitory trend in several independent experiments. This might be due to the presence of endogenous OPGL in the system, (either from the cell preparation, or the serum) that excess OPG would inhibit. Dempster reported similar observations at a recent meeting (Dempster et al., 1998, The American Society for Bone and Mineral Research. Abstract F087. *Bone.* 23:S432). Nonetheless, even in the presence of excess OPG, OCs retain a low level of basal resorbing activity suggesting that something other than OPGL is responsible for regulating the basal level of OC resorption. We cannot, however, rule out the possibility that some residual ex vivo OC activity is due to prior OPGL exposure of the OCs in vivo. The marked stimulation of bone resorption in these cultures by OPGL does not appear to be mediated by increases in the number of multinucleate OCs as their numbers did not significantly change after the various treatments. In contrast, Fuller et al. (1998) have recently reported that OPGL (TRANCE) acts as an OC survival factor in vitro, as has been well documented for CSF-1 (Fuller et al., 1993). This apparent discrepancy may be explained by the fact that our OC survival measurements were performed on OCs cultured on bone slices, while those of Fuller et al., were on OCs cultured on untreated glass coverslips (even in their hands, OCs plated on bone do not require OPGL for survival, see Fig. 2, Fuller et al., 1998). It seems likely that under certain conditions, OPGL can act as a survival factor. Finally, in contrast to the action of OPGL on OC differentiation, activation of mature OCs on bone, or stimulation of actin ring formation on glass by OPGL occurs in the absence of added CSF-1.

Initial observations that OPG treatment of growing mice induced a very rapid (3 d) increase in bone density, led us to consider that OPG might act to inhibit OC activity in addition to being an antagonist of OC differentiation (Simonet et al., 1997). Furthermore, OPGL caused hypercalcemia within 2 d in vivo, possibly due to the activation of preformed OCs (Lacey et al., 1998). By investigating mature OCs in culture and performing very short-term in vivo experiments, we have tried to distinguish between the role of OPGL on OC differentiation and the action of OPGL on stimulating mature OCs in culture, and preexisting OCs in vivo to resorb bone: OPGL clearly plays a role in both OC differentiation (Lacey et al., 1998; Yasuda et al., 1998b) and OC activation in vitro (Lacey et al., 1998, Fuller et al., 1998 and present data). The interpretation of the 1 h in vivo treatment of mice with OPGL is complex, as we cannot rule out the kidney as the source of the increase in blood ionized calcium. However, we found that mice maintained on a low calcium diet for 48 h still show a significant and dose-dependent elevation in blood ionized calcium in response to OPGL (see Materials and Methods; data not shown), thus ruling out the gut absorption as the source of calcium. Given the robust and rapid activation of OCs in vitro by OPGL as evinced by both bone resorption and actin ring formation shown here, it seems most likely that OC activation is involved in vivo as well.

OPGL is identical to RANKL/TRANCE, (Wong et al., 1997), and it has been previously suggested that RANK is its receptor on OC progenitors (Lacey et al., 1998; Yasuda et al., 1998b). Recently Hsu et al. (1999) and Nakagawa et al. (1998) provided direct evidence that OPGL exerts its activity on OC progenitors via its receptor RANK. We show here that a monospecific antibody to RANK bound to isolated multinucleate OCs demonstrating that RANK is expressed at the surface by mature OCs. In support of our result, Hsu et al. (1999) recently demonstrated that RANK mRNA is expressed by mature OCs in situ. The anti-RANK polyclonal antibody was found to activate OCs as evinced by OC polarization and formation of actin rings, in an apparently not independent manner to OPGL. The most likely explanation is that the anti-RANK antibody was acting as an agonist by binding RANK, causing receptor aggregation and signal transduction (see Nakagawa et al., 1998). Together these pieces of evidence implicate RANK as the relevant receptor for OPGL mediated cytoskeletal rearrangements and osteoclast activation.

At this time, it is unknown how liganding RANK leads to cytoskeletal rearrangement and ultimately to activation of bone resorption in the OC, however, several signaling molecules have been specifically implicated in the cytoskeletal rearrangements associated with OC activation that may also play a role in OPGL activation of OCs. $pp60^{c\text{-}src}$ is clearly a central and key component involved in activation of mature OCs. $pp60^{c\text{-}src}$ is highly expressed in OCs (Horne et al.; Tanaka et al., 1992) and c-src$-/-$ knockout mice exhibit profound osteopetrosis (Soriano et al., 1991) due to an inability of c-src$-/-$ OCs to become polarized, form actin rings or ruffled borders; all of which are necessary for bone resorption (Soriano et al., 1991; Boyce et al., 1992; Lowe et al., 1993). More recent evidence links the engagement of $\alpha_v\beta_3$ integrin via $pp60^{c\text{-}src}$ (translocation and activation) to PI3 kinase activation (Hall et al., 1995; Hruska et al., 1995; Nakamura et al., 1995; Chellaiah and Hruska, 1996; Hruska et al., 1996; Lakkakorpi et al., 1997; and Nakamura et al., 1997), and association with the F-actin capping/severing protein, gelsolin (Chellaiah et al., 1998). Thus for the first time, a specific cytoskeletal protein (gelsolin) and mechanism (reversal of actin capping to support further F-actin polymerization) have been implicated in OC activation by receptor engagement and cell attachment. The stimulation of RANK by OPGL appears to enhance cytoskeletal rear-

rangements beyond those induced by OC attachment and integrin engagement, and leads to marked stimulation of bone resorption. It will be very interesting to determine if further enhancement of this signaling pathway involving $pp60^{c\text{-}src}$ and PI3 kinase or a completely separate path is responsible for OPGL-RANK–induced actin ring formation and OC activation. Recent data from several groups, (Darnay et al., 1998; Wong et al., 1998; Hsu et al., 1999) suggest that signaling through RANK is mediated by binding to TRAF (TNFR-associated factor) family members. Data from Hsu et al. (1999) further suggests that JNK activation downstream of RANK/TRAF interactions may be important for OC-like cell differentiation. Events downstream of OPGL-RANK–mediated OC cytoskeletal changes remain to be investigated.

In summary, OPGL binds to individual mature OC-inducing cytoskeletal changes indicative of OC activation and stimulates multiple spatially associated cycles of robust bone resorption in vitro. These effects of OPGL are very selective as they can be inhibited by the natural soluble decoy receptor, OPG, or mimicked by agonistic antibodies to the OPGL receptor, RANK. In addition, OPGL given intravenously induces a rapid increase in blood ionized calcium in mice suggesting that preexisting OCs are activated by OPGL in vivo. Based on these many pieces of evidence, we conclude that in addition to its role in OC differentiation, OPGL is a potent and direct regulator of OC activity in vitro and in vivo.

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