Development of bone depends on a continuous supply of bone-degrading osteoclasts. Although several factors such as the matrix metalloproteinases and the integrins have been shown to be important for osteoclast recruitment, the mechanism of action remains poorly understood. In this study we investigated the molecular mechanisms homing osteoclasts to their future site of resorption during bone development. We show that RANKL and VEGF, two cytokines known to be present in bone, possess chemotactic properties toward osteoclasts cultured in modified Boyden chambers. Furthermore, in ex vivo cultures of embryonic murine metatarsals, a well established model of osteoclast recruitment, antagonists of RANKL and VEGF reduced calcium release, showing that both cytokines play roles during bone development. In cultures of purified osteoclasts both RANKL and VEGF induced phosphorylation of ERK1/2, a well-known chemotactic mediator of osteoclast, also induced activation of ERK1/2, although this activation followed a kinetic pattern differing from that of RANKL and VEGF. RANKL and VEGF-induced, but not M-CSF-induced, osteoclast invasion was completely blocked by the specific inhibitor of ERK1/2 phosphorylation, PD98059. In addition, PD98059 was able to inhibit calcium release in cultures of embryonic metatarsals. In contrast, PD98059 was unable to abrogate the RANKL-induced calcium release in the tibia model, demonstrating that only some of the RANKL functions on osteoclast physiology are regulated through the ERK1/2 pathway. Taken together, these results show that RANKL and VEGF, in addition to their role in osteoclast differentiation and activation of resorption, are important components of the processes regulating osteoclast chemotaxis.

The development and continuous remodeling of the skeleton demand a tightly regulated balance between the bone-forming and bone-resorbing processes. Although many aspects of the resorptive processes remain elusive, there is general consensus that the hematopoietically derived osteoclast is the pivotal cell in the degradation of the bone matrix (reviewed in Ref. 1). Inevitably, the focus centers on the factors that direct the resorptive activity of the osteoclasts and in particular the recruitment of the immature osteoclasts to the future site of resorption. Understanding the control of osteoclast recruitment to the future resorption site is of great importance, as incorrect regulation of recruitment it likely to constitute at least part of the underlying cause of the majority of bone metabolic disorders.

During embryonic development of long bones, osteoclast precursors appear in the mesenchymal tissue surrounding the primitive bone collar. Along with endothelial cells they are recruited into the calcified tissue where they mature and begin formation of the marrow cavity (2). Cytokines are likely to constitute the molecular mediator through which the osteoclasts are directed to their future site of resorption. We, as well as others (3–7), have previously shown that cytokines expressed in the cellular environment of the bone surface are able to act at various stages of the osteoclast lifecycle. Macrophage colony-stimulating factor (M-CSF) has long been shown to play an important role in osteoclast physiology. At least part of that role may be attributable to its well-documented stimulatory action on osteoclast migration (8, 9). In in vitro cultures, osteoclasts have been shown to migrate toward sources of M-CSF, marking the cytokine as one of the potential mediators of osteoclast recruitment. Other cytokines that are expressed in the immediate vicinity of the bone surface and whose receptors are present on the osteoclast include vascular endothelial growth factor (VEGF) and receptor activator of nuclear factor κB ligand (RANKL). VEGF has been shown to partially rescue M-CSF deficiency in the op/op mice (10). Furthermore, systemic injection of a soluble chimeric VEGF receptor (mFlt-1-IgG) induced morphological changes in the growth plate suggesting impaired chondroclast (a subset of osteoclasts present during bone development) function (11). Finally, we have previously mentioned an effect of VEGF on osteoclast migration (3). Similarly, RANKL has been shown to have numerous effects on osteoclast physiology, including induction of differentiation, activation of resorption and stimulation of survival (12–14).

RANKL, VEGF, and M-CSF all exert their functions through multiple signal transduction pathways, which have not yet been completely characterized. Especially, the pathways involving the mitogen-activated protein kinases (MAPK) have attracted considerable interest in recent years due to their central role in a range of osteoclastic activities. The p38 MAPK was shown to be essential for osteoclast differentiation in vitro (15, 16). The extracellular signal-regulated kinases 1 and 2 (ERK1/2) are activated by M-CSF, the interleukins IL-1α and IL-1β and are likely involved in osteoclast survival (17, 18). In addition, the ERK1/2 kinases have also been implicated in fibroblast growth factor-2 (FGF-2) and Gas6-induced resorp-
tion (19, 20). Although, RANKL activation of ERK1/2 in mature osteoclasts has been demonstrated, the relevance of this activation has yet to be clarified (21).

Here, we used a combination of in vivo and in vitro cell migration assays to study the role of RANKL and VEGF in osteoclast recruitment during bone development. We demonstrate that addition of antagonists of RANKL and VEGF, osteoprotegerin (OPG) and endostatin, respectively, led to reduced recruitment of osteoclasts in vivo cultures of embryonic bones. Moreover, both RANKL and VEGF dose-dependently induced osteoclast recruitment in the Boyden chamber invasion assay. We explored cytokine activation of the MAPKs in cultures of purified osteoclasts and found that RANKL and VEGF, as well as M-CSF, activated the ERK1/2, but failed to activate p38 MAPK. Further studies of the ERK1/2 kinases demonstrated that ERK1/2 is indeed involved in osteoclast recruitment in embryonic bones. Interestingly, we discovered that RANKL and VEGF utilize an ERK1/2-dependent pathway for inducing osteoclast migration; whereas M-CSF-mediated migration is independent of the ERK1/2 cascade. Finally, we demonstrate that RANKL-induced bone resorption is independent of ERK1/2 activity, further underlining the important differences in the signaling cascades employed by these two pathways for inducing osteoclast migration; whereas M-CSF-RANKL and VEGF, as well as M-CSF, activated the ERK1/2, p38, and p44/p42 MAPKs.

MATERIALS AND METHODS

Reagents and Mouse—All animal experiments were performed according to approved protocols following guidelines at Nordic Bioscience A/S, Denmark.

Recombinant murine RANKL, human VEGF, human M-CSF, and recombinant human Flt-Fc chimeric protein were from R&D Systems. Recombinant human OPG-Fc chimeric protein was from Alexis Corporation. Endostatin was purchased from Calbiochem. Antibodies toward human Flt-1, human M-CSF receptor, human RANK, rat RANKL, human VEGF, human M-CSF, and phospho-ERK1/2 and phospho-p38 MAPK antibodies were purchased from Cell Signaling Technology. The MAP kinase inhibitors PD98059 and U0126 were from Calbiochem.

Preparation of Osteoclast-like Cells and Spleen Cell-derived Osteoclasts—Osteoclast-like cells were generated in the co-culture system (22). Briefly, primary calvarial osteoblasts were isolated by mincing calvariae from 1 day old BalbC CF1 mice and then culturing them in αMEM containing 10% fetal bovine serum. Hematopoietic cells were isolated by dissecting the femur and tibiae of 6-week-old BalbC CF1 mice, and the cells were cultured in αMEM containing 10% fetal bovine serum and 10 μM 125-i-dihydroxy-vitamin D3 (1-25(D3)). Spleen cell-derived osteoclasts were generated as described (12). Briefly, spleens were isolated from BalbC CF1 mice and mashed through a 70-μm filter (Invitrogen A/S). The cells were placed on a 0.1 M filter (Invitrogen A/S). The filters were coated with 10 μg/ml reconstituted type I collagen (Nitta Collagen) at a concentration of 2.4 mg/ml. 0.1 μM proteolytic cavities were created from bone. These cavities were then cultured for 22 h with or without additions as described under “Results.” The cells were fixed in 3.7% formaldehyde, and OCLs were visualized by staining for osteoclast-specific enzymes.

Chemoattractant Assays—Chemoattractant assays were performed from previous procedures (23). The assays were performed in modified Boyden chambers with polycarbonate filters containing 12-μm pore membranes (Corning Costar). The filters were coated with 10 μl of reconstituted type I collagen (Nitta Collagen) at a concentration of 2.4 mg/ml. Murine osteoclast-like cells (OCLs) were prepared from osteoclast/osteoblast co-cultures by sequential treatment with collagenase 0.1% (w/v) and dispase 0.1% (w/v) to remove osteoblastic cells. The remaining cells were loosened by trypsin treatment and then gently lifted off the plates with a rubber policeman. The OCLs were seeded in the culture inserts in αMEM containing 0.1% (w/v) albumin. The inserts were placed in 12-well plates, and the cells were cultured for 22 h with or without additions as described under “Results.” The cells were fixed in 3.7% formaldehyde, and OCLs were visualized by staining for a leukocyte acid phosphatase kit (Sigma-Aldrich). In all assays where inhibitors were used, the OCLs were allowed to attach for 1 h before the addition of the inhibitor and cytokines to reduce any activity of the inhibitor on attachment of the cells. Invasion was determined as the ratio of OCLs that had invaded through the collagen gel to reach the lower side of the membrane compared with the total number of OCLs in the insert.

Bone Resorption Models—Bone resorption was evaluated with well established models (2), on littersmates. Timed pregnant mothers were mated copiously with 100 μg of 45CaCl2 (American Radiolabeled Chemicals) at day 16 after the confirmation of the vaginal plug. The middle three metatarsals (preserved as a triad) and the tibiae were isolated from each hindlimb of day 17 embryos. One metatarsal triad or tibia from each embryo was used for treatments and the other as a control. The bone explants were cultured floating on Millipore filter membrane in 400 μl of BGJb medium supplemented with NaHCO3 (0.2 g/liter) NaCl (0.9 g/liter), albumax (1 g/liter), glutamax (0.5 g/liter), ascorbate (50 mg/liter), and the factors/inhibitors specified in the figure legends. The bones were cultured for 4 days, with measurement of 45Ca release every day. On day 4 the 45Ca remaining in the tissue was released by formic acid treatment measured by scintillation counting. The demineralization of the bone explant was then calculated as the percentage of total 45Ca amount.

Immunoblotting—Spleen cell-derived osteoclasts were starved for 2 h in αMEM containing 0.1% (w/v) albumin to minimize the activation of kinases induced by the presence of serum and cytokines in the cultures. This was followed by incubation with the cytokines for various times as specified in the figure legends.

After incubation with or without stimuli the spleen cell-derived osteoclasts were washed twice in ice-cold phosphate-buffered saline, followed by lysis in modified radioimmunoprecipitation assay buffer (30 mM NaCl, 50 mM Tris, 5 mM EDTA, 1% Nonidet P-40, 1% deoxycholic acid, 0.1% SDS, 5 mM NaF, 1 mM NaVO3, and protease inhibitor mixture from Calbiochem). Cell lysates were centrifuged for 30 min at 4 °C at 15,000 × g. 10 μg of total protein were loaded and electrophoresed on 10% SDS-PAGE gels. The proteins were transferred to a nitrocellulose membrane (BA85; pore size: 0.45 μm from Schleicher & Schuell). The quality of the protein loading was confirmed by staining with 0.2% Ponceau S in trichloroacetic acid. Nonspecific binding was blocked by incubation of the membrane in TBS-T buffer (50 mM Tris, 150 mM NaCl, 1% Tween 20) containing 5% skim milk powder for 1 h, followed by incubation with the appropriate primary antibody overnight at 4 °C, and then a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. All blots were visualized using the ECL kit from Amersham Biosciences.

Statistics—The statistical analysis of the migration experiments was performed using the two-tailed Student’s t test. The statistical analysis on the 45Ca release experiments was performed using a paired two-tailed Student’s t test. * p < 0.05; ** p < 0.01; *** p < 0.001.

RESULTS

RANKL and VEGF Induce Osteoclast Invasion—Through their function in osteoclastogenesis and angiogenesis, respectively, RANKL and VEGF have already been ascribed important but distinct roles in the development of bone. We hypothesized an additional and overlapping role for the two growth factors as integral parts of the osteoclast recruitment machinery. To study the effect of RANKL and VEGF on osteoclast motility we used the modified Boyden chambers coated with a type I collagen gel. When RANKL was exclusively added to the lower chamber we observed a dose-dependent increase in OCL invasion from the upper chamber through the type I collagen matrix into the lower chamber (Fig. 1A). Maximal activation was obtained at RANKL concentrations of 100 ng/ml or higher, resulting in an increase of 70–90% of the invasion. Addition of RANKL to the upper chamber did not significantly activate OCL invasion, showing that RANKL induces invasion in a chemotactic manner. Addition of 300 ng/ml of hOPG-Fc completely abrogated the RANKL-induced invasion, without affecting the basal level of migration. Importantly, we did not observe any differences in osteoclast number between the treated and non-treated conditions (data not shown).

We have previously mentioned that VEGF stimulates osteoclast invasion (3). Here we observed a biphasic dose-dependent induction of invasion against the VEGF concentration gradient (Fig. 1B), with optimal stimulation at 1 ng/ml where the invasion is increased by 40–50% when compared with the non-stimulated controls. Similar to RANKL, addition of VEGF to the upper chamber did not result in a significant activation, showing that VEGF also stimulates OCL invasion in a chemo-
tactic manner. Addition of soluble VEGF-receptor sFlt-Fc completely inhibited VEGF-induced invasion, whereas basal invasion was unaffected.

Several VEGF receptors exist, of which both Flt-1 and Flk-1 have been observed in osteostats (10, 11, 24). Addition of the Flt-1-specific ligand placenta growth factor-2 (PIGF-2) to the modified Boyden chambers resulted in increased invasion similar to addition of VEGF, indicating at least a partial role of Flt-1 in the process.

M-CSF has previously been described as a powerful chemo-tactic factor for osteostats (8, 25); however, studies with M-CSF in the modified Boyden Chamber have never been reported. In our system M-CSF acted as a chemoattractant, activating OCL invasion to levels comparable with RANKL and VEGF stimulation, with maximal activation (50–60% increase when compared with baseline levels) of motility being reached at 5 ng/ml of M-CSF and higher.

**Endostatin Inhibits VEGF-stimulated Osteoclast Migration**—Endostatin is a known antagonist of VEGF-mediated endothelial cell migration and proliferation (26, 27). Its effects on other cell types and cytokine-mediated signals, however, still remain to be studied.

We tested the effect of endostatin on VEGF-, RANKL-, M-CSF-, and PIGF-2-stimulated osteoclast invasion through type I collagen in the modified Boyden chamber. Endostatin completely inhibited VEGF (Fig. 2A) and PIGF-2 (Fig. 2D)-mediated invasion, whereas no effect was observed on RANKL (Fig. 2B) or M-CSF- (Fig. 2C) mediated invasion, suggesting it is specific for VEGF receptor-mediated signaling. We did not observe any differences in osteoclast number between the treated and non-treated conditions (data not shown). Preincubation of cells with endostatin for 30 min was necessary for endostatin to exert its inhibitory effect (data not shown), corresponding with previous findings in endothelial cell migration studies (26).

**Antagonists of RANKL and VEGF Inhibit Calcium Release from Cultured Primitive Long Bones**—The murine metatarsal system provides an excellent model for studying osteoclast recruitment during bone development. When metatarsals are isolated from 17-day old embryos, mature actively resorbing osteoclasts are already present on the bone surface of the marrow cavity, and no further recruitment is necessary for resorption (2, 28). In order to investigate whether RANKL plays a physiologically relevant role in osteoclast recruitment during bone development, we studied the effect of recombinant hOPG-Fc in the two bone models. Addition of 500 ng/ml of hOPG-Fc led to complete inhibition of the 45Ca release from the metatarsals showing that RANKL indeed plays a role in bone development (Fig. 3A). Although it can not be ruled out that part of this inhibition is due to the effect of hOPG-Fc on osteoclastic differentiation, we observed the presence of osteoclasts inside, as well as outside the periosteum of the hOPG-Fc treated metatarsals by histological analysis (data not shown), thus confirming that recruitment and not only osteoclastogenesis is impaired.

We also found that hOPG-Fc inhibited the demineralization of the tibia to ~50% of the control levels, thus confirming previous findings (29). We have previously reported that addition of a soluble VEGF receptor led to a decrease in osteoclast recruitment in the metatarsals (3). Here we find that, although not as efficient as hOPG-Fc, addition of 60 ng/ml endostatin inhibits osteoclast recruitment in this system (Fig. 3B), thus further supporting an important role of VEGF in bone development. Interestingly we found that endostatin did not inhibit Ca2+ release in the tibia model, suggesting that inhibition of VEGF does not affect resorption, but only recruitment.

**RANKL, VEGF, and M-CSF induce MAPK (ERK1/2) Activation**—The MAP kinases ERK1/2 and p38 are involved in several cellular processes, such as proliferation, cell survival, differentiation, and cytokine-activated migration. We hypothesized that the MAPK- and VEGF-mediated osteoclast recruitment might involve MAPK phosphorylation. In order to have cell populations devoid of contaminating cell types such as stromal cells we studied MAPK activation in spleen cell-derived osteoclasts (SOCs) instead of OCLs.

Addition of RANKL led to activation of ERK1/2 within 30 min after stimulation (upper panels, Fig. 4A). This activation was sustained throughout the 2-hour period tested. Total level
of ERK1/2 protein was unchanged for the entire period. No activation of p38 was observed (lower two panels, Fig. 4A). VEGF induces ERK1/2 activation with kinetics highly similar to those of RANKL (Fig. 4B). Correspondingly, VEGF stimulation did not lead to activation of p38 (Fig. 4B). As expected fromRefs. 17 and 25, M-CSF activated ERK1/2. However, with a maximal activation already after 10 min, followed by a reduction in the activation level, as previously described (17). Thus, the biphasic kinetic profile of M-CSF differed from those of RANKL and VEGF. Again, no effect on p38 activation was observed (Fig. 4C). Finally, we found that PlGF-2 activated ERK1/2 with a kinetic pattern similar to VEGF (Fig. 4D), indicating that Flt-1 is responsible for the ERK1/2 activation by VEGF.

Inhibition of ERK1/2 Activation by PD98059 in Cytokine-activated Osteoclast Migration—To study whether the cytokine-induced ERK1/2 activation is involved in osteoclast migration, we used PD98059, which specifically blocks the ERK1/2, but not the p38 MAP kinase pathways (30, 31), in the modified Boyden chamber assay. Addition of PD98059 completely abrogated both RANKL- and VEGF-stimulated OCLs invasion in the modified Boyden chamber (Fig. 5, A and B), without affecting the osteoclast number (data not shown), suggesting that ERK1/2 play an important role in osteoclast recruitment induced by RANKL and VEGF. PD98059 had no effect on the basal level of invasion. Interestingly, we observed no effect of PD98059 on M-CSF-induced invasion (Fig. 5C), indicating that other signal transducers are likely to mediate this effect. To rule out that inhibition of M-CSF-induced osteoclast invasion simply requires higher PD98059 concentrations than RANKL and VEGF-induced invasion respectively, we tested PD98059 in concentrations up to 50 μM. Although we, as expected from (17), observed reduced viability of the OCLs at 50 μM PD98059, M-CSF still activated invasion through the type I collagen matrix, and PD98059 had no effect on this activation (data not shown). These data show that M-CSF activated chemotaxis is a good control for nonspecific inhibition by PD98059, when performing osteoclast chemotaxis assays.

Inhibition of ERK1/2 Activity by PD98059 in Bone Explants—To determine whether ERK1/2 activation plays a role in osteoclast recruitment in developing long bones, we tested the effect of PD98059 in the ex vivo bone explants cultures. Inhibition of the activation of ERK1/2 by PD98059 led to a significant reduction in 45Ca release from cultured embryonic metatarsals from E17-day-old embryos (Fig. 6A) showing that the mechanism regulating invasion of osteoclasts into the marrow cavity does to some extent depend on ERK1/2 activity. Interestingly, no effect of the inhibitor was observed in cultures...
of tibiae (Fig. 6B), indicating that resorption is independent of ERK1/2 activity.

Effect of PD98059 on RANKL-stimulated Bone Resorption—The data described in the previous section, showed that demineralization of embryonic tibia by osteoclasts is independent of ERK1/2. In the experimental setup addition of VitD3 is used to induce resorption in the tibia model. Since the demineralization is sensitive to hOPG-Fc (see Fig. 3A), RANKL must at least to some extent mediate the VitD3-induced resorption, although the influence of other cytokines cannot be excluded. This implies that only part of the effects which RANKL exert on osteoclasts are mediated through ERK1/2. In order to specifically test whether PD98059 inhibits RANKL-mediated resorption, we first tested whether RANKL could replace VitD3 in the tibia system, and as seen in Fig. 7A, RANKL induced demineralization of the tibiae to the same extent as VitD3. Interestingly, neither VEGF nor M-CSF was able to induce demineralization of the tibiae. This is in correlation with our own findings with the inability of endostatin to inhibit demineralization and previous findings for M-CSF (32). As shown in Fig. 7B, addition of PD98059 to RANKL-stimulated tibia cultures did not inhibit the demineralization, thus indicating that RANKL-mediated bone resorption is independent of ERK1/2 activity.

DISCUSSION

The lifecycle of an osteoclast comprises a series of consecutive highly specialized steps. The mature giant multinucleated osteoclast is formed from the hematopoietically derived pre-osteoclastic monocytes. Along the process of maturation the osteoclast is recruited to the future site of resorption where at least one round of bone solubilization is initiated. Ultimately, the osteoclast undergoes apoptosis in order to allow new bone formation to take place on the newly resorbed bone surface (reviewed in Ref. 33). The signals that initiate the recruitment of the osteoclasts to their future site of resorption are of major interest, since incorrect regulation of these is likely to be involved in pathological situations. Cultures of embryonic metatarsals, where resorption is dependent on osteoclast invasion into the developing marrow cavity, provide a useful model to study the processes governing osteoclast recruitment (2, 3, 28, 32).

Among the factors governing other forms of cell recruitment are the cytokines. In this work, we have studied the role of RANKL and VEGF, two cytokines known to be present in the immediate bone environment (6, 11, 12), on osteoclast recruitment. We show that both cytokines work as chemoattractants on the osteoclast in a similar manner with respect to kinetics and signal transduction pathway. Furthermore, RANKL and VEGF stimulate osteoclast recruitment through a signaling pathway differing from that of M-CSF, a well characterized chemoattractant for osteoclasts. Finally, we showed that bone resorption induced by RANKL involves a different signaling pathway compared with the chemotactic properties of the cytokine.

The entire process by which pre-osteoclasts becomes destined to migrate to the future resorption site, where they join with other pre-osteoclasts to form mature osteoclast before
commencing resorption, remains largely unknown, despite the central role in bone remodeling. One of the main hypotheses implies that controlling cells, present at the future site of resorption, initiates a homing signal, which attracts the osteoclast by means of chemoattractants. Whether the controlling cells are of the stromal/osteoblastic lineage or not remains an open question. Likewise the molecular identity of the mediators is unknown. Two particular cytokines attracted our attention due to their inherent presence during bone development. Through its osteoclastogenic properties RANKL, expressed by, among others, the osteoblasts (12), is a critical component of the osteoclast maturation pathway. Studies have shown that RANKL can be proteolytically cleaved from the surface of the expressing cells and released into the surrounding environment (34). In addition, the released soluble fragment of RANKL has been shown to retain its osteoclastogenic function (12). VEGF is expressed by osteoblasts and in the hypertrophic cartilage of developing bones (6, 11). Furthermore, VEGF was previously implicated in osteoclast differentiation (10) and resorptive activity (24). Both cytokines are thus in a potential position to play a role in osteoclast recruitment. The receptor for RANKL (RANK) is expressed on the surface of osteoclasts, precursors as well as fully matured (35, 36). Similarly, the two major VEGF receptors, Flk-1 and Flt-1, are expressed by osteoclasts (10, 11, 24). Functional data, however, indicate that Flt-1 is the main VEGF receptor in osteoclasts (10).

In the Boyden’s modified chambers we found that both RANKL and VEGF were able to stimulate osteoclast invasion against a concentration gradient of the cytokines with efficacy comparable to that of M-CSF. Interestingly, the fact that the Flt-1 ligand PlGF-2 induced invasion in a similar manner to VEGF confirmed previous studies (10) that Flt-1 is the major VEGF receptor in osteoclasts. The physiological relevance of these chemotactic properties was demonstrated in the ex vivo metatarsal model of osteoclast recruitment where antagonists of each cytokine, OPG, and endostatin respectively, blocked dissolution of the calcified matrix in the developing marrow cavity. Correspondingly, we have previously shown that soluble Flt-1 blocks invasion of osteoclast into the marrow cavity in developing long bones (3). Although, it is well known that RANKL induces osteoclast differentiation and bone resorption this is to our knowledge the first study to demonstrate a direct chemotactic ability and potential role of RANKL in osteoclast recruitment. Our findings are indirectly supported by a study by Burgess et al. (13), who reported altered shape of pits formed by osteoclasts when cultured on bone slices in the presence of RANKL compared with cultures without RANKL. We speculated that the altered shape of the pits observed by Burgess et al. was due to an increased motility of the osteoclasts.

The fact that antagonists of VEGF block 45Ca release in the metatarsal model where bone resorption is contingent on preceding osteoclast invasion into the developing marrow cavity, but not in the tibia model where resorbing osteoclasts are already present on the bone surface indicates that the role of VEGF is restricted to recruitment. In contrast, RANKL is involved in both recruitment and activation of resorption.

The matrix metalloproteinases (MMPs) have been shown to play important roles in osteoclast recruitment and invasion (2, 4).
Although the exact mechanism of action remains controversial. We have previously pointed to an integrated action of MMP-9 and VEGF in osteoclast recruitment based on the regulatory role of MMP-9, as well as its ability to solubilize VEGF bound to the ECM (3, 38). Similarly, RANKL was released proteolytically from the cell surface, at least in vitro (34). Although, Lum et al. (34) focused on TACE, they speculate that other metalloproteinases could perform similar actions, hence releasing RANKL into the surrounding environment thus extending its sphere of influence. The present data, thus further

![Fig. 5. Involvement of the ERK1/2 kinase in cytokine-stimulated invasion of mouse osteoclasts.](image)

![Fig. 6. Effect of PD98059 on the demineralization of metatarsals and tibiae.](image)
support the integrated action between MMPs at or near the future site of resorption and the cytokines able to attract osteoclasts when released in the surroundings.

Activation of RANK, Flt-1, and c-fms, by RANKL, VEGF, and M-CSF, respectively, has been shown in turn to lead to activation of downstream signaling molecules, including the Src family kinases, the PI 3-kinase, and the MAP kinases in different cell culture models (21, 39–43). In this study, we showed that RANKL and VEGF resulted in similar activation patterns of the ERK1/2 kinases, whereas p38 was not affected. Activation with M-CSF also led to phosphorylation of ERK1/2, although following a different time course, possibly indicating a different role of M-CSF-mediated ERK1/2 activation. The ERK1/2 kinases have previously been implicated in regulation of osteoclast survival (17). Furthermore, studies have demonstrated ERK1/2 activation in osteoclasts in response to stimulation by various cytokines, including RANKL and M-CSF (17, 21, 25). So far, the physiological role of the ERK1/2 activation in osteoclasts has not been elucidated. Here we demonstrate that RANKL and VEGF induce ERK1/2 activation in osteoclasts, and that this activation is necessary for recruitment of the osteoclasts to the future site of resorption. In contrast, we found that M-CSF utilizes a different signaling pathway to activate recruitment. Two previous reports looked at the involvement of MAPKs in M-CSF induced osteoclast migration. Pilkington et al. (44) showed that M-CSF-induced migration of rat osteoclasts is dependent on ERK1/2 activity (44). In contrast, Nakamura et al. (25) concluded that activation of murine osteoclast by M-CSF was dependent on a complex consisting of \( \alpha_\beta_9 \) integrin, Pyk2, c-Cbl, and the PI 3-kinase, but independent of c-Src and ERK1/2. The reason for this discrepancy remains to be found; however, as discussed by Nakamura et al. (25) it could depend on the substrate used for the experiments. Another likely contributing factor is the species of the osteoclast, since Pilkington et al. used rat cells, and Nakamura et al. and we used murine cells. In addition, Nakamura et al. found that, although not related to cell migration, M-CSF could induce ERK1/2 activation in murine osteoclasts via c-Src, thus, by combining these data with a study demonstrating that RANKL stimulates c-Src activation (21), we speculate that RANKL- and VEGF-mediated ERK1/2 activation and cell migration in osteoclast could be dependent on c-Src.

The essential role of RANKL in bone resorption is well established, and as our data show, RANKL may perfectly well substitute VitD3 in the tibia cultures, as expected from both Tsukii et al. (29) and our finding that OPG-FC inhibited the \( ^{45} \text{Ca} \) release. PD98059 did not inhibit the RANKL induced bone resorption in tibiae suggesting that RANKL induced osteoclast recruitment differs in terms of signaling pathway from RANKL induced osteoclastic bone resorption. Although both VEGF and M-CSF have been reported to be able to stimulate bone resorption, neither of the cytokines was able to compensate for the lack of VitD3 in our systems.

In summary, we propose that the range of functions of VEGF

![Fig. 7. Effect of RANKL, VEGF, M-CSF, and the ERK1/2 kinase inhibitor PD98059 on the demineralization of embryonic tibiae.](image)

![Fig. 8. Proposed model for the role of RANKL, VEGF, M-CSF, and ERK1/2 in the activation of osteoclastic migration.](image)
and RANKL during bone development is expanded to include osteoclast recruitment through chemotactic properties. Furthermore, their chemotactic properties share a signaling pathway, which in turn differs from that of M-CSF. Finally, our findings suggest that RANKL employs different signaling pathways for mediating its activation of bone degradation and its chemotactic properties toward the osteoclast, respectively. We hypothesized signaling pathway is summarized in Fig. 8.

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