EGF-like Ligands Stimulate Osteoclastogenesis by Regulating Expression of Osteoclast Regulatory Factors by Osteoblasts

IMPLICATIONS FOR OSTEOLYTIC BONE METASTASES*

Received for publication, June 20, 2007 Published, JBC Papers in Press, July 17, 2007, DOI 10.1074/jbc.M705064200

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Epidermal growth factor (EGF)-like ligands and their receptors constitute one of the most important signaling networks functioning in normal tissue development and cancer biology. Recent in vivo mouse models suggest this signaling network plays an essential role in bone metabolism. Using a coculture system containing bone marrow macrophage and osteoblastic cells, here we report that EGF-like ligands stimulate osteoclastogenesis by acting on osteoblastic cells. This stimulation is not a direct effect because osteoclasts do not express functional EGF receptors (EGFRs). Further studies reveal that EGF-like ligands strongly regulate the expression of two secreted osteoclast regulatory factors in osteoblasts by decreasing osteoprotegerin (OPG) expression and increasing monocyte chemoattractant protein 1 (MCP1) expression in an EGFR-dependent manner and consequently stimulate TRAP-positive osteoclast formation. Addition of exogenous OPG completely inhibited osteoclast formation stimulated by EGF-like ligands, while addition of a neutralizing antibody against MCP-1 exhibited partial inhibition. Coculture with bone metastatic breast cancer MDA-MB-231 cells had similar effects on the expression of OPG and MCP1 in the osteoblastic cells, and those effects could be partially abolished by the EGFR inhibitor PD153035. Because a high percentage of human carcinomas express EGF-like ligands, our findings suggest a novel mechanism for osteolytic lesions caused by cancer cells metastasizing to bone.

The adult human skeleton continuously undergoes remodeling, namely, being resorbed by osteoclasts and renewed by osteoblasts. The maintenance of the skeleton requires the coordinated activity and constant generation of these cells. Osteoblasts are derived from mesenchymal stromal stem cells and, along with their precursors, are the major sources for regulating osteoclast formation by producing several cytokines, including receptor activator of NF-κB ligand (RANKL), osteoprotegerin (OPG), and macrophage colony-stimulating factor (M-CSF). RANKL is the key mediator for osteoclast formation and activation (1, 2). OPG acts as a soluble decoy receptor by blocking the interaction of RANKL with its receptor (RANK) on osteoclasts, thereby inhibiting osteoclastogenesis (2, 3). M-CSF mainly contributes to proliferation, survival, and differentiation of early osteoclast precursors (4). Recently, monocyte chemoattractant protein 1 (MCP1), a CC chemokine, was shown to have the ability to induce tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclastic cells and to stimulate osteoclast fusion and activity (5, 6). Studies in our laboratory indicate that MCP1 is expressed in osteoblasts and exhibits chemoattractant activity toward osteoclasts.

The epidermal growth factor (EGF)-like ligands and their cognate receptors constitute one of the best-studied signaling networks. This network modulates cell functions in a variety of ways, including proliferation, survival, adhesion, migration, and differentiation. There are four distinct receptors in this family: EGF receptor (EGFR, also known as ErbB-1/HER1), ErbB-2 (HER2), ErbB-3 (HER3), and ErbB-4 (HER4). The EGF-like ligands consist of EGF, amphiregulin, and transforming growth factor α (TGFα), which only bind to the EGFR, and heparin-binding EGF (HB-EGF), betacellulin, and epiregulin, which can bind to both the EGFR and ErbB4. The EGFR is a receptor-tyrosine kinase. Upon ligand binding, it undergoes dimerization and phosphorylation at tyrosine residues in its intracellular domain, thus activating several important cellular signal transduction pathways, such as Ras-Raf-MAP-kinase, PI3-kinase-Akt, and PLCγ-PKC pathways etc (reviewed in Ref. 8).

Epidermal growth factor receptor signaling contributes to bone metabolism by affecting both osteoblasts and osteoclasts. Although most EGFR-null mice die within the first postnatal week, surviving animals display craniofacial alterations and cleft palate (9). Overexpression of EGF-like ligands in mouse, receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG), and macrophage colony-stimulating factor (M-CSF).

The abbreviations used are: RANKL, receptor activator of nuclear factor-κB ligand; EGF, epidermal growth factor; HB-EGF, heparin binding-EGF; EGFR, EGF receptor; OPG, osteoprotegerin; M-CSF, macrophage colony-stimulating factor; MCP1, monocyte chemoattractant protein 1; PI 3-kinase, phosphoinositide 3-kinase; PMA, phorbol 12-myristate 13-acetate; ELISA, enzyme-linked immunosorbent assay; BMM, bone marrow macrophages; TGF, transforming growth factor; TRAP, tartrate-resistant acid phosphatase.

2 The abbreviations used are: RANKL, receptor activator of nuclear factor-κB ligand; EGF, epidermal growth factor; HB-EGF, heparin binding-EGF; EGFR, EGF receptor; OPG, osteoprotegerin; M-CSF, macrophage colony-stimulating factor; MCP1, monocyte chemoattractant protein 1; PI 3-kinase, phosphoinositide 3-kinase; PMA, phorbol 12-myristate 13-acetate; ELISA, enzyme-linked immunosorbent assay; BMM, bone marrow macrophages; TGF, transforming growth factor; TRAP, tartrate-resistant acid phosphatase.

3 Xin, L., Qin, L., Bergenstock, M., Bevelock, L. M., Novack, D. V., and Partridge, N. C. (2007) J. Biol. Chem., in press.
such as betacellulin, EGF, and TGFα, all resulted in stunted growth (10–12). Histology studies revealed there is abnormal overproliferation of osteoblasts in EGF transgenic mice (12). On the other hand, mice humanized for the EGFR (the endogenous mouse EGF gene was replaced by human EGFR cDNA) exhibit low EGFR activity in bone and display accelerated osteoblast differentiation (13). We have shown that amphiregulin-null mice displayed significantly less tibial trabecular bone than wild-type mice (14). Consistent with these in vivo results, in vitro cell culture experiments suggest that EGF and amphiregulin stimulate proliferation of preosteoblastic cells but inhibit their further differentiation into osteoblastic cells (14–17).

Beside these effects on osteoblasts, EGF and TGFα have the ability to strongly stimulate bone resorption in cultured fetal rat long bones, newborn mouse calvarial cultures, and long term human marrow culture (18–20), suggesting these growth factors participate in regulating osteoclastogenesis and bone resorption. A recent study of neonatal EGFR-null mice revealed that EGFR deficiency causes delayed primary ossification of the cartilage anlage and delayed osteoclast and osteoblast recruitment. Further studies have suggested that primary osteoclastic cultures express the EGFR (21). However, the detailed mechanism of how EGF-like ligands stimulate bone resorption is still largely unknown.

Bone is the preferred metastasis site for many cancer cells, such as breast, lung, and prostate cancers, etc. The metastasized tumors result in osteolytic lesions, osteoblastic lesions, or both. In all cases, especially in osteolytic lesions, the osteoclast formation and activity are greatly stimulated by tumor invasion. The prevailing view is that PTH-related protein (PTHrP), secreted by tumor cells, plays a major role in this process by acting on osteoblasts in bone to increase the expression of RANKL (reviewed in Ref. 22). The EGFR family plays indispensable roles in the pathogenesis of many human carcinomas. Many studies suggest that the majority of cancers express at least one, or in many cases coexpress, several EGF-like ligands. The autocrine loop consisting of tumor-derived EGF-like ligands and their overexpressed receptors on tumor cells are essential for tumor growth and progression (reviewed in Ref. 23). It raises the question of whether bone-metastasized tumors utilize EGF-like ligands to facilitate bone destruction by osteoclasts.

Here we report that EGF-like ligands strongly stimulate osteoclast formation in the coculture of osteoblastic cells and bone marrow macrophages (BMMs), the precursors for osteoclasts, by regulating the expression of OPG and MCP-1 in osteoblastic cells. Because coculture with bone metastatic breast cancer MDA-MB-231 cells had similar effects on the expression of OPG and MCP1 in the osteoblastic cells, and those effects could be partially abolished by EGFR inhibitor, we reason that EGF-like ligands, similar to PTHrP secreted by tumors cells, may contribute to osteolytic lesions in bone metastases.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Recombinant human EGF, TGFα, HB-EGF, amphiregulin, heregulin were purchased from R&D Systems (Minneapolis, MN). PD153035 was obtained from Calbiochem. Murine secreted RANKL (sRANKL), M-CSF, human OPG, and neutralizing antibody against mouse MCP-1 were obtained from Peprotech (Rocky Hill, NJ). Human PTHrP and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Antibodies against RANKL (Santa Cruz Biotechnology, Santa Cruz, CA), EGFR, and β-actin (Cell Signaling Technology, Danvers, MA) were used for immunoblotting experiments.

**Cell Culture**—MC3T3-E1 subclone 4 cells (24, 25) were maintained in growth medium (αMEM supplemented with 1% fetal bovine serum plus 100 international units/ml penicillin and 100 μg/ml streptomycin). For experiments, MC3T3 cells were seeded in either growth medium or osteoblast differentiation medium (growth medium with 50 μg/ml L-ascorbic acid) at a density of 50,000 cells/cm². Media were changed every 2 days. To obtain primary osteoclastic cultures, bone marrow cells were flushed out from femora and tibiae of a 1–2-month-old mouse, plated in coculture medium (αMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 international units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine) in a 100-mm dish, and incubated at 37 °C in 5% CO₂ overnight. The next day, the nonadherent cells were pelleted and seeded at a density of 200,000 cells/cm². These cells are considered as BMM, the osteoclast precursors, and cultured in the presence of sRANKL (30 ng/ml) and M-CSF (30 ng/ml) for 5 days with a medium change at day 3 to obtain mature osteoclasts. In the presence of M-CSF but not RANKL, these cells became preosteoclastic cells, which would further differentiate into mature osteoclastic cells after addition of RANKL. To obtain primary bone marrow osteoclastic cultures, bone marrow cells were flushed out from femora and tibiae of 1–2-month-old mice and plated at a density of 300,000 cells/cm² in growth medium. Media were changed to differentiation medium on day 5 and every 2–3 days afterward. For coculture experiments of MC3T3 cells and osteoclasts, MC3T3 cells were seeded on day 0 in coculture medium plus 50 μg/ml l-ascorbic acid. On day 2, freshly isolated BMMs were seeded on top of MC3T3 cells with addition of sRANKL (30 ng/ml) to promote osteoclast formation. Media were changed 3 days later, and cells were either harvested for RNA or stained to detect TRAP-positive osteoclastic cells on day 7. For coculture experiments of primary osteoblastic cells and osteoclasts, primary osteoblastic cells were cultured as described above. On day 12, BMMs were seeded on top of primary osteoblastic cells along with sRANKL (10 ng/ml). TRAP staining was performed 2 and 4 days later with one medium change on day 14. Breast cancer cell line MDA-MB-231 was obtained from ATCC and maintained in coculture medium. To coculture MC3T3, MDA-MB-231, and BMM cells, MC3T3 and MDA-MB-231 cells were seeded at the same density of 50,000 cells/cm² in coculture medium at day 0. Two days later, freshly isolated BMMs were seeded in the same well in the presence of sRANKL (30 ng/ml). TRAP staining was performed on day 7 with a medium change at day 5. The animal protocol was approved by Robert Wood Johnson Medical School Institutional Animal Care and Use Committee.

**TRAP Staining**—The osteoclast preparations were stained for TRAP activity using a leukocyte acid phosphatase kit from Sigma.
EGF-like ligands stimulate osteoclastogenesis in bone.

**RESULTS**

**EGF-like Ligands Stimulate Osteoclastogenesis in Cocultures of Osteoblastic Cells and BMMs**—To study the ability of osteoblasts to support osteoclast formation, we cocultured mouse BMMs with differentiating MC3T3-E1 subclone 4 cells. MC3T3 is a preosteoblastic cell line, which undergoes osteoblastic differentiation in the presence of L-ascorbic acid. However, there were almost no TRAP-positive osteoclastic cells formed in the coculture system even in the presence of exogenous RANKL (Fig. 1A, left panel). Interestingly, addition of EGF-like ligands, such as EGF, TGFα, HB-EGF, and amphiregulin, strongly stimulated formation of TRAP-positive osteoclastic cells in this coculture system (Fig. 1A, right four panels). Real-time RT-PCR experiments further demonstrated that there were about 28-fold and 39-fold increases in TRAP mRNA expression levels in the EGF and TGFα-treated cocultures, respectively (Fig. 1B).

To further confirm this phenomenon, we repeated this coculture experiment using mouse primary bone marrow osteoblastic cells instead of MC3T3 cells. In our hands, it takes about 2–3 weeks for the primary bone marrow osteoblastic cells to form bone nodules as shown by von Kossa staining and to abundantly express osteoblastic markers, such as osteocalcin, integrin-binding bone sialoprotein, and alkaline phosphatase (data not shown). In this experiment, the primary osteoblastic cells were treated with or without TGFα from day 5, and BMMs were added into the culture at day 12. Fig. 1, C and D show that there were significantly more TRAP-positive osteoclastic cells formed in the TGFα-treated coculture system than in the control coculture 2 or 4 days later. Other EGF-like ligands exhibited similar effects (data not shown).

The above results clearly indicate a dramatic increase in osteoclastogenesis in the osteoblast-osteoclast coculture system when EGF-like ligands are present. Note that exogenous

**Real-time RT-PCR**—Total RNA from cell cultures was isolated using Tri Reagent (Sigma). TaqMan® Reverse Transcription kit (Applied Biosystems, Foster City, CA) was used to reverse-transcribe mRNA into cDNA. Following this, PCR was performed on Opticon (MJ Research) using a SYBR® Green PCR Core kit (Applied Biosystems). The primers used for the RT-PCR are summarized in the supplemental Table S1. Mouse β-actin was used as an internal control for mouse genes. In the coculture experiments of MC3T3 and MDA-MB-231 cells in the same well, mouse ATF4 was used as an internal control because its primers only detect the mouse gene and not the human homolog, and ATF4 expression was not regulated by EGF-like ligands (data not shown).

**EGF Ligand Binding and Autoradiography**—Cells were cultured in 24-well plates and incubated with 0.8 μl/well mouse 125I-EGF (80–120 μCi/μg, 50 μCi/ml, GE Healthcare) with or without 250-fold cold EGF in αMEM containing 1 mg/ml bovine serum albumin for 2 h at 37 °C. After washing twice with phosphate-buffered saline, cells were lysed in 0.5 M NaOH and counted in a gamma counter (1282 CompuGamma CS, LKB Wallac). Alternatively, cells were cultured in 4-well chamber slides. After 125I-EGF binding as described above, slides were fixed, dried, and dipped into NTB emulsion solution (Eastman Kodak Company). After 6 weeks of exposure, slides were developed to view the silver grains and counterstained with cresyl violet.

**Mineral Dissolution Assay**—Mouse BMMs were plated onto 16-well BD BioCoat™ Osteologic™ Discs (BD Biosciences, Franklin Lakes, NJ) seeded at a density of 200,000 cells/cm² in coculture medium plus sRANKL (30 ng/ml) and M-CSF (30 ng/ml). Two days later, media were changed to 50% fresh coculture medium and 50% conditioned medium from either control or EGF-treated MC3T3 cells for 4 days plus sRANKL. Similar medium changes were performed every 2 days until day 8 for quantitation of resorption areas using SPOT Advanced software under microscopy.

**ELISA**—The OPG and TGFα protein levels in media were determined using the mouse OPG Quantikine ELISA kit and human TGFα Quantikine ELISA kit from R&D Systems.

**Statistical Analysis**—All results are expressed as means ± S.E. of triplicate measurements with all experiments being repeated at least three times. Statistical analyses were carried out using the Student’s t test (Microsoft Excel 2002).

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**FIGURE 1.** EGF-like ligands stimulate osteoclastogenesis in cocultures of osteoblasts and BMMs. A, TRAP staining of cocultures of MC3T3 cells and mouse BMMs. MC3T3 cells were cultured in differentiation medium with or without EGF-like ligands for 2 days. Then, BMMs harvested from mouse long bones were seeded on top of MC3T3 cells along with 30 ng/ml RANKL. Cells were stained for TRAP activity after 5 days with a medium change at day 3. The concentration of all EGF-ligands was 8 × 10^−11 M in this report unless otherwise specified. White arrows point to TRAP-positive osteoclastic cells. AR, amphiregulin. B, real-time RT-PCR analyses of TRAP mRNA levels in the above cocultures after 5 days. C, TRAP staining of cocultures of primary bone marrow osteoblastic cells and BMMs. Bone marrow cells harvested from mouse long bones were cultured in osteoblastic differentiation medium for 12 days. Control- or TGFα-containing media were added from day 5, with media changes every 2 days. Then, BMMs were seeded on top of these osteoblastic cells with RANKL (10 ng/ml). Cells were stained for TRAP activity after another 2 or 4 days. D, densitometric quantitation of TRAP staining in C.
RANKL is indispensable for this phenomenon because both MC3T3 cells (Fig. 5B) and primary osteoblastic cells (data not shown) express very low levels of endogenous RANKL and those levels are not sufficient to support osteoclast formation.

**EGF-like Ligands Do Not Have Direct Effects on Osteoclastic Cells** — The effects of EGF-like ligands on the osteoblast and osteoclast coculture could be caused by two possibilities: 1) EGF-like ligands have direct effects on osteoclastic cells, or 2) these peptides have indirect effects on osteoclastic cells by acting through osteoblastic cells. To investigate the first possibility, we studied whether osteoclastic cells express functional EGF binding sites. A previous report suggested that primary osteoclastic cells express EGFR mRNA (21). Our RT-PCR analyses with either bone marrow primary osteoclastic cells or RAW264.7 cells, a preosteoclast cell line, also indicated the existence of EGFR mRNA albeit at low abundance (data not shown). However, EGFR protein cannot be detected by Western blot (Fig. 2D, last lane). To further clarify it, we used radioisotope-labeled ligand binding experiments to detect the existence of functional EGFR in osteoclasts. When cells were incubated with $^{125}$I-EGF ligand, we found no strong binding in RAW264.7 cells, primary preosteoclastic cells (obtained by culturing BMMs with M-CSF), or primary mature osteoclastic cells (obtained by culturing BMMs with both M-CSF and RANKL) (Fig. 2A). In addition, the presence of 250-fold unlabeled EGF did not further decrease the $^{125}$I-EGF binding with the above cells, suggesting the binding observed with these cells is most likely due to nonspecific binding. ROS17/2.8 cells, an EGFR-deficient osteoblastic osteosarcoma cell line, were used as a negative control. In contrast, $^{125}$I-EGF bound strongly to Saos2 cells, an EGFR-positive osteoblastic cell line, and this binding was dramatically decreased by 250-fold unlabeled EGF.

To further confirm that osteoclasts lack functional EGFR binding sites, we performed $^{125}$I-EGF ligand binding experiments with cells cultured on chamber slides. After exposing cells to emulsion for 6 weeks, we observed abundant silver grains, indicators of $^{125}$I-EGF binding, in the Saos2 cells, but few or no silver grains over either RAW264.7 or mature osteoclastic cells (Fig. 2B).

In summary, the above results demonstrate that both pre- and mature osteoclastic cells exhibit very low levels of EGF binding sites, namely, EGFRs. Our further experiments showed that EGF-like ligand treatment had no effects on osteoclast for-
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EGF-like Ligands Regulate the Expression of Secreted Osteoclast Regulatory Factors in Osteoblastic Cells—To study how EGF-like ligands regulate osteoclast formation indirectly, conditioned media were collected from either control or EGF-treated MC3T3 cells after 4 days of differentiation and were used to culture mouse BMMs for 5 days with one medium change on day 3. As shown in Fig. 3, A and B, multinucleated osteoclasts formed in control-treated conditioned medium were generally small and had less than 30 nuclei per cell. In contrast, osteoclasts formed in EGF-treated MC3T3-conditioned medium were much larger and many of them had more than 30 nuclei per cell. Mineral dissolution assay further indicates that osteoclasts cultured with EGF-treated conditioned medium resorbed about 5-fold more mineralized surface on osteologic discs than that cultured in control-treated conditioned medium (Fig. 3, C and D). Note that exogenous RANKL was required in this experiment because MC3T3 cells do not express sufficient RANKL and most RANKL molecules exist in a transmembrane form. This result clearly indicates that EGF regulates expression of certain secreted factor(s) in MC3T3 cells, and those factors have the ability to regulate osteoclastogenesis.

FIGURE 3. EGF stimulates osteoclastogenesis through regulating secreted factors from osteoblasts. Differentiating MC3T3 cells were treated with either control or EGF for 4 days with medium change at day 2. Then, conditioned media were collected from the above cultures and used for culturing BMMs with the addition of RANKL (30 ng/ml). The media were changed after 3 days with new conditioned media. On day 5, cells were stained for TRAP activity (A), and TRAP-positive osteoclast cell numbers were counted (B). C, similar experiment was performed with BMMs seeded on BioCoatosteologic discs and resorbed mineral areas were measured on day 8. Representative images of resorption pits in osteologics (white areas) are shown. D, quantitation of resorption areas. *, p < 0.05 versus control.

One possibility for why osteoclasts do not bind 125I-EGF while MC3T3 cells do, is that osteoclasts might express much more EGF-like ligands and therefore have already saturated their EGFR sites. However, real-time RT-PCR showed both osteoclasts and MC3T3 cells express low and comparable amounts of EGF-like ligands (supplemental Fig. S2). Taken together, these results clearly demonstrate that MC3T3 cells, but not osteoclasts, express functional EGFR and therefore EGF-like ligands must have indirect effects on osteoclastic cells by acting through osteoblastic cells. The existence of functional EGFR in osteoblasts has been well documented in osteoblastic cell lines, such as UMR 106-01, primary osteoblastic cell cultures (26) and in vivo osteoblasts using in situ hybridization and immunohistochemistry (27).
cells secreted high amounts of OPG (135, 165, 150 ng/ml at days 4, 6, and 8, respectively) but EGF treatment strongly inhibited OPG production to 6, 18, and 19 ng/ml at days 4, 6, and 8, respectively. Apart from EGF, other EGF-like ligands, HB-EGF, and amphiregulin, also exhibited similarly strong inhibitory effects on OPG production (Fig. 4C). Because OPG is a decoy receptor for RANKL, a decrease in OPG expression by EGF-like ligands will definitely lead to an increase in osteoclast formation. We also measured RANKL expression in differentiating MC3T3 cells. Real-time RT-PCR indicated RANKL was expressed at low levels (the relative mRNA abundance to β-actin is about $6 \times 10^{-6}$) and neither L-ascorbic acid nor TGFα treatment affected its expression (Fig. 5A). Western blotting did not reveal any RANKL protein in cell lysates of MC3T3 cells with or without TGFα treatment while the positive control did show RANKL protein (Fig. 5B). Therefore, the above results demonstrate that EGF-like ligands mainly regulate OPG, but not RANKL, expression in these osteoblastic cells. This is also consistent with our previous observation that EGF regulates osteoclastogenesis by regulating secreted factors from osteoblastic cells while RANKL is a transmembrane protein.

In addition to OPG, we also analyzed the mRNA levels of MCP1, M-CSF, and IL-6 in MC3T3 cells after EGF treatment using real-time RT-PCR. Fig. 6 shows that both MCP1 and M-CSF expression were increased by EGF treatment. MCP1 expression was increased about 2–3-fold during the whole culture period, and M-CSF expression was increased about 3–5-fold. We did not find EGF treatment altered IL-6 mRNA levels (data not shown).

To study whether OPG, MCP1, and M-CSF are target genes for EGF-like ligands in primary osteoblastic cells, we examined their expression in mouse bone marrow primary osteoblastic cultures. Treatment of differentiating primary osteoblastic cultures with EGF-like ligands dramatically inhibited OPG expression both at the mRNA (Fig. 7A) and protein (Fig. 7C) levels. Furthermore, EGF-like ligands also significantly increased MCP1 expression in primary osteoblastic cells (Fig. 7B). However, we found that these ligands had no effect on M-CSF expression (data not shown), indicating that up-regulation of M-CSF by EGF may not be a ubiquitous event.

**EGF-like Ligands Regulate OPG and MCP1 Expression in an EGFR-dependent Manner**—Four ErbB receptors can form either homodimers or heterodimers to transduce signaling. To test whether the EGFR is the main receptor involved in the regulation of OPG and MCP1 expression by EGF-like ligands, we added EGFR-specific tyrosine kinase inhibitor PD153035 in the medium before TGFα treatment of MC3T3 cells. As shown in Fig. 8, PD153035 completely abolished the effects of TGFα on OPG and MCP1 expression. Furthermore, heregulin, a peptide that activates ErbB3 and ErbB4 but not EGFR (8), had no

![FIGURE 4. EGF-like ligands inhibit OPG production in MC3T3 cells.](image)

**FIGURE 5. RANKL expression is not stimulated by EGF-like ligands in MC3T3 cells.** MC3T3 cells were cultured in growth medium or differentiation medium with or without TGFα. A, real-time RT-PCR analyses were performed with RNA harvested on indicated times to measure RANKL expression. The amount of RANKL expression in RNA harvested at day 2 in growth medium was set as 1. B, immunoblotting of RANKL protein expression in MC3T3. A1.1, a murine T cell hybridoma, was treated with anti-CD3 for 6 h and used as a positive control for RANKL protein (7). Each lane was loaded with equal amounts of protein (40 μg).
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Effect on OPG and MCP1 expression in MC3T3 cells. These data suggest that regulation of OPG and MCP1 requires EGFR tyrosine kinase activity.

 Regulation of Osteoclastogenesis by EGF-like Ligands Is Mainly through OPG and Partially through MCP1 Pathways—To investigate whether regulation of OPG and MCP1 expression in osteoblasts by EGF-like ligands results in stimulation of osteoclastogenesis in the coculture system, we blocked the OPG decrease by adding exogenous OPG or prevented MCP1 action by adding neutralizing anti-MCP1 antibody to the cocultures of MC3T3 and BMM cells. Similar to our previous results, EGFR treatment greatly stimulated osteoclast formation in the coculture system, while there were almost no TRAP-positive cells in the absence of EGF (Fig. 9A). Addition of exogenous OPG completely eliminated EGF effects, resulting in no TRAP-positive cells formed. Note the concentration of OPG used in this experiment is 30 ng/ml, which is much lower than the medium OPG concentration in MC3T3 cells after 4 days of differentiation (140–170 ng/ml). In contrast, addition of neutralizing anti-MCP1 antibody in the coculture significantly decreased, but did not completely eliminate, the number of TRAP-positive cells in the coculture compared with the IgG control. Analyses of TRAP mRNA levels further confirmed the above observation (Fig. 9B). In summary, it seems that EGF stimulation of osteoclast formation in the coculture system is mainly through inhibiting OPG and partially through stimulating MCP1.

EGFR Signaling Contributes to Regulation of OPG and MCP1 in MC3T3 Cells by MDA-MB-231 Cells—EGF-like ligands are expressed or overexpressed in most human tumors. Because osteolytic bone metastases strongly stimulate osteoclast formation, we reasoned that EGF-like ligands expressed by metastasizing tumor cells might contribute to this process by acting on osteoblasts and regulating their expression of OPG and MCP1 to stimulate osteoclast formation. To test this hypothesis, we studied the effects of coculturing MDA-MB-231 cells on the osteoclastogenesis stimulated by osteoblasts. We chose MDA-MB-231 cells because these breast cancer cells have strong bone metastatic osteolytic activity. Preliminary real-time RT-PCR revealed that these cells express high levels of TGFα, HB-EGF and amphiregulin mRNA but low levels of EGF and PTHrP mRNA (Fig. 10A). We also used ELISA to measure TGFα levels in the culture medium (Table 1). While conditioned medium from MDA-MB-231 cells contained TGFα (about 6 pg/ml), 4 h of PMA treatment caused release of cleaved transmembrane TGFα into the medium and increased its concentration to about 55 pg/ml, indicating the majority of TGFα exists in a transmembrane form. PMA was used previously for TGFα shedding (28) and itself had no effect on ELISA measurement.
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TABLE 1

| Conditioned medium | TGFα (pg/ml) |
|--------------------|--------------|
| Medium only        | 0            |
| Conditioned medium | 5.7 ± 2.5    |
| Conditioned medium + PMA | 3.4 ± 1.6 |
| Me2SO 1 h treated conditioned medium | 5.6 ± 0.2 |
| PMA 1 h treated conditioned medium | 32.8 ± 1.4 |
| PMA 4 h treated conditioned medium | 55.5 ± 5.4 |

* Conditioned medium collected from MDA-MB-231 culture.
* Conditioned medium collected from MDA-MB-231 culture and then addition of PMA (30 ng/ml).
* Conditioned medium collected from MDA-MB-231 culture that was treated with Me2SO for 1 h.
* Conditioned medium collected from MDA-MB-231 culture that was treated with PMA (30 ng/ml) for 1 h.

Note: PD153035 itself has no effect on either EGF-like ligand production in MDA-MB-231 or OPG and MCP1 expression in MC3T3 cells (data not shown). These results imply the importance of EGF signaling in mediating regulation of osteoclast regulatory factors in osteoblasts by breast cancer cells and also indicate that other unknown signaling pathways are involved.

DISCUSSION

In this report, we demonstrate that EGF-like ligands have the ability to strongly stimulate osteoclastogenesis by regulating OPG and MCP1 expression in osteoblastic cells in an EGF-dependent manner. We also provide evidence that cancer cells that can metastasize to bone may utilize this EGF signaling pathway to promote osteoclast formation. Therefore, these data suggest a novel mechanism for osteolytic lesions caused by cancer bone metastases. Because of a relatively large selection of EGF antagonists available as pharmaceuticals, we think that this finding is particularly worthy of further investigation.

EGF and TGFα have long been recognized as bone resorption factors. In mouse neonatal calvarial cultures, EGF or TGFα-stimulated resorption is dependent on prostaglandin (PG) synthesis and is inhibited by indomethacin (20, 29). In contrast, in fetal long bone cultures, EGF or TGFα-stimulated resorption is not inhibited by indomethacin (29, 30). EGF has been shown to prominently enhance endogenous PGE2 synthesis in the parental cell line (MC3T3-E1) of the MC3T3-E1 subclone 4 used in this current study (31). The RANKL/OPG ratio is the ultimate determinant of osteoclastogenesis in bone. Like other bone resorption hormones and cytokines, such as 1,25(OH)2D3, PTH, and IL-11 etc, PGE2 regulates osteoclastogenesis through stimulation of RANKL and inhibition of OPG expression in osteoblast/stromal cells (32). Our data clearly demonstrate that EGF-like ligands dramatically decrease OPG

MB-231 cells strongly inhibited mouse OPG expression (about 10-fold) and dramatically increased mouse MCP1 expression (about 50-fold) by MC3T3 cells (Fig. 10C). PD153035, a specific inhibitor of the EGFR tyrosine kinase, was added to the coculture medium to test whether this regulation is EGF-dependent or not. This compound partially eliminated inhibition of OPG expression (92% decrease in OPG in the presence of Me2SO versus 80% decrease in the presence of PD153035, Fig. 10C). The stimulation of MCP1 expression was also significantly decreased by addition of PD153035 (about 50-fold increase in MCP1 in the presence of Me2SO versus 35-fold increase in the presence of PD153035, Fig. 10C). Note PD153035 itself has no effect on either EGF-like ligand production in MDA-MB-231 or OPG and MCP1 expression in MC3T3 cells (data not shown). These results imply the importance of EGF signaling in mediating regulation of osteoclast regulatory factors in osteoblasts by breast cancer cells and also indicate that other unknown signaling pathways are involved.
production but have no apparent effect on RANKL expression in osteoblastic cells and hence result in stimulation of osteoclast formation. One possibility is that EGF down-regulates OPG through the PGE2 pathway.

In addition, our study also indicates that MCP1 partially mediates the osteoclastogenic effect of the EGF-like ligands. MCP1 was recently identified as an important factor acting directly on pre/osteoclasts to stimulate osteoclast formation and activity in the presence of RANKL (5). Moreover, MCP1 is a chemoattractant for pre/osteoclasts. Because RANKL is a transmembrane protein, this chemoattractant feature of MCP1 could be useful to attract osteoclast precursors to osteoblasts and to locate the resorption site. Interestingly, we found that the breast cancer cell line MDA-MB-231 is able to dramatically stimulate MCP1 expression about 50-fold in MC3T3 cells through both EGF-dependent and -independent pathways, implying MCP1 might play an important role in mediating osteoclast activation in breast cancer bone metastases.

Bone is a common site for cancer metastasis, especially for breast cancer cells. PTHrP is considered to be the major cancer-secreted factor that mediates the osteolytic lesions by stimulating RANKL expression in osteoblasts (22). In addition, another tumor-produced factor, IL-8, has been reported to be correlated with metastatic potential of cancer cell lines in vivo and its action is not through the RANKL/OPG pathway (33). A majority of solid neoplasms overexpress EGF-like ligands and their cognate receptors (23). The expression of TGFα has been demonstrated in all carcinoma types, with many tumors showing overexpression of this peptide as compared with normal tissue (34). In particular, 40–70% breast cancers, 60–100% lung cancers, 50–90% colon cancers, 55–100% ovarian cancers, 40–100% head and neck cancers, and 50–90% head and neck cancers, have been found to express TGFα (23). It is worth noting that MDA-231 cells express much more EGF-like ligands than PTHrP at mRNA level (Fig. 10A). These facts, taken together with our data that EGF-like ligands stimulate osteoclastogenesis through osteoblastic cells, lead us to hypothesize that EGFR signaling contributes to osteolytic bone metastasis. Because we did not observe that RANKL expression is enhanced by EGF-like ligands, we consider the role of EGFR signaling as facilitating PTHrP action. Indeed, we find that cotreatment of osteoblastic cells with TGFα and PTHrP has additive or synergistic effects on decreasing OPG and increasing MCP1 expression (data not shown).

Interestingly, a recent report suggests a similar role for EGFR signaling in bone metastasis but with different mechanisms. In this report (35), it was found that gefitinib, an EGFR tyrosine kinase inhibitor, inhibited M-CSF and RANKL expression in two human mesenchymal stem cell-like cell lines, HDS-1 and -2. The discrepancy on RANKL expression may be caused by the different differentiation stages of the cells used in this report. HDS cells are more like mesenchymal stem cells and are able to differentiate into both adipocytes and osteoblasts. However, we performed our experiments in osteoblastic differentiating MC3T3 cells, which have undetectable levels of RANKL. Nevertheless, both reports suggest that EGFR signaling contributes to cancer cell-mediated osteolytic lesions through acting on osteoblasts. EGFR signaling plays multiple roles in cancer pathogenesis. EGF-like ligands and their receptors are important to the growth and survival of tumor cells via paracrine and/or autocrine pathways. All these proteins exhibit transforming ability both in vivo and in vitro (23). A recent study demonstrated that EGFR signaling is involved in tumor invasive ability by cross-talk with the urokinase-type plasminogen activator (uPA)/uPAR receptor (uPAR) system (36). EGFR signaling has also been shown to be involved in tumor angiogenesis (37, 38). Here, our results implicate another important role of EGFR signaling on bone metastasis. In addition to their indirect effects on osteoclastogenesis, EGF-like ligands may also have direct effects on osteoblastogenesis because they are potent inhibitors of osteoblast differentiation (14, 39). Dramatically decreased mature osteoblast number was recently recognized as a phenomenon associated with osteolytic lesions (40). Previous clinical studies mostly focused on the importance of EGFR signaling in tumor cells. Our studies highlight the role of EGFR signaling in osteoblasts and therefore suggest a novel mechanism for the application of anti-EGFR drugs in the treatment of bone metastasis of cancer cells.

Acknowledgments—We thank Dr. Eric Richfield at UMDNJ-Robert Wood Johnson Medical School for help with autoradiography. We thank Liying Zhang at UMDNJ-Robert Wood Johnson Medical School for providing cell lysates of A1.1 cells treated with anti-CD3.

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