Amphiregulin Is a Novel Growth Factor Involved in Normal Bone Development and in the Cellular Response to Parathyroid Hormone Stimulation

Received for publication, August 26, 2004, and in revised form, October 20, 2004
Published, JBC Papers in Press, October 27, 2004, DOI 10.1074/jbc.M409807200

Ling Qin, Joseph Tamasi, Liza Raggatt, Xin Li, Jean H. M. Feyen, David C. Lee, Emanuel DiCicco-Bloom, and Nicola C. Partridge

From the Departments of Physiology and Biophysics and Neuroscience and Cell Biology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854; Bristol-Myers Squibb Pharmaceutical Research Institute, Pennington, New Jersey 08534; and the Department of Biochemistry and Biophysics and University of North Carolina Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

Parathyroid hormone (PTH), an 84-amino acid polypeptide hormone secreted by the parathyroid glands, plays an essential role in calcium and phosphate homeostasis and bone remodeling. Paradoxically, PTH has two opposite effects on bone if administered in different ways. Although continuous administration causes bone loss, intermittent injection increases bone volume and bone density (1–3). To date, PTH is the only osteoporosis treatment that promotes bone formation. The primary target of PTH in bone is the osteoblast, which expresses a single PTH receptor (PTH1R), a G protein-coupled seven-transmembrane domain receptor (4). Upon PTH binding, both protein kinase A and protein kinase C signaling pathways are activated, and consequently the expression of many genes are altered (5). Recently, we performed microarray experiments to study the gene expression profile changes in PTH-treated UMR 106-01 cells, a rat osteoblastic osteosarcoma cell line (6). A total of 125 known genes were identified as PTH-regulated. Amphiregulin (AR), a member of the epidermal growth factor (EGF) family, was one of those genes.

The EGF family members are divided into two classes (7). The first class, also known as EGF-like ligands, all bind to the EGF receptor (EGFR/ErbB1), which includes EGF, AR, and transforming growth factor alpha (TGF-α), which bind to EGFR exclusively, and heparin-binding EGF (HB-EGF), betacellulin, and epiregulin, which bind both EGFR and ErbB4. The second class, collectively termed neueregulins, bind directly to the receptors ErbB3 and/or ErbB4. The EGFR (ErbB1) is a receptor tyrosine kinase and lies at the beginning of a complex signal transduction cascade that modulates cell proliferation, survival, adhesion, migration, and differentiation (8). Upon ligand binding, the EGFR undergoes dimerization and phosphorylation at tyrosine residues in its intracellular domain, thus activating several important cellular signal transduction pathways. The major signaling routes are the Ras-Raf-mitogen-activated protein kinase (9) and phosphatidylinositol 3-kinase-Akt pathways (10). It is now known that ErbB2 is the preferred co-receptor for the EGFR, and the heterodimeric receptor complex signals more potently than a homodimer of the EGFR (8).

AR was first isolated from conditioned medium of MCF-7 human breast carcinoma cells exposed to phorbol 12-myristate 13-acetate (11, 12). AR is bifunctional because it inhibits the growth of many human tumor cells but stimulates the proliferation of other cells such as normal fibroblasts and keratinocytes (13, 14). Similar to EGF, AR is produced as a precursor transmembrane protein that undergoes proteolytic cleavage to yield the mature protein. So far, there have been no reports of AR production or function in bone. However, EGF has been shown to have several effects on bone cells or on bone: it stimulates osteoblast proliferation (15), decreases alkaline phosphatase (16) and collagen production (17), changes bone nodule formation (18), and yet, has catabolic effects on bone metabolism.
(19), i.e. similar to AR, bifunctional effects. Nevertheless, the production, detailed mechanism, and the significance of the EGF signaling pathway in bone are not well understood.

In this paper, we demonstrate that AR is a general immediate response gene for PTH action in bone. It has profound effects on osteoblasts because it strongly stimulates the growth of preosteoblasts while it inhibits the differentiation and mineralization of mature osteoblasts. Its role in normal bone development and in PTH function will be discussed.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**Synthetic human PTH(1–38) was purchased from Bachem (Torrance, CA). Compounds 32, compound 56, wortmannin, and 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃) were purchased from Calbiochem. Rat PTH(1–34), recombinant human AR, human EGF, prostaglandin E₂ (PGE₂), and cycloheximide were obtained from Sigma. Antibodies for ERK1/2 and phospho-ERK1/2 were obtained from Santa Cruz (Santa Cruz, CA). Antibodies for Akt and phospho-Akt were obtained from Cell Signaling (Beverly, MA).

**Cell Culture—**UMR 106-01 cells were maintained in Eagle’s minimal essential medium (MEM) supplemented with 5% (v/v) fetal bovine serum until confluence at day 7. Then the medium was switched to differentiation medium (BGJb medium containing 10% fetal bovine serum) until day 10. Cells were cultured in MEM containing 10% fetal bovine serum for 2 days before the addition of appropriate agents. Rat primary calvarial osteoblastic cells were obtained from neonatal rat calvariae by sequential digestions with collagenase and trypsin as described previously (20). Cells were cultured in MEM containing 10% fetal bovine serum until confluence at day 7. Then the medium was switched to differentiation medium (BGJb medium containing 10% fetal bovine serum, 10 μM β-glycerophosphate, and 50 μg/ml ascorbic acid). MC3T3 cells were maintained in MEM plus 10% (v/v) fetal bovine serum. After confluence, cells were cultured in the same differentiation medium. Before PTH treatment, both types of cells were serum starved for 1 day.

**In Vivo Injection of PTH—**Four-week-old male Sprague-Dawley rats, about 75 g, were purchased from Hilltop (Scottdale, PA). Rats were injected subcutaneously with vehicle (0.9% saline solution) or hPTH(1–38) (6 μg/100 g) and euthanized using CO₂ at 0.5, 1, 4, or 8 h after injection. The primary spongiosa samples from distal femur were harvested as described previously (21). The animal protocols were approved by Robert Wood Johnson Medical School Institutional Animal Care and Use Committee.

**Analysis of mRNA Abundance by Real Time RT-PCR—**Cells or tissues were harvested at the indicated time points after hormone treatments. Total RNA was isolated using TRI Reagent (Sigma) followed by an RNeasy kit (Qiagen). A TaqMan Reverse Transcription kit (Applied Biosystems) was used to reverse transcribe mRNA into cDNA. Following this, PCR was performed on Option (MJ Research) using a SYBR Green I kit (Applied Biosystems). Each analysis was performed two or three times with independent sets of cells or tissues from hormone treatment to RT-PCR to obtain the mean ± S.E. shown in the figures. The primers used for the RT-PCR are summarized in the supplemental table. For UMR 106-01 and MC3T3 cells, rat and mouse β-actin was used as an internal control.

**DNA Synthesis Measurement—**Incorporation of [³H]thymidine into macromolecules was used to assess DNA synthesis. Rat primary calvarial osteoblastic cells were seeded at 4 × 10⁴ cells/well in 24-well plates. Cells were then serum starved for 1 day before being treated with appropriate agents overnight. Cells were incubated with [³H]thyidine (1 μCi/ml) for the final 3 h of incubation. DNA containing incorporated radiolabel was collected onto glass fiber filters using a semiautomated cell harvester (Skatron), whereas unincorporated [³H]thyidine was removed by exhaustive water elution. The incorporation was assayed by liquid scintillation counting. Experiments were performed with three samples/group and repeated twice. Statistical analysis was performed by Student’s t test.

**Cell Cycle Analysis—**Rat primary calvarial osteoblastic cells were seeded into 100-mm dishes at 5 × 10⁵ cells/dish. Cells were serum starved the next day for 1 day before treatment with AR or EGF inhibitors overnight. One million cells were trypsinized, washed once with phosphate-buffered saline, and fixed in 70% ethanol for at least 1 h on ice. Fixed cells were washed with phosphate-buffered saline and incubated with propidium iodine solution containing ribonuclease A. The cell cycle of stained cells was analyzed by Beckman Coulter XL.

**Alizarin Red Staining and Alkaline Phosphatase Activity Staining—**For alizarin red staining, cells were fixed in cold 70% ethanol for 1 h and stained with 40 μM alizarin red-S for 10 min. Then cells were washed five times for 20 min each with water and then photographed. For alkaline phosphatase activity staining, cells were stained with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (Sigma) for 10 min, and the reaction was stopped by washing with water.

**Immunoblotting—**Preparation of cell lysates and Western blot analyses were performed as described previously (22).

**Microcomputer Tomography (microCT) Measurement of AR Null and Wild-type Mice—**AR null mice (B6/129) were described previously (23). A breeding colony was established using heterozygous mice to provide littermate controls. The femora and tibiae were harvested from 4-week-old AR null mice and their wild-type littermates and were subjected to microCT analysis. The analysis was performed by Robert Wood Johnson Medical School Institutional Animal Care and Use Committee.

**RESULTS**

**PTH Stimulates AR Expression Both in Vitro and in Vivo—**Recently we identified 125 PTH-regulated genes in UMR 106-01 cells using microarray technology (6). AR (also known as schwannoma-derived growth factor) mRNA was shown to be stimulated more than 2-fold after 4 h and 12 h with 10⁻¹⁰ M rat PTH(1–34) treatment. Real time RT-PCR quantitatively proved that AR is indeed an early PTH-responsive gene in this osteoblastic cell line (Fig. 1A). The mRNA expression of AR dramatically increased about 23-fold after 1 h of 10⁻¹⁰ M rPTH(1–34) treatment. This stimulation then decreased, as the fold change was about 11-fold at 4 h of PTH treatment, but was maintained at a significant level even at 12 h (8-fold). As shown in Fig. 1B, the stimulation of AR expression is PTH dose-dependent. Significant stimulation was observed at 10⁻¹⁰ M rPTH(1–34) (4-fold) and higher concentrations (15-fold at 10⁻⁹ M and 23-fold at 10⁻⁸ M). No obvious PTH effect was detected at 10⁻¹¹ and 10⁻¹² M. The real time RT-PCR product was sequenced, and the result completely matches the rat AR cDNA sequence (data not shown).

To study whether AR is a ubiquitous PTH target gene, we tested two other PTH-responsive osteoblastic cell preparations: rat calvarial primary osteoblastic cells (Fig. 1C) and mouse MC3T3 cells (Fig. 1D). The primary osteoblastic cells undergo proliferation, differentiation, and mineralization phases in vitro. Rat PTH(1–34) strongly induced AR expression in these cells, with the highest induction (23-fold) in the mineralization phase and the lowest induction (5-fold) in the proliferation phase. In all phases the strongest induction of AR occurred at
It is worth noting that the basal expression of AR did not change among three phases (data not shown). In MC3T3 cells, a significant increase in AR expression was also observed at early time points (4-fold at 1 h and 3-fold at 2 h) of PTH treatment.

It is known that intermittent injection of PTH into young rats increases bone and mineral density (24). Expression of several genes, such as c-fos, IL-6, and myc, which are important for PTH actions in bone, have been found to be regulated by acute PTH injection in rat osteoblast-enriched femoral metaphyseal primary spongiosa (21). To investigate whether PTH regulates AR in vivo, we injected human PTH(1–38) (8 μg/100 g) into young male rats and harvested the femoral metaphyses at various time points. As shown in Fig. 1E, the level of AR mRNA was elevated dramatically to about 12-fold after 1 h of PTH injection and decreased to about 2-fold after 4 h, demonstrating that amphiregulin is actually regulated by PTH in vivo in a very rapid fashion similar to that seen in culture.

**PTH Induction of AR Is a Primary Response—**To determine whether PTH induction of AR requires new protein synthesis, UMR 106-01 cells were treated with 10^{-8} M rat PTH(1–34) for 1 h. Cells were then treated with 10^{-8} M rat PTH(1–34) for another 1 h before harvesting RNA for real time RT-PCR analyses. The AR mRNA level in the cells treated only with ethanol was set as 1.

**AR Is a Target Gene for Other Osteotropic Hormones—**In addition to PTH, 1α,25-(OH)_{2}D_{3} and PGE_{2} are other agents that play important roles in bone remodeling via osteoblasts. Next, we investigated whether these hormones could also regulate AR expression. The fact that there was no superinduction of AR in the cycloheximide-treated cells also tends to exclude the possibility that PTH regulates AR by stabilizing its mRNA. In addition, in a separate study, we have shown that PTH stimulates activity of the AR promoter in a cAMP-response element-binding protein-independent manner.2

---

2 L. Qin and N. C. Partridge, manuscript submitted.
starved for 1 day then treated with either 1α,25-(OH)_{2}D_{3} or PGE_{2} for various time periods. RNAs were harvested to analyze the AR mRNA level by real time RT-PCR (Fig. 3). Interestingly, both hormones have strong effects on AR induction, albeit the expression patterns are different from those induced by PTH. The AR induction by 1α,25-(OH)_{2}D_{3} peaked to 24-fold at 12 h but was low at earlier time points (3-fold at 1 h and 2-fold at 4 h) (Fig. 3A). Note that AR has been recently found to be up-regulated by 1α,25-(OH)_{2}D_{3} in squamous cell and breast carcinoma cells in a similar time-dependent pattern (25). PGE_{2} quickly and continuously elevated the expression of AR, resulting in 13-, 12-, and 11-fold increases at 1, 4, and 12 h, respectively (Fig. 3B).

Expression of EGF-like Ligands and Their Receptors in Osteoblastic Cells—To date, there is no report of the expression of EGF family ligands and receptors in osteoblastic cells. Because we have demonstrated that AR is regulated by several osteotropic hormones, it became important to determine the profile of other EGF ligands and their receptors in osteoblastic cells. Using RT-PCR, we were able to detect the expression of EGF, TGF-α, HB-EGF, betacellulin, epiregulin, EGFR, and ErbB2 in both UMR 106-01 and rat calvarial primary osteoblastic cells (data not shown). Furthermore, we investigated whether expression of those ligands and receptors is regulated by PTH. Real time RT-PCR revealed that although the expression of EGFR, ErbB2, EGF, epiregulin, and betacellulin was unaffected by PTH treatment in UMR 106-01 cells, TGF-α and HB-EGF expression showed more than 2-fold increases at 12 h and 1 h of 10^{-8} M rPTH(1–34) treatment, respectively (supplementary figure). Those results are consistent with our previous microarray analysis results in which the expression of TGF-α showed a 2.5-fold increase at 12 h of PTH treatment, and the expression of HB-EGF was not detected under any conditions. Nevertheless, these fold inductions by PTH are much lower than that of AR as shown above (23-fold at 1 h), suggesting that PTH mainly regulates AR but not other EGF-like ligands and receptors in bone.

AR Has Strong Proliferative Effects on Osteoblastic Cells, and the EGFR Signaling Pathway Is Important for Normal Growth of Osteoblastic Cells—AR is a bifunctional growth factor because it stimulates proliferation in some cells and inhibits growth in others (13, 14). Next we investigated the effect of AR on the proliferation of osteoblastic cells. Rat calvarial osteoblastic cells in the proliferation phase were used for all of the following experiments. Those cells are considered as committed preosteoblasts because they do not express differentiation-specific markers. These primary cells were obtained from calvariae of neonatal rats and cultured in medium with various amounts of AR for 4 days. Fig. 4A clearly indicates that the cell number/well increased as the medium AR concentration increased. Even in the presence of the lowest AR concentration (5 ng/ml), a significant increase in cell number (35%) was observed (p < 0.001). [3H]Thymidine incorporation into DNA showed a similar result (Fig. 4B). There was a concentration-dependent increase in DNA synthesis of rat primary osteoblastic cells. Specifically, 50 ng/ml (5 nM) AR produced a 2.7-fold increase in [3H]thymidine incorporation over untreated cells. This effect was slightly higher than 25 ng/ml (4 nM) EGF and comparable with 10% fetal bovine serum.

AR binds to and signals through the EGFR in breast cancer cell lines, fibroblasts, and keratinocytes. The addition of EGFR-specific inhibitors, 1 μM compound 32 (C32) or compound 56 (C56), not only eliminated the proliferative effect of AR on rat primary osteoblastic cells, but decreased incorporation of [3H]thymidine to almost 50% of control cells (Fig. 4C, left part), suggesting that this effect of AR was through the EGFR. Similar to a previous report that PTH inhibits the proliferation of osteoblastic cells (26, 27), 10^{-8} M rPTH(1–34) inhibited DNA synthesis by 50% in primary osteoblastic cells. The addition of C32 or C56 had an additive effect with PTH, further inhibiting [3H]thymidine incorporation to only 25% of control (Fig. 4C, middle part). Moreover, 1 μM C32 or C56 on its own strongly inhibited [3H]thymidine incorporation to about 50% of control cells (Fig. 4C, right part). Note these cells have been serum-depleted for 1 day before addition of inhibitors. This result clearly indicates that EGFR-like ligands, including AR, produced by osteoblastic cells could have autocrine or paracrine effects on normal growth of osteoblastic cells through EGFR signaling pathways. Similar results were also observed with UMR 106-01 cells (data not shown).

Flow cytometry experiments were performed to study the effect of AR on the regulation of the cell cycle of primary osteoblastic cells. As shown in Table I, after serum starvation, 80.6% of cells were in G_{1} phase of the cell cycle. AR treatment decreased cells in G_{1} to about 68.5% and increased cells in both S phase and G_{2}/M phase. Simultaneous addition of C32 completely abolished the effect of AR on cell cycle regulation. However, C32 itself showed no effect on cell cycle regulation under these conditions.

In summary, the above experiments demonstrate that AR has potent stimulatory effects on the proliferation of preosteoblastic cells. This is consistent with previous findings that EGF stimulates the growth of osteoblastic cell lines in vitro (15).
day 20 cultures to analyze the expression of several bone markers. As shown in Fig. 5C, the mRNA levels of MMP-13, alkaline phosphatase, osteocalcin and osteonectin were decreased about 3-, 7-, 50-, and 2-fold, respectively, in AR-treated osteoblastic cells compared with untreated cells. Note that the difference in the starting time of AR treatment (AR_d1 versus AR_d7) had no effect on preventing differentiation.

AR Stimulates Akt Phosphorylation, ERK Phosphorylation, and c-fos and c-jun Expression in Osteoblastic Cells—The phosphorylated EGFR transmits signals through a variety of intracellular substrates, depending partly on the cell type and the bound ligand. To study the intracellular events subsequent to AR-binding osteoblastic cells, we analyzed Akt and ERK phosphorylation, two major signal pathways activated by the AR-bound ligand. To study the intracellular events subsequent to AR treatment (Fig. 6A), we next investigated whether AR could stimulate c-fos and c-jun expression. As shown in Fig. 6C, AR quickly and transiently increased c-fos (30-fold) and c-jun (about 12-fold) mRNA levels with a peak between 15 and 30 min in UMR 106-01 cells. This stimulation was mediated by both MEK and EGFR because the MEK inhibitor PD98059 (lanes 3 and 4) and the EGFR inhibitor C32 (lanes 8 and 12) eliminated ERK phosphorylation by AR.

Because phosphorylated ERK is known to translocate into the nucleus and activate the transcription of c-fos and c-jun (28), we next investigated whether AR could stimulate c-fos and c-jun expression. As shown in Fig. 6D, AR quickly and transiently increased c-fos (30-fold) and c-jun (about 12-fold) mRNA levels with a peak between 15 and 30 min in UMR 106-01 cells. This stimulation required a functional EGFR because the MEK inhibitor PD98059 (lanes 3 and 4) and the EGFR inhibitor C32 (lanes 8 and 12) eliminated ERK phosphorylation by AR.

AR Null Mice Have Less Trabecular Bone Than Wild-type Mice—Mice lacking functional AR reveal a critical role for AR in ductal morphogenesis in the developing mammary gland, but initial experiments showed no growth abnormalities in these mice (23). We harvested tibiae and femurs from 4-week old AR null mice and their wild-type siblings to study their skeletal parameters. MicroCT measurement revealed that AR null mice have significantly less bone in the trabecular bone compartment in the proximal tibia because parameters such as percent bone volume, trabecular number and thickness, and connectivity density are decreased significantly by 26, 17, 8, and 36%, respectively, in the null mice, whereas trabecular separation is increased significantly by 21% (p < 0.05) (Table II). These results strongly suggest that AR plays a critical role
in bone metabolism. Meanwhile, microCT studies with cortical bone in the mid shaft femur revealed that there are no differences in skeletal parameters of this area of bone between AR null and wild-type mice (Table III). There was no significant difference in the body weight between these two groups (data not shown).

**DISCUSSION**

In the present study, we have provided both *in vitro* and *in vivo* evidence demonstrating that AR, an EGF-like ligand, is expressed differentially in osteoblasts after PTH treatment. PTH rapidly stimulated the expression of AR mRNA, reminiscent of a classical immediate early response. In addition, AR expression was induced by other osteotropic agents, such as 1α,25-(OH)2D3 and PGE2. This is the first report suggesting an important role for AR in bone development and metabolism and implicates it as the major EGF-like ligand regulated by osteotropic hormones in bone.

Our investigations with primary osteoblastic cell cultures indicate that AR stimulates preosteoblast proliferation but inhibits its further differentiation. The overall effect could be to expand the pool of preosteoblasts and limit the number of mature osteoblasts. According to our current data, a model is proposed to depict the role of AR in PTH action in bone (Fig. 7). The pluripotent mesenchymal stem cells residing in the bone marrow give rise to osteoblastic precursors in addition to differentiation into chondrocytes, adipocytes, myocytes, and endothelial cells. Within an appropriate environment, the committed preosteoblast further differentiates into the mature osteoblast, a cell that deposits bone matrix proteins and finally becomes the osteocyte embedded in mineralized bone. PTH functions mainly on the mature osteoblast and osteocyte because the preosteoblast has a low response to PTH (Fig. 1C). PTH treatment appears to facilitate the final differentiation of the osteoblast and inhibit its apoptosis, thus increasing its bone formation activities. Meanwhile, PTH-treated osteoblasts produce various cytokines and growth factors influencing its surrounding environment. Because the bone marrow is a very heterogeneous and hence complicated system, the targeted cells could include mesenchymal stem cells, hematopoietic stem cells, various stages of precursors for osteoclasts, osteoblasts, chondrocytes, adipocytes, and myocytes and mature osteoclasts and osteoblasts. A classic example is that PTH-treated osteoblasts increase RANKL expression to trigger osteoclastogenesis, thus stimulating bone resorption (29). A recent interesting finding demonstrates that PTH injection into mice expands the hematopoietic stem cell pool through increasing expression of the Notch ligand Jagged1 on the osteoblast membrane (30). Our microarray studies reveal that more than 10 paracrine or autocrine factors are regulated in osteoblastic cells by PTH treatment (6). Therefore, it seems that PTH has great power to manipulate the microenvironment in bone through its actions on the osteoblast. Some of the manipulations, such as increasing expression of RANKL and Jagged1, may not be directly involved in or may even be contradictory to
FIG. 6. AR activates Akt phosphorylation (A), ERK phosphorylation (B), and c-fos and c-jun expression (C and D) in osteoblastic cells. UMR 106-01 cells (lanes 1–5 in A and lanes 1–4 in B), primary osteoblastic cells at day 6 (lanes 6–9 in A and lanes 5–8 in B) and at day 14 (lanes 10–13 in A and lanes 9–12 in B) were serum starved for 1 day and pretreated with the following for 1 h: dimethyl sulfoxide (0.1% v/v) (lanes 1, 2, 6, 7, 8, 10, 11, and 12 in A and lanes 1, 2, 5, 6, 7, 9, 10, and 11 in B), wortmannin (wort); A, lane 3: 0.03 μM; lane 4: 0.3 μM; lane 5: 3 μM, 1 μM C32 (lanes 9 and 13 in A; lanes 8 and 12 in B), PD98059 (PD, B, lane 3: 50 μM; lane 4: 100 μM). Cells were then treated with the following for 10 min before harvesting cell lysates for Western blot analysis: 50 ng/ml AR (lanes 3, 9, 11, and 13 in A; lanes 2, 3, 4, 6, 8, 10, and 12 in B), 40 ng/ml EGF (lanes 8 and 12 in A; lanes 7 and 11 in B). A, immunoblot analysis using antiphosphorylated Akt antibody (top panel) and anti-Akt antibody (bottom panel). B, immunoblot analysis using antiphosphorylated ERK antibody (top panel) and anti-ERK antibody (bottom panel). C, UMR 106-01 cells were serum starved for 2 days and then treated with 50 ng/ml AR for the indicated time points. The levels of c-fos and c-jun expression were assessed by real time RT-PCR. D, UMR 106-01 cells were pretreated with the following for 1 h: dimethyl sulfoxide (0.1% v/v), 1 μM C32, or 1 μM C9t. Cells were then treated with control or 50 ng/ml AR for 30 min. The levels of c-fos and c-jun expression were assessed by real time RT-PCR.

TABLE II

Structural parameters of trabecular bone in the proximal tibia of 4-week-old AR null mice and wild-type littermates measured by microCT

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type</th>
<th>AR null</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent bone volume (%)</td>
<td>21.44 ± 1.47</td>
<td>15.76 ± 1.19*</td>
</tr>
<tr>
<td>Trabecular number (1/mm)</td>
<td>5.75 ± 0.27</td>
<td>4.77 ± 0.23*</td>
</tr>
<tr>
<td>Trabecular thickness (μm)</td>
<td>44.49 ± 1.23</td>
<td>40.93 ± 1.08*</td>
</tr>
<tr>
<td>Trabecular separation (μm)</td>
<td>178.01 ± 9.82</td>
<td>215.71 ± 10.32*</td>
</tr>
<tr>
<td>Connectivity density (1/mm)</td>
<td>281.68 ± 23.34</td>
<td>179.60 ± 14.02*</td>
</tr>
</tbody>
</table>

TABLE III

Structural parameters of cortical bone in the mid shaft femur of 4-week-old AR null mice and wild-type littermates measured by microCT

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type</th>
<th>AR null</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent bone volume (%)</td>
<td>46.27 ± 0.68</td>
<td>46.01 ± 0.95</td>
</tr>
<tr>
<td>Periosteal perimeter (mm)</td>
<td>7.18 ± 0.21</td>
<td>7.16 ± 0.16</td>
</tr>
<tr>
<td>Cortical thickness (mm)</td>
<td>0.138 ± 0.005</td>
<td>0.131 ± 0.003</td>
</tr>
</tbody>
</table>

PTH anabolic actions. The latter has come to be defined as an increase in bone formation through the possible mechanisms of a stimulation of osteoblast proliferation, differentiation and inhibition of apoptosis. We think that AR is one of those means for PTH to manipulate the microenvironment in bone. Our data suggest its possible role in osteoblastogenesis, increasing preosteoblast proliferation but inhibiting its maturation (Fig. 7). Because bone marrow mesenchymal stem cells express EGFPR (31) and can proliferate and form colonies in a serum-deprived medium as long as EGF is present (32), it is possible that AR has a role in regulating the mesenchymal stem cell pool and therefore influencing several other cell lineages (Fig. 7, dashed arrow).

Our studies also suggest detailed mechanisms about how AR stimulates proliferation and inhibits differentiation. AR treatment of preosteoblastic cells rapidly stimulates Akt and ERK phosphorylation and c-fos and c-jun expression. Because all of those signals lead to cell proliferation, we reason that this is the mechanism for AR to stimulate preosteoblast proliferation and to facilitate the progression of cells from G1 to S and G2 phases. Previously EGF was found to oppose the BMP2 induction of osteogenic differentiation markers (33). Later studies demonstrated that BMP stimulates phosphorylation of Smad1, the mediator for BMP signals, and induces its nuclear accumu-
tion. The phosphorylation of Smad1 by ERK in response to EGF inhibits its nuclear accumulation (34). Because AR stimulates phosphorylation of ERKs in both proliferating and differentiating osteoblastic cells, it may use a similar mechanism involving Smad1 to inhibit differentiation.

AR is a member of the EGF family. It is expressed in many human tissues and acts as an autocrine factor for a variety of cancer cell lines and normal cells. Compared with the EGF peptide, the AR mature peptide has a N-terminal extension, but both peptides have amino acid homology and three-dimensional configuration homology and bind to the same receptor (EGFR), indicating that these two peptides or even other EGF family members share redundant functions in vivo. There are several lines of evidence to date implying that EGF-like ligands and the EGFR signal cascade play important roles in bone metabolism, especially in bone formation. First, our studies showed that all EGF-like ligands and their receptors are present in osteoblastic cells. Second, although AR is the only one that is highly regulated by PTH, TGF-α and betacellulin were also stimulated by PTH about 2-fold. A previous study observed about a 2-fold increase in EGFR mRNA in UMR 106-01 cells after 48 h of PTH treatment (35). Third, previous (15) and current work in our laboratory has demonstrated that EGF, AR, and TGF-α strongly stimulate osteoblastic cell proliferation (data for TGF-α are not shown). AR also inhibited osteoblast differentiation. Fourth, blocking the EGFR by using inhibitors C32 and C56 significantly inhibits basal DNA synthesis of osteoblastic cells. Fifth, EGF/AR/TGF-α triple knock-out mice are growth-retarded, having a 40% reduction in body weight compared with wild-type mice at weaning (36). EGFR null mice are either embryonic lethal or display growth retardation (50–70%) in neonates depending on the genotype background (37–39). Furthermore, mice humanized for EGFR have a low level expression of EGFR in bone and display accelerated osteoblast differentiation and hindered osteoblast proliferation (40). These results suggest an important role of EGF-like ligands and their receptors in bone metabolism.

Because of the multiple members of the EGF family, AR function in bone may be compensated by other members in AR null mice. Consequently, we observed only a mild osteoporosis phenotype in those mice. Our current studies on EGF/AR/TGF-α triple knock-out mice will definitely provide more information about the roles of the EGF family in bone metabolism.

Acknowledgments—We thank Dr. Jude Onyia and Dr. John Vahle for kind advice on the in vivo PTH injection protocols and Dr. Huizhou Fan for discussion of the manuscript. We thank Numan Rashid for technical assistance on cell cycle analysis.

REFERENCES


Downloaded from http://www.jbc.org/ at UQ Library on September 5, 2016
Supplementary table

Table I: Sequences of primers used for RT-PCR.

<table>
<thead>
<tr>
<th>gene</th>
<th>5’ primer</th>
<th>3’ primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR*</td>
<td>TTTGGTGAACGGTGGAGAA</td>
<td>CGAGGATGATGGCAGAGACAA</td>
</tr>
<tr>
<td>β-actin*</td>
<td>TCCTCCTGAGCGCAAGTACTCT</td>
<td>CGGACTCATCGTACTCCTGCTT</td>
</tr>
<tr>
<td>AR</td>
<td>TATTGGCATCATTACCCGG</td>
<td>GCATGTGCACTCCCGTTTTC</td>
</tr>
<tr>
<td>EGFR</td>
<td>ACCAGCAGGACTTTCTTCCCA</td>
<td>TAAAACACTGCTTGGCAGG</td>
</tr>
<tr>
<td>ErbB2</td>
<td>ACCGGGCACAGACATGAAGTTG</td>
<td>AAGTTGCCCTGCACCTCCCTGAC</td>
</tr>
<tr>
<td>EGF</td>
<td>AAAGAGGTGGCATCGGTTGA</td>
<td>AGGCCCTGACACCATGTACTCTCA</td>
</tr>
<tr>
<td>epieregulin</td>
<td>ATGGAAGACGATCCCGGTTG</td>
<td>CGCTCATGTTCCACCAGGTAGAT</td>
</tr>
<tr>
<td>TGF-α</td>
<td>GCCCAGATTCGCCACACTCAGTA</td>
<td>TCACAGCAGAACCACCCACCTGA</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>ATGACCACACTACCTGCTTTGGA</td>
<td>TCCTGCGCTATGGTACTCTGAAC</td>
</tr>
<tr>
<td>betacellulin</td>
<td>TCCTGCATCTGTGAGAAAGGCT</td>
<td>ACAGCACCATGAACGCTATCA</td>
</tr>
<tr>
<td>alkaline phosphatase</td>
<td>ACAATGAGATGCGCCCAGAG</td>
<td>ACATGTACTCCCGGACCA</td>
</tr>
<tr>
<td>MMP-13</td>
<td>GCCCTATCCCTTGATGCCATT</td>
<td>ACAGTTCCAGGCTCAACCTGCTG</td>
</tr>
<tr>
<td>osteonectin</td>
<td>AACATTGCACCACCTGCTTCTTT</td>
<td>TGATGTCCCTGCTTGGATGC</td>
</tr>
<tr>
<td>osteocalcin</td>
<td>CTGCATTCTGTCCCTCTGACCT</td>
<td>GCCGGAGTCTATCCACCTACCTT</td>
</tr>
<tr>
<td>c-fos</td>
<td>CTGCCTTTCTCAATGACCCTG</td>
<td>GCCGGGAAAACGAAAGTCATCAA</td>
</tr>
<tr>
<td>c-jun</td>
<td>AAACGCACCTTCTACACGTGC</td>
<td>CAAGGTATGCTCTGCTCAGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AACCCTACCCATCTCCCAAAGG</td>
<td>GCCTCTCCATGTTGGTGA</td>
</tr>
<tr>
<td>β-actin</td>
<td>TCCTGAGCGCAAGTACTCCTG</td>
<td>CGGACTCATCGTACTCCTGCTT</td>
</tr>
</tbody>
</table>

All primers were designed against rat RNA sequence except that those labeled with * were against mouse sequence.
**Supplementary figure**

**PTH regulation of EGF-like ligands and their receptors in osteoblasts.**
UMR 106-01 cells were treated with control or $10^{-8}$ M PTH(1-34) for 1, 4, 12, 24 h. Messenger RNA levels of EGF-like ligands [epiregulin, TGF-α, HB-EGF, betacellulin (BTC) and EGF] and their receptors (EGFR and ErbB2) were analyzed by real-time RT-PCR.
Amphiregulin Is a Novel Growth Factor Involved in Normal Bone Development and in the Cellular Response to Parathyroid Hormone Stimulation
Ling Qin, Joseph Tamasi, Liza Raggatt, Xin Li, Jean H. M. Feyen, David C. Lee, Emanuel DiCicco-Bloom and Nicola C. Partridge

doi: 10.1074/jbc.M409807200 originally published online October 27, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M409807200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/11/09/M409807200.DC1.html

This article cites 40 references, 12 of which can be accessed free at
http://www.jbc.org/content/280/5/3974.full.html#ref-list-1