Transforming Growth Factor-β Stimulates the Production of Osteoprotegerin/Osteoclastogenesis Inhibitory Factor by Bone Marrow Stromal Cells*

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Osteoprotegerin (OPG)/osteoclastogenesis inhibitory factor (OCIF) is a recently identified cytokine that belongs to the tumor necrosis factor receptor superfamily and regulates bone mass by inhibiting osteoclastic bone resorption. The present study was undertaken to determine whether OPG/OCIF is produced in bone microenvironment and how the expression is regulated. A transcript for OPG/OCIF at 3.1 kilobases was detected in bone marrow stromal cells (ST2 and MC3T3-G2/PaA6) as well as in osteoblastic cells (MC3T3-E1). Transforming growth factor-β1 (TGF-β1) markedly increased the steady-state level of OPG/OCIF mRNA in a dose-dependent manner, while TGF-β1 suppressed the mRNA expression of tumor necrosis factor-related activation-induced cytokine (TRANCE)/receptor activator of NF-κB ligand (RANKL), a positive regulator of osteoclastogenesis to which OPG/OCIF binds.

The effect of TGF-β1 on the expression of OPG/OCIF mRNA was transient, with a peak level at 3–6 h. The up-regulation of OPG/OCIF mRNA by TGF-β1 in ST2 cells did not require de novo protein synthesis and involved both a transcriptional and a post-transcriptional mechanism. Western blot analysis and an enzyme-linked immunosorbent assay revealed that TGF-β1 significantly increased the secretion of OPG/OCIF protein by ST2 cells at 6–24 h. In murine bone marrow cultures, TGF-β1 markedly inhibited the formation of tartrate-resistant acid phosphatase-positive multinucleated osteoclast-like cells in the presence of 1,25-dihydroxyvitamin D3, whose effect was significantly reversed by a neutralizing antibody against OPG/OCIF.

These results suggest that TGF-β1 negatively regulates osteoclastogenesis, at least in part, through the induction of OPG/OCIF by bone marrow stromal cells and that the balance between OPG/OCIF and TRANCE/RANKL in local environment may be an important determinant of osteoclastic bone resorption.

Osteoclasts are multinucleated, giant cells that are primarily responsible for bone resorption (1, 2). In normal bone remodeling cycles, osteoclastic bone resorption is followed by osteoblastic bone formation to continuously replace old bone with new bone. These two processes are temporally and spatially coordinated to maintain the skeletal integrity, and excessive bone resorption, typically seen in association with estrogen deficiency after menopause, causes osteoporosis (3, 4).

It is generally accepted that osteoclasts are hematopoietic in origin and derived from cells of monocyte-macrophage lineage (1, 2). In addition, accumulating evidence suggests an important role of the bone marrow microenvironment, especially bone marrow stromal cells, in the regulation of osteoclast formation and bone resorption by mature osteoclasts (1, 2). Recent gene knockout experiments and genetic analysis of osteopetrotic animals have disclosed the involvement of various molecules in the formation and the function of osteoclasts, including PU1 (5), macrophage-colony stimulating factor (6), c-Fos (7), NF-κB (8, 9), c-Src (10), and cathepsin-K (11). However, complex, multistep processes of osteoclastogenesis and cross-talks between osteoclast progenitors and bone marrow microenvironment have not been fully understood (1, 2).

Simonet et al. (12) have recently identified a novel member of the tumor necrosis factor (TNF)1 receptor superfamily, termed osteoprotegerin (OPG), that regulates bone mass through an inhibitory effect on osteoclastogenesis. Independently, we have purified from the conditioned medium of human embryonic lung fibroblasts (IMR-90) a cytokine, termed osteoclastogenesis inhibitory factor (OCIF), which inhibits the formation of osteoclasts in vitro (13). Molecular cloning of human OCIF revealed that OCIF and OPG are identical (14). Although it is evident that OPG/OCIF is expressed in a wide variety of tissues and inhibits bone resorption in vivo as well as in vitro (12–14), it remains unclear which cell types in the bone marrow microenvironment produce OPG/OCIF, how the expression is regulated, and how it acts in the process of osteoclastogenesis. In order to approach these important issues, we have now studied the expression of the OPG/OCIF gene and its regulation in several bone- and bone marrow-derived cell lines. The results indicate that the expression of the OPG/OCIF gene is markedly induced by transforming growth factor-β1 (TGF-β1) in osteoclastogenesis-supporting stromal cells, through both a transcriptional and a post-transcriptional mechanism, and that

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§ The abbreviations used are: TNF, tumor necrosis factor; OPG, osteoprotegerin; OCIF, osteoclastogenesis inhibitory factor; TGF-β1, transforming growth factor-β1; 1,25(OH)2D3, 1α,25-dihydroxyvitamin D3; CHX, cycloheximide; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazol; PPAR, peroxisome proliferator activated receptor; TRANCE, tumor necrosis factor-related activation-induced cytokine; RANKL, receptor activator of NF-κB ligand; ELISA, enzyme-linked immunosorbent assay.

‡ We therefore refer to this cytokine as OPG/OCIF in this paper.
locally produced OPG/OCIF plays a functional role in the negative feedback regulation of osteoclastic bone resorption.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human TGF-β1 was purchased from Boehringer Mannheim. Other reagents were obtained from Sigma. 1α,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) was dissolved in 99.5% ethanol, and stock solutions of cycloheximide (CHX) and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazol (DRB) were prepared in dimethyl sulfoxide.

**Cell Culture**—Mouse bone marrow stromal cell line ST2 (15) was obtained from Riken Cell Bank (Hatsakazi, Japan) and cultured in RPMI 1640 supplemented with 10% fetal calf serum. Cells were propagated in a humidified incubator at 37°C in 5% CO₂.

**Expression Analysis by Northern Blotting**—Total cellular RNA was purified by the acid guaniadinium phenol chloroform method (18), and Northern blot analysis was performed as described previously (19). First strand cDNA was synthesized with total RNA from MC3T3-E1 cells using Molony murine leukemia virus reverse transcriptase (Stratagene, La Jolla, CA), and cDNA was amplified by polymerase chain reaction (PCR) with oligonucleotide primers designed on the basis of the reported mouse OPG/OCIF cDNA sequence (12): sense primer (5′-CTGGCAGCATCTGGAATGAG-3′) and antisense primer (5′-AATTAGCAGGCCCAAATG-3′). Mouse tumor necrosis factor-related activation-induced cytokine (TRANCE) receptor was amplified by PCR with sense (5′-GACTCGACTTGGGACTGAGT-3′) and antisense (5′-GAGAACTTGTTGATTGTAGCC-3′) primers, which were designed on the basis of the reported cDNA sequence (20, 21). Mouse osteoclast receptor activator of NF-κB (RANKL) cDNA was amplified by PCR with sense (5′-CAGGACAGCAGGACATC-3′) and antisense (5′-TTGGGTTGCTCCTTTGCGT-3′) primers. EF1α mRNA served as an internal control in Northern blot analysis to verify the integrity and quantity of the applied RNA (22).

**Enzyme-linked Immunosorbent Assay (ELISA) for OPG/OCIF**—Aliquots of conditioned medium from MC3T3-E1 and ST2 cells were centrifuged at 5,000 g for 5 min to remove cell debris. The resulting supernatant was concentrated 20-fold with Centricon-50 (Amicon, Beverly, MA). Samples were electrophoresed on 7.5% SDS-polyacrylamide gels under a nonreducing condition and transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) by the random primer technique (Multiprime DNA labeling systems, Amersham Pharmacia Biotech). OPG/OCIF protein was visualized using the horseradish peroxidase-conjugated rabbit anti-OPG/OCIF polyclonal antibody (R&D Systems, Minneapolis, MN) and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, England) by exposure to autoradiography film. A regulated mouse OPG/OCIF cDNA sequence (12) was subcloned into pCR-script Amp(+) (Stratagene), and cDNA probe was labeled with [32P]dCTP (Amersham Pharmacia Biotech, Buckinghamshire, England) by the random primer technique (Multiprime DNA labeling systems, Amersham Pharmacia Biotech).

**RESULTS AND DISCUSSION**

TGF-β1 Induces the Expression of OPG/OCIF mRNA in Bone Marrow Stromal Cells—It has been demonstrated that OPG/OCIF mRNA is expressed in a number of normal human tissues, including lung, kidney, placenta, and heart, and that systemic administration of recombinant OPG/OCIF increases bone density and protects against bone loss due to estrogen deficiency in mice (12). However, it remains to be clarified whether OPG/OCIF is produced in bone cells, how its expression is regulated by systemic calciotropic hormones as well as local cytokines, and whether OPG/OCIF produced in bone microenvironment plays any significant role in normal bone remodeling and in pathological conditions, such as osteoporosis. In order to address these issues, we set out to examine the expression of OPG/OCIF in osteoblastic cells as well as bone marrow stromal cells that play an important role in supporting the differentiation and maturation of bone-resorbing osteoclasts (25).

As shown in Fig. 1, OPG/OCIF mRNA was constitutively expressed in osteoblastic MC3T3-E1 cells and bone marrow stromal cell lines, ST2 and PA6. Parathyroid hormone (100 nM) and 1,25(OH)₂D₃ (10 nM), calcium-regulating hormones that stimulate osteoclastic bone resorption, caused a modest but reproducible decrease in OPG/OCIF mRNA levels in these cells (data not shown). In contrast, TGF-β1, an important local regulator of bone metabolism (26), caused a marked and transient induction of OPG/OCIF mRNA in ST2, MC3T3-E1, and PA6 cells (Fig. 1). TGF-β1 also induced the expression of OPG/OCIF mRNA in bone marrow stromal cells freshly isolated from mouse long bones (data not shown).

We next examined a time course and a dose response of the effects of TGF-β1 on the expression of OPG/OCIF mRNA in ST2 cells in more detail. An increase in OPG/OCIF mRNA was apparent as early as 1.5 h after the addition of TGF-β1, and the steady-state mRNA level peaked between 3 and 6 h and then returned to a base-line level by 48 h (Fig. 2). As shown in Fig. 3A, the effect of TGF-β1 on OPG/OCIF mRNA was dose-dependent. A clear cut increase in OPG/OCIF mRNA was observed at 0.2 ng/ml, and the maximal response was obtained at 2 ng/ml of TGF-β1 (Fig. 3B).

Recently, a molecule that is induced on the surface of bone marrow stromal cells by bone-resorbing hormones and supports the formation of osteoclasts has been identified as TRANCE/RANKL (20, 21). OPG/OCIF binds to TRANCE/ RANKL and inhibits osteoclastogenesis by interfering with the interaction between TRANCE/RANKL and its receptor molecule on osteoclast precursors, possibly receptor activator of NF-κB (RANKL) (27). We examined whether TGF-β1 modulates the expression of TRANCE/RANKL as well as OPG/OCIF in...
bone marrow stromal cells. As shown in Fig. 3A, TGF-β1 suppressed the expression of TRANCE/RANKL mRNA in a dose-dependent manner in ST2 cells, which was exactly the mirror image of the effect on OPG/OCIF mRNA.

Mechanism of Induction of OPG/OCIF mRNA by TGF-β1—In order to obtain some insights into the mechanism by which TGF-β1 up-regulates OPG/OCIF mRNA, we examined the effect of CHX, a protein synthesis inhibitor. As shown in Fig. 4A, the simultaneous addition of CHX (10 ng/ml) to ST2 cells did not affect the increase in OPG/OCIF mRNA in response to TGF-β1, suggesting that new protein synthesis is not required for the up-regulation of OPG/OCIF mRNA by TGF-β1. We also examined the effect of DRB, an inhibitor of RNA polymerase II, on the TGF-β1-induced accumulation of OPG/OCIF mRNA. The addition of DRB markedly attenuated the increase in OPG/OCIF mRNA level by TGF-β1, suggesting that the up-regulation occurred, at least in part, at a transcriptional level (Fig. 4B).

To examine if TGF-β1 also affects the stability of OPG/OCIF mRNA, ST2 cells were first treated with TGF-β1 for 2 h, after which DRB was added to assess the disappearance rate of OPG/OCIF mRNA. In the absence of TGF-β1, the level of OPG/OCIF mRNA decreased rapidly after RNA synthesis was inhibited by DRB, with an apparent half-life of approximately 3–4 h (Fig. 5A and B). In contrast, pretreatment with TGF-β1 substantially stabilized OPG/OCIF mRNA, suggesting that a post-transcriptional mechanism is also involved (Fig. 5A and B). The rapid turnover of OPG/OCIF mRNA may be attribut-
able to the presence in the 3′-untranslated region of the “AUUUA” instability motif (28), and a post-transcriptional mechanism involving the stability of the transcript may represent an important point in the regulation of OPG/OCIF gene expression.

**TGF-β1 Stimulates the Secretion of OPG/OCIF Protein by ST2 Cells—**OPG/OCIF is a secreted protein and has been shown to exist as both a 60-kDa monomer and a 120-kDa disulfide-linked homodimeric form in the conditioned medium of human fibroblasts (13, 14). To examine if TGF-β1 actually induces the production and secretion of OPG/OCIF protein by ST2 cells, the conditioned medium was collected at various times after stimulation with TGF-β1, and the amount of OPG/OCIF protein was determined by Western blot analysis and ELISA, using a specific antibody raised against murine OPG/OCIF. The results of Western blot analysis revealed that OPG/OCIF protein was constitutively secreted by ST2 cells and that the amount in the culture medium was markedly increased 6–24 h after TGF-β1 treatment (Fig. 6A). Mature protein of OPG/OCIF consists of 380 amino acids and was detected in the conditioned medium of ST2 cells mainly as a 100-kDa protein (Fig. 6A), which presumably represents a dimeric form. It is likely that the minor difference in the molecular mass between human (120 kDa) and murine (100 kDa) OPG/OCIF molecules is due to a difference in the number of potential N-linked glycosylation sites (four in human and three in murine OPG/OCIF) (12).

The ELISA system we constructed also detected low levels (0.1–0.2 ng/ml) of immunoreactive OPG/OCIF in the conditioned medium of ST2 cells. As shown in Fig. 6B, again, TGF-β1 caused a marked and transient increase in the concentration of OPG/OCIF. The amount of OPG/OCIF reached a maximal level at 12 h (nearly 2.5 ng/ml) and returned to a base-line level at 48 h, suggesting that the protein is also quite labile. These results are in good agreement with those of RNA analysis in both the kinetics and the magnitude of the effect and indicate that the production and secretion of OPG/OCIF protein is transiently induced by TGF-β1.

**Role of OPG/OCIF in the Inhibition of Osteoclastogenesis by TGF-β1**—It is widely recognized that TGF-β plays a pivotal role in the local regulation of bone metabolism (26). TGF-β, produced by osteoblasts and abundantly stored in the bone matrix, is released during bone resorption and is activated by the acidic microenvironment created by the vacuolar type proton pump of osteoclasts (26). TGF-β stimulates the synthesis and secretion of matrix proteins, inhibits matrix mineralization, and modulates the proliferation and differentiation of osteoblasts. In vivo, local injection of TGF-β has been shown to
stimulate bone formation (29, 30). Thus, it is thought that TGF-\(\beta\) is involved in the coupling between bone resorption and formation. However, conflicting results have been reported concerning the effects of TGF-\(\beta\) on bone resorption (31–36). TGF-\(\beta\) was initially shown to stimulate bone resorption in mouse calvaria organ cultures (31), while the cytokine inhibited bone resorption in long term human marrow cultures (32) and in fetal rat long bone assays (33). In addition, biphasic effects of TGF-\(\beta\) on the formation of osteoclast-like cells have been reported in mouse bone marrow cultures (34, 35), with stimulatory and inhibitory responses being observed at low (10–100 pg/ml) and high (4 ng/ml) concentrations, respectively (34).

In agreement with the reported observations (34–36), TGF-\(\beta\) at 2 ng/ml caused a marked suppression of the formation of osteoclast-like cells in the murine bone marrow culture system (Fig. 7). We then attempted to determine whether OPG/OCIF produced by bone marrow stromal cells in response to TGF-\(\beta\) plays a role in the regulation of osteoclastic bone resorption, utilizing a neutralizing antibody against OPG/OCIF. As expected (12–14), recombinant OPG/OCIF at 30 ng/ml markedly reduced the number of tartrate-resistant acid phosphatase-positive multinucleated osteoclasts formed in the presence of 1.25(OH)\(_2\)D\(_3\), and the neutralizing polyclonal antibody against OPG/OCIF (5 \(\mu\)g/ml) blocked the biological effect of OPG/OCIF (Fig. 7). In addition, the blocking antibody significantly reversed the inhibitory effect of TGF-\(\beta\) on osteoclastogenesis in a dose-related fashion (Fig. 7), suggesting that TGF-\(\beta\) inhibits osteoclastogenesis, at least in part, through the induction of OPG/OCIF in bone marrow stromal cells. The reported observations that high concentrations (4 ng/ml) of TGF-\(\beta\) inhibited the formation of osteoclasts in the same culture system (34–36) may be consistent with our results that approximately 2 ng/ml TGF-\(\beta\) was sufficient to inhibit osteoclastogenesis (Fig. 7). On the other hand, the fact that anti-OPG/OCIF antibody did not completely reverse the suppressive effect of TGF-\(\beta\) on osteoclastogenesis suggests the involvement of other mechanisms than the induction of OPG/OCIF as well. Possible mechanisms include 1) the suppression of positive regulators of osteoclastogenesis by TGF-\(\beta\), such as TRANCE/RANKL, shown in the current study (Fig. 3); 2) a direct inhibitory effect of TGF-\(\beta\) on osteoclast precursors; and 3) the effects of TGF-\(\beta\) on the growth and differentiation of osteoclastogenesis-supporting stromal cells. Further studies are required to address these points.

It has been reported that administration of recombinant OPG/OCIF to normal mice or rats increases bone mineral density and produces a pattern of cartilage retention within the bone trabeculae, suggesting that OPG/OCIF acts as an antiresorptive humoral factor (12, 14). In addition, treatment of ovariectomized rats with OPG/OCIF resulted in an increase in bone volume, suggesting that OPG/OCIF is capable of protecting against excessive bone resorption associated with estrogen deficiency (12). Systemic overproduction of OPG/OCIF in the liver of transgenic mice caused severe osteoporosis, and histological examination of such mice revealed that the number of osteoclasts, but not of osteoclast precursors, was markedly reduced, suggesting a defect in later stages of osteoclast differentiation (12). These findings implicate OPG/OCIF as a secreted protein that regulates bone density through negative regulation of osteoclastogenesis.

According to the results of our clinical study,\(^\text{3}\) the serum concentration of OPG/OCIF seems to increase with age in both healthy men and women, and it is significantly higher in postmenopausal women with osteoporosis than in age-matched control subjects. Thus, circulating OPG/OCIF concentrations may reflect age-related changes in bone metabolism, and the increased serum levels in osteoporotic women may represent a physiological response to the excessive bone resorption. Most importantly, these observations are consistent with the concept that OPG/OCIF actively participates in the regulation of bone remodeling under physiological as well as pathological conditions.

In conclusion, the results of the present study indicate that TGF-\(\beta\) induces the expression of the OPG/OCIF gene in bone marrow stromal cells, through a transcriptional as well as a post-transcriptional mechanism, and that OPG/OCIF secreted into the local environment in response to TGF-\(\beta\) mediates the inhibition of osteoclastogenesis, at least partially. Based on these results, we propose a negative feedback model for the local regulation of bone remodeling by TGF-\(\beta\) and OPG/OCIF (Fig. 8). During bone resorption, the acidic microenvironment generated by osteoclasts releases and activates matrix-bound TGF-\(\beta\), which in turn induces the production and secretion of OPG/OCIF by stromal cells while suppressing the expression of TRANCE/RANKL. OPG/OCIF is a potent cytokine capable of inhibiting the formation of osteoclasts through disruption of the functional interaction between TRANCE/RANKL on osteoclast precursors (20). Thus, the balance between OPG/OCIF and TRANCE/RANKL may represent an important determinant of bone resorption. Further studies are required to elucidate the molecular mechanisms by which TGF-\(\beta\) regulates the expression of OPG/OCIF and TRANCE/RANKL genes (37) and the precise mechanism of action of OPG/OCIF and TRANCE/RANKL in the complicated network of various cell types in the bone microenvironment.

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