Transforming Growth Factor-β Induces Expression of Receptor Activator of NF-κB Ligand in Vascular Endothelial Cells Derived from Bone*

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Vascular endothelial cells in bone are thought to have significant roles on pathological bone resorption such as bone metastasis and hypercalcemia because this resorption is often seen where blood vessels are abundant. However, the detailed mechanisms have not yet been elucidated. Here, we focused on transforming growth factor-β (TGF-β) and studied its effects on vascular endothelial cells because TGF-β is abundantly stored in bone matrix and is released and activated during bone resorption. We found that TGF-β up-regulated the expression of receptor activator of NF-κB ligand (RANKL) mRNA and protein in bone marrow-derived endothelial cells and in primary vascular endothelial cells but not in osteoblasts. Further analysis revealed that TGF-β promoted phosphorylation of cAMP response element-binding protein and p38. Protein kinase A inhibitor KT5720 and p38 inhibitor SB203580 significantly reduced the TGF-β-induced RANKL expression. Moreover, we found two CRE-like domains in murine RANKL promoter region that were critical for TGF-β-dependent RANKL expression. Therefore, protein kinase A and p38 signaling pathways are involved in TGF-β-induced RANKL expression by stimulating transcription factors that bind to the CRE-like domains. Our findings indicate that TGF-β stimulates osteoclastogenesis by promoting RANKL expression in endothelial cells under pathological conditions.

The interactions of cells within the bone microenvironment play important roles in bone remodeling. Osteoblasts are involved in the bone remodeling through the production of soluble factors that regulate proliferation and differentiation of osteoclasts and by means of cell-cell interaction (1). Many soluble osteotropic factors have been identified; however, it is not clear what kinds of adhesion molecule(s) are involved in the interaction between osteoblasts and osteoclasts. Recently, the receptor activator of NF-κB ligand (RANKL),1 which is also known as the osteoclast differentiation factor, was cloned (2, 3). RANKL is essential for full osteoclastic differentiation of hematopoietic precursor cells into mature multinucleated bone-resorptive osteoclasts in the presence of macrophage colony-stimulating factor (4, 5). It is highly expressed on the surface of osteoblasts or bone marrow stromal cells in the areas of trabecular bone remodeling and of excessive osteolysis (6). Its expression is up-regulated in marrow stromal cells or osteoblasts by osteotropic hormones or cytokines such as parathyroid hormone, 1,25-dihydroxyvitamin D3, interleukin (IL)-11, and prostaglandin E2 (PGE2). RANKL interacts with a cell surface receptor, RANK, present at a certain stage of osteoclasts differentiation and on mature osteoclasts to stimulate their fusion, development, and bone resorption (7). Osteoprotegerin (OPG), which is also known as osteoclastogenesis inhibitory factor, is a soluble member of the tumor necrosis factor receptor superfamily and is widely expressed in multiple tissues and binds to RANKL, thereby neutralizing its function. Therefore, OPG acts as a secreted decoy receptor to negatively regulate osteoclast differentiation, activity, and survival both in vivo and in vitro (8).

It is also remarkable that bone has a substantial amount of blood vessels, and histological studies have shown that vascular endothelial cells might have an impact on osteolytic bone disease (9). For instance, most metastatic foci in bone occur near the epiphyseal plate where microvasculature is abundant (10). Moreover, osteolytic bone diseases are often accompanied with excessive angiogenesis (11, 12). However, no reasonable explanation for the role of vascular endothelial cells on pathological bone diseases has yet been found. We previously established bone-derived endothelial cells (BDECs) to study their roles in bone disease (13). We found that they play an important role in bone metabolism by producing IL-11 and PGE2 (13, 14). These facts indicate that endothelial cells in bone play an important role in osteoclastogenesis.

Bone is unique because its matrix serves as a reservoir of growth factors. Transforming growth factor-β (TGF-β) is one of the most abundantly stored in bone matrix (15). Because TGF-β is released and activated during the breakdown of the bone matrix in pathological conditions, the released TGF-β might affect the behavior of cells within the bone microenvironment. It has been reported that released TGF-β stimulates tumor growth and further bone breakdown by stimulating cancer cell production of parathyroid hormone-related protein (16).

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1 The abbreviations used are: RANKL, receptor activator of NF-κB ligand; BDECs, bone marrow-derived endothelial cells; CRE, cyclic AMP response element; CREE, CRE-binding protein; HUVECs, human umbilical vein endothelial cells; MAPF, mitogen-activated protein kinase; PGE2, prostaglandin E2; PKA, protein kinase A; TGF-β, transforming growth factor-β; OPG, osteoprotegerin; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; DMEM, Dulbecco’s modified Eagle’s medium; EBM, essential basal medium; WT, wild-type; DN, dominant-negative; RT, reverse transcription; COX, cyclooxygenase.

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TGF-β-mediated RANKL Expression in Endothelial Cells

| Primer          | Cycle | Nucleotides | Annealing temperature |
|-----------------|-------|-------------|-----------------------|
| Mouse RANKL     | 30    | 434–1034 (601 bp) | 67°C                  |
| Mouse G3PDH     | 23    | 51–1033 (983 bp) | 65°C                  |
| Mouse EP4       | 23    | 715–1556 (842 bp) | 65°C                  |
| Human RANKL     | 30    | 533–1086 (556 bp) | 65°C                  |
| Human OPG       | 30    | 55–483 (409 bp) | 65°C                  |
| Human β-actin   | 23    | 468–1128 (661 bp) | 68°C                  |

Therefore, it is possible that released active TGF-β modifies the nature of endothelial cells in bone to promote osteoclastogenesis.

In this report, we examined the effects of TGF-β on endothelial cell function. We found that TGF-β stimulated the expression of RANKL both in our previously established BDECs and in human umbilical vein endothelial cells (HUVECs), although TGF-β down-regulated its expression in bone marrow stroma ST2 cells. Further analysis revealed that p38 mitogen-activated protein kinase (MAPK) and protein kinase A (PKA) signaling pathways were involved in the TGF-β-induced RANKL expression. We also discovered that mouse RANKL promoter contains two cyclic AMP response element (CRE)-like domains in the TGF-β-induced RANKL expression. Therefore, endothelial cells might be involved in osteoclastogenesis via RANKL expression during the pathological bone resorption.

**Experimental Procedures**

**Reagents**—TGF-β, was obtained from Roche Molecular Biochemicals. Vitamin D3 was purchased from Sigma. COX-2 inhibitor NS-398 was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). PKA inhibitor KT5720 and p38 MAPK inhibitor SB203580 were obtained from Calbiochem (La Jolla, CA). MEK1 inhibitor PD98059 was obtained from New England Biolabs (Cambridge, MA).

**Cell Culture Conditions**—The mouse bone marrow-derived endothelial cell line BM-3 was established in our laboratory as described previously (15). BM-3 cells were cultured on gelatin-coated dishes (Iwaki, Tokyo, Japan) and fed with Dulbecco’s modified Eagle’s medium (DMEM) (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 2 mM L-glutamine, 100 μg/ml kanamycin (Meiji Seika Co., Ltd., Tokyo, Japan), and 10% heat-inactivated fetal bovine serum (Invitrogen) (DMEM growth medium) at 37°C in a humidified atmosphere of 5% CO2, 95% air. Mouse bone marrow stroma ST2 cells were obtained from Riken Cell Bank (Ibaraki, Japan) and cultured in RPMI 1640 medium (Nissui Pharmaceutical Co.) supplemented with 2 mM L-glutamine, 100 μg/ml kanamycin, and 10% fetal bovine serum (Invitrogen) (RPMI growth medium). HUVECs, culture medium (essential basal medium (EBM)), and medium supplements (packaged as Single-Quots containing human recombinant epidermal growth factor, hydrocortisone, gentamicin, bovine brain extract, and fetal bovine serum) were purchased from Sanko Junyaku (Tokyo, Japan).

**RNA Extraction, cDNA Synthesis, and PCR**—Total cellular RNA was purified by the acid-guanidium-phenol-chloroform method; first strand cDNA was synthesized with the 1.0 μg of the total RNA using Moloney murine leukemia virus reverse transcriptase; and 1.5 μg of the cDNA was amplified by PCR in the conditions as shown in Table I. Oligonucleotides were designed to amplify and detect human RANKL (GenBank™ accession number AF190947), murine RANKL (GenBank™ accession number AF019048), human OPG (GenBank™ accession number U94322), human β-actin (GenBank™ accession number NM001101), murine G3PDH (GenBank™ accession number M22999), and murine EP4 (GenBank™ accession number NM008985).

**Western Blot Analysis**—Western blot analysis was performed as described previously (17). Briefly, the cells were harvested and solubilized with lysis buffer containing Nonidet P-40 and 0.2% SDS. The cell lysates (25 μg/lane) were applied to a 4–20% gradient polyacrylamide gel. The electrophoresed proteins were transblotted onto a nitrocellulose membrane. After blocking, the membranes were incubated with antibodies to COX-1 or COX-2 (Cayman Chemical Co., Ann Arbor, MI); antibodies to CREB, phospho-CREB (Ser133), p38 MAPK, phospho-p38 MAPK (Thr180/Thr182), ATP-2, or phospho-ATF-2 (Thr239/Thr242) (Cell Signaling Technology, Beverly, MA); antibodies to mouse RANKL or human RANKL (R&D Systems). MIA an antibody to β-actin (Sigma); or an antibody to active-MAPK/ERK (pTEpY; Promega, Madison, WI). The membranes were then incubated with appropriate peroxidase-conjugated secondary antibodies and developed with enhanced chemiluminescence mixture (Amersham Biosciences). Western blot analysis of RANKL was performed according to the method described previously (18).

**Measurement of the PGE2 Concentration**—BM-3 cells were plated onto six-well gelatin-coated dishes and grown to confluence in DMEM. The cells were washed twice with phosphate-buffered saline and incubated in serum-free DMEM for 24 h, and then TGF-β was added to the culture. The conditioned medium was filtrated with 0.22-μm filters and stored at −80°C until use. The concentration of PGE2 in each sample was determined by using an enzyme immunoassay according to the manufacturer’s instructions (Cayman Chemical Co.).

**Promoter Assay**—BM-3 cells were seeded onto six-well gelatin-coated dishes and grown to 50–80% confluence in DMEM growth medium. ST2 cells were seeded at the same density onto six-well dishes and grown in RPMI 1640 growth medium. After incubation for 18–24 h, 1 μg of pGL3-basic vector containing pGL3–1005 and 1 μg of pGL3–300 containing CRE reporter sequences were added into cells using 3 μg of Fugene6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. After incubation for 4 h, the medium was replaced with serum-free medium. After incubation for an additional 12 h, the cells were treated with TGF-β. Luciferase activity in the lysate was measured using a luciferase assay system as per the manufacturer’s instructions (Promega). Luciferase activity in the lysate was measured using MicroLumat LB96P luminometer (Berthold, Bad Wildbad, Germany). The activities were normalized by measuring protein concentrations in each sample with a BCA protein assay kit (Pierce) or by co-transfection of control thymidine kinase-driven Renilla luciferase plasmid pRL-TK (Promega).

**Transient Transfection Assay**—BM-3 cells that had been seeded at 1.5 × 105 were transfected with appropriate plasmids using Fugene6 (Roche Molecular Biochemicals). We typically transfected at least 50% of cells as judged using control pcDNA3 vector expressing enhanced green fluorescent protein under fluorescence microscopy. The wild type murine CREB and dominant-negative human CREB cDNA (DN CREB133) in a pCMV vector were purchased from CLONTECH (Palo Alto, CA). The p38 MAPK cDNA was generated by RT-PCR using mRNA isolated from C7 cells as the template. The sense and antisense primers for murine p38 MAPK were 5'-AGAGGGGGCAAGAGGAGGCAAGG-3' and 5'-CTAGGCTCAGTCTTCTGTGTCAGAGGTTG-3', respectively. The PCR products were cloned into a pBluescriptII KS+ vector (Invitrogen) and Sma I restriction fragments of the internal EcoRI site in p38 MAPK cDNA were accomplished by converting GAAATTG to GATCT by PCR mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). After restriction with EcoRI, the EcoRI-EcoRI fragment cDNA was subcloned into an EcoRI site of the pcDNA3 vector in which the sequence between the BamHI and EcoRI sites had been replaced with a FLAG epitope containing the translation initiation codon ATG at the 5’ end (pcFLAG vector) (20).

**Electrophoretic Mobility Shift Assay**—Nuclear extracts of BM-3 cells were prepared with the NE-PER nuclear extraction reagent (Pierce). Biotin end-labeled double-stranded oligonucleotides 5'-biotin-TGGATT-TGAGGCTACAGCTCTGG-3' (−945 to −926) and 5'-biotin-CCTACAGTCATGTTGG-3', respectively. The PCR products were cloned into a pBluescriptII KS+ vector (Invitrogen) and Sma I restriction fragments of the internal EcoRI site in p38 MAPK cDNA were accomplished by converting GAAATTG to GATCT by PCR mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). After restriction with EcoRI, the EcoRI-EcoRI fragment cDNA was subcloned into an EcoRI site of the pcDNA3 vector in which the sequence between the BamHI and EcoRI sites had been replaced with a FLAG epitope containing the translation initiation codon ATG at the 5’ end (pcFLAG vector) (20).

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DMEM for 24 h. The cells were serum-starved for an additional 24 h. Then the cells were cultured in serum-free DMEM containing the indicated concentrations of TGF-β for 3 h (A) or in serum-free DMEM containing 10 ng/ml TGF-β for the indicated time periods (B). Total RNA (1 μg) of each sample was extracted, and RT-PCR was carried out using murine RANKL or murine G3PDH primer sets. The amplified products of murine RANKL (601 bp) or murine G3PDH (983 bp) were electrophoresed in a 1% agarose gel plate stained with ethidium bromide and visualized by UV illumination. Repeated experiments gave similar results. C, BM-3 cells were seeded at a density of 1.0 × 10^5 cells/60-mm dish and cultured in DMEM for 48 h. The cells were serum-starved for an additional 24 h. Then the cells were cultured for the indicated time periods in serum-free DMEM medium containing 10 ng/ml TGF-β. The cells were scraped off and lysed in lysis buffer. The cell lysates were electrophoresed and immunoblotted with an anti-mouse RANKL antibody or an anti-β-actin antibody. ODF, osteoclast differentiation factor.

RESULTS

TGF-β Induces RANKL mRNA and Protein Expression in Endothelial Cells—Some osteolytic bone diseases are known to be associated with excessive angiogenesis in bone (11, 12). Hence, it is possible that bone endothelial cells participate in the regulation of bone remodeling as osteoblasts. After establishing BDECs, we found that they promoted bone resorption in vitro by secreting IL-11 (13) or PGE2 (14) in response to several osteotropic factors. Recently, RANKL was cloned from stromal and osteoblasts and has been reported to be essential for osteoclastogenesis (3). We thus examined the expression of RANKL in BDECs. Because TGF-β is stored in bone matrix and is released and activated during the osteoclast-mediated bone resorption (15), we examined the expression of RANKL mRNA in BDECs after treatment with TGF-β. As shown in Fig. 1A, TGF-β induced RANKL mRNA expression in a dose-dependent manner in bone-derived endothelial BM-3 cells. Time course analysis revealed that RANKL mRNA expression reached the peak at 3 h (Fig. 1B). Then the amount of RANKL mRNA declined slightly in a time-dependent manner. In contrast, the expression level of G3PDH mRNA was not changed by TGF-β treatment (Fig. 1, A and B). To confirm the results, we performed Western blot analysis using an anti-RANKL antibody. BM-3 cells were treated with 10 ng/ml TGF-β and then harvested and subjected to SDS-PAGE. Consistent with the previous results, TGF-β induced RANKL protein expression in a time-dependent manner in BM-3 cells (Fig. 1C). Under this condition, β-actin protein expression was not changed by TGF-β treatment.

BM-3 cells were established by transformation with SV40 virus (13). To exclude the possibility that the established BM-3 cells might respond differently than endothelial cells in vivo, we investigated the effect of TGF-β on RANKL mRNA expression in HUVECs. Consistent with the established BM-3 cell line, TGF-β enhanced the expression of RANKL mRNA in HUVECs in a time-dependent (Fig. 2A) and a dose-dependent manner (Fig. 2B). We also performed Western blot analysis with an anti-RANKL antibody and found that TGF-β induced RANKL protein expression in HUVECs (Fig. 2C). The results indicate that TGF-β induces RANKL expression in cells of endothelial lineage.

OPG is a soluble decoy receptor of RANKL, thereby inhibiting the RANK binding to RANKL and negatively regulating osteoclast differentiation, activity, and survival both in vivo and in vitro (8). Recently, TGF-β was reported to up-regulate OPG expression in osteoblasts (21, 22). Interestingly, TGF-β down-regulated OPG mRNA expression in HUVECs in a dose-dependent manner (Fig. 2B). This result indicates that TGF-β stimulates osteoclastogenesis not only by inducing RANKL expression but also by decreasing OPG expression in endothelial cells.

Increase in RANKL Promoter Activity after Treatment with TGF-β in Endothelial Cells but Not in Osteoblasts—To further quantify the TGF-β-mediated transcriptional regulation of RANKL, we performed transient luciferase assays. BM-3 cells were transiently transfected with RANKL promoter-luciferase fusion gene containing 1005 bases of 5'-flanking sequence (pGL3–1005) (19). Consistent with the RT-PCR analysis (Fig. 1A), the addition of TGF-β increased the luciferase activity in pGL3–1005-transfected BM-3 cells in a dose-dependent manner (Fig. 3A). In pGL3–1005-transfected ST2 cells, no such luciferase activity was seen even in the presence of 10 ng/ml TGF-β (Fig. 3B). Consistent with the previous reports (19), treatment of ST2 cells with 1,25-dihydroxyvitamin D3 raised the RANKL promoter activity about 2-fold (Fig. 3B). RT-PCR analysis also suggested that TGF-β could not induce RANKL mRNA expression in ST2 cells (data not shown).

PGE2 Is Not Involved in the TGF-β-induced RANKL Expression in Endothelial Cells—PGE2 is a potent stimulator of bone
resorption in vitro and induces osteoclast formation in mouse bone marrow culture (23). PGE2 synthesis is regulated by cyclo-oxygenases COXs, which catalyze the production of prostaglandins from arachidonic acids (24). COX-1 is constitutively expressed, and COX-2 is inducibly expressed by a variety of mitogen and inflammatory stimuli (25–28). It is also reported that COX-2 is up-regulated in BDECs by cytokine stimulation such as IL-1a and basic fibroblast growth factor (14). Moreover, TGF-β was seen to stimulate COX-2 expression and PGE2 release in a dose-dependent manner. We screened the COX-2 specific inhibitor NS-398. Pretreatment of the cells with 10 or 30 μM NS-398 completely inhibited the PGE2 synthesis (Fig. 4D). Therefore, we speculated that COX-2 expression and its product, PGE2 secretion, could be the cause of RANKL up-regulation. To investigate whether PGE2 induction was critical for TGF-β-mediated RANKL expression, we examined the expression in BM-3 cells that had previously been treated with the COX-2-specific inhibitor NS-398. Pretreatment of the cells with 10 or 30 μM NS-398 completely inhibited the PGE2 synthesis (Fig. 4E). The TGF-β-induced RANKL mRNA expression was not affected even in the presence of NS-398. Therefore, TGF-β-induced RANKL expression in BM-3 cells was not due to COX-2 protein expression or to PGE2 secretion.

Involvement of p38 MAPK and PKA in TGF-β-induced RANKL Expression in Endothelial Cells—We screened the drugs that could interfere with TGF-β-induced RANKL expression. It revealed that pretreatment of cells with the p38 MAPK inhibitor SB203580 and the PKA inhibitor KT5720, but not the MAPK/ERK inhibitor PD98059, decreased RANKL mRNA expression (Fig. 5A). These results suggest that PKA and p38 MAPK may participate in the TGF-β-induced RANKL expression.

Because PKA promoted the phosphorylation of transcription factor CREB, we performed Western blot analysis with an anti-phospho-CREB antibody. As shown in Fig. 5B, the treatment of BM-3 cells with TGF-β induced the phosphorylation of CREB without affecting CREB protein amount. The phosphorylation of CREB was seen within 15 min after TGF-β was added and then declined slightly in a time-dependent manner (data not shown). Because TGF-β did not rise the level of cAMP

![Image](http://www.jbc.org/)

**Fig. 3. Induction of RANKL promoter activity by TGF-β in BM-3 cells but not in ST2 cells.** BM-3 (A) and ST2 (B) cells were transiently transfected with the luciferase reporter vector pGL3-1005 containing nucleotides −1005 to +1 of the murine RANKL gene. After incubation for 4 h in serum-containing medium, the cells were refed with serum-free medium for 12 h. The cells were then incubated with serum-free medium containing the indicated concentrations of TGF-β or 1,25-dihydroxyvitamin D3 for 3 h. The luciferase activity in the cell lysate was measured, as described under “Experimental Procedures.” The luciferase activity was represented as relative light units (RLU) and was normalized by dividing the protein amount in the cell lysate. Each point represents a mean ± S.D. of triplicate determinations. Repeated experiments gave similar results.

![Image](http://www.jbc.org/)

**Fig. 4. Effects of TGF-β-induced PGE2 secretion on RANKL expression in BM-3 cells.** A, BM-3 cells were serum-starved for 24 h. Then the cells were incubated for 3 h in serum-free DMEM containing the indicated concentrations of TGF-β. The cells were then incubated for 3 h in serum-free DMEM containing no additions (−) or 1,25-dihydroxyvitamin D3 for 3 h. The luciferase activity in the cell lysate was measured, as described under “Experimental Procedures.” B, BM-3 cells were serum-starved for 24 h. Then the cells were incubated in serum-free DMEM containing the indicated concentrations of TGF-β for 4 h. PGE2 concentrations in the culture medium were measured by enzyme immunoassay, as described under “Experimental Procedures.” The total RNA (1 μg) of each sample was extracted, and RT-PCR was carried out using murine RANKL or G3PDH primer sets. D, total RNA (1 μg) from BM-3 cell lysates or ST2 cell lysates was extracted, and RT-PCR was carried out using murine RANKL or G3PDH primer sets. E, BM-3 cells were serum-starved for 24 h. Then the cells were incubated for 3 h in serum-free DMEM containing no additions (−) or 10 ng/ml TGF-β (+). In some experiments, the cells were pretreated with the indicated concentrations of NS-398 for 6 h before TGF-β was added. PGE2 concentrations in the culture medium were measured by enzyme immunoassay as described under “Experimental Procedures.” The total RNA (1 μg) of each sample was extracted, and RT-PCR was carried out using murine RANKL or G3PDH primer sets. The amplified products of murine RANKL (601 bp), murine EP4 (893 bp) were electrophoresed in 1% agarose gel plate stained with ethidium bromide and visualized by UV illumination. Repeated experiments gave similar results. ODF, osteoclast differentiation factor.
in BM-3 cells (data not shown), TGF-β may directly activates PKA as it did in mesangial cells (32). When BM-3 cells were incubated with PKA activator forskolin, it induced RANKL mRNA expression in a time-dependent manner (Fig. 5C). Thus, PKA activation is involved in RANKL mRNA expression. To confirm the involvement of the PKA-CREB signaling pathway in RANKL expression, we transiently transfected pCMV vector containing WT and DN CREB cDNA into BM-3 cells. As shown in Fig. 5D, overexpression of WT CREB protein alone induced the RANKL mRNA expression. In contrast, overexpression of DN CREB protein did not up-regulate but rather decreased the RANKL mRNA expression. Moreover, TGF-β-induced RANKL mRNA expression was suppressed by transfecting DN CREB cDNA into BM-3 cells (Fig. 5E). These results strongly indicate that the PKA-CREB signaling pathway plays an important role in the TGF-β-induced RANKL expression.

Because the p38 MAPK inhibitor SB203580 could also inhibit the TGF-β-induced RANKL mRNA expression in BM-3 cells (Fig. 5A), we examined the role of p38 MAPK signaling on RANKL expression. The p38 MAPK is activated by phosphorylation via upstream kinases, such as MKK3 and MKK6 (33), so we checked the amount of the phosphorylated form of p38 MAPK after TGF-β addition. As shown in Fig. 6A, TGF-β promoted the phosphorylation of p38 MAPK but not MAPK/ERK. Because p38 MAPK phosphorylates and activates ATF-2 (34), which belongs to CREB/ATF bZIP family of transcription factors, we examined the phosphorylation of ATF-2 after TGF-β treatment. As shown in Fig. 6A, TGF-β promoted ATF-2 phosphorylation in a dose-dependent manner. Thus, p38 MAPK was activated by TGF-β in BM-3 cells. To confirm the role of p38 MAPK, we transiently transfected p38 MAPK cDNA into BM-3 cells. As shown in Fig. 6B, overexpression of p38 MAPK increased the amount of RANKL mRNA but not as significantly as CREB overexpression (Fig. 5D). Recently, it was reported that p38 MAPK regulates CREB activity in B cells (34). To examine this possibility, we examined CREB phosphorylation after p38 MAPK transfection. Interestingly, overexpression of p38 MAPK raised RANKL expression without CREB activation (Fig. 6B). Because ATF-2 was phosphorylated by TGF-β treatment (Fig. 6A), ATF-2 might independently play a role in RANKL transcription. These results demonstrate that PKA and p38 MAPK are involved in TGF-β-induced RANKL expression in endothelial cells.

**Identification of CRE-like Domains in Murine RANKL Promoter Region**—We then checked whether murine RANKL promoter contains sequences that have homology to CRE consensus sequence (TGACGTCA). We found two CRE-like domains in murine RANKL promoter (−939 to −932, TGAGGTC; and −478 to −471, TGAAGTC) (Fig. 7A). To confirm the DNA-protein interaction, we employed electrophoretic mobility shift assay using double-stranded oligonucleotides containing 20 bp of mouse RANKL promoter sequences, −945 to −926 and −484 to −465, and nuclear extracts prepared from BM-3 cells treated with or without TGF-β (Fig. 7B). We observed the formation of a single complex when both oligonucleotides were incubated with nuclear extracts from TGF-β-treated BM-3 cells. The DNA-protein complex was not found when cells were treated with 10 or 30 μM SB203580 or 10 μM KT5720 before TGF-β treatment (Fig. 7C). Moreover, the formation of DNA-protein complex was efficiently competed with 200-fold molar excess of unlabeled CRE consensus oligonucleotide. Thus, complex formation is mediated by the interaction with CREB/ATF transcription factors. To determine whether the CRE-like element is involved in RANKL gene expression by binding to these CRE-like domains, a construct containing both CRE-like domains (pGL3−1005-Luc) or a construct containing one CRE-
like domain (pGL3–723-Luc) was transfected into BM-3 cells. Addition of TGF-β resulted in an almost 2-fold increase in transcription of pGL3–1005-Luc (Fig. 7D). However, the induction was reduced to about 1.3-fold in the presence of one CRE-like domain in the reporter construct (pGL3–723-Luc), and no reporter activity was observed in pGL3–256-Luc containing no CRE-like domain, further suggesting the involvement of CREB/ATF transcription factors in RANKL gene regulation.
Therefore, CREB/ATF transcription factors might be involved in RANKL expression via PKA and p38 MAPK in endothelial cells.

DISCUSSION

Until now, formation of osteoclasts has been studied by focusing on the interaction with osteoblasts. Especially, cell-cell contact between the two cell types is reported to be essential for osteoclastogenesis (35). Recent research revealed that RANKL was expressed on the surface of osteoblasts and bound to RANK, which is expressed on the surface of osteoclast precursors. Many reports indicate that RANK binding to RANKL plays the main role for osteoclastogenesis. Therefore, cell-cell contact is most certainly involved in osteoclastogenesis via RANK-RANKL system. However, RANKL is expressed not only on osteoblasts but also on dendritic cells. RANK is also expressed on the surface of T-cells, and the binding of RANK to RANKL regulates T-cell activation (36). Therefore, RANKL expression is not restricted to osteoblastic cells.

It has long been suggested that vascular endothelial cells contribute to osteolysis by producing such mediators as IL-1, IL-6, fibroblast growth factors, and colony-stimulating factors (9, 37). Histological studies of bone metastasis especially revealed that most metastatic foci occur near the epiphyseal plate where microvascularules are abundant (10) and that osteoclasts are formed around blood vessels of metastatic tumors. Therefore, the contribution of vascular endothelial cells to osteoclastogenesis is almost evident, whereas detailed mechanisms have not been elucidated. Recently, we established BDEC cell lines and found that they produced IL-11 when stimulated by inflammatory cytokines (13). We also found that basic fibroblast growth factor promoted PGE2 production from BDECs (14). Because both IL-11 and PGE2 were known to induce RANKL expression in osteoblasts (7), BDECs might contribute to RANKL expression indirectly.

In this study, we found that BDECs themselves expressed RANKL when cultured with TGF-β (Fig. 1). The same result could be observed in human primary endothelial cells, HUVECs (Fig. 2). These findings, together with a recent report that human microvascular endothelial cells expressed RANKL when stimulated by IL-1α or tumor necrosis factor α (38), convinced us that vascular endothelial cells express RANKL as well. When we examined the effects on BDECs on osteoclast formation in vitro, we found that TGF-β promoted osteoclastogenesis only in the presence of BDECs (data not shown). Therefore, BDECs would contribute to osteoclastogenesis by expressing RANKL. We focused on TGF-β for the following two reasons: TGF-β is abundantly stored in bone matrix and released during osteolysis, and some cancer cells were known to release TGF-β by themselves. We have previously reported that the breast cancer cell line MDA-MB-231 and the lung cancer cell line A549 released TGF-β, but the prostate cancer cell line PC3 did not (13). TGF-β is known to inhibit osteoclastogenesis in the murine neonatal calvaria organ culture system (39). TGF-β might inhibit osteoclastogenesis because of the up-regulation of OPG and the down-regulation of RANKL in osteoblasts (21). However, TGF-β-producing MDA-MB-231 and A549 cells formed osteolytic bone metastasis in vivo. Therefore, TGF-β may give us a hint about the relationship between osteolytic bone metastasis and vascular endothelial cells. Those cancer cells reach bone through blood flow and first have contact with vascular endothelial cells. In contrast to its role in osteoblasts, TGF-β down-regulated OPG expression in HUVECs in addition to up-regulating RANKL expression (Fig. 2).

Thus, in pathological conditions, especially osteolytic bone metastasis, vascular endothelial cells might work dominantly and accelerate bone resorption in vivo. Moreover, TGF-β promoted the growth of BDECs (data not shown), which is consistent with the previous reports that it induced angiogenesis (40, 41); it would lead to further osteolysis, following tumor growth. TGF-β is also known to induce parathyroid hormone-related protein production in breast cancer cells, which in turn up-regulates RANKL expression in osteoblasts (16). Our results support the notion that TGF-β promotes osteolytic bone metastasis.

Recently, it was reported that BMP-2, a member of the TGF-β superfamily, induced RANKL expression in osteoblasts and that it was inhibited by the COX-2-specific inhibitor NS-398 (42). However, TGF-β-induced RANKL expression in vascular endothelial cells could not be inhibited by NS-398, even though PGE2 production was significantly lowered (Fig. 4E). Therefore, to search for COX-2-independent mechanisms of RANKL induction by TGF-β to find a solution to vascular endothelium related to excessive osteolysis, we evaluated inhibitors of TGF-β-induced RANKL expression. We found that KT5720, a specific inhibitor of PKA, eliminated RANKL expression (Fig. 5A). The role of PKA is to activate CREB, and we speculated that TGF-β-activated CREB via PKA activation because TGF-β has been reported to activates PKA and its downstream transcription factor CREB in mesangial cells (32). TGF-β phosphorylated CREB within 30 min in BDECs (Fig. 5B). Moreover, the PKA activator forskolin induced RANKL mRNA expression in a time-dependent manner (Fig. 5C). The results of dominant-negative CREB transfection (Fig. 5, D and E) clearly showed that CREB regulates RANKL transcription. In contrast to BDECs, TGF-β could not induce CREB phosphorylation in ST2 cells (data not shown). The difference of CREB activation provoked by TGF-β can explain why TGF-β up-regulates RANKL in vascular endothelial cells but not in osteoblastic cells. Down-regulation in osteoblasts (21) may involve another pathway of TGF-β.

In this report, we showed that SB203580, a specific inhibitor of p38 MAPK, also reduced RANKL expression stimulated by TGF-β (Fig. 5A). The p38 MAPK is phosphorylated and activated by MKK3 or MKK6 (43). We revealed that p38 MAPK is not required for TGF-β-induced CREB phosphorylation (Fig. 6B). p38 MAPK phosphorylates cellular transcription factors, such as c-Jun, JunD, Elk-1, and ATF-2 (43–45). ATF-2 belongs to the CREB/ATF bZip family of transcription factors that binds to nucleotide sequences homologous to the palindromic CRE, TGACGTC(A). Because TGF-β treatment promoted ATF-2 phosphorylation (Fig. 6A), ATF-2 may be able to substitute for the main role of CREB to induce RANKL expression. This may be the reason why a higher dose of SB203580 was required to eliminate RANKL expression induced by TGF-β. Further analysis of RANKL promoter region revealed two CRE-like domains (Fig. 7A). Because TGF-β treatment promoted the interaction between CREB/ATF transcription factors and the CRE-like domains (Fig. 7B) and the complex formation was blocked by pretreatment of the cells with p38 MAPK inhibitor and PKA inhibitor (Fig. 7C), activation of CREB and ATF-2 might be involved in the RANKL gene expression. We also found that inhibitors to PKA and p38 MAPK suppressed the TGF-β-induced osteoclastogenesis in the presence of BDECs (data not shown). These results strongly suggested that the CREB/ATF bZip family of transcription factors are involved in TGF-β-induced RANKL expression in endothelial cells. Recently, it was reported that the p38 MAPK pathway plays an important role in RANKL-induced osteoclast differentiation of precursor bone marrow cells (48). Together with this result, inhibiting p38 MAPK pathway may have dual effects that prevent RANKL expression from endothelial cells and block RANK signaling. These results may be useful for...
treat ing osteolytic bone metastasis accelerated by vascular endothe lial cells.

We conclude that vascular endothelial cells might be in volved in the acceleration of osteoly sis in pathological condi tions because of the up-regulation of RANKL expression, which is the result of the activation of TGF-β-induced CREB/ATF bZIP family of transcrip tion factors via PKA and p38 MAPK.

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