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Basic Fibroblast Growth Factor Induces Osteoclast Formation by Reciprocally Regulating the Production of Osteoclast Differentiation Factor and Osteoclastogenesis Inhibitory Factor in Mouse Osteoblastic Cells

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Basic fibroblast growth factor (bFGF) induced osteoclast formation in co-cultures of mouse spleen cells and osteoblasts. Osteoclastogenesis inhibitory factor (OCIF) and a selective cyclooxygenase-2 (COX-2) inhibitor, NS-398, abolished bFGF-induced osteoclast formation. bFGF did not affect spleen cells, but it did affect osteoblasts, to stimulate osteoclast formation. Northern blot analysis revealed that bFGF up-regulated the expression of osteoclast differentiation factor (ODF) and COX-2 and down-regulated the expression of OCIF in primary osteoblastic cells. NS-398 abolished the increase of ODF mRNA, but it had no effect on the decrease of OCIF mRNA. NS-398 suppressed the binding of ^{125}I-labeled OCIF to osteoblastic cells treated with bFGF. Enzyme-linked immunosorbent assay showed that bFGF inhibited OCIF production by osteoblastic cells, and the inhibition was not affected by NS-398. We conclude that bFGF induces osteoclast formation by stimulating ODF production through COX-2-mediated prostaglandin synthesis and by suppressing OCIF production through a mechanism independent of prostaglandin synthesis.

Abbreviations used: bFGF, basic fibroblast growth factor; OCIF, osteoclastogenesis inhibitory factor; ODF, osteoclast differentiation factor; sODF, soluble ODF; COX-2, cyclooxygenase-2; OCL, osteoclast-like cells; IL, interleukin; PTH, parathyroid hormone; PGE_2, prostaglandin E_2; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; OPG, osteoprotegerin; OPGL, OPG ligand; TRANCE, TNF-related activation-induced cytokine; RANKL, receptor activator of NF-κB ligand; TNF, tumor necrosis factor; M-CSF, macrophage colony-stimulating factor; RANK, receptor activator of NF-κB; PG, prostaglandin; TRAP, tartrate-resistant acid phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, Enzyme-linked immunosorbent assay; SE, standard errors.

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Bone remodeling is regulated by bone-forming osteoblasts and bone-resorbing osteoclasts, both of which are modulated by a variety of hormones and local factors. An imbalance between bone formation and bone resorption causes various diseases affecting bone metabolism (1–3). Osteoclasts are multinucleated cells that derive from hematopoietic cells of the monocyte/macrophage lineage. A co-culture system of spleen cells with osteoblasts or bone marrow stromal cells has been established to produce osteoclasts (4, 5). In the co-cultures, osteoclast-like cells (OCLs) are formed from spleen cells in the presence of such stimulators of bone resorption as interleukin 6 (IL-6), IL-11, parathyroid hormone (PTH), prostaglandin E_2 (PGE_2), and 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] (1, 2, 6). The cell-to-cell interaction between osteoblasts/stromal cells and osteoclast progenitors in the co-cultures has been found to be essential for the OCL formation. Suda *et al.* (1, 2, 6) hypothesized that a membrane-bound factor, designated as "osteoclast differentiation factor (ODF)," is expressed on osteoblasts/stromal cells in response to osteotropic factors, and that it transduces a signal essential for osteoclastogenesis to osteoclast progenitors through cell-to-cell interaction.

We recently purified and molecularly cloned osteoclastogenesis-inhibitory factor (OCIF) (7, 8) (also called osteoprotegerin [OPG] (9)). OCIF/OPG is a secreted member of the tumor necrosis factor receptor (TNFR) family, and it inhibits osteoclastogenesis *in vitro* and *in vivo* (9–12). Subsequently, we succeeded in molecular cloning of ODF as a ligand for OCIF/OPG (10). ODF (also called OPG ligand [OPGL] (13), TNF-related activation-induced cytokine [TRANCE] (14), and receptor activator of NF-κB ligand [RANKL] (15)) is a member of the membrane-associated tumor necrosis factor (TNF) ligand family and it induces osteoclast differen-

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tiation from progenitor cells co-treated with macrophage colony-stimulating factor (M-CSF) in the absence of osteoblasts/stromal cells and osteotropic factors (10, 13). ODF is a long-sought ligand expressed on osteoblasts/stromal cells in response to osteotropic factors, and it mediates an essential signal to osteoclast progenitors for their differentiation into active osteoclasts (10, 16). Furthermore, we demonstrated that the receptor activator of NF-κB (RANK) (15) is the signaling receptor essential for ODF-mediated osteoclastogenesis (17). It is believed that ODF, RANK, and OCIF/OPG play essential roles in osteoclastogenesis (18, 19).

Basic fibroblast growth factor (bFGF) is a potent regulator of both bone formation (20–22) and bone resorption (23). bFGF is produced by bone cells (24, 25) and stored in extracellular matrix (26). It is a potent mitogen for a wide variety of cells, including osteoblasts/stromal cells (22, 27, 28) and a major inhibitor of type I collagen synthesis in osteoblastic cells (29). Little is known about the physiological roles of bFGF in bone remodeling, because of its multiple effects on bone cells. Clarification of the roles of bFGF in both physiological and pathological conditions is essential for the application of bFGF to such metabolic bone diseases as osteopetrosis and osteoporosis.

Hurley *et al.* (30) recently reported that bFGF induces OCL formation in mouse bone marrow cultures by a mechanism dependent on prostaglandin (PG) synthesis. However, the precise mechanisms by which bFGF induces OCL formation are unknown. In the present study, we demonstrate that bFGF-mediated OCL formation depends not only on PG synthesis but also on the ODF-RANK signaling in co-cultures of mouse spleen cells and osteoblastic cells. The target cells mediating the effects of bFGF on OCL formation in the co-cultures were found to be osteoblastic cells, but not osteoclast progenitors. We therefore investigated the mRNA expression and production of ODF and OCIF in osteoblastic cells treated with bFGF. Here we report that bFGF-mediated osteoclast formation is dependent on the up-regulation of ODF expression through PG synthesis mediated by cyclooxygenase-2 (COX-2), and on the down-regulation of OCIF expression independent of PG synthesis.

MATERIALS AND METHODS

Reagents. Recombinant human and mouse OCIF were prepared as described previously (8, 31). Soluble mouse ODF (sODF) was prepared as described previously (10, 16). bFGF was purchased from AUSTRAL Biologicals (San Ramon, CA). NS-398 was from Cayman CHEMICAL (Ann Arbor, MI).

Osteoclast formation assays. Spleen cells (1×10^5 cells) prepared from normal male ddY mice (6 to 15 weeks old) and primary osteoblasts (4×10^3 cells) prepared from calvaria of newborn ddY mice, as described previously (32), were co-cultured for a week in a 96-well plate in α-MEM (Gibco BRL) containing 10% fetal calf serum (FCS), with or without various concentrations of bFGF, in the presence or absence of mouse OCIF (100 ng/ml) and/or NS-398 (1 μM). NS-398 was added 1 hr before the addition of bFGF. Culture medium was replaced every 2 or 3 days. After treatment, the cells were subjected to tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts) staining or a TRAP solution assay, as described previously (5, 7).

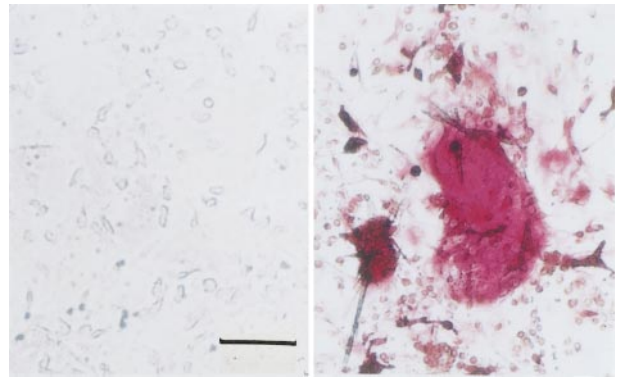

FIG. 1. Morphology of OCLs induced from the co-cultures by bFGF. Mouse spleen cells and primary osteoblastic cells were cultured for 7 days in the presence (right panel) or absence (left panel) of 10 ng/ml of bFGF. The cells were stained for TRAP. TRAP-positive cells appeared as red cells. Bar = 100 μm.

Northern blot analysis. Isolation of total RNA and hybridization were done as described previously (10, 33). Mouse primary osteoblasts were pre-cultured for 24 hr in T-75 flasks in α-MEM containing 10% FCS, and then treated with bFGF (10 ng/ml), in the presence or absence of NS-398 (1 μM). NS-398 was added 1 hr before the addition of bFGF. Culture medium was replaced every 2 or 3 days. A blot containing 10 μg of total RNA per lane from osteoblasts treated as above was probed with ODF, COX-2, OCIF, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. GAPDH mRNA was used as an internal control.

Analysis of the binding of ^{125}I-OCIF to mouse osteoblasts. Radioiodination of human OCIF was performed as described (8). Mouse primary osteoblasts (2.4×10^4 cells) were treated with bFGF (10 ng/ml), in the presence or absence of NS-398 (1 μM), in 24-well plates for 6 days, as described above. The binding analysis was performed as described previously (8).

Enzyme-linked immunosorbent assay (ELISA) for mouse OCIF. Mouse primary osteoblasts were treated with or without bFGF (10 ng/ml) and/or NS-398 (1 μM) in T-25 flasks, as described above. Conditioned media obtained from the cultures were stored at −80°C until use. OCIF concentrations in the conditioned media were determined with an ELISA system as described previously (34), with a modification in which anti-mouse OCIF monoclonal antibodies (mAbs) were used in place of anti-human OCIF mAbs.

RESULTS

bFGF Induces Osteoclast Formation in Co-Cultures of Spleen Cells and Osteoblasts

We first examined whether bFGF induces OCL formation in co-cultures of mouse spleen cells and calvariae-derived osteoblastic cells (Fig. 1). There were no TRAP-positive cells in untreated co-cultures. In contrast, numerous TRAP-positive cells were observed in bFGF-treated co-cultures, some of which developed to be multinucleated giant cells.

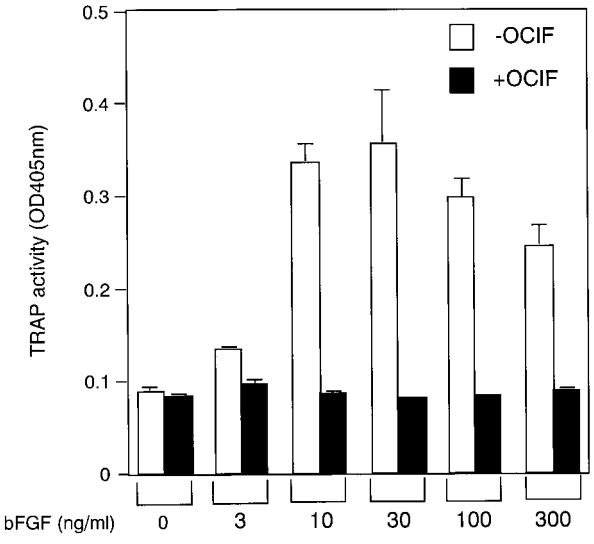

FIG. 2. Effect of OCIF on bFGF-induced OCL formation in co-cultures of mouse osteoblastic cells and spleen cells. The co-cultures were treated for 7 days with various concentrations of bFGF (0 to 300 ng/ml) in the presence (closed boxes) or absence (open boxes) of mouse OCIF (100 ng/ml). OCL formation was assessed by measuring TRAP activity. Data are expressed as means ± standard errors (SE) of three cultures.

OCIF Inhibits OCL Formation Induced by bFGF

Since such bone-resorbing factors as $1,25(OH)_2D_3$, PTH, PGE_2, and IL-11 induce OCL formation through the ODF-RANK signaling pathway in the co-cultures (18, 19), we examined whether bFGF induces OCL formation through the same signaling pathway. When the co-cultures were treated with various concentrations of bFGF (3 to 300 ng/ml) for 7 days, bFGF-mediated OCL formation, expressed as TRAP activity, was observed, in a dose-dependent manner (3 to 30 ng/ml) (Fig. 2). The induction was maximal at 30 ng/ml, and it decreased at higher concentrations (100 and 300 ng/ml). Mouse OCIF (100 ng/ml) concurrently added to the co-cultures completely inhibited bFGF-mediated OCL formation at any dose of bFGF (Fig. 2).

bFGF-Mediated OCL Formation Depends on PG Synthesis

It is known that bFGF induces COX-2 production in osteoblastic cells and stimulates the synthesis of PGE_2 (35), a potent stimulator of osteoclast formation in mouse bone marrow cultures or co-cultures of mouse osteoblastic cells and spleen cells (5, 36, 37). We examined the involvement of PG synthesis in the bFGF-mediated OCL formation in the co-cultures. When the co-cultures were treated for 7 days with 10 ng/ml of bFGF or 10 nM $1,25(OH)_2D_3$, in the presence or absence of NS-398, NS-398 inhibited the bFGF-mediated OCL formation (Fig. 3), but not the $1,25(OH)_2D_3$-mediated OCL formation (data not shown).

bFGF Up-regulates ODF Expression and Down-regulates OCIF Expression in Osteoblastic Cells

To elucidate whether bFGF directly acts on osteoclast progenitors, we examined the effects of bFGF on OCL formation, using an osteoblast/stromal cell-free culture system. Addition of bFGF (3 to 100 ng/ml) to 6 day-spleen cell cultures induced no OCLs in the presence or absence of 20 ng/ml of M-CSF (data not shown). When spleen cells were cultured for 7 days with 10 or 100 ng/ml of sODF and 20 ng/ml of M-CSF, numerous OCLs were formed, irrespective of the addition of bFGF (data not shown). These results indicate that bFGF does not act on spleen cells, including osteoclast progenitor cells, but it does act on osteoblastic cells to induce osteoclastogenesis under the conditions tested in this study. Therefore, we focused on osteoblastic cells to understand the mechanism by which bFGF induces osteoclastogenesis, and examined the effects of bFGF on the expression of mRNAs for ODF and OCIF, which are key regulators in osteoclastogenesis, in mouse primary osteoblastic cells, in the presence or absence of NS-398. ODF mRNA was weakly expressed under normal conditions. The mRNA expression increased slightly at 4 hr of culturing, remained elevated until 72 hr, and then markedly increased to a maximum at 96 and 120 hr (Fig. 4). Concurrent addition of NS-398 suppressed the bFGF-mediated elevation of ODF mRNA level at 72, 96, and 120 hr, but not at early time points (4 to 48 hr) (Fig. 4). On the other hand, OCIF mRNA was abundantly expressed under normal conditions. The mRNA level decreased at 24 hr of culturing, further decreased at 48 hr, and then reached a minimum at 120 hr (Fig. 4). Concurrent addition of NS-398 did not affect the bFGF-mediated decrease of OCIF mRNA at any time points (Fig. 4). We also examined the effect of bFGF on the expression of COX-2

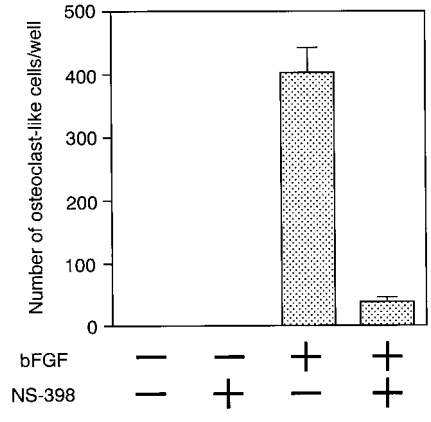

FIG. 3. Effect of NS398 on bFGF-induced OCL formation in co-cultures of mouse osteoblastic cells and spleen cells. The co-cultures were treated with NS-398 or bFGF, alone or in combination, for 7 days, followed by fixing and staining for TRAP. TRAP-positive cells per each well were scored. Data are expressed as means ± SE of five cultures.

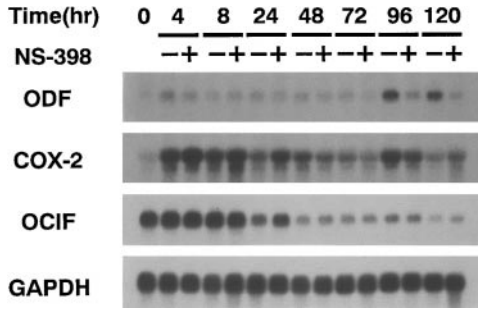

FIG. 4. Time course of the effects of bFGF on the expression of mRNAs for ODF, COX-2, and OCIF in mouse osteoblastic cells. Mouse primary osteoblastic cells were pre-cultured for 24 hr and treated with bFGF (10 ng/ml) in the presence or absence of NS-398 (1 μM), for the indicated periods. A blot loaded with 10 μg of total RNA from mouse osteoblastic cells was probed with ODF, COX-2, OCIF, or GAPDH cDNA.

mRNA in the osteoblastic cells. COX-2 mRNA expression drastically increased after 4 hr of bFGF treatment, gradually decreased to the initial level at 72 hr, and then increased again at 96 hr (Fig. 4).

bFGF Stimulates the Production of ODF by Osteoblasts

The increase in ODF production by mouse osteoblastic cells after treatment with bFGF was monitored by the binding of ^{125}I-OCIF to the cells. ^{125}I-OCIF bound the osteoblastic cells cultured in the presence of 10 ng/ml of bFGF for 6 days, but it did not bind untreated cells (Fig. 5). Concurrent addition of NS-398 with bFGF suppressed the increase in ODF production by 60%, but not to the control level. bFGF slightly stimulated the proliferation of osteoblastic cells (control, $7.2 \times 10^4 \pm 0.2$ cells/well; bFGF, $9.8 \times 10^4 \pm 0.6$ cells/well; bFGF plus NS-398, $1.0 \times 10^5 \pm 0.1$ cells/well), but NS-398 did not show any effects on the proliferation of osteoblastic cells.

bFGF Suppresses the Production of OCIF by Osteoblasts

We examined OCIF concentrations in the conditioned media of osteoblastic-cell cultures treated with or without bFGF by an ELISA (Fig. 6). OCIF concentration in the control medium after 24 hr of preculturing (0 time) was about 0.5 ng/ml. The concentration of OCIF gradually increased and reached a maximum (3.5 ng/ml) at 120 hr. In contrast, bFGF added to the culture increased the OCIF concentration up to 24 hr, and kept it around 1 ng/ml thereafter. Concurrent addition of NS-398 did not restore OCIF concentrations decreased by bFGF.

DISCUSSION

In previous studies, we have demonstrated that ODF expressed on osteoblasts/stromal cells mediates an essential signal to osteoclast progenitors for their differentiation into active osteoclasts through RANK (ODF receptor) and that OCIF, a decoy receptor for ODF, inhibits the ODF signaling as a competitor of RANK (18). Hurley *et al.* (30) recently reported that bFGF induces OCL formation in mouse bone marrow cultures by mechanisms that require PG synthesis. We suspected that the bFGF-induced OCL formation requires the ODF-RANK signaling. To demonstrate the hypothesis, we initially examined effects of OCIF on bFGF-induced OCL formation. OCIF inhibited the dose-dependent effect of bFGF on OCL formation in the co-cultures of mouse spleen cells and osteoblastic cells, indicating that the ODF-RANK signaling was involved in the bFGF-induced OCL formation.

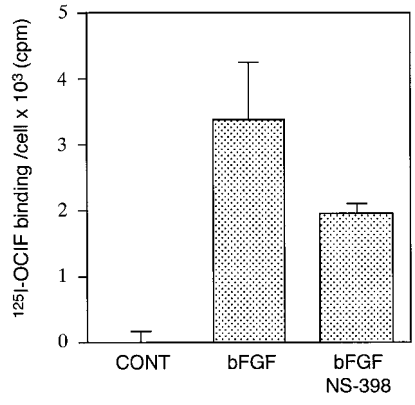

FIG. 5. Binding of ^{125}I-OCIF to osteoblastic cells. Mouse osteoblastic cells were treated with bFGF (10 ng/ml) for 6 days, in the presence or absence of NS-398 (1 μM), and were incubated with ^{125}I-OCIF as described previously (8). CONT represents untreated cells. Specific binding per cell is expressed as means ± SE of three cultures.

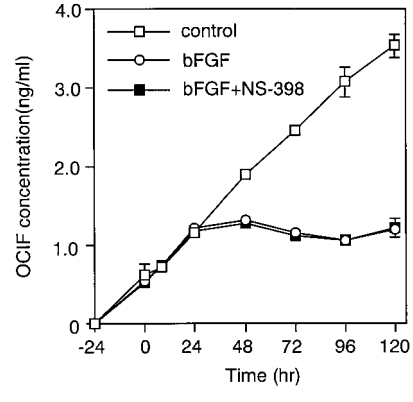

FIG. 6. Time course of OCIF accumulation by mouse osteoblast cells. Mouse osteoblastic cells were pre-cultured for 24 hr and treated with bFGF (10 ng/ml), in the presence or absence of NS-398 (1 μM), for the indicated periods. Open square, control; open circle, bFGF; closed square, bFGF and NS-398. Time 0 is the time bFGF was added. The concentrations of OCIF in the culture media were measured by an ELISA, as described under Materials and Methods. Data are expressed as means ± SE of three cultures.

Hurley *et al.* (30) showed that NS-398 inhibited the bFGF-mediated OCL formation by 95%, but not the PGE_2-mediated OCL formation, using mouse bone marrow cultures. We confirmed that NS-398 also inhibited the bFGF-mediated OCL formation by 90% in the co-culture system, indicating the involvement of PG synthesis. In contrast, NS-398 neither affected $1,25(OH)_2D_3$-mediated OCL formation nor the proliferation of osteoblastic cells in the present study. These data indicate that $1,25(OH)_2D_3$-mediated OCL formation is independent of PG synthesis and the inhibitory effect of NS-398 is specific for the bFGF-mediated OCL formation. Kawaguchi *et al.* (35) demonstrated that bFGF induces COX-2 mRNA expression in osteoblastic cells and stimulates PGE_2 synthesis. Northern blot analysis showed that the expression of mRNAs for ODF and COX-2 was similarly up-regulated by bFGF in osteoblastic cells. NS-398 did not affect the weak elevation of ODF mRNA levels at 4 hr, but it suppressed the strong elevation at 96 and 120 hr after bFGF treatment. Consistent with the results in the Northern blot analysis, ^{125}I-OCIF binding analysis revealed that treatment of osteoblastic cells with bFGF for 6 days induced ODF production in the cells, and the production was markedly inhibited by addition of NS-398. Taken together with our previous findings that PGE_2 stimulates ODF mRNA expression and ODF production in mouse primary osteoblastic cells cells (10), these results suggest that bFGF stimulates ODF production in osteoblastic cells through COX-2-mediated PG synthesis, and induces osteoclastogenesis in the co-cultures.

In contrast, bFGF suppressed OCIF expression in osteoblastic cells. Since 3.5 ng/ml of OCIF shows nearly half inhibitory effect in *in vitro* OCL formation assay (7), the decrease in OCIF production would support OCL formation. The decrease in OCIF mRNA expression and OCIF production was not recovered by addition of NS-398, suggesting that the bFGF–induced suppression of OCIF expression was not mediated by PG synthesis. We also found that PGE_2 did not affect the OCIF expression in mouse primary osteoblastic cells (our unpublished observations). On the other hand, the expression of OCIF mRNA in human bone marrow stromal cells has been shown to be down-regulated by PGE_2 (38). The inconsistency may be due to a difference in species or cell types. Since ODF molecules on osteoblastic cells can be occupied with OCIF produced by the cells, an increase in the ^{125}I-OCIF binding appears to represent either an increase of ODF production or a decrease of OCIF production. The observation that NS-398 did not completely inhibit the bFGF-induced ^{125}I-OCIF binding to osteoblastic cells would indicate that a decrease in OCIF production by the cells is involved in the increase of free ODF on the cells.

It is known that bFGF stimulates resorption by a mechanism independent of PG synthesis in fetal rat long bone cultures (23). In contrast, in mouse calvaria organ cultures, bFGF-induced bone resorption is dependent on PG synthesis (35). Administration of bFGF to animals induces bone synthesis rather than bone resorption (39, 40). There is another report showing the complicated effects of bFGF on bone remodeling. Jimi *et al.* (41) previously reported that bFGF inhibits OCL formation induced by $1,25(OH)_2D_3$, PGE_2, and IL-11. We observed that bFGF inhibits OCL formation induced by vitamin D_3 through suppressing ODF production by osteoblasts (manuscript in preparation). It is of interest to elucidate the mechanisms by which bFGF affects osteoclastogenesis positively or negatively in the different conditions (e.g., in the presence or absence of one of bone-resorbing factors such as $1,25(OH)_2D_3$, PGE_2, and IL-11) to understand the complicated functions of bFGF on bone remodeling.

In bone, bFGF produced by osteoblastic cells is deposited in bone matrix (24–26). Once bone resorption is initiated, bFGF may be released from the bone matrix and have effects on bone remodeling. Transforming growth factor β1 (TGF-β1), which is also released from bone matrix, was shown to suppress bone resorption in long-term human marrow cultures (42) and in fetal rat long bone assays (43). Takai *et al.* (44) recently reported that TGF-β1 inhibits osteoclastogenesis via stimulation of OCIF production in bone marrow stromal cells. TGF-β1 also down-regulates ODF expression in the cells. In the present study, we demonstrated that bFGF had effects opposite to TGF-β1 in regulation of the expression of OCIF and ODF in osteoblastic cells in inducing osteoclastogenesis. Together with the previous reports showing that reciprocal gene expression of OCIF and ODF in osteoblasts/stromal cells is important in inducing OCL formation (8, 10, 44, 45), these results support the hypothesis that bFGF, as well as TGF-β1, plays an important role in bone remodeling by modulating the production of ODF and OCIF by osteoblastic cells.

In the present study, we showed that bFGF stimulated OCL formation in co-cultures of mouse osteoblastic cells and spleen cells, and that bFGF stimulated ODF production through COX-2-mediated PG synthesis and reduced OCIF production through a mechanism independent of PG synthesis. Further studies are required to elucidate the molecular mechanisms by which bFGF regulates the expression of OCIF and ODF. This line of studies will shed light on understanding the precise role of bFGF in bone remodeling *in vivo*.

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