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Basic Fibroblast Growth Factor Inhibits Osteoclast Formation Induced by 1α,25-Dihydroxyvitamin D_3 through Suppressing the Production of Osteoclast Differentiation Factor

Nobuaki Nakagawa, Hisataka Yasuda,[1] Kazuki Yano, Shin-ichi Mochizuki, Naoki Kobayashi, Hitoshi Fujimoto, Kyoji Yamaguchi, Nobuyuki Shima, Tomonori Morinaga, and Kanji Higashio
Research Institute of Life Science, Snow Brand Milk Products Co. Ltd., Tochigi 329-0512, Japan

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Basic fibroblast growth factor (bFGF) inhibited osteoclast-like cell (OCL) formation in cocultures of mouse spleen cells with either osteoblasts or a stromal cell line, ST2, in the presence of 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. bFGF directly acted on osteoblasts/stromal cells, but not osteoclast progenitors, to inhibit 1,25$(OH)_2D_3$-induced OCL formation. bFGF suppressed the mRNA expression of osteoclast differentiation factor (ODF) but did not affect that of osteoclastogenesis inhibitory factor (OCIF) in ST2 cells treated with 1,25$(OH)_2D_3$ and dexamethasone. Enzyme-linked immunosorbent assay showed that bFGF hardly affected OCIF production in the treated ST2 cells. A genetically engineered soluble form of ODF, but not anti-OCIF neutralizing antibody, abolished bFGF-mediated inhibition of OCL formation. bFGF suppressed the binding of ^{125}I-labeled OCIF to both ST2 cells and osteoblasts treated with 1,25$(OH)_2D_3$. These findings indicate that bFGF inhibits 1,25$(OH)_2D_3$-induced OCL formation via suppression of ODF production by osteoblasts/stromal cells.

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.

[1] To whom correspondence should be addressed at Research Institute of Life Science, Snow Brand Milk Products Co., Ltd., 519 Ishibashi-machi, Shimotsuga-gun, Tochigi 329-0512, Japan. Fax: 81-285-53-1314. E-mail: fvbd7042@mb.infoweb.ne.jp.

Bone remodeling is regulated by the balance of bone-forming osteoblasts and bone-resorbing osteoclasts. Both osteoblasts and osteoclasts are regulated by a variety of hormones and local factors. An imbalance between bone formation and bone resorption causes such metabolic bone diseases as osteopetrosis and osteoporosis (1–3). Osteoclasts are multinucleated giant cells that develop from hematopoietic cells of the monocyte/macrophage lineage. A coculture system of spleen cells with osteoblasts or bone marrow stromal cells has been established to produce osteoclasts (4, 5). In the cocultures, 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 1α,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 cocultures has been found to be essential for the OCL formation. These osteotropic factors seem to act on osteoblasts/stromal cells to induce osteoclastogenesis. 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, to osteoclast progenitors, a signal essential for osteoclastogenesis, 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 stimulated by 1,25$(OH)_2D_3$, PTH, or IL-11. Analyses of rats injected with OCIF/OPG (9, 10), transgenic mice overexpressing OCIF/OPG (9), and OCIF/OPG knockout mice (11, 12) demonstrated that OCIF/OPG plays a central role as an inhibitor of osteoclastogenesis *in vivo.* Subsequently, we succeeded in molecular cloning

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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 differentiation from progenitor cells cotreated with macrophage colony-stimulating factor (M-CSF) in the absence of osteoblasts/stromal cells and osteotropic factors (10, 13). In a fetal mouse long bone culture system, a genetically engineered soluble-form ODF (sODF) induces bone resorption (16). Both anti-ODF antibody and OCIF/OPG negated bone resorption elicited by osteotropic factors, such as $1,25(OH)_2D_3$, PTH, and PGE_2. The results established that ODF is a long-sought ligand expressed on osteoblasts/stromal cells in response to osteotropic factors, and that 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 the regulation 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.

Jimi *et al.* (30) previously reported that basic fibroblast growth factor (bFGF) inhibited $1,25(OH)_2D_3$-induced OCL formation in cocultures of mouse bone marrow cells and a mouse stromal cell line, ST2. However, little is known about the mechanism by which bFGF regulates osteoclast formation. Since $1,25(OH)_2D_3$ up-regulates ODF expression and down-regulates OCIF expression in osteoblasts/stromal cells, it is suspected that bFGF regulates the expression of these two factors. In the present study, we analyzed the effects of bFGF on the expression of ODF and OCIF in ST2 cells and/or osteoblasts cultured in the presence of $1,25(OH)_2D_3$, using Northern blot analysis, enzyme-linked immunosorbent assay (ELISA), and ^{125}I-OCIF binding assay. We also examined whether sODF or anti-OCIF neutralizing antibody abolishes bFGF-mediated inhibition of osteoclast formation in the presence of $1,25(OH)_2D_3$. The present study demonstrated, for the first time, that bFGF inhibits $1,25(OH)_2D_3$-induced osteoclastogenesis in cocultures of osteoblasts/stromal cells and spleen cells, via suppression of ODF production.

MATERIALS AND METHODS

Reagents. Recombinant human and mouse OCIF were prepared as described previously (8, 31). sODF was prepared as described previously (10, 16). A neutralizing polyclonal antibody against OCIF was prepared as described previously (32, 33). Control antibody was prepared by purifying immunoglobulin from rabbit preimmune serum. Human bFGF was purchased from AUSTRAL Biologicals (San Ramon, CA); NS-398 was from Cayman CHEMICAL (Ann Arbor, MI); human M-CSF was from Yoshitomi Pharmaceutical Co. (Osaka, Japan); dexamethasone (Dex) and $1,25(OH)_2D_3$ were from Wako Pure Chemical Co. (Osaka, Japan), and ST2 cells were from RIKEN CELL BANK (Ibaraki, Japan).

Osteoclast formation assays. Primary osteoblastic cells were prepared from calvaria of newborn ddY mice as described previously (34). Spleen cells (8×10^4 cells) prepared from normal male ddY mice (6 to 15 weeks old) were cocultured with osteoblastic cells (4×10^3 cells) for a week in 96-well plates in α-MEM supplemented with 10% fetal calf serum (FCS), in the presence of $1,25(OH)_2D_3$ (10 nM), with or without bFGF (10 ng/ml). Spleen cells were similarly cocultured with ST2 cells (4×10^3 cells), in the presence of $1,25(OH)_2D_3$ (10 nM) and Dex (100 nM), with or without various concentrations of bFGF, anti-OCIF antibody, mouse OCIF (mOCIF), sODF, or M-CSF. Dex was used to enhance the effect of $1,25(OH)_2D_3$ on OCL formation. 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 (5, 7).

Enzyme-linked immunosorbent assay (ELISA) for mouse OCIF. ST2 cells were pre-cultured for 24 h in T-25 flasks in α-MEM containing 10% FCS, and then treated with or without bFGF (10 ng/ml), in the presence of $1,25(OH)_2D_3$ (10 nM) and Dex (100 nM). 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 (35), with a modification in which anti-mouse OCIF monoclonal antibodies (mAbs) were used in place of anti-human OCIF mAbs.

Analysis of the binding of ^{125}I-OCIF to ST2 cells. Radioiodination of human OCIF was performed as described (8). ST2 cells (2.4×10^4 cells) were cultured in 24-well plates, in the presence of $1,25(OH)_2D_3$ (10 nM) and Dex (100 nM), with or without bFGF (10 ng/ml), as described above. The binding analysis was performed as described previously (8).

Northern blot analysis. Isolation of total RNA and hybridization were done as described previously (10, 36). ST2 cells (9.4×10^5 cells) were pre-cultured for 24 h in T-75 flasks in α-MEM containing 10% FCS, and then treated with or without bFGF (10 ng/ml), in the presence of $1,25(OH)_2D_3$ (10 nM) and Dex (100 nM). A blot containing 10 μg of total RNA per lane from the cells 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.

RESULTS

bFGF inhibits the $1,25(OH)_2D_3$-induced OCL formation in the cocultures. TRAP-positive cells were formed in cocultures of mouse spleen cells with either mouse osteoblastic cells or ST2 cells after 7 days of culturing in the presence of $1,25(OH)_2D_3$. bFGF added to each culture markedly inhibited the TRAP-positive

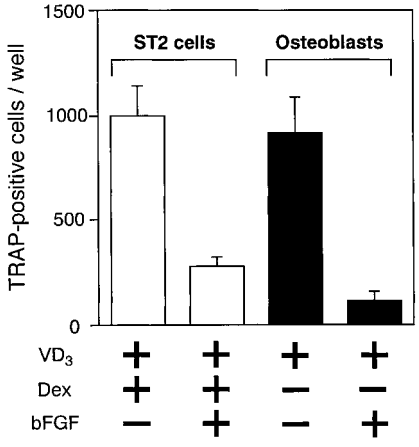

FIG. 1. Effect of bFGF on $1,25(OH)_2D_3$-induced OCL formation. Mouse spleen cells and either ST2 cells or osteoblastic cells were cocultured for 7 days, with or without 10 ng/ml of bFGF, in the presence of 10 nM $1,25(OH)_2D_3$ and 100 nM Dex (only for ST2 cells), fixed and stained for TRAP. TRAP-positive cells per each well were scored. ST2 cells, open boxes; osteoblastic cells, closed boxes. Data are expressed as means ± SD of five cultures.

cell formation (Fig. 1). To understand the molecular mechanism by which bFGF inhibits the $1,25(OH)_2D_3$-induced OCL formation, we used the coculture system of spleen cells and ST2 cells.

Although Jimi *et al.* (30) reported that the target cells for bFGF in the inhibition of osteoclast formation are not likely to be osteoclast progenitors but osteoblast/stromal cells, they could not completely exclude the possibility that bFGF directly acts on the osteoclast progenitors. We therefore examined this possibility. When spleen cells were cultured for 7 days with 10 or 100 ng/ml of sODF and 20 ng/ml of M-CSF, in the presence or absence of bFGF, numerous osteoclasts were formed, irrespective of the addition of bFGF (data not shown). The results indicate that bFGF does not affect osteoclast progenitors in the inhibition of osteoclast formation in the cocultures.

bFGF-mediated inhibition of OCL formation does not depend on the production of OCIF. We first examined whether bFGF exerts its inhibitory effect through production of OCIF. As shown in Fig. 2, bFGF inhibited the $1,25(OH)_2D_3$-induced OCL formation, which was expressed as TRAP activity, in a dose-dependent manner, and gave a half-maximal inhibition at 1 ng/ml, and a maximal inhibition at 10 ng/ml (Fig. 2). As we have previously reported, mouse OCIF added to the cocultures completely inhibited the OCL formation. The inhibitory effect of bFGF was comparable to that of OCIF. The OCL formation reduced by bFGF was not recovered by the addition of anti-OCIF neutralizing antibody, which abolished OCIF activity, suggesting that OCIF is not involved in the bFGF-mediated inhibition of OCL formation (Fig. 2). To confirm that bFGF does not induce OCIF production by ST2 cells, we measured OCIF concentrations in the conditioned media of the cell cultures with an ELISA. The OCIF concentration in the control ST2 cell culture was 3.5 ng/ml after 24 h of preculturing, and gradually increased and reached a maximum (7.8 ng/ml) at 72 h. In contrast, when the cells were cultured in the presence of $1,25(OH)_2D_3$ and Dex, the OCIF concentration in the conditioned medium was decreased, in a time dependent manner (Fig. 3). bFGF added to the culture together with $1,25(OH)_2D_3$ and Dex had little effect on the decrease of OCIF concentration (Fig. 3).

sODF abolishes the inhibitory effect of bFGF on $1,25(OH)_2D_3$-induced OCL formation. We next examined whether sODF and M-CSF, the two factors essential for osteoclastogenesis, abolish the inhibitory effect of bFGF on $1,25(OH)_2D_3$-induced OCL formation. Concurrent addition of sODF with bFGF to the cocultures in the presence of $1,25(OH)_2D_3$ and Dex recovered the reduced OCL formation, in a dose-dependent manner, and completely abolished the inhibitory effect of bFGF at 30 and 100 ng/ml (Fig. 4). In contrast, M-CSF did not recover the reduced OCL formation at all (Fig. 4). Neither sODF nor M-CSF, added to the cocultures with bFGF, showed any additive or synergistic effect (Fig. 4).

bFGF suppresses $1,25(OH)_2D_3$-induced production of ODF by osteoblasts/stromal cells Production of ODF by ST2 cells was analyzed by measuring the binding of

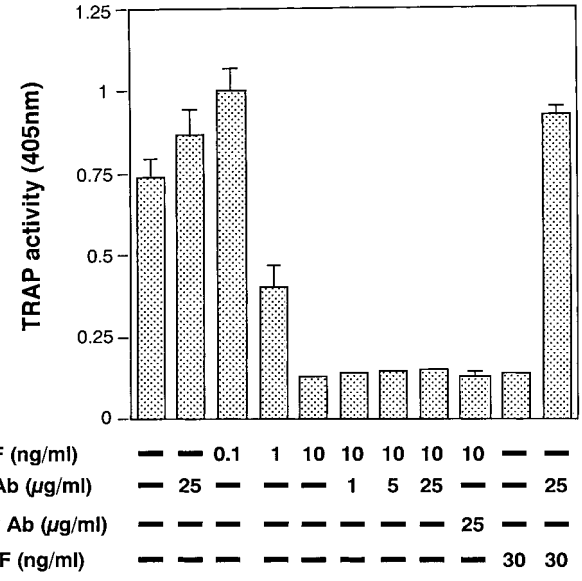

FIG. 2. Effect of anti-OCIF antibody on bFGF-mediated inhibition of OCL formation. Mouse spleen cells and ST2 cells were cocultured in the presence of $1,25(OH)_2D_3$ and Dex for 7 days, with various concentrations of cytokines or antibodies, as indicated. OCIF Ab, a neutralizing anti-OCIF polyclonal antibody; Control Ab, immunoglobulin purified from preimmune serum. OCL formation was evaluated by measuring TRAP activity. Data are expressed as means ± SD of four cultures.

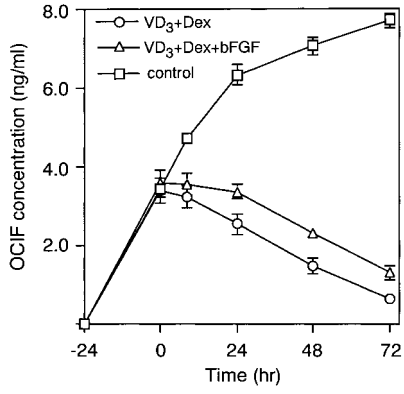

FIG. 3. Time course of OCIF accumulation in culture media of ST2 cells. ST2 cells were precultured for 24 h, and then treated for the indicated time with or without 10 ng/ml of bFGF, in the presence or absence of $1,25(OH)_2D_3$ and Dex. The concentrations of OCIF were measured by ELISA as described under Materials and Methods. Square, control; circle, $1,25(OH)_2D_3$ and Dex; triangle, $1,25(OH)_2D_3$, Dex and bFGF. Data are expressed as means ± SD of five cultures.

^{125}I-OCIF to the cells treated with or without bFGF, in the presence of $1,25(OH)_2D_3$ and Dex. As we have previously reported, $1,25(OH)_2D_3$ and Dex induced ODF production by ST2 cells in a few days. Concurrent addition of bFGF with $1,25(OH)_2D_3$ and Dex markedly decreased the ODF production on days 3, 4, and 5 (Fig. 5). Similar results were obtained from an experiment using mouse osteoblastic cells (data not shown). Northern blot analysis revealed that ODF mRNA level rapidly increased in ST2 cells cultured in the presence of

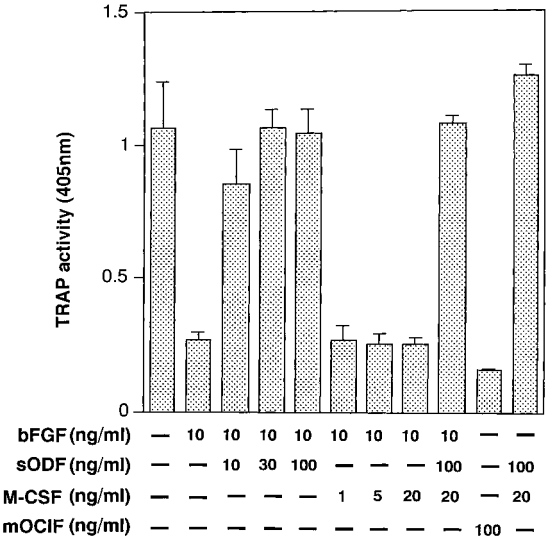

FIG. 4. Effect of sODF and M-CSF on bFGF-mediated inhibition of OCL formation. Spleen cells and ST2 cells were cocultured for 7 days with the indicated concentrations of sODF, M-CSF, and mOCIF, in the presence of $1,25(OH)_2D_3$ and Dex. OCL formation was evaluated by measuring TRAP activity. Data are expressed as means ± SD of four cultures.

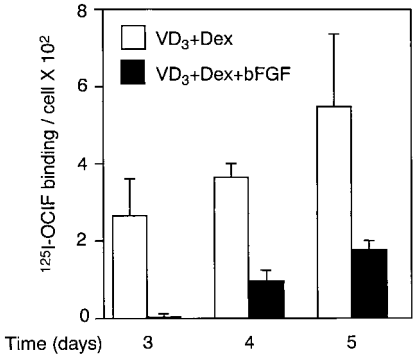

FIG. 5. bFGF suppressed the binding of OCIF to ST2 cells. ST2 cells were cultured with (closed boxes) or without (open boxes) 10 ng/ml of bFGF, in the presence of $1,25(OH)_2D_3$ and Dex, for the indicated periods, and they were incubated with ^{125}I-OCIF as described (8). Specific binding per cell is expressed as means ± SD of three cultures.

$1,25(OH)_2D_3$ and Dex, reached a maximum on day 3, and then gradually decreased (Fig. 6). Concurrent addition of bFGF to the cell culture markedly reduced the ODF expression at any time point (Fig. 6). In contrast, bFGF had little effect on the $1,25(OH)_2D_3$-induced decrease of OCIF mRNA (Fig. 6). bFGF also suppressed the $1,25(OH)_2D_3$-induced ODF expression in osteoblastic cells (data not shown).

DISCUSSION

Jimi *et al.* (30) previously reported that bFGF inhibits OCL formation in cocultures of mouse bone marrow cells and ST2 cells, and suggested that bFGF may stimulate or inhibit the production of a factor(s), which respectively blocks or facilitates OCL formation, from ST2 cells. In the present study, we demonstrated that bFGF-mediated inhibition of OCL formation induced by $1,25(OH)_2D_3$ was due to the suppression of ODF production.

In the coculture system, osteoclast formation is modulated by reciprocal expression of ODF and OCIF in osteoblasts/stromal cells (8, 10, 18, 19, 37). We previously reported that $1,25(OH)_2D_3$ up-regulates the ODF

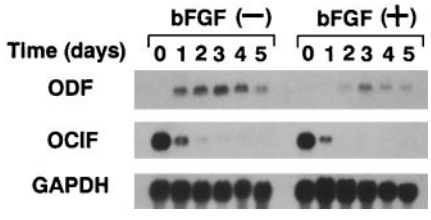

FIG. 6. Downregulation of ODF mRNA expression by bFGF in ST2 cells in the presence of $1,25(OH)_2D_3$ and Dex. A blot loaded with 10 μg of total RNA from ST2 cells cultured, for the indicated periods, in the presence of $1,25(OH)_2D_3$ and Dex, with (right panel) or without (left panel) bFGF, was probed with ODF, OCIF, or GAPDH cDNA.

expression and down-regulates the OCIF expression in osteoblasts/stromal cells (8, 10). In the present study, Northern blot analysis, ELISA, and ^{125}I-OCIF binding assay revealed that bFGF suppressed the ODF expression but had little effect on the OCIF expression in osteoblasts/stromal cells cultured in the presence of $1,25(OH)_2D_3$. Consistent with these observations, sODF, but not anti-OCIF neutralizing antibody, recovered the OCL formation reduced by bFGF in the presence of $1,25(OH)_2D_3$. Taken together, these results indicate that bFGF inhibits $1,25(OH)_2D_3$-induced OCL formation via suppression of ODF production by osteoblasts/stromal cells. On the other hand, it is known that M-CSF produced by osteoblasts/stromal cells is also essential for osteoclast formation (38). In the present study, we showed that concurrent addition of M-CSF with bFGF to the cocultures did not abolish the inhibitory effect of bFGF. Jimi *et al.* (30) reported that bFGF suppresses neither the M-CSF-induced colony formation of bone marrow cells nor the expression of M-CSF in stromal cells. These results indicate that M-CSF is not involved in the bFGF-mediated inhibition of OCL formation.

There is a discrepancy in the effects of bFGF on OCL formation. We and Jimi *et al.* (30) showed that bFGF inhibits $1,25(OH)_2D_3$-induced OCL formation in the cocultures. On the other hand, Hurley *et al.* (39) previously reported that bFGF induces osteoclast formation in mouse bone marrow cultures by mechanisms that require prostaglandin synthesis. Kawaguchi *et al.* (40) reported that bFGF induces cyclooxygenase-2 (COX-2) mRNA expression in osteoblastic cells and stimulates the synthesis of PGE_2, which is known to induce osteoclast formation in mouse bone marrow cultures or the cocultures of mouse osteoblastic cells and spleen cells (5, 41, 42). We examined the involvement of prostaglandin synthesis in the inhibition of $1,25(OH)_2D_3$-induced OCL formation by bFGF. A selective COX-2 inhibitor, NS398, had no effect on the inhibition of OCL formation, when added to the cocultures of spleen cells and ST2 cells, together with bFGF in the presence of $1,25(OH)_2D_3$ and Dex (our unpublished observations). The results suggest that the inhibitory effect of bFGF on $1,25(OH)_2D_3$-induced OCL formation in the cocultures does not depend on prostaglandin synthesis, and that the effect is independent of the mechanism by which bFGF induces OCL formation in bone marrow cultures. In fetal rat long bone cultures, bFGF stimulates resorption by a mechanism independent of prostaglandin synthesis (23). In contrast, in mouse calvaria organ cultures, bFGF-induced bone resorption is dependent on prostaglandin synthesis (40). Administration of bFGF to animals induces bone synthesis rather than bone resorption (43, 44). Recently, we found that bFGF up-regulates the ODF expression and down-regulates the OCIF expression by osteoblastic cells while inducing osteoclastogenesis in the absence of $1,25(OH)_2D_3$ (manuscript in preparation). 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 affects bone remodeling. 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, 33, 37, 45), these results support the hypothesis that bFGF plays an important role in bone remodeling by modulating the production of ODF and OCIF by osteoblastic cells. The different effects of bFGF both on osteoclast formation and the expression of ODF and OCIF in different experimental conditions (e.g., in the presence or absence of $1,25(OH)_2D_3$), may indicate the complicated functions of bFGF in bone remodeling.

In the present study, we showed that bFGF inhibits the $1,25(OH)_2D_3$-induced OCL formation in cocultures of osteoblasts/stromal cells and spleen cells via the suppression of ODF production by osteoblasts/stromal cells. These findings may provide important information for the use of bFGF in the treatment of osteoporosis. Further studies are required to elucidate the molecular mechanisms by which bFGF regulates the expression of OCIF and ODF in osteoblasts/stromal cells treated with such bone-resorbing factors as $1,25(OH)_2D_3$ and PTH. This line of studies will shed light on identification of the cytokine network modulating the expression of OCIF and ODF in osteoclastogenesis.

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