Regulatory Mechanism of 1α,25$(OH)_2D_3$-Induced Osteoclastogenesis by FGF (Fiboblast Growth Factor)-2

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Regulatory Mechanism of 1α,25$(OH)_2D_3$-Induced Osteoclastogenesis by FGF (Fibroblast Growth Factor)-2

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Table of Contents

Acknowledgements

Table of Contents ··· i

List of Figures ··· iii

Abstract ··· 1

I. Introduction ··· 4

II. Materials and methods ··· 10

1. Materials ··· 10
2. Methods ··· 10
   1) In vitro formation assay of osteoclast ··· 10
   2) MTT assays ··· 13
   3) Measurement of alkaline phosphatase activity (ALPase activity) ··· 13
   4) RT-PCR ··· 14
   5) Data analysis ··· 15

III. Results ··· 16

1. Effect of FGF-2 on the osteoclast formation in co-cultures 16
2. Changes in ALPase activity in osteoblastic cells ··· 19
3. Alteration of RANKL, OPG and M-CSF mRNA expression by FGF-2 ··· 19
4. ADAM and MMP13 mRNA expression by in osteoblastic cells ··· 23

Ⅳ. Discussion ········ 29
VI. References ········ 37
국문요약 ········ 46

List of Figures

Fig. 1. A model of cellular interactions for osteoclastogenesis. ··· 7

Fig. 2. Coculture system of osteoblast and bone marrow cells. · 11

Fig. 3. Osteoclast differentiation in the presence of FGF-2 at various concentrations. ··· 17

Fig. 4. Formation of multinucleated osteoclast in the presence of FGF-2. ··· 18

Fig. 5. Cell proliferation in the presence of FGF-2 at various concentrations. ··· 20

Fig. 6. Effect of FGF-2 on alkaline phosphatase activity of osteoblastic cells. ··· 21

Fig. 7. Effect of FGF-2 on the RANKL expression of osteoblastic cells. ··· 22

Fig. 8. Effect of FGF-2 on the OPG expression of osteoblastic cells. ··· 24

Fig. 9. Effect of FGF-2 on the M-CSF expression of osteoblastic cells. ··· 25

Fig.10. Relative density of RANKL and OPG mRNA expressions. ··· 26

Fig.11. Effect of FGF-2 on the ADAM8 expression of osteoblastic cells. ··· 27

Fig.12. Effect of FGF-2 on the MMP13 expression of osteoblastic cells. ··· 28

Abstract

Regulatory Mechanism of 1α,25$(OH)_2D_3$-Induced Osteoclastogenesis by FGF-2

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(Directed by Prof. Syng-Ill Lee, D.D.S., Ph.D.)

This study investigated the action of FGF (fibroblast growth factor)-2, a potent regulator on the functions of bone and cartilage cells. Of FGFs, FGF-2 acts like an autocrine/paracrine factor for bone cells. With regards to the effects of FGF-2 on bone cells, the response is still controversial in terms of bone metabolism, bone formation and bone resorption. To clarify the role of FGF-2, osteoclast formation was monitored in coculture system, mouse cavarial osteoblastic cells/bone marrow cells in the presence of 1α,25$(OH)_2D_3$ ($VitD_3$). Numerous TRAP-positive cells (80~498) induced by $VitD_3$ were observed in cocultures. When FGF-2 (0.1, 1, 10, and 100 ng/ml) was treated to the coculture system, $VitD_3$-induced osteoclast formation was inhibited by FGF-2 at low concentrations (0.1, 1 ng/ml) while at high concentrations (10, 100 ng/ml) stimulated or not changed. It was

also confirmed that exposure of FGF-2 to cocultures did not show a toxic effect in the entire range of concentration (0.1, 1.0, 10, and 100 nM), suggesting that the effect of FGF-2 on osteoclast formation was caused by its changing the physiological activity, not by its direct toxic effect upon the cells. To see the effect of FGF-2 on the osteoblastic cells, expressions of RANKL, OPG and M-CSF mRNAs were monitored by RT-PCR after incubation for 96 hrs. The RANKL mRNA expression level was reduced at low concentration. However, FGF-2 did not make any remarkable change in OPG and M-CSF mRNA expression. Consequently FGF-2, at low concentrations, resulted in the decrement of RANKL/OPG ratio. In addition, FGF-2 decreased the alkaline phosphatase activity which indicates the osteoblast differentiation. A disintegrin and metallo-proteinase (ADAM8), and matrix metallo-proteinase 13 (MMP13) which are responsible for osteoclast differentiation and degradation of extracellular matrix (ECM) respectively were examined by RT-PCR in the presence of FGF-2. FGF-2 did not exert any changes in ADAM8 while decreased the MMP13 mRNA expression at low concentration. Taken all together, these findings suggest that FGF-2 seems to have a dual effects on osteoclastogenesis, especially, FGF-2 at low concentration decreased osteoclast formation via the down-regulation of the RANKL and MMP13 mRNA expressions

in osteoblastic cells which in turn inhibits the osteoclast formation.

Key words : FGF-2, osteoclastogenesis, RANKL, dual effect

I. Introduction

There are two major groups of cells which are involved in skeletal maintenance, osteoblasts and osteoclasts. They are equal but opposite function during normal bone remodeling and skeletal homeostasis. Osteoblast and osteoclast receives the systemic signals from the metabolic products and mechanical strain, which follows to mediate the bone formation and resorption. Histologically, osteoblast stems from the mesenchymal stem cells through a series of progenitor stages to form mature, matrix secreting osteoblasts. Osteoclasts are multinucleated cells that derive from hematopoietic cells of the monocyte/macrophage lineage, and attach to the bone surface. Then the osteoclasts exert their own function, secreting hydrogen ions into lacunae via ruffled border and an osteoclastic specific organelle, which dissolved the bone matrix. As mentioned the above, Osteoblast and osteoclast seems to act independently each other, In fact, it is generally accepted that osteoclastic bone resorption precedes osteoblastic bone formation, suggesting that these two cells are closely coupled together.

Question is what signals governs the coupling between osteoblast and osteoclast? Of course, this coupling phenomenon is more sophisticated than physiological. Recently, it has been

revealed that regulatory interaction occurs in which cells in osteoblastic lineage regulate osteoclastic differentiation (Roodman, 1996; Suda *et. al.*, 1997; Takahashi *et. al.*, 1988). This interaction was demonstrated in coculture system which osteoblast or osteoblast precursors in the form of stromal cells were cultured with bone marrow or spleen cells as sources of osteoclast precursors. This resulted in the formation of osteoclast. However, when these cells were separated by membrane which is permeable to soluble factors but impermeable to cells, no osteoclasts were formed. Suda *et al.* (1992, 1995, 1996) 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. Recently, osteoclastogenesis-inhibitory factor (OCIF, also called osteoprotegerin:OPG) was purified and cloned (Tsuda *et al.*, 1997; Yasuda *et al.*, 1998). OPG which was initially identified as a novel secreted member of the tumor necrosis factor receptor (TNFR) family, and it inhibits osteoclastogenesis induced by $1\alpha,25(OH)_2D_3$, PTH, or IL-11. And receptor activator of NF-kB ligand (RANKL, also known ODF) is a member of the membrane-associated tumor necrosis factor (TNF) ligand family, inducing osteoclast differenciation from

progenitor cells co-treated with macrophage colony stimulating factor (M-CSF). Furthermore, receptor activator of NF-*k*B (RANK) is the signaling receptor essential for RANKL-mediated osteoclastogenesis (Nakagawa *et. al.*, 1998). Consequently it is believed that RANKL, RANK and OPG play crucial roles in the regulation in osteoclastogenesis (Yasuda *et. al.*, 1998; Suda *et. al.*, 1999; Fig 1).

As mentioned previously, a coculture system of spleen cells with osteoblasts or bone marrow stromal cells has been established to produce osteoclasts (Takahashi *et al.*,1988: Udagawa *et al.*,1989). 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 1 α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] (Suda *et al.*, 1992 ; Suda *et al.*,1995 ; Suda *et al.*,1996). Of growth factors, FGF is involved in bone remodeling (Canalis *et al.*,1988). Members of the fibroblast growth factor (FGFs) family have been shown to be important in both normal bone remodeling and in pathologic disorders of bone (Baird *et al.,*1989: Gospodarowicz *et al.,* 1990: Noda *et al.,*1991). Basic fibroblast growth factor (bFGF) is a potent regulator of both bone formation (Hurley *et al.*,1992; Canalis *et al.*,1988) and bone resorption (Simmons *et al.*,1991).

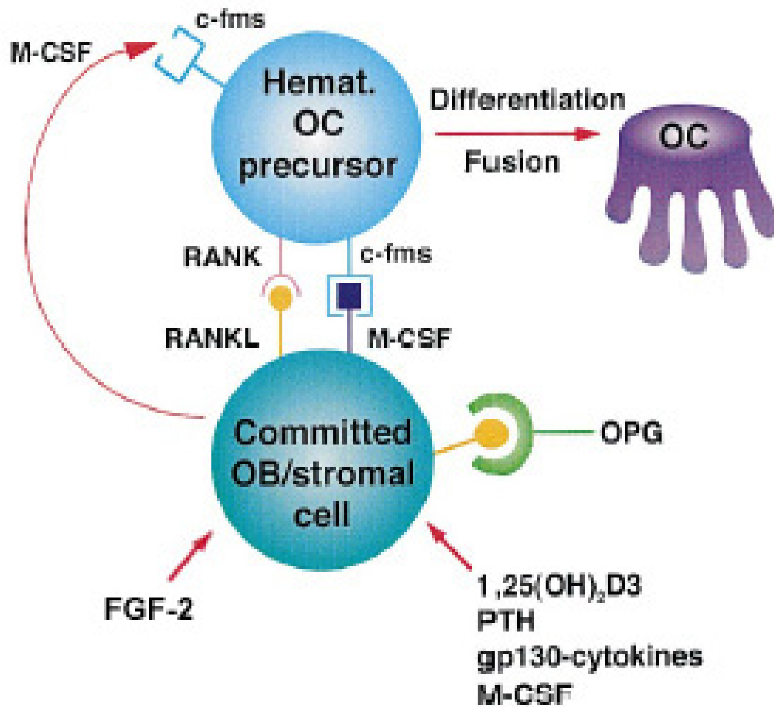


Fig. 1. A model of cellular interactions for osteoclastogenesis. Stromal cells and osteoblasts (committed OB: stromal cell) express RANKL on their cell surface. RANKL can be regulated 1α,25$(OH)_2D_3$ ($VitD_3$), parathyroid hormone (PTH), and cytokines which use gp130 as part of their receptor, like IL-6 and Oncostatin M (gp130 cytokines).

bFGF is produced by bone cells (Globus *et al.*,1989; Hurly *et al.*,1994) and stored in extracellular matrix (Hauschka *et al.*,1986). In addition, it is a potent mitogen for a wide variety of cells, including osteoblasts/stromal cells (Canalis E. *et al.*,1988; Globus *et al.*,1988; Oliver *et al.*,1990), and a major inhibitor of type I collagen synthesis in osteoblastic cells (Hurley MM. *et al.*,1993). Nevertheless, little is known about the physiological roles of bFGF in bone remodeling because of its multiple effects on bone cells.

Of FGFs, FGF-2 is well known as a potent regulator of functions of bone and cartilage cells. It acts as an autocrine/paracrine factor for bone cells (Canalis *et al.*, 1988; Globus *et al.*, 1988; Oliver *et al.*, 1990). In regards to the effects of FGF-2 on bone cells, it has been reported that the exogenous application of FGF-2 has stimulatory effects on bone formation in several vivo model. Paradoxically, FGF-2 is also known as a potent stimulator of bone resorption (Simmons *et al.*, 1991; Kawagushi *et al.*, 1995; Hurley *et al.*, 1998) and is involved in joint destruction of rheumatoid arthritis patients (Manabe *et al.*, 1999). The stimulatory effect of FGF-2 on osteoclast formation is mediated by the induction of cyclooxygenase-2, a main regulatory enzyme for prostaglandin production in bone, and receptor activator of nuclear factor-kB ligand (RANKL), a key

membrane-associated molecule regulating osteoclast differentiation in osteoblasts (Lacey *et al.*, 1998: Yasuda *et al.*, 1998; Wong *et al.*, 1997). On the other hand, there is another possibility that FGF-2 acts directly on mature osteoclasts to stimulate bone resorption (Kawaguchi *et al.*,2000; Chikazu *et al.*, 2000) Conclusively, the response of FGF-2 to bone cell is still controversial as far as osteoclast differentiation concerns. Therefore, the understanding about the roles of FGF-2 in both physiological and pathological conditions are required for the application of FGF-2 to such metabolic bone diseases as osteopetrosis and osteoporosis. Theoretically, since $1\alpha,25(OH)_2D_3$ up-regulates RANKL expression and down-regulates OPG expression in osteoblasts/stromal cells, it is suspected that bFGF regulates the expression of these two factors as well. Furthermore, In the present study, we analyzed the effects of FGF-2 on 1) osteoclast formation, 2) alkaline phosphatase activity, 3) the expression of RANKL, M-CSF and OPG mRNA and 4) ADAM8, MMP13 mRNA expression to evaluate the whether FGF-2 is to do with the extracellular matrix metabolism in coculture system.

Ⅱ. Materials and methods

1. Materials

All routine cell culture media were obtained from GIBCO/BRL (Grand Island, NY). The Tartrate-resistant acid phosphatase staining kit was purchased from the Sigma Chemical Co., Ltd. (St. Louis, MO). FGF-2 was purchased from Promega. The ICR mice were purchased from Samtacho Co., Ltd. (Seoul, Korea). All other chemicals were of the highest grade commercially available.

2. Methods

1) *In vitro* formation assay of osteoclast

The osteoblast formation assay was carried out as previously reported Choi *et al.* (2001) (Fig. 2). Briefly, the osteoblasts were isolated from 1～2 day-old newborn mice. 30～50 calvariae were digested in 10 ml of an enzyme solution containing 0.2% collagenase (Wako, Japan) and 0.1% dispase (GiBCO BRL, U.S.A) for 20 minutes at 37 ℃ in a shaking water bath. The supernatant was discarded and 10 ml of the enzyme solution was added. After shaking at 37 ℃ for 20 minutes, the supernatant was collected carefully and transferred to a new tube. This digestion of calvariae by collagenase-dispase was repeated three

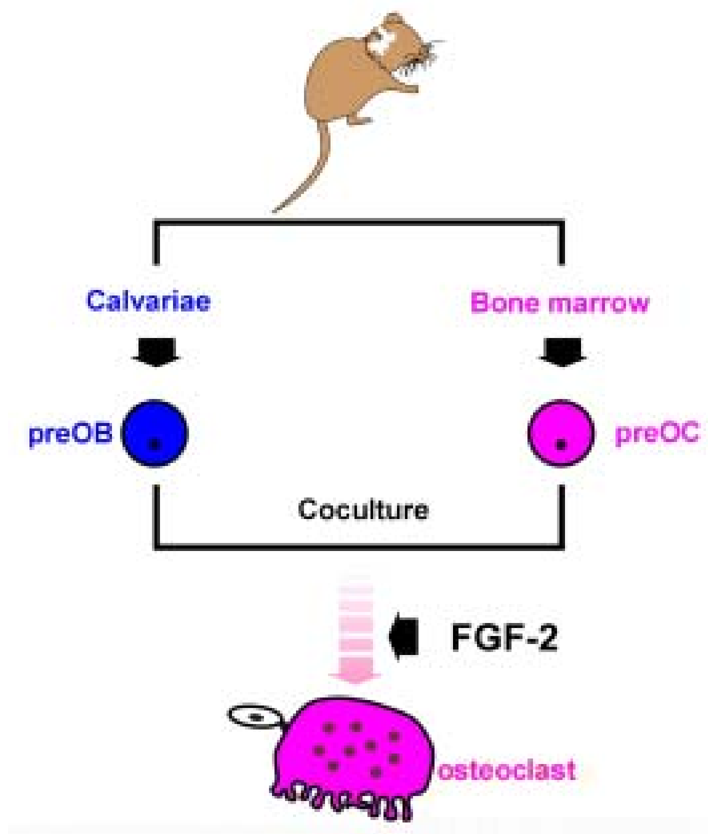


Fig. 2. Coculture system of osteoblast and bone marrow cells. Mouse calvarial cells (1×10^4 cells/well) were cocultured with bone marrow cells (1×10^5 cells/well) in α-MEM containing 10 % fetal calf serum in 48-well plates. All cultures were maintained at 37。C in a humidified atmosphere of 5 % CO_2 in air. After treatment, the cells were subjected to tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts) staining.

times. The collected was placed in a centrifuge at 1,500×g for 10 minutes to collect the osteoblastic cells. The cells were resuspended in α-MEM containing 10% fetal calf serum (FCS) and cultured in 10 cm culture dishes at a concentration of 10^5 cells/dish to confluence. The cells were then detached from the culture dishes by trypsin-EDTA, suspended in α-MEM with 10% FCS and used for the coculture as osteoblastic cells.

Femorial and tibial bone marrow cells were collected from 4-week-old mice. The tibiae and femora were removed and dissected free of adhering tissues. The bone ends were removed and the marrow cavity was flushed by slowly injecting media at one end using a 25-gauge needle. The calvariae and bone marrow cells collected were washed and used for the coculture.

Mouse calvarial cells (1×10^4 cells/well) were cocultured with bone marrow cells (1×10^5 cells/well) in α-MEM containing 10% fetal calf serum in 48-well plates (Corning Inc., Corning, NY). The culture volume was made up to 400 ㎕ per well with α-MEM supplemented with 10% fetal calf serum (FCS), in the presence of FGF-2. All cultures were maintained at 37 ℃ in a humidified atmosphere containing 5% CO_2 in air. After treatment, the cells were subjected to tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts) staining.

2) MTT assays

MTT (3-[4,5-dimethylthiazol-2-yl-]-2,5-diphenyltetrazolium bromide) test uses the principle that tetrazolium salts are reduced by a reducing enzyme (succinate dehydrogenase) from the mitochondria so that the toxicity of the viable cells and the level of cellular proliferation can be measured. MTT was dissolved in phosphate-buffered saline (PBS) at 5 mg/ml and filtered to remove any insoluble residue. The MTT solution was added directly to the assay plates at a rate of 10 ㎕ to 100 ㎕ of the cell culture medium. The cells were subsequently incubated for an additional 4 hours at 37 ℃. The purple formazan crystals formed were dissolved in DMSO(dimetyl sulfoxide) followed by thorough mixing. The plates were subsequently read on a spectrophotometer at 570 nm (measurement) and 630 nm (reference).

3) Measurement of alkaline phosphatase activity (ALPase activity)

We tested the effect of FGF-2 on osteoblast differentiation. ALPase activity as a marker enzyme of osteoblast was measured to evaluate the osteoblast differenciation. Cells were cultured in 6-well plates for 4 days. The cultured cells which removed from plates at every 4 days, washed 2 times with ice-cold phosphate-buffered saline (PBS), and the cell layer in each well

was scraped into 1 ml of ice-cold PBS. After the addition of 1 ml of 1% Triton X-100 at 4 ℃, stood on the ice. The temperature of the reaction mixture (Sigma 387-A) maintained at the assay temperature of 30 ℃. The conversion of substrate by ALPase was measured at 405 nm using a microtiter plate reader. All samples were blanked against reaction wells previously containing conditioned media alone. Protein concentration was determined by using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with BSA as the standard ALPase activities which were expressed as p-nitrophenol produced in millimoles per minute per milligrams of protein.

4) RT-PCR

Mouse calvarial cells were cultured on 100 mm dishes in α-MEM containing 10% FBS, 10 ng/ml of $VitD_3$, and varies concentration of FGF-2 for days. The expressions of RANKL, OPG, M-CSF and β-actin were evaluated by RT-PCR using total RNA isolated from murine osteoblastic cells. The primers used were : for RANKL (750 bp) 5'-ATC AGA AGA CAG CAC TCA CT-3' (forward), 5'-ATC TAG GAC ATC CAT GCT AAT GTT C-3' (reverse); for OPG (636 bp) 5'-TGA GTG TGA GGA AGG GCG TTA C-3' (forward), 5'-TTC CTC GTT CTC TCA ATC TC-3' (reverse); for M-CSF 5'-ATG ACA AGG CCT GCG

TCC GA-3' (forward), 5'-AAG CTC TGG CAG GTG CTC CTG-3' (reverse, 395 bp) and 5'-GCC GCC TCC ACC TGT AGA ACA-3' (reverse, 286 bp); for β-actin (366 bp) 5'-GGA CTC CTA TGG TGG GTG ACG AGG-3' (forward), 5'-GGG AGA GCA TAG CCC TCG TAG AT-3' (reverse). In addition, ADAM8 and MMP13 mRNA expression were also analysed. The following primer were used for ADAM8 (350 bp) 5'-CAT AGT GAA ACC AAA GAG GCC-3' (forward), 5'-ATA GGA GCA GTG GTA TCT CC-3' (reverse).

5) Data analysis

The statistical analyses for the differences between the groups were carried using one way analysis of the Mann-Whitney U test. For all statistical tests, a p value < 0.05 was considered to be statistically significant.

Ⅲ. Results

1. Effect of FGF-2 on the osteoclast formation in co-cultures

We examined whether FGF-2 induces osteoclast formation in cocultures of mouse calvariae-derived osteoblastic cells and bone marrow cells (Fig. 3 and 4). There were no TRAP-positive cells in untreated cocultures (in the absence of $VitD_3$). In contrast, numerous TRAP-positive cells were observed in $VitD_3$ or FGF-2-treated co-cultures, some of which developed to be multinucleated cells. To induce the mature osteoclast from the osteoclast precursor cells, 10 nM of $1\alpha,25(OH)_2D_3$ was administrated to the coculture system. As shown in Fig. 4, multinucleated osteoclasts (MNC) were formed when treated with $VitD_3$ in cocultures. When several concentrations of FGF-2 (0.1, 1, 10, and 100 ng/ml) were added to the coculture system, osteoclastogenesis is inhibited at the low concentration of FGF-2 (0.1, 1 ng/ml) while stimulated or not changed at the high concentration of FGF-2 (10, 100 ng/ml). This implies FGF-2 induced or inhibited osteoclastogenesis, depending upon the dose to be treated. However, it might be possible that FGF-2 causes cell damage directly without interrupting the normal maturation of osteoclasts. To confirm this possibility, we used a viability test using MTT method. As shown

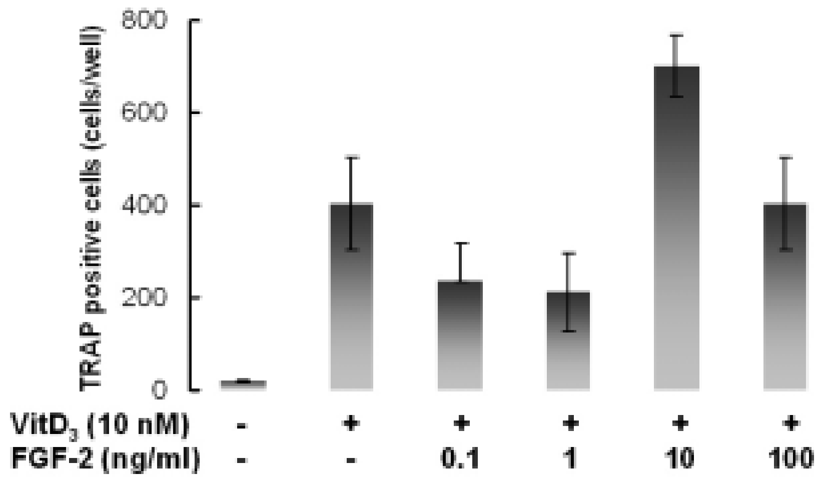


Fig. 3. Osteoclast differentiation in the presence of FGF-2 at various concentrations. FGF-2 was added to the osteoblast/bone marrow coculture with 10 nM of 1α,25$(OH)_2D_3$. After incubation for additional 4 days, cells were stained with TRAP staining method. TRAP positive multinucleated cells that have more than 3 nucleus were counted. Vertical bars represent mean±standard error.

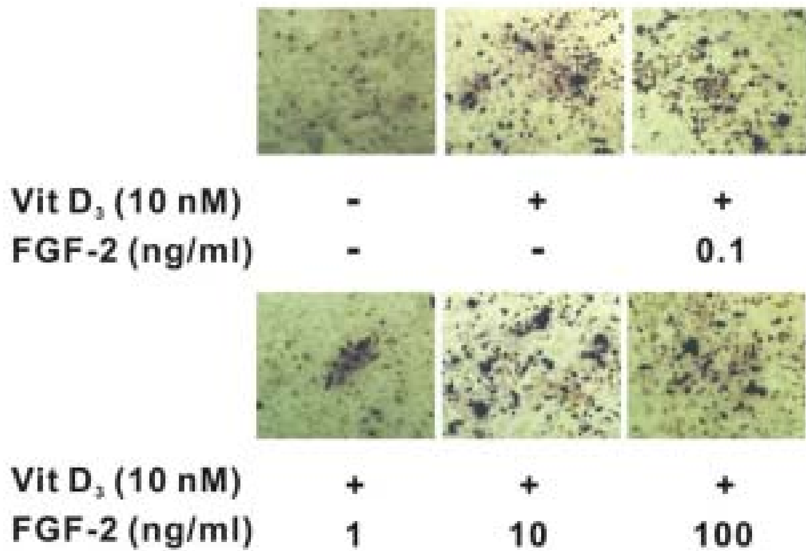


Fig. 4. Formation of multi-nucleated osteoclast in the presence of FGF-2. Cells were treated with FGF-2 and/or $1\alpha,25(OH)_2D_3$. After incubation for additional 4 days, cells were stained with TRAP staining method.

in Fig. 5, FGF-2 did not exert a toxic effect in the entire range of concentration (0.1, 1, 10, and 100 nM). This results suggest that the effect of FGF-2 on differentiation was caused by changing its physiological activity, not by its direct toxic effect upon the cells.

2. Changes in alkaline phosphatase activity in osteoblastic cells

To investigate whether the FGF-2 induce the changes in osteoblast differentiation, ALPase activity was measured. As shown in Fig. 6, FGF-2 decreased the ALPase activity in osteoblastic cells.

3. Alteration of RANKL, OPG and M-CSF mRNA expression by FGF-2

Osteoclastogenesis is in part mediated and modulated by RANKL, OPG and M-CSF secreted from the osteoblastic cells. To confirm the effect of FGF-2 on bone cells, FGF-2 was added to the osteoblastic cell, then expressions of RANKL, OPG and M-CSF mRNAs were monitored by RT-PCR after 96 hours of incubation. FGF-2 at the low concentration (1 ng/ml) resulted in the reduction of the RANKL expression level (Fig. 7). On the other hand, OPG expression (Fig. 8), a decoy receptor, which binds to RANKL and blocks RANK

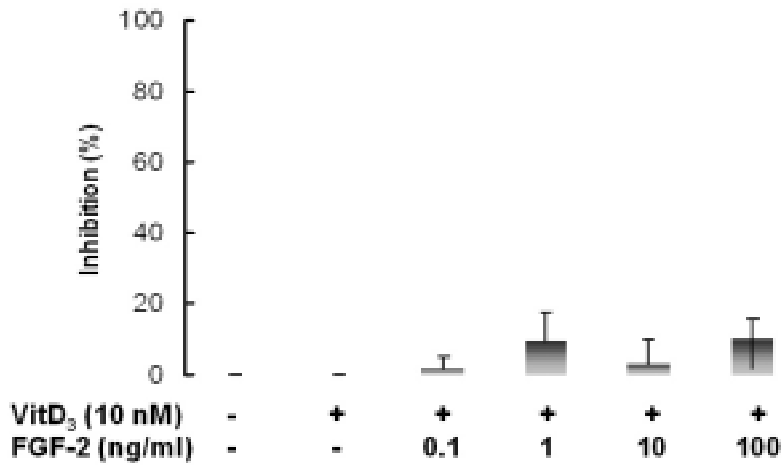


Fig. 5. Cell proliferation in the presence of FGF-2 at various concentrations. FGF-2 was added to the osteoblast/bone marrow coculture with 10 nM of $1\alpha,25(OH)_2D_3$. MTT assays were carried out according to the method described in materials and methods. Vertical bars represent mean± standard error.

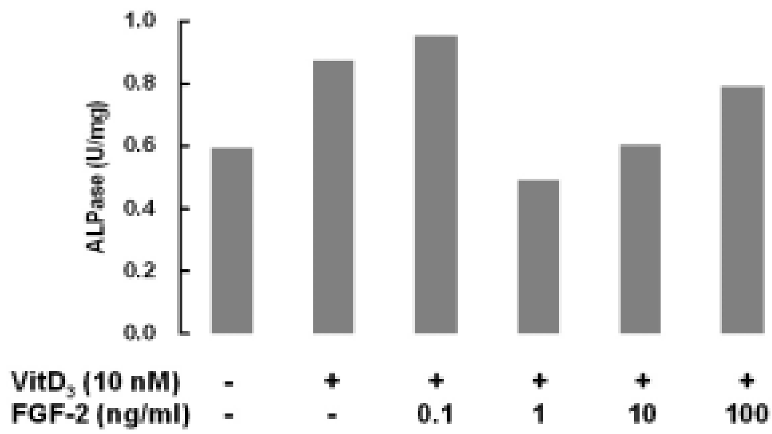
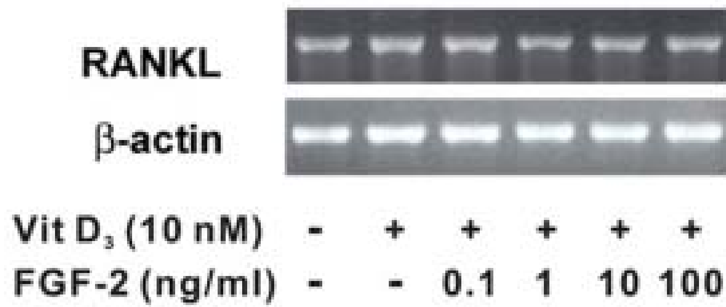


Fig. 6. Effect of FGF-2 on alkaline phosphatase activity of osteoblastic cells. Various concentrations of FGF-2 were added to the osteoblast culture with 10 nM $1\alpha,25(OH)_2D_3$. After incubation for additional 4 days, whole cell broth was harvested and ALPase activity was assayed according to the method described in materials and methods. This is a representative data of the experiments.

A



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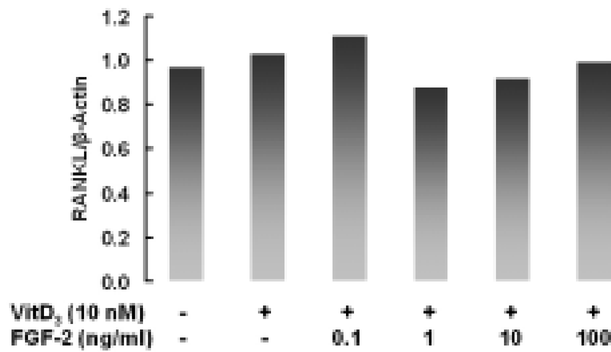


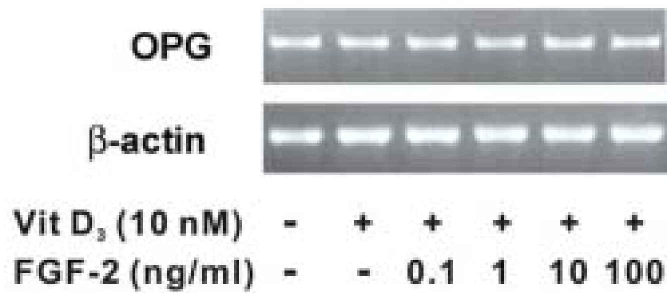
Fig. 7. Effect of FGF-2 on the RANKL expression of osteoblastic cells. Various concentrations of FGF-2 were added to the osteoblast culture with 10 nM $1\alpha,25(OH)_2D_3$. After incubation for additional 4 days, total RNA was isolated. mRNAs were amplified according to the method described in materials and methods. Panels A, agarose gel electrophoresis; B, Relative density of RANKL mRNA expression. This is a representative data of the experiments.

stimulation, and M-CSF (Fig. 9) did not change as the FGF-2 concentration was increased. The mRNA expression ratio of RANKL/OPG ratio was decreased at 1 ng/ml of FGF-2 but increased at higher concentrations (Fig. 10).

4. ADAM and MMP13 mRNA expression in osteoblastic cells

With osteoblastic cells, the changes in ADAM8 and MMP13 mRNA expressions were monitored. Although the concentration of FGF-2 that was added to the medium increased the expression of ADAM8 mRNA did not show any significant change (Fig. 11). In case of MMP13, mRNA expression was repressed only at the concentration of 100 ng/ml (Fig. 12). However, any correlation between FGF-2 and MMP13 was not detected.

A



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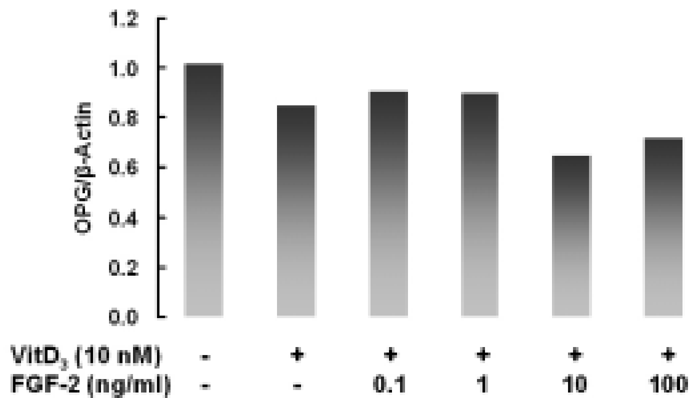
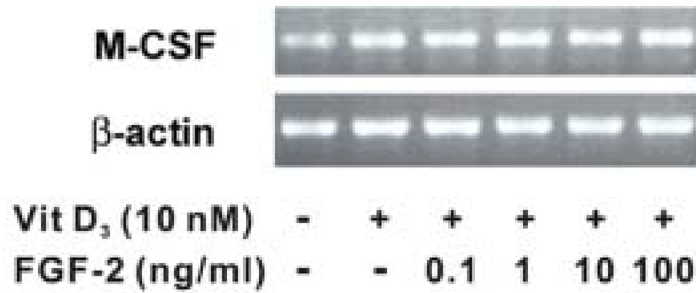


Fig. 8. Effect of FGF-2 on the OPG expression of osteoblastic cells. Various concentrations of FGF-2 were added to the osteoblast culture with 10 nM 1α,25$(OH)_2D_3$. After incubation for additional 4 days, total RNA was isolated. mRNAs were amplified according to the method described in materials and methods. Panels A, agarose gel electrophoresis; B, Relative density of OPG mRNA expression. This is a representative data of the experiments.

A



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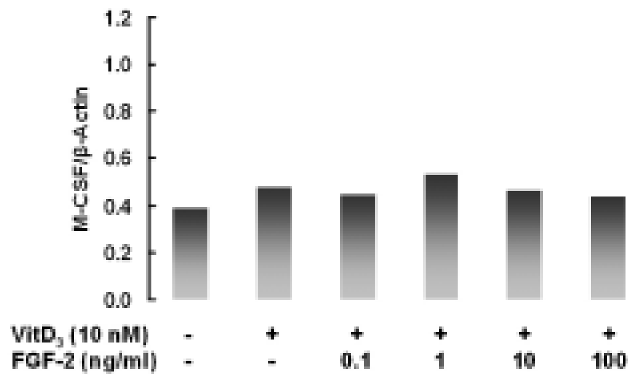


Fig. 9. Effect of FGF-2 on the M-CSF expression of osteoblastic cells. Various concentrations of FGF-2 were added to the osteoblast culture with 10 nM $1\alpha,25(OH)_2D_3$. After incubation for additional 4 days, total RNA was isolated. mRNAs were amplified according to the method described in materials and methods. Panels A, agarose gel electrophoresis; B, Relative density of M-CSF mRNA expression. This is a representative data of the experiments.

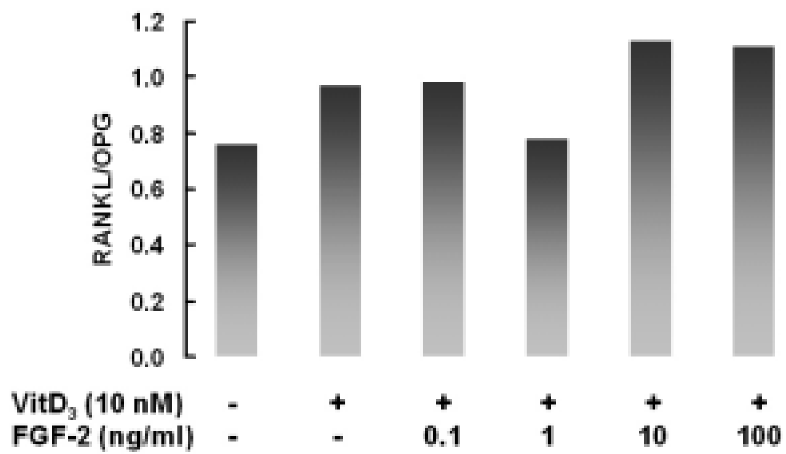
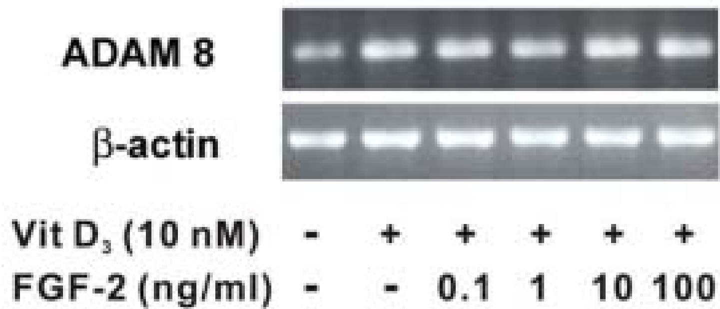


Fig. 10. Relative density of RANKL and OPG mRNA expressions. Agarose gel electrophoresis was carried out and densities of the bands were analyzed with densitometer. Relative ratio of RANKL and OPG were calculated based on the density of each band. This is a representative data of the experiments.

A



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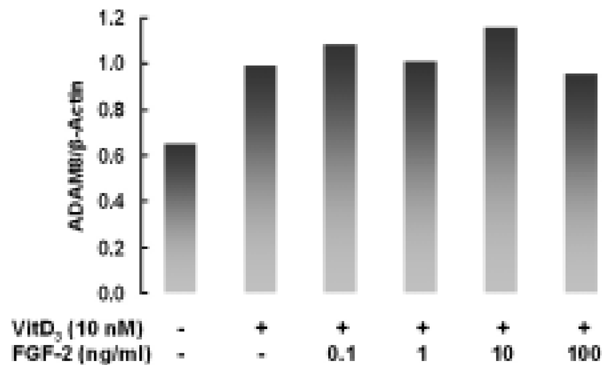
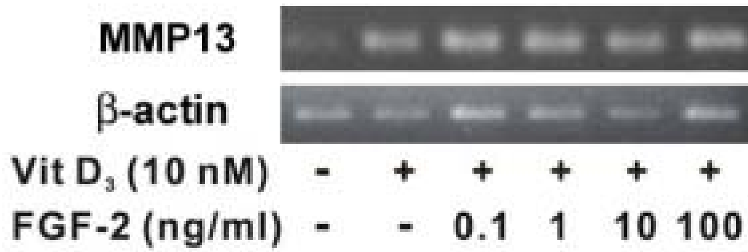


Fig. 11. Effect of FGF-2 on the ADAM8 expression of osteoblastic cells. Various concentrations of FGF-2 were added to the osteoblast culture with 10 nM $1\alpha,25(OH)_2D_3$. After incubation for additional 4 days, total RNA was isolated. mRNAs were amplified according to the method described in materials and methods. Panels A, agarose gel electrophoresis; B, Relative density of ADAM8 mRNA expression. This is a representative data of the experiments.

A



B

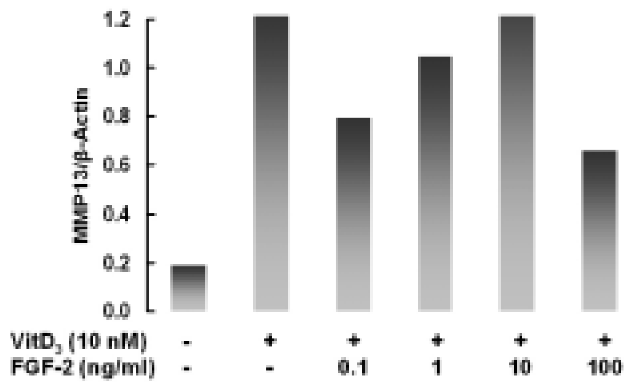


Fig. 12. Effect of FGF-2 on the MMP13 expression of osteoblastic cells. Various concentrations of FGF-2 were added to the osteoblast culture with 10 nM $1\alpha,25(OH)_2D_3$. After incubation for additional 2 days, total RNA was isolated. mRNAs were amplified according to the method described in materials and methods. Panels A, agarose gel electrophoresis; B, Relative density of MMP13 mRNA expression. This is a representative data of the experiments.

Ⅳ. Discussion

It has been recognized that RANKL expressed on osteoblasts/stromal cells mediates an essential signal to osteoclast progenitors for their differentiation into active osteoclasts through RANK and OPG, a decoy receptor for RANKL, inhibits the RANKL signaling as a competitor of RANK (Lacey *et al.*, 1998; Hofbauer *et al.*, 2000). With regards to the regulation of osteoclastogenesis, the FGF-2 response is controversal or not consistent in terms of bone formation and bone resorption. In fact, the effect of FGF-2 on the osteoclastogenesis based on OPG/RANKL/RANK system is not understood well. Therefore, we examined the changes in $VitD_3$-induced osteoclast formation by the FGF-2 and evaluate its response by monitoring the OPG/RANKL/RANK system in this study. Firstly, a variety of FGF-2 concentration (1-100 ng/ml) were treated in coculture system. As shown in Fig 3, $VitD_3$-induced osteoclastogenesis was inhibited by the low concentration of FGF-2 (0.1, 1 ng/ml), while stimulate rather than the inhibition of osteoclastogenesis in the case of high concentration of FGF-2 (10 ng/ml). Obviously, the $VitD_3$-induced osteoclastogenesis was regulated by FGF-2, showing the opposite effects (inhibition or stimulation of osteoclasto-genesis) at a low or high concentration of FGF-2. Such a dual

effect of FGF-2 on osteoclastogenesis makes the investigators confused. For instances, a series of bone metabolism studies *in vivo* have indicated that FGF-2 has stimulatory effect on bone formation (Aspenberg *et al.,* 1989: Kawaguchi *et al.,* 1994: Kawaguchi *et al.,* 2001). On contrary, Hurley *et al.* (1998) recently reported that FGF-2 (10^{-8} M/l) induces osteoclast formation in mouse bone marrow cultures. Moreover, several lines of evidences suggest that FGF-2 (10^{-9} M) can generate the bone resorption (Kawaguchi *et al.,* 2000: Chikazu *et al.*, 2000), reflecting the complexity and dual effects of osteoclastogenesis.

From the all of previous results, FGF-2 might have dual effect on bone metabolism (bone formation and bone resorption), showing the different response on osteoclastogenesis as a function of concentration (Fig. 3). We confirmed the different effects of FGF-2 on osteoclastogenesis from the individual repetitive trials which showed the same patterns with the dual responses. Tentatively, It might be speculated that FGF-2 responses on osteoclastogenesis depend upon the concentration to be administrated in co-culture system. Although FGF-2 concentration is critical to modulate the osteoclastogenesis, we focused on the regulation of osteoclastogenesis by low concentration of FGF-2 which could be near to the ideal concentration *in vivo*. Again, as shown in Fig. 3, FGF-2 at low concentrations inhibited the

osteoclastogenesis on the basis of osteoclast formation. Thus we have been in search of the regulation in OPG/RANKL/RANK system which is essential for the typical osteoclastogenesis.

The question was what mechanism is involved in the inhibition of osteoclastogenesis by FGF-2 at low concentrations. Here, FGF-2 decreased the RANKL mRNA expression (Fig. 7), while no significant differences on OPG and M-CSF mRNA expression were monitored in RT-PCR. With quantitative aspects, the inhibitory effect of FGF-2 on RANKL mRNA expression did not seem to be consistent with the blocking ability of osteoclast formation in response to FGF-2 (Fig. 3). These findings indicate other possible routes might be speculated as regulatory pathways of osteoclastogenesis as well as OPG/RANKL/RANK system. Recently, alternative pathway with the new concept on the regulation of osteoclastogenesis began to be in consideration since the possibility for OPG/RANKL/RANK-independent pathway was also accepted to explain the osteoclastogenesis induced by bacterial LPS (Jiang *et al.*, 2002). Consequently, the osteoblast may govern the osteoclastogenesis via not only OPG/RANKL/ RANK system, but also RANKL-independent system (for example, LPS) by which osteoclastogenesis is introduced.

Unlike RANKL mRNA, OPG and M-CSF mRNA was not changed by the FGF-2. Of cause, FGF-2 reduced OPG mRNA

expression at high concentration, being consistent with stimulation of osteoclast formation (Fig. 8). However, we could not found any changes in M-CSF at a given FGF-2 concentration (Fig. 9). Conclusively FGF-2 decreases the osteoclastogenesis, down-regulating primarily the RANKL mRNA in osteoblastic cells, not affecting the osteoclast maturation. In regard to osteoblast differentiation, ALPase activity also was analysed (Fig. 6). As shown in Fig. 6, FGF-2 decreased the ALPase activity in osteoblastic cells, suggesting that FGF-2 is not the essential factor for the osteoblast differentiation. But it still does not clear how the osteoblast differentiation is coupled to osteoclast formation in terms of regulation of osteoclastogenesis.

As described the above, FGF-2 inhibits the RANKL mRNA expression in osteoblastic cells. Nevertheless, Kawaguchi *et al.* (2000) demonstrated that the expression of RANKL mRNA was up-regulated by FGF-2 in osteoblastic cells. Consistent with the results in the Northern blot analysis, ^{125}I-OPG binding analysis revealed that treatment of osteoblastic cells with FGF-2 for 6 days induced RANKL production in the cells, and the production was markedly inhibited by addition of NS-398 which is an inhibitor of cyclooxygenase-2 (COX-2). Taken together with these previous findings, PGE_2 stimulates RANKL mRNA expression and RANKL production in mouse primary osteoblastic cells,

suggesting that FGF-2 stimulates RANKL production in osteoblastic cells through COX-2-mediated PG synthesis, and induces osteoclastogenesis in the co-cultures. Furthermore, it was shown that FGF-2 suppressed OPG expression in osteoblastic cells (Kawaguchi *et al.,* 2000). The decrease in OPG mRNA expression and OPG production was not recovered by addition of NS-398, indicating that the FGF-2-induced suppression of OPG expression was not mediated by PG synthesis. In contrast to these results, no remarkable changes were observed in OPG mRNA expression with various FGF-2 concentrations in this study (Fig. 8). Accordingly, FGF-2 response on osteoclastogenesis might be controversal when it comes to gene expression of RANKL in osteoblasts/stromal cells. With the above results, it is clear that FGF-2 plays an important role in bone remodeling by modulating the production of RANKL in osteoblastic cells. But, further studies are required to elucidate the molecular mechanisms by which FGF-2 regulates the expression of RANKL and OPG.

In general, fibroblast growth factors (FGFs) are potent mitogens for a wide variety of cells of mesenchymal and neuroectodermal origin. FGFs also play a role in the differentiation of a variety of cells and are involved in morphologenesis, angiogenesis, and development. The FGF family now consists of 23 members, FGF-1 to FGF-23, and there are 4

structurally related high-affinity receptors (FGFR1 to FGFR4) belonging to receptor tyrosine kinases that have an intrinsic protein tyrosine kinase activity and elicit tyrosine auto-phosphorylation of the receptor. Recent reports showing that mutations of FGFRs cause several genetic diseases with severe impairment of bone and cartilage formation, such as achondroplasia and thana-tophoric dysplasia type II, indicate the essential role of FGF signalings on bone and cartilage metabolism. The target cells mediating the effects of FGF-2 on osteoclast formation in the co-cultures were found to be osteoblastic cells, but not osteoclast progenitors. However, some studies suggest that fibroblast growth factor -2 (FGF-2) has an osteogenic effect. FGF-2 is synthesized by osteoblasts and deposited in bone matrix. This growth factor enhances the proliferation of osteoprogenitor and osteoblast-like cells and induces formation of bone-like nodules in cultures of bone marrow cells. Similar skeletal effects were observed in vivo in young and aged rats treated with bFGF. For example, systemic injections of the growth factor increased the osteoblast population and cancellous bone mass in intact rats. In addition, new bone spicules were observed in the marrow cavity of these animals. Like the above results, bone metabolism seems to be very complicated, and controversal as discussed previously, depending

upon the cell type, *in vivo* or *in vitro* experiment, and culture types. In spite of that, we can evaluate the final destination of bone remodeling (bone formation or bone resorption) as a RANKL/OPG ratio at a given circumstances. In Fig 10, at a low concentration FGF-2 decreased RANKL/OPG ratio, whereas at high concentrations increased RANKL/OPG ratio. Besides the OPG/RANKL/OPG system, A disintegrin and metallo-proteinase (ADAM8), and matrix metalloproteinase 13 (MMP13) which are responsible for osteoclast differentiation and degradation of extracellular matrix (ECM) respectively were examined by RT-PCR in the presence of FGF-2. MMP13 mRNA expression was reduced by the treatment of low concentration of FGF-2 suggesting that FGF-2 could be in charge of the protection of collagenase activity. However, no remarkable changes in ADAM8 was noticed.

In summary, the balance between osteoblast and osteoclast functions is regulated systematically by a variety of hormone and locally by the production of paracrine factors by osteoblast or bone marrow stromal cells that regulate osteoclast functions. The development of osteoclast requires the intimate contact between osteoblastic stromal cells and osteoclast precursors. RANKL, RANK, and OPG recently identified as regulators of osteoclastogenesis. Here, FGF-2 at a low concentration resulted in

the inhibition of osteoclastogenesis by the decrement of RANKL/OPG ratio, but FGF-2 at a high concentration stimulates the osteoclastogenesis through the enhancement of RANKL/OPG, showing the dual effects. Taken all together, these findings suggest that FGF-2 on osteoclastogenesis seems to have a dual effects by the modulating the RANKL/OPG ratio including the MMP13. However, underlying mechanism for the dual effect of FGF-2 remains to be solved.

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국문요약

Regulator Mechanism of 1α,25$(OH)_2D_3$-Induced Osteoclastogenesis by FGF-2

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김현아

이 연구는 골세포와 연골세포의 기능에 있어 강력한 조절인자인 FGF-2의 작용을 규명하였다. 여러 종류의 FGF 중 FGF-2는 골세포에서 autocrine, 또는 paracrine 방식으로 작용하며, 이것이 골 대사, 골 형성과 흡수에 미치는 반응에 관해서는 논쟁의 여지가 있다. 따라서, 쥐의 두개골에서 분리한 조골세포와 골수세포와의 혼합배양에서 유도되는 파골세포 형성과정 중 FGF-2의 작용을 검토하였다. $VitD_3$에 의한 파골세포 형성 유도시 다수의 TRAP 양성세포를 관찰할 수 있었으며, FGF-2 (0.1, 1,10 and 100 ng/ml) 첨가시 낮은 농도 (0.1, 1 ng/ml)에서는 $VitD_3$에 의하여 유도된 파골세포 형성이 억제되었고 높은농도 (10, 100 ng/ml)에서는 변화를 보이지 않거나 촉진되었다. 또한, 실험에 사용된 FGF-2의 모든 농도에서 어떠한 세포독성도 나타내지 않았다. 따라서, 이는 파골세포 형성의 억제는 직접적인 독성작용에 의하지 않고 생리적 작용의 변화에 기인한 것임을 나타낸다.

FGF-2를 조골세포에 첨가하여 RANKL, OPG 및 M-CSF의 mRNA 발현을 측정한 결과 낮은 농도에서는 RANKL mRNA발현이 감소하였으나 OPG와 M-CSF는 큰 변화를 보이지 않았다. 그밖에도 FGF-2

는 조골세포의 분화정도를 가늠하는 alkaline phosphatase와 세포외기질의 분해에 관련된 효소인 MMP13 (matrix metalloproteinase 13)의 mRNA발현을 감소시키고 파골세포 분화와 관련 있는 ADAM8 (A disintegrin and metalloprotease)의 발현에는 영향을 미치지 않는 것으로 나타났다. 이상의 결과로부터, FGF-2는 파골세포 형성과정에 있어 이중 효과 (dual effect)를 보이며, 특히 낮은 농도에서는 조골세포의 RANKL과 MMP13의 mRNA발현을 감소시킴으로써 파골세포의 형성을 억제한다.

핵심되는 말 : FGF-2, 파골세포형성, RANKL, 이중효과