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Effect of Osteotropic Agents on the Expression of RANKL and OPG in Saos-2 Cells

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Various osteotropic agents that influence bone resorption are known to act primarily via osteoblasts/stromal cells. Recently, receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) have been suggested to be key molecules that regulate osteoclast differentiation and activation. RANKL induces osteoclastogenesis and activates mature osteoclasts while OPG acts as a physiologic inhibitor of RANKL. It is conceivable, therefore, that change in RANKL and OPG expression in osteoblasts/stromal cells affect their ability to support osteoclast formation, activity and survival. In this study, we examined the effects of several osteotropic agents on RANKL and OPG mRNA expression in Saos-2 human osteoblastic cells. Cells were exposed to parathyroid hormone (PTH, 10^{-8} M), 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$, 10^{-8} M), dexamethasone (10^{-8} M), interleukin-1β (IL-1β, 5 ng/ml), tumor necrosis factor-α (TNF-α, 5 ng/ml), transforming growth factor-β (TGF-β, 1 ng/ml), or insulin-like growth factor-I (IGF-I, 10 ng/ml) for 2, 4, 8, and 24 h, and mRNA levels were analyzed by semi-quantitative reverse transcription-polymerase chain reaction. All the tested osteotropic agents more or less regulated both RANKL and OPG mRNA level during the examined period. RANKL/OPG ratio was up-regulated by PTH, 1,25$(OH)_2D_3$, dexamethasone, TGF-β, IGF-I, and increased RANKL/OPG ratio was maintained up to 24 h. IL-1β and TNF-α transiently up-regulated RANKL/OPG ratio at 2 h but thereafter they greatly decreased RANKL/OPG ratio. These results showed that RANKL and OPG could be potential targets for bone resorption regulation by osteotropic hormones, cytokines, and growth factors. However, regulatory patterns were not always coincident with *in vivo* or *in vitro* effects on osteoclastogenesis, implying that RANKL and OPG are not the sole mediators of their action.

Keywords: RANKL, OPG, osteotropic agents, osteoblast

Introduction

In the bone microenvironment, there is a dynamic balance between resorption and formation that maintains skeletal homeostasis. Osteoclasts, which are present only in bone, are multinucleated giant cells with the capacity to resorb mineralized tissues. The development of active osteoclasts *in vitro* requires intimate contact between osteoblastic stromal cells and osteoclast precursors of the monocyte/macrophage lineage (Takahashi *et al.*, 1988). This process is influenced by a variety of osteotropic factors including parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), glucocorticoids, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and insulin-like growth factor I (IGF-I) (Roodman, 1996). Most of these factors have been known to affect osteoclast differentiation by acting on the osteoblast population, rather than on the osteoclast precursors (Suda *et al.*, 1992).

Recently, two regulatory molecules produced by osteoblast lineage cells were suggested to play important roles in osteoclastogenesis. One of these is receptor activator of nuclear factor-κB ligand (RANKL), which is identical to osteoprotegrin ligand (OPGL), TNF-related activation-induced cytokine (TRANCE) and osteoclast differentiation

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factor (ODF) (Anderson *et al.*, 1997; Wong *et al.*, 1997; Lacey *et al.*, 1998; Yasuda *et al.*, 1998b). RANKL is a membrane-associated protein and stimulates osteoclast formation and activation by binding to its specific receptor, RANK, on the surface of osteoclast (Hsu *et al.*, 1999). The other molecule is OPG (Simonet *et al.*, 1997), which is identical to osteoclastogenesis inhibitory factor (OCIF) (Tasuda *et al.*, 1997). OPG is a secretory protein that acts as a decoy receptor by binding RANKL and blocking the interaction between RANKL and RANK. Thus, OPG inhibits not only formation of osteoclast but also bone resorption by osteoclasts both *in vitro* and *in vivo* (Simonet *et al.*, 1997; Yasuda *et al.*, 1998a). Based on these reports, it has been suggested that RANKL and OPG produced by osteoblastic cells are key factors in osteoclast formation and function, regulated by osteotropic agents (Horwood *et al.*, 1998; Nagai and Sato, 1999). Moreover, osteotropic agents have been documented to modulate osteoclast formation through the regulation of the ratio of RANKL/OPG expression rather than increasing or decreasing RANKL and/or OPG alone (Nagai and Sato, 1999; Hofbauer *et al.*, 2000).

In the present study, we examined the effects of several osteotropic agents on RANKL and OPG mRNA expression in Saos-2 cells to investigate whether these agents regulate bone metabolism through the regulation of RANKL and OPG expression. Our results suggested that RANKL and OPG could be targets for bone resorption regulation by osteotropic hormones, cytokines, and growth factors, though they might not be the sole mediators.

Materials and Methods

Materials

DMEM, fetal bovine serum (FBS), Superscript™ First-Strand Synthesis System, and other culture reagents were purchased from Gibco-BRL (Grand Island, NY, USA). Recombinant human (rh) IL-1β, rh TNF-α, rh TGF-β1 and rh IGF-I from R&D systems (Minneapoils, MN, USA); 1,25$(OH)_2D_3$ from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA); dexamethasone from Sigma (St. Louis, MO, USA). Human PTH (1-84) was kindly provided by Park at Mogam Biotechnology Research Institute (Yongin-kun, Korea). TRI reagent was purchased from Molecular research center (Cincinnati, OH, USA). TaKaRa Taq™ was purchased from TaKaRa (Otsu, Shiga, Japan).

Cell Culture

Saos-2 osteoblastic cells, obtained from American Type Culture Collection (Rockville, USA), were cultured in 100 mm tissue culture dishes containing Dulbeccos modified Eagles medium (DMEM) supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were cultured at 37°C in 95% humidified air plus 5% CO_2. Culture media were changed every 2-3 days. When the cells became 70-80% confluent, culture media were changed with DMEM supplemented with 1% FBS and indicated osteotropic agents.

Treatment of osteotropic agents

To observe the regulatory effects of osteotropic agents on RANKL and OPG mRNA levels in osteoblastic cells, cells were incubated in the presence of the following osteotropic agents for 2, 4, 8, and 24 h : IL-1β (5 ng/ml), TNF-α (5 ng/ml), TGF-β (1 ng/ml), IGF-I (10 ng/ml), 1,25$(OH)_2D_3$ (10^{-8} M), PTH (10^{-8} M), dexamethasone (10^{-8} M).

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

After the indicated culture period, cells were lysed with TRI reagent for the isolation of total cellular RNA. cDNA was synthesized from 1 μg of total RNA by extension of random primers with 1 U of Superscript RT. PCR was performed in a final volume of 50 μl containing 2 U of Takara Taq, 1× PCR buffer, 0.8 mM dNTP mixture, and 100 pmol of specific primers. The reactions were: denaturation at 95°C for 30 sec, annealing at 55°C (for RANKL) or 60°C (for OPG and β-actin) for 30 sec, and extension at 72°C for 1 min. Gene-specific PCR primers were as following: RANKL (product 404 bp) forward 5'-GTTTCCCATAAAGTGAGTCTGT-3', reverse 5'-TTAAAAGCCCCAAAGTATGTT-3'; OPG (product 406 bp) forward 5'-GGGGACCACAATGAACAAGTTG-3', reverse 5'-AGCTTGCACCACT-CCAAATCC-3'; β-actin (product 712 bp) forward 5'-CGGGAAATCGTGCGTGACAT-3', reverse 5'- AACTTTGGGGGATGCTCGC-3'. All PCRs were within the exponential amplification range. PCR products were electrophoresed on a 1.2% agarose gel and visualized under the UV light after ethidium bromide staining. For semi-quantitative estimation, the gel was analyzed with Quantity One (BIO-RAD. Hercules, CA, USA).

Results and Discussion

Various molecules, including locally produced growth factors, cytokines, and systemic osteotropic hormones, have been suggested as important regulators of osteoclast function for the last decades. Extensive *in vitro* and *in vivo* studies have definitely verified RANKL and OPG as the key regulatory molecules for all steps of the osteoclast life. Moreover, there were increasing evidences to support the idea that these two molecules act as the distal effector molecules onto which the regulatory effects of many growth factors, cytokines, and osteotropic hormones on bone resorption may converge.

In this regard, our results showing that PTH, 1,25$(OH)_2D_3$, and dexamethasone regulated RANKL and OPG mRNA expression level in favor of osteoclastogenesis, are consistent

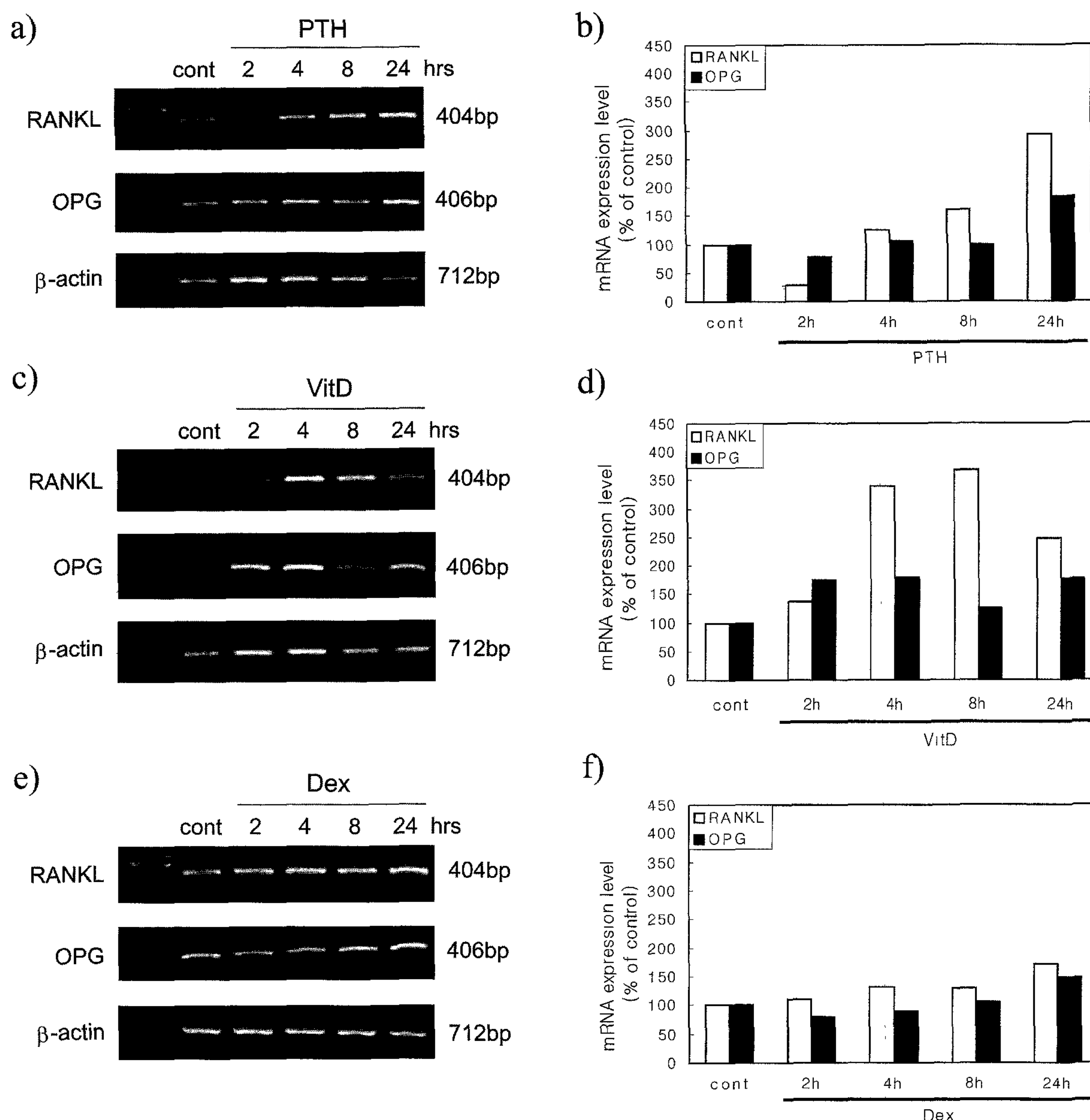

Fig. 1. Effects of PTH, $1,25(OH)_2vitD_3$ and dexamethasone on the RANKL and OPG mRNA expression in Saos-2 cells. Cells were treated with PTH (10^{-8} M), $1,25(OH)_2vitD_3$ (10^{-8} M), or dexamethasone (10^{-8} M) for 2, 4, 8, and 24 h and lysed for total RNA isolation. Semi-quantitative RT-PCR was performed as described in "Materials and Methods". a, c, e) 10 µl of PCR products were analyzed by electrophoresis on a 1.2% agarose gel and visualized under the UV light after ethidium bromide staining.; b, d, f) Relative RANKL and OPG mRNA expression level was plotted as the percentage of control after normalization to β-actin mRNA levels. PTH: Parathyroid hormone, VitD: $1,25(OH)_2vitD_3$, Dex: dexamethasone

with the suggested roles of them in osteoclast biology (Fig. 1). PTH and $1,25(OH)_2D_3$ increased RANKL expression up to more or less 3 times of control. Although there was some concomitant increase in OPG mRNA level, the overall change of RANKL/OPG ratio remained increased during the indicated period (Fig. 1 & 4). These results are compatible with the effects of $1,25(OH)_2D_3$, PTH, and glucocorticoids on bone metabolism *in vivo* and *in vitro* (Suda *et al.*, 1992; Roodman, 1996). Previously, it has been reported that these osteotropic hormones induce changes of OPG expression in favor of osteoclastogenesis (Hofbauer *et al.* 1998, 1999; Horwood *et al*, 1998; Lee and Lorenzo, 1999). However, considering the patterns and time courses of response to these hormones, previous reports have some differences from our results, which is thought to be attributable to the discrepancy in cell types and treatment schemes employed in these studies.

IL-1β and TNF-α are inflammatory cytokines that are suggested to be important mediators of bone destruction in inflammatory disease and multiple myeloma (Roodman, 1996). They are potent stimulators of bone resorption and osteoclast-like cell formation *in vitro*. In this study,

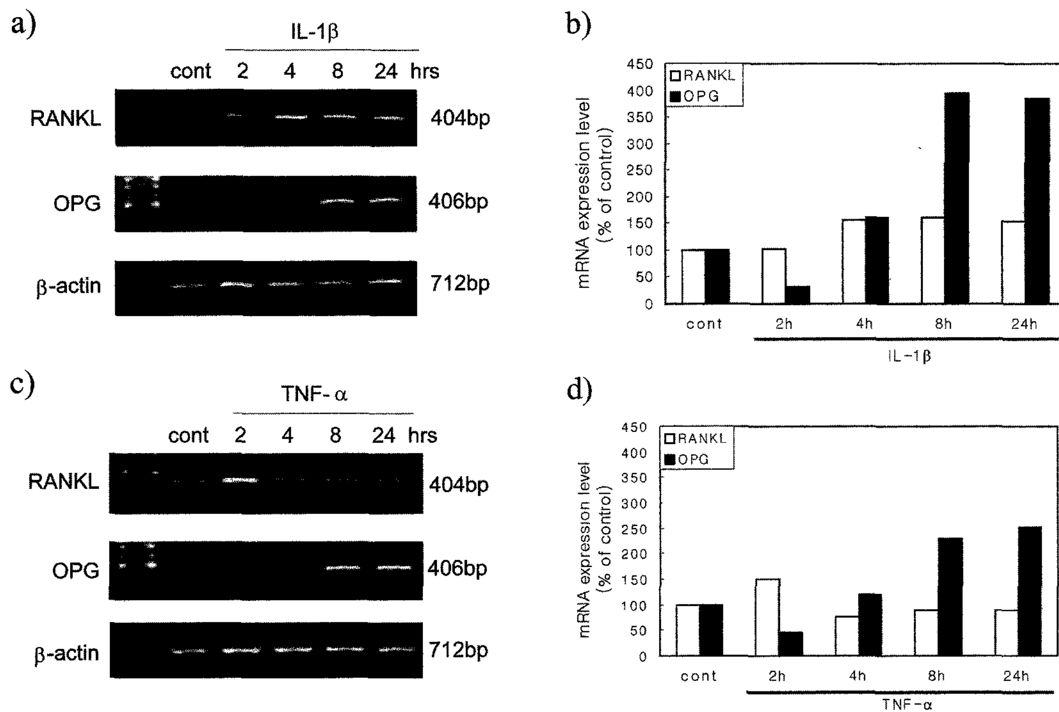

Fig. 2. Effects of IL-1β and TNF-α on the RANKL and OPG mRNA expression in Saos-2 cells. Cells were incubated with IL-1β (5 ng/ml) or TNF-α (5 ng/ml) for 2, 4, 8 and 24 h and lysed for total RNA isolation. Semi-quantitative RT-PCR was performed as described in "Materials and Methods". a, c) 10 μl of PCR products were analyzed by electrophoresis on a 1.2% agarose gel and visualized under the UV light after ethidium bromide staining.; b, d) Relative RANKL and OPG mRNA expression level was plotted as the percentage of control after normalization to β-actin mRNA levels.

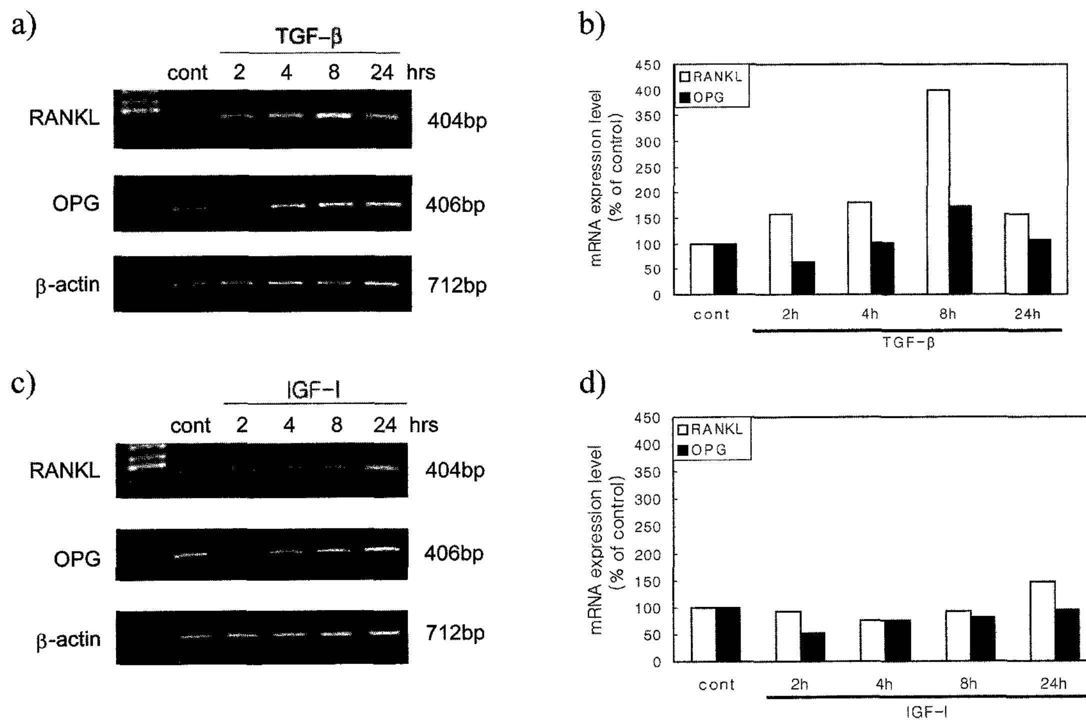

Fig. 3. Effects of TGF-β and IGF-I on the RANKL and OPG mRNA expression in Saos-2 cells. Cells were incubated with TGF-β (1 ng/ml) or IGF-I (10 ng/ml) for 2, 4, 8 and 24 h and lysed for total RNA isolation. Semi-quantitative RT-PCR was performed as described in "Materials and Methods". a, c) 10 μl of PCR products were analyzed by electrophoresis on a 1.2% agarose gel and visualized under the UV light after ethidium bromide staining.; b, d) Relative RANKL and OPG mRNA expression level was plotted as the percentage of control after normalization to β-actin mRNA levels.

RANKL/OPG ratio was highly increased after 2 h treatment of IL-1β or TNF-α because these cytokines decreased OPG mRNA level. Thereafter, however, both IL-1β and TNF-α greatly increased OPG mRNA expression while only slightly changed the RANKL expression, resulting in decrease in RANKL/OPG ratio much below that of control (Fig. 2 & 4). Brandstrom *et al.* (1998) and Vidal *et al.* (1998) have reported similar results concerning OPG mRNA expression in MG-63 cells in which they suggested that up-regulation of OPG might implicate a negative feedback mechanism for rapid reduction of osteolytic activity induced by inflammatory cytokines. Recently, however, it has been demonstrated that osteoclasts can be formed or activated in RANKL-independent fashion by the proinflammatory cytokines such as IL-1 β (Jimi *et al.*, 1999) and TNF-α (Kobayashi *et al.*, 1999). These suggest the possibility that these cytokines might circumvent the RANKL-OPG system and induce osteoclast differentiation in an alternate pathway, especially in inflammatory bone diseases. Therefore it is implied that these cytokines might employ more complex strategy rather than straight forward in order to exhibit their bone resorbing activity.

TGF-β and IGF-I are present in a large amount in bone matrix and suggested to play an important regulatory role during bone remodeling (Bonewald, 1996; Conover, 1996). In this study, TGF-β highly increased RANKL expression, reaching peak level at 8 h (Fig. 3a, b). Although OPG expression was also increased at that time, the degree was much less than that of RANKL, resulting in increase of RANKL/OPG ratio (Fig. 3 & 4). Previously, it has been reported that TGF-β markedly increases OPG mRNA level in a dose-dependent manner (Takai *et al.*, 1998; Murakami *et al.*, 1998). Because they did not observe the effect on RANKL expression at the same time, it is not clear whether our result is similar to their results. Recently, there were reports that TGF-β has direct stimulatory effects on osteoclastogenesis in addition to indirect effects via osteoblasts

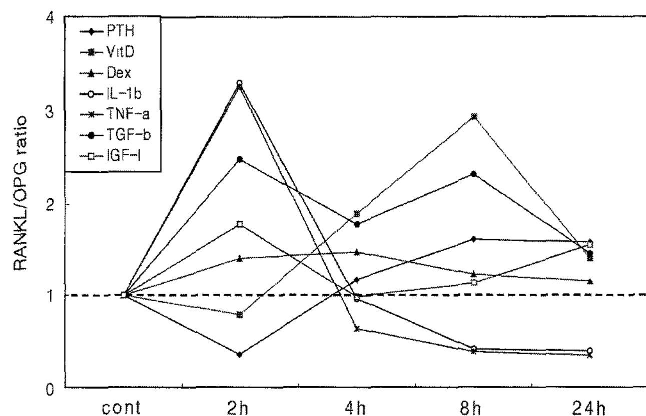

Fig. 4. Effects of osteotropic agents on the RANKL/OPG ratio in Saos-2 cells. RANKL/OPG ratio = RANKL (% of control)/OPG (% of control)

(Quinn *et al.*, 2001; Koseki *et al.*, 2002). This implies that the regulation of RANKL and OPG expression may play a partial role in regulation of osteoclast by TGF-β.

IGF-I has been shown to promote the formation of osteoclasts from mononuclear precursors and to stimulate activity of preexisting osteoclasts, which represents an indirect action through osteoblastic cells (Mochizuki *et al.*, 1992; Hill *et al.*, 1995). In this study, IGF-I induced only slight changes in RANKL and OPG mRNA level (Fig. 3c, d). This result suggests that IGF-induced stimulation of osteoclastogenesis and bone resorbing activity may largely be mediated by other mechanisms than RANKL-OPG regulation.

Taken together, these results showed that RANKL and OPG could be potential targets for bone resorption regulation by osteotropic hormones, cytokines, and growth factors. However, regulatory patterns were not always coincident with *in vivo* or *in vitro* effects on osteoclastogenesis, indicating that other targets may exist.

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