The effect of five osteotropic factors on osteoprotegerin mRNA expression in gingival fibroblasts

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ABSTRACT

Purpose: Osteoprotegerin (OPG) is a secreted glycoprotein and a member of the tumor necrosis factor (TNF) receptor family that inhibits bone resorption by suppressing osteoclastogenesis. Gingival fibroblasts (GF) play a role in periodontal disease progression, and the purpose of this experiment was to evaluate influence of osteotropic factors on the expression of osteoprotegerin mRNA in these cells.

Materials and Methods: In this experiment, the influence of osteoclastogenic factors, interleukin-1 beta (IL-1β), TNF-α, prostanglandin E2 (PGE_2). parathyroid hormone (PTH) and 1α, 25-dihydroxyvitamin D_3 on the expression of osteoprotegerin mRNA in GF was studied by Northern blot hybridization.

Results: As expected, PGE_2 tended to inhibit OPG levels and this was most prominent at 24 hours of culture with 10^{-7}M of PGE_2. TNF-α at 10ng/ml and also at 25ng/ml decreased OPG levels to almost 30% of the control at 24 hours. This contrasts with reports of increased OPG levels from osteoblast/stromal cells and gingival fibroblasts stimulated by TNF-α. Decrease of OPG levels with PGE_2 and TNF-α suggests a pathway whereby these mediators exert their resorptive effects. However, OPG levels were increased almost 3-fold at 24 hours with IL-1β(1 to 15ng/ml) and increased 1.4 fold with 24-hour treatment of 10^{-7}M PTH.

Conclusion: Increase of OPG levels suggests that these 'osteoclastogenic' factors act in more complex ways and may act to inhibit bone resorption in inflammatory periodontitis. This result supports the role of OPG as a negative feedback mechanism in osteoclastic activity. ***(J Korean Acad Periodontol 2008;38:395-404)***

KEY WORDS: gingival fibroblast; IL-1; osteoclastogenesis; osteoprotegerin.

Introduction

Bone is a specialized form of connective tissue that serves as a load-bearing structure and as a mineral reservoir. Four major types of cells are found in bone. Osteoblasts are cells that are responsible for synthesis of the organic matrix and regulate the mineralization of the bone matrix. Osteocytes are surrounded by the bone matrix and maintain the bone matrix. Bone lining cells cover bone surfaces that are inactive, undergoing neither bone formation nor bone resorption. Osteoclasts are large, multinucleated cells that function to resorb both the mineral and organic phases of bone. Bone is constantly undergoing remodeling. It is being resorbed by osteoclasts and actively formed by osteoblasts. In health, the amount of bone formed by osteoblasts is equivalent to the amount of bone resorbed by osteoclasts. In some disease states, this balance between resorption and formation can be broken.

Inflammatory periodontitis is one disease that affects the balance of bone metabolism negatively. Chronic periodontitis is defined as inflammation of the gingiva extending into the adjacent attachment apparatus. This is characterized by loss of clinical attachment due to destruction of the periodontal ligament and loss of the adjacent supporting bone. This supporting bone (alveolar bone) is like any other bone and methods used for elucidating mechanisms of bone

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destruction can be used to study the disease process in periodontitis. Ultimately, the alveolar bone loss in periodontitis is caused by bone resorption by the osteoclast. This cell degrades bone matrix proteins by producing collagenases, cathepsins and other hydrolases. The osteoclast also resorbs the mineral phase of bone with a specialized proton generating mechanism.

Of the four main cells in bone, osteoclasts differs in that it does not originate from local progenitor cells, rather it arises from the fusion of mononuclear progenitors of the monocyte/macrophage family of hematopoietic precursor cells. Udagawa et al.[1] showed that osteoclast precursors required cell-to-cell contact with osteoblast/stromal cells to develop into osteoclasts which implied that a membrane-bound molecule existed on the osteoblast/stromal cells. At permissive concentrations of macrophage colony stimulationg factor (M-CSF), the presence of this ligand which was named osteoclast differentiation factor (ODF)[2] (also known as receptor for activation of nuclear factor kappa B ligand (RANKL)[3], osteoprotegerin ligand (OPGL)[4] and tumor necrosis factor-related, activation-induced cytokine (TRANCE)[5] is sufficient for osteoclastogenesis. RANKL is a membrane-bound homotrimeric protein found on osteoblastic and activated T-cells[6]. Activated T-cells have also been found to secrete it[6].

The membrane receptor for this ligand is synthesized on osteoclasts and osteoclast precursors and is identical to a receptor discovered in immune cells (RANK)[3,7]. When RANKL binds to RANK of osteoclast precursors, several signaling pathways such as nuclear factor kappa B (NF-κB) and c-Jun NH_2-terminal kinase (JNK) are activated and differentiation of osteoclast precursors into osteoclasts begins[3,5].

For the osteoclast to be fully developed, NF-κB, c-Fos, and nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1) must be sequentially expressed[8].

Osteoprotegerin (OPG) is a secreted glycoprotein and a member of the tumor necrosis factor (TNF) receptor superfamily that inhibits bone resorption by suppressing osteoclastogenesis[9]. It is now known that osteoprotegerin acts as a soluble "decoy" receptor that competes with RANKL for RANK. The ratio of the expression level of RANKL molecule to that of the OPG molecule seems to be the decisive factor in osteoclastogenesis[10]. Factors that influence RANKL expression by osteoblasts also regulate OPG expression[11]. Usually, when RANKL expression is increased, OPG expression is down-regulated, or not up-regulated to the same degree as RANKL so that RANKL/OPG ratio changes in favor of osteoclastogenesis[12].

Periodontitis, if left unchecked, can ultimately result in loss of teeth due to alveolar bone resorption by osteoclasts. Gingival fibroblasts play a role in periodontal inflammation and the expression of osteoprotegerin mRNA has been observed in the gingival fibroblast[13]. Many cellular events that are involved in bone resorption, both systemic and alveolar, are modulated by a group of osteotropic agents that exert extremely potent effects on bone cells[14]. It would be one more step in elucidating the mechanism of bone resorption, and of alveolar bone resorption in particular, if their effects on gingival fibroblasts could be observed.

In this experiment, the effects of five osteotropic factors, IL-1β, TNF-α, PGE_2, parathyroid hormone (PTH), and 1α,25-dihydroxyvitamin D_3, on osteoprotegerin mRNA expression was studied by Northern blot hybridization.

Materials and Methods

1. Cytokines and hormones

Human recombinant (hr) IL-1β and hrTNF-α were purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). PGE_2, PTH, and 1α, 25-dihydroxyvitamin D_3 were purchased from Sigma Co.(St. Louis, MO, USA).

2. Preparation and culture of cells

Human gingival fibroblasts were obtained from explant cultures of healthy gingiva of a 27-year-old systemically healthy volunteer who visited the Seoul National University Hospital for esthetic crown lengthening. Gingival tissue was finely minced and placed on culture dish and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL, Grand Island, NY, USA), penicillin (100U/ml), streptomycin (100U/ml) (GIBCO BRL., Grand Island, NY, USA), and Fungizone (2.5U/ml) (GIBCO BRL., Grand Island, NY, USA) (at 37°C, 5% CO_2). Cells grown from the gingival tissue were collected after trypsin (0.05%) + EDTA (0.53mM)(GIBCO BRL., Grand Island, NY, USA) treatment and washed with Dulbecco's phosphate-buffered saline(DPBS) and grown in a plastic cell culture dish. Cultures at 5th to 10th passage were treated with various osteotropic hormones in DMEM/2% FBS. In the first part of the experiment, the cells were exposed to a given concentration each of IL-1β, TNF-α, PGE_2, PTH, and 1α, 25-dihydroxyvitamin D_3 for 2, 4, 8, or 24 hours. In the second part of the experiment, the cells were exposed to various concentrations of IL-1β, TNF-α, PTH, PGE_2 and 1α,25-dihydroxyvitamin D_3 for 24 hours.

3. RNA preparation

Total RNA from each culture was isolated using RNeasy Mini Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's protocol. Cells grown in a monolayer in dishes were trypsinized and collected as cell pellets prior to lysis. RLN buffer {[50 mM Tris-Cl, pH 8.0 (Sigma Co., St Louis, MO, USA)], [1.15mM $MgCl_2$ (Sigma Co., St Louis, MO, USA)], [0.5% NP40 (Sigma Co., St Louis, MO, USA)]} with 1mMDTT was added and to the cell pellets and carefully resuspended and incubated for 5 minutes on ice to lyse the plasma membrane. This was centrifuged at 300×g for 2 minutes then the supernatant was moved to a new tube. Proprietary RLT buffer was added. The sample was homogenized vigorously and 70% ethanol was added. 700μl of sample was applied to the RNeasy mini spincolumn sitting in a 2-ml collection tube, and was centrifuged for 15 seconds at 8000×g. The RNA column was transferred to a new 2-ml collection tube and 500μl of proprietary RPE buffer was added to the column and this was centrifuged at 8000g for 15 seconds. 500μl of RPE buffer was added once more to the column and centrifuged for 2 minutes at maximum speed. The column was transferred to a new collection tube and 60μl of RNAse-free distilled water was added. This was centrifuged for a minute at 8000×g to elute the RNA.

4. Northern blot analysis

Total RNA from each cell was denatured with heat at 65°C for 10 minutes and resolved by electrophoresis in a 1.2% agarose gel with 5% formaldehyde (Merck, Darmstadt, Germany) and 10X MOPS (0.2M MOPS, 50mM NaOAc, 1mM EDTA)(Sigma Co., St. Louis, MO, USA) in 1X MOPS buffer. The samples were mixed well with 2μl RNA sample buffer and loaded on the gel. Gel running was done at 80V. The gel was stained with ethidium bromide solution for 10 minutes and was observed under UV. This was transferred to a nylon membrane (NYTRAN-PLUS, Schleicher & Schuell, Inc., Keene, NH, USA) in 20X SSC buffer. After the transfer, the membrane was dried on 3MM paper.

North2South-Direct HRP Labeling and Detection Kit purchased from Pierce (Rockford, IL, USA) was used to label the cDNA probe and to detect the RNA bands of OPG and β-actin. Probe labeling was accomplished according to manufacturer's instructions and labeled with horseradish peroxidase (HRP). Prehybridization was done at 55°C for 1 hour with the hybridization buffers provided in the kit. After the prehybridization, probe was added to the hybridization solution and the membrane was incubated for 2 hours. The membranes

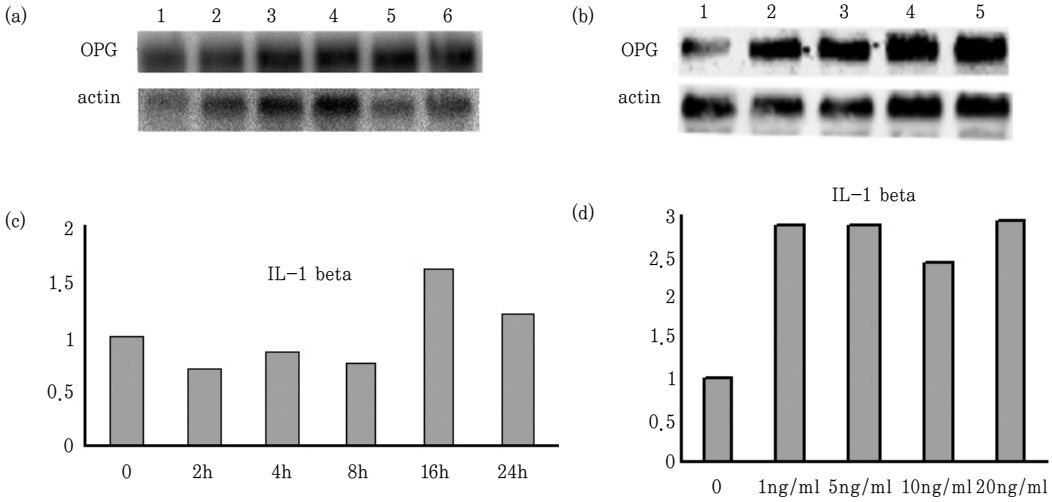

Figure 1. *Effects of IL-1β on the levels of OPG mRNA in HGF cultures. HGF was exposed to 5ng/ml of IL-1β for varying hours of time (a, c) and different concentrations of IL-1β were added for 24 hours (b, d). Total RNA (10μg/lane) was analyzed by Northern blotting using human OPG and β-actin cDNA probes. The mRNA levels of OPG (upper panel) and β-actin (lower panel) are shown. The graphs indicate the ratio of OPG to β-actin as a percentage of the control (normalized to 100%).*

were washed three times in 2X SSC/0.1% SDS at 55℃ for 15 minutes. The membranes were also washed 3 times in 2X SSC for 15 minutes each at room temperature. The chemiluminescent substrate solution was prepared and the membrane was incubated in the solution for 10 minutes at room temperature. The northern images were visualized with LAS^{1000Plus} Luminescent Image Analysis System (Fujifilm Medical Systems USA, Stamford, Conn, USA). The results were analyzed with TINA 1.0 program.

The dose-and time-dependent effects of PGE_2 on OPG mRNA expression are shown in Fig. 2. A decrease to 63% and to 58% of the control could be seen at 2 to 4 hours and a slight increase to 103% at 24

Results

The dose- and time-dependent effects of IL-1β on OPG mRNA expression in gingival fibroblasts are shown in Fig. 1. OPG levels were slightly decreased at 2 to 8 hours but at 16 and 24 hours, 60% and 20% increase was seen with 5 ng/ml of IL-1β. At 24 hours, greater than 200% increase in OPG levels was seen at all dosage levels. At 1, 5, 10, 25ng/ml level, greater than 250% increase could be observed.

hours could be seen with 10^{-6}M of PGE_2. At 24 hours of culture with 10^{-7}M of PGE_2, this decrease reached maximum level (46%).

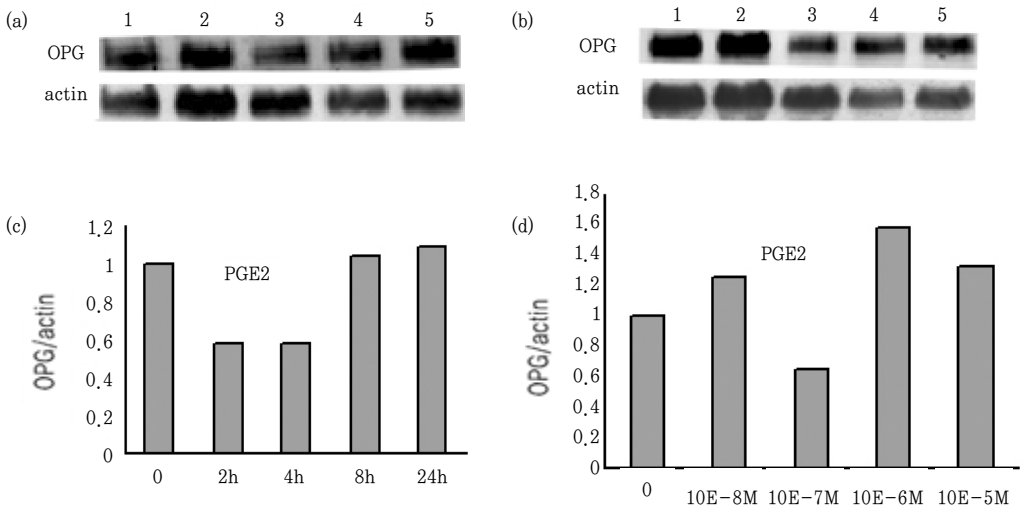

Figure 2. *Effects of PGE_2 on the levels of OPG mRNA in HGF cultures. HGF was exposed to 10^{-6}M of PGE_2 for varying hours of time (a, c) and to different concentrations of PGE_2 for 24 hours (b, d). Total RNA (10 μg/lane) was analyzed by Northern blotting using human OPG and β-actin cDNA probes. The mRNA levels of OPG (upper panel) and β-actin (lower panel) are shown. The graphs indicate the ratio of OPG to β-actin as a percentage of the control (normalized to 100%).*

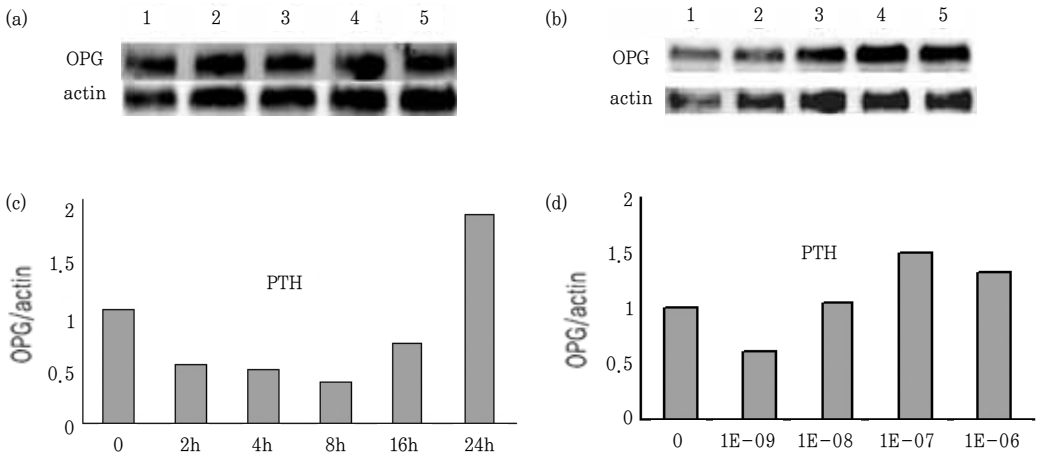

Figure 3. *Effects of PTH on the levels of OPG mRNA in HGF cultures. HGF was exposed to 10-8M of PTH for varying hours of time and to different concentrations of PTH for 24 hours. Total RNA (10 μg/lane) was analyzed by Northern blotting using human OPG and β-actin cDNA probes. The mRNA levels of OPG (upper panel) and β-actin (lower panel) are shown. The graphs indicate the ratio of OPG to β-actin as a percentage of the control (normalized to 100%).*

Effect of PTH on the expression of OPG in gingival fibroblasts is seen in Fig. 3. PTH (10^{-8}M) tended to decrease OPG mRNA levels during 2 to 16 hours of culture, and this was greatest at 8 hours where OPG expression was lowered to 35% of control. But almost 2-fold increase was seen at 24 hours. At 24 hours, 1.5-fold increase was seen in OPG levels with 10^{-8} and 10^{-7}M of PTH, but OPG mRNA levels decreased to 63% of control with 10^{-10}M of PTH.

TNF-α tended to decrease OPG mRNA expression levels (Fig. 4). This decrease was slight with 5ng/ml of TNF-α, which reached 81% at 24 hours. With increasing doses of TNF-α at 24 hours, this decrease was increased and became less than one-third of control at 10 and 15ng/ml.

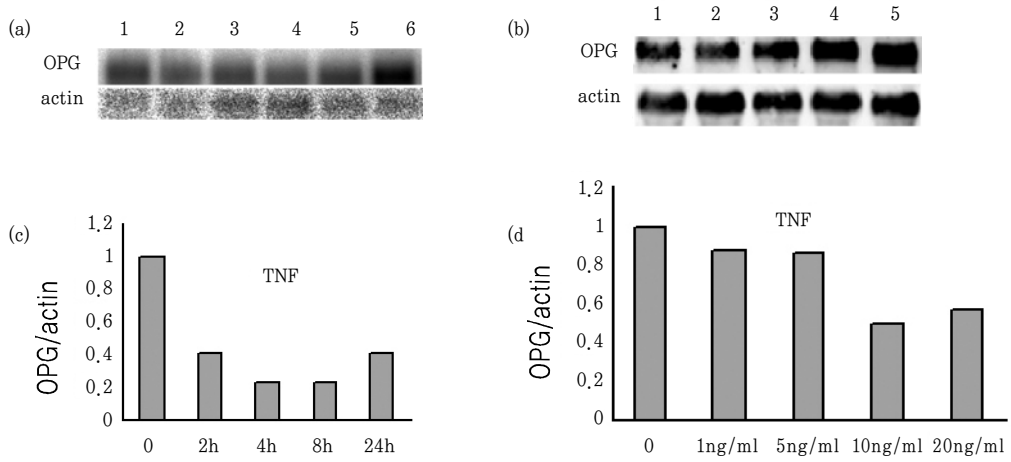

Figure 4. *Effects of TNF-α on the levels of OPG mRNA in HGF cultures. HGF was exposed to 5ng/ml of TNF-α for varying hours of time (a, c) and to different concentrations of TNF-α for 24 hours (b, d). Total RNA (10μg/lane) was analyzed by Northern blotting using human OPG and β-actin cDNA probes. The mRNA levels of OPG (upper panel) and β-actin (lower panel) are shown. The graphs indicate the ratio of OPG to β-actin as a percentage of the control (normalized to 100%).*

Effects of 1α, 25-dihydroxyvitaminD_3 on OPG mRNA expression levels are shown in Fig. 5. 1α, 25-dihydroxyvitaminD_3 (10^{-8}M) tended to decrease OPG mRNA expression levels at 2 to 24 hours. At 24 hours, greater concentrations of 1α, 25-dihydroxyvitaminD_3 (10^{-8}M and 10^{-7}M) tended to decrease OPG levels but slight increase was seen at lower concentrations of OPG.

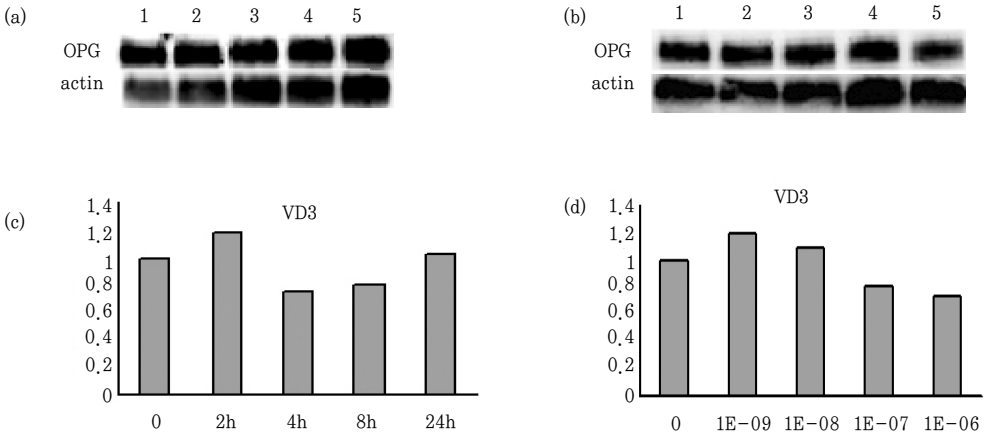

Figure 5. *Effects of 1α,25-dihydroxyvitamin D_3 on the levels of OPG mRNA in HGF cultures. HGF was exposed to 10-8M of 1α,25-dihydroxyvitamin D_3 for varying hours of time(a, c) and to different concentrations of 1α,25-dihydroxyvitamin D_3 for 24 hours(b, d). Total RNA (10μg/lane) was analyzed by Northern blotting using human OPG and β-actin cDNA probes. The mRNA levels of OPG (upper panel) and β-actin (lower panel) are shown. The graphs indicate the ratio of OPG to β-acti n as a percentage of the control (normalized to 100%).*

Discussion

It has been shown in many studies that OPG is produced in various tissues, such as lung, heart, and kidney[9,15], and numerous cells, such as bone marrow stromal cells, osteoblastic cells, and lung fibroblasts[15]. Also, human dental mesenchymal cells, such as pulpal cells, periodontal ligamental cells, and gingival fibroblasts have been shown to produce OPG transcripts[14,16-17]. Rani and MacDougall[16] have reported that odontoblasts and pulpal cell lines also produced RANKL.

In inflammatory periodontitis, osteoclastic activity is promoted and resulting bone resorption may lead to tooth loss and debilitation. The fact that this destruction of alveolar bone is initiated by bacterial assault is well documented, but there is much to be elucidated about the coordinated regulatory mechanism of the formation and the activity of the osteoclasts. It has been suggested that OPG can suppress osteoclast maturation and activity by binding RANKL/ODF and thereby preventing its interaction with RANK on osteoclasts and osteoclast progenitor cells. Teng et al.[18] have reported that injection of recombinant human osteoprotegerin-fragment crystallizable region (OPG-Fc) fusion protein inhibited alveolar bone resorption and reduced the number of osteoclasts after challenge with *Aggregatibacter actinomycetemcomitans* in mice. A wide array of hormones and cytokines involved in the regulation of bone resorption do not act directly on osteoclasts but exerts its effects via other cells such as osteoblasts. In this study, the effects of 5 osteotropic molecules on OPG mRNA expression levels in the gingival fibroblasts have been studied.

IL-1β is a principal mediator of inflammatory reactions acting on many cell types and this cytokine is increased in the gingiva of adult periodontitis subjects compared with clinically healthy gingiva. Also, IL-1β levels are elevated in active periodontitis sites compared to stable inflamed sites[19]. IL-1β has the potential to initiate tissue destruction and bone loss in periodontal diseases. It is the most potent known inducer of bone demineralization and synergizes with tumor necrosis factor alpha in stimulating bone resorption as well as major changes in the connective tissue matrix[20]. One study has shown that the rate of IL-1β messenger RNA was higher in the connective tissue furthest from the pocket epithelium, suggesting a role in the alveolar bone resorption that occurs in periodontal disease[21]. Another study has demonstrated that administration of IL-1β accelerated alveolar bone destruction in ligature-induced periodontal tissue inflammation in rats over a 2-week period[22].

Contrary to expectations as cytokines stimulating osteoclast-ogenesis were usually reported to decrease OPG levels albeit in different cell types[23], IL-1β is known to increase levels of OPG mRNA expression in various cells. Sakata et al[13] have reported this increase with gingival fibroblasts, Hofbauer et al's[24] s study also shows increased OPG mRNA expression in human osteoblastic cells. The result of this study also show that stimulation with Il-1β increased OPG mRNA expression in gingival fibroblasts. This suggests that Il-1β acts not only to stimulate resorption of bone but may act to also inhibit it and it also supports the role of OPG as a negative feedback mechanism for the reduction of osteoclastic activity[24].

Prostaglandins play a role in inflammatory periodontitis. Of the prostaglandins, PGE_2 is the most potent stimulator of bone resorption. This shows a wide range of proinflammatory actions and its effects are enhanced by synergisms with other inflammatory mediators. PGE_2 levels reflect the disease status of the periodontium. PGE_2-induced bone resorption may be explained by this mechanism. Also PGE_2 inhibited OPG formation in various cell types such as mouse calvarial cells[25] and human bone marrow stromal cells[26]. Other studies have shown PGE_2 having a crucial role in RANKL-dependent osteoclastogenesis induced by LPS or bacterial sonicates[27].

PTH tended to increase OPG mRNA levels in this experiment. It was reported by Ogata et al[28] that

gingival fibroblasts lack PTH receptors as these cells did not produce cAMP when stimulated by PTH. This is by no means conclusive, as different density of PTH receptors may result in different signal transduction pathways being stimulated. This result is puzzling as PTH acts to increase extracelluar ionized calcium levels directly through its actions on kidney and bone. PTH tended to inhibit production of OPG activity in mouse calvarial bone[25] and also in various osteoblastic cell lines[29].

TNF-α decreased OPG mRNA levels in this study. This result, while expected, is contrary to the results reported by Sakata et al[13]. In their study, 3ng/ml of TNF-α increased OPG levels to 210% at 24 hours. Studies by Nakashima et al[23,24] show that OPG mRNA levels in stromal cells increased when stimulated with TNF-α. Wada's study with PDL fibroblasts stimulated with LPS shows induction of TNF-α results in increase of both OPG and RANKL levels, but increase of OPG production was greater[30]. Brandstrom's study[26] shows OPG levels decreasing when stimulated with TNF-α. Results from this study suggest that TNF-α, a potent stimulator of bone resorption may exert its effects by this way.

Vitamin D is a principal factor required for the development and maintenance of bone as well as for maintaining normal calcium homeostasis. In this study, 1α,25-dihydroxyvitaminD_3 tended to inhibit OPG mRNA expression slightly at greater than 10^{-8}M concentration. It is difficult to say if this observed change is meaningful or not. In a study by Hofbauer et al[24] 1α,25-dihydroxyvitaminD_3 increased OPG mRNA levels by 90 and 50% in fetal osteoblastic cells and normal trabecular osteoblastic cell but did not affect OPG mRNA levels in marrow stromal preosteoblastic cells. Some have reported decreased OPG levels when dermal fibroblasts were stimulated with both 1α,25-dihydroxyvitaminD_3 and dexamethasone[31] and when stromal cells were stimulated with 1α,25-dihydroxyvitaminD_3 and dexamethasone[32]. Horwood has reported that 1α,25-dihydroxyvitaminD_3 decreased OPG mRNA formation in osteoblastic cell lines[29]. Suda[33] et al has reported that OPGmRNA in bone did not change appreciably in 1-3 weeks at physiologic doses of 1α,25-dihydroxyvitaminD3 Vitamin D at physiologic doses tended to decrease PTH-induced bone resorption and this effect was by suppressing PTH-induced RANKL mRNA exression.

The fact that many different cell types express OPG mRNA makes it difficult to analyze the effect of numerous osteotropic factors on OPG formation by these cells. Also, basal level differences between individuals may exist and this should be kept in mind when established cell lines are not used. The results of this study suggests that production of OPG by gingival fibroblasts which may act as a local suppressor of bone resorption, is influenced by IL-1β, PGE_2, PTH, TNF-α, and 1α,25-dihydroxyvitaminD_3. Control of OPG levels may be one way that these mediators of bone resorption exert its effects. In addition, further study into the relative levels of OPG and RANKL in the periodontium would offer more insight into the regulatory mechanism of bone metabolism in inflammatory periodontitis. Also, basal level differences of OPG production in individuals may offer explanations for differences in host response. It would have helped to clarify the results if more samples from different individuals were compared.

Many therapies have been advocated for the treatment of periodontitis. Mechanical debridement is usually enough to arrest the disease in most cases but other cases may benefit from adjunctive therapies of which non-steroidal anti-inflammatory drugs (NSAIDs) to modulate arachidonic acid metabolites, anti-collagenase therapies, and cytokine receptor antagonists are some examples. As final effector molecules of bone resorption, control of RANKL, RANK, and OPG is a way that offers therapeutic possibilities in many different bone diseases[34,35] and in case of osteoporosis and rheumatoid arthritis, clinical trials are being undertaken[36-38]. Further study is required before this novel approach may be used in periodontal

therapy.

In this experiment, OPG mRNA expression by gingival fibroblasts which may act as a local suppressor of bone resorption, was influenced by IL-1β, PGE_2, PTH, TNF-α, and 1α,25-dihydroxyvitaminD_3. These osteotropic factors may influence bone resorption by control of OPG levels.

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