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Osteoprotegerin induces osteopontin via syndecan-1 and phosphoinositol 3-kinase/Akt in human periodontal ligament cells

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Background and Objective: Our previous study found that thrombin induced osteoprotegerin synthesis in human periodontal ligament cells. As elevated levels of osteoprotegerin can exert biological effects on various cell types, in the present study we investigated the effect of osteoprotegerin on the expression of osteopontin in human periodontal ligament cells.

Material and Methods: Cultured human periodontal ligament cells were treated with recombinant human osteoprotegerin (0–100 ng/mL) for 24–48 h. The expression of osteopontin mRNA and protein was analyzed using reverse transcription–polymerase chain reaction and western blot analyses, respectively. Phosphoinositol 3-kinase inhibitor, Akt inhibitor, heparinase, neutralizing antibody against receptor activator of nuclear factor-κB ligand (RANKL) and syndecan-1, and small interfering RNA against syndecan-1, were used to determine the mechanism involved.

Results: Osteoprotegerin up-regulated the mRNA and protein expression of osteopontin in human periodontal ligament cells in a dose-dependent manner. Addition of neutralizing antibody against RANKL attenuated the inductive effect of osteoprotegerin on osteopontin expression. In addition, the inductive effect of osteoprotegerin was abolished by phosphoinositol 3-kinase and Akt inhibitors, as well as by syndecan-1 antibody or syndecan-1 small interfering RNA. None of the inhibitors had any effect on the background level of osteopontin expression.

Conclusion: An increased level of osteoprotegerin can generate signals via a RANKL/syndecan-1/phosphoinositol 3-kinase/Akt pathway. The results also suggest that osteopontin is one of the downstream targets of the pathway mediated by osteoprotegerin in human periodontal ligament cells. Thus, in addition to counteracting RANKL in the RANKL–osteoprotegerin system, osteoprotegerin may play a role in periodontal tissue remodeling through modulation of the extracellular matrix.

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Human periodontal ligament cells have been considered to play an important role in alveolar bone remodeling. They are responsible for homeostasis of the periodontal tissue through controlling of the levels of receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin, and have been shown to increase RANKL expression in response to inflammatory cytokines or mechanical stress (1,2) and to increase osteoprotegerin expression in response to tensile stress (3). Our previous study found that human periodontal ligament cells also secreted high amounts of osteoprotegerin when stimulated with thrombin (4). In addition, Wada *et al*. (5) demonstrated that osteoprotegerin secreted by human periodontal ligament cells inhibited osteoclastogenesis.

Osteoprotegerin and RANKL have been shown to play significant roles in bone remodeling (6,7). Regarding the bone system, RANKL is expressed on osteoblast lineage cells and exerts its biological effect by binding to receptor activator of nuclear factor-κB (RANK), the receptor at the surface of osteoclasts. Osteoprotegerin is produced by osteoblasts and acts as a decoy receptor for RANKL, preventing it from binding to and activating RANK on the osteoclast surface (8). Thus, it has been widely accepted that osteoprotegerin regulates osteoblast-mediated bone resorption.

Several studies have shown that osteoprotegerin is capable of exerting several biological effects on various cell types. It induces the expression of matrix metalloproteinase (MMP)-9, MMP-2 and tissue inhibitor of matrix metalloproteinase-1 in osteoclast cultures (9). In addition, it regulates pulmonary artery smooth muscle cell proliferation and migration (10). These data suggest that osteoprotegerin modulates its biological effects not only via the RANKL–osteoprotegerin system but also via a RANKL-independent pathway (9,11,12).

In regard to periodontal tissue, up-regulation of osteoprotegerin mediated by human periodontal ligament cells in response to tensile stress has been reported (13). We also found that human periodontal ligament cells increased osteoprotegerin synthesis in response to thrombin (4), the molecule that plays multiple roles in the early stages of healing. Osteopontin is a matricellular protein that participates in both tissue regeneration and remodeling. It is an osteogenic differentiation marker and can facilitate wound healing in the initial stage of mineralization (14,15). Therefore, it is of interest to investigate the effect of an increased amount of osteoprotegerin on the expression of osteopontin. In the present study we showed that osteoprotegerin up-regulates osteopontin expression in human periodontal ligament cells, possibly through the syndecan-1 and phosphoinositol 3-kinase/Akt signaling pathway.

Material and methods

Cell culture

Human periodontal ligament cells were obtained from healthy periodontal ligament tissue of noncarious, freshly extracted third molars, or from teeth removed for orthodontic reasons. The protocol was approved by the Ethical Committee of the Faculty of Dentistry, Chulalongkorn University, and informed consent was obtained from each patient. Briefly, teeth were rinsed with sterile phosphate-buffered saline and the periodontal ligament was removed from the middle third of the root. The explants were harvested on 60-mm culture dishes and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL of penicillin, 100 μg/mL of streptomycin and 5 μg/mL of amphotericin B, in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. Each preparation was prepared from one molar. Cells from passage 3 of three different donors were used in the experiments, which were run in triplicate. Media and all supplements used were from Gibco BRL (Carlsbad, CA, USA).

Treatment of cells

Human periodontal ligament cells were seeded in six-well plates, at a density of 25,000 cells/cm^2. Cells were stimulated with 25, 50 and 100 ng/mL of recombinant human osteoprotegerin (R&D Systems, Minneapolis, MN, USA) in 0.5% serum-containing medium. After 24 h, the RNA was extracted for reverse transcription–polymerase chain reaction analysis. Cell protein extracts for western blot analysis were collected from a parallel set of cultures after 48 h of stimulation with osteoprotegerin. The effective dose was selected and used for the rest of the experiments.

Inhibition

The inhibitors used were 1.4 μM LY294002, a phosphatidylinositol 3′-kinase inhibitor (Cayman Chemical, MI, USA), 10 μM Akt inhibitor II (Calbiochem, EMD Biosciences, Inc., San Diego, CA, USA), 0.5 U/mL of heparinase III (Sigma-Aldrich Chemical, St Louis, MO, USA), 0.02 μg/mL of anti-RANKL neutralizing antibody (R&D Systems), 2 μg/mL of syndecan-1 antibody (DL-101) and syndecan-1 small inhibitory RNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were treated with each inhibitor for 30 min (24 h for syndecan-1 small inhibitory RNA or control oligonucleotide) prior to the addition of 100 ng/mL of osteoprotegerin.

Reverse transcription–polymerase chain reaction

After treatment, total cellular RNA was extracted using Tri reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. One microgram of each RNA sample was converted to cDNA by avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) for 1.5 h at 42°C. Subsequently, polymerase chain reaction amplification was performed. The primers were prepared following the reported sequences from GenBank. The oligonucleotide sequences of the primers used are: osteopontin forward, 5′-AGT ACC CTG ATG CTA CAG ACG-3′ and reverse, 5′-CAA CCA GCA TAT CTT CAT GGC-3′; and glyceraldehyde-3-phosphate dehydrogenase forward, 5′-TGA

AGG TCG GAG TCA ACG GAT-3′ and reverse, 5′-TCA CAC CCA TGA CGA ACA TGG-3′.

Polymerase chain reaction amplification was performed using Taq polymerase (Qiagen, Hilden, Germany) in a reaction volume of 25 μL containing 25 pmol of primers and 1 μL of reverse transcription product. The amplification profile was one cycle at 94°C for 1 min, 30 cycles at 94°C for 1 min, hybridization at 60°C for 1 min and extension at 72°C for 2 min (30 cycles for osteopontin and 22 cycles for glyceraldehyde-3-phosphate dehydrogenase), followed by one extension cycle of 10 min at 72°C. The polymerase chain reaction was performed in a DNA thermal cycler (Biometra, Göttingen, Germany). The amplified DNA was then electrophoresed on a 2% agarose gel and visualized by ethidium bromide fluorostaining. All bands were scanned and analyzed using Scion image-analysis software (Scion Corporation, Frederick, MD, USA).

Protein extraction and western blot analysis

Protein was extracted into radioimmunoprecipitation buffer (50 mM Tris/HCL, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate) containing cocktail protease inhibitors or phosphatase inhibitors (1 mM sodium vanadate, 50 mM NaF) (Sigma-Aldrich Chemical). For detection of phospho-Akt or total Akt, cells were treated with recombinant osteoprotegerin for 5 min before protein extraction. Protein concentrations were measured using the BCA™ protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of protein samples were subjected to electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel and subsequently transferred onto nitrocellulose membrane. The membrane was incubated with primary antibody against osteopontin (Chemicon International, Temecula, CA, USA), phospho-Akt, total Akt (Cell Signaling Technology, Danvers, MA, USA), syndecan-1 (R&D Systems) or beta-actin (Chemicon International) at 4°C. The dilution used of all primary antibodies was 1:1000. The membranes were then incubated with biotinylated secondary antibody, followed by peroxidase-labeled streptavidin. The signal was captured by chemoluminescence (Pierce Biotechnology) and the relative intensity of bands was measured using Scion image analysis.

Transfection of small inhibitory RNA

Human periodontal ligament cells were grown in six-well plates, in medium without antibiotics, to 70–80% confluence. Cells were added with the mixed solution of small interfering RNA oligonucleotides specific to syndecan-1 according to the manufacturer's instructions (Santa Cruz). Cells were transfected with the small inhibitory RNA for 24 h before treatment. In the control culture, control small interfering RNA (Santa Cruz) was added.

Statistical analysis

Results were expressed as mean ± standard deviation. Statistical differences were determined using one-way analysis of variance followed by *post-hoc* analysis (Scheffe's test). Differences with a p-value of < 0.05 were considered statistically significant.

Results

Human periodontal ligament cells were treated with osteoprotegerin for 24 and 48 h. After treatment, the RNA or protein extracts were collected as described in the Material and methods. The results showed that osteoprotegerin up-regulated the expression of osteopontin at both mRNA and protein levels in a dose-dependent manner, profoundly at 100 ng/mL (Fig. 1). The effective dose of osteoprotegerin (100 ng/mL) was used for the rest of the experiments. All relative band densities from each experiment are presented as bar charts.

The intracellular signaling pathway was investigated by means of inhibitors. Phosphoinositol 3-kinase inhibitor, as well as Akt inhibitor, exhibited an inhibitory effect on the osteoprotegerin-induced production of osteopontin, at both transcriptional and translational levels (Fig. 2).

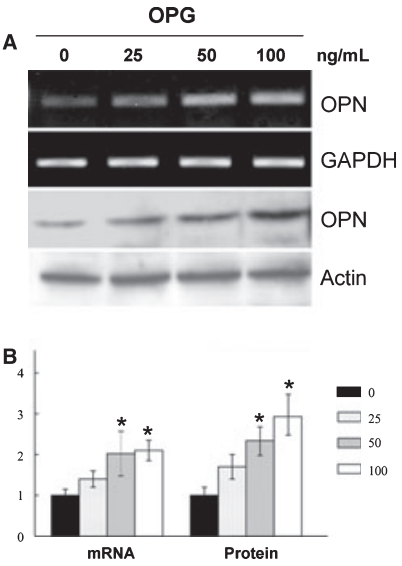

Fig. 1. Effects of osteoprotegerin on the mRNA expression and protein synthesis of osteopontin in human periodontal ligament cells. Cells were treated with 0, 25, 50 or 100 ng/mL of osteoprotegerin for 24 h before analysis of mRNA expression or for 48 h before analysis of protein expression. (A) The polymerase chain reaction products (upper two row in panel A) and western blot analysis (lower two rows in panel A) revealed the up-regulation of osteopontin in a dose-dependent manner. (B) The relative density was presented as graphs. The results are expressed as mean ± standard deviation from three different experiments. $*p < 0.05$ compared with the control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OPG, osteoprotegerin; OPN, osteopontin.

To determine whether the inductive effect was mediated by the binding of osteoprotegerin to membranous RANKL, cells were pre-incubated with neutralizing antibody against RANKL for 30 min prior to the addition of osteoprotegerin. An equal amount of goat immunoglobulin G was added to the control culture. The result showed that anti-RANKL immunoglobulin G inhibited the ability of osteoprotegerin to induce osteopontin and to activate Akt (Fig. 3).

Osteoprotegerin contains a heparin-binding domain that is able to bind heparan sulfate proteoglycans. Therefore, we further investigated the

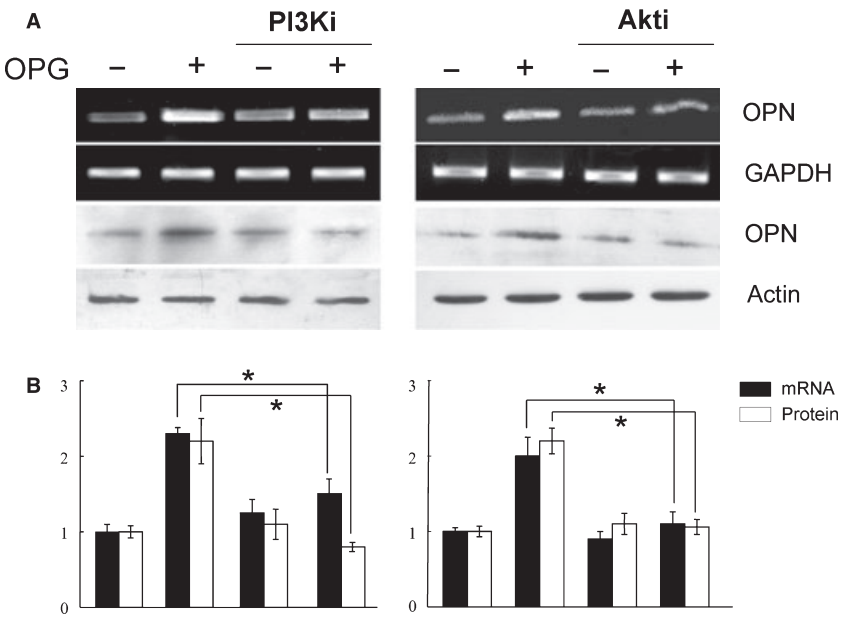

Fig. 2. Effects of phosphatidylinositol 3-kinase inhibitor and Akt inhibitor on the osteoprotegerin-induced production of osteopontin. Human periodontal ligament cells were pre-incubated with 1.4 μM phosphoinositol 3-kinase inhibitor or with 10 μM Akt inhibitor for 30 min prior to the addition of osteoprotegerin. (A) Both phosphoinositol 3-kinase inhibitor and Akt inhibitor exerted an inhibitory effect on the up-regulation of osteopontin induced by osteoprotegerin, at both mRNA and protein levels. (B) The relative density was is shown by bar charts. The results are expressed as mean ± standard deviation from three different experiments. *Significant difference ($p < 0.05$). Akti, Akt inhibitor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OPG, osteoprotegerin; OPN, osteopontin; PI3Ki, phosphatidylinositol 3-kinase inhibitor.

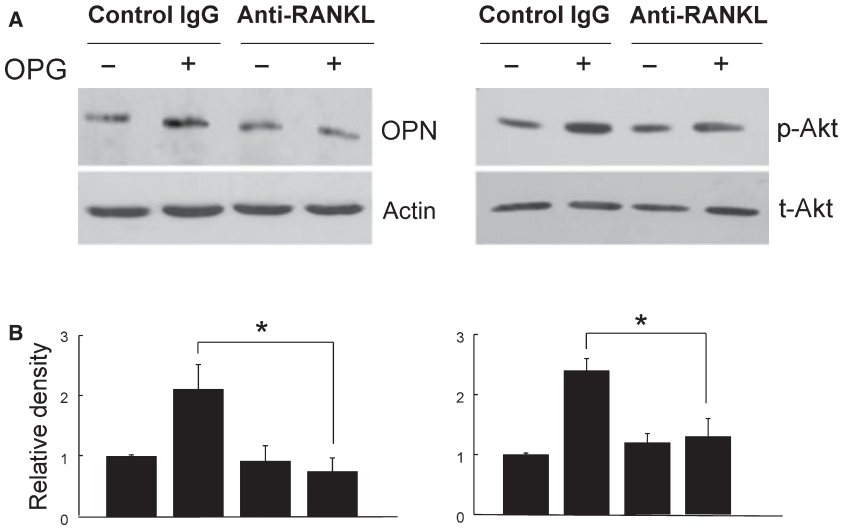

Fig. 3. Inhibitory effects of receptor activator of nuclear factor-κB ligand (RANKL) neutralizing antibody (anti-RANKL) on the osteoprotegerin-induced synthesis of osteopontin. Anti-RANKL was applied to the cultures 30 min before the treatment, and the same amount of goat immunoglobulin G was added to the control (control IgG). (A) Western blot analysis showed that anti-RANKL abolished the increase of osteopontin as well as the increase of phospho-Akt. (B) The relative density normalized to actin or total Akt is shown in bar charts. The results are expressed as mean ± standard deviation from three different experiments. *Significant difference ($p < 0.05$). OPG, osteoprotegerin; OPN, osteopontin; p-Akt, phosphor-Akt; t-Akt, total-Akt.

involvement of cell-surface heparan sulfate proteoglycans and syndecan-1. Heparinase III, an enzyme that cleaves heparan sulfate proteoglycans from the cellular surface, was added to the cultures 30 min prior to treatment with osteoprotegerin. The results showed that heparinase completely abolished the increase of osteopontin induced by

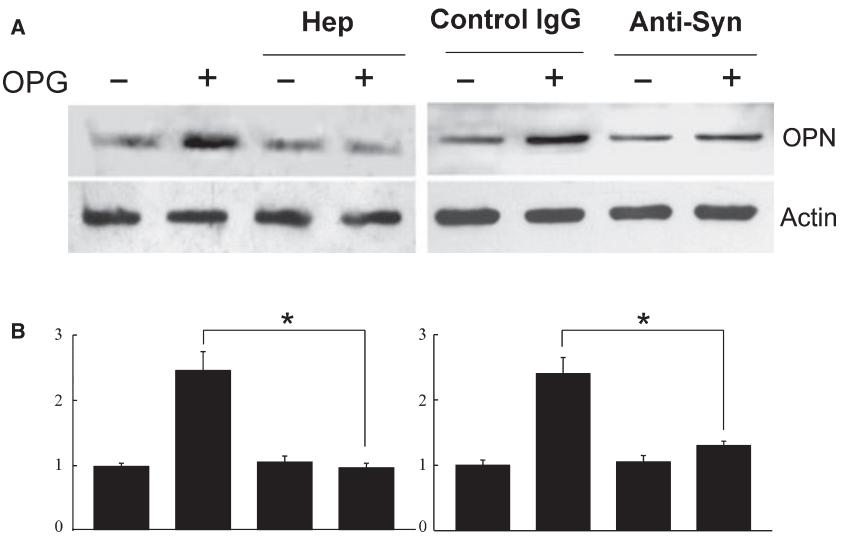

Fig. 4. Inhibitory effects of heparinase and syndecan-1 antibody on the osteoprotegerin-induced synthesis of osteopontin. Heparinase or syndecan-1 antibody was applied to the cultures 30 min before the treatment. (A) Western blot analysis showed that heparinase as well as syndecan-1 antibody abolished the increase in osteopontin. (B) The normalized relative density is shown as bar charts. The results are expressed as mean ± standard deviation from three different experiments. *Significant difference ($p < 0.05$). Anti-Syn, syndecan-1 antibody; Hep, heparinase; IgG, immunoglobulin G; OPG, osteoprotegerin; OPN, osteopontin.

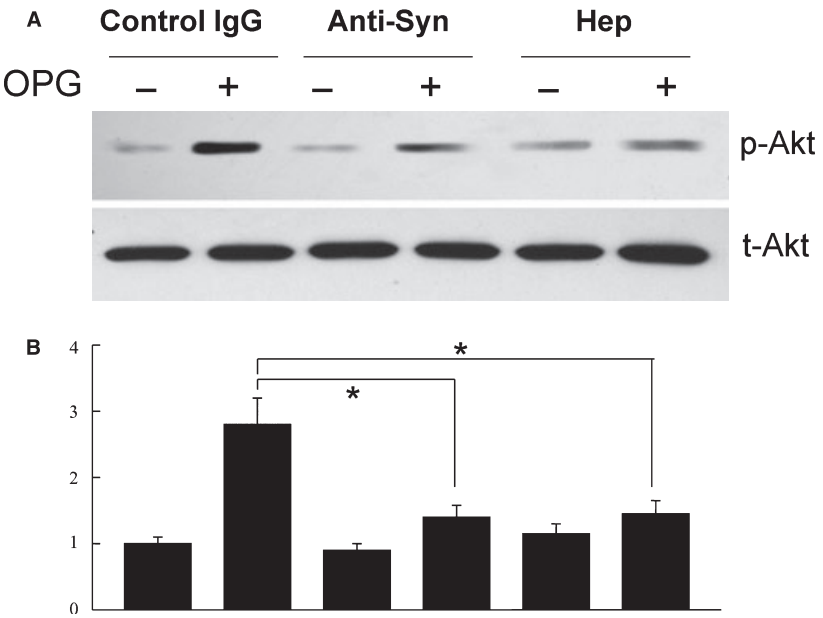

Fig. 5. Effects of heparinase and syndecan-1 antibody on the increase of phospho-Akt. Human periodontal ligament cells were pre-incubated with heparinase or syndecan-1 antibody 30 min prior to the addition of osteoprotegerin. (A) Western blot analysis revealed that osteoprotegerin stimulated phospho-Akt and the stimulating effect was abolished by heparinase as well as by syndecan-1 antibody. (B) The relative density is shown by bar charts. The results are expressed as mean ± standard deviation from three different experiments (*significant difference; $p < 0.05$). Anti-Syn, syndecan-1 antibody; Hep, heparinase; OPG, osteoprotegerin; p-Akt, phospho-Akt; t-Akt, total Akt.

osteoprotegerin (Fig. 4). In addition, syndecan-1 antibody was used to clarify whether syndecan-1, a member of a family of cell-surface heparan sulfate proteoglycans, participates in the induction. We found that syndecan-1 antibody also attenuated the inductive effect of osteoprotegerin on osteopontin protein synthesis (Fig. 4). Likewise, the activation of phospho-Akt was examined. The results showed that the increased level of phospho-Akt stimulated by osteoprotegerin was abolished by heparinase III and syndecan-1 antibody (Fig. 5).

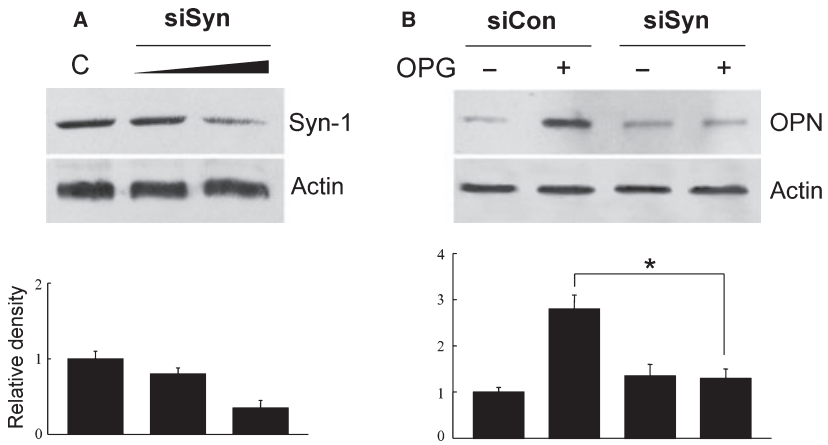

Fig. 6. Effects of syndecan-1 small interfering RNA on osteoprotegerin-induced osteopontin synthesis. (A) Human periodontal ligament cells were transfected with syndecan-1 small interfering RNA for 24 h, following which the cell extracts were collected. Western blot analysis showed that syndecan-1 synthesis decreased after small interfering RNA transfection. (B) Cells were transfected with syndecan-1 small interfering RNA or with control small interfering RNA for 24 h prior to the addition of osteoprotegerin. The results shows that syndecan-1 small interfering RNA suppressed the increase of osteopontin induced by osteoprotegerin. The relative density is shown by bar charts. The results are expressed as mean ± standard deviation from three different experiments. *Significant difference ($p < 0.05$). OPG, osteoprotegerin; OPN, osteopontin; siCon, control small interfering RNA; siSyn, syndecan-1 small interfering RNA.

To confirm the results obtained using syndecan-1 antibody, we used syndecan-1 small interfering RNA in another approach. Syndecan-1 small interfering RNA and control small interfering RNA were transfected into human periodontal ligament cells 24 h before treatment with osteoprotegerin. The results showed that syndecan-1 small interfering RNA was able to reduce syndecan-1 protein expression and inhibit the osteopontin synthesis induced by osteoprotegerin (Fig. 6).

Discussion

We report here that osteoprotegerin up-regulates osteopontin synthesis in human periodontal ligament cells via interacting with cell-surface syndecan-1. In addition, our results revealed the involvement of phosphoinositol 3-kinase/Akt in the signaling pathway. The up-regulation of osteopontin mediated by osteoprotegerin supports our hypothesis that osteoprotegerin can exert a biological effect on human periodontal ligament cells in addition to counteracting RANKL in the RANKL–osteoprotegerin system.

Elevated levels of osteoprotegerin have been found in subjects with chronic inflammatory diseases such as rheumatoid arthritis (16), coronary artery disease (17) and primary biliary cirrhosis (18). This may reflect a mechanism of response to osteoclast activity or a bone remodeling balance. The impact of the increased level of osteoprotegerin on cell behavior is not clear. However, there are reports supporting the view that osteoprotegerin influences cell activity. Osteoprotegerin induces protease and protease expression (9), activates MMP-9 and forms tertiary complex RANK–RANKL–osteoprotegerin on osteoclasts to regulate osteoclast function (11). These data support our finding, that osteoprotegerin up-regulates osteopontin in human periodontal ligament cells.

Up-regulation of osteopontin expression, as well as the activation of Akt, was inhibited by neutralizing antibody against RANKL, suggesting the involvement of RANKL in the induction. As there is no evidence that membranous RANKL is able to generate intracellular signals, we hypothesized that the signal may have been mediated through RANKL-associated receptor(s). Because osteoprotegerin contains a heparan sulfate-binding domain, we hypothesized that cell-surface receptors containing heparan sulfate proteoglycans, such as syndecan, might be involved in this phenomenon. This hypothesis was supported by the inhibitory effect of heparinase, syndecan-1 antibody and syndecan-1 small interfering RNA. As the results showed that both RANKL and syndecan-1 are involved in osteoprotegerin-induced osteopontin expression, it is possible that a trimolecular complex of RANKL–osteoprotegerin–syndecan-1 occurs on the cell surface through the osteoprotegerin heparin sulfate-binding domain and that the cytoplasmic domain of syndecan-1 takes part in signaling initiation. However, the detailed mechanism of the association between RANKL and syndecan-1 needs further investigation.

We also investigated the effect of osteoprotegerin on the expression of genes that might be involved in the balance of periodontal tissue homeostasis, such as type I collagen, RANKL, osteoprotegerin and periostin (data not shown). The results showed that only osteopontin expression was increased, suggesting the selective effect of osteoprotegerin on human periodontal ligament cells. However, further investigation is needed.

The inhibitory effect of heparinase, syndecan-1 antibody or syndecan-1 small interfering RNA, on the inductive effect of osteoprotegerin, suggested

that osteoprotegerin mediated its effect through a cell-surface proteoglycan, syndecan-1, in human periodontal ligament cells. This result is in agreement with previous reports, showing that osteoprotegerin is bound to syndecan-1 via an interaction with heparan sulfate in multiple myeloma cells (19) and in human peripheral blood monocytes (12). Involvement of intracellular signaling through phosphoinositol 3-kinase/Akt was also demonstrated in a study of human monocyte migration (12). In the present study, we found that osteoprotegerin activated Akt and up-regulated osteopontin. The phosphorylation of Akt requires phosphoinositol 3-kinase-dependent generation of phosphatidylinositol 3,4,5- triphosphate and therefore serves as an indicator of phosphoinositol 3-kinase activity (20). Heparinase, syndecan-1 antibody and syndecan-1 small interfering RNA inhibited both osteoprotegerin-induced osteopontin and the activation of Akt. Taken together, the results suggest that the upstream signal of phosphoinositol 3-kinase/Akt should be associated with the surface receptor, syndecan-1.

In the present study, it was found that the inductive effect occurred only upon the addition of a high concentration of osteoprotegerin, because none of the inhibitors had any effect on the control of osteopontin expression. This result suggests that the physiological level of osteoprotegerin in periodontal tissue should not affect the expression of osteopontin in human periodontal ligament cells.

Elevation of the levels of osteopontin by increasing the amount of osteoprotegerin in human periodontal ligament cells could be a mechanism of regeneration or remodeling in periodontal tissue. Osteopontin was found to be localized in periodontal ligament cells adjacent to deciduous tooth roots, in association with odontoclasts in resorption lacunae (21). We also found that the expression of osteopontin mRNA and protein was up-regulated in human periodontal ligament cells in response to mechanical stress (22,23). These results suggest that osteopontin is involved in bone remodeling mediated by human periodontal ligament cells.

Although osteopontin participates in the process of bone resorption by promoting the attachment and migration of osteoclasts (24), it also plays a role in assisting bone formation. Osteopontin has been shown to regulate osteoblast adhesion and to induce the expression of alkaline phosphatase and osteocalcin (25,26). In periodontal regeneration, osteopontin expression was intense at the border of newly formed cementum and bone (27). In addition, the expression of osteopontin was found to be increased in tissue treated using a guided tissue-regeneration technique (28). Therefore, it is possible that an increased amount of osteoprotegerin may be involved in periodontal tissue remodeling through modulation of extracellular matrix molecules such as osteopontin.

In conclusion, our present study shows that a high level of osteoprotegerin could up-regulate osteopontin expression in human periodontal ligament cells. The results also suggest that osteoprotegerin can generate signals via the RANKL/syndecan-1/phosphoinositol 3-kinase/Akt pathway, in addition to being a decoy receptor for RANKL.

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