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Thrombin induces osteoprotegerin synthesis via phosphatidylinositol 3′-kinase/mammalian target of rapamycin pathway in human periodontal ligament cells

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Background and Objective: Thrombin influences the biological behavior of periodontal ligament cells and plays multiple roles in the early stages of bone healing. Osteoprotegerin (OPG) is one of the key molecules that regulate bone homeostasis and prevent osteoclastogenesis. The purpose of this study was to evaluate the biological effects of thrombin on OPG synthesis in human periodontal ligament (HPDL) cells *in vitro*.

Material and Methods: Cells were treated with various concentrations (0.001, 0.01 and 0.1 U/mL) of thrombin. The mRNA expression and protein synthesis of OPG, as well as of receptor activator of nuclear factor κB ligand (RANKL), were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis, respectively. The influence of thrombin on OPG synthesis and its signaling pathway were investigated using inhibitors.

Results: Thrombin profoundly induces protein synthesis of OPG at 0.1 U/mL. The inductive effect was inhibited by cycloheximide, but not by indomethacin. The phosphatidylinositol 3′-kinase (PI3K) inhibitor, LY294002, and the mammalian target of rapamycin (mTOR) inhibitor, rapamycin, exerted an inhibitory effect on the thrombin-induced OPG synthesis. In addition, the effect was inhibited by protease-activated receptor (PAR)-1 antagonist. Activation of phospho-Akt (p-Akt) was observed and the effect was abolished by LY294002.

Conclusion: Thrombin induces OPG synthesis in HPDL cells post-transcriptionally, possibly through PAR-1. The regulation was through the PI3K/Akt and mTOR pathway. This finding suggests that thrombin may play a significant role in alveolar bone repair and homeostasis of periodontal tissue, partly through the OPG/RANKL system.

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Thrombin is a serine protease which plays a central role in blood coagulation through the cleavage of fibrinogen. In addition to the coagulant effect, it also exerts specific receptor-mediated influences on cell function. Thrombin promotes endothelial cell adhesion (1) and induces proliferation of vascular smooth muscle cells (2,3). Thrombin also plays a part in regulating connective tissue growth factors (4), and induces the expression and synthesis of interleukin-8 (IL-8) (5) and matrix metalloproteinase-9 in fibroblasts (6). These effects indicate that thrombin may play important roles in tissue repair and wound healing.

In mineralised tissue, thrombin is able to influence the biological behavior of osteoblasts and plays multiple roles in the early stages of bone healing (7). It has been shown to stimulate proliferation, migration, prostaglandin release and calcium mobilization in osteoblast-like cell lines or primary cultures of osteoblasts (8–11). These results suggest a role of thrombin in bone repair.

Thrombin exerts its action through proteolytic activation of its receptors, including protease-activated receptors (PARs; 12,13). Proteolytic activation of PARs involves cleavage of the N-terminal extracellular domain, resulting in a new N-terminus which can interact with the extracellular loop of the receptor and activate intracellular signaling. To date, four PARs have been identified. However, only PAR-1, -3 and -4 can be activated by thrombin (12).

In periodontal disease, thrombin is likely to be generated via the coagulation cascade on the surface of local damaged vessels of periodontal tissue. Recently, thrombin-rich plasma and platelet-rich plasma, which contain thrombin, have been used successfully for periodontal regeneration (14). However, the action of thrombin in periodontal tissue is still uncertain. Thrombin has been shown to stimulate proliferation and protein synthesis in human periodontal ligament (HPDL) cells (15). In addition, it could promote collagen gel contraction in gingival fibroblasts (16). Although there are few reports regarding the role of thrombin in periodontal tissue, the data suggest the possibility that thrombin may play a role in repair and healing of periodontal tissue.

Receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) are among the molecules which are involved in the regulation of bone resorption (17,18). The RANKL, upon binding to RANK on the cell surface of osteoclasts, functions to induce differentiation, activation and survival of osteoclasts, whereas OPG, a decoy receptor for RANKL, functions to inhibit osteoclastogenesis (19). Thus, the balance of RANKL and OPG in the tissue is one of the factors significant in the mechanism of hard tissue destruction and remodeling (20,21).

A hallmark of periodontal disease is the destruction of alveolar bone. An increase in the mRNA expression of RANKL has been found in patients with periodontitis (22–24). In addition, application of OPG has been demonstrated to reduce bone destruction, suggesting an important role for OPG/RANKL in the homeostasis of alveolar bone (25). Although a function of thrombin in bone metabolism has been suggested, the exact role of thrombin in periodontal disease has not been elucidated.

Since both thrombin and the OPG/RANKL ratio are important components of the process of tissue repair, we hypothesized that thrombin could affect the balance of OPG and RANKL in HPDL cells. The present study was undertaken to investigate the effect of thrombin on OPG synthesis. The participation of PAR-1 and the signaling pathways mediated by thrombin are highlighted.

Material and methods

Cell culture

Human periodontal ligament cells were obtained from healthy periodontal ligament tissue of non-carious, freshly extracted third molars, or tooth removed for orthodontic reasons. All patients gave written informed consent. The protocol was approved by the Ethical Committee of the Faculty of Dentistry, Chulalongkorn University. Briefly, the explant was taken from the middle third of the root, cut into pieces, placed into 35 mm culture dishes (Corning, New York, NY, USA) and grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin and 0.25 μg/mL of amphotericin B, and incubated in an atmosphere of 5% CO_2 (95% air) at 37°C. When the cells growing from explants had reached confluence, they were trysinized and subcultured. Cells at passage 3–5 were used for the experiments. Cells from three preparations were used. Each preparation was from one molar. Media and all supplements were from Gibco (Grand Island, NY, USA).

Treatment of cells

Human periodontal ligament cells were seeded in six-well plates, at a density of 25,000 cells cm^{-2}. After deprivation of serum for 16 h, cells were stimulated with 0.001, 0.01 or 0.1 U/mL of thrombin. After 24 h, the RNA was extracted for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Cell protein extracts and the conditioned medium were collected from the parallel set of cultures for Western blot analysis after 48 h of treatment. The effective dose was selected and used for the rest of the experiments. To examine whether the effect induced by thrombin was similar to that mediated through PAR-1, cultures were exposed to PAR-1 agonist peptide, SFLLRN (Peptides International Inc., Louisville, KY, USA), which mimics the first six amino acids of the new amino terminus unmasked by receptor cleavage, functions as an agonist for PAR-1 and activates the receptor independently of thrombin and proteolysis. Medium was collected for analysis.

Inhibition

The inhibitors used were indomethacin (10 μM; Sigma-Aldrich Chemical Co., St Louis, MO, USA), actinomycin D (0.5 μg/mL; Calbiochem, EMD Bio-

sciences, San Diego, CA, USA), cycloheximide (1 μg/mL; Sigma), phosphatidylinositol 3′-kinase (PI3K) inhibitor, LY294002 (1.4 μM; Cayman Chemical, Ann Arbor, MI, USA), rapamycin (100 pM; Calbiochem) and PAR-1 antagonist, SCH 79797 dihydrochloride (100 nM; Tocris Bioscience, Bristol, UK). Cells were treated with each inhibitor for 30 min prior to the addition of 0.1 U/mL of thrombin. After the treatment, cell protein extraction and collection of medium were performed for further analyses.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Cells were treated with thrombin, as described above. 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, PCR amplification was performed. The primers were prepared following the reported sequences from GenBank. The oligonucleotide sequences of the primers used are: RANKL forward 5′-CCA GCA TCA AAA TCC CAA G-3′ and reverse 5′-CCC CTT CAG ATG ATC CTT C-3′; OPG forward 5′-TGC AGT ACG TCA AGC AGG A-3′ and reverse 5′-TGA CCT CTG TGA AAA CAG C-3′; and GAPDH forward 5′-TGA AGG TCG GAG TCA ACG GAT-3′and reverse 5′-TCA CAC CCA TGA CGA ACA TGG-3′.

PCR 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 RT 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 for RANKL (26 cycles for OPG and 22 cycles for GAPDH), followed by one extension cycle of 10 min at 72°C. The PCR 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 Corp., Frederick, MD, USA).

Western blot analysis

Osteoprotegerin was prepared from the culture medium. The lyophilized medium was dissolved in sample buffer, boiled and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis to detect OPG. To detect RANKL, phospho-Akt or total Akt, cell protein extracts were prepared on ice using radioimmunoprecipitation buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 and 0.25% sodium-deoxycholate) containing cocktail protease inhibitors (10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 1 mM NaF and 1 mM Na_3VO_4) (Sigma) or phosphatase inhibitors (1 mM sodium vanadate and 50 mM NaF). Protein concentrations were measured using a BCA™ protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein from each sample were boiled and subjected to electrophoresis, under reducing conditions, on a 12% polyacrylamide gel, along with prestained high molecular weight standards (Bio-Rad, Hercules, CA, USA). The proteins were then transferred onto the nitrocellulose membrane (Immobilon-P, Millipore Corp., Bedford, MA, USA). The membrane was stained overnight with any of the following primary antibodies: RANKL (R&D System, Inc., Minneapolis, MN, USA), OPG (R&D System), β-actin (Chemicon International, Temecula, CA, USA), phospho-Akt or total Akt (Cell Signaling Technology, Inc., Danvers, MA, USA) at a dilution of 1:1000 at 4°C, followed by biotinylated secondary antibody and peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA, USA). The protein bands were detected using a commercial chemiluminescence system (Pierce) and were exposed on CL-X Posture film (Pierce). The band intensity was determined by Scion image-analysis software.

Statistical analysis

Results are expressed as means ± SD. Statistical differences were determined by one-way ANOVA followed by *post hoc* analysis (Scheffé's *post hoc* test). Differences at $p < 0.05$ were considered statistically significant.

Results

Thrombin induces the protein expression of OPG

The effective dose of thrombin for OPG expression was determined. Human periodontal ligament cells were stimulated with 0.001–0.1 U/mL of thrombin. After the treatment, the RNA or protein extracts and conditioned medium were collected as described in the Materials and methods. The results demonstrated that thrombin increased the mRNA expression of RANKL, but not that of OPG, in a dose-dependent manner (Fig. 1A). The relative density compared with control cultures is shown in Fig. 1B. We also found that the protein expression of both RANKL and OPG increased (Fig. 1C). It is noticeable that the protein level of OPG did not correlate with that of the mRNA. The level of OPG protein increased in a dose-dependent manner and was profound at the concentration of 0.1 U/mL. The ratio of OPG/RANKL intensity was calculated and the result demonstrated that the OPG/RANKL ratio increased significantly above the control value, as shown in Fig. 1D. The thrombin concentration of 0.1 U/mL was then selected for the rest of the experiments.

To examine whether thrombin regulated RANKL and OPG expression differently, indomethacin, a non-specific cyclo-oxygenase (COX) inhibitor, was applied to the cultures 30 min before addition of thrombin. The result demonstrated that indomethacin was capable of blocking thrombin-induced expression of RANKL, but not that of OPG (Fig. 2). This indicates that regulation of RANKL and OPG by thrombin is mediated through different pathways. Regulation of RANKL via COX and prostaglandin E_2 (PGE_2) has

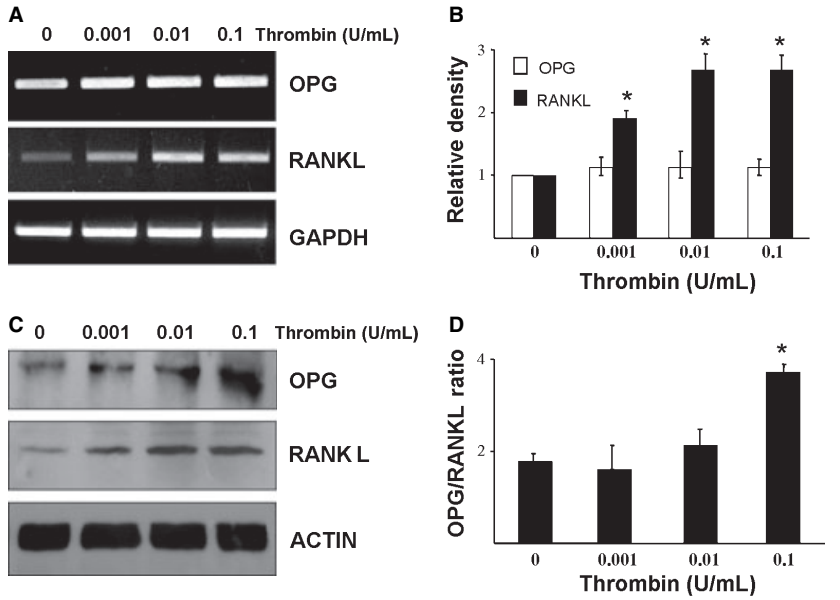

Fig. 1. Effect of thrombin on mRNA expression of OPG and RANKL (A) and protein synthesis (C) in HPDL cells. Cells were treated with 0.001, 0.01 or 0.1 U/mL of thrombin for 24 h in serum-free conditions. (A) The PCR products revealed upregulation of RANKL, but not of OPG, in a dose-dependent manner. The mean ± SD density of the PCR product from three gels is shown in the bar graph (B). (C) Western blot analysis reveals a dose-dependent increase of RANKL and OPG protein after 48 h of treatment. The OPG/RANKL ration relative density is presented as a bar graph in (D). The results are expressed as means + SD from three different experiments. $*p < 0.05$ compared with the non-treated condition. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

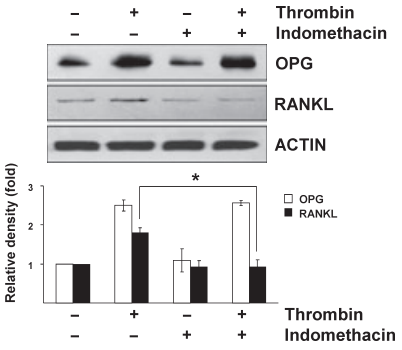

Fig. 2. Effect of indomethacin on protein expression of OPG and RANKL. Human periodontal ligament cells were exposed to 10 μM of indomethacin for 30 min before addition of 0.1 U/mL of thrombin for 48 h. Western blot analysis shows that indomethacin exerts an inhibitory effect on thrombin-induced RANKL, but not on thrombin-induced OPG. The bar graph represents the mean ± SD density of each band normalized to actin, from three independent experiment. $*p < 0.05$ compared with thrombin-treated control culture. −, without thrombin or indomethacin; +, with thrombin or indomethacin.

been established (26,27); we therefore focus only on the regulation of OPG synthesis.

To determine whether the effect of thrombin on OPG synthesis was post-transcriptional, actinomycin D and cycloheximide, inhibitors of mRNA transcription and protein biosynthesis, respectively, were used. Human periodontal ligament cells were exposed to thrombin in the absence or presence of either actinomycin D or cycloheximide for 24 h. We found that the increased OPG induced by thrombin was abolished by cycloheximide (Fig. 3), suggesting post-transcriptional regulation.

Thrombin induces OPG synthesis through the PI3K/Akt and mTOR pathways

To investigate the kinase signaling pathways involved in thrombin-induced OPG synthesis, either LY294002, the specific PI3K inhibitor, or rapamycin, a potent inhibitor of mTOR, was applied to quiescent cells

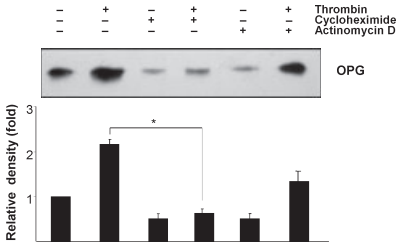

Fig. 3. Effect of cycloheximide and actinomycin D on thrombin-induced protein expression of OPG in HPDL cells. Cells were pre-incubated with either cycloheximide (1 μg/mL) or actinomycin D (0.5 μg/mL) for 30 min prior to treatment with 0.1 U/mL of thrombin for 24 h. The thrombin-induced protein expression of OPG was attenuated by cycloheximide, but not by actinomycin D. The OPG protein relative density is presented in the bar graph graph and the results are expressed as means ± SD from three different experiments. $*p < 0.05$ compared with thrombin-treated control culture. −, without thrombin or inhibitor; +, with thrombin or inhibitor.

for 30 min before addition of thrombin. The results showed that both LY294002 and rapamycin exerted an inhibitory effect on the thrombin-induced OPG synthesis (Fig. 4A,B). Phosphorylation of Akt (p-Akt) was also examined and was found to be increased in response to thrombin. In addition, the increase of p-Akt could be inhibited by LY294002 (Fig. 4C), providing evidence that thrombin mediated its effect through PI3K/Akt and mTOR signaling pathway.

Thrombin stimulation involves PAR-1

Since proteolytic cleavage of PARs by thrombin has been proposed (12), the existence of PARs on HPDL cells was explored. We found that HPDL cells expressed PAR-1, PAR-2 and PAR-3 (data not shown). A PAR-1 antagonist was used to investigate the involvement of PAR-1. The result in Fig. 5A showed that PAR-1 antagonist could diminish the increase of OPG. To examine whether PAR-1 contributes to the regulation of OPG synthesis, PAR-1 agonist peptide was used to mimic the effect of thrombin. The PAR-1 agonist appeared to enhance the protein expression of OPG. In addition,

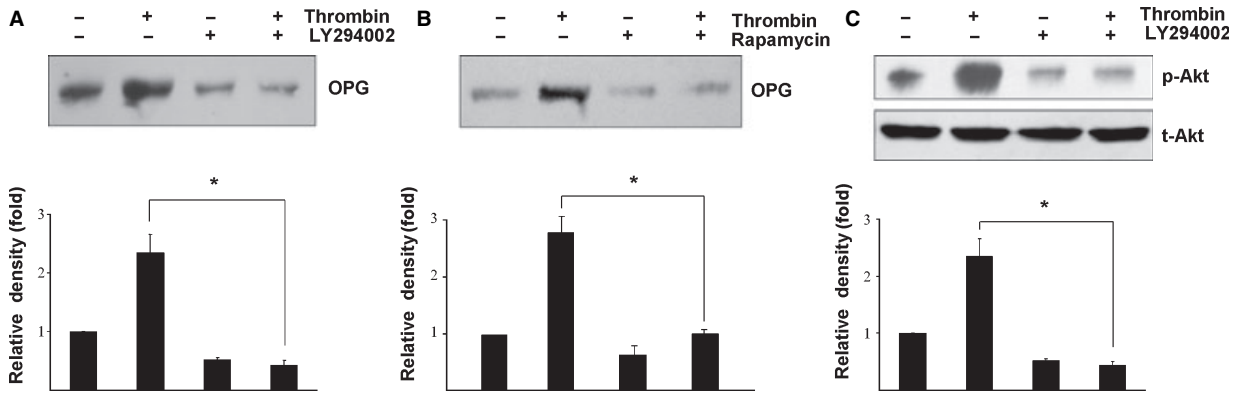

Fig. 4. Inhibitory effect of LY294002 (A,C) and rapamycin (B) on thrombin-induced protein expression of OPG in HPDL cells. Cells were pre-incubated with LY294002 or rapamycin for 30 min before the addition of 0.1 U/mL of thrombin for 48 h (for OPG detection) or 5 min (for Akt detection). After 48 h, the medium was collected, lyophilized and prepared for Western blot analysis. The LY294002 (A) and rapamycin (B) could inhibit the increase of thrombin-induced OPG. (C) Cell protein extracts were collected under ice-cold conditions for Western blot analysis after 5 min of treatment. The activation of phospho-Akt (p-Akt) mediated by thrombin was observed and the activation was inhibited by LY294002. The bar chart (C) represents the average band density of p-Akt normalized to its total protein (t-Akt). $*p < 0.05$ compared with thrombin-treated control culture. −, without thrombin or LY294002; +, with thrombin or LY294002.

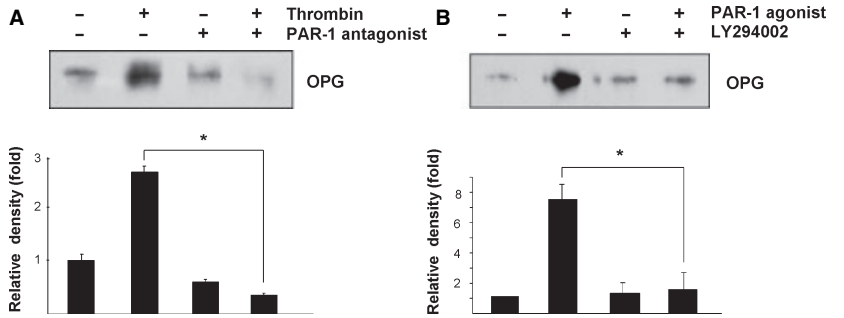

Fig. 5. Inhibitory effect of PAR-1 antagonist on the thrombin-induced protein expression of OPG in HPDL cells (A) and the effect of LY294002 on PAR-1-induced protein expression of OPG (B). (A) Cells were exposed to PAR-1 antagonist (100 nM) for 30 min before addition of thrombin for 48 h. The medium was collected for Western blot analysis. The PAR-1 antagonist inhibited the increase in OPG mediated by thrombin. (B) Cells were incubated with LY294002 for 30 min before addition of PAR-1 agonist (100 nM) for 48 h. Western blot analysis revealed that PAR-1 stimulated protein expression of OPG and thsi stimulation was abolished by LY294002. The bar graphs represent the average relative density of OPG. $*p < 0.05$ compared with thrombin-treated or PAR-1-treated control culture. −, without treatment or inhibitor; +, with treatment or inhibitor.

the effect could be blocked by LY294002 (Fig. 5B). These data suggest that PAR-1 could be involved in the enhancement of OPG synthesis mediated by thrombin.

Discussion

The balance of RANKL and OPG plays a significant role in bone homeostasis. They are two key molecules that regulate osteoclast formation, activation and survival. In this study, we demonstrated that thrombin may be involved in the homeostasis of alveolar bone and periodontium by regulating the balance of RANKL and OPG synthesized by HPDL cells.

Our results clearly demonstrated that thrombin differentially regulated RANKL and OPG expression. Upon activation, thrombin increased both RANKL mRNA and protein synthesis. No change in the level of OPG mRNA was observed at 24 h (nor at 48 h; data not shown), but the protein synthesis was increased, by a post-transcriptional mechanism. Although both RANKL and OPG synthesis increased in response to thrombin, the ratio of OPG to RANKL was higher than the control value, suggesting an anabolic role of thrombin in alveolar bone homeostasis via HPDL cells.

By means of inhibitors, the effect of thrombin on OPG synthesis was demonstrated to involve both PI3K and mTOR signaling pathways. Signaling pathways of thrombin through the protease-activated receptor (PAR), a G-protein-coupled receptor, have been described in several kinds of cells. A variety of signaling molecules have been reported to be activated by thrombin, including mitogen-activated protein kinase (MAPK), Rho kinase, protein kinase C (PKC) and janus kinase-signal transducer and activator of transcription (JAK-STAT; 28). In this study, inhibitors of MAPK, Rho kinase and PKC had no effects on thrombin-mediated OPG synthesis (data not shown). The different response could depend on the cell type studied. However, the involvement of PI3K is consistent with previous results regarding to the effect of thrombin in several cell types, such as the induction of vascular endothelial growth factor (VEGF) in retinal pigment epithelial cells (29) or the induction of IL-8 in prostate cancer cells (30). In addition, the inhibitory effect of rapamycin on OPG synthesis indi-

cates that mTOR participates in OPG regulation. Mammalian target of rapamycin is a multidomain protein that functions as a regulator of the translational machinery, and the signal from the PI3K and mTOR pathways has been shown to regulate cell growth, size and proliferation in many cell types (31).

The increased ratio of OPG to RANKL supports the concept that thrombin may participate in bone repair. A high amount of OPG may be significant in the reduction of bone resorption, as demonstrated in the model of osteoporosis (32). However, in the present study, thrombin did not exhibit a mitogenic effect, although it has been shown to act as a mitogen for osteoblasts and marrow stromal cells (33,34). The difference is possibly due to the dose of thrombin used in our study, which is lower than that of previous studies, which were approximately 1–10 U/mL.

The increased ratio of OPG to RANKL by thrombin in HPDL cells seems to be in contrast with previous reports on the catalytic effect of thrombin. For instance, thrombin increased release of ^{45}Ca from mouse calvarial bone explants (35). The difference may result from either different cell types or different models used in the experiments. Induction of bone resorption by thrombin was mediated by the release of prostaglandins, and this effect could be inhibited by indomethacin, a COX inhibitor. Since PGE_2 has been reported to be a potent inducer of RANKL in osteoblasts (36), the resorbing activity found after the thrombin application may have resulted from the increased level of RANKL.

In this study, indomethacin did not suppress OPG synthesis, which indicates that regulation of OPG synthesis induced by thrombin in HPDL cells is a COX-independent mechanism. This result is different from the previous one reported by Marklund *et al*. (37), whose report suggested that thrombin at 10 U/mL stimulated release of PGE_2. It is possible that the response of HPDL cells depends on the dose of thrombin. Our present results suggest that low doses of thrombin (0.1 U/mL or less) increase OPG synthesis. This finding supports our hypothesis that thrombin may play a role in periodontal tissue repair, partly through the balance of OPG and RANKL. However, the significance of the thrombin-induced OPG *in vivo* requires further investigation.

With regard to receptors, thrombin has been shown to communicate with cells through its proteolytic action on PAR-1, which was considered to be a thrombin receptor (12). Protease-activated receptor -3 and PAR-4, but not PAR-2, also function as thrombin receptors. However, function of PAR-3 required the presence of PAR-4 (38). In addition, a non-PAR thrombin receptor was reported (39). In this study, addition of PAR-1 antagonist abolished the thrombin-induced OPG synthesis. Taking into account that HPDL cells express PAR-1 and PAR-3, but not PAR-4, we suggest that PAR-1 is involved in the OPG synthesis mediated by thrombin.

In conclusion, thrombin can stimulate OPG synthesis in HPDL cells post-transcriptionally, resulting in an increase of the OPG/RANKL ratio. Thrombin is likely to exert its action through PAR-1 and the PI3K/Akt and mTOR signaling pathways. These results suggest that thrombin could play a significant role in alveolar bone repair and maintenance of homeostasis of periodontal tissue, partly through the balance of RANKL and OPG.

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