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Adenosine triphosphate stimulates RANKL expression through $P2Y_1$ receptor–cyclo-oxygenase-dependent pathway in human periodontal ligament cells

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Background and Objective: Our previous study showed that human periodontal ligament cells responded to mechanical stress by increasing adenosine triphosphate (ATP) release, accompanied by the increased expression of RANKL and osteopontin. We found that the signaling pathway of mechanical stress-induced osteopontin was mediated through ATP/$P2Y_1$ receptor and Rho kinase activation but that of mechanical stress-induced RANKL was different. In this study, we further investigated the effect of extracellular ATP on the expression of RANKL and the mechanism involved.

Material and Methods: Human periodontal ligament cells were treated with ATP (10–40 μM). The expressions of RANKL and cyclo-oxygenase 2 (COX-2) were examined by RT-PCR and western blot analysis. The level of prostaglandin E_2 was determined using ELISA. Signaling pathways were investigated by using inhibitors and antagonist.

Results: Adenosine triphosphate induced the expression of RANKL. Indomethacin, an inhibitor of COX, could abolish the induction of RANKL expression, suggesting a COX-dependent mechanism. A cAMP-dependent protein kinase inhibitor, H89, and a nuclear factor κB (NFκB) inhibitor, pyrrolidine dithiocarbamate, inhibited RANKL expression, prostaglandin E_2 production and NFκB translocation. In addition, a specific $P2Y_1$ receptor antagonist, MRS2179, and $P2Y_1$ small interfering RNA diminished the effect of ATP.

Conclusion: Extracellular ATP stimulates RANKL expression in human periodontal ligament cells through a pathway dependent on the $P2Y_1$ receptor, cAMP-dependent protein kinase, NFκB and COX. Our results suggest that, among the molecules responsible for the effect of mechanical stress, ATP participates in bone resorption or bone homeostasis by mediating its signal through the $P2Y_1$ receptor and the NFκB–COX–RANKL axis in periodontal tissue.

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Adenosine triphosphate (ATP) and related nucleotides are recognized as important and ubiquitous extracellular messengers that are released from various kinds of tissues (1,2). Adenosine triphosphate is often released from different cell types in response to mechanical perturbations such as shear stress, membrane stretch or hypo-osmotic swelling (1,3–5).

Upon the mechanical stress or biological activation, ATP can be released via lytic or non-lytic mechanisms involving vesicle-mediated secretion, carrier-mediated transport or plasma membrane channels (6,7). The released ATP activates plasma membrane receptors known as purinergic (P2) receptors, which are classified broadly within two families, based on their mode of signal transduction, as the ligand-gated ion channels (P2X) and the G-protein-coupled metabotropic receptors (P2Y; 8).

Adenosine triphosphate is also one of the regulators of bone homeostasis. It has been demonstrated that ATP can stimulate osteoclast activity via an up-regulation of the expression of RANKL in osteoblasts (9). Extracellular ATP stimulated the resorptive activity of rat osteoclasts (10). Pit formation *in vitro* was observed when ATP was added to cultures of human osteoclasts isolated from a giant cell tumor (11). These lines of evidence indicate that ATP participates in bone homeostasis through the regulation of RANKL.

Receptor activator of nuclear factor κB ligand is a molecule essential for osteoclastogenesis. It is expressed by osteoblasts as a membrane-associated factor. When RANKL binds to RANK, a receptor expressed on the cell surface of osteoclast precursors, it stimulates the differentiation of those precursors into mature osteoclasts. In contrast, an interaction of RANKL with RANK can be inhibited by osteoprotegerin, a soluble factor secreted by osteoblasts. Osteoprotegerin acts as a decoy receptor for RANKL and thus prevents osteoclast differentiation. Therefore, RANKL and osteoprotegerin are considered as major factors that regulate bone homeostasis.

It is of interest that human periodontal ligament cells also express RANKL and osteoprotegerin and that their balance could be altered by mechanical stress (12,13). Our previous study found that cells responded to mechanical stress by increasing the release of ATP, as well as the expression of RANKL and osteopontin, at both the mRNA and the protein level (14). We also reported that the mechanical stress up-regulated osteopontin via ATP/$P2Y_1$ receptor and Rho kinase activation (15). However, the elevation of RANKL expression by ATP has not been elucidated. In the present study, we hypothesized that the up-regulation of RANKL in human periodontal ligament cells was a consequence of release by ATP. The role of ATP in RANKL expression and the mechanism involved was demonstrated in human periodontal ligament cells.

Material and methods

Cell culture

Human periodontal ligament cells were obtained from extracted healthy third molars for orthodontic reason with informed consent. The protocol was approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University. Teeth were rinsed with sterile phosphate-buffered saline, and the periodontal tissue was removed from the middle third of the root surface and grown in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal calf serum (Hyclone), 2 mM L-glutamine (Gibco BRL, Carlsbad, CA, USA), 100 units/mL penicillin (Gibco BRL), 100 μg/mL streptomycin (Gibco BRL) and 5 μg/mL amphotericin B (Gibco BRL), and incubated in a humidified atmosphere of 95% of air, 5% CO_2 at 37°C. Each preparation was from one molar. Cells from passage 3 of three different preparations were used in the experiments.

Treatment of cells

Cells were seeded in six-well plates, at a density of 25,000 cells cm^{-2}, and grown to subconfluence. After deprivation of serum for 6 h, cells were stimulated with ATP (Sigma-Aldrich Chemical, St Louis, MO, USA). To examine the effect of ATP on RANKL, cyclo-oxygenase (COX) mRNA expression and prostaglandin E_2 (PGE_2) secretion, cells were treated with 0, 10, 20 or 40 μM ATP in serum-free medium. The RNA was extracted for RT-PCR analysis after 16 h of treatment, and the medium was collected for ELISA at 2 h after exposure to ATP. Cell protein extracts and culture medium were collected from a parallel set of cultures after 48 h of stimulation for western blot analysis. The effective dose was selected and used for the rest of the experiments.

To confirm the activity of adenylyl cyclase on RANKL expression and PGE_2 release, forskolin (Sigma-Aldrich Chemical) at 0.1 and 1 mg/mL was used.

Inhibitors

The inhibitors used were 10 μM indomethacin (Sigma-Aldrich Chemical), 20 μM pyrrolidine dithiocarbamate (PDTC; Sigma-Aldrich Chemical), 2 μM phospholipase C inhibitor U73122, 5 nM cAMP-dependent protein kinase inhibitor H89 dihydrochloride hydrate (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 5 μM $P2Y_1$ receptor antagonist MRS2179 (Sigma-Aldrich Chemical) and small interfering RNA or control oligonucleotide (Santa Cruz Biotechnology). Cells were treated with each inhibitor for 30 min (24 h for small interfering RNA or control oligonucleotide) prior to the addition of 40 μM ATP.

Extraction of RNA and semi-quantitative RT-PCR

Total cellular RNA was extracted with reagent (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 reverse transcription using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) for 1.5 h at 42°C. Subsequent

to reverse transcription, PCR was performed. The primers were prepared following the reported sequences from GenBank. The oligonucleotide sequences of the primers were as follows:

The PCR was performed using Taq polymerase (Qiagen, Hilden, Germany) with a PCR volume of 25 μl. The amplification profile for RANKL (32 cycles) was one cycle at 94°C for 1 min, hybridization at 60°C for 1 min and extension at 72°C for 2 min, followed by one extension cycle at 72°C for 10 min. The same profile was also used for osteoprotegerin (24 cycles), COX-1 (30 cycles), COX-2 (27 cycles) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 22 cycles). The PCR was performed in the DNA thermal cycler (Biometra, Gottingen, Germany). The amplified DNA was electrophoresed on a 1% agarose gel and visualized by ethidium bromide fluorostaining. The relative intensities of the gel bands were measured by imaging software analysis (Scion Image; Scion, Frederick, MD, USA).

RANKL	Forward, 5′ CCA GCA TCA AAA TCC CAA GT 3′ Reverse, 5′ CCC CTT CAG ATG ATC CTT C 3′
Osteoprotegerin	Forward, 5′ TGC AGT ACG TCA AGC AGG A 3′ Reverse, 5′ TGA CCT CTG TGA AAA CAG C 3′
COX-1	Forward, 5′ GCA GCT GAG TGG CTA TTT CC 3′ Reverse, 5′ ATC TCC CGA GAC TCC CTG AT 3′
COX-2	Forward, 5′ TTC AAA TGA GAT TGT GGG AAA ATT GCT 3′ Reverse, 5′ AGA TCA TCT CTG CCT GAG TAT CTT 3′
$P2Y_1$	Forward, 5′ CGG TCC GGG TTC GTC C 3′ Reverse, 5′ CGG ACC CCG GTA CCT 3′
Glyceraldehyde 3-phosphate dehydrogenase	Forward, 5′ TGA AGG TCG GAG TCA ACG GAT 3′ Reverse, 5′ TCA CAC CCA TGA CGA ACA TGG 3′

Western blot analysis

Osteoprotegerin was prepared from the culture medium. The lyophilized medium was dissolved in sample buffer, boiled and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to detect osteoprotegerin. To detect RANKL, $P2Y_1$ receptor and actin, protein was extracted with radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich Chemical). Protein concentrations were measured using a protein assay kit (BCA protein assay kit; Pierce Biotechnology, Rockford, IL, USA). Equal amouts of protein samples were subjected to electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel and subsequently transferred onto nitrocellulose membrane. For the reduction of non-specific binding, the membrane was incubated in 5% non-fat milk for 1 h before being incubated in the primary antibody. The antibody raised against RANKL (dilution 1:50; R&D Systems, Minneapolis, MN, USA), osteoprotegerin (dilution 1:250; R&D Systems), $P2Y_1$ (dilution 1:300; Abcam, Cambridge, MA, USA) or actin (dilution 1:1000; Chemicon International, Temecula, CA, USA) was used as the primary antibody. The membranes were incubated in biotinylated secondary antibody, followed by peroxidase-labeled streptavidin. The signal was captured by chemoluminescence. The relative intensities of bands were measured by imaging software analysis (Scion Image; Scion).

Measurement of PGE_2

Measurement of PGE_2 from the media was performed using PGE_2 ELISA kits (Parameter PGE_2 Immunoassay; R&D Systems), according to the manufacturer's instructions. The experiments were performed in triplicate.

Detection of nuclear factor κB (NFκB) localization by immunofluorescence

Cells were seeded in a chamber slide (Lab-TEK® II Chamber slide™ System; Nalge Nunc International Corp., Rochester, NY, USA) at a density of 50,000 cells per chamber and grown to subconfluence. After being starved in serum-free conditions for 6 h, cells were treated with ATP in the presence or absence of inhibitors. At the indicated time, cells were washed in phosphate-buffered saline (for 5 min, twice) and fixed with cold methanol (Merck KGAA, Darmstadt, Germany) for

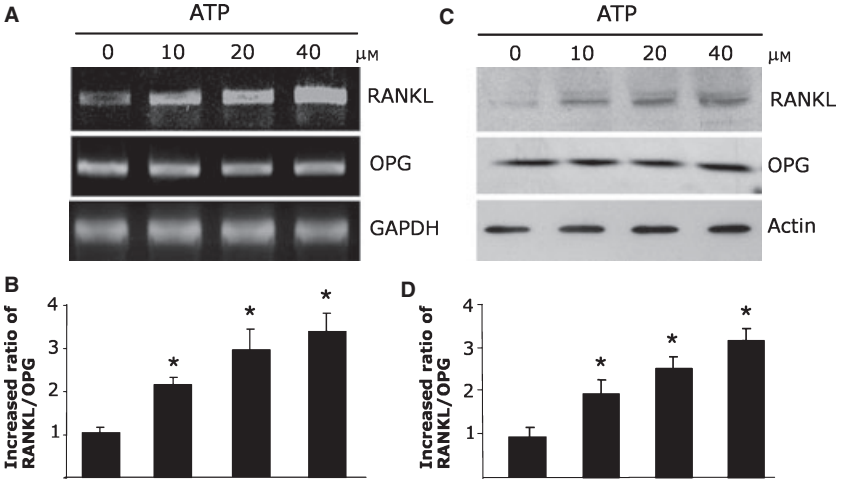

Fig. 1. Adenosine triphosphate induced RANKL mRNA and protein expression. Human periodontal ligament cells were treated with various concentrations of ATP ranging from 0 to 40 μM for 16 h for RT-PCR and 48 h for western blot analysis in serum-free conditions. The results from both RT-PCR (A) and western blotting (C) showed that ATP increased the expression of RANKL, but not that of osteoprotegerin (OPG), in a dose-dependent manner. The graphs represent the ratio of RANKL to osteoprotegerin from RT-PCR (B) and western blot analysis (D). The results are expressed as means ± SD from three different experiments. *Significant difference, $p < 0.05$.

10 min. Cells were incubated overnight at 4°C with primary antibody against NFκB p50 (NLS; dilution 1:200 in 10% fetal bovine serum, sc-114; Santa Cruz Biotechnology), followed by biotinylated secondary antibody (dilution 1:1000; Zymed Laboratories Inc., South San Francisco, CA, USA) and streptavidin–fluorescein isothiocyanate (dilution 1:1000; Sigma-Aldrich Chemical) for 40 min. The chamber slide was then washed with phosphate-buffered saline and mounted. Cells were observed using a scanning photographic system (MIRAX MIDI, Carl Zeiss MicroImaging Inc., Jena, Germany).

Transfection of small interfering RNA

Human periodontal ligament cells were grown in six-well plates, in medium without antibiotics, to 70–80% confluence. Cells were treated with a mixed solution of small interfering RNA oligonucleotides specific to P2Y$_1$ according to the manufacturer's instructions (Santa Cruz Biotechnology) and were transfected with the small interfering RNA for 24 h before treatment with ATP. For the control culture, control small interfering RNA was added.

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) using statistical software (SPSS, Chicago, IL, USA). Scheffe's test was used for *post hoc* analysis ($p < 0.05$).

Results

Our previous results indicated that an increased level of RANKL expression as well as increased ATP release was a response of human periodontal ligament cells to mechanical stimulation (14,15); the effect of ATP on RANKL expression was therefore further investigated in the present study. Human periodontal ligament cells were activated with 0, 10, 20 or 40 μM ATP, and the expressions of RANKL mRNA and protein were analyzed at 16 and 48 h, respectively. The mRNA expression of RANKL was normalized to the expression level of GAPDH, while the amount of RANKL protein was normalized to the amount of actin. The results showed that ATP increased the expression of RANKL in a concentration-dependent manner at both transcriptional and translational levels (Fig. 1A,C). The effective concentration (40 μM) of ATP was used for the rest of the experiments. However, neither mRNA nor protein expression of osteoprotegerin was observed (Fig. 1A,C). The relative band densities of RANKL/osteoprotegerin from each experiment are depicted as histograms (Fig. 1B,D).

To determine the intracellular pathway involved in the regulation of RANKL by ATP, indomethacin was used as an inhibitor. We found that the elevation of RANKL induced by ATP was suppressed by indomethacin, a non-specific inhibitor of COX activity (Fig. 2A,B). To confirm that ATP mediated its signal via the COX pathway, the expressions of COX-1 and COX-2 and the production of PGE_2 were examined after stimulating the cells with 0–40 μM ATP. The result showed that ATP increased the expression of COX-2 but not COX-1 (Fig. 2C). Production of PGE_2 increased remarkably at concentrations of 20 and 40 μM (Fig. 2D).

We also investigated which molecules are involved in the ATP-induced RANKL expression. Inhibitors were used to explore the signals involved. The toxicity of the inhibitors was monitored using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Pretreatment with U73122, an inhibitor of phospholipase C, resulted in neither blockade of the PGE_2 release nor RANKL

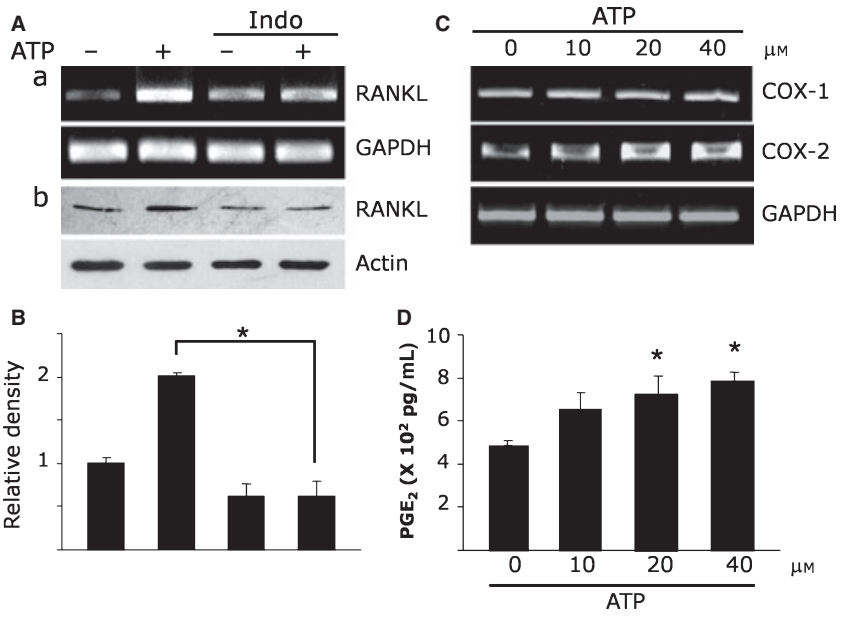

Fig. 2. Adenosine triphosphate-induced RANKL expression was inhibited by indomethacin. (A) Human periodontal ligament cells were treated with 40 μM ATP in the presence or absence of indomethacin, a non-specific COX inhibitor, to determine the role of COX in the mechanism. The inhibitor was added 30 min before the treatment. The RNA was extracted at 16 h, and the cell protein extract was collected at 48 h after the treatment. The results showed that application of indomethacin could inhibit the up-regulation of RANKL expression induced by ATP at both the mRNA (Aa) and the protein levels (Ab). The graph (B) represents the band density from western blot analysis when control as marked as 1. The results are expressed as means ± SD from three different experiments. (C) Adenosine triphosphate induced COX-2 mRNA after treatment with various concentrations of ATP. The results revealed that the expression of COX-2 increased in a concentration-dependent manner, but not COX-1. (D) The amount of PGE_2 in the culture medium was also measured by ELISA after 2 h of exposure to ATP. We found that ATP increased PGE_2 production significantly in a dose-dependent manner. The graph shows the mean ± SD of band density from three separate experiments. *Significant difference, $p < 0.05$.

expression in response to ATP stimulation (data not shown). In contrast, pre-incubation with either the NFκB inhibitor PDTC or H89 dihydrochloride hydrate, a potent inhibitor of cAMP-dependent protein kinase (PKA), inhibited the elevation of both RANKL (Fig. 3A) and PGE_2 (Fig. 3B), suggesting that cAMP and NFκB are the upstream signals of the PGE_2–RANKL axis in response to ATP in human periodontal ligament cells. To confirm the involvement of cAMP, an adenylyl cyclase activator, forskolin, was used. It appeared that forskolin increased the synthesis of RANKL and increased the release of PGE_2 from human periodontal ligament cells (Fig. 3C,D). Immunofluorescent staining was also used to explore the NFκB nuclear translocation. The results showed that ATP induced NFκB translocation and the effect was attenuated by H89 as well as by PDTC (Fig. 4).

To investigate whether $P2Y_1$ is the receptor through which ATP mediates its signal to up-regulate COX-2 and RANKL expression, a specific P2Y1 antagonist, MRS2179, was applied to the cultures prior to addition of ATP. It appeared that MRS2179 inhibited the stimulatory effect of ATP on COX-2 expression (Fig. 5A) and PGE_2 production (Fig. 5B). In addition, the expression of RANKL mRNA and protein were attenuated (Fig. 5C,D). The results suggested that ATP could act through the $P2Y_1$ receptor.

Small interfering RNA was used as another approach to confirm the results obtained using $P2Y_1$ receptor antagonist. Control small interfering RNA and $P2Y_1$ small interfering RNA (120, 140 and 180 pM) were transfected into human periodontal ligament cells for 24 h before application of ATP. The results confirmed that $P2Y_1$ small interfering RNA reduced $P2Y_1$ mRNA and protein expression effectively at 120 pM (Fig. 6A) and that it exerted an inhibitory effect on both PGE_2 release (Fig. 6B) and RANKL expression (Fig. 6C,D) similar to that exerted by $P2Y_1$ receptor antagonist.

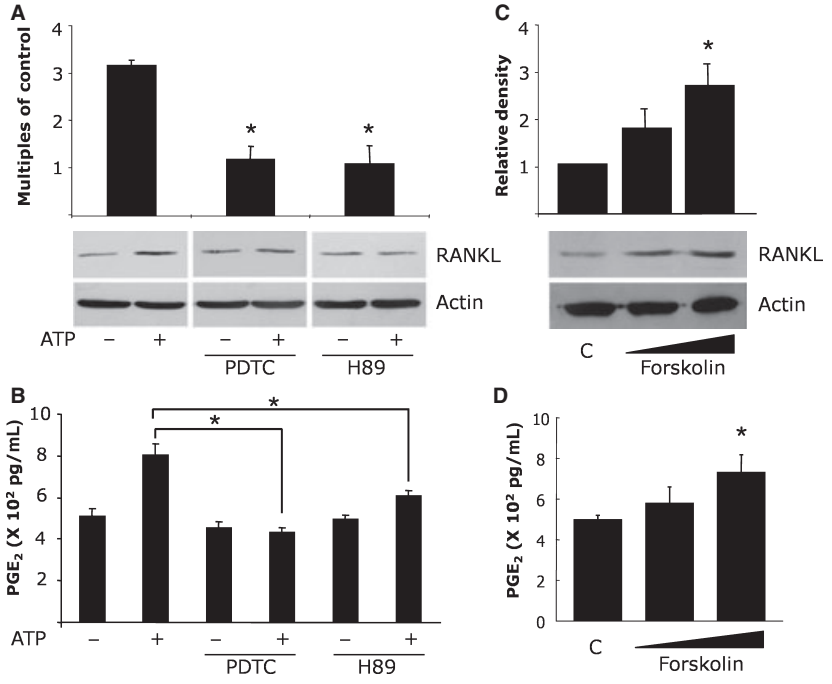

Fig. 3. The stimulatory effect of ATP was abolished by PDTC and H89. Cells were treated with PDTC, an NFκB inhibitor, and H89, a cAMP-dependent protein kinase inhibitor, 30 min before the addition of ATP. The protein extract was collected at 48 h for western blot analysis and the culture medium was collected at 2 h for ELISA after the treatment. The results showed that ATP-induced RANKL (A) and PGE_2 production (B) were inhibited by both PDTC and H89. The effects of forskolin, an activator of adenylyl cyclase, on RANKL expression and PGE_2 release are shown in (C) and (D), respectively. The graph (C) represents the band density from the ratio of RANKL to actin when control as marked as 1. *Significant difference, $p < 0.05$.

Discussion

The results revealed that extracellular ATP stimulates RANKL production through the COX-dependent pathway. In addition, the stimulatory effect is mediated through the $P2Y_1$ receptor in human periodontal ligament cells.

The role of ATP on the up-regulation of RANKL has been reported mostly in osteoblasts. Buckley *et al.* (9) demonstrated that ATP stimulated human osteoclast activity via the up-regulation of osteoblast-expressed RANKL. Jin-Man Kim and co-workers (16) also demonstrated that blocking ATP generation significantly decreases RANKL-stimulated osteoclast differentiation. However, the increase in the level of RANKL generated by ATP in human periodontal ligament cells has not been elucidated. Although our previous study showed that mechanical stress evoked RANKL expression as well as ATP secretion, the impact of ATP on RANKL expression has not been clarified. The results of this study provided a clearer picture showing that the increase of stress-induced RANKL could be a consequence of ATP release.

The involvement of COX/PGE_2 in the regulation of RANKL expression has been reported by Kanzaki and co-workers (12). They demonstrated that compressive force stimulated osteoclastogenesis in periodontal ligament cells by increasing the expression of RANKL and PGE_2 production. We considered the possibility that PGE_2 might be an intermediate mediator of the ATP-induced RANKL expression. The expression of COX-1 and COX-2 and the production of PGE_2 were examined, and the results showed that COX-2 and PGE_2 responded to ATP stimulation. The supporting evidence is that the stimulatory effect of ATP was

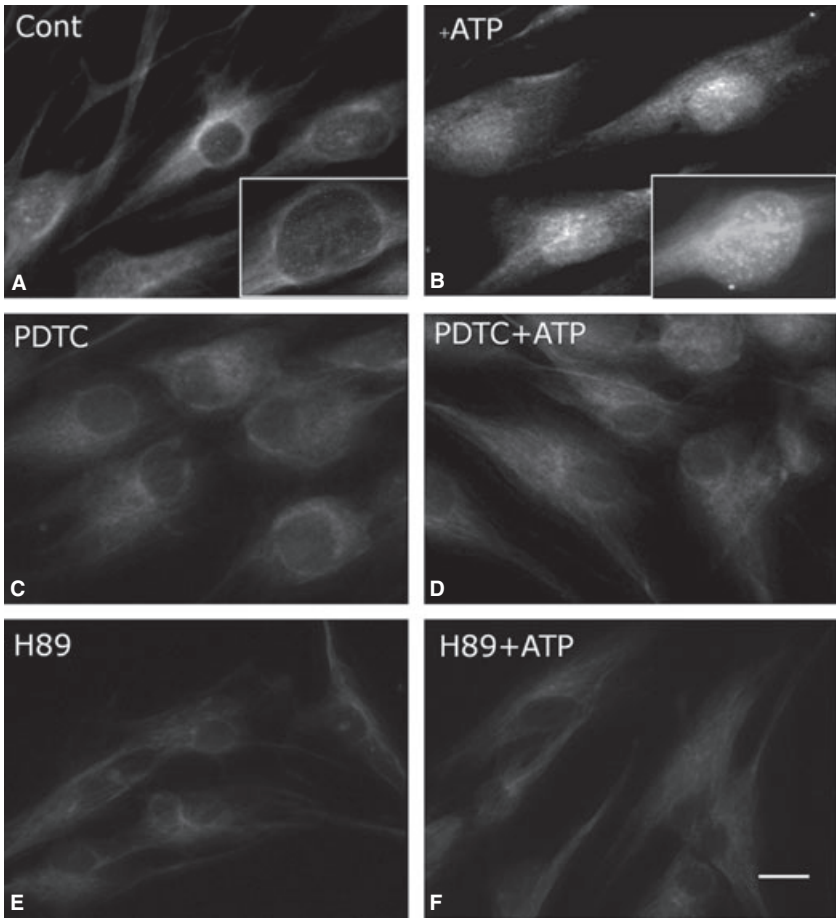

Fig. 4. Nuclear localization of NFκB enhanced by ATP was abolished by PDTC and H89. Human periodontal ligament cells were incubated with or without inhibitor, PDTC or H89, for 30 min before being treated with ATP for 2 h. Immunofluorescent staining showed that ATP enhanced nuclear localization of NFκB (B) when compared with the control treatment (A). The effect of ATP was abolished by PDTC (D) and H89 (F) when compared with their corresponding control cultures (C,E). Scale bar represents 10 μm.

abrogated by indomethacin, an inhibitor of prostanoid synthesis. In addition, these results were consistent with previous reports demonstrating the effect of ATP on the release of PGE_2 in many cell types, such as endothelial cells (17), astrocytes (18) and epithelial cells (19). We conclude that, in human periodontal ligament cells, the up-regulation of RANKL by ATP is via a COX-dependent pathway.

The blocking effect exerted by NFκB inhibitor and PKA inhibitor on the release of PGE_2 and the expression of RANKL also suggests that NFκB and cAMP are the upstream regulators of the PGE_2 release. Our study indicated that ATP induced the translocation of NFκB and the translocation was suppressed by the inhibitor of cAMP-dependent protein kinase. The results suggest that cAMP could be the upstream signal of NFκB. It is tempting to speculate that ATP induces the formation of cAMP and causes activation of NFκB. Subsequently, activation of NFκB will activate cyclo-oxygenase, resulting in the release of PGE_2.

The involvement of cAMP in ATP-induced RANKL expression was further confirmed by the use of forskolin. Addition of forskolin without ATP could increase the expression of RANKL at both the mRNA and the protein level.

The finding that NFκB is one of the downstream targets of the P2 receptor agreed with the report by Korcok *et al.* (20), which demonstrated that nucleotides acted through $P2Y_6$ receptors to initiate NFκB signaling in osteoclasts. In addition, the role of NFκB in controlling the release of PGE_2 had been reported (21). However, our data suggest that the increase of PGE_2 and RANKL stimulated by ATP are not a consequence of PLC activity. This finding is different from those found in other cell types including epithelial cells (19,22), which proposed that ATP-induced release of PGE_2 was dependent on intracellular Ca^{2+} and phospholipase C activity.

In regard to the ATP receptor, ATP is able to act through almost all subtypes of P2 receptors to exert various effects (23). It has been shown to initiate NFκB signaling and enhance survival through $P2Y_6$ (20) and $P2X_7$ receptors (24) in osteoclasts, to activate DNA synthesis by acting on P2X receptors in human osteoblast-like MG-63 cells (25) and to generate resorption pits on dentin disks by elevating RANKL expression in osteoblast-like UMR-106 cells, where the $P2Y_1$ receptor is predominantly expressed (9). These results indicate that locally acting ATP may play a pivotal role in osteoclast activation at bone-resorbing sites by inducing elevated expression of RANKL ligament in bone cells. For human periodontal cells, we found in the previous study that perturbing the function of the $P2Y_1$ receptor using MRS2179, a specific inhibitor of the $P2Y_1$ receptor, attenuated the inductive effect of ATP on osteopontin expression (15). In the present study, a similar blocking effect on ATP-induced RANKL expression was seen using MRS2179 as well as small interfering RNA. In addition, it is notable that interference with the function of the $P2Y_1$ receptor exerted an almost complete inhibition on the stimulatory effect of ATP, indicating that the $P2Y_1$ receptor could be the main receptor involved in the regulation of both RANKL and osteopontin expression by ATP in human periodontal ligament cells.

Since mechanical stress induces expression of PGE_2 (12) as well as proinflammatory cytokines such as interleukin-1β (26), which is able to stimulate the release of PGE_2, it is possible that the release of PGE_2 is a

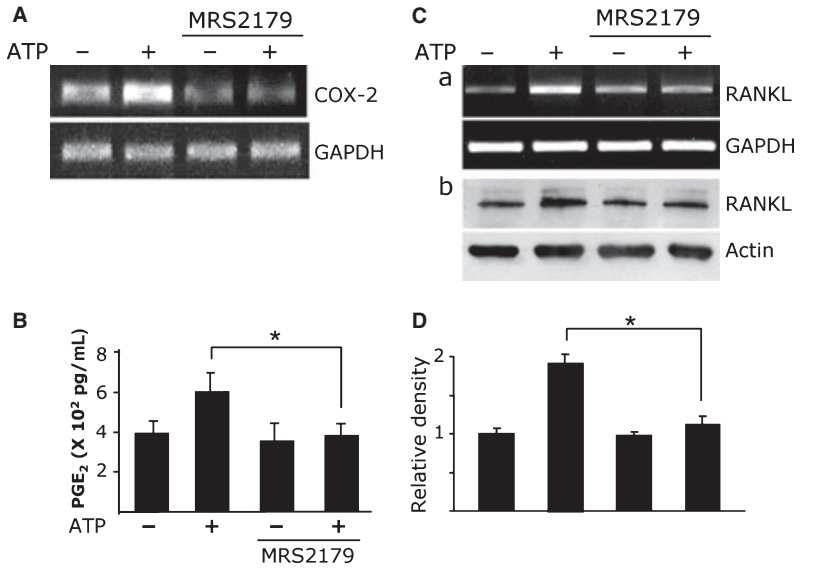

Fig. 5. The effect of MRS2179 on ATP-induced RANKL expression. Cells were pre-incubated with MRS2179, a specific $P2Y_1$ receptor antagonist, for 30 min prior to addition of ATP. After 16 and 48 h of exposure to ATP, the RNA and protein extract were collected for RT-PCR and western blot analysis, respectively. The culture medium from a parallel set of experiments was collected after 2 h of treatment for ELISA. The results showed that MRS2179 exerted an inhibitory effect on the ATP-induced COX-2 expression (A) and PGE_2 production (B). MRS2179 attenuated the ATP-induced RANKL expression at both the mRNA (Ca) and the protein level (Cb). The graph (D) represents the band density from western blot analysis when control as marked as 1. The results are expressed as means ± SD from three different experiments. *Significant difference, $p < 0.05$.

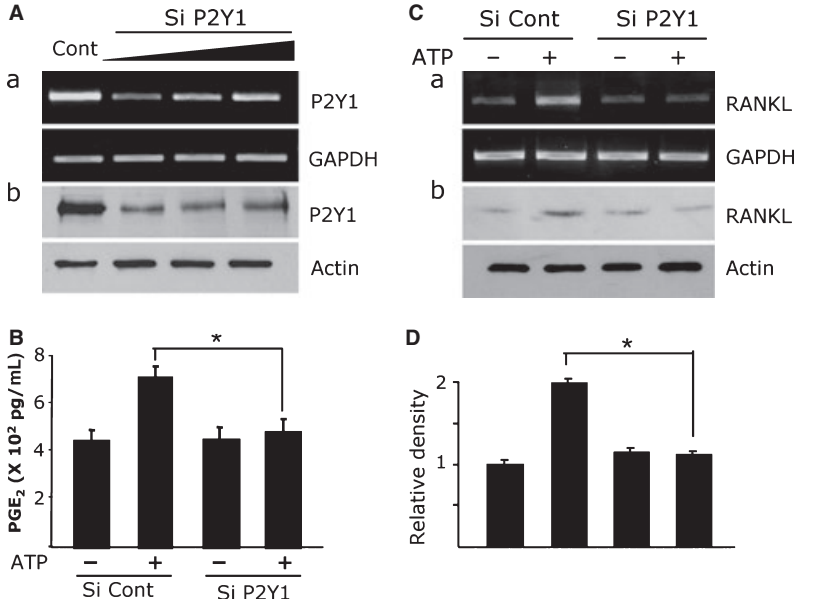

Fig. 6. Adenosine triphosphate-induced up-regulation of RANKL may involve the $P2Y_1$ receptor. Human periodontal ligament cells were transfected with $P2Y_1$ small interfering (Si) RNA. (A) The $P2Y_1$ small interfering RNA could interfere with the expression of the P2Y1 receptor at both the mRNA (Aa) and the protein level (Ab). The $P2Y_1$ small interfering RNA inhibited the release of PGE_2 (B) as well as the expression of RANKL (C). The graph represents the band density from western blot analysis when control as marked as 1 (D). The results are expressed as means ± SD from three different experiments. *Significant difference, $p < 0.05$.

result of the action of those pro-inflammatory cytokines. We demonstrated in the previous report that mechanical stress increased the level of ATP (15) and revealed in the present study that ATP could cause a rise in PGE_2 production within 2 h. Our results advance the understanding that, besides proinflammatory cytokines, ATP is one of the upstream signaling molecules for PGE_2 release in response to mechanical stress in human periodontal ligament cells and plays a role in periodontal tissue homeostasis through the $P2Y_1$ receptor.

In conclusion, extracellular ATP stimulates RANKL production by human periodontal ligament cells through $P2Y_1$ receptor–COX–PGE_2 pathways (Fig. 7). Adenosine triphosphate may thereby play an important physiological role in periodontal remodeling.

Acknowledgements

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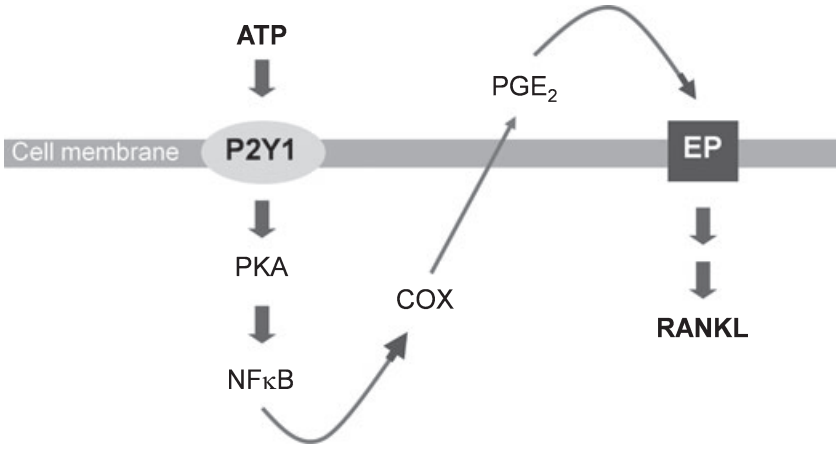

Fig. 7. Proposed model of ATP-induced RANKL expression. The schematic drawing proposes that ATP activates the $P2Y_1$ receptor in human periodontal ligament cells, which mediates its signal through protein kinase A (PKA) and nuclear factor κB (NFκB). Activation of NFκB causes the release of PGE_2 via a COX-dependent pathway. The activation of RANKL expression is a consequence of the released PGE_2, after which the cascade of signaling molecules involved requires further clarification. EP, E prostanoid receptor.

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