จุฬาลงกรณ์มหาวิทยาลัย
ทุนวิจัย
กองทุนรัชดาภิเษกสมโภช

รายงานวิจัย

อิทธิพลของแรงกดเชิงกลต่อการแสดงออกของออสติโอพอนทินในเซลล์เอ็นยึดปริทันต์

โดย

ประสิทธิ์ ภวสันต์
ทัศนีย์ ยงชัยระกูล

ตุลาคม 2551

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กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณ อาจารย์และบุคลากรของภาควิชาศัลยศาสตร์ คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ที่ให้ความช่วยเหลือในการเก็บเนื้อเยื่อฟันจากผู้ป่วย ขอบคุณ คุณจีรนันท์ มโนกวินโชค ทันตแพทย์ สุชาติ วงศ์ขันตี และบุคลากรในหน่วยปฏิบัติการเนื้อเยื่ออนินทรีย์ ที่ให้ความช่วยเหลือในการเพาะเลี้ยงเซลล์และงานด้านเทคนิค และขอขอบคุณคณะอนุกรรมการบริหารทุนวิจัย กองทุนรัชดาภิเษกสมโภชที่ให้การสนับสนุนเงินทุนวิจัย

ชื่อโครงการวิจัย: อิทธิพลของแรงกดเชิงกลต่อการแสดงออกของออสติโอพอนทินในเซลล์เอ็นยึดปริทันต์

ชื่อผู้วิจัย: ประสิทธิ์ ภวสันต์ ทัศนีย์ ยงชัยตระกูล

เดือนและปีที่ทำวิจัยเสร็จ: กรกฎาคม 2551

บทคัดย่อ

วัตถุประสงค์: แรงกดเชิงกล เช่นแรงจากการจัดฟัน สามารถก่อให้เกิดการทำลายและการอักเสบของเนื้อเยื่อปริทันต์ ออสติโอพอนทีน เป็นไซโตไคน์ที่มีความสัมพันธ์กับการลุกลามของโรคปริทันต์จากการที่แรงกดมีผลต่อเอ็นยึดปริทันต์ และเซลล์เอ็นยึดปริทันต์เองก็มีบทบาทในการทำลายและการปรับเปลี่ยนของเนื้อเยื่อ คณะผู้วิจัยจึงต้องการศึกษาผลของแรงกดเชิงกลต่อการแสดงออกและการควบคุมการสร้างออสติโอพอนทีนในเซลล์เอ็นยึดปริทันต์ของมนุษย์

วิธีการวิจัย: ใช้แรงกดชนิดต่อเนื่องต่อเซลล์ ตรวจสอบการแสดงออกและการสร้างออสติโอพอน-ทีนด้วยรีเวอร์สทรานสคริปชัน โพลีเมอเรสเชน รีแอกชัน (อาร์ที-พีซีอาร์) และเวสเทอร์น อนาไลสิส และตรวจสอบกลไกการกระตุ้นด้วยการใช้สารยับยั้ง

ผล: อาร์เอ็นเอและโปรตีนของออสติโอพอนทีนเพิ่มขึ้นตามแรงกด พร้อมกับมีการเพิ่มของรีเซบเตอร์ แอกติเวเตอร์ออฟนิวเคลียแคปปาบี ไลแกนส์ (แรงค์ไลแกนส์) อินโดเมธาซินสามารถยับยั้งการเพิ่มของแรงค์ไลแกนส์ได้ แต่ยับยั้งการเพิ่มของออสติโอพอนทีนไม่ได้ แสดงว่ากลไกการกระตุ้นออสติโอพอนทีนไม่ได้เกิดผ่านไซโคลอ็อกซิจิเนส ผลปรากฏว่า การเพิ่มของออสติโอพอนทีนถูกยับยั้งได้ด้วยสารยับยั้งต่อโรไคเนส แต่ไม่สามารถยับยั้งได้ด้วยไซโตคาลาซินบี

สรุปและวิจารณ์: แรงกดเชิงกลกระตุ้นออสติโอพอนทีนในเซลล์เอ็นยึดปริทันต์โดยผ่านโรไคเนส ผลการศึกษานี้แสดงให้เห็นถึงความสำคัญของออสติโอพอนทีนที่ถูกเหนี่ยวนำด้วยแรงกดเชิงกลต่อการละลายและการปรับเปลี่ยนของกระดูกเบ้าฟัน

คำสำคัญ: ออสติโอพอนทีน แรงกดเชิงกล แรงกด เซลล์เอ็นยึดปริทันต์ โรไคเนส

Project title: Influence of mechanical stress on the expression of osteopontin in human periodontal ligament cells.

Name of investigation: Prasit Pavasant, Tussanee Yongchaitrakul

Year: July 2008

Abstract

Background: Mechanical stress such as orthodontic forces can produce mechanical damage and inflammatory reaction in periodontium. Osteopontin (OPN) is a multifunctional cytokine that found to be correlated with periodontal disease progression. As periodontal ligaments (PDL) can be affected by stress and PDL cells are involved in periodontal destruction and remodeling, we aimed to investigate the influence of mechanical stress on the expression and regulation of OPN in human PDL (HPDL) cells.

Methods: The mechanical stress was generated by continuous compressive force and the expression of OPN was examined by reverse transcription polymerase chain reaction (RT-PCR) and Western analysis. The application of inhibitors was used to examine the mechanism involved.

Results: Both mRNA and protein expression of OPN significantly increased in a force-dependent manner. Increase of receptor activator of nuclear factor kappa B ligand (RANKL) was also observed. Interestingly, application of indomethacin could abolish the induction of RANKL but not that of OPN, suggesting the cyclooxygenase (COX)-independent mechanism for stress-induced OPN expression. In addition, the up-regulation of OPN was diminished by Rho kinase inhibitor, but not by cytochalasin B.

Conclusions: Mechanical stress affects OPN expression in HPDL cells via Rho kinase pathway. Since OPN participates in bone resorption and remodeling induced by mechanical and biological signals, these results suggest the significance of stress-induced OPN in HPDL cells in alveolar bone resorption and remodeling.

Keywords: Osteopontin, mechanical stress, compressive force, periodontal ligament cells, Rho kinase

สารบัญ

รายการภาพประกอบ

หน้า

Introduction

Periodontal ligaments (PDL) function to support and form a shock absorbing system in order to mitigate mechanical forces such as masticatory or orthodontic forces. Human PDL (HPDL) cells are responsible to the mechanical stress transduced from the tooth, which is significant for the process of periodontal tissue remodeling and repair.[1-2]

Mechanical stress can induce secretion of several inflammatory cytokines which are detectable in gingival crevicular fluid (GCF).[3-4]. The elevation of the cytokines due to mechanical force may result in the inflammation of periodontal tissue. In addition, mechanical force is able to damage the periodontal tissue resulting in alveolar bone resorption and tooth loss.[5-6]

Mechanical stress can also induce bone loss through the imbalance of receptor activator of nuclear kappa B ligand (RANKL) and osteoprotegerin (OPG). Previous studies demonstrated the relationship of RANKL and OPG expression stimulated by mechanical stress in human PDL cells.[7-8] The expression of membrane bound and soluble form of RANKL increased in a force-dependent manner while the OPG expression decreased or unaltered.[7-8] As RANKL and OPG are the key proteins of proliferation, differentiation, and activation of osteoclasts,[9] increase of RANKL expression in PDL cells due to mechanical stress supports that PDL plays a role in the regulation of alveolar bone resorption and remodeling.

Osteopontin (OPN) has also been accounted for bone remodeling. The expression of OPN was increased in response to the mechanical stress in bone cells.[10-11] OPN is one of a major non-collageneous bone matrix protein. It is a highly phosphorylated protein containing an arginine-glycine-aspartate (RGD) motif, which binds to integrin and enable bone cells to adhere to the mineralized matrix.[12] It is known to be produced by

osteoblasts,[13-14] as well as osteoclasts,[14-15] and participates in the function of both cell types such as bone formation, resorption and remodeling.[12]

OPN is thought to regulate the adhesion, attachment, and spreading of osteoclasts to the bone surface.[16] It has been proposed that OPN is a mechano-responsive gene in bone cell.[17] Up-regulation of OPN mRNA was detected in osteocytes, osteoblasts, and bone lining cells in the pressure side of the experimental tooth movement in rats.[10,18] Impairment of bone resorption in unloading-induced bone loss in OPN-deficient mice[19] indicates that OPN plays a role in response to mechanical loading.

Moreover, OPN plays a role in tissue inflammation.[20] It can act as a regulator of macrophages infiltration and facilitates the adhesion and migration of macrophages and leukocytes.[21-22] The level of OPN expression may also be used as an indicator of inflammatory response in periodontal tissue.

The expression of OPN in HPDL cells and PDL tissues had been reported.[23-26] The results from immunohistochemical analysis showed that OPN was primarily localized in PDL cells adjacent to deciduous tooth roots, with the greatest prevalence in association with odontoclasts in resorption lacunae.[25] The expression of OPN was regulated by several growth factors and extracellular matrix protein.[26-27] Recent studies by Kido et al. and Sharma and Pradeep showed that the increasing level of OPN in the GCF corresponded with the severity of periodontitis and suggested that the level of OPN may be a marker of periodontal disease severity.[28-29] However, the source of OPN in periodontal disease is still unclear.

As mechanical stress can cause inflammation and periodontal destruction, we hypothesized that mechanical stress could induce OPN expression in HPDL cells. Therefore, the aim of this study was to investigate the influence of compressive force-

generated stress on OPN expression in HPDL cells and to examine the signaling pathway involved in OPN induction.

Material & Methods

Human periodontal ligament cell culture

Human periodontal ligament (PDL) cells were obtained from healthy third molars extracted for orthodontic reasons and prepared as previously described. The protocol was approved from The Ethics Committee, Faculty of Dentistry, Chulalongkorn University. Informed consent was obtained from each patient. Briefly, teeth were rinsed with sterile phosphate buffer saline and the PDL were scraped out from the middle third of the root. The explants were harvested on a 60-mm culture dishes and grown in Dulbecco's Modified Eagle Medium[†] (DMEM) supplemented with 10% fetal calf serum,[‡] 2mM L-glutamine,[§] 100 units/ml penicillin,[‖] 100 μg/ml streptomycin,[¶] and 5 μg/ml amphotericin B[#] at 37 °C in humidified atmosphere of 95% air, 5% CO_2. Cells from the third to the fifth passage were used. All experiments were performed triplicate using cells prepared from three different donors.

Application of mechanical stress to PDL cells

The method for mechanical stress application was applied from Kanzaki et al.[7] Briefly, PDL cells were seeded in 6-well-plates at a density of 200,000 cells/well for 16 hours. A plastic cylinder containing metal coins was placed over the culture in order to generate compressive forces ranging from 0 to 2.5 g/cm^2.

For inhibitory experiments, each inhibitor was added into the medium 30 min prior to the experiment. The inhibitors used included 1.27 nM Rho-kinase Inhibitor,** 40 μM Cytochalasin–B[††] and 10 μM indomethacin[‡‡].

RNA extraction and semi-quantitative reverse transcription polymerase chain-reaction assay (RT-PCR)

Total cellular RNA was extracted with Tri-reagent[§§] according to manufacturer's instructions. One μg of each RNA sample was converted to cDNA by a reverse transcription using an AMV (Avian myeloblastosis virus) reverse transcriptase[‖‖] for 1.5 hours at 42°C. Subsequent to the reverse transcription, a polymerase-chain reaction was performed. The primers were prepared following the reported sequences from GenBank. The oligonucleotide sequences of the primers are: GAPDH, forward, 5'-TGAAGGTCGGAGTCAACGGAT-3', and reverse, 5'-TCACACCCATGACGAACATGG-3'; OPN, forward, 5'-AGTACCCTGATGCTACAGACG-3', and reverse, 5'-CAACCAGCATATCTTCATGGC-3'; RANKL, forward, 5'-CCAGCATCAAAATCCCAAGT-3', and reverse, 5'-CCCCTTCAGATGATCCTTC-3'; COX-2, forward, 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3', and reverse, 5'-AGATCATCTCTGCCTGAGTATCTT-3'.

The PCR was performed using Taq polymerase[¶¶] with a PCR volume of 25 μl. The amplification profile for OPN was 1 cycle at 94°C for 1 min, 30 cycles of 94°C for 1 min, hybridization at 60°C for 1 min, and extension at 72°C for 2 min, followed by 1 extension cycle at 72°C for 10 min. The same profile was also used for RANKL (32 cycles), COX-2 (32 cycles) and GAPDH (22 cycles). The PCR was performed in the DNA thermal cycles.[##] The amplified DNA was then electrophoresed on a 2% agarose gel and visualized by ethidium bromide fluorostaining. The relative intensities of the gel bands were measured by Scion Image analysis.

Protein extraction and Western blot analysis

Protein was extracted with RIPA buffer. Protein concentrations were measured using BCA protein assay kit (Pierce, Rockford, IL, U.S.A.). Equal amounts of protein samples were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and subsequently

transferred onto nitrocellulose membrane. The membrane was incubated with primary antibody against OPN,*** RANKL[†††] or actin[‡‡‡]. The membranes were then incubated with biotinylated secondary antibody, followed by peroxidase-labeled streptavidin. The signal was captured by chemoluminescence.[§§§] The relative intensities of bands were measured by Scion Image analysis.

Statistical Analysis

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

† Hyclone Logan, Utah.

‡ Hyclone.

§ Gibco BRL, Carlsbad, CA.

‖ Gibco BRL.

¶ Gibco BRL.

Gibco BRL.

** Calbiochem, EMD Biosciences, Inc, San Diego, CA.

†† Calbiochem,

‡‡ Sigma-Aldrich Chemical, St Louis, MO

§§ Molecular Research Center, Cincinnati, OH.

‖‖ Promega, Madison, WI.

¶¶ Qiagen, Hilden, Germany

Biometra GmH, Göttingen, Germany

*** Chemicon International, Temecula, CA.

††† R&D system, Minneapolis, MN.

‡‡‡ Chemicon International.

§§§ Pierce Biotechnology, Rockford, IL.

Results

The results in Fig. 1 showed the response of HPDL cells after activated with mechanical stress generated by compressive forces for 24 hours. Cells were cultured under continuous compressive force varying from 0 to 1.25 g/cm^2. The results from both RT-PCR and Western analysis indicated that OPN expression increased depending on the stress applied.

The results in Fig. 2 showed that mechanical stress generated from 1.25 g/cm^2 of force could increase OPN mRNA expression within 1 hour after stimulation and reach the maximum level at around 24 hours after treatment.

The signaling pathway involved in the OPN induction was examined by means of inhibitors. Previous reports showed that mechanical stimuli increased RANKL expression in HPDL cells via a cyclooxygenase-2 (COX-2) dependent pathway.[7] To examine whether COX is involved in stress-induced OPN expression, cells were incubated with indomethacin, a non-specific COX inhibitor, for 30 minutes before activated with 1.25 and 2.5 gm/cm^2 of force. The results indicated that both levels of force significantly increased RANKL and OPN mRNA and protein expression. Application of indomethacin inhibited the up-regulation of RANKL expression at 2.5 gm/cm^2 but not at 1 gm/cm^2. No inhibitory effect was observed in OPN expression. In addition, the level of COX-2 was examined by RT-PCR and the results revealed that COX-2 was up-regulated at 2.5 gm/cm^2 but not at 1.25 gm/cm^2 (Fig.3).

Induction of OPN expression was inhibited by Rho kinase inhibitor as shown in Fig. 4. Rho kinase inhibitor abolished the mechanical stress-induced OPN expression in both mRNA and protein levels. Interestingly, cytochalasin B, an inhibitor of actin polymerization, had no inhibitory effect on the stress-induced OPN expression.

Discussion

The results from the present study reveal that mechanical stress generated from compressive forces induces OPN expression in human PDL cells in a force dependent manner. The results also indicate an involvement of Rho kinase in stress-induced OPN expression in human PDL cells.

An increase of OPN mRNA expression in stress-induced human PDL cells was observed within an hour. This finding is in agreement with the work by Toma et al., who proposed that OPN might be a mechano-responsive gene.[17] Fujihara et al.[30] also showed a shear stress responsive element (SSRE; GAGACC) in OPN promoter, supporting that OPN could be turned on by mechanical stress.

It has been shown that the number of tartrate-resistant acid phosphatase positive (TRACP+) cells as well as OPN expression increased predominantly at the pressure side of bone and teeth.[6,10-11] In vivo and in vitro studies indicated that bone cells synthesized OPN in response to mechanical stimulation.[10-11,17] The results in this study showed that PDL cells could act in the similar fashion. Increase of OPN is believed to facilitate the function of osteoclasts in bone resorption. Results from OPN-deficient mice indicated that OPN participated in the process of bone resorption and/or remodeling induced by mechanical and biological signals. Unloading-induced bone loss and bone loss due to ovariectomy were impaired in the absence of OPN.[19,31-32] In addition, OPN deficient mice could not respond to parathyroid induced bone resorption.[33]

OPN also plays a role in migration and attachment of osteoclast. OPN stimulates osteoclast migration through $\alpha v\beta 3$ integrin and CD44.[34-35] Furthermore, OPN acts as a chemotactic factor for osteoclasts in the process of bone resorption.[10] Therefore, induction of OPN by mechanical stress in PDL cells may enhance the recruitment and migration of osteoclasts, resulting in alveolar bone resorption.

Increase of OPN may also correspond with the increase of osteoclastogenesis. Although OPN does not seem to be an essential factor in the development of osteoclasts during normal development,[36] it plays an important role in osteoclastogenesis in pathological conditions.[33] It has been shown that neutralizing antibody to OPN suppressed osteoclastogenesis, whereas addition of OPN enhanced osteoclastogenesis in marrow stromal cells.[37-38] Ishii et al. showed that OPN could influence osteoclastogenesis by enhancing RANKL and decreasing OPG expression in stromal cells.[38] Whether OPN influence the expression of RANKL and OPG in human PDL cells requires further investigation.

The suggested function of OPN in bone resorption corresponded with the reports showing that OPN was increased in GCF of patients with periodontitis.[28-29] Increase of OPN may facilitate both the function of osteoclasts and the recruitment of macrophages and leukocytes which results in periodontal tissue inflammation. The observation by Sharma and Pradeep that periodontal treatment could reduce OPN in GCF[29] supports the importance of the increased-OPN in periodontal tissue on the progression of periodontal disease.

Our results also showed the up-regulation of RANKL in stress-induced PDL cells. RANKL is one of a key protein that functions in the differentiation, activation and survival of osteoclasts. Stress-induced RANKL expression in PDL cells was reported to be a COX-2 dependent mechanism.[7] In this study, application of indomethacin, a non-specific COX inhibitor, abolished the induction of RANKL when PDL cells were activated with force at 2.5 gm/cm^2 but not at 1.25 gm/cm^2. The inhibitory effect of indomethacin was corresponded to the level of COX-2 induced by mechanical stress (Fig. 3). These findings indicate that stress-induced RANKL expression in PDL cells involves more than one signaling pathway, depending upon the level of compressive force. In term of OPN expression, indomethacin

could not inhibit the up-regulation of OPN in PDL cells at both levels of pressure, suggesting a COX-independent pathway in OPN induction.

Stress-induced OPN expression was inhibited by Rho kinase inhibitor. The function of Rho kinase involves in the dynamic of cytoskeleton, such as formation of focal adhesion, actin stress fiber, and redistribution of cytoskeletal components.[39] Cytoskeleton dynamic has been reported to play a role in mechanical-induced OPN expression in chick osteoblasts.[17] However, cytoskeletal component may not involve in the up-regulation of OPN induced by mechanical stimulation in human PDL cells since cytochalasin B could not inhibit the stress-induced OPN expression.

The involvement of Rho kinase in the regulation of OPN expression has been reported in smooth muscle cells.[40-41] Induction of OPN was observed when smooth muscle cells were activated with UTP or high glucose occurred via Rho kinase, suggesting the association of Rho kinase in OPN expression. However, the exact mechanism is unclear.

Conclusion

In conclusion, the present study demonstrates that mechanical stress induces OPN expression via Rho kinase pathway in human PDL cells. We propose that the increased OPN possibly plays an important role in the mechanism of pressure-induced alveolar bone resorption.

References

1. Lekic P, McCulloch CA. Periodontal ligament cell population: the central role of fibroblasts in creating a unique tissue. *Anat Rec* 1996;245:327-341.

2. McCulloch CA, Lekic P, McKee MD. Role of physical forces in regulating the form and function of the periodontal ligament. *Periodontol 2000* 2000;24:56-72.

3. Ren Y, Maltha JC, Van't Hof MA, Von den Hoff JW, Kuijpers-Jagtman AM, Zhang D. Cytokine levels in crevicular fluid are less responsive to orthodontic force in adults than in juveniles. *J Clin Periodontol* 2002;29:757-762.

4. Yamaguchi M, Kasai K. Inflammation in periodontal tissues in response to mechanical forces. *Arch Immunol Ther Exp (Warsz)* 2005;53:388-398.

5. Verna C, Zaffe D, Siciliani G. Histomorphometric study of bone reactions during orthodontic tooth movement in rats. *Bone* 1999;24:371-379.

6. Kaku M, Uoshima K, Yamashita Y, Miura H. Investigation of periodontal ligament reaction upon excessive occlusal load – osteopontin induction among periodontal ligament cells. *J Periodont Res* 2005;40:59-66.

7. Kanzaki H, Chiba M, Shimizu Y, Mitani H. Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappaB ligand up-regulation via prostaglandin E2 synthesis. *J Bone Miner Res* 2002;17:210-220.

8. Yamaguchi M, Aihara N, Kojima T, Kasai K. RANKL increase in compressed periodontal ligament cells from root resorption. *J Dent Res* 2006;85:751-756.

9. Khosla S. Minireview: the OPG/RANKL/RANK system. *Endocrinology* 2001;142:5050-5055.

10. Terai K, Takano-Yamamoto T, Ohba Y, et al. Role of osteopontin in bone remodeling caused by mechanical stress. *J Bone Miner Res* 1999;14:839-849.

11. Morinobu M, Ishijima M, Rittling SR, et al. Osteopontin expression in osteoblasts and osteocytes during bone formation under mechanical stress in the calvarial suture in vivo. *J Bone Miner Res* 2003;18:1706-1715.

12. Denhardt DT, Guo X. Osteopontin: a protein with diverse functions. *FASEB J* 1993;7:1475-1482.

13. Weinreb M, Shinar D, Rodan GA. Different pattern of alkaline phosphatase, osteopontin, and osteocalcin expression in developing rat bone visualized by in situ hybridization. *J Bone Miner Res* 1990;5:831-842.

14. Merry K, Dodds R, Littlewood A, Gowen M. Expression of osteopontin mRNA by osteoclasts and osteoblasts in modeling adult human bone. *J Cell Sci* 1993;104:1013-1020.

15. Tezuka K, Sato T, Kamioka H, et al. Identification of osteopontin in isolated rabbit osteoclasts. *Biochem Biophys Res Commun* 1992;186:911-917.

16. Reinholt FP, Hultenby K, Oldberg A, Heinegard D. Osteopontin – a possible anchor of osteoclasts to bone. *Proc Natl Acad Sci USA* 1990;87:4473-4475.

17. Toma CD, Askar S, Gray ML, Schaffer JL, Gerstenfeld LC. Signal transduction of mechanical stimuli is dependent on microfilament integrity: identification of osteopontin as a mechanically induced gene in osteoblasts. *J Bone Miner Res* 1997;12:1626-1636.

18. Takano-Yamamoto T, Takemura T, Kitamura Y, Nomura S. Site-specific expression of mRNAs for osteonectin, osteocalcin, and osteopontin revealed by in situ hybridization in rat periodontal ligament during physiological tooth movement. *J Histochem Cytochem* 1994;42:885-896.

19. Ishijima M, Tsuji K, Rittling SR, et al. Resistance to unloading-induced three-dimesional bone loss in osteopontin-deficient mice. *J Bone Miner Res* 2002;17:661-667.

20. Singh M, Ananthula S, Milhorn DM, Krishnaswamy G, Singh K. Osteopontin: a novel inflammatory mediator of cardiovascular disease. *Front Biosci* 2007;12:214-221.

21 Giachelli CM, Lombardi D, John RJ, Murry CE, Almeida M. vidence for a role of osteopontin in macrophage infiltration in response to pathological stimuli in vitro. *Am J Pathol* 1998;152:353-358.

22. Ophascharoensuk V, Giachelli CM, Gordon K, et al. Obstructive uropathy in the mouse: Role of osteopontin in interstitial fibrosis and apoptosis. *Kidney Int* 1999;56:571-580.

23. Nohutcu RM, McCauley LK, Koh AJ, Somerman MJ. Expression of extracellular matrix proteins in human periodontal ligament cells during mineralization in vitro. *J Periodontol* 1997;68:320-327.

24. Li H, Bartold PM, Young WG, Xiao Y, Waters MJ. Growth hormone induces bone morphogenetic proteins and bone-related proteins in the developing rat periodontium. *J Bone Miner Res* 2001;16:1068-1076.

25. Lee A, Schneider G, Finkelstein M, Southard T. Root resorption: the possible role of extracellular matrix proteins. *Am J Orthod Dentofacial Orthop* 2004;126:173-177.

26. Chutivongse N, Sumrejkanchanakij P, Yongchaitrakul T, Pavasant P. Insulin-like growth factor-I attenuates the inhibitory effect of type I collagen through beta1 integrin receptor. *Biochem Biophys Res Commun* 2005;336:836-841.

27. Saygin NE, Tokiyasu Y, Giannobile WV, Somerman MJ. Growth factors regulate expression of mineral associated genes in cementoblasts. *J Periodontol* 2000;71:1591-1600.

28. Kido J, Nakamura T, Asahara Y, Sawa T, Kohri K, Nagata T. Osteopontin in gingival crevicular fluid. *J Periodontal Res* 2001;36:328-333.

29. Sharma CG, Pradeep AR. Gingival crevicular fluid osteopontin levels in periodontal health and disease. *J Periodontol* 2006;77:1674-1680.

30. Fujihara S, Yokozeki M, Oba Y, Higashibata Y, Nomura S, Moriyama K. Function and regulation of osteopontin in response to mechanical stress. *J Bone Miner Res* 2006;21:956-964.

31. Ishijima M, Rittling SR, Yamashita T, et al. Enhancement of osteoclastic bone resorption and suppression of osteoblastic bone formation in response to reduced mechanical stress do nor occur in the absence of osteopontin. *J Exp Med* 2001;193:399-404.

32. Yoshitake H, Rittling SR, Denhardt DT, Noda M. Osteopontin-deficient mice are resistant to ovariectomy-induced bone resorption. *Proc Nat Acad Sci USA* 1999;96:8156-8160.

33. Ihara H, Denhardt DT, Furuya K, et al. Parathyroid hormone-induced bone resorption does not occur in the absence of osteopontin. *J Biol Chem* 2001;276:13065-13071.

34. Chellaiah MA, Hruska KA. The integrin αvβ3 and CD44 regulate the actions of osteopontin on osteoclast motility. *Calcif Tiss Int* 2002;72:197-205.

35. Chellaiah MA, Kizer N, Biswas R, et al. Osteopontin deficiency produces osteoclast dysfunction due to reduced CD44 surface expression. *Mol Biol Cell* 2003;14:173-189.

36. Rittling SR, Matsumoto HN, McKee MD, et al. Mice lacking osteopontin show normal development and bone structure but display altered osteoclast formation in vitro. *J Bone Miner Res* 1998;13:1101-1111.

37. Yamate T, Mocharla H, Taguchi Y, Igietseme JU, Manolagas SC, Abe E. Osteopontin expression by osteoclast and osteoblast progenitors in the murine bone marrow:

demonstration of its requirement for osteoclastogenesis and its increase after ovariectomy. *Endocrinology* 1997;138:3047-3055.

38. Ishii T, Ohshima S, Ishida T, et al. Osteopontin as a positive regulator in the osteoclastogenesis of arthritis. *Biochem Biophys Res Commun* 2004;316:809-815.
39. Nobes CD, Hall A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 1995;81:53-62.
40. Chaulet H, Desgranges C, Renault MA, et al. Extracellular nucleotides induce arterial smooth muscle cell migration via osteopontin. *Circ Res* 2001;89:772-728.
41. Kawamura H, Yokote K, Asaumi S, et al. High glucose-induced upregulation of osteopontin is mediated via Rho/Rho kinase pathway in cultured rat aortic smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2004;24:276-281.

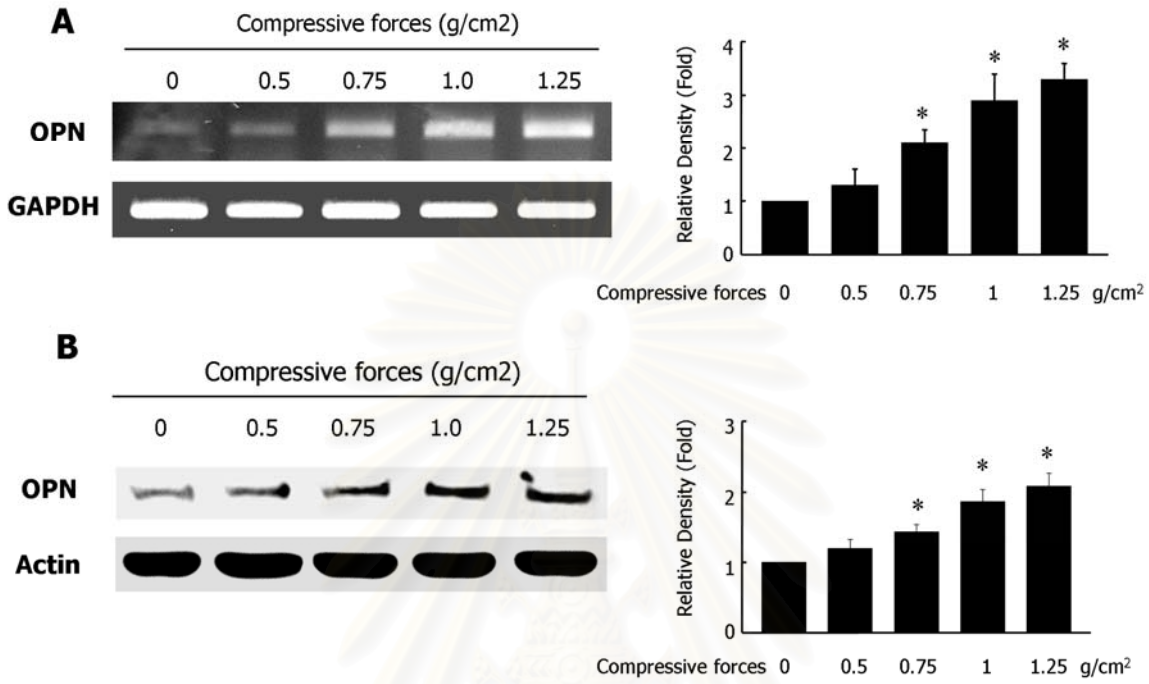

Fig. 1 Compressive forces induced OPN expression. Human PDL cells were stimulated with 0-1.25 g/cm^2 of force as described in Materials and Methods. Fig. 1A showed the change in OPN mRNA expression after 24 hours stimulation. Increase in OPN expression was significantly increased at 0.75 g/cm^2 of forces and above. Similar profile of protein level was observed (Fig. 1B). Graph on the right showed average $\pm$ standard deviation (S.D.) of band density from three separated experiments. (* denoted the significant difference; $p < 0.05$).

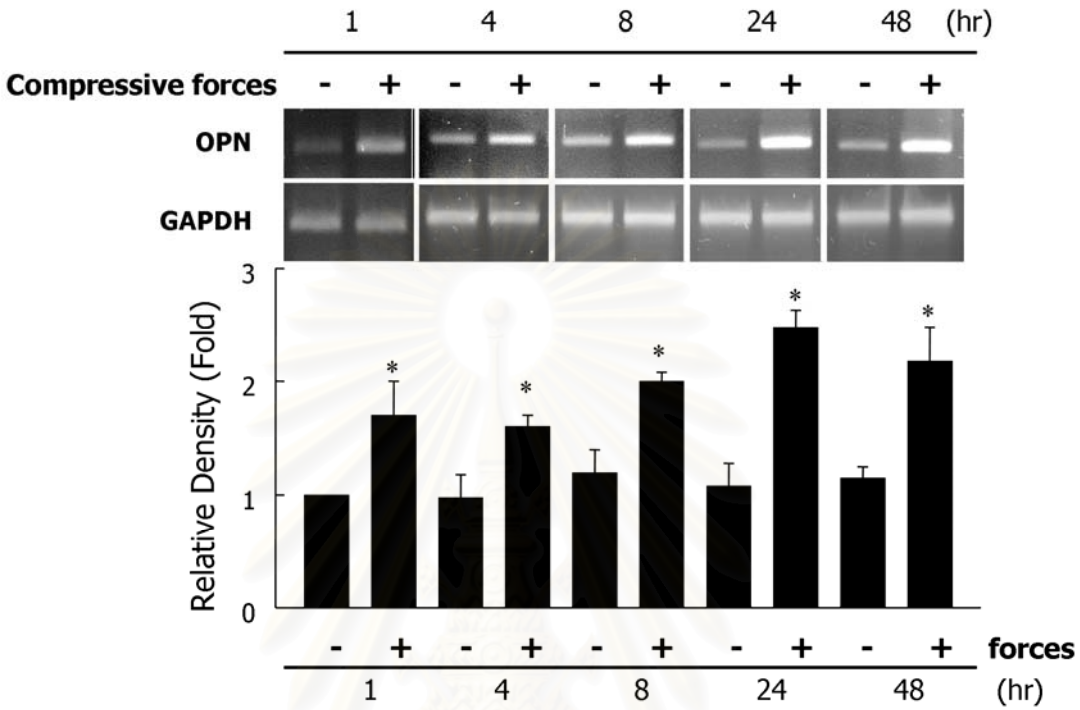

Fig. 2 Time-course experiment of stress-induced OPN expression. HPDL cells were stimulated with 1.25 g/cm^2 of force and the expression of OPN mRNA was determined at 1, 4, 8, 24 and-48 hours. Graphs showed average $\pm$ S.D. of band density from three separated experiments. (* denoted the significant difference; $p < 0.05$).

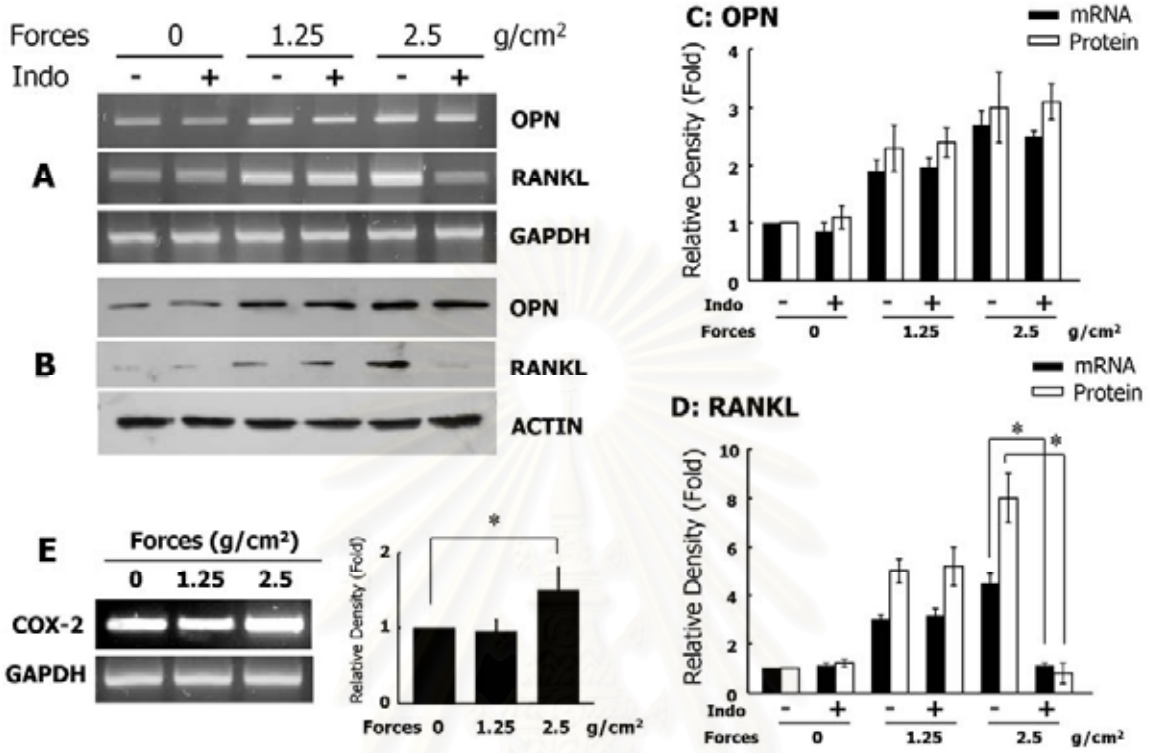

Fig. 3 The effect of indomethacin on stress-induced OPN and RANKL expression. HPDL cells were pre-incubated with indomethacin (Indo) for 30 minutes prior to the stimulation with either 1.25 or 2.5 g/cm^2 of force. Fig. 3A and B showed the changes in mRNA and protein level of OPN and RANKL after 24-hour stimulation, respectively. Graphs in Fig. 3C and D revealed the average $\pm$ S.D. of band density of OPN and RANKL from Fig. 3A and B. The expression of COX-2 mRNA was shown in Fig. 3E. (* denoted the significant difference; $p < 0.05$)

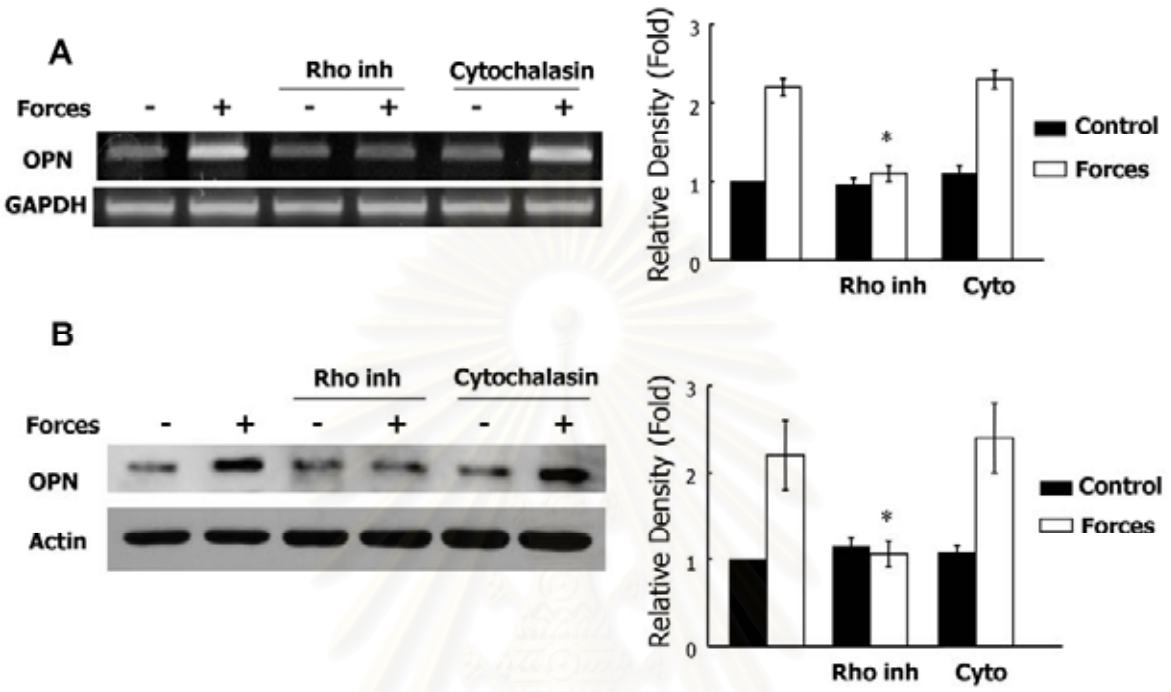

Fig. 4 Rho kinase inhibitor inhibited the up-regulation of OPN. Cells were pre-incubated with Rho kinase inhibitor (Rho inh) or cytochalasin B (Cyto) for 30 minutes before application of force at 1.25 g/cm^2. Fig. 4A and 4 B showed the results from RT-PCR and Western analysis, respectively. Graphs on the right showed average $\pm$ S.D. of band density from three separated experiments. (* denoted the significant difference; $p < 0.05$).