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เมื่อถูกกระตุ้นด้วยแรงดัน

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THE REGULATION OF OSTEOPONTIN IN STRESS-INDUCED HUMAN PERIODONTAL LIGAMENT CELLS

Mr. Suchart Wongkhantee

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Oral Biology
Faculty of Dentistry
Chulalongkorn University
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สุชาติ วงศ์ขันตี : การควบคุมการแสดงออกของออสทีโอพอนทินในเซลล์เอ็นยึดปริทันต์ มนุษย์เมื่อถูกกระตุ้นด้วยแรงดัน (THE REGULATION OF OSTEOPONTIN IN STRESS-INDUCED HUMAN PERIODONTAL LIGAMENT CELLS) อ.ที่ปรึกษา : รศ.ทพ.ดร. ประสิทธิ์ ภวสันต์, อ.ที่ปรึกษาร่วม : รศ.ทพญ. ทัศนีย์ ยงชัยตระกูล, 105 หน้า.

แรงกดเชิงกลที่กระทำต่อฟัน เช่น แรงจากการจัดฟัน อาจก่อให้เกิดการทำลายและการอักเสบต่ออวัยวะปริทันทันต์ได้ ในกระบวนการทำลายอวัยวะปริทันต์นี้ โปรตีนตัวหนึ่งที่มีความเกี่ยวข้อง คือ ออสทีโอพอนทิน ซึ่งเป็นโปรตีนที่มีหลายบทบาทในขบวนการต่างๆของเซลล์ รวมทั้งมีหน้าที่เกี่ยวข้องกับการเคลื่อนที่ และการยึดเกาะของเซลล์ทำลายกระดูกหรือเซลล์ออสทิโอคลาสท์ การศึกษานี้มีวัตถุประสงค์ เพื่อ ศึกษาผลของแรงกดเชิงกลต่อการควบคุมแสดงออกของออสทีโอพอนทินรวมถึงกลไกการถ่ายสัญญาณที่เกิดขึ้นในเซลล์เอ็นยึดปริทันต์มนุษย์

การสร้างแรงกดเชิงกลในห้องปฏิบัติการ ทำได้โดยกดทับเซลล์เอ็นปริทันต์ในจานเลี้ยงเซลล์ ด้วยน้ำหนักกดทับอย่างต่อเนื่อง จากนั้นตรวจวัดระดับ เอ็มอาร์เอ็นเอของออสทีโอพอนทินด้วยเทคนิค รีเวอร์สทรานสคริบชัน-โพลีเมอร์เรสเชนรีแอคชัน และวัดระดับโปรตีนของออสทีโอพอนทินด้วยเทคนิคเวสเทิร์น ส่วนขบวนการส่งถ่ายสัญญาณที่เกิดขึ้น จะตรวจสอบโดยการใช้สารยับยั้งชนิดต่างๆ

ผลการศึกษา พบการเพิ่มขึ้นของออสติโอพอนทิน ทั้งในระดับเอ็มอาร์เอ็นเอและระดับโปรตีน แปรผันโดยตรงตามปริมาณแรงกดที่เพิ่มขึ้น นอกจากนั้นยังพบว่าแรงกดเชิงกล สามารถกระตุ้นการเพิ่มขึ้นของ รีเซบเตอร์ แอคทิเวเตอร์ ออฟ นิวเคลียร์ แฟคเตอร์-แคปปา บี ลิแกน ด้วย ผลการใช้สารอินโดเมทาซิน สามารถยับยั้งการเพิ่มขึ้นของ รีเซบเตอร์ แอคทิเวเตอร์ ออฟ นิวเคลียร์ แฟคเตอร์-แคปปา บี ลิแกน แต่ไม่สามารถยับยั้งการเพิ่มขึ้นของออสทีโอพอนทิน แสดงให้เห็นว่าการส่งถ่ายสัญญาณการเพิ่มขึ้นของออสทีโอพอนทิน ไม่เกี่ยวข้องกับเอนไซม์ไซโคลออกซิจิเนส แต่การใช้สารยับยั้งชนิดโรห์ ไคเนส สามารถยับยั้งการเพิ่มขึ้นของออสทีโอพอนทินได้ แสดงให้เห็นว่าโมเลกุลในโรห์ ไคเนส มีความสัมพันธ์กับกระบวนการนี้

ในการทดลองใช้ อาหารเลี้ยงเซลล์ที่เก็บหลังจากใช้น้ำหนักกดทับ พบว่าสามารถกระตุ้นการแสดงออกของออสทีโอพอนทินได้เช่นเดียวกับการกดทับโดยตรง ซึ่งผลการทดลองนี้ถูกยับยั้งได้ด้วยสารยับยั้งชนิดสุรามิน และ เอ็นเอฟ 449 แสดงให้เห็นถึงความสัมพันธ์ของนิวคลีโอไทด์และ พี 2 รีเซบเตอร์ กับการเพิ่มขึ้นของออสทิโอพอนทินในเซลล์เอ็นยึดปริทันต์เมื่อถูกกระตุ้นด้วยแรงกดเชิงกล และสอดคล้องกับการเพิ่มขึ้นของสารนิวคลีโอไทด์ชนิด เอทีพี จากเซลล์เอ็นยึดปริทันต์หลังการกระตุ้นด้วยแรงกดเชิงกล และการเติมเอทีพี สามารถเพิ่มออสติโอพอนทินในเซลล์เอ็นยึดปริทันต์ผ่านทางโรห์ ไคเนส

โดยสรุป แรงกดเชิงกลกระตุ้นการเพิ่มขึ้นของออสทีโอพอนทินในเซลล์เอ็นยึดปริทันต์มนุษย์ได้ โดยกระตุ้นให้เซลล์เอ็นยึดปริทันต์หลั่งเอทีพีออกมา และกระตุ้น พี 2 รีเซบเตอร์ที่ผิวเซลล์เองหรือเซลล์ข้างเคียง ก่อนส่งถ่ายสัญญาณไปยังโมเลกุลของโรห์ ไคเนส และการเพิ่มขึ้นของออสทีโอพอนทิน มีความสัมพันธ์กับภาวะการทำลายกระดูกเบ้าฟันในทางคลินิก โดยการเหนี่ยวนำการเคลื่อนที่และการเกาะของเซลล์ทำลายกระดูก

สาขาวิชา ชีววิทยาช่องปาก

ปีการศึกษา 2550

ลายมือชื่อนิสิต..

ลายมือชื่ออาจารย์ที่ปรึกษา..

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม..

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KEY WORDS : CELLS / MECHANICAL STRESS / OSTEOPONTIN / PERIODONTAL LIGAMENT Rho KINASE / ADENOSINE 5'-TRIPHOSPHATE / P2 RECEPTORS

SUCHART WONGKHANTEE: THE REGULATION OF OSTEOPONTIN IN STRESS-INDUCED HUMAN PERIODONTAL LIGAMENT CELLS. THESIS ADVISOR: ASSOC. PROF. PRASIT PAVASANT, Ph.D. THESIS CO-ADVISOR: ASSOC. PROF TUSSANEE YONGCHAITRAKUL, M.S. 105 pp.

Mechanical stress such as orthodontic forces can produce mechanical damage and inflammatory reaction in the periodontium. Osteopontin (OPN) is a multifunctional cytokine, function as a chemotactic factor and enhance the spreading and the attachment of osteoclasts to the bone surface. This study aimed to examine the influence of mechanical stress on the expression and regulation of OPN as well as the signaling pathway involved in human periodontal ligament (HPDL) cells.

The *in vitro* mechanical stress was generated by continuous compressive force to HPDL cell culture, the expression of OPN mRNA and protein was examined by reverse transcription-polymerase chain reaction and Western analysis, respectively. The application of inhibitors was used to investigate the involved mechanism.

The results of this study demonstrated the increased of OPN in a force dependent manner in both mRNA and protein levels. Interestingly, the increased of receptor activator of nuclear factor-kappa B ligand (RANKL) was also observed. Application of indomethacin could abolish the induction of RANKL but not that of OPN, suggesting the cyclooxygenase-independent mechanism for stress-induced OPN expression. The upregulation of OPN was diminished by Rho kinase inhibitor suggesting of the involvement of Rho kinase pathway involved in this mechanism

The condition media (CM) collected from stress-induced HPDL cell also increased OPN expression similar to the direct stress application. Application of suramin and NF449, a purinergic P2 receptor inhibitor, abolished both stress-induced and CM-induced OPN expression, indicating the involvement of nucleotides and P2 receptor in the inductive mechanism. In addition, the increase of adenosine 5'-triphosphate (ATP), the universal nucleotide ligand of P2 receptor family, was also found in CM after stress stimulation and the application of exogenous ATP also induced OPN expression via Rho kinase pathway.

In the conclusion, mechanical stress affects OPN expression in HPDL cells by stimulating the releasd of ATP into the medium, subsequently, ATP acts through P2 receptor located at cell surface, and then activated Rho kinase pathway. The increase of OPN participates in clinical alveolar bone resorption and remodeling by inducing the migration and the attachment of osteoclasts.

Field of study : Oral Biology

Academic year 2007

Student's signature..

Advisor's signature..

Co-advisor's signature..

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Hope this thesis is worth reading and practically useful for every one who has been fascinated by the excitement of oral biology research like me.

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LIST OF ABBREVIATIONS

ADP	adenosine 5’-diphosphate
AMP	adenosine 5’-monophosphate
AP-1	actvated protein 1
ATP	adenosine 5’-triphosphate
Ca^{2+}	calcium ion
DAG	diacylglyceral
DMEM	Dulbecco’s Modified Eagle Mediun
FAK	focal adhesion kinase
H^{+}	hydrogen ion
IL-1	interleukin-1
IP3	inositol triphosphate
JNK	c-Jun N-terminal kinase
K^{+}	potassium ion
M-CSF	macrophage colony-stimulating factor
mDia1	mammalian diaphanous protein 1
OPG	osteoprotegerin
OPN	osteopontin
P receptors	purines and pyrimidines receptors
PDL	periodontal ligament
PIP	phosphatidylinositol phospate
PIPK	PIP kinase
PKC	protein kinase C
PKN1	protein kinase N1
PTH	parathyroid hormone
RANK	Receptor Activator of Nuclear factor Kappa B
RANKL	Receptor Activator of Nuclear factor Kappa B ligand
TNFα	tumor necrosis factor-alpha
UDP	uridine 5’-diphosphate
UTP	uridine 5’-triphosphate

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CHAPTER I

INTRODUCTION

Background and rationale

The periodontal ligament (PDL) is always exposed to mechanical loading during occlusion and mastication in healthy periodontal tissue. Under normal physiologic condition, the homeostasis of PDL tissues is maintained by appropriate mechanical loading (Tsuji et al., 2004). However, excessive mechanical force and/or tissue inflammation can become manifest as periodontal tissue damage including alveolar bone resorption and tooth loss (Verna et al., 1999). When the mechanical stress loaded onto a tooth, the force is transduced to the PDL; then, cell in the PDL respond to the mechanical stress to regulate the resorption and formation of bone matrix by signaling the surrounding cells (Lekic and McCulloch, 1996).

Previous study demonstrated the relationship of receptor activator of nuclear kappa B ligand (RANKL) and osteoprotegerin (OPG) expression stimulated by mechanical stress in PDL cells, that there was an up-regulation of RANKL expression while the OPG expression was not affected (Kanzaki et al., 2002) or was decreased (Yamaguchi et al.,2006). It is well-documented that RANKL and OPG are the key proteins of proliferation, differentiation, and activation of osteoclasts. The increased ratio of RANKL/OPG resulted in osteoclastic bone resorption (Khosla, 2001). But the

functions of osteoclasts might not be completed if these cells are not recruited to the resorption site and attach to the bone surface. In previous studies, osteopontin (OPN) was reported to involve in osteoclast function. An *in vitro* migration assay demonstrated the chemotactic effect of OPN toward osteoclasts (Terai et al.,1999). Furthermore, OPN might enhanced the attachment of osteoclasts to bone surface, then osteoclastic bone resorption started (Denhardt and Guo, 1993). Several studies have shown the expression of osteopontin in both mRNA level and protein level in PDL cells (Nohutcu, 1997; Li et al., 2001; Lee et al., 2004), but no study has reviewed the expression pattern of OPN after stimulate with mechanical stress as well as the signaling pathway involvement in stress-induced OPN expression in the HPDL cells.

Review of related literatures

Mechanical stress

Mechanical stress is a measure of force per unit area. In body, it is a body's internal distribution of force per area that reacts to external applied loads. Mechanical stress was classified into:

1. Dynamic stress
2. Static stress
3. Other mechanical stress

1. **Dynamic stress** is a measure of the dynamic force (F in [N]) acting externally is calculated by multiplying the mass (m in [kg]) by acceleration (a in [m/s^2]):

$F = m \times a$ [N=kgm/s^2]

Acceleration (a) is calculated by dividing velocity (v in [m/s]) by time (t in [s]):

$a = v / t$ [m/s2] or

Distance (s in [m]) divided by time squared (t^2 in [s^2]):

$a = s / t^2$ [m/s^2]

Dynamic stress was divided into:

1.1 Impact stress are mechanical/dynamic stress, which are caused by acceleration or deceleration of masses, and, though not generally repeated at regular intervals, do occur more or less frequently and with varying intensity.

Vertical impact: Free fall (dropping), tipping (by exceeding tilting moment), tumbling (rolling edge over edge), bumping (abrupt setting down)

Horizontal impact: jolting impact, bumping against vehicle wall

Impact stresses are generally stated as a multiple of acceleration due to gravity (g=9.81 m/s2)

1.2 Vibratory stress, measure as Hz, are the vibrations of subjects.

1.3 Other dynamic stresses are localized impact stresses: abrasion(dragging, pushing, pulling) or chafing (friction).

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2. Static stress is a measure of pressure (p in [N/m^2]) acting externally on the subjects is calculated by divided static force (F in [N]) and area (A in [m^2]):

$$p = F / A \ [N/m^2], \text{ wherein } F = m \times g \ [N=kgm/s^2]$$

Compressive stress arise as a result of stacking items of subject on top of one another.

3. Other mechanical stresses such as electrostatic charging, sound wave stress, radioactivity: bangs, noise, radiation. (Wikipedia, the free encyclopedia, 2007)

Bone biology

Bone is a highly specialized from other connective tissue that, together with cartilage, makes up the skeletal system. It is composed of inorganic mineral salts deposited within an organic collagen matrix, and three major cell types: osteoclasts, osteoblasts and osteocytes (Table 1.1). Bone is a dynamic, living tissue; continuous modelling and remodelling by bone cells allow the skeleton to grow and adapt. Abnormalities of bone remodelling can produce a variety of skeletal disorders.

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Table 1.1 **Characteristics of bone cells** (Hoebertz et al., 2003)

	Osteoblasts (bone-forming cells)	Osteoclasts (bone-resorbing cells)
Origin	Derived from precusors in periosteum and bone marrow stroma; common stromal precusors also gives rise to fibroblasts, adipocytes and chondrocytes	Derived from pro-monocytic precusors in bone marrow, spleen and peripheral blood; genarally multinucleated
Function	Secrete and mineralize bone organic matrix (~90% type I collagen)	Unique ability for extracellular resorption of mineralized; rapidly excavate characteristic pits and troughs on bone surfaces
Markers	Alkaline phosphatase, osteocalcin and osteonectin	Tartrate resistance acid phosphatase, carbonic anhydrase II, vitronectin receptor and calcitonin receptor
Differentiation and proliferation	Differentiate into osteocytes (network of strian-detecting cells) when engulfed by bone matrix; primary cells (e.g. from rodents or humans) proliferate readily for a few weeks in culture; form mineralized collagenous bony nodules at high density in long-term cultures (~3 weeks)	Primary cultures containing precursors (e.g. from mouse bone marrow or human peripheral blood) can differentiate into multinucleated functional osteoclasts in vitro (~2 weeks)
Cell lines	Many transformed 'osteoblast-like' cell lines are available, mostly derived from osteosarcomas (e.g. ROS 17/2.8, MG63 and UMR106); cell lines have limited osteogenic potential in vitro	Some transformed macrophage-likecelllines (e.g. RAW 264.7) differentiate into 'osteoclast-like' cells but appear to be unable to resorb bone in vitro

Osteoblasts are mononuclear cells of mesenchymal origin that are responsible for bone formation. They are able to secrete an extracellular matrix consisting mainly of type I collagen, which they later mineralize. The periosteum and bone marrow are important sources of mesenchymal osteoprogenitor cells. Osteoblasts that are actively secreting bone matrix are large cuboidal mononuclear cells with a prominent protein synthesizing apparatus, whereas the quiescent osteoblasts that cover most adult bone surfaces have a flat morphology. Some osteoblasts become incorporated into osteocytes, which form a regular, interconnected network of cells that is thought to mediate responses to mechanical loading. In contrast to cartilage, bone is highly vascular; the blood vessels and nerve fibers that ramify though bone constitute an important, albeit poorly understood, regulatory system.

Osteoclasts have the unique ability to resorb bone extracellularly, a process that entails excavation of characteristic pits and troughs on bone surfaces. Osteoclasts are multinucleated cells formed by the porliferation of haematopoietic, mononuclear progenitors of monocyte and macrophage lineage and their subsequent fusion into multinucleated osteoclasts. The most characteristic feature of osteoclasts is the presence of ruffled borders and clear zone. Vacuolar H^+-ATPase exists in the ruffled border membrane of osteoclasts, and acidifies resorbing area under the ruffled border. The ruffled border is surrounded by a clear zone, which serves for the attachment of osteoclasts to the bone surface to maintain a microenvironment favorable for bone resorption. (Katagiri and Takahashi, 2002).

Bone remodeling and mechanical stress

The shape of bone changes corresponding to the physical circumstances such as mechanical stress (Chambers et al., 1993). The change is a result of bone remodeling with a series of cellular events that occur in the remodeling site through the activation of osteoclasts and osteoblasts, followed by resorption and formation of bone, respectively (Terai et al., 1999).

When mechanical stress is loaded on bone, the bone matrix receives the stress efficiently. Numerous osteocytes are located in the bone matrix and they communicate with each other and with osteoblasts on the bone surface via processes and gap junction connections (Aarden et al., 1994). Such a cellular network is speculated to certain important functions for adaptation to a mechanical load environment by the detection of mechanical stress (Turner and Forwood, 1995). previous studies revealed that osteocytes act as mechanosensitive cells in the early stage of bone remodeling. However, the relationship among mechanical stress, cellular reactions, and bone remodeling has not been cleared.

Role of osteoblast/stromal cells in osteoclast differentiation

Biological model of *in vitro* osteoclast differentiation have been developed that have facilitated detailed study of numerous factors involved in the regulation of this process. The most common models are cultures of mouse bone marrow or cocultures of haematopoietic cells with bone-derived stromal cells, which give rise to large numbers

of bone resorbing osteoclasts (Suda, 1992). Studies based on these models have found that mesenchymal derived stromal cells play a critical role in supporting and stimulating osteoclast differentiation, a process that probably necessitates cell-cell contact between osteoclast precusors and stromal cells (Takahashi et al., 1988; Quinn et al., 1994). In some human models, however, a cellular interaction between osteoclast precusors and stromal cells is not always required (Kurihara et al., 1991; Matayoshi et al., 1996; Roux et al., 1996).

Local and hormonal factors involved in osteoclast differentiation

Bone resorption is closely controlled *in vivo* by cellular and hormonal factors, which affect not only osteoclast activity, but also osteoclast formation. Parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3[1,25$(OH)_2D_3$] increase bone resorption, primarily via an indirect mechanism that is mediated by osteoblasts (Suda, 1992). Oestrogens have a negative impact on osteoclast differentiation, and oestrogen deficiency leads to increased osteoclast differentiation and activation (de Vernejoul, 1993). The cytokines IL-1, IL-6 and TNF-alpha are known to increase bone resorption by stimulating both osteoclast activity and differentiation. This effect involves, at least in part, prostaglandin production (Roodman, 1992). The major role of macrophage colony-stimulating factor (M-CSF) has been pointed out in M-CSF-deficient mice, which develop an osteopetrosis that is characterized by the absence of osteoclasts (Yoshida et al., 1990). Studies using murine coculture have shown that M-CSF acts both on

proliferation and on differentiation of precursor cells (Tanaka et al., 1993). Local injection of M-CSF in rat metaphyseal bone also increase in situ osteoclast differentiation and bone resorption (Orcel et al., 1990). Others cytokines stimulate bone resorption, such as leukaemia inhibiting factor and IL-11. (Ishimi et al., 1992) Conversely some cytokines such as IL-4 and IFN-gamma have been shown to inhibit osteoclast differentiation *in vitro* (Lacey et al., 1995). The role of transforming growth factors-ß is more complex; it decreases osteoclast precursor proliferation and bone resorption activity (Oreffo et al., 1990), but it also increases the expression of two osteoclastic markers – vitronectin receptor and calcitonin receptor (Mbalaviele et al., 1992).

The discovery of new members of the TNF receptor-ligand family; RANK, RANKL and OPG, has clarified the molecular mechanism of osteoclast differentiation regulated by osteoblasts/stromal cells (Katagiri and Takahashi, 2002).

RANKL (Receptor Activator of Nuclear factor Kappa B Ligand)

RANKL, also called osteoclast differentiation factor (ODF), TNF-related induced cytokine (TRANCE), or osteoprotegerin ligand (OPGL)

Because osteoblast-stromal cell interactions with osteoclast precursors are required for subsequent osteoclast differentiation, an ODF expressed by these cells and recognized by osteoclast precursors was suspected. Such a factor was identified as RANKL(Lacey et al., 1998).

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Human RANKL is a 317-amino acid peptide that has aproximately 30% homology to the TNF-related apoptosis-inducing ligand and to CD40, and approximately 20% homology to Fas ligand. It has now been shown to exist in two forms: a 40- to 45-kDa cellular, membrane-bound form and a 31-kDa soluble form derived by cleavage of the full length form at position 140 or 145. RANKL mRNA is expressed in highest levels in bone and bone marrow, as well as in lymphoid tissues (lymph node, thymus, spleen, fetal liver, and Peyer's patches). It major role in bone is the stimulation of osteoclast differentiation, activity (Lacey et al., 1998), and inhibition of osteoclast apoptosis (Fuller et al., 1998). Indeed, in the presence of low levels of M-CSF, RANKL appears to be both necessary and sufficient for the complete differentiation of osteoclast precursor cells into mature osteoclasts (Lacey et al., 1998). In addition, it is clear that RANKL has a number of effects on immune cells, including activation of c-Jun N-terminal kinase (JNK) in T cell (Wong et al., 1997), inhibition of apoptosis of dendritic cells, induction of cluster formation by dendritic cells, and effects on cytokine-activated T cell proliferation (Anderson et al., 1997).

Consistent with this finding, RANKL knockout mice have severe osteopetrosis with defects in tooth eruption (Kong et al., 1999). They also have a complete absence of osteoclasts. In addition, they exhibit defects in early differentiation of T and B cells, lack lymph node, have defects in thymic differentiation, but have a normal splenic structure and Peyer's patches (Kong et al., 1999). A somewhat unexpected finding in this mice is that they also have defects in mammary gland development. In particular, they fail to

form lobulo-alveolar structures during pregnancy, resulting in death of the newborns (Feta et al., 2000).

RANK (Receptor Activator of Nuclear factor Kappa B)

Human RANK is a 616-amino acid peptide, with a 28-amino acid signal peptide, an N-terminal extracellular domain, a short transmembrane domain of 21 amino acid, and a large C-terminal cytoplasmic domain. It is expressed primarily on cells of the macrophage/monocyte lineage, including preosteoclastic cells, T and B cells, dendritic cells, and fibroblasts (Anderson et al., 1997). The ultimate proof that RANK expressed on preosteoclastic cells was the sole receptor on these cells for RANKL came with the demonstration that RANK knockout mice had profound osteopetrosis due to an absence of osteoclast (Li et al., 2000). Moreover, osteoclastogenesis could be initiated in these mice by transfering of the RANK cDNA back into hematopoietic precursors. Interestingly, similar to the RANKL knockout mice, RANK knockout mice also lacked peripheral lymph nodes and had defective T and B cell maturation but differed from the RANKL knockout mice in having normal thymic development (Li et al., 2000).

OPG (Osteoprotegerin)

OPG (or osteoclastogenesis inhibitory factor, OCIF) was found to be initially synthesized as a 401 amino acid peptide, with a 21-amino acid propeptide that was cleaved, resulting in a mature protein of 380 amino acid. In contrast to all other TNF

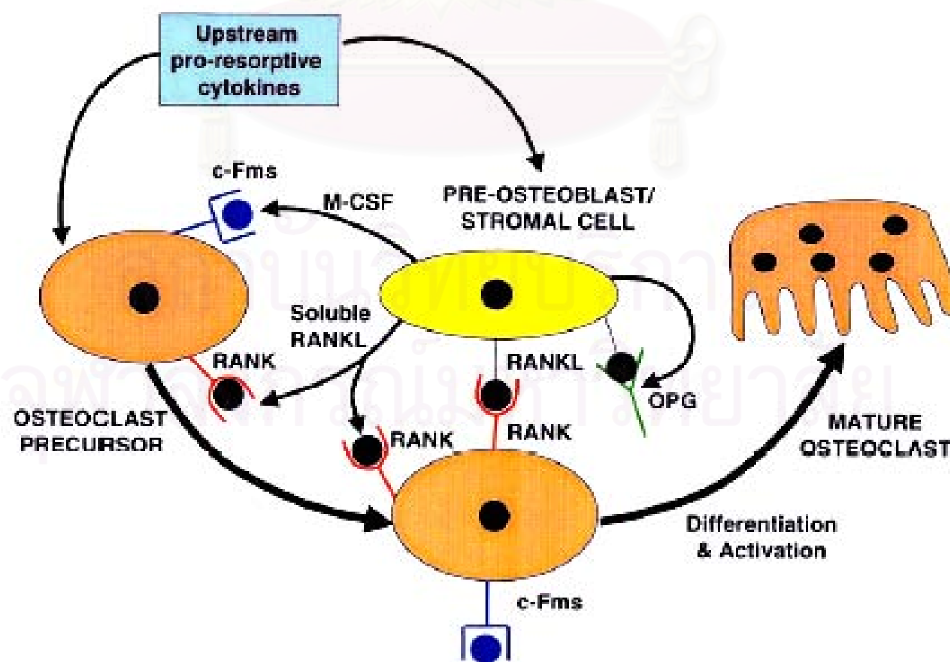
receptor superfamily members, OPG lacks transmembrane and cytoplasmic domains and is secreted as a soluble protein. The N-terminal region contains four cysteine-rich domains (D1-D4) and most closely relates to TNF-receptor-2 and CD40. The C-terminal region contains two death domain homologous region (D5 and D6) as well as a region (D7) containing a heparin binding site and a cysteine residue necessary for homodimerization (Simonet et al., 1997).

OPG mRNA was found to be expressed in a number of tissues, including lung, heart, kidney, liver, stomach, intestine, brain and spinal cord, thyroid gland and bone (Yasuda et al., 1998). Because the major biological action of OPG described to date has been to inhibit osteoclast differentiation and activity (Simonet et al., 1997), the potential role of OPG in these other tissues remains to be established. However, mice with target ablation of OPG not only develop severe osteoporosis due to markedly increased osteoclast formation and subsequent bone resorption, but also have profound calcification of the large arteries, marked intimal and medial proliferation, and partial aortic dissection by the age of 4 months. Thus, OPG likely also plays a significant role in the vasculature (Bucay et al., 1998).

Figure 1.1 summarizes the control of osteoclastogenesis that has emerged in the post RANK / RANKL / OPG era. RANKL, expressed on the surface of preosteoblastic/stromal cells, binds to RANK on the osteoclastic precursor cells. M-CSF, which binds to its receptor, c-Fms, on the preosteoclastic cells, appears to be necessary for osteoclast development because it is the primary determinant of the pool

of these precursor cells (Udagawa et al., 1990). RANKL, however, is critical for the differentiation, fusion into multinucleated cells, activation and survival of osteoclasts. OPG, a decoy receptor, puts a brake on the entire system by blocking the effects of RANKL. A number of preresorptive cytokines, such as TNF-alpha and IL-1, modulate this system primarily by stimulating M-CSF production (thereby increase the pool of preosteoclastic cells) and by directly increasing RANKL expression (Hofbaucer et al., 1999).

Figure 1.1 Osteoclast differentiation and RANK, RANKL, and OPG system (Hofbaucer et al., 1999)



OPN (Osteopontin)

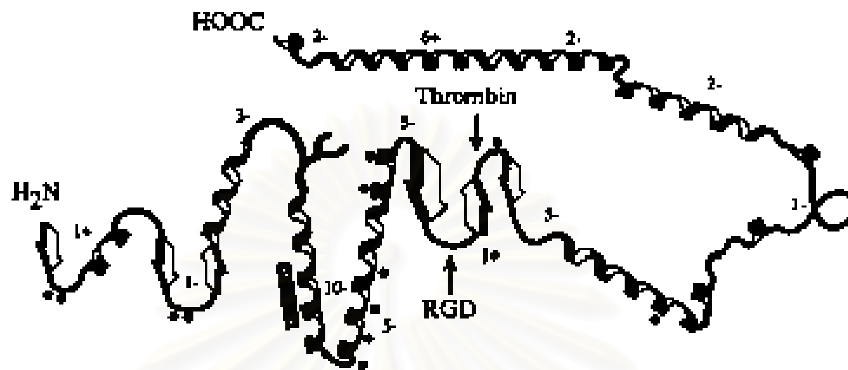


Figure 1.2 The mouse OPN (Denthardt and Guo, 1993). Regions of predicted α-helix and β-sheet structure are indicated by helices and arrows in the cartoon representation of the protein; the shade box and solid circles indicate the stretch of aspartic acid residues and putative sites of serine/threonine phosphorylation, respectively; the conserved RGD and thrombin cleavage sites are marked; the negative and positive numbers along the chain indicate at 20-residue intervals the net charge in that region of the polypeptide backbone at neutral pH. (Denthardt and Guo., 1993)

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OPN is a major noncollageneous bone matrix protein, that is synthesized as a 34-kDa protein (Sodek et al., 2002). A sialic acid-rich phosphorylated glycoprotein originally isolated from bone, it contains a motif of glycine-arginine-glycine-aspartic acid

serine (GRGDS) amino acid sequence that promotes cell attachment via cellular $\alpha_v\beta_3$ integrin (Denhardt and Guo, 1993). OPN is thought to promote or regulate the adhesion, attachment, and spreading of osteoclasts to the bone surface during bone resorption (Reinholt et al., 1990). Highly conserved regions of protein sequence induce an aspartate-rich sequence and sites of Ser/Thr phosphorylation, which mediate binding to hydroxyapatite, and RGD motif that mediates cell attachment / signaling, and a thrombin-susceptible site, which can modulate the integrin mediated interaction (Turner and Forwood, 1995). It is known to be produced by osteoblasts (Weinreb et al., 1990), as well as osteoclasts (Merry et al., 1993), and is considered to play important roles in bone formation, resorption, and remodeling (Denhardt and Guo, 1993).

OPN expression is regulated by calcitropic cytokines and hormones as well as mechanical stress both *in vitro* and *in vivo*. Intermittent hydrostatic compression, four point bending, and experimental tooth movement enhance OPN expression in osteoblasts or osteoclasts *in vitro* or *in vivo* (Butler, 1989; Dodds et al., 1993; McKee et al., 1993; Takano-Yamamoto et al., 1994). Mechanical forces have been suggested to act at the site of cell attachment, possibly involving extracellular matrix proteins including OPN, to generate a shear stress at adhesion plaques, to transmit signals via integrins to the cytoskeleton, and to modify cell shape and gene expression (Nomura et al., 1988).

From the two experiments of the same group, they investigated the spatial gene expression of bone matrix proteins in periodontal ligament and in the interradicular

septum (IRS) during physiological tooth movement in rats (Takano-Yamamoto et al., 1993). Osteonectin and Osteocalcin mRNAs were expressed in preosteoblasts and osteoblasts in the bone formation site of the IRS. In contrast, osteopontin mRNA (Opn) was expressed in osteoclasts and osteocytes only in the resorption site of the septum (Takano-Yamamoto et al., 1993). These finding suggested that OPN participates in bone resorption during experimental tooth movement. Then, they demonstrated that mechanical stress induced expression of OPN by immunohistochemistry technique in almost all osteocytes and in some of the osteoblasts and bone lining cells in the resorption site in the early stage of experimental tooth movement. Following this event, numerous osteoclasts were recruited to the bone surface. An in vitro migration assay demonstrated the chemotactic effect of OPN toward osteoclastic cells (Terai et al., 1999).

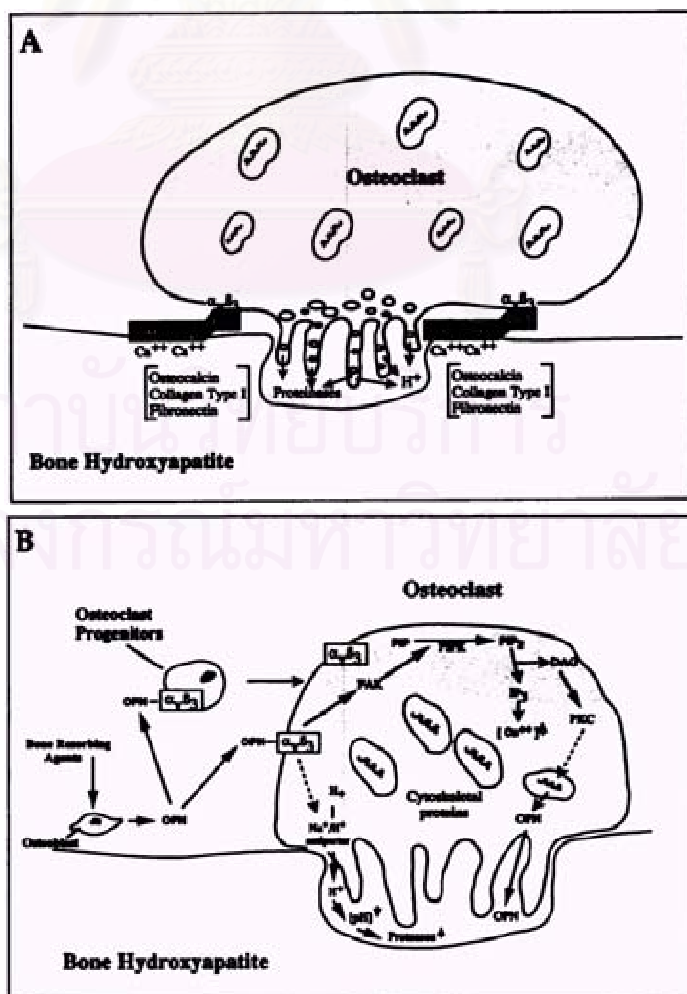
The possible roles of OPN in osteoclast function

When osteoclasts adhere to bone during bone resorption, a specialized structure is form at the bone-cell interface that consists of a clear zone surrounding an area where the plasma membrane appears in a ruffled configuration interdigitating with bone matrix. The clear zone is a region of adherence between the osteoclasts and the bone surface that appeared to seal the ruffled membrane region from the extracellular environment., thus creating an isolated microenvironment that resembles an

extracellular lysosome in which acidic condition conducive to dissolution of the calcium phosphate matrix are created (Denhardt and Guo, 1993).

There is controversy of the roles of OPN in the osteoclastic bone resorption. In general agreement, an OPN- $\alpha_v\beta_3$ integrin interaction is important in adherence of the osteoclasts to bone (Ross et al., 1993), but Jin et al. (1990) have suggested that OPN, which is produced by osteoblasts in response to agents that cause bone resorption (interleukin 1α and β, TNFα, lypopolysaccharide, and 1α,25-$(OH)_2$ vitamin D3), promotes the bone-resorbing activity of osteoclasts via the $\alpha_v\beta_3$ integrin. Figure 1.3 show the possible roles of OPN in osteoclast function.

Figure 1.3 The possible roles of OPN in osteoclast function. A) OPN promotes attachment of osteoclasts to the bone surface via interactions on the one hand with the hydroxyapatite crystal, and on the other hand with the cell surface, possibly $\alpha_v\beta_3$ integrin. Proteins that OPN may interact include osteocalcin, type I collagen, and fibronectin. OPN may also contribute to isolating the absorption lacuna from the external environment. B) OPN promote changes in gene expression via signal transduction cascade initiate by an interaction of OPN with a cell surface receptor such as $\alpha_v\beta_3$ integrin. FAK,focal adhesion kinase; DAG, diacylglyceral; PIP, phosphatidylinositol phospate; PIPK, PIP kinase; IP3, inositol triphosphate; PKC, protein kinase C. (Denhardtand Guo, 1993)



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Purines and pyrimidines receptors

The idea that purines could act as extracellular signalling molecules was first proposed more than 80 years ago (Cheek et al., 2000). Extracellular nucleotides has since been implicated in a wide range of biological processes, including smooth muscle contraction, inflammation, platelet aggregation and pain, among many others (Ralevic and Burnstock, 1998). Receptor of purines and pyrimidines have been classified into two groups;-

1. P1 receptors with adenosine as the main ligand
2. P2 receptors with ATP, ADP, UTP and UDP as the main ligands. On the basis of pharmacology, cloning and transduction studies, ionotropic ligand-gated ion channels $P2X_{1-7}$ and metabotropic G-protein-coupled $P2Y_{1,2,4,6,11,12,13,14}$ receptor families were established (Table 1.2) (Figure 1.4)

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Figure 1.4 ionotropic ligand-gated ion channels P2X (right), and metabotropic G-protein-coupled P2Y receptor (left). P2X receptor subunits consist of two hydrophobic transmembrane domains, a large N-glycosylated extracellular loop and intracellular N- and C-termini. At least three or four subunits are thought to form a functional P2X receptor channel. P2Y receptors consist of seven transmembrane domains, with an extracellular N-terminus and an intracellular C-terminus.(Acris Antibodies web page, 2007)

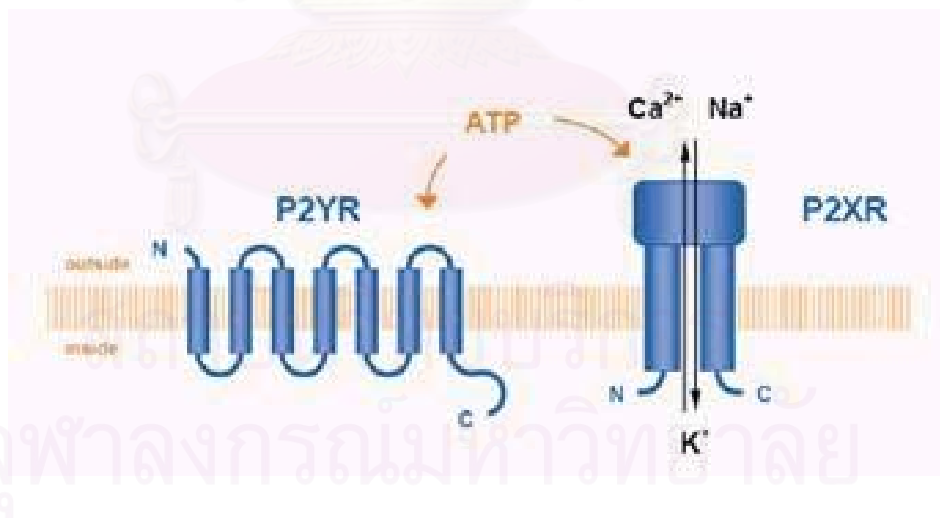

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Table 1.2 Characteristics of P2 receptors (Hoebertz et al., 2003)

Receptor	Agonists[a]	Special characteristics
P2X		
$P2X_1$	αβmeATP = ATP = 2meSATP	Rapid desensitization
$P2X_2$	ATP ≥ ATPγS ≥ 2meSATP >>αβmeATP	Sensitive to pH and Zn^{2+}
$P2X_3$	2meSATP ≥ ATP ≥ αβmeATP	Rapid desensitization
$P2X_4$	ATP >> αβmeATP	-
$P2X_5$	ATP >> αβmeATP	-
$P2X_6$	-	does not function as a homomultimer
$P2X_7$	BzATP > ATP ≥ 2meSATP >> αβmeATP	large pore with prolonged activation
P2Y		
$P2Y_1$	2meSADP > 2meSATP = ADP > ATP	-
$P2Y_2$	UTP = ATP	-
$P2Y_4$	UTP ≥ ATP	-
$P2Y_6$	UDP > UTP >> ATP	-
$P2Y_{11}$	ARC67085MX > BzATP ≥ ATPγS > ATP	-
$P2Y_{12}$	ADP	-
$P2Y_{13}$	2meSADP ≥ ADP	-
$P2Y_{14}$	UDP-gluclose	-

[a] Abbreviation: ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; ATPγS, adenosine 5'-3-O-thiotriphosphate; BzATP, 2',3'-O-(4-benzoyl-benzoyl)ATP; αβmeATP, αβ-methylene ATP; 2meSADP, 2'-methylthioADP; 2meSATP, 2-methylthioATP; UDP, uridine 5'-diphosphate; UTP, uridine 5'-tri phosphate.

Role of P2 receptors in osteoclast biology

The first evidence that osteoclasts respond to nucleotides came from studies using rabbit osteoclasts. Adenosine 5'-triphosphate (ATP) was shown to elicit an increase in the concentration of intracellular Ca^{2+} $[Ca^{2+}]_i$ in these cells via influx of Ca^{2+} from internal stores (Yu and Ferrier, 1993). Subsequent electrophysiological studies provided evidence for the coexistence of both P2X and P2Y receptors on octeoclasts (Weidema et al., 1997). More recent studies reported that the adenosine 5'-diphosphate (ADP) analoque ADPβS elicited a Ca^{2+}-dependent K^+ current in rabbit osteoclasts, consistent with the presence of $P2Y_1$ receptor on osteoclasts (Naemsch et al.,1999).

Using immunocytochemistry and in situ hybridization techniques on rat bone sections and cultured rat bone cells, evidence for the expression of $P2X_2$, $P2X_4$, $P2Y_1$, $P2Y_2$ receptor subtypes on osteoclasts was found (Hoebertz et al., 2000). Detection of the $P2X_4$ receptor was consistent with reverse-transcription polymerase chain reaction (RT-PCR) evidence (Naemsch et al.,1999). In addition, cultured rat osteoclasts show nuclear staining for the $P2X_7$ receptor (Hoebertz et al., 2000; Naemsch et al.,2001). A recent study using RT-PCR found evidence for the expression of a wider range of P2 receptors ($P2X_{1,4,5,6,7}$ and $P2Y_{1,2,4,6,11}$ receptors) on normal human osteoclasts, but no evidence for $P2X_2$ receptor expression (Buckly et al.,2002).

A potential role for P2 receptors in osteoclast biology was first proposed in 1995 when ATP was shown to stimulate bone resorption by cells derived from a human osteoclastoma or 'giant cell tumor' (Bowler et al., 1995). Although it was proposed that

this action might be mediated by the $P2Y_2$ receptor (Bowler et al., 1995), this could not be confirm in a follow-up study because the potent $P2Y_2$ agonist uridine 5'-triphosphate (UTP), in contrast to ATP failed to stimulate bone resorption (Bowler et al., 1998). Subsequently, ATP at low concentration was shown to stimulate not only the resorptive activity of osteoclasts but also the information of rodent osteoclasts. The stimulatory effect of ATP on resorption was amplified greatly when rat osteoclasts were co-activated by culture in acidified medium (Morrison et al., 1998). This suggested the possible involvement of the acid-sensitive $P2X_2$ receptor, the only P2 receptor that elicits a significant increase in ATP-evoked currents when the pH is lowered to below 7.0 (Wildman et al., 1998).

The first evidence to link a specific P2 receptor to the action of nucleotides on bone resorption was reported (Hoebertz et al., 2001). Extracellular ADP and 2-methylthioADP (2-meSADP), a selective $P2Y_1$ receptor agonist, were shown to be potent stimulators of bone resorption at nanomolar to low micromolar concentrations, as assessed by three independent methods (Hoebertz et al., 2001). The action of ADP on resorption pit formation by mature rat osteoclasts were biphasic: no effects were evident at higher concentrations (20-200 μM), which is agreement with a bell-shaped response curve observed earlier for ADP at the $P2Y_1$ receptor (Sak et al., 2000). Adenosine 5'-monophosphate (AMP) and adenosine had no effect on resorption, which suggests that ADP effect could be blocked in a non-toxic manner by the compound MRS2179 (2'-deoxy-N^6-metyladenosine-3',5'-bisphosphate) one of the most potent $P2Y_1$ receptor

antagonists reported to date (Boyer et al., 1998). The experiments indicated that extracellular ADP could stimulate resorption directly via the $P2Y_1$ receptor expressed on mature osteoclasts or indirectly via receptors expressed on osteoblasts, which in turn release pro-resorptive local factors, or both direct and indirect mechanism. A recent study on human osteoclasts suggested that the effect of ATP on resorption is indirect through upregulation of RANKL in osteoblasts (Buckley et al.,2002). Experiments using mouse marrow cultures also indicated that ADP stimulates osteoclast formation from haematopoietic precursors, in addition to activating mature osteoclasts. In mouse calvarial bone organ cultures, resorption stimulated by ADP was blocked by the cyclooxygenase inhibitor, indomethacine, suggesting a requirment for endogenous prostaglandin synthesis in this culture system. A similar dependency on prostaglandins has been observed for other osteolytic agents, such as protons, in culvarial organ cultures (Rabadjija et al., 1990). Two earlier studies also investigated the actions of ADP on osteoclasts, but at much higher concentrations: ADP at 50 µM increased $[Ca^{2+}]_i$ and ADP at 100 µM induced a decrease in the intracellular pH in rabbit osteoclast (Yu and Ferrier, 1994), whereas interestingly ADP appears to exert its major pro-resorptive action on osteoclasts at much lower concentrations of between 20 nM and 2 µM.

As mentioned above, ATP is also a potent stimulator of activation and formation of rodent osteoclasts, an effect only evident at low pH (~6.9), which suggests the involvment of the $P2X_2$ receptor (Morrison et al., 1998). However, a similarly low pH is also required for the stimulatory effect of ADP, acting though the non-acid-sensitive $P2Y_1$

receptor (Hoebertz et al., 2001). This is consistent with earlier studies showing that the pro-resorptive effects of other agents are acid dependent (Arnett and Dampster, 1990), and thus suggests that there is a universal dependency of osteolytic agents on slight local acidification for their action. Whether the $P2X_2$ receptor plays a general role in mediating this process remains to be determined.

Osteoclasts have been reported to undergo cell death when exposed to high concentrations of ATP (1-2 mM) (Morrison et al., 1998). Apart from initiating active cell death,activation of $P2X_7$ receptors could also be involved in a different process in osteoclast biology: the receptor has been implicated in the formation of giant cells by mediating the fusion of murine macrophage-like cells (Chiozzi et al., 1997). It is therefore conceivable that the fusion of osteoclast precursors is also initiated by $P2X_7$-receptor-mediated pore formation in the membranes of adjacent cells, leading to the development of cytoplasmic bridges. However, high concentrations of ATP can also cause the development of a slowly activating inward current that is permeable only to small cations; this rules out pore formation and suggests another unknown role for the $P2X_7$ receptor in osteoclast biology (Naemsch et al., 2001). Table 1.4 summerizes the evidence and possible function of P2 receptors in osteoclasts.

Role of P2 receptors in osteoblast biology

Several studies have shown that nucleotides act though P2 receptors to induce formation of inositol (1,4,5)-tris-phosphate [Ins(1,4,5)P_3] and transiently elevate $[Ca^{2+}]_i$ in osteoblasts (Kumagai et al., 1989; Kumagai et al., 1991). Studies on rat osteoblast-like cells demonstrated that extracellular nucleotides interact with at least two receptor subtypes; the phamocological profiles were characteristic of $P2Y_1$- and $P2Y_2$-like receptors (Yu and ferrier, 1993; Sistare et al., 1994). Studies on single cells and populations of human osteoblasts revealed heterogeneity of receptor expression with one cell culture (Dixon et al., 1997). This might indicate that expression of P2 receptors changes during the osteoblast life cycle, depending on the differentiation state.

The first molecular evidence for the expression of P2Y receptors by osteoblasts came with the localization by in situ hybridization of $P2Y_2$ receptors (Bowler et al., 1995) in human osteoblasts and RT-PCR evidence for $P2Y_1$,$P2Y_2$,$P2Y_4$ and $P2Y_6$ receptors in human osteosarcoma cell lines (Maier et al., 1997). More recently, evidence for the expression of $P2X_2$, $P2X_5$, $P2Y_1$ and $P2Y_2$ receptors, at protein and mRNA level, on rat osteoblasts was reported (Hoebertz et al., 1997). P2X receptor expression has also been described in human osteoblasts, and the $P2X_5$ receptor has been implicated in the stimulation of DNA synthesis by ATP (Nakamura et al., 2000). Earlier studies showed that $P2X_5$ receptor immunoreactivity was indeed restricted to the metabolically active, differentiating cell layers in epithelium and hair follicles (Groschel-Stewart et al., 1999).

Thus, $P2X_5$ receptor might parcicipate in the regulation of osteoblastic proliferation and differentiation.

There are conflicting data on the expression of the $P2X_7$ receptor in osteoblasts. The presence of $P2X_7$ receptors in primary human osteoblasts was found on rat osteoblasts (Hoebertz et al., 2000). An earlier study reported that high ATP concentrations caused formation of pores in murine osteoclasts macrophages, but not in osteogenic or chondrogenic cells (Modderman et al., 1994). Thus, the potential role and presence of $P2X_7$ the receptor in osteoblasts remains to be clarified.

Activation of $P2Y_1$ and $P2Y_2$ receptors has been shown to shown to potentiate subsequent parathyroid hormone (PTH) receptor-mediated Ca^{2+}signalling (Buckley et al., 2001, Bowler et al, 1999). For example, it has been suggested that PTH receptors are capable of activating adenylyl cyclase but might be unable to activate phospholipase C until cells receive a signal as a consequence of P2 receptor activation (Bowler et al, 2001). These synergies suggest a mechanism through which systemic PTH could initiate bone remodelling at specific site in the skeleton by cooperating with the localized of nucleotides. Thus, one could speculate that in damaged bone tissues increased local levels of PDGF and nucleotides released from activated platelets, endothelial cells and other cells, attract osteogenic cells to lesional site and stimulate their proliferation. Extracellular nucleotides present in the bone microenvironment might thus be capable of modulating bone cells and controlling the remodelling process by

interacting with, and potentiating, both systemic hormones, such as PTH, and local growth factors (Bowler et al, 2001).

Furthermore, extracellular nucleotides have been shown to reduced the amount of bone formed by primary rat osteoblasts in a novel in vitro appositional bone formation model (Jones et al., 1997). This study used relatively high concentration of ATP (50-500 µM) and results for the effects of UTP were equivocal, so that it was not possible to infer which receptor subtypes might be involved. The actions of nucleotides on bone formation by osteoblasts were re-examined using a different, more conventional model. Both UTP and ATP, at concentrations as low as 1-10 µM, but not adenosine or ADP, caused strong inhibition of mineralized bone nodule formation by cultured rat osteoblasts (Hoebertz et al., 2002). The potent inhibitory actions of ATP and UTP point to the involvement of either $P2Y_2$ or $P2Y_4$ receptors. No expression of $P2Y_4$ receptor suptype on rat osteoblast was found, which suggests that the $P2Y_2$ receptor might mediated these inhibitory effects (Hoebertz et al., 2000). The earlier observation by Jones et al. that adenosine 5'-O-3-thiotriphosphate (ATPγS), a potent agonist at the $P2Y_2$ receptor, also inhibited osteoblastic formationis consistent with the notion that the effect is mediated via the $P2Y_2$ receptor (Jones et al., 1997).

$P2Y_2$ receptors have been shown to mediate oscillatory fluid flow-induced Ca^{2+} mobilization in murine osteoblasts (You et al., 2002). Mechanically stimulated human osteoblasts have also been shown to propagate fast intercellular Ca^{2+} waves via autocrine activation of $P2Y_2$ receptors (Jorgensen et al., 1997). This is interest in view of

the coordinated cell activity needed for the control of bone remodelling. Intercellular signal propagation might represent a mechanism by which mechanically initiated signals, possibly by osteocytes, diffuse through the bone tissue to surface osteoblasts and osteoclasts. In a follow-up study, signalling between osteoblasts and osteoclasts was investigated. Surprisingly, signalling to osteoclasts was not mediated by P2Y receptors but appeared to require the $P2Y_7$ receptor (Jorgensen et al., 2002).

The observation that the functionally effective concentration of ATP, UTP and ADP are in the low micromolar range could be relevant to the bone microenvironment, where low-level fluctuations of extracellular nucleotide concentrations are likely to occur. Cultured osteoblasts are capable to release ATP, resulting in nanomolar concentrations in the medium (Romanello et al., 2001). However, concentration measured in tissue cultures medium are unlikely to reflect accurately concentration occurring at the cell surface and in the small volumes of the extracellular microenvironment in intact tissues. So far, release of UTP has been reported for several cell types, although not for osteoblast. However, UTP could easily be generated extracellular from other nucleotides through the action of acto-nucleotidase (Lazarowski et al., 2000). UTP can also act through P2Y receptors to upregulate ATP release from human osteoblasts, providing a possible positive feedback mehanism (Bowler et al., 2001). Table 1.3 summarizes evidence and possible functions of P2 receptors in osteoblasts. (A speculative model for the role and interactions of P2 receptors on bone cells is shown in Fig1.5)

Table 1.3 Evidence and possible functions for P2 receptors in osteoblasts (Hoebertz et al., 2003)

Receptor subtype	Species	Evidence	Proposed function
Osteoblasts			
$P2X_2$	Rat	Immunolabeling and insitu hybridization	-
$P2X_5$	Rat Human	Immunolabelling RT-PCR	Proliferation, differentiation
$P2X_6$	Human	RT-PCR	-
$P2X_7$	Human	RT-PCR	Active cell death at high ATP concentrations
	Human	Immunolabelling and RT-PCR	
P2Y	Rat	Ca^{2+} release from stores	-
$P2Y_1$	Rat Human	In situ hybidization RT-PCR	Enhance PTH-induced ca2+ signalling; release of pro-resorptive factors (e.g. prostaglandins and RANKL)
$P2Y_2$	Rat Human	In situ hybridization RT-PCR	Inhibition of bone formation; intercellular communication between osteoblasts
$P2Y_4$	Human	RT-PCR	-
$P2Y_6$	Human	RT-PCR	-

Table 1.4 Evidence and possible functions for P2 receptors in osteoclasts (Hoebertz et al., 2003)

Receptor subtype	Species	Evidence	Proposed function
Osteclasts			
P2X	Rat	Ca^{2+} influx	-
$P2X_2$	Rat	Immunolabelling and in situ hybridization	Increase osteoclast activity
$P2X_4$	Rat	Immunolabelling and in situ hybridization	-
	Rat	Nonselective cation current	
	Rabbit	RT-PCR and cation current	
$P2X_7$	Rat	Immunolabelling	Intercellular communication between osteoblasts and osteoclasts; fusion of osteoclast progenitors; active cell death (at high concentrations)
	Mouse	Permeabilization	
	Rabbit	Nonselective cation current	
	Human	RT-PCR	
P2Y	Rabbit	Ca^{2+} release from stores	-
	Rat	Ca^{2+} release from stores	
$P2Y_1$	Rat	In situ hybridization	Increased osteoclast formation; increased resorptive activity
$P2Y_2$	Rat	In situ hybridization	-
	Human	RT-PCR	
	Human	In situ hybridization	

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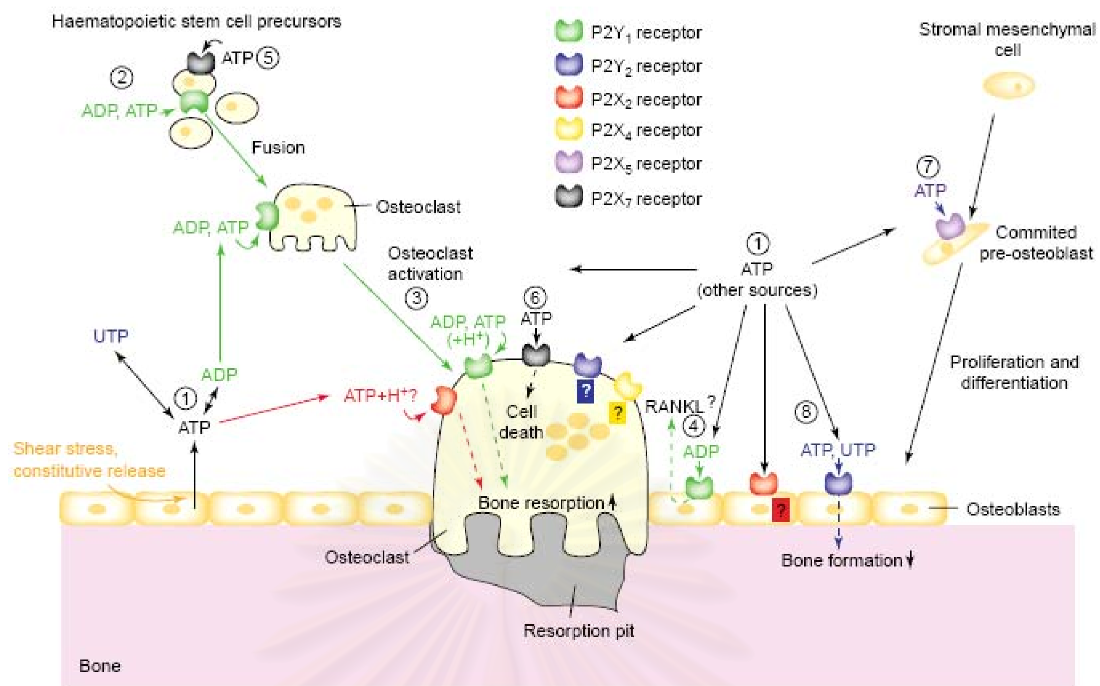

Figure 1.5 Schematic diagram illustrating the potential roles played by extracellular nucleotides and P2 receptors in modulating bone cell function. (Hoebertz et al., 2003) Adenosine 5'-triphosphate (ATP), released from osteoblasts (e.g. though shear stress or constitutively) or from other sources, can be degraded to adenosine 5'-diphosphate (ADP) or converted into uridine 5'-triphosphate (UTP) via ecto-nucleotidase (1). All three nucleotides can act separately on specific P2 receptor subtypes, as indicated by the color coding. ATP is a universal agonist, whereas UTP is only active at the $P2Y_2$ receptor and ADP is only active at the $P2Y_1$ receptor. ADP, via $P2Y_1$ receptor appears to stimulate both the formation (i.e. fusion) of osteoclasts from haematopoietic precursors (2) and the resorptive activity of mature osteoclast (3). For the latter, a synergistic action of ATP and protons has also been proposed via the $P2X_2$receptor. ADP could also stimulate resorption indirectly through actions of osteoblasts, which in turn release pro-resorptive factors (e.g. RANKL)(4). ATP at high concentration might facilitate fusion of osteoclast

progenitors through $P2X_7$ receptor pore formation (5) or induce cell death of mature osteoclasts via $P2X_7$ receptors (6). In osteoblasts, ATP, via $P2X_5$ receptors, might enhance proliferation and/or differentiation (7). In contrast, UTP, via $P2Y_2$ receptors, is a strong inhibitor of bone formation by osteoblasts (8). For some receptors (e.g. $P2X_4$ and $P2Y_2$ receptors on osteoclasts or $P2X_2$ receptors on osteoblasts) evidence for expression has been found but their role is still unclear (question marks). Dash lines indicate signalling events in the cell (Hoebertz et al., 2003).

Adenosine 5'-triphosphate (ATP)

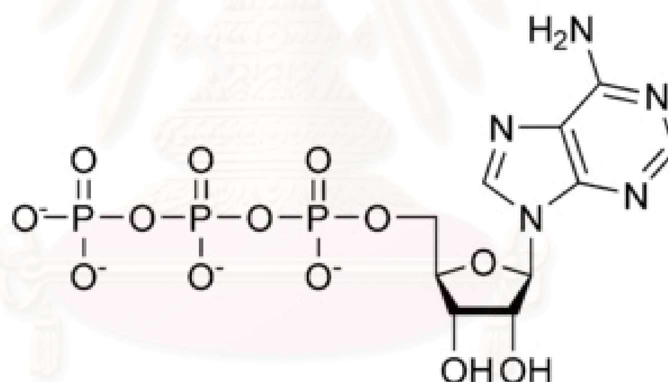

Figure 1.6 Molecular structure of ATP (Wikipedia free encyclopedia, 2007)

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Adenosine 5'-triphosphate (ATP) is a multifunctional nucleotide that is most important as a "molecular currency" of intracellular energy transfer. In this role ATP transports chemical energy within cells for metabolism. It is produced as an energy source during the processes of photosynthesis and cellular respiration and consumed by many enzymes and a multitude of cellular processes including biosynthetic

reactions, motility and cell division. ATP is also incorporated into nucleic acids by polymerases in the processes of DNA replication and transcription. In signal transduction pathway, ATP is used as a substrate by kinases that phosphorylate proteins and lipids, as well as by adenylate cyclase, which uses ATP to produce the second messenger molecular cyclic AMP.

The structure of this molecule consists of a purine base (adenine) attached to the 1' carbon atom of a pentose (ribose). Three phosphate groups are attached at the 5' carbon atom of pentose sugar (Fig. 1.6). When ATP is used in DNA synthesis, the ribose sugar is first converted to deoxyribose by ribonucleotide reductase. ATP was discovered in 1929 by Karl Lohmann and was proposed to be the main energy-transfer molecule in cell by Fritz Albert Lipman in 1941 (Hoebertz, 2003).

Physical and chemical properties

ATP consist of adenosine – itself composed of an adenine ring and ribose sugar – and three phosphate groups (triphosphate). The phosphoryl groups, starting with the group closest to the ribose, are refered to as the alpha (α), beta (β), and gamma (γ) phosphates. ATP is highly soluble in water and is quite stable in solutions between pH 6.8-7.4, but is rapidly hydrolysed at extreme pH, consequently ATP is best stored as an anhydrous salt (Stecher, 1968).

The system of ATP and water under standard conditions and concentrations is extremely rich in chemical energy; the bond between the second and third phosphate

groups is loosely said to be particularly high in energy. Strictly speaking, the bond itself is not high in energy (like all chemical bonds its required energy to break), but energy is produced when the bond is broken and water is allowed to react with the two products. Thus, energy is produced from the new bond formed between ADP and water, and between phosphate and water (Romero and de Meis, 1989). The net change in enthalpy at standard temperature and pressure of the decomposition of ATP into hydrated ADP and hydrated inorganic phosphate is -20.5 kJ/mole (Gajewski et al., 1986). This large release in energy makes the decomposition of ATP in water extremely exergonic, and hence useful as a means for chemically storing energy.

Biosynthesis

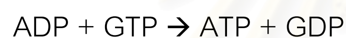
ATP can be produced by redox reactions using simple and complex sugars (carbohydrates) or lipids as an energy source. For ATP to be synthesized from complex fuels, they first need to be broken down into their basic components. Carbohydrates are hydrolysed into simple sugars, such as glucose and fructose. Fats (triglycerides) are metabolized to give fatty acids and glycerol.

The overall process of oxidizing glucose to carbon dioxide is known as cellular respiration and can produce up to 30 molecules of ATP from a single molecule of glucose. ATP can be produced by a number of distinct cellular processes; the three main pathways used to generate energy in eukaryotic organisms are glycolysis, the citric acid cycle/oxidative phosphorylation, and beta-oxidation. The majority of this ATP

production by a non-photosynthetic aerobic eukaryote takes place in the mitochondria, which can make up nearly 25% of the total volume of typicall cell (Wong and Lodish, 2006).

ATP replenishment by nucleoside diphosphate kinases

ATP can also be synthesized through several so-called “replenishment” reactions catalyzed by the enzyme families of nucleoside diphosphate kinases (NDKs), which use other nucleoside triphosphates as a high-energy phosphate donor, and the ATP:guanido-phosphotransferase family, which uses creatine.



$$ADP + GTP \rightarrow ATP + GDP$$

ATP recycling

The total quantity of ATP in the human body is about 0.1 mole. The majority of ATP is not usually synthesised de novo, but is generated from ADP by the aforementioned processes. Thus, at any given time, the total amount of ATP + ADP remains fairly constant.

The energy used by human cells requires the hydrolysis of 100 to 150 moles of ATP daily which is around 50 – 75 kg. Typically, a human will use up their body weight of ATP over the course of the day (carlo et al., 2001). This means that each ATP molecule is recycled 1000 to 1500 times during a single day (100/0.1=1000). ATP cannot be stored, hence its comsumption being followed closely by its synthesis.

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Functions in cells

ATP is generated in the cell by energy-releasing processes and is broken down by energy-consuming processes, in this way ATP transfer energy between spatially-separate metabolic reaction. ATP is the main energy source for the majority of cellular functions. This includes the synthesis of macromolecules, including DNA and RNA, and proteins. ATP also plays a critical role in the transport of macromolecules across cell membranes, e.g. exocytosis and endocytosis.

ATP is critically involved in maintaining cell structure by facilitating assembly and disassembly of elements of the cytoskeleton. In a related process, ATP is required for the shortening of actin and myosin filament crossbridges required for muscle contraction. This latter process is one of the main energy requirments of animals and is essentail for locomotion and respiration.

Cell signaling

1. Extracellular signaling

ATP is also a signaling molecule. ATP, ADP, or adenosine are recognized by purinergic receptors. In humans, this signaling role is important in both central and peripheral nervous system. Activity-dependent release of ATP from synapses, axons and glia activates purinergic membrane receptors known as P2 (Fields and Brurnstock, 2006).

2. Intracellular signaling

ATP is critical in signal transduction processes. It is used by kinases as the source of phosphate groups in their phosphate transfer reactions. Kinase activity on substrates such as proteins and membrane lipids are a common form of signal transduction. Phosphorylation of protein by a kinase can activate or inhibit the target's activity, these proteins may themselves be kinases, and form part of a signal transduction cascade such as the mitogen-activated protein kinase cascade (Mishra et al., 2006).

ATP is also used by the adynylate cyclase and is transformed to the second messenger molecule cyclic AMP, which is involved in triggering calcium signals by the release of calcium from intracellular stores (kamenetsky et al., 2006). This form of signal tranduction is particularly important in brain function, although it is involved in the regulation of multitude of other cellular processes.

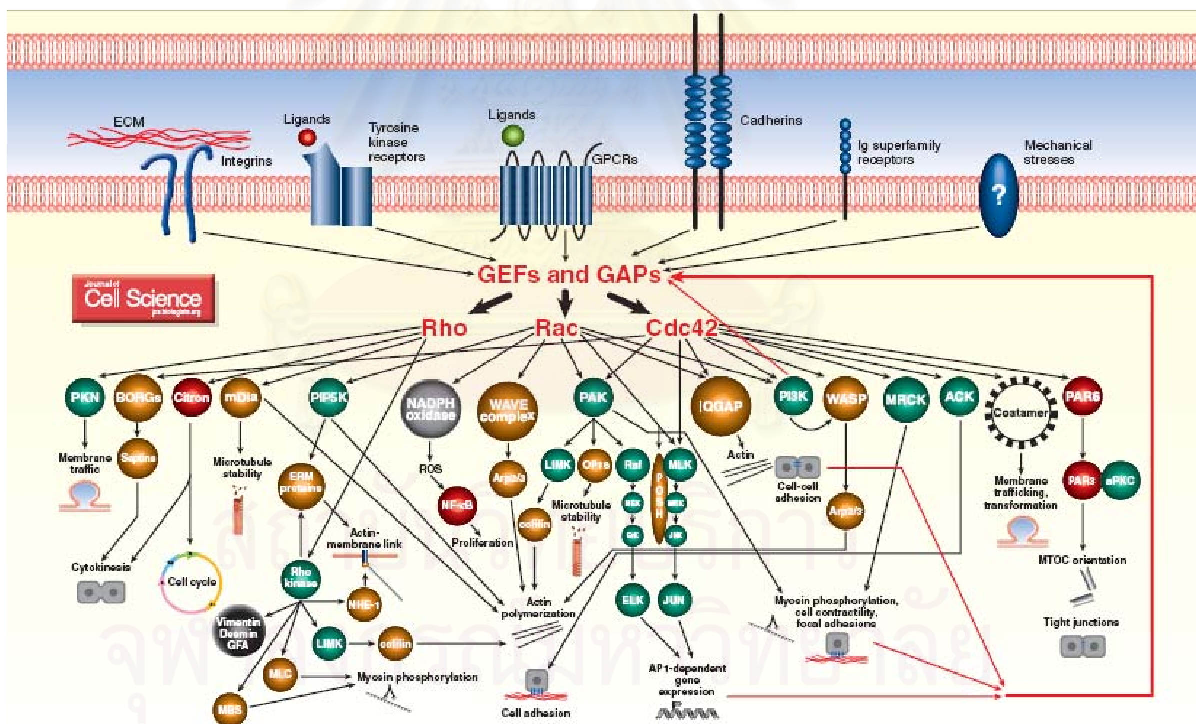
Rho GTPase signaling

Rho GTPase belong to the Ras superfamily of small GTPases and control a wide variety of cellular processes such as actin cytoskeleton rearrangement, microtubule dynamics, cell adhesion and polarity. Like all member of the Ras superfamily, Rho GTPases function as conformational switches by cycling active GTP- and inactive GDP bound forms. The cycle is regulated by two classes of protein, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs catalyze

nucleotide exchange. This results in the activation of Rho GTPases due to the concentration of GTP than GDP in cells. GAPs stimulate GTP hydrolysis, which results in the inactivation of Rho GTPases. Furthermore, Rho GTPases are regulated by guanine nucleotide dissociation inhibitors (GDIs). These extract GDP-bound forms from the membrane to the cytosol and inhibit the release of GDP from the GTPases. Rho GTPases in the GTP-bound form are able to bind a variety of downstream target proteins, called effectors, which initiate a variety of cellular responses (Hakoshima et al., 2003).

In mammalian cells, all members of the Rho family divided into 3 subgroups; Rho regulates a signal transduction pathway linking growth factor receptors to the assembly of focal adhesions and actin stress fibers, Rac regulates the formation of lamellipodia and membrane ruffles and Cdc42 controls the formation of filopodia (Kozma et al., 1995). Rho, Rac, and Cdc42 have also been shown to regulate changes in gene transcription and in particular there have been numerous reports that Rac and Cdc42 activate the JNK and p38 MAP kinase pathways (Coso et al., 1995). It is likely, therefore that these three GTPases play a key regulatory role in cell movement and in morphogenetic processes where changes in the actin cytoskeleton are coordinated with changes in gene transcription (Hall, 1998).

Figure 1.7 Rho signalling pathway. (Schwartz, 2004) Cells receive extracellular stimuli in the form of soluble molecules (growth factors, cytokines and hormones) that bind to cell surface receptors, adhesive interactions (extracelluar matrix and cell-cell adhesion) or mechanical stresses (tension, compression and fluid shear stress). These stimuli act upon guanine-nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs) to control the activation stae of the small GTPases Rho, Rac and Cdc42. Once activated, the GTPases bind to a sprectrum of effectors to stimulate downstream signaling pathway



In fig 1.6, Potein kinase N1 (PKN1, also known as PRK) and PKA2 are Rho effectors involved in endosomal trafficking. Citron is ROCK-related kinase thatis critical for citokinesis and is also implicated in other aspects of cell cycle progression.

Mammalian diaphanous protein 1 (mDia1, alsoknown as dia-relatedformin or DRF), mDia2 and mDia mediated both actin polymerization through a profiling-dependent mechanism and stabilization of microtubule plus end in cell migration.

Rho kinase 1 (also known as ROCK1) and ROCK2 are key Rho effectors that have multiple substrates. A partial list includes the myosin-binding subunit of the myosin phosphatase (MBS), which leads to inhibition of phosphatase activity, increased myosin light chain phosphorylation and hence increased tension generation; LIM kinase (LIMK), which phosphorylates cofilin to release actin monomers and promote actin polymerization; and myosin regulatory light chain itself, which again promotes contractility. Rho kinase phosphorylates ezrin-radixin-moesin (ERM) family proteins to activate their function as linkers between actin and the plasma membrane. Rho kinases also phosphorylate the Na/H+ antiporter NHE-1 to promote actin-membrane interactions and several intermediate filament proteins (desmin, vimentin and glial fibrillary acidic protein) to regulate intermediate filament structure.

Both Rac and Rho bind to phosphatidylinositol-4-phosphate 5-kinase (PIP5K); activation of PIP5K by Rho also requires Rho kinases. Production of phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)*P*2] contributes to the activation of ERM proteins and to actin polymerization through WASP, profilin and multiple actin-capping proteins. Other reported substrates for Rho kinases that are not pictured include MARCKS, EF-1α, calponin, CPI-17 and collapsin-response mediator protein 2.

Rac has numerous effectors that mediate effects on the cytoskeleton and gene expression. Rac binds p67 PHOX to increase activation of the NADPH oxidase system and production of reactive oxygen species (ROS), which mediate activation of NF-kB-dependent gene expression, effects of Rac on cell cycle progression and inhibition of Rho activity. Rac binds the WAVE complex (also containing Abi and IRSp53/58), to release active WAVE, which promotes actin polymerization in lamellipodia through activation of the Arp2/3 complex. Both Rac and Cdc42 bind and activate the kinases PAK1, PAK2 and PAK3. PAKs have multiple substrates, including LIM kinase, which leads to actin polymerization; OP18/stathmin, which stabilizes microtubule plus ends;

and Raf-1 and MEK1, whose phosphorylation by PAK enhances transmission of the signal to ERK. PAK activity also regulates myosin phosphorylation and cell contractility through several pathways, including myosin light chain kinase, myosin regulatory light chain, myosin heavy chain and caldesmon. Other pathways not listed on the diagram include filamin A, which cooperates with PAK to promote membrane ruffling; components of the paxillin-GIT/PKL-P1X complex, which regulates cell adhesion and motility; and the apoptotic regulator BAD, which promotes cell survival.

Rac and Cdc42 are important activators of Jun N-terminal kinase (JNK) and p38, which stimulate AP-1-dependent gene expression. Mixed lineage kinases (MLKs) appear to be the major Rac/Cdc42 effectors leading to JNK and p38 activity. Rac and Cdc42 also bind to the actin-binding protein IQGAP, which is implicated in regulation of cell-cell adhesion. Both Rac and Cdc42 also bind and stimulate PI 3-kinase. The

resultant 3'-phosphorylated lipids bind to and stimulate Rac GEFs, creating a positive feedback loop that maintains cell migration.

WASP (and the more widely expressed N-WASP) are critical downstream effectors of Cdc42 that mediate formation of filopodia. These effectors also require PtdIns(4,5)*P*2 and interact directly with Arp2/3 complex to promote actin polymerization. Cdc42 activates the serine/threonine kinase MRCK, which is related to Rho kinases and can promote myosin phosphorylation. Cdc42 also activates the tyrosine kinases ACK1 and ACK2; the latter regulates focal adhesion formation and organization of the actin cytoskeleton. Cdc42 promotes oncogenic transformation in part by binding to the α and γ coatamer proteins and regulating membrane trafficking. Finally, Cdc42 is the critical determinant of polarity in a wide variety of developmental systems; this involves binding of Cdc42 to PAR6. PAR6 is a component of a complex containing PAR3 and atypical protein kinase C. This complex regulates positioning of the microtubule microtubule organizing center (MTOC) in migrating cells and early embryos, and tight junction formation in epithelia. Borg proteins are Cdc42 effectors that connect to septins, structural proteins that can polymerize to form filaments involved in cytokinesis in yeast and mammalian cells, and that probably carry out additional structural roles in mammalian cells as well (Joberty et al., 2001).

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Research questions and objectives

Several studies demonstrated the expression of osteopontin in both mRNA level and protein level in PDL tissue (Nohutcu, 1997, Li et al. 2001, Lee et al. 2004). Lee et al. using immunohistochemical analysis found that OPN was primarily localized in the PDL cells adjacent to deciduous tooth roots, with the greatest prevalence in association with odontoclasts in resorption lacunae (Lee at al. 2004). Furthermore, the osteocytes, osteoblasts, and bone lining cells in the pressure side of the experimental tooth movement in rats expressed OPN (Terai et al. 1999). Additionally, unloading did not reduce bone in the OPN-deficient mice (Ishijima et al. 2001).

Although earlier studies have demonstrated the presence of OPN in PDL tissue, no study have shown the expression pattern of OPN in the PDL cells after induction with mechanical stress. The purposes of this study are;

1. To assess the relationship between the expression of OPN in the PDL cells and mechanical stress.
2. To compare the change of OPN and RANKL/OPG expression
3. To examine the pathway involved in the expression of OPN in the PDL cells after stimulation with mechanical stress.

CHAPTER II

MECHANICAL STRESS INDUCES OSTEOPONTIN EXPRESSION IN HUMAN PERIODONTAL LIGAMENT CELLS THROUGH Rho KINASE.

Summary

Background: Mechanical stress such as orthodontic forces can produce mechanical damage and inflammatory reaction in the periodontium. Osteopontin (OPN) is a multifunctional cytokine that has been correlated with periodontal disease progression. Because the periodontal ligament (PDL) can be affected by stress and PDL cells are involved in periodontal destruction and remodeling, we aimed to study 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 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-independent

mechanism for stress-induced OPN expression. In addition, the upregulation of OPN was diminished by Rho kinase inhibitor but not by cytochalasin B.

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

Introduction

Periodontal ligament (PDL) functions to support and form a shock absorbing system in order to mitigate mechanical forces such as masticatory or orthodontic forces. HPDL cells are responsible to the mechanical stress transduced from the tooth, which is significant for the process of periodontal tissue remodeling and repair (Lekic and McCulloch,1996).

Mechanical stress can induce secretion of several inflammatory cytokines that are detectable in gingival crevicular fluid (GCF) (Ren et al., 2002; Yamaghuchi and Kansai, 2005). The elevation of the cytokines caused by 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 (Verna et al., 1999; Kaku et al., 2005).

Mechanical stress can also induce bone loss through the imbalance of receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG). Previous studies (Kanzaki et al., 2002; Yamaghuchi et al., 2006) have shown the relationship of RANKL and OPG expression stimulated by mechanical stress in human PDL cells. The expression of the membrane-bound and soluble forms of RANKL increased in a force-dependent manner, whereas OPG expression decreased or was unaltered (Kanzaki et al., 2002; Yamaghuchi et al., 2006). Given that RANKL and OPG are the key proteins of proliferation, differentiation, and activation of osteoclasts (Khosla, 2001), increase of RANKL expression in PDL cells caused by mechanical stress supports that PDL plays a role in the regulation of alveolar bone resorption and remodeling.

Osteopontin (OPN) has also been associated with bone remodeling. The expression of OPN was increased in response to the mechanical stress in bone cells (Terai and Takano-Yamamoto, 1999; Morinobu et al., 2003). OPN is one of the major non-collageneous bone matrix proteins. It is a highly phosphorylated protein containing an arginine-glycine-aspartate (RGD) motif, which binds to integrin and enables bone cells to adhere to the mineralized matrix (Denhardt and Guo, 1993). It is known to be produced by osteoblasts (Weinreb et al., 1990; Merry et al., 1993) and osteoclasts (Merry et al., 1993; Tezuka et al., 1992) and participates in the functions of both cell types, such as bone formation, resorption, and remodeling (Denhardt and Guo, 1993).

OPN is thought to regulate the adhesion, attachment, and spreading of osteoclasts to the bone surface (Reinholt et al., 1990). It has been proposed that OPN is a mechano-responsive gene in bone cell (Toma et al., 1997). Up-regulation of OPN was detected in osteocytes, osteoblasts, and bone lining cells in the pressure side of the experimental tooth movement in rats (Terai et al., 1999; Takano-Yamamoto et al., 1994). Impairment of bone resorption in unloading-induced bone loss in OPN-deficient mice (Ishijima et al., 2002) indicates that OPN plays a role in response of bone to mechanical loading.

Moreover, OPN plays a role in tissue inflammation (Singh et al., 2007). It can act as a regulator of macrophage infiltration and facilitates the adhesion and migration of macrophages and leukocytes (Giachelli et al., 1998; Opascharoensak et al., 1999). 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 has been reported (Nohutcu et al., 1997; Li et al., 2001; Lee et al., 2004; Chutivongse et al., 2005). 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 (Lee et al., 2004). The expression of OPN was regulated by several growth factors and extracellular matrix protein (Chutivongse et al., 2005; Saygin et al., 2000). Recent studies by Kido et al (2001). and Sharma and Pradeep (2006) showed that the increasing level of OPN in the GCF corresponded with

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the severity of periodontitis and suggested that the level of OPN may be a marker of periodontal disease severity. However, the source of OPN in periodontal disease is still unclear.

In view of the fact that 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.

Experimental Procedure

HPDL cell culture

Human periodontal ligament (PDL) cells were obtained from healthy third molars extracted for orthodontic reasons and prepared as previously described. The protocol is approved from The Ethics Committee, Faculty of Dentistry, Chulalongkorn University. The study was conducted from April 2006 to March 2007. Informed consent was obtained from each patient (two females and one male; 18-22 year of age). Briefly, teeth were rinsed with sterile phosphate buffer saline, and the PDL was removed 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)(Hyclone, Logan, UT.) supplemented with 10% fetal calf serum (Hyclone), 2mM L-glutamine (Gibco BRL,

Carlsbad, CA.), 100 units/ml penicillin, 100 µg/ml streptomycin (Gibco BRL), and 5 µg/ml amphotericin B (Gibco BRL) at 37 ^{o}C in humidified atmosphere of 95% air, 5% CO2. 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.(2002). Briefly, PDL cells were seeded in six-well plates at a density of 200,000 cells/well for 16 hours. A plastic cylinder containing metal coins was placed over the culture to generate compressive forces ranging from 0 to 2.5 g/cm^2.

To study the involvement of cytoskeleton and cyclo-oxygenase in stress-induced OPN expression in HPDL cells, 1.27 nM of Rho-kinase inhibitor (Calbiochem, EMD Biosciences, San Diego, CA.), 40 mM of cytochalasin-B (Calbiochem, EMD Biosciences) and 10 mM of indomethacin (Sigma-Aldrich Chemical, St. Loiuis, MO.) were used in the inhibitory experiments. Each inhibitor was added into the medium 30 minutes before the experiment.

RNA Extraction and Semiquantitative Reverse Transcription-Polymerase Chain Reaction Assay (RT-PCR)

Total cellular RNA was extracted with reagent (Tri-reagent, Molecular Research center, Cincinnati, OH.) according to manufacturer's instructions. One microgram of

each RNA sample was converted to cDNA by RT using avian myeloblastosis virus (AMV) reverse transcriptase for 1.5 hours at 42°C. Subsequent to RT, PCR was performed. The primers were prepared following the reported sequences from GenBank. The oligonucleotide sequences of the primers are as follows: 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'; and cyclooxygenase (COX)-2, forward, 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3', and reverse, 5'- AGATCATCTCTGCCTGAGTATCTT-3'.

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

Protein Extraction and Western Blot Analysis

Protein was extracted with radioimmunoprecipitation assay (RIPA) buffer. Protein concentrations were measured using a protein assay kit (BCA protein assay kit, Pierce Biotechnology, Rockford, IL.). 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 OPN (Chemicon International, Temacula, CA.; dilution 1:1,000), RANKL (R&D System, Minneapolis, MN.; dilution 1:200), or actin (Chemicon International; dilution 1:1,000). The membranes were incubated with biotinylated secondary antibody, followed by peroxidase-labeled streptavidin. The signal was captured by chemiluminescence (Pierce Biotechnology). The relative intensities of bands were measured by imaging software analysis (Scion Image, Scion).

Statistical Analysis

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

Results

The results in Figure 2.1 show the response of HPDL cells after they were 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.

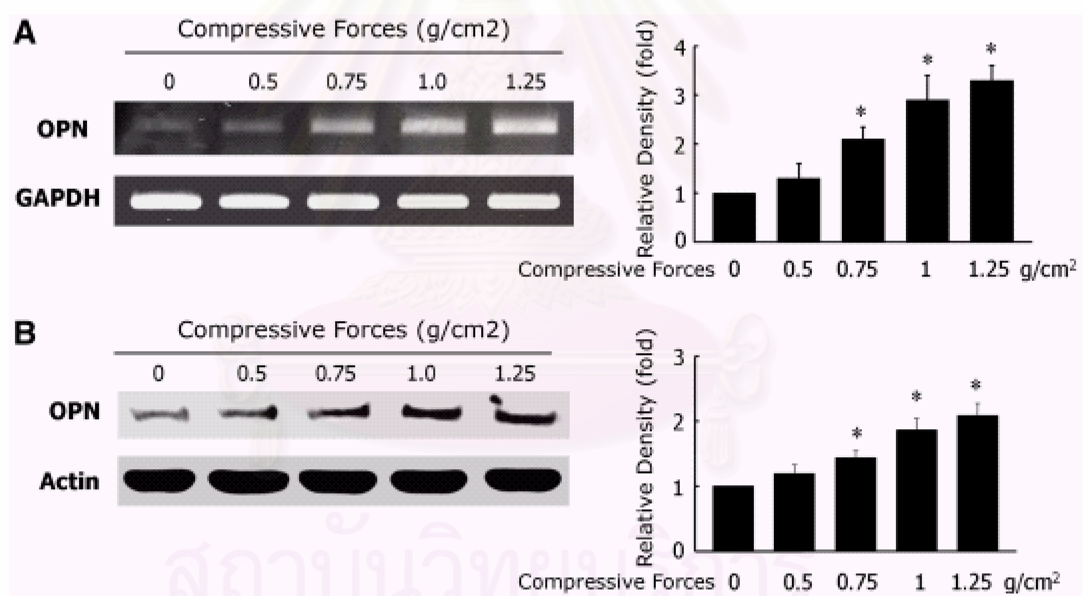

Figure 2.1 Compressive forces induced OPN expression. Human PDL cells were stimulated with 0 to 1.25 g/cm^2 of force as described in Materials and Methods. A) The change in OPN mRNA expression after 24-hour stimulation. An increase in OPN expression was significantly increased at 0.75 g/cm^2 of force and above. A similar

profile of protein level was observed (B). The graph on the right shows the average ± SD of band density from three separate experiments. *Significant difference, P <0.05.

The results in Figure 2.2 show that mechanical stress generated from 1.25 g/cm^2 of force could increase OPN mRNA expression within 1 hour after stimulation and reach maximum levels at ~24 hours after treatment.

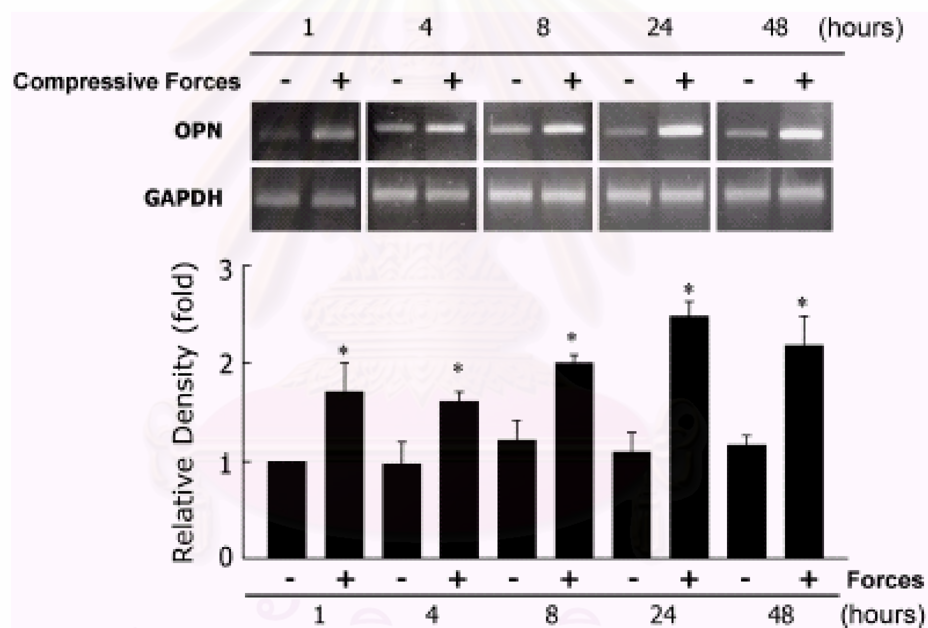

Figure 2.2 Time-course experiment of stress-induced OPN expression. HPDL cells were stimulated with 1.25 g/cm^2 of force, and the expression of OPN mRNA was determined at 1, 4, 8, 24, and 48 hours. Graphs show the average ±SD of band density from three separate experiments. *Significant difference, P <0.05.

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 through a COX-2 dependent pathway (Kanzaki et al., 2002). 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 being activated with 1.25 and 2.5 g/cm^2 of force. The results indicated that both levels of force significantly increased RANKL and OPN mRNA and protein expressions. Application of indomethacin inhibited the upregulation of RANKL expression at 2.5 g/cm^2 but not at 1 g/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 upregulated at 2.5 g/cm^2 but not at 1.25 g/cm^2 (Fig. 2.3).

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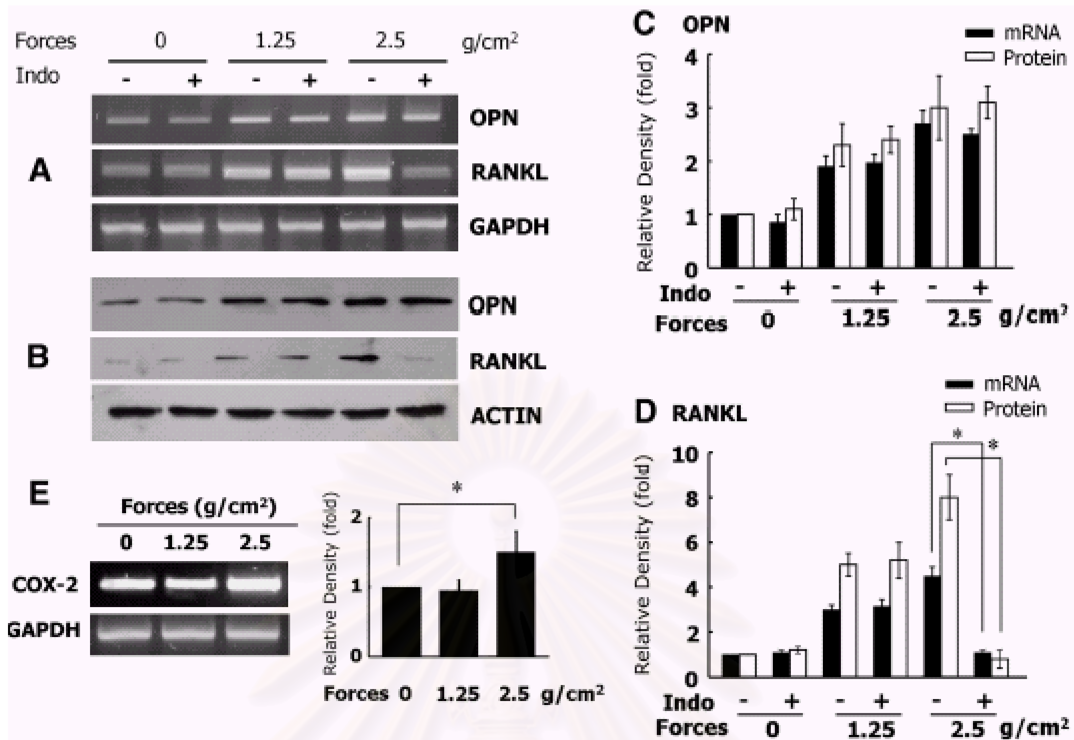

Figure 2.3 The effect of indomethacin on stress-induced OPN and RANKL expression. HPDL cells were preincubated with indomethacin (Indo) for 30 minutes before stimulation with either 1.25 or 2.5 g/cm^2 of force (A and B). Changes in mRNA and protein level of OPN and RANKL after 24-hour stimulation, respectively. Graphs in C and D show the average ± SD of band density of OPN and RANKL from A and B. The expression of COX-2 mRNA is shown in E. *Significant difference, $P < 0.05$.

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Induction of OPN expression was inhibited by the Rho kinase inhibitor as shown in Figure 2.4. The 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.

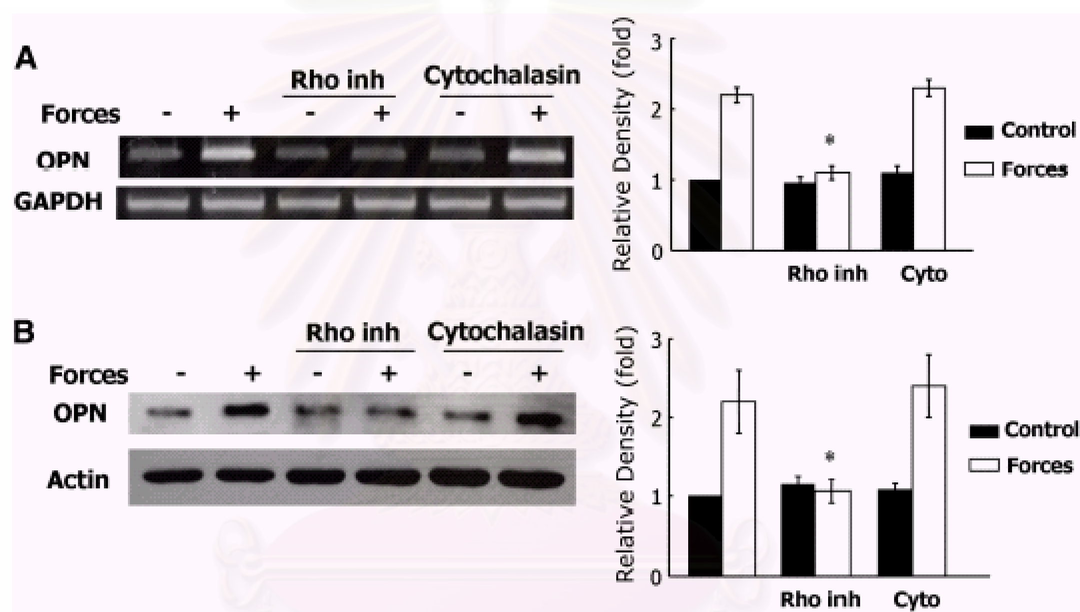

Figure 2.4 Rho kinase inhibitor inhibited the upregulation of OPN. Cells were preincubated with Rho kinase inhibitor (Rho inh) or cytochalasin B (Cyto) for 30 minutes before the application of force at 1.25 g/cm^2. (A and B) Results from RT-PCR and Western analysis, respectively. Graphs on the right show the average ± SD of band density from three separate experiments. *Significant difference, $P < 0.05$.

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 (1997), who proposed that OPN might be a mechanoresponsive gene. Fujihara et al.(2006) also showed a shear stress responsive element (SSRE; GAGACC) in the 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 (TRAP+) cells and OPN expression increased predominantly at the pressure side of bone and teeth (Kaku et al., 2005; Terai et al., 1999; Morinobu et al., 2003). *In vivo* and *in vitro* studies (Terai et al., 1999; Morinobu et al., 2003; Toma et al., 1997) indicated that bone cells synthesized OPN in response to mechanical stimulation. The results in this study show that PDL cells can act in a 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 biologic signals. Unloading induced bone loss and bone loss caused by ovariectomy were impaired in the absence of OPN (Ishijima et

al., 2002; Ishijima et al., 2001; Yoshitake et al., 1999). In addition, OPN-deficient mice could not respond to parathyroid-induced bone resorption (Ihara et al., 2001).

OPN also plays a role in migration and attachment of osteoclasts. OPN stimulates osteoclast migration through $\alpha v \beta 3$ integrin and CD44 (Chellaiah et al., 2002; Chellaiah et al., 2002). Furthermore, OPN acts as a chemotactic factor for osteoclasts in the process of bone resorption (Terai et al., 1999). Therefore, induction of OPN by mechanical stress in PDL cells may enhance the recruitment and migration of osteoclasts, resulting in alveolar bone resorption.

An increase of OPN may also correspond with an increase in osteoclastogenesis. Although OPN does not seem to be an essential factor in the development of osteoclasts during normal development (Rittling et al., 1998), it plays an important role in osteoclastogenesis in pathologic conditions (Ihara et al., 2001). It has been shown that neutralizing antibody to OPN suppressed osteoclastogenesis, whereas addition of OPN enhanced osteoclastogenesis in marrow stromal cells (Yamate et al., 1997; Ishii et al., 2004). Ishii et al.(2004) showed that OPN could influence osteoclastogenesis by enhancing RANKL and decreasing OPG expression in stromal cells. Whether OPN influences the expression of RANKL and OPG in human PDL cells needs further study.

The suggested function of OPN in bone resorption corresponded with reports showing that OPN was increased in GCF of patients with periodontitis (Kido et al., 2001; Sharma et al., 2006). 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 (2006) that periodontal treatment could reduce OPN in GCF supports the importance of the increased OPN in periodontal tissue on the progression of periodontal disease.

Our results also show the upregulation of RANKL in stress-induced PDL cells. RANKL is one of the key proteins 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 (Kanzaki et al., 2002). 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 g/cm2 but not at 1.25 g/cm2. The inhibitory effect of indomethacin corresponds to the level of COX-2 induced by mechanical stress (Fig. 2.3). These findings indicate that stress-induced RANKL expression in PDL cells involves more than one signaling pathway, depending on the level of compressive force. In terms of OPN expression, indomethacin cannot inhibit the upregulation of OPN in PDL cells at both levels of pressure, suggesting a COX-independent pathway in OPN induction.

Stress-induced OPN expression is inhibited by a Rho kinase inhibitor. The function of Rho kinase is involved in the cytoskeleton dynamics, such as formation of focal adhesion, actin stress fiber, and redistribution of cytoskeletal components (Nobes et al., 1995). Cytoskeleton dynamics have been reported to play a role in mechanical-induced OPN expression in chick osteoblasts (Toma et al., 1997). However, cytoskeletal

components may not be involved in the upregulation of OPN induced by mechanical stimulation in human PDL cells because cytochalasin-B cannot inhibit stress-induced OPN expression.

The involvement of Rho kinase in the regulation of OPN expression has been reported in smooth muscle cells (Chaulet et al., 2001; Kawamuraet al., 2004). Induction of OPN was observed when smooth muscle cells were activated with uridine triphosphate (UTP) or high glucose occurred through Rho kinase, suggesting the association of Rho kinase in OPN expression. However, the exact mechanism is unclear.

Conclusion

The present study showed that mechanical stress induces OPN expression through the Rho kinase pathway in human PDL cells. We propose that increased OPN plays an important role in the mechanism of pressure-induced alveolar bone resorption.

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CHAPTER III

STRESS-INDUCED OSTEOPONTIN VIA $P2Y_1$ RECEPTOR IN PDL CELLS

Introduction

Human periodontal ligament (HPDL) cells are responsible for the mechanical stress transduced from the tooth, which is significant for the process of periodontal tissue remodeling and repair (Lekic and McCulloch,1996). Improper mechanical stress can induce bone loss through the elevation of inflammatory cytokines (Ren et al., 2002; Yamaguchi and Kasai, 2005) and imbalance of receptor activator of nuclear kappa B ligand (RANKL) and osteoprotegerin (OPG) (Kanzaki et al., 2002; Yamaguchi et al., 2006). Our previous study demonstrated that mechanical stress also induced the expression of OPN through Rho kinase pathway. However, the exact mechanism is unclear.

In the present study, the molecules involved in the stimulation of OPN expression are investigated. The involvement of Rho kinase in the regulation of OPN expression has been reported in smooth muscle cells (Chaulet et al., 2001; Kawamura et al., 2004). Induction of OPN was observed when smooth muscle cells were activated with nucleotides or high glucose occurred via Rho kinase, suggesting the role of nucleotides in OPN expression. Among the nucleotides, ATP has been recognized as an important and ubiquitous extracellular messenger in various kinds of tissues.

(Brambilla and Abbracchio, 2001; Burnstock and Knight, 2004). ATP is released by mechanical stimulations and activates surrounding cells via P2Y (G-protein coupled) and P2X (ion channel) ATP receptors (Schwiebert, 2000). The released ATP works as autocrine and paracrine mediators, and play roles in the mechano-transduction in several cell types. (Nakano et al., 1997; Yamamoto et al., 2000; Furuya et al., 2005). In bone cells, ATP has a potent stimulatory action on IL-6 secretion but an inhibitory action on OPG expression. ATP also stimulates human osteoclast activity via up-regulation of RANKL. [Buckley et al., 2002]. These evidences emphasize the important role of ATP as one of the regulators of bone homeostasis.

Recent study reported that ATP caused growth arrest in HPDL cells, suggesting the influence of ATP in periodontal tissue regeneration (Kawase et al., 2007). In the present study, we found that HPDL cells expressed P2Y receptors, leading to the hypothesis that these receptors may be significant for the response of cells to mechanical stress. We thus hypothesized that the up-regulation of OPN expression induced by mechanical stress was caused by the release of ATP, which acted through P2 receptors in HPDL cells.

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Materials and Methods

Cell culture

HPDL cells were obtained from healthy third molars extracted for orthodontic reasons and prepared as previously described (Pattamapun et al., 2003). The protocol was approved by The Ethics Committee, Faculty of Dentistry, Chulalongkorn University and the informed consent was obtained from each patient. Briefly, teeth were rinsed with sterile phosphate buffer saline and the PDL was removed 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) (Hyclone, Logan, UT) supplemented with 10% fetal calf serum (Hyclone), 2mM L-glutamine (Gibco BRL, Carlsbad, CA), 100 units/ml penicillin, 100 µg/ml streptomycin (Gibco BRL), and 5 µg/ml amphotericin B (Gibco BRL) in humidified atmosphere of 95% air, 5% CO2 at 37 ^{o}C. 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

The method for mechanical stress application was modified from Kanzaki et al.(2002). Briefly, cells were seeded in 6-well-plates at a density of 200,000 cells/well for 16 h. A plastic cylinder containing metal coins was placed over the culture in order to generate compressive forces ranging from 0 to 2 g/cm^2.

For inhibitory experiments, each inhibitor was added into the medium 30 min prior to the experiment. The inhibitors used included 15 μM suramin, 0.18 μM NF449, 1 unit/ml apyrase, 5 μM MRS2179 (all are from Sigma-Aldrich Chemical, St. Loiuis, MO), and 1.25 nM Rho-kinase inhibitor (Calbiochem, EMD Biosciences, San Diego, CA).

Application of ATP

Exogenous ATP (0.1, 1, 10 μM) (Sigma-Aldrich) was applied to HPDL cell culture for 2 h before total RNA extraction and 24 h for protein extraction. RNA extraction and semi-quantitative transcription polymerase chain-reaction assay (RT-PCR).

Total cellular RNA was extracted (Tri-reagent, Molecular Research center, Cincinnati, OH) according to manufacturer's instructions. One microgram of each RNA sample was converted to cDNA by using avian myeloblastosis virus (AMV) for 1.5 hours at 42 C. Subsequently to RT, PCR was performed. The primers were prepared following the reported sequences from GenBank. The oligonucleotide sequences of the primers were shown in table 3.1.

The PCR product was performed using Taq polymerase (Qiagen, Hilden, Germany) with a PCR volume of 25 μl. The amplification profile for OPN was one cycle at 94°C for 1 minute, 30 cycles at 94°C for 1 minute, hybridization at 60°C for 1 minute and extension at 72°C for 2 minutes, followed by one extension cycle at 72°C for 10 minutes. The same profile was also used for $P2X_1$, $P2X_3$, $P2Y_1$, $P2Y_2$ (35 cycles) and GAPDH (22 cycles) ,the oligonucleotide sequences of the primers were shown in table

3.1). The PCR was performed in the DNA thermal cycler (Biometra, GÖttingen, Germany). The amplified DNA was electrophoresesed on a 2% agarose gel and visualized by ethidium bromide fluorostaining and the relative intensities of the bands were measured by imaging software analysis.

Protein extraction and Western blot analysis

Protein was extracted with radioimmunoprecipitation assay (RIPA). Protein concentrations were measured using BCA protein assay kit (Pierce, Rockford, IL). 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 (Chemicon International, Temacula, CA; dilution 1:1,000), P2Y1 (Abcam; dilution 1:300), P2Y2 (Abcam; dilution 1:300), or actin (Chemicon International; dilution 1:1,000). The membranes were then incubated with biotinylated secondary antibody, followed by peroxidase-labeled streptavidin. The signal was captured by chemiluminescence (Pierce Biotechnology). The relative intensities of bands were measured by imaging software analysis (Scion Image, Scion)

Luciferin-Luciferase bioluminescence assay

The extracellular ATP concentration was determined using a kit from ENLITEN® ATP Assay System Bioluminescence Detection kit for ATP measurement (Promega).

During analysis 100 μl of Enliten Luciferase/Luciferin (L/L) medium (rL/L reagent, reconstitution buffer) were added to 100 μl of sample into the microplate (PACKARD). The resulting light signal was immediately measured by a luminometer (Victor Light Luminesence Counter, PerkinElmer Ltd., MA). A calibrating curve was generated for each luciferase assay using serial dilution of an ATP standard.

Statistical Analysis

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

Table 3.1 the oligonucleotide sequences of the primers

Primers	Sources	Sequences	Predicted length (bp)
GAPDH	NM002046.3	forward, 5'-TGAAGGTCGGAGTCAACGGAT-3' reverse,5'-TCACACCCATGACGAACATGG-3'	396
OPN	NM000582.2	forward,5'-AGTACCCTGATGCTACAGACG-3' reverse,5'-CAACCAGCATATCTTCATGGC-3'	321
$P2X_1$	NM002558.2	forward,5'-CTGTGAAGACGTGTGAGATCTTTGG-3' reverse,5'-TTGAAGTGACGGTAGTTGGTCC-3'	456
$P2X_3$	NM002559.2	forward,5'-ATCAACCGAGTAGTTCAGC-3' reverse,5'-GATGCACTGGTCCCAGG-3'	696
$P2Y_1$	NM002563.2	forward,5'-CGGTCCGGGTTCGTCC-3' reverse,5'-CGGACCCCGGTACCT-3'	528
$P2Y_2$	NM002564.2	forward,5'-CTAAAGCCAGCCTACGGGAC-3' reverse,5'-TCCTATCCTCTGCATGTC-3'	373

Results

HPDL cells were activated with mechanical stress (1.5 g/cm^2) for 24 h. The results showed that stress induced the up-regulation of OPN at both mRNA (Fig. 3.1A, upper panel) and protein level (Fig. 3.1A, lower panel). The induction effect was inhibited by suramin, an antagonist for P2 receptor family (Fig. 3.1A), and NF449, an antagonist for $P2X_1$, $P2X_3$, $P2Y_1$, and $P2Y_2$ (Fig. 3.1B). These results lead to the hypothesis that nucleotide receptors, especially $P2X_1$, $P2X_3$, $P2Y_1$, and $P2Y_2$ were involved in the induction of OPN. The band density measured from Western blot analysis was represented by graph.

To further analyze the involvement of nucleotide, the conditioned medium (CM-S) was collected from the culture after activation with stress for 2 hours and transferred to another set of cultures for 24 hours. The results in Fig. 3.2A revealed that application of CM-S, but not the conditioned medium from control (CM-C), induced the OPN expression. The results suggested the effect of the secreting molecule from stress-treated cultures in the OPN induction.

We further examined the amount of ATP in the culture medium and found that stress increased ATP secretion into the medium as shown in Fig. 3.2B. Application of apyrase, ecto-ATPase, could partially inhibit the inductive effect of CM-S. The evidence supported that ATP in the CM-S was significant in OPN induction (Fig. 3.2C).

Since ATP exerted its effect through P2 receptor, we investigated the expression of P2 receptor in HPDL cells. The results in Fig. 3.3A indicated that both $P2Y_1$ and $P2Y_2$ receptors were expressed in HPDL cells. However, we could not detect the expression of $P2X_1$ and $P2X_3$ receptors (data not shown). To elucidate the kind of P2Y receptors that was involved in stress-induced OPN expression, MRS2179, the specific P2Y1 antagonist, was used. The results from Fig. 3.3B showed that MRS2179 could completely inhibit stress-induced OPN expression, indicating the role of P2Y1 receptor.

To confirm the role of ATP, exogenous ATP (0, 0.1, 1, and 10 μM) was applied to the cultures. The results showed that ATP induced the expression of OPN in a dose dependent manner (Fig. 3.4A). In addition, the inductive effect of ATP was abolished by Rho kinase inhibitor (Fig. 3.4C) similarly to the results obtained from mechanical stress application. However, Rho kinase inhibitor had no effect on the stress-induced release of ATP (Fig. 3.4B). These results suggested that Rho kinase was involved in the stress/ATP-induced OPN expression but not in the stress-induced ATP release.

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Figure 3.1

Stress-induced OPN expression was inhibited by suramin and NF449. HPDL cells were stimulated with 1.5 g/cm^2 of force for 24 hour. Increase of OPN mRNA (upper panels) and protein (lower panels) expressions were observed. The stress-induced OPN expression was significantly abolished by both suramin (A) and NF449 (B). The graph represents the band density from Western blot analysis. The results are expressed as mean ± SD from three different experiments. *p<0.05.

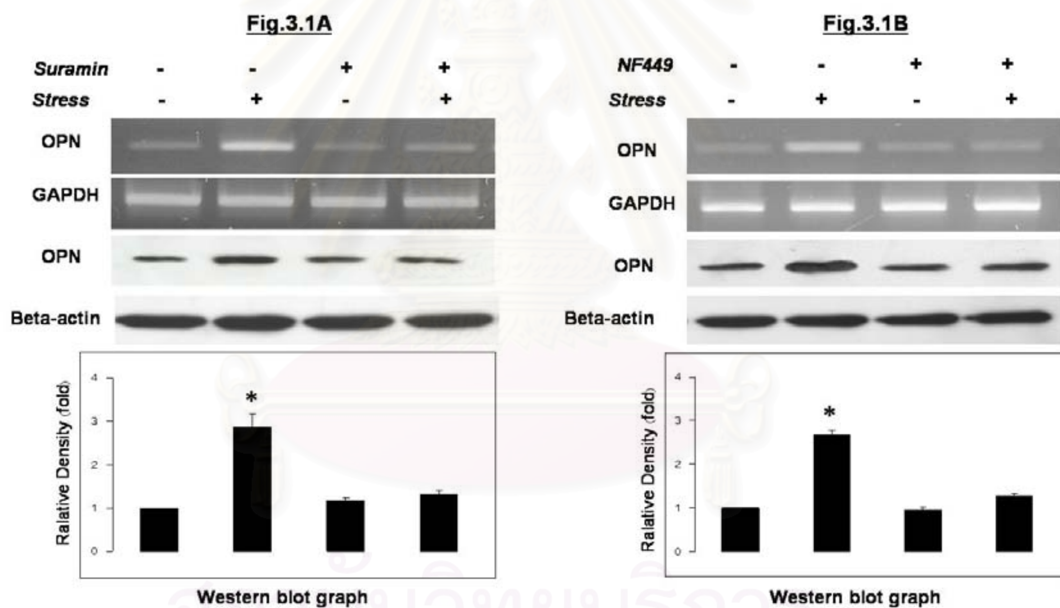

Figure 3.2

The conditioned medium (CM-S) induced the expression of OPN. (A) Cells were incubated for 24 h with the medium transferred from the stress-stimulated (CM-S) and non-stimulated (CM-C) cultures. CM-S induced OPN expression when compared with control (CM-C). The inductive effect exerted by CM-S was similar to that influenced by stress (S) and could be inhibited by NF449. (B) Luciferin-Luciferase bioluminescence assay revealed that the amount of ATP in the medium collected from stress-stimulated cultures (1, 1.5, 2 g/cm^2) was obviously increased. (C) Apyrase partially inhibited the CM-S-induced OPN expression. The graph shows the band density from Western blot analysis. The results are expressed as mean ±SD from three separate experiments. *p<0.05; a, mRNA expression; b, protein expression.

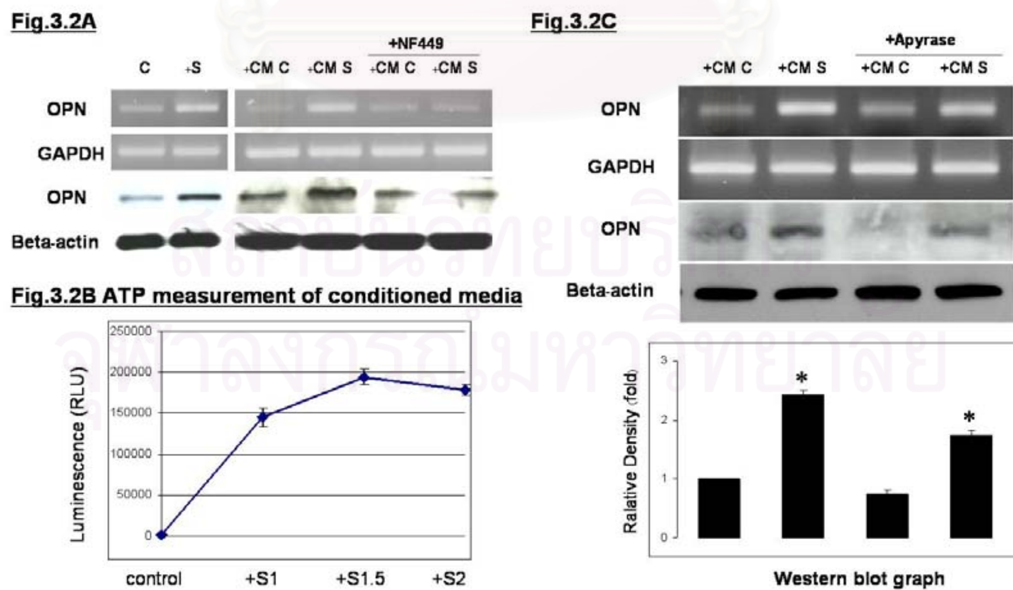

Figure 3.3

HPDL cells expressed P2Y1 and P2Y2 ATP receptors. (A) HPDL cells expressed $P2Y_1$ and $P2Y_2$ receptors detectable at both mRNA and protein levels. (B) MRS2179, a specific $P2Y_1$ antagonist, exerted the inhibitory effect on the stress-induced OPN expression. The graph represents the mean ±SD of the band density from Western blot analysis. The data was from three separate experiments. *$p<0.05$; a, mRNA expression; b, protein expression.

Fig.3.3A

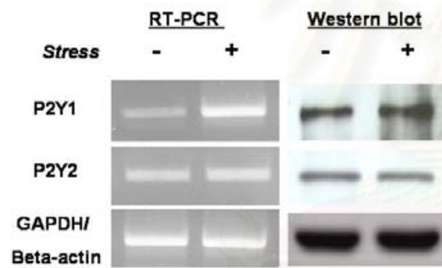

Fig.3.3B

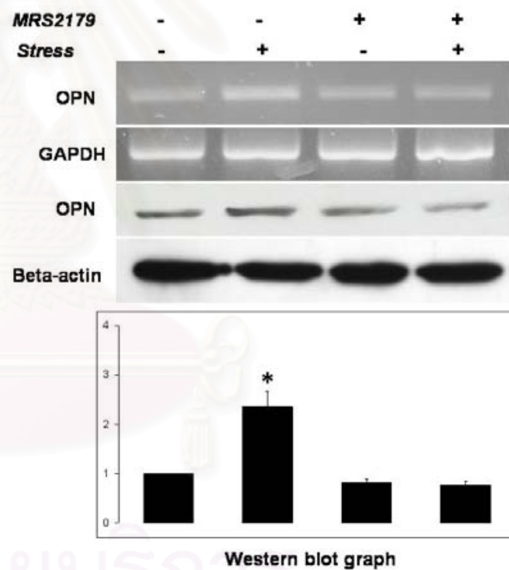

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Figure 3.4

ATP induced the up-regulation of OPN via Rho kinase. (A) HPDL cells were incubated with exogenous ATP (0.1, 1 and 10 µM) for 24 h. The result showed that ATP stimulated the expression of OPN in a dose-dependent manner. Cells were incubated with Rho kinase inhibitor (Rhoi) for 30 min before applying ATP (1 µM) or stress (1.5 g/cm^2) for 24 h. The results demonstrated that the increase of OPN expression (B), but not that of ATP (C), induced by stress was attenuated by Rhoi. The graph in (B) represents the band density from Western blot analysis. The results are expressed as mean ±SD from three separate experiments. *p<0.05; a, mRNA expression; b, protein expression.

Fig.3.4A

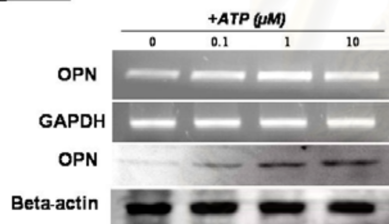


Fig.4C

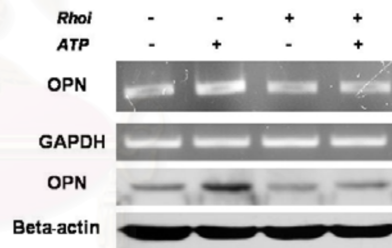
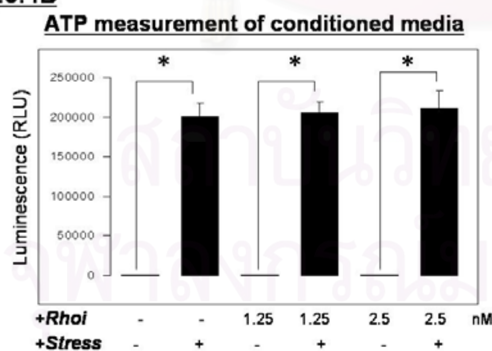
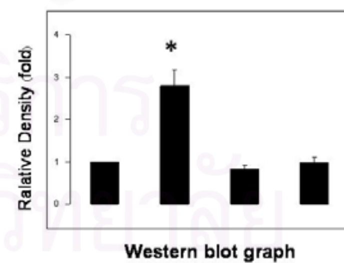


Fig.3.4B





Discussion

We have previously reported that mechanical stress induced OPN expression in HPDL cells (Wongkhantee et al., 2007). In this study, we further demonstrate that the increased expression of OPN is mediated by the release of ATP, which exerts its stimulating effect through $P2Y_1$ receptors.

Increase of the release of ATP in response to mechanical stress has been shown in several cell types, such as subepithelial fibroblasts (Furuya et al., 2005), chondrocytes (Graff et al., 2000) and osteoblasts (Romanello et al., 2001; Homolya et al., 2000). In the present study, we show that HPDL cells are able to release ATP in response to mechanical stress. However, the exact mechanism of stress-induced ATP release required further investigation.

This is the first report showing that ATP induces osteopontin expression. Our previous study showed that mechanical stress induced OPN occurred via Rho kinase pathway. Rho kinase inhibitor could inhibit the action of ATP-induced OPN but not the stress-induced ATP release. These findings suggest that stress induces the release of ATP, which in turn mediates Rho kinase activation and results in the up-regulation of OPN. The function of ATP in the conditioned medium was confirmed by apyrase, a potent ecto-ATPase for ATP degradation. However, the incomplete blockade exerted by apyrase on the conditioned medium-induced OPN expression suggests that there might be other molecule(s) involved in the mechanism.

We found that HPDL cells expressed both $P2Y_1$ and $P2Y_2$ receptors. Although ATP is able to act through almost all subtypes of P2 receptors (Hoebertz et al., 2003), the fact that MRS2179, the specific P2Y1 receptor, completely inhibited the action of stress-induced OPN indicate that P2Y1 is the main receptor involved in the induction of OPN.

The stimulating effect of ATP on the expression of OPN could affect the homeostasis of periodontium. OPN has been shown to facilitate migration and adhesion of osteoclasts (Denhardt and Guo, 1993, Teria et al., 1999). OPN null mice exhibited the lack of bone remodeling (Hoebertz et al., 2000). It is possible that ATP release from HPDL cells influence the behavior of both osteoblasts and osteoclasts, which express P2 receptors, since ATP can potently enhance the activation and formation of osteoclasts (Morrison et al., 1998) and stimulates cell proliferation in osteoblasts (Orriss et al., 2006). Therefore, the released ATP by HPDL cells may influence the behavior of both PDL and bone cells and subsequently affects the homeostasis of periodontium.

In conclusion, the present study shows that mechanical stress induces OPN expression via ATP, which mediates the signal through $P2Y_1$ receptor in HPDL cells. We propose that the increase of ATP plays an important role in the mechanism of pressure-induced alveolar bone resorption.

CHAPTER IV

GENERAL DISCUSSIONS AND FUTURE STUDIES

The results from present study suggest the new model of the upregulation of OPN in stress-induced HPDL cells as shown in Fig. 4.1.

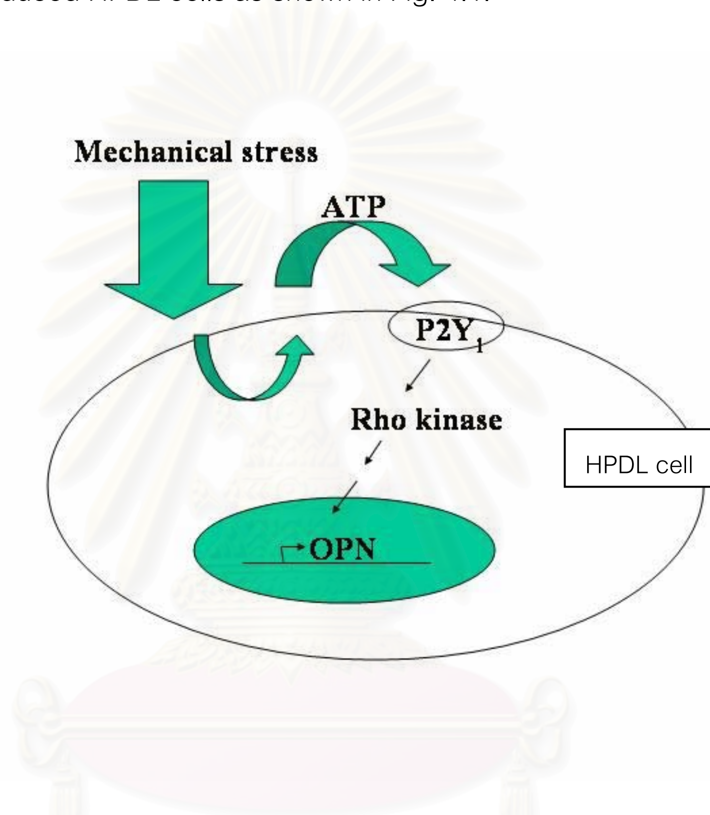


Figure 4.1 The regulation mechanism of OPN expression induced by mechanical stress in HPDL cells.

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As shown in the figure, mechanical stress induces ATP release from HPDL cells. The released ATP acts through $P2Y_1$ receptor at the cell surface and then activats Rho kinase downstream and subsequently induce OPN expression. This was the first report of the relationship between mechanical-induced ATP releasing and the regulation of

OPN expression in HPDL cells. However, the detailed molecular mechanisms of ATP-released mechanism as well as the target molecules of Rho kinase are still unclear. However, there were studies showing that NF-kB and AP1 were involved in UTP-induced OPN expression in arterial smooth muscle cells (Renault et al., 2003, Renault et al., 2005). It is interesting to investigate the involvement of NF-kB and AP1 in ATP-induced OPN expression in HPDL cells in our future study.

The released of ATP and other nucleotides by mechanical stimulation have been reported in many cell types such as human osteoblasts (Romanello et al., 2001) and porcine chondrocytes (Graff., 2000). Furuya et al. (2005) demonstrated that mechanical stimulation evoked Ca^{2+} increased in the rat duodenal villi subepithelial fibroblastss and ATP released from the cells. The released ATP activated P2Y receptors particularly $P2Y_1$ receptors and they suggested that subepithelial fibroblasts functioned as the mechano-sensor in intestine. In this study, the fact that PDL cells can respond to stress suggested that PDL cells acted as the mechano-sensor in periodontal tissues.

The stress application model in this study was applied from Kanzaki et al.(2002). The stress was generated adding the metal coins in the plastic lid of 50 ml tube instead of using moveable granule lead in loading process. It is an effective apparatus because of the perfect fitting in both diameter and height to the 6-well plates but do not contact the floor and surrounding wall of the wells. The limitation of this stress application model is the weight limit that should not over 4 g/cm^2 since it will cause the efflux of medium from the wells.

In previous studies, many types of mechanical stimulation models were proposed in PDL cells cultures. These models include 'the special hydrostatic pressure apparatus' that used water as the pressure medium and pressure transmitted to the cells through the packed Teflon pouch (Yamamoto et al., 2006), 'the flexercell strain unit' that simulated the tension side of root in the orthodontic tooth movement (Kanzaki et al., 2006). The animal models were also applied in the studies such as the experimental tooth movement in rat using the orthodontic closed coil springs (Fujihara et al., 2006), or using the piece of elastic bands inserted interproximally between rat teeth (Teria et al., 1999). The sample of a very simply technique to apply mechanical stress was just 'touching' cells with a fine glass rod (Furaya et al., 2005). Each model had the different advantages and disadvantages, and were selected according to the objectives and the applications of the experiments.

In this present study, the static stress was applied model in order to mimic the continuously loading of forces in orthodontic treatment. Because of the limitation of the weight limit in our model as described above, and the study of Kanzaki et al., (2002) showed the partially damage of HPDL cells when applied weight more than 4 g/cm^2. For these reasons, the weight applied could not reach to the normal orthodontic forces (around 50 -100 g) in cell culture system. In the more complicated orthodontic pateints, there are factors that affected the force applied such as the frictional resistant between the orthodontic wire and bracket or the elastic band and bracket, which caused the reduction of orthodontic force at the interface of tooth surface and periodontal ligament.

Otherwise, there was no report of the force level occurred in root surface but the similarity of clinical situation and the laboratory cell culture is cell damage when expose to the unadaptive high forces. The three dimensional collagen cell cultured model was set up by de Araujo et al., (2006), and they could loaded up to 9.5 g/cm^2. This model may suggested the more simulation of cell culture system and should be used in the future.

Mechanical stress plays an important role in many parts of our body in order to adapt to the environment. In the skeletal tissues, the movement of body caused stress to in bone and cartilage as demonstrated in previous studies (Romanello et al., 2001, Graff et al., 2000). In respiratory system, the moving air caused stress to the epithelium lining in respiratory tracts as shown in the study of mice nasal epithelium cell line (Homolya et al., 2000). In vasculation system, the blood flow caused mechanical stress to endothelium and smooth muscle cells of blood vessels, demonstrated with the experiments by Renault et al. (2005) and Liang et al. (2002). Mechannical stress also play roles in periodontal tissue, it is directly absorb the forces from the tooth root before transduced to the alveolar bone. Many researchers tried to investigate mechanism of PDL cell response to stress (Kanzaki et al., 2002, 2006, Yamamoto et al., 2006, Yamaguchi et al., 2006) and focus in RANK-RANKL-OPG system. In our experiments, we also tried to elucidate this mechanism focusing on OPN, the chemotactic factor for osteoclasts that promotes the attachment of osteoclasts to bone surface (Terai et al., 1999, Denhardt and Guo, 1993).

Mechanical stress had the pros and cons. In our pilot study, we found that low level of stress induced HPDL cell proliferation (data not shown), suggesting the roles of stress in PDL tissue regeneration. The releasing of ATP, the extracellular signaling molecules induced by mechanical stress, in this study suggested that mechanical stress was the important factor in cell communication. But the high level of stress application caused HPDL cell death (Kanzaki et al., 2002). The study of the mechanism of differential mechanical stress in HPDL cells is needed in future study.

The interesting topics for future investigation include:

- To find the relationship between p2 receptor subtypes expression and PDL cell differentiation.
- To investigate the influence of mechanical stress on other nucleotides releasing such as adenosine, AMP, ADP or UTP.
- To investigate the influence of these nucleotides in the regulation of OPN, RANKL, and OPG.
- To elucidate the mechanism of ATP releasing by mechanical stress in HPDL cells.
- To find the down stream signaling pathway to Rho kinase in the regulation of OPN expression induced by mechanical stress.
- To examine the effect of strain in the regulation of OPN or other bone marker proteins including the signaling molecules involved.

The clinical significance and the benefits of this study are to provide the understanding of PDL cell behaviors in response to mechanical stress and to provide the information of the relationship between mechanical stimulation and the orthodontic treatment. In addition, the more understanding of this pathway may useful in the development of the inhibitor for reduce the severity of periodontal disease.

In conclusion, this study demonstrated that the regulation of OPN expression induced by mechanical stress caused by the released of ATP into the medium and acted through $P2Y_1$ receptor and then activated Rho kinase. We propose that the released ATP and the increased OPN are the important factors in the processes of periodontal tissue remodeling and repair.

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