ผลของอินเตอร์ลิวคิน-6 ต่อการแสดงออกของรีเซ็ปเตอร์แอคติเวเตอร์ออฟเอ็นเอฟ แคบปา-บีไลแกน ออสทีโอโปรทีเจริน และ มาโครฟาจ-โคโลนีสติมูเลติงแฟคเตอร์ ในเซลล์โพรงประสาทฟันของมนุษย์

นางสาวทราย พินิจไพฑูรย์

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THE EFFECT OF INTERLEUKIN-6 ON THE EXPRESSION OF RECEPTOR ACTIVATOR OF NF-κB LIGAND, OSTEOPROTEGERIN AND MACROPHAGE COLONY STIMULATING FACTOR IN HUMAN DENTAL PULP FIBROBLAST

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ทราย พินิจไพฑูรย์ : ผลของอินเตอร์ลิวคิน-6 ต่อการแสดงออกของรีเซ็ปเตอร์แอคติเวเตอร์ออฟเอ็นเอฟแคปปา-บีไลแกน ออสทีโอโปรทีเจริน และ มาโครฟาจ-โคโลนีสติมูเลติงแฟคเตอร์ในเซลล์โพรงประสาทฟันของมนุษย์ (THE EFFECT OF INTERLEUKIN-6 ON THE EXPRESSION OF RECEPTOR ACTIVATOR OF NF-KB LIGAND, OSTEOPROTEGERIN AND MACROPHAGE COLONY STIMULATING FACTOR IN HUMAN DENTAL PULP FIBROBLAST) อ. ที่ปรึกษา : ผศ.ทพ.ดร.ทิพวรรณ ธราภิวัฒนานนท์, อ.ที่ปรึกษาร่วม รศ.ทพ.ดร. ประสิทธิ์ ภวสันต์, 51 หน้า. ISBN 974-14-1874-4.

อินเตอร์ลิวคิน-6 เป็นไซโตไคน์ที่มีหน้าที่เกี่ยวข้องกับการอักเสบ และการตอบสนองทางภูมิคุ้มกัน นอกจากนั้นยังเกี่ยวข้องกับการสร้างเซลล์สลายกระดูก และการละลายของกระดูกด้วย วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาผลของอินเตอร์ลิวคิน-6 และอินเตอร์ลิวคิน-6กับรีเซปเตอร์ชนิดหลั่งออกจากเซลล์ที่จำเพาะต่ออินเตอร์ลิวคิน-6 ต่อการแสดงออกของโมเลกุลที่เกี่ยวข้องกับการเกิดเซลล์สลายกระดูก 3 ชนิด รีเซ็ปเตอร์แอคติเวเตอร์ออฟเอ็นเอฟแคปปา-บีไลแกน ออสทีโอโปรทีเจริน และมาโครฟาจ-โคโลนีสติมูเลติงแฟคเตอร์ในเซลล์โพรงประสาทฟันของมนุษย์ เซลล์โพรงประสาทฟันจะถูกเลี้ยงในอาหารเลี้ยงเชื้อที่มีอินเตอร์ลิวคิน-6 ที่ความเข้มข้นตั้งแต่ 0-10 นาโนกรัมต่อมิลลิลิตร และอินเตอร์ลิวคิน-6 ร่วมกับรีเซปเตอร์ชนิดหลั่งจากเซลล์ที่มีความเข้มข้นตั้งแต่ 0-10 นาโนกรัมต่อมิลลิลิตร ทำการศึกษาการแสดงออกของโมลกุลทั้งสามในระดับเอ็มอาร์เอ็นเอ และโปรตีน โดยวิธีพีซีอาร์ เวสเทอร์นบลอท และอีไลซา ผลการทดลองพบว่าอินเตอร์ลิวคิน-6 มีผลเพิ่มการแสดงออกของรีเซปเตอร์แอคติเวเตอร์ออฟเอ็นเอฟแคปปา-บีไบแกนทั้งในระดับเอ็มอาร์เอ็นเอ และโปรตีน แต่ไม่มีผลต่อออสทีโอโปรทีเจริน และมาโครฟาจ-โคโลนีสติมูเลติงแฟคเตอร์ และเมื่อเติมรีเซปเตอร์ชนิดหลั่งออกจากเซลล์ที่จำเพาะต่ออินเตอร์ลิวคิน-6 พบว่ามีผลเพิ่มการแสดงออกของรีเซปเตอร์แอคติเวเตอร์ออฟเอ็นเอฟแคปปา-บีไลแกนในเฉพาะระดับเอ็มอาร์เอ็นเอ แต่เมื่อศึกษาในระดับโปรตีนพบว่ามีผลลดการแสดงออกของทั้งรีเซปเตอร์แอคติเวเตอร์ออฟเอ็นเอฟแคปปา-บีไลแกนและออสทีโอโปรทีเจริน แต่ไม่มีผลต่อระดับของมาโครฟาจ-โคโลนีสติมูเลติงแฟคเตอร์ ผลการศึกษานี้ชี้ให้เห็นว่าการเพิ่มของอินเตอร์ลิวคิน-6 ในขณะที่มีการอักเสบของโพรงประสาทฟันอาจนำไปสู่การละลายของเนื้อเยื่อแข็งในโพรงประสาทฟันได้โดยผ่านกลไกความสมดุลย์ของรีเซปเตอร์แอคติเวเตอร์ออฟเอ็นเอฟแคปปา-บีไลแกนและออสทีโอโปรทีเจริน และรีเซปเตอร์ชนิดหลั่งออกจากเซลล์มีผลทำให้การสร้างโปรตีนของรีเซปเตอร์แอคติเวเตอร์ออฟเอ็นเอฟแคปปา-บีไลแกนและออสทีโอโปรทีเจรินลดลง

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ลายมือชื่อนิสิต.....ทราย พินิจไพฑูรย์.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
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KEY WORD : HUMAN DENTAL PULP (HDP) CELLS, INTERLEUKIN-6 (IL-6), INTERLEUKIN-6 RECEPTOR (IL-6R) AND SOLUBLE IL-6R (sIL-6R), RECEPTOR ACTIVATOR OF NF-ΚB (RANK) AND LIGAND (RANKL), MACROPHAGE COLONY-STIMULATING FACTOR (M-CSF), OSTEOPROTEGERIN (OPG). SAI PINIJPAITOON: THE EFFECT OF IL-6 ON THE EXPRESSION OF RANKL, OPG AND M-CSF ON HUMAN DENTAL PULP FIBROBLAST. THESIS ADVISOR: ASST. PROF. THIPAWAN THARAPIWATTANANON, THESIS COADVISOR: ASSOC. PROF. PRASIT PAVASANT. 51 pp. ISBN 974-14-1874-4.

Interleukin-6 (IL-6) is a highly pleiotropic cytokine that mediates the host inflammation and known to influence osteoclast formation and bone resorption. The aim of this study was to investigate the effects of IL-6 and combination of IL-6 and soluble IL-6 receptor (sIL-6R) on the expression of Receptor activator of NF kappa B ligand (RANKL), Osteoprotegerin (OPG) and Macrophage colony stimulating factor (M-CSF) in human dental pulp (HDP) cells cultures. Culture of HDP cells was established from the explant of pulp tissue. HDP cells were cultured in the presence of 0-10 ng/ml of IL-6. In another experiment sIL-6R ranging from 1-10 ng/ml was added in combination with IL-6. The expression of RANKL, M-CSF and OPG were determined using RT-PCR, western blot analyses and ELISA.

Results indicated that IL-6 significantly increased RANKL expression at both mRNA and protein levels in dose-dependent manner but had no effect on the expression of M-CSF and OPG. IL-6+sIL-6 increased RANKL in mRNA level but had not affect on OPG and M-CSF expression. In contrast, RANKL and OPG protein were decreased following increased of sIL-6R. This study may lead to the implication that increasing IL-6 level in inflamed dental pulp may affect on mineralized tissue resorption due to the imbalance of RANKL/OPG system. Moreover, the combination of IL-6 and sIL-6R had decreased RANKL and OPG expression in protein level.

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CHAPTER I

INTRODUCTION

Background and rationale

Interleukin-6 (IL-6) is a highly pleiotropic cytokine that mediates the host response to injury and infection. It regulates various aspects of the immune response, acute phase reaction, hematopoiesis, and has effects on B- and T-cell growth and differentiation. IL-6 is produced locally in bone following stimulation by IL-1 and tumor necrosis factor (TNF) (Feyen et al, 1989; Kurihara et al, 1990). IL-6 and IL-6 mRNA are produced by numerous cells types, including macrophages, monocytes, endothelial cells, and smooth muscle cells (Kishimoto et al, 1995 for review). Furthermore, IL-6 can be secreted by dental cell such as HDP, gingival fibroblast, periodontal ligament cells (Hosoya et al, 1996; Belibasakis et al, 2005; Ogura et al, 1994). IL-6 is involved in the pathogenesis of various inflammatory diseases such as Rheumatoid arthritis, multiple myeloma and osteoporosis (Hirano et al., 1988, Kawano et al, 1988, Jilka et al, 1992).

IL-6 is a proinflammatory cytokine that possesses activities that involve the process of osteoclastogenesis. IL-6 may enhance or suppress inflammatory bone destruction ((Jilka et al, 1992, Balto et al, 2001). IL-6 stimulates the formation of osteoclast precursors from colony-forming unit–granulocyte-macrophage and increases osteoclast numbers in vivo leading to systemic increases in bone resorption (Jilka et al, 1992). IL-6 can suppress bone resorption, Balto (Balto et al, 2001) demonstrate that mice lacked IL-6 have significantly increased bone resorption following pulpal infection, compared to wild-type controls. They concluded that IL-6 suppress bone resorption due to the anti-inflammation effect. Gorny (Gorny et al, 2004) also suggest that IL-6 in particular in murine cells may have a direct inhibitory action on osteoclast forming hematopoietic cells in vitro.

Osteoclast is an important cell responsible for bone resorption. The cytokines proximally responsible for the formation and activation of osteoclasts are RANKL

(receptor activator of nuclear factor kappa B ligand) and OPG (osteoprotegerin) (Suda et al, 1997). RANKL has been shown to both activate mature osteoclasts and mediate osteoclastogenesis in the presence of M-CSF (macrophage colony stimulating factor) (Tanaka et al, 1993). OPG acts as a decoy receptor for RANKL, preventing it from binding to and activating RANK. It also inhibits the development of osteoclasts and down regulates the RANKL signaling through RANK.

IL-6 exerts its activity via a cell surface receptor which consists of two components, a ligand-binding 80-kDa glycoprotein chain (IL-6R, gp80) and a non-ligand-binding but signal transducing 130-kDa glycoprotein chain (gp130) (Taga et al, 1989). It was reported that the genetically engineered human and mouse soluble IL-6R (sIL-6R), which lacks transmembrane and cytoplasmic regions, could also mediate the IL-6 signal through gp130 (Taga et al, 1989). IL-6 can stimulate osteoclast-like cell formation in human marrow cultures in both absence and presence of sIL-6R (Lowik et al, 1989, Moonga et al, 2002). From the study of Tamura et al (1993) suggests that increased circulating or locally produced sIL-6R induces osteoclast formation in the presence of IL-6. This is in agreement with investigations of Palmqvist (Palmqvist et al, 2002), where it has been shown that IL-6 does not stimulate releasing of mineral and bone matrix of bone explant unless sIL-6R is present. Furthermore, Irwin also suggest that the combination of IL-6 and sIL-6R can activated expression of MMP-1, the molecule responsible for degrade extracellular matrix in bone resorption process (Irwin et al, 2002). There are inconsistent with the study of Lowik (Lowik et al, 1989) and Ishimi (Ishimi et al, 1990) that IL-6 can be stimulated in vitro bone resorption in fetal mouse metacarpals and calvaria without adding exogenous sIL-6R. There are the studies showed that IL-6 can stimulate the expression RANKL in both membrane-bound and soluble (Nakashima et al, 2000). ELISA and RT-PCR results showed that the combination of IL-6 and sIL-6R can stimulate the releasing of both mineral and matrix from bone explants and activate expression of RANKL and OPG in calvarial bones (Palmqvist et al, 2002). Further more, IL-6 also enhances RANKL sensitivity of pagetic osteoclast precursors and so contributes to elevation of osteoclast numbers in Paget's disease (Menaa et al, 2000).

Many studies showed dental cell can express RANKL and OPG that involved in bone resorption (Rani and MacDougall, 2000; Lossdorfer et al, 2002). Moreover, Teng et al (2000) found that Actinobacillus actinomycetem Comitan can upregulate CD4+ T lymphocytes to increase RANKL production and to enhance alveolar osteoclastic bone resorption in periodontitis murine. Kanzaki et al (2002) showed that PDL cells under mechanical stress increase expression of RANKL and osteoclast formation during orthodontic tooth movement.

Dental pulp is a loose mesenchymal tissue enclosed in the mineralized tissue dentin. Dental caries is the most common cause of pulpal inflammation which allows pulp cells to be exposed with oral bacteria or bacterial products and may result in pulpitis. The inflammatory process in pulpitis is basically similar to that in connective tissue elsewhere in the body. When the inflammatory exudates leave the vessel because of an increase in hydrostatic pressure, there is a corresponding rise in the interstitial pressure and releasing many inflammatory cytokines such as prostaglandin, collagenase, Interleukin-1β (IL1β), Interleukin-6 (IL-6) and etc (Hosoya and Matsushima, 1996; Matsushima et al, 1998; O'Boskey and Panagakos, 1998). The sequelae of pulpal inflammation are varying such as pulp necrosis, external root resorption, internal root resorption, bone resorption and etc. IL-6 is not spontaneously produced by intact normal cells; in general, its secretion requires a stimulus, for example, following cellular activation by Peptidoglycan from Lactobacillus casei (Matsushima et al, 1998), Methyl mercaptan (CH3SH) (Coil et al, 2004) and etc. The study of Barkhordar (Barkhordar et al, 1999) also found more intense IL-6 protein in inflamed human pulp tissue than in healthy pulpal tissue. There are showed on the study above that IL-6, RANKL, OPG and M-CSF can present in the dental pulp in many situation. Although, several studies have also shown that IL-6 can activate expression of RANKL and OPG on many cells but on HDP has not been elucidated. Increased IL-6 on HDP cells may play important role in the kinetics of internal root resorption. Objective of this study are to evaluate the effect of IL-6 and combination of IL-6 and IL-6R on the production of RANKL, OPG and M-CSF in HDP cells and also on the osteoclast differentiation.

Research questions

1. Whether IL-6 can affect RANKL, OPG, M-CSF expression in human dental pulp cell (HDP) cells.
2. Whether combination of IL-6 and IL-6R can affect RANKL, OPG, M-CSF expression in HDP cells.

Objective

1. To determine the level of RANKL, OPG and M-CSF expression in HDP cells modulated by IL-6
2. To determine the level of RANKL, OPG and M-CSF expression in HDP cells modulated by the combination of IL-6 and sIL-6R.

Hypothesis

IL-6 and sIL-6R could affect RANKL, OPG and M-CSF expression in HDP cells.

Research design

Laboratory experimental research

- To detect mRNA level use RT-PCR analysis
- To detect protein level use Western blot analysis and ELISA

Keyword

Human dental pulp (HDP) cells

Interleukin-6 (IL-6)

Interleukin-6 receptor (IL-6R) and soluble IL-6R (sIL-6R)

Receptor activator of NF-κB (RANK) and ligand (RANKL)

Macrophage colony-stimulating factor (M-CSF)

Osteoprotegerin (OPG).

Osteoclast

Limitation of research

In vitro study

Benefit

We anticipate that the results from this line of investigation would provide a better understanding of the internal root resorption process. With proper knowledge of the biological processes, manipulation of the response of the cellular network may be possible, thus leading us to new and specific therapies for internal root resorption in the future.

Ethical consideration

None

CHAPTER II

LITURATURE REVIEWS

Bone is a specialized connective tissue formed by a mineralized matrix that confers its elastic and strength properties. Bone remodeling involves synthesis of organic matrix by osteoblasts, and bone resorption by osteoclasts. This equilibrium is tightly regulated by many physical parameters (ie, mechanical stimulations) and numerous polypeptides (hormones, cytokines) (Heymann and Rouesselle, 2000 for review). Receptor activator of NF-κB ligand (RANKL) (Suda et al, 1997) is an important cytokine for regulate osteoclastogenesis that acts through its osteoclastic receptor, receptor activator of NF-κB (RANK) in the present of M-CSF (Tanaka et al, 1993), while osteoprotegerin (OPG) serves as a decoy receptor that binds RANKL and prevents activation of RANK (Suda et al, 1997).

IL-6, one of the important cytokines, also involve in bone resorption process. IL-6 is an instrumental in amplification of the immune response and may directly be involved in tissue destruction in many diseases. IL-6 can induce osteoclast differentiation depends on IL-6 receptors that expressed on osteoblast (Udagawa et al, 1995). The background and interactions between these cytokines that responsible in osteoclastogenesis are presented in this review literature.

Interleukin-6 (IL-6)

IL-6 is a multifunctional cytokine that plays a central role in host defense due to its wide range of immune and hematopoietic activities and its potent ability to induce the acute phase response. IL-6 is a member of the family of cytokines that includes interleukin-11, leukemia inhibitory factor, oncostatin M, cardiotrophin-1, and ciliary neurotrophic factor (Heinrich et al, 1998). They activate target genes involved in differentiation, survival, apoptosis and proliferation. The IL-6 gene is located at chromosome 7p21 (Sehgal et al., 1986) and 5 (Mock et al., 1989) in the human and

mouse genomes, respectively. IL-6 and IL-6 mRNA are produced by numerous cells types, including macrophages, monocytes, fibroblast, endothelial cells, and smooth muscle cells. IL-6 also has a wide range of biological activities on various target cells (figure A). In accordance with its functional pleiotropy, IL-6 has been implicated in the pathology of many diseases including multiple myeloma (Kawano et al, 1988), rheumatoid arthritis (Hirano et al, 1988), Castleman's disease (Yoshizaki et al., 1989), psoriasis (Grossman et al., 1989), and osteoporosis (Jilka et al 1992).

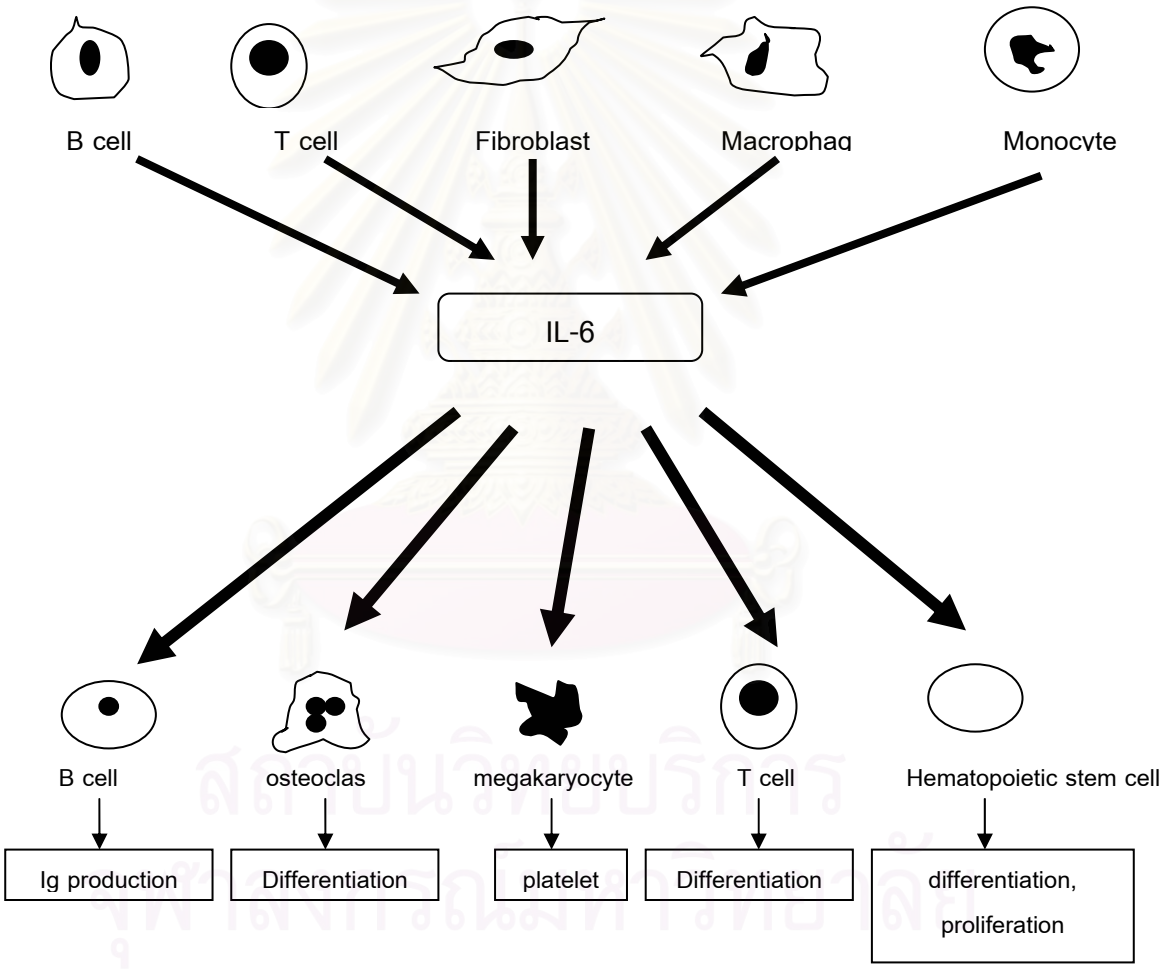


Figure A IL-6-producing cells and biological activities of IL-6. IL-6 is produced by lymphoid and nonlymphoid cells, such as T cells, B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangial cells, and several kinds of tumor cell (top of figure). IL-6 also has a wide range of biological activities on various target cells (bottom of figure). (modify from Naka et al, 2000)

Biological activities of IL-6 are mediated by the IL-6 receptor system, which comprises two membrane proteins, the ligand binding α-subunit receptor (IL-6R or gp80) and the signal transducing β-subunit (gp130) (Taga et al, 1989). Although the human IL-6 receptor (hIL-6R) shows a broad distribution (e.g. activated B cells, resting T cells, B lymphoblastoid cell types, hepatoma lines, myeloma and monocyte cell lines), some cell types lack this receptor. Normal cells usually express between 10^2 and 10^3 receptors.

The gp80, cytoplasmic segment of the IL-6R is relatively short (82 amino acids) and lacks intrinsic catalytic activity (Yamasaki et al, 1988). The cDNA of the human IL-6R (hIL-6R) encodes a protein of 468 amino acids, including a signal peptide of 19 amino acids, an extracellular region of 339 amino acids, a transmembrane domain of 28 amino acids, and a short cytoplasmic domain of 82 amino acids (Yamasaki et al., 1988). There are six potential N-linked glycosylation sequences in the extracellular domain. (Figure B)

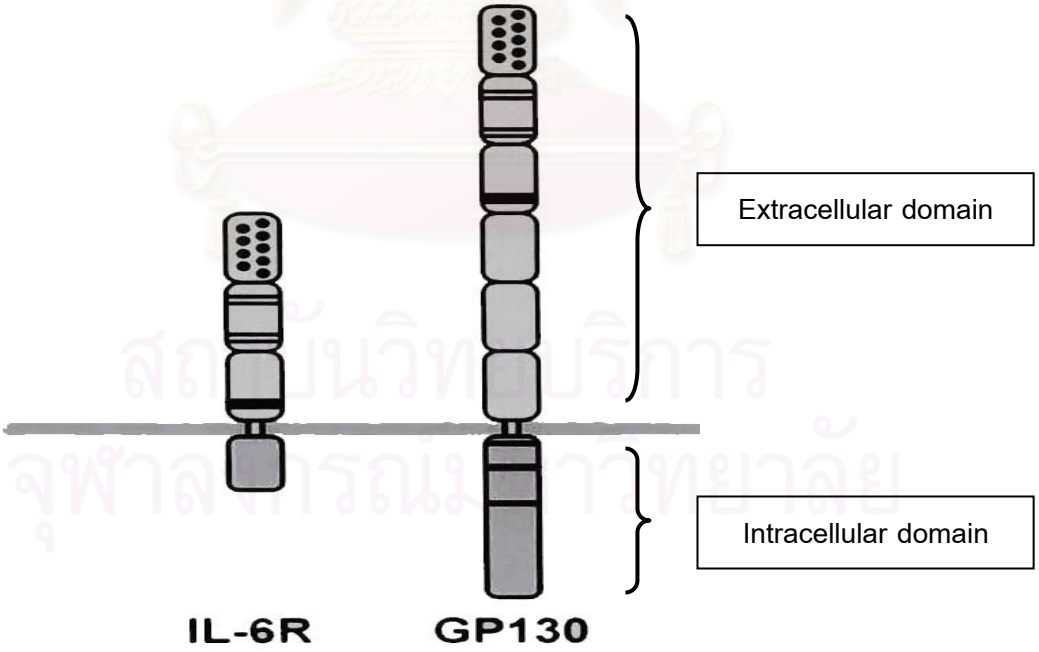


Figure B Structure of IL-6R and gp130. Schematic representation of the IL-6R and gp130. The shaded horizontal line represents a cell membrane. (apply from Richard et al, 1997)

Furthermore, IL-6R has a soluble (sIL-6R) form that can act like the membrane-bound form. sIL-6R may be generated by a proteolytic shedding of membrane bound IL-6R or from an alternatively spliced of mRNA species (Lust et al, 1992; Horiuchi et al, 1994). A natural secreted soluble form of IL-6R that is capable of binding IL-6 has been detected in low abundance in human urine (Novick et al, 1989) and serum (Narazaki et al, 1993; Frieling et al, 1994).

The gp130 (130 kD protein), which does not bind directly to IL-6, is the molecule that responsible for signal transduction. The cDNA of human gp130 encodes a protein consisting of 918 amino acids, which includes a leader sequence of 22 amino acids, an extracellular domain of 597 amino acids, a transmembrane domain of 22 amino acids, and a cytoplasmic domain of 277 amino acids (Hibi et al, 1990) (figure 2). Gp 130 has the soluble form lik gp80 receptor (Kishimoto et al, 1995 for review).

Binding of IL-6 to its receptor initiates cellular events including activation of JAK kinases and activation of ras-mediated signaling (figure C). Activated JAK kinases phosphorylate and activate STAT transcription factors, particularly STAT3, that move into the nucleus to activate transcription of genes containing STAT3 response elements. The ras-mediated pathway, acting through Shc, Grb-2 and Sos-1 upstream and activating Map kinases downstream, activates transcription factors such as ELK-1 and NF-IL-6 (Heinrich et al, 2003 for review)

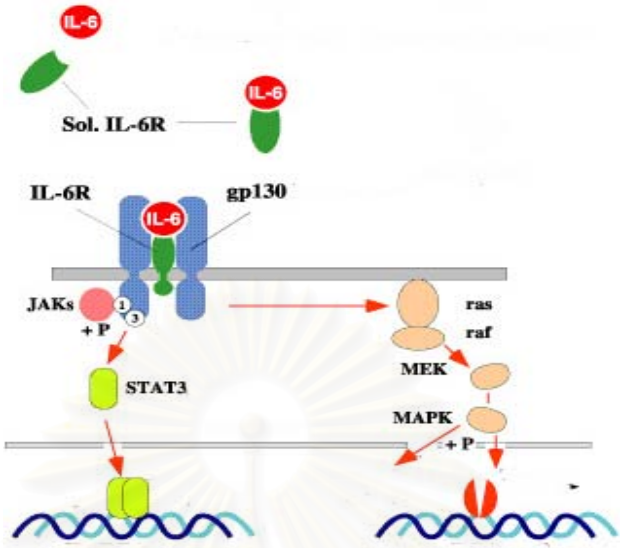


Figure C The JAK–STAT signal transduction pathway and MAPK pathway were induced by IL-6

IL-6 involve in the bone resorption process and osteoclastogenesis. IL-6 may enhance or suppress inflammatory bone destruction ((Jilka et al, 1992, Balto et al, 2001). IL-6 stimulates the formation of osteoclast precursors from colony-forming unit–granulocyte-macrophage and increases osteoclast numbers in vivo leading to systemic increases in bone resorption (Jilka et al, 1992). Littlewood (Littlewood et al, 1991) showed that human osteoblasts produced IL-6 in response to several external stimuli. This could be implied for the role of IL-6 in bone remodeling and systemic responses to local injury. IL-6 can suppress bone resorption, Balto (Balto et al, 2001) demonstrate that mice lacked IL-6 have significantly increased bone resorption following pulpal infection, compared to wild-type controls. They concluded that IL-6 suppress bone resorption due to the anti-inflammation effect. Gorny (Gorny et al, 2004) also suggest that IL-6 in particular in murine cells may have a direct inhibitory action on osteoclast forming hematopoietic cells in vitro. Moreover Huang et al show in their study that the lack of IL-6 enhanced periapical inflammation as evidenced by the finding that lesions developed faster at an earlier phase in the IL-6 knock-out mice than the lesions developed in the IL-6 wild-type mice. However, IL-6 depletion did not prevent bone

resorption; in fact, it promoted the resorption as the result of enhanced inflammation (Huang et al, 2001).

In vitro studies in various cells culture have demonstrated that the addition of sIL-6R in conjunction with IL-6 induced effects on bone resorption but not seen when IL-6 was added alone. From the investigations of Palmqvist (Palmqvist et al, 2002), where it has been shown that IL-6 does not stimulate releasing of mineral and bone matrix of bone explant unless sIL-6R is present. Furthermore, Irwin also suggest that the combination of IL-6 and sIL-6R can activated expression of MMP-1, the molecule responsible for degrade extracellular matrix in bone resorption process (Irwin et al, 2002). This is in agreement with investigations in mouse bone marrow cultures, where it has been shown that IL-6 does not stimulate osteoclast formation unless sIL-6R is present (Tamura et al, 1993). However, there are many studies show IL-6 can stimulate bone resorption in the absence of sIL-6R. For example Lowik and Ishimi have suggested that IL-6 can be stimulated in vitro bone resorption in fetal mouse metacarpals and calvaria without adding exogenous IL-6R (Lowik et al, 1989, Ishimi et al, 1990).

There are some studies suggest that IL-6 may contribute to bone loss caused by estrogen deficiency (Jilka et al, 1992). Estrogen can decrease the levels of the IL-6 mRNA. In addition, estrogen inhibited TNF-α, the cytokine that can induced IL-6 production in primary bone cell cultures derived from neonatal murine calvaria (Girasole et al, 1992). Furthermore, Girasole et al also show in their study, TNF-stimulated osteoclast development was suppressed by a neutralizing monoclonal anti-IL-6 antibody.

Bone is a specialized connective tissue formed by a mineralized matrix that confers its elastic and strength properties. Bone remodeling allows to adapt bone tissue to mechanical constraints and to maintain phosphocalcic homeostasis through coordinated phases of formation and resorption. Thus, bone remodeling involves synthesis of organic matrix by osteoblasts, and bone resorption by osteoclasts. This equilibrium is tightly regulated by physical parameters (ie, mechanical stimulations) and numerous polypeptides (hormones, cytokines). Any disturbance between these effectors leads to the development of skeletal abnormalities, characterized by

decreased (osteoporosis) or increased (osteopetrosis) bone mass. Osteoblasts and osteoclasts are responsible for the maintenance of the dynamic balance between bone formation and resorption which is essential for the integrity of the skeleton. Osteoclast, the multinucleated cells that resorb bone, develops from hematopoietic cells of the monocyte/macrophage lineage (Greenfield et al, 1999, Yasuda et al, 1999). Mature osteoclasts are multinucleated giant cells that express high levels of tartrate-resistant acid phosphatase (TRAP), the most noticeable feature of the osteoclast. Situated on the endosteal bone surfaces, these cells are polarized, furnished with a ruffled border surrounded by the sealing zone on their base, the basolateral membrane and the apical functional secretory domain. The role of osteoclasts is to degrade mineralized bone matrix. This mechanism involves dissolution of crystalline hydroxyapatite and proteolytic cleavage of the organic matrix. Dissolution of mineral is achieved by secretion of HCl via the action of ATP-dependent vacuolar proton pumps across the ruffled border membrane as well as in intracellular vacuoles. Actin and the $\alpha v\beta 3$ integrin facilitated the attachment of the osteoclast to bone. Carbonic anhydrase II (CAII) generates proton and bicarbonate from carbon dioxide and water to achieve acidification of the resorption lacunae and begin the process of bone demineralization. Proton is then actively transported through the action of ATP6i across the membrane of the ruffled border. Chloride channels balance the charge of ions across the membrane through coupling with the proton pump. Finally, excess bicarbonate is removed through the basolateral membrane by passive exchange with chloride. RANKL, the critical gene of osteoclast differentiation, activation and survival of mature osteoclasts, binds to RANK and activates at least five distinct signaling cascades: inhibitor of NF-kB kinase (IKK), c-Jun N-terminal kinase (JNK), p38, and NFAT2. (see figure D)

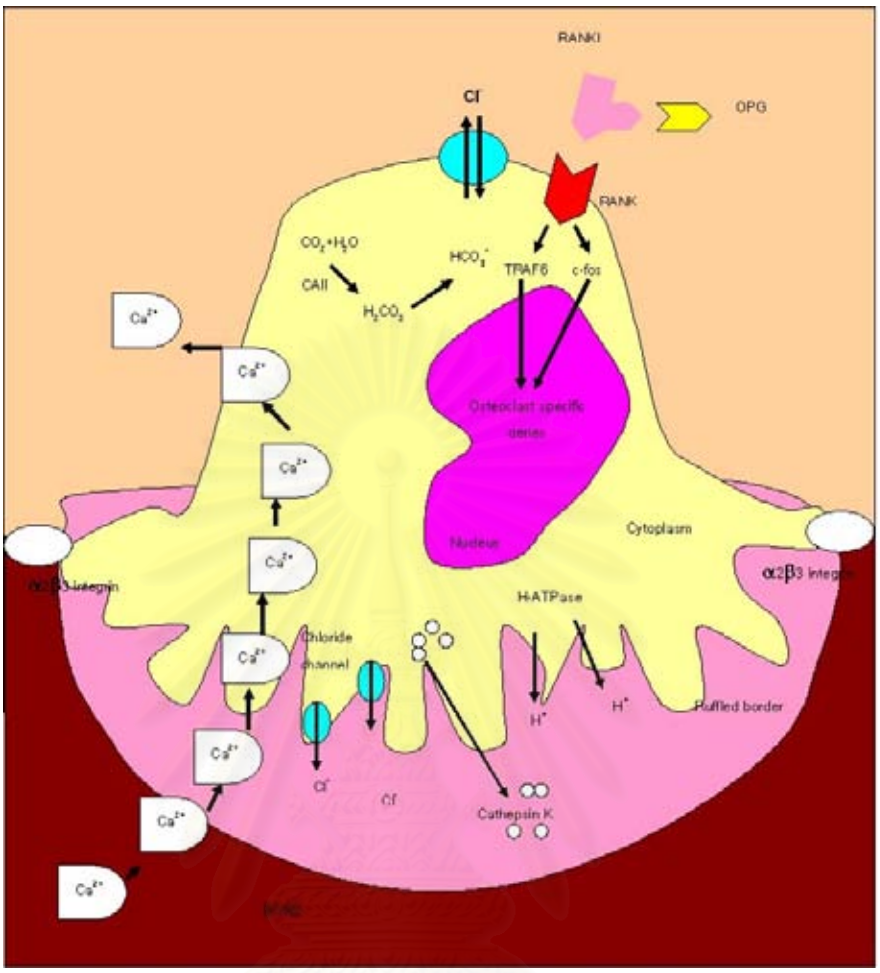


Figure D Model of a resorbing osteoclast.

Osteoclastogenesis depends on several local factors secreted by pluripotent bone marrow stromal cells and on mature osteoblasts. It has been demonstrated that osteoblasts or stromal cells play a role in osteoclast differentiation, and interaction of these cells with osteoclast precursors is important for the osteoclastogenesis. The cytokines proximally responsible for the formation and activation of osteolasts have recently been identified. RANKL and OPG have been identified as members of a ligand-receptor system that directly regulates osteoclast differentiation and bone resorption. RANKL has been shown to both activate mature osteoclasts and mediateo steoclastogenesis in the presence of M-CSF. The balance RANKL/OPG was systematically analyzed, compared to healthy controls and correlated to the severity of osteolysis.

Receptor activator of NF-κB (RANK) and ligand (RANKL)

RANKL is a member of the TNF superfamily, is expressed by a variety cell types, including osteoblast/stromal (Horwood et al, 1998), T cell line (Horwood et al., 1999) , endothelial cell (Ishida et al., 2002), odontoblasts, odontoclasts, human pulp fibroblast and periodontal ligament cell from both deciduous and permanent teeth (Lossdorfer et al., 2002, Hasegawa et al., 2002a,b). RANKL is involved in many diseases such as rheumatoid arthritis (Takayanagi et al, 1997), multiple myeloma (Giuliani et al, 2001), breast cancer (Kitazawa, 2002) and etc. RANKL gene product is a 401 amino acid polypeptide. The receptor of RANKL is RANK. RANK is a transmembrane protein of 616 amino-acids which belongs to the TNF receptor (TNFR) superfamily. This natural receptor of RANKL is expressed primarily on the cells of the monocyte/macrophage lineage including osteoclastic precursors, B and T cells, dendritic cells and fibroblasts. Binding of RANKL to its receptor, expressed on the surface of osteoclasts and their precursors promote osteoclast differentiation and activation. A ligand binding of RANK to the receptor leads to an activation of NF-κB, c-Jun N-terminal kinase (JNK), calcium signaling, and induction of gene expression programs in these cells (Hofbauer and Heufelder, 2001 for review). RANKL exists both in a soluble and a membranous forms, which are differently modulated by agents such as 1,25-dihydroxyvitamin D3 and dexamethasone (Ikeda et al, 2001). The soluble form, which corresponds to the c-terminal part of membranous RANKL, may be produced either directly by the cell through an alternative splicing followed by an excretion in the extra-cellular medium, or by a proteolytic cleavage of membranous RANKL. Nakashima (Nakashima et al, 2000) showed in the present study that IL-6 can stimulating osteoclast development via the expression of RANKL in both soluble and membrane-bound forms but membrane-bound RANKL works more efficiently than soluble RANKL in the osteoclastogenesis development. Jimi (Jimi et al, 1999) also found in their study that the secreted form of RANKL has an effect on the survival, multinucleation and pit-forming activity of murine osreoclasts.

Macrophage colony-stimulating factor (M-CSF)

M-CSF has been identified both in vitro and vivo by its ability to support the clonal growth and differentiation of precursor cells of monocytes macrophage lineage. The result from many studies showed that M-CSF is indispensable for both the proliferative phase and the differentiation phase of osteoclast development (Kodama et al., 1991, Suda et al., 1999). M-CSF was also identified in extracts from a variety of tissues, and the supernatants from various cell and organ cultures (Das et al, 1980, Stanley et al, 1981). Maximum stimulation of M-CSF-mediated proliferation can be observed at concentrations of 250 pM, and in the case of bone-marrow colony-forming cells, proliferation can be detected at concentrations as low as 1 pM (Stanley et al, 1981). The normal range for serum M-CSF concentration is between 150 and 500 U/ml or 3–8 ng/ ml (Barreda et al, 2004 for review). M-CSF is synthesized by a variety of cell types including endothelial cells, fibroblasts, bone marrow/stromal cells, osteoblasts, thymic epithelial cells, keratinocytes, astrocytes, myoblasts, mesothelial cells, endometrial gland cells, and the placentatrophoblast decidual stroma (Barreda et al, 2004 for review). Stimulation of M-CSF production can also be achieved following activation of cells including monocytes, macrophages, microglial cells, endothelial cells, T lymphocytes, B lymphocytes, fibroblasts, chondrocytes, and mesangial cells. The human M-CSF gene is localized in the short arm of chromosome 1 at positions p13-p21, and includes 10 exons and nine introns that span an area of about 21 kb. The biological effects of M-CSF are mediated via the high affinity M-CSF receptor (M-CSFR, CD115). The c-fms receptor, which is the M-CSF receptor, a member of the class III receptor tyrosine kinase family (RTK), is a large integral plasma membrane glycoprotein (140–150 kDa) with an N-terminal glycosylated extracellular portion containing five repeated immunoglobulin (Ig) domains, a short single transmembrane domain, a juxtamembrane domain, two intracellular kinase domains divided by a unique kinase insert, and a C-terminal domain. The M-CSF receptor is expressed primarily on cells on the macrophage lineage and, as such, is a useful marker to discriminate between monocytic and granulocytic progenitor cells and their differentiated progeny. The human

M-CSFR is located on chromosome 5q33.3. The M-CSFR gene is 58 kb in length and is composed of 22 exons and 21 introns. It is interesting to note that a variety of inflammatory cytokines can up-regulate mononuclear phagocyte expression of M-CSF including GM-CSF (Horiguchi et al, 1987), IL-4, TNF-α (Besse et al, 2000), IL-1 (Falkenburg, 1990), and IFN-γ (Horiguchi et al, 1986). (Barreda et al, 2004 for review). There are many studies both in vivo and vitro revealed that M-CSF is very important in osteoclastogenesis (Jimi et al, 1995, Tanaka et al, 1993). Studies in the op/op osteopetrotic mouse, which has a point mutation in the M-CSF gene and has severe osteopetrosis due to an absence of osteoclast (Yoshida et al, 1990). From this study has clearly shown an important role for M-CSF in murine osteoclast development. Futhermore, Sundquist found that the injection of M-CSF into op/op mice improves the skeletal sclerosis in osteopetrotic mutation mice by in crease osteoclast (Sundquist et al, 1995). M-CSF also may affect mature osteoclasts, as well as osteoclast precursors. There is a study also identified a role for M-CSF in maintaining the survival and chemotactic behavior of mature osteoclasts (Suda et al, 1999 for review). In their studies showed, M-CSF prevent apoptosis of osteoclasts and enhanced osteoclast motility. To confirm the involvement of M-CSF in osteoclast development, normal spleen cells were cocultured with osteoblasts derived from op/op mice (Tanaka et al, 1993). When M-CSF was added throughout the 6-day coculture period, osteoclasts were formed in response to 1α,25(OH)2D_3. However, the lack of M-CSF either for the first 4 days or for the final 2 days failed to result in osteoclast formation. These results confirm that M-CSF is indispensable for both the proliferative phase and the differentiation phase of osteoclast development. Similarly, Biskobing (Biskobing et al, 1995) showed that M-CSF plays important roles in proliferation and differentiation of osteoclast progenitors in mouse bone marrow cultures.

Osteoprotegerin (OPG).

OPG plays a pivotal role in bone metabolism regulation by inhibiting osteoclast differentiation and activation and increasing osteoclast apoptosis (Yasuda et al, 1998).

OPG is identified as a member of the TNF receptor family, but, unlike all other members of the family, it lacks a transmembrane domain and represent a secreted TNF receptor. OPG mRNA is detected in the calavaria, skin, liver, lung, and heart of the adult mouse, and peaks at days 7 and 15 in fetal tissue (Simonet et al, 1997). OPG is expressed primarily by bone marrow stromal cells, but can be induced in B lymphocytes, dendritic cells, and follicular dendritic cells. It acts as a decoy receptor for RANKL to inhibit osteoclastogenesis (Yasuda et al, 1998). The OPG gene product is a 401 amino acid polypeptide, which is signal peptidase-cleaved to 380 amino acids (44 kDa), N-linked glycosylated (55 kDa), and secreted as a disulfide-linked 110 kDa homodimer (Hofbauer and Heufelder, 2001 for review).

In vitro, both native and recombinant OPG inhibit the formation of multinucleate osteoclast-like cells and decrease the expression of osteoclast markers, i.e., the calcitonin receptor, integrin, $\alpha v\beta 3$, and TRAP. In vivo administration of OPG resulted in an increase in bone mineral density and bone volume associated with a decrease of active osteoclast number and blood calcium in normal and ovariectomized rats (Yamamoto et al, 1998, Akatsu et al, 1998). Ablation OPG by targeted gene deletion in mice leads to early onset osteoporosis, demonstrating for the first time a critical requirement for endogenous OPG in the maintenance of normal postnatal bone mass and skeletal architecture. Many OPG−/− mice exhibit gross skeletal abnormalities from shortly after birth up to 5–6 months of age (Bucay et al, 1998). Thus, OPG is an important factor negatively regulating osteoclastogenesis both in vivo and vitro. The expression of OPG is regulated by growth factors, cytokine and steroid hormones: thus, TGFβ, TNFα, IL-1, IL-6 (Palmqvist et al, 2002), estrogen and BMP-2, which promote osteoblast differentiation, can induce OPG expression. In addition, calcium-modifying agents (vitamin D, prostaglandins E2, and hydrocortisone), which increase bone resorption, also decrease OPG expression by osteoblasts (Walsh and Choi, 2003).

Modulation of RANKL, RANK, OPG and IL-6

Based on extensive studies on RANKL and OPG in osteoclast biology, it had concluded that these two cytokines act as the distal effectors molecules onto which

many growth factors, cytokines, peptide and steroid hormones, and drugs known to affect bone metabolism converge. Undifferentiated (preosteoblastic) marrow stromal cells strongly express RANKL and secret only low levels of OPG. Increased RANKL-to-OPG ratio is correlated with their capacity to support osteoclast formation and activation. Once these cells differentiate towards the mature osteoblastic phenotype, the RANKL-to-OPG ratio declines, and their osteoclastogenetic capacity is suppressed or lost (Deyama et al, 2000). Regulation of RANKL and OPG gene expression as well as OPG secretion by human and rodent osteoblastic lineage cells has been extensively studied. Many known osteotropic cytokines and hormones have been found to regulate either RANKL or OPG, or both, and either in comparable or opposite directions. Hormones that regulate RANKL and OPG include steroid hormones (glucocorticoids), 1,25-dihydroxyvitamin D3, 17β-estradiol, and peptide hormones (parathyroid hormone) (Walsh and Choi, 2003 for review). Other modulators include peptide growth factors, transforming growth factor β, bone morphogenetic protein 2, basic fibroblast growth factor and proinflammatory cytokines (IL-1, IL-6, and IL-11, TNF-$\alpha$and TNF-β) , prostaglandin (Walsh and Choi, 2003 for review).

There are many studies showed the effect of IL-6 on osteoclastogenesis via RANKL-OPG system. For example, Liu et al (2005) showed the effect of IL-6 and prostaglandin E2 signaling systems in the enhancement of osteoclastogenesis. IL-6 increased PGE2 secretion and COX-2 in bone cell. PGE2, in turn, stimulated osteoclastogenesis through inhibition of OPG secretion, stimulation of RANKL production on osteoblasts, and up-regulation of RANK expression on osteoclasts. In addition, IL-6 was the mediator of PGE2-induced suppression of OPG production by osteoblasts. Furthermore, there are the studies showed that IL-6 can stimulate the expression RANKL in both membrane-bound and soluble (Nakashima et al, 2000). ELISA and RT-PCR results showed that the combination of IL-6 and sIL-6R can stimulate the releasing of both mineral and matrix from bone explants and activate expression of RANKL and OPG in calvarial bones (Palmqvist et al, 2002). Further more, IL-6 also

enhances RANKL sensitivity of pagetic osteoclast precursors and so contributes to elevation of osteoclast numbers in Paget's disease (Menaa et al, 2000).

IL-6, RANKL, OPG and M-CSF in human dental pulp cells

Human pulp fibroblasts (HPF) are the predominant cells in pulpal tissue can be activated to produce IL-6 and factors which mediate inflammation. Dental caries is the most common cause of pulpal inflammation which allows pulp cells to be exposed with oral bacteria or bacterial products and may result in pulpitis. The inflammatory process in pulpitis is basically similar to that in connective tissue elsewhere in the body. When the inflammatory exudates leave the vessel because of an increase in hydrostatic pressure, there is a corresponding rise in the interstitial pressure and releasing many inflammatory cytokines such as prostaglandin, collagenase, Interleukin-1β (IL1β), Interleukin-6 (IL-6) and etc (Hosoya and Matsushima, 1997; Matsushima et al, 1998; O'Boskey and Panagakos, 1998). The sequelae of pulpal inflammation are varying such as pulp necrosis, external root resorption, internal root resorption, bone resorption and etc. IL-6 is not spontaneously produced by intact normal cells; in general, its secretion requires a stimulus, for example, following cellular activation by Peptidoglycan from Lactobacillus casei (Matsushima et al, 1998), Methyl mercaptan (CH3SH) (Coil et al, 2004) and etc. The study of Barkhordar (Barkhordar et al, 1999) also found more intense IL-6 protein in inflamed human pulp tissue than in healthy pulpal tissue. The study of Huang et al (2001) demonstrated the effect of IL-6 depletion on the formation of periapical lesions after pulpal infection. Yamaguchi (Yamaguchi et al, 2004) also found that Il-6 was produced by human pulp fibroblasts in the inflammatory process of pulp tissue during orthodontic tooth movement following stimulation with neuropeptides.

Rani and MacDougall has demonstrated their study that dental tissue, including HDP and odontoblast express OPG, RANKL and M-CSF, factor that are crucial for the regulation of osteoclast formation (Rani and MacDougall, 2000). Suzuki et al (Suzuki et al, 2004) have reported RANKL mediate osteoclastogenesis during mouse tooth germ development. Hasegawa et al (2002a) showed in their study that human PDL cells can

express RANKL-OPG and could regulate the differentiation of osteoclasts in from deciduous teeth. This study is inconsistent with their study in PDL cells in permanent teeth (Hasegawa et al, 2002b). Although PDL cells from permanent teeth can express RANKL-OPG like PDL from deciduous teeth but they can not stimulate bone resorption in normal situation. These observations concludes that osteoclastogenesis in the alveolar bone, which is essential for the accommodation of normal tooth development, is mediated by RANK-RANKL signaling (Hasegawa et al, 2002a; Fukushima et al, 2003).

Taken together, these data suggest that IL-6 plays an important role in stimulating bone resorption in some physiological and/or pathological conditions. Although, several studies have also shown that IL-6 can activate expression of RANKL and OPG on many cells but on HDP has not been elucidated. This is an objective of my study to examine the effect of IL-6 on HDP cell to expression of molecule that related with bone resorption.

CHAPTER III

MATERIALS AND METHODS

Material

1. surgical blade number 15
2. forceps
3. 35 mm, Nunc, Denmark
4. 60 mm tissue culture dish, Nunc, USA
5. 24-well tissue culture plate, Nunc, USA
6. 48-well tissue culture plate, Nunc, USA
7. Laminar flow hood
8. transfer pipette
9. Hemocytometer
10. Cuvette
11. Dulbecco's Modified Eagle Medium (DMEM Gibco BRL, USA)
12. Dulbecco's Modified Eagle Medium without phenol red (DMEM Gibco BRL, USA)
13. 0.25 % trypsin – EDTA
14. Fetal bovine serum ; FBS, Gibco BRL, USA
15. Penicillin G, Gibco BRL, USA
16. Streptomycin, Gibco BRL, USA
17. Amphotericin B, Gibco BRL, USA
18. L-glutamine, Gibco BRL, USA
19. Phosphate buffer saline ; PBS
20. 0.01 molar Borate buffer
21. 1% Methylene blue
22. 4% formaldehyde
23. eluted dye
24. nitrocellulose membrane

25. Tris/HCL
26. NaCl
27. EDTA
28. 1% NP-40
29. 0.25% Na-deoxycholate
30. proteinase inhibitors (10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4)
31. 1.5 ml eppendorf tube
32. 0.65 ml eppendorf tube
33. PCR tube
34. Trizol
35. agarose
36. TAE
37. transfer buffer
38. 75% ethanol
39. isopropanol
40. chloroform
41. nuclease free water
42. TWEEN-20
43. M-CSF ELISA kit (Quantikine® R&D System Inc., MN, USA)

Equipment

1. CO_2 Incubator
2. autoclave
3. centrifuged machine
4. Phase-contrast light microscope
5. Spectrophotometer
6. thermocycler
7. ultraviolet light

Methods

Explantation and Toxicity test

Explantation procedure

Human PDL cells were obtained from healthy third molars extracted for orthodontic reasons. Triplicate from 3 different people. Informed consent was obtained from each patient before the cell culture was performed. All teeth were stored in Dulbecco's modified Eagle's medium (DMEM, GIBBCO BRL, USA), containing 10% FBS, 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml amphotericin B, immediately after extraction. Wash the teeth with phosphate-buffered saline (PBS) and remove pulp tissue by broke the teeth with hammer. Pulp tissue were remove from each extracted tooth by forceps, cut into 1x1 mm by surgical blade and placed on the culture dish (35 mm, Nunc, Denmark). Tissue explants were maintained in DMEM supplemented with 10% fetal bovine serum. All of the cultures were incubated at 37° C in a humidified atmosphere of 5% CO_2 in air. When HDP cells migrated from the explant had become confluent, they were detached with 0.2% trypsin and 0.02% EDTA 4 Na in PBS and subcultured in 1:3 split ratio to passage 1^{st} on 60 mm dishes. All of the experiment uses only cells from passage 3^{rd}- 5^{th}.

Toxicity test

HDP fibroblasts were plated in triplicate into 24 well plates, at initial density of 50,000 cell/cm^2 in DMEM plus 10% fetal bovine serum (FBS), 10% FBS, Penicillin G (100 U/ml), Streptomycin sulfate(100 μg/ml), Amphotericin B (25 μg/ml), L-glutamine (2 mM) overnight. Then the medium was changed to serum free media twice, 3 hours each round.

After 6 hours, culture medium were changed from serum free mediu to serum free media containing various concentration of IL-6 range from 0, 0.1, 1, 10 ng/ml and

culture for another 48 hours. Cell numbers under all conditions were measured by methylene blue staining assay method.

Methylene blue staining assay (MB assay)

Briefly, after remove cells from CO_2 incubator, HDP were washed with PBS 2 times for remove all condition media. Then HDP were fixed with 4% formaldehyde solution for 30 minutes, wash with PBS 1 time and borate buffer (0.01 molar, pH 8.5)1 time respectively. Add 100 µl of 1% methylene blue, left 30 minutes. Vigorously wash all wells with borate buffer many times for removing the excess dye, left in room temperature until absolutely evaporated. Dissolve the dye with eluted dye 1 ml per well and measure the quantity of cells, using the spectrophotometer at wavelength 667 nm.

Determination of M-CSF, OPG, RANKL RNA level

Fibroblasts were plated out in 6-well plates at a density of 200,000 cells per well in DMEM supplemented with 10% FBS, for 16 hours. Then the medium was changed to serum free media twice, 3 hours each round. After staffing, HDP cell were treated with various concentration of IL-6 and sIL-6R (see diagram) at the appropriated concentration from growth study above in serum free media without phenol red supplemented with 0.5% FBS and incubated for 72 hours. After 72 hours, condition media were collected for western blot analysis and HDP cell were extracted for RNA isolation.

RT-PCR

RNA isolation

After the treatment, total cellular RNA was extracted with TRI Reagent (Molecular Research Center, Cincinnati, Ohio, USA) according to manufacturer's instructions.

Brirfly, 1 ml. of TRI reagent was added to each well, scraped the cell layer and incubate for 5 minutes and then transfer TRI reagent into 1.5 ml. tube, 200 μl of chloroform was added and shaken vigorously. The mixture was then centrifuged at 15,000 rpm for 15 minutes. The aqueous phase was collected and added isopropanol to precipitate RNA. After centrifugation, RNA pellet was dissolved in nuclease free water and the amount of RNA was determined by the absorption at 260/280 nm. using a spectrophotometer (Thermospectronic, Roche, NY, USA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

One μg of each RNA sample was converted to cDNA by a reverse transcription using an AMV (Avian myeloblastosis virus) reverse transcriptase (Promega, Madison, WI, USA) for 1.5 hours at 42°C. Subsequent to the reverse transcription, a polymerase-chain reaction was performed. The primers were prepared following the reported sequences from GenBank. The oligonucleotide sequences of the primers are

M-CSF	forward	5' CTA AGC TGG ACG CAC AGA CCA 3'
	reverse	5' TCT CAG GCT GCA CAC CTT 3'
RANKL	forward	5' CCA GCA TCA AAA TCC CAA GT 3'
	reverse	5' CCC CTT CAG ATG ATC CTT C 3'
OPG	forward	5'TGCAGTACGTCAAGCAGGAG 3'
	Reverse	5'TGACCTCTGTGAAAACAGC 3'
GAPDH	forward	5' TGA AGG TCG GAG TCA ACG GAT 3'
	reverse	5' TCA CAC CCA TGA CGA ACA TGG 3'

The PCR was performed using Tag polymerase (Qiagen, Hilden, Germany) with a PCR volume of 25 μl. The mixtures contained 25 pmol of primers and 1μl of RT product. The PCR working conditions were set at a denaturation for 1 min at 94 °C, primer annealing for 1 min at 60°C, and chain elongation for 1.45 min at 72°C on a DNA thermal cycler (Tpersonal, Whatman Biometra, Goettingen, Germany). The amplified DNA was then electrophoresed on a 2% agarose gel and visualized by ethidium

bromide fluorostaining. An intensity of each band was determined by Scion Image analysis software (Scion Corporation, Frederick, Maryland).

Enzyme-linked immunosorbent assay (ELISA) for M-CSF

An ELISA was used for the quantification of M-CSF (Quantikine® R&D System Inc., MN, USA). Two monoclonal antibodies with specificity to different epitopes on each cytokine molecule of interest were used.

Assay procedure (briefly)

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for M-CSF has been pre-coated onto microplate. Samples were pipetted into the wells and any M-CSF presented in the sample bound by the immobilized antibody. After washing away any unbounded substance by a wash buffer, an enzyme-lined polyclonal antibody specific for M-CSF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of M-CSF bound in the initial step. The color development is stopped and the intensity of the color is measured at wave length 450 nm within 30 minutes by spectrophotometer.

Western analysis for OPG and RANKL

The amount of OPG was determined from the culture medium. The medium was collected and lyophilized to concentrate. The lyophilized was dissolved in sample buffer, boiled and subject to SDS-PAGE.

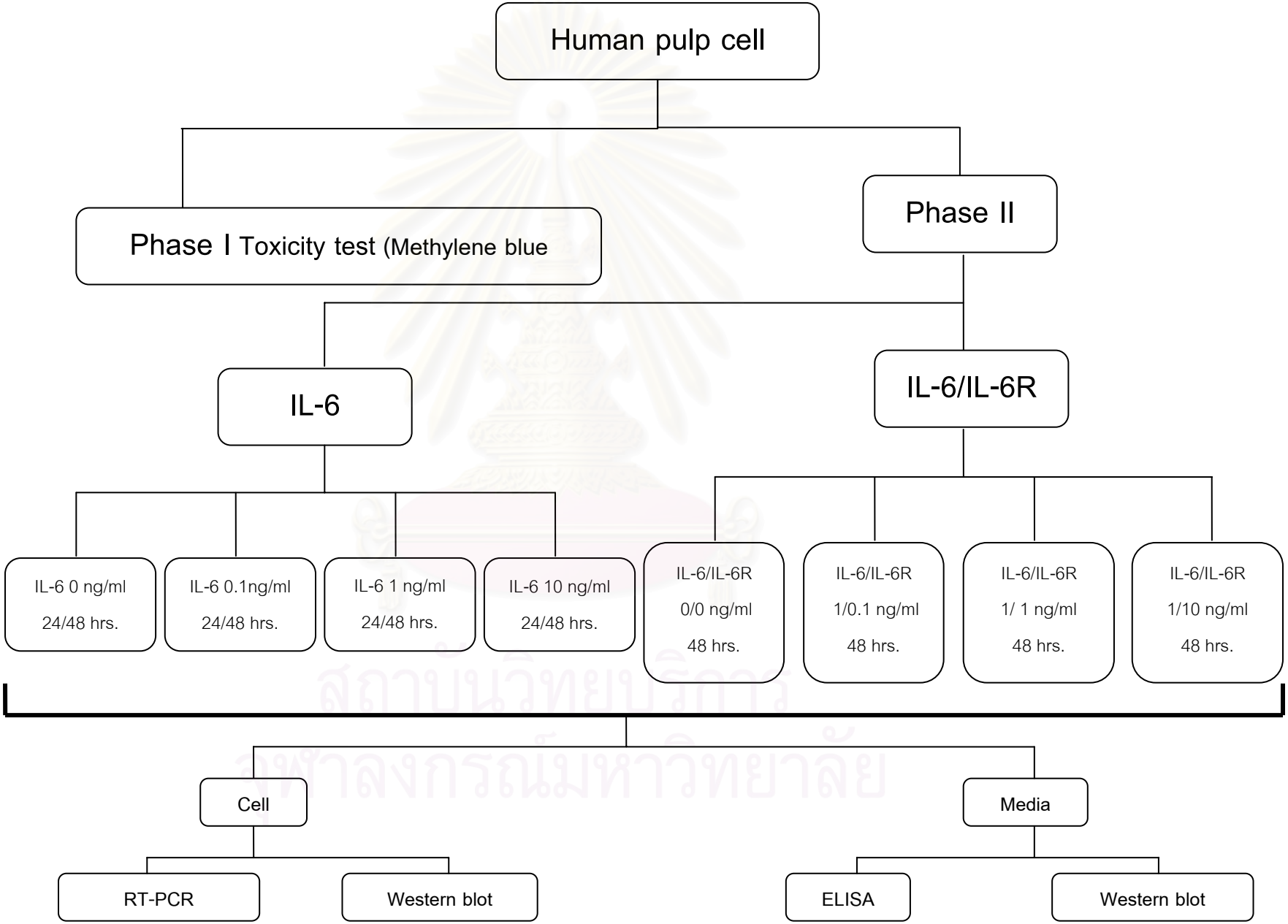
Due to RANKL was membrane-bounded molecules, protein level should be assayed from the cells by following procedure. HDP cells culture in 6-well plates, which incubated in appropriate concentration, were washed with PBS 2 times and lysed directly with RIPA buffer (50 mM Tris/HCL, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate) containing cocktail protease inhibitors (Sigma Chemical Co., St.

Louis, MO, USA).; 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4.

The amount of proteins was determined using BCA protein assay kit (Pierce, Rockford, IL, USA). Protein extraction from each sample was separated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the nitrocellulose membrane (Immobilon-P, Millipore Corporation, Bedford, MA). The membrane was incubated in blocking buffer (5% non-fat dry milk, 0.1% Tween 20 in deionized water) at room temperature for 1 hour. Then, the membrane was stained overnight with primary antibody for RANKL (Chemicon International, Temecula, CA), OPG or β-actin (Chemicon international, Temecula, CA) at the dilution of 1:500 in blocking buffer at 4°C. After extensively washed with PBS, the membrane was incubated with biotinylated-secondary antibody (Sigma Chemical Co., St. Louis, MO) for 30 minutes at room temperature, and peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA) for 30 minutes, respectively. The protein bands were detected using a commercial chemiluminescence system (Pierce, Rockford, IL) and were exposed on CL-X Posture film (Pierce, Rockford, IL). The band intensity was determined by Scion Image analysis software.

Statistical analysis

Results are expressed as mean ±SD. Statistical difference between groups were determined using one-way analysis of variance and LSD's test was used for post-hoc analysis. A p value of less than 0.05 was considered statistically significant.


Human pulp cell
Phase I Toxicity test (Methylene blue
Phase II
IL-6
IL-6/IL-6R
IL-6 0 ng/ml
24/48 hrs.
IL-6 0.1ng/ml
24/48 hrs.
IL-6 1 ng/ml
24/48 hrs.
IL-6 10 ng/ml
24/48 hrs.
IL-6/IL-6R
0/0 ng/ml
48 hrs.
IL-6/IL-6R
1/0.1 ng/ml
48 hrs.
IL-6/IL-6R
1/ 1 ng/ml
48 hrs.
IL-6/IL-6R
1/10 ng/ml
48 hrs.
Cell
Media
RT-PCR
Western blot
ELISA
Western blot

CHAPTER IV

RESULT

Toxicity test

Methylene blue staining assay shows IL-6 had no significant effect on fibroblast growth at any of the concentrations studied. Cell counts under each condition are shown in figure 1.

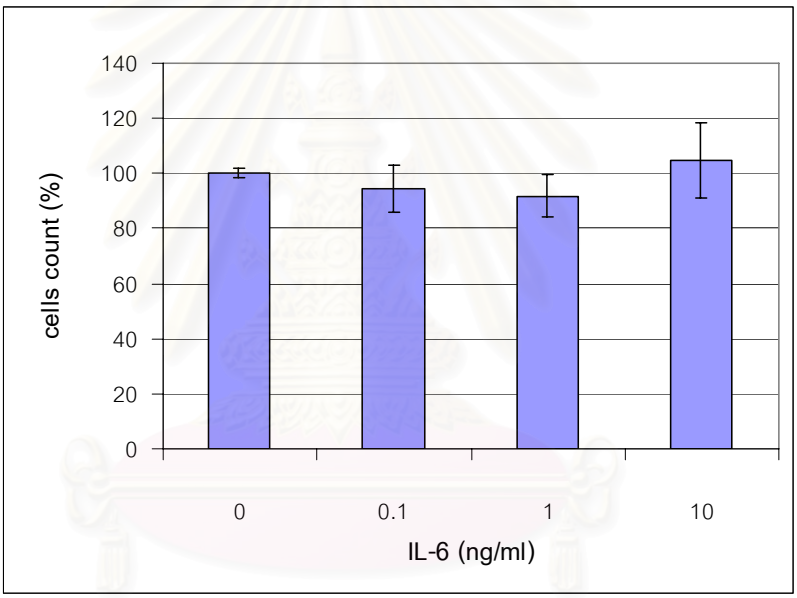


Figure 1 Cell counts after 24 hours in culture under each condition. All values are indicated by the average ±SD relative to the control from three independent experiments. No significantly different from control ($p < 0.05$).

Effects of IL-6 on RANKL, OPG and M-CSF expression

HDP cells were cultured in the presence of 0, 0.1, 1, 10 ng/ml IL-6 for 24 hours and the cell extracts were collected.

Effect on RANKL

RT-PCR analysis demonstrated that RANKL expression was increased in dose dependent manner after treated with various doses of IL-6 (Fig. 2). IL-6 also had effect on RANKL protein (Fig. 3), which harmony with the result from RT-PCR.

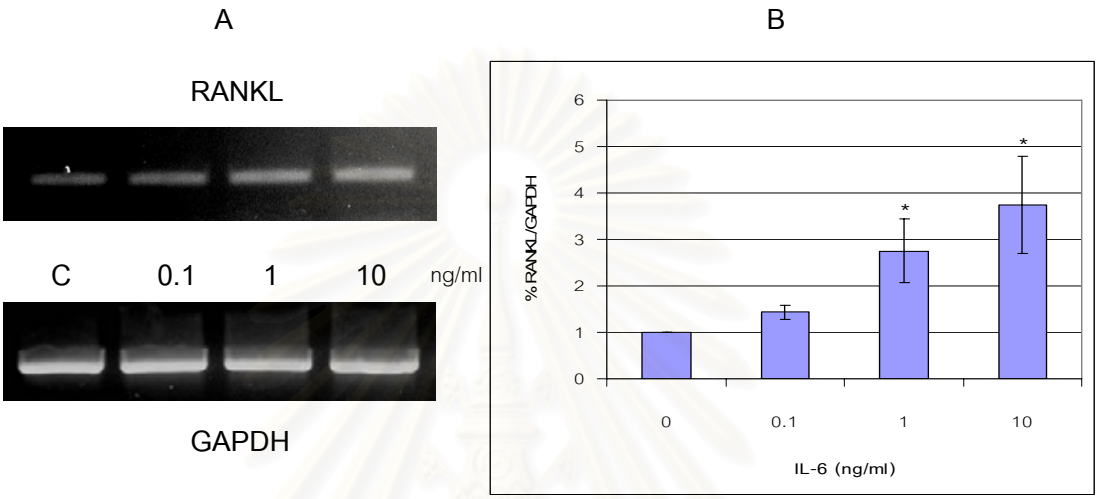


Figure 2 A: Semi-quantitative RT-PCR analysis of RANKL mRNA expression in HDP modulated with IL-6. B: The quantification of RANKL expression which normalized to the GAPDH. All values are indicated by the average ±SD relative to the control from three independent experiments. * Significantly different from control ($p < 0.05$).

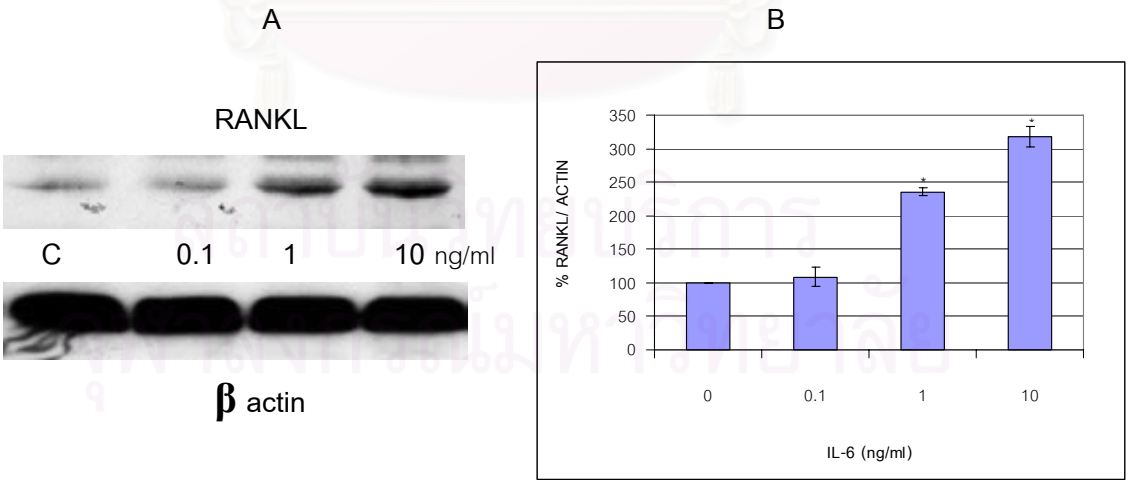


Figure 3 A: Western blot analysis of RANKL protein modulated by IL-6. B: The quantification of RANKL expression which normalized to the β actin. All values are indicated by the average ±SD relative to the control from three independent experiments. *Significantly different from control ($p < 0.05$).

Effect on OPG

The result show OPG expression on HPD cells modulated by IL-6 is not changed comparing to control in both mRNA and protein level (Fig. 4,5).

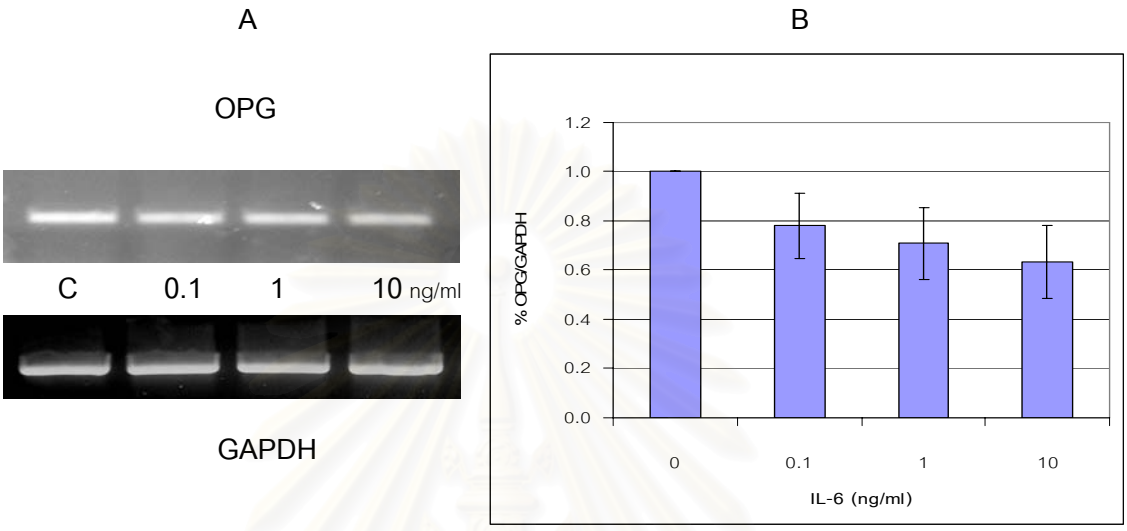


Figure 4 A: Semi-quantitative RT-PCR analysis of OPG mRNA expression in HDP modulated with IL-6. B: The quantification of OPG expression which normalized to the GAPDH. All values are indicated by the average ±SD relative to the control from three independent experiments. No significantly different from control ($p < 0.05$).

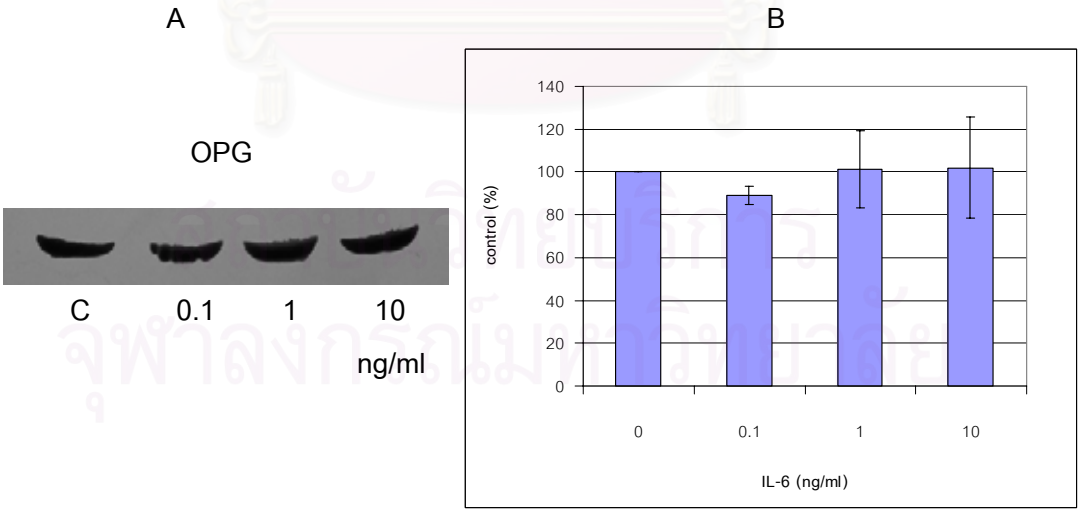


Figure 5 A: Western blot analysis of OPG protein modulated by IL-6. B: The quantification of OPG expression. All values are indicated by the average ±SD relative to the control from three independent experiments. No significantly different from control ($p < 0.05$).

Effect on M-CSF

The changing of M-CSF expression on HDP cells does not observe in mRNA level (Fig. 6). The data was confirmed by ELISA for protein measurement (Fig. 7)

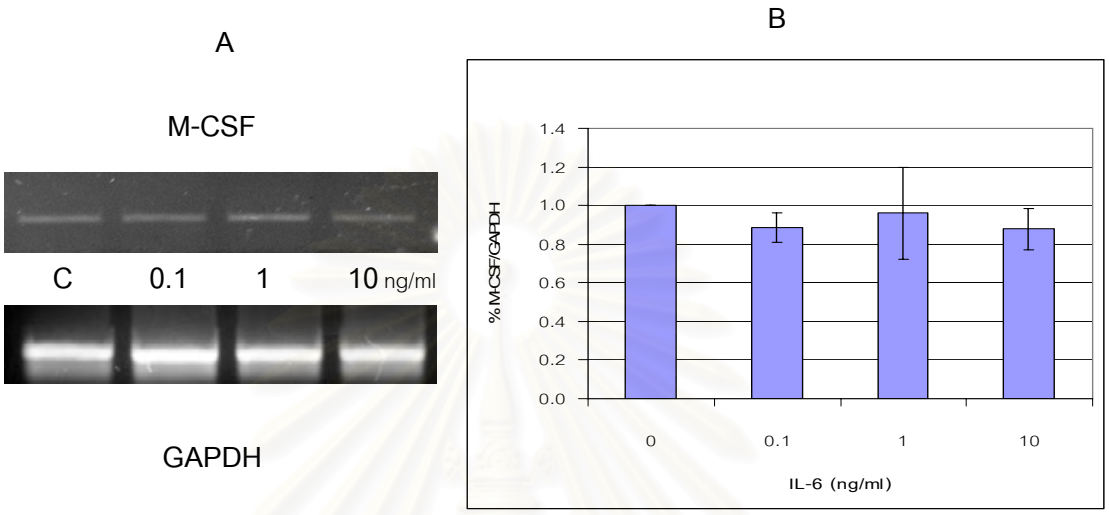


Figure 6 A: Semi-quantitative RT-PCR analysis of M-CSF mRNA expression in HDP modulated with IL-6. B: The quantification of M-CSF expression which normalized to the GAPDH. All values are indicated by the average ±SD relative to the control from three independent experiments. No significantly different from control ($p < 0.05$).

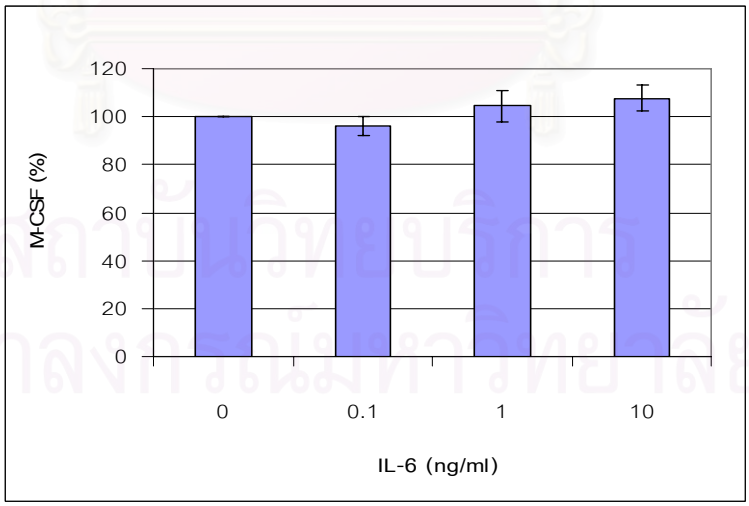


Figure 7 The quantification of M-CSF expression by ELISA. All values are indicated by the average ±SD relative to the control from three independent experiments. No significantly different from control ($p < 0.05$).

Effects of combination of IL-6 with sIL-6R on RANKL, OPG and M-CSF formation

HDP cells were cultured in the presence of 1 ng/ml IL-6 and varying dose of sIL-6R (0, 0.1, 1, 10 ng/ml) for 24 hours and the cell extracts were collected.

Effect on RANKL

RT-PCR analysis (Fig. 8) indicates that RANKL expression was increased in dose dependent manner. In contrast, the result of protein level which analyzed by western blot analysis reveal the decreasing of RANKL expression following the increase of sIL-6R concentration (Fig. 9).

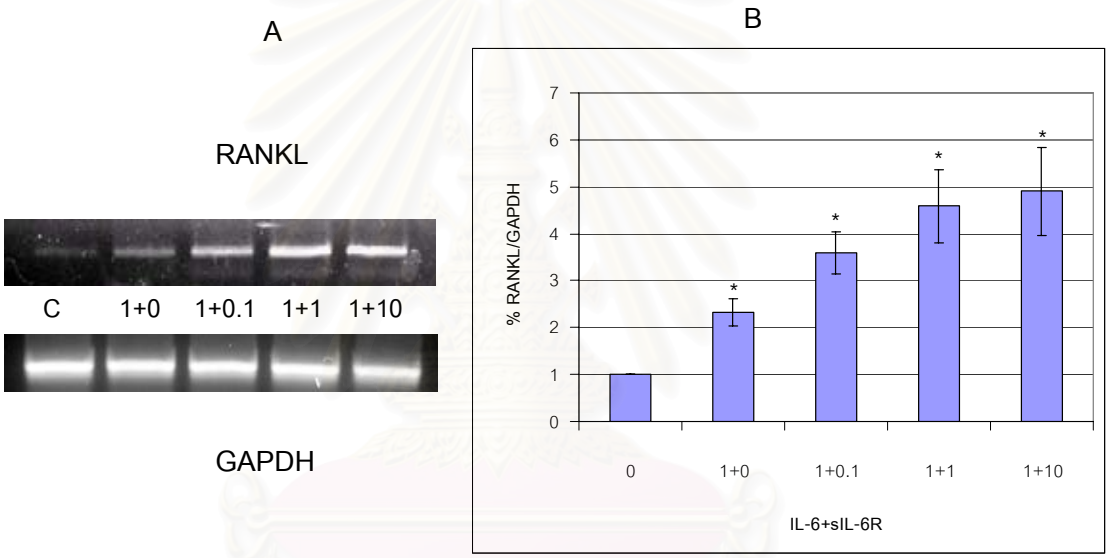


Figure 8 A: Semi-quantitative RT-PCR analysis of RANKL mRNA expression in HDP cells modulated with IL-6 plus sIL-6R. B: The quantification of RANKL expression which normalized to the GAPDH. All values are indicated by the average ±SD relative to the control from three independent experiments. *Significantly different from control ($p < 0.05$).

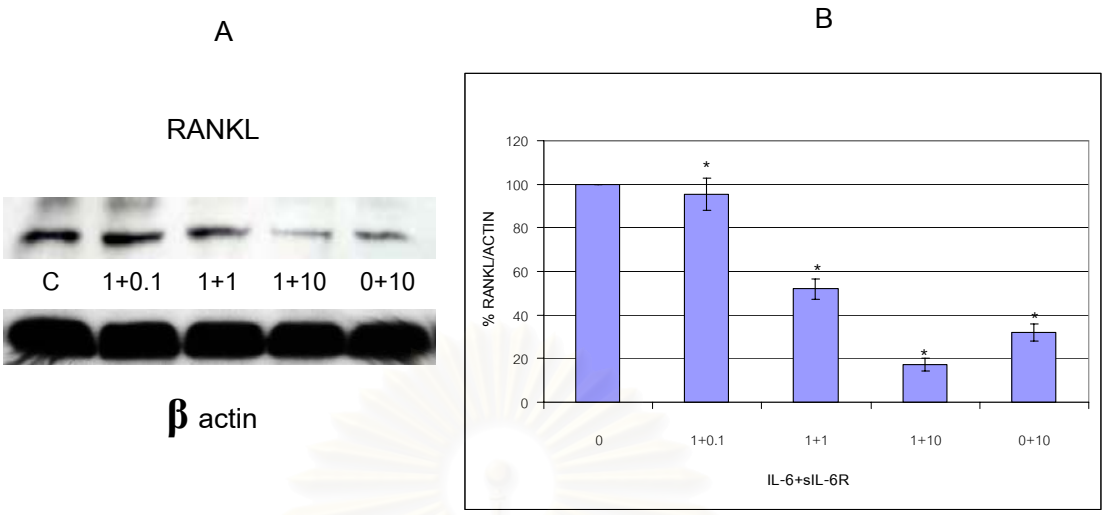


Figure 9 A: Western blot analysis of RANKL protein modulated by combination of IL-6 plus sIL-6R. B: The quantification of RANKL expression which normalized to the β actin. All values are indicated by the average ±SD relative to the control from three independent experiments. *Significantly different from control ($p < 0.05$).

Effect on OPG

OPG was analysis by semi-quantitative PCR and western blot. The results show that IL-6 plus sIL-6R does not change OPG expression in mRNA level (Fig. 10) but in protein level is decreased (Fig. 11).

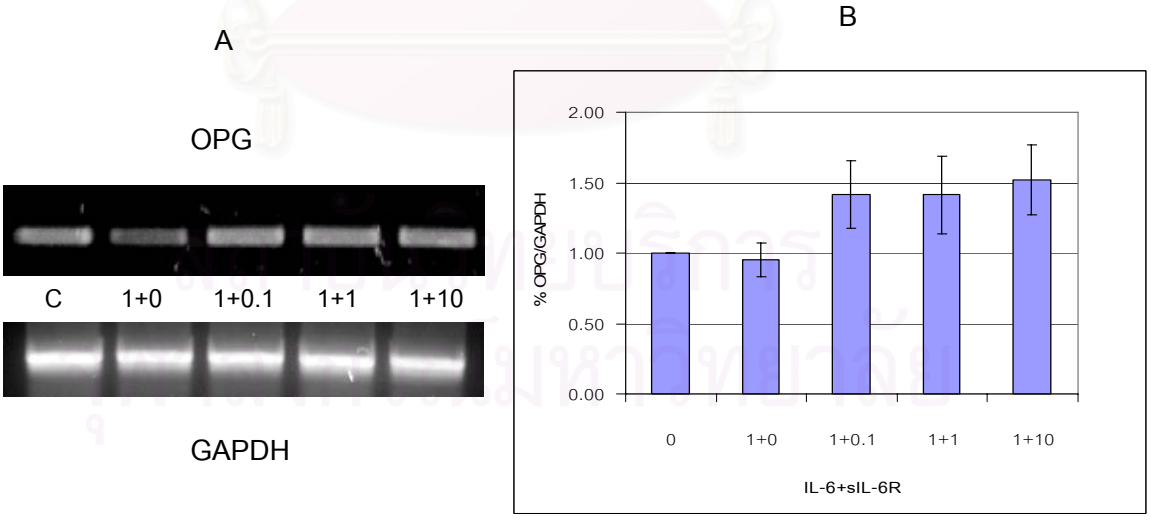


Figure 10 A: Semi-quantitative RT-PCR analysis of OPG mRNA expression in HDP modulated with IL-6 plus sIL-6R. B: The quantification of OPG expression which normalized to GAPDH. All values are indicated by the average ±SD relative to the control from three independent experiments. No significantly different from control ($p < 0.05$).

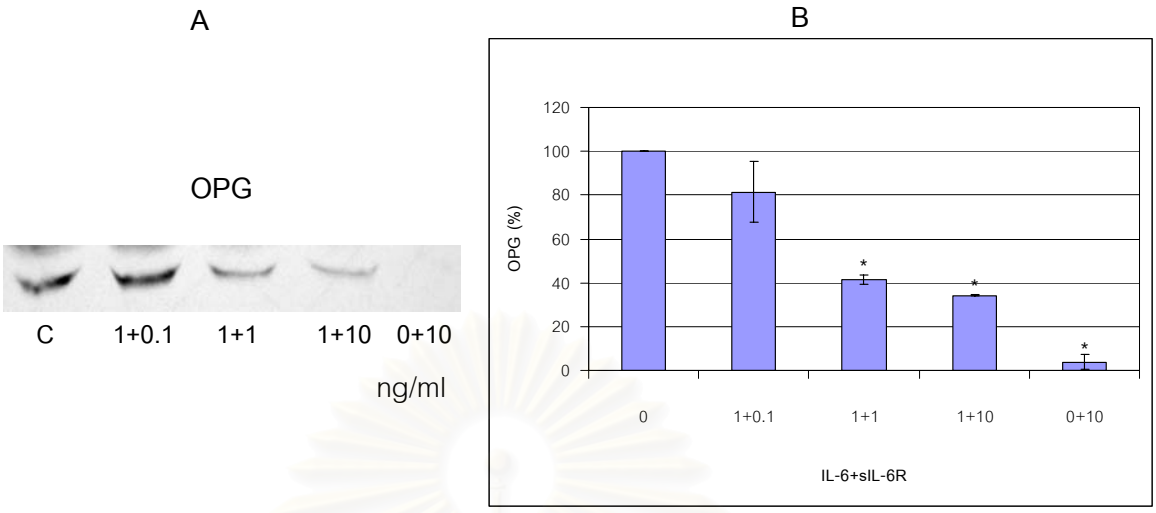


Figure 11 A: Western blot analysis of OPG protein modulated by combination of IL-6 plus sIL-6R. B: The quantification of OPG expression. All values are indicated by the average ±SD relative to the control from three independent experiments. *Significantly different from control ($p < 0.05$).

Effect on M-CSF

M-CSF was analyzed after modulated with IL-6 plus sIL-6R. The result (Fig. 12, 13) shows M-CSF expression does not change in any concentration.

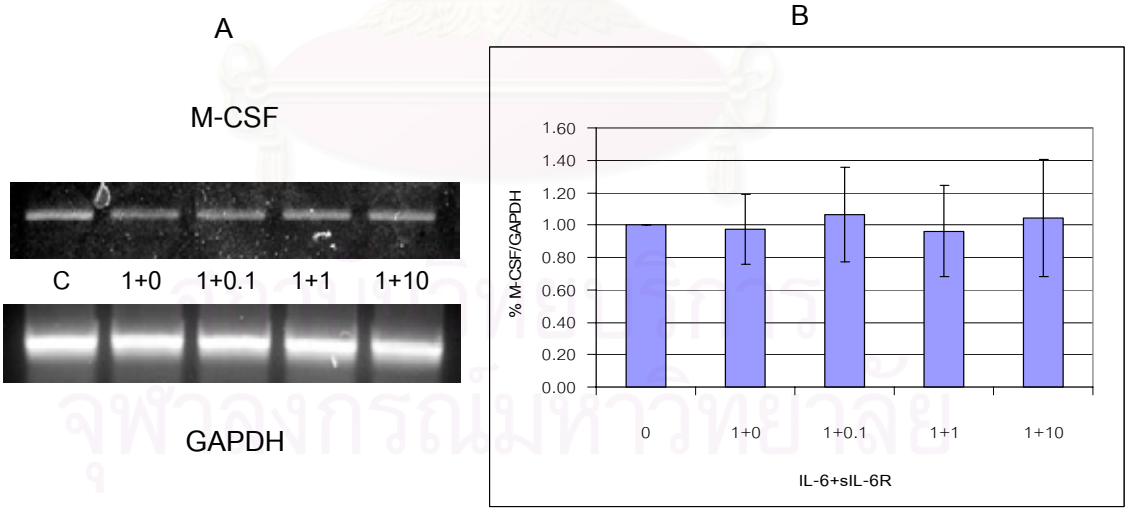


Figure 12 A: Semi-quantitative RT-PCR analysis of M-CSF mRNA expression in HDP modulated with IL-6 plus sIL-6R. B: The quantification of M-CSF expression which normalized to GAPDH. All values are indicated by the average ±SD relative to the control from three independent experiments. No significantly different from control ($p < 0.05$).

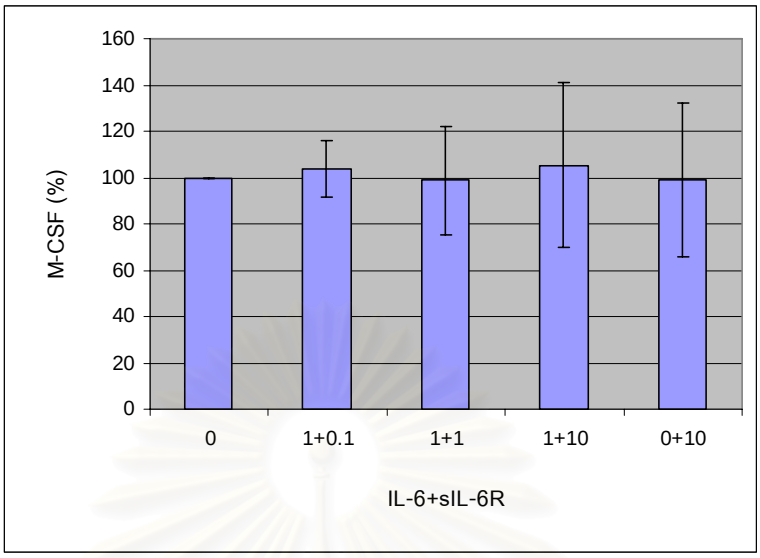


Figure 13 The quantification of M-CSF protein modulated by IL-6+sIL-6R. All values are indicated by the average ±SD relative to the control from three independent experiments. No significantly different from control ($p < 0.05$).

CHAPTER V

DISCUSSION AND CONCLUSION

The present study demonstrates that IL-6 can up-regulate RANKL formation in both mRNA and protein level in HDP cells. Furthermore, IL-6 had no effect on M-CSF and OPG production both in mRNA and protein level. Although, IL-6 had not changed the expression of OPG significantly in statistical analysis but from the trend from quantitative analysis, OPG level is likely reductive following increase of IL-6 concentration. The result may intimate the imbalance of RANKL/OPG on HDP cell modulated by IL-6. Imbalance of this complex may lead to the odontoclastogenesis which is an essential process in mineralized tissue resorption. Many investigations have been reported the effect of RANKL on mineralized tissue resorption. Abnormalities of this system have been implicated in the pathogenesis of some diseases. For example, Teng (Teng et al, 2000) showed increasing of RANKL stimulated by *A. actinomycetemcomitans* in periodontitis is the cause of alveolar bone resorption. In addition, Fukushima (Fukushima et al, 2002) showed the increasing of RANKL/OPG ratio during physiologic root resorption of deciduous teeth. Moreover, RANKL was found increasedly in systemic diseases that relate in bone resorption, i.e. Rheumatoid arthritis (Takayanagi et al, 1997), Multiple myeloma (Giuliani et al, 2001) and breast cancer (Kitazawa, 2002). This phenomenon might be used to explain the mechanism of internal root resorption in dental pulp due to increasing expression of RANKL on HDP cells in this experiment. IL-6 can adjust the balance RANKL/OPG ratio on HDP cells like does on other cells.

Although, M-CSF is an important molecule to support the clonal growth and differentiation of precursor cells of monocytes macrophage lineage (Kodama et al., 1991, Suda et al., 1999). However, in the present study IL-6 did not enhance the expression of M-CSF. The unchanged of M-CSF expression should not affect the activation of osteoclast because M-CSF may be released from inflammatory cells in

environment such as monocytes, macrophages, T lymphocytes, and B lymphocytes (Barreda et al, 2004 for review) during inflammation.

HDP cells that used in the present study have been investigate the expression of IL-6R by PCR method. The result showed HDP cells can express IL-6R (data shown in appendix). In addition, HDP cells can response to IL-6 by increasing in RANKL expression, so that HDP cells should possess IL-6R on their cell membrane. Recent evidence indicates that another form of IL-6R, sIL-6R, is generated via both cell surface proteolysis (shedding) and alternative mRNA splicing (Lust et al, 1992; Horiuchi et al, 1994). Even though, the IL-6R is not involved in signaling, soluble form of the IL-6R (sIL-6R) is still able to bind IL-6 and the complex of IL-6 and the sIL-6R activates target cells expressing gp130 in a process termed ‘‘transsignalling’’ (Rose-John and Heinrich, 1994). It has been shown that IL-6/sIL-6R complex could bind to membrane gp130 with high affinity. However the activity of the IL-6/sIL-6R complex is limited by the presence of a soluble form of gp130 (sgp130) which competes effectively with membrane bound gp130 for the sIL-6/IL-6 complex.

In general, the majority of known soluble cytokine, and hormone receptors act as an antagonist activity with its receptors. However, the function of soluble form of the IL-6R (sIL-6R), which lacks the transmembrane and cytoplasmic parts, is different; the sIL-6R shows agonistic activity when complexed with its ligand. Tamura et al (1993) suggests that increased circulating or locally produced sIL-6R induces osteoclast formation in the presence of IL-6. This is in agreement with investigations of Palmqvist (Palmqvist et al, 2002) that IL-6 does not stimulate releasing of mineral and bone matrix of bone explant unless sIL-6R is present. According to these results, I further investigate the combination effect of sIL-6 and IL-6 in HDP cells. In the experiment, sIL-6 was added in condition media with IL-6 in varying doses (0, 0.1, 1, 10 ng/ml). The outcome show the combination of IL-6 and sIL-6R resulted in significant up-regulate RANKL expression only in mRNA level. Interestingly, the result from western blot analysis indicated that RANKL and OPG protein was decreased in dose dependent manner. Moreover, addition of sIL-6R alone can decrease the level of RANKL expression. This result lead to a speculation that sIL-6R might act as an antagonistic mediator of IL-6 by

diminishing RANKL expression both background and IL-6 –induced condition on HDP cells. These resulted are in contrast with the others. The hypothesis of this phenomenon might be defined in some ways.

In support of previous researches (Bellido et al, 1998; O'Brien et al, 1999), this study found that sIL-6R act as up-regulator on RANKL expression in transcriptional process by increasing RANKL mRNA. Moreover, the results were in concurrent with the investigations of Palmqvist (Palmqvist et al, 2002) in neonatal mouse calvaria that IL-6 plus sIL-6R stimulate RANKL expression in mRNA level. However, when analyze in protein level, the result partially disagree with previous studies. The present result show IL-6 plus sIL-6R decrease both RANKL and OPG protein following increased of sIL-6R concentration. It may be implicated that sIl-6R act as an antagonistic agent of Il-6 on RANKL and OPG expression only in protein synthesis. sIL-6R might inhibit or dawdling the translational process of protein production of these proteins. However, others possible factors involved in this result should be proven in further study.

In case of mRNA stability, it is possible that sIL-6R may influent on mRNA stability after transcriptional process. The stability of mRNA might be blocked by binding of sIL-6R to the system. Thus, the protein levels are decreased due to degradation of mRNA following the increasing of sIL-6R concentration. However, the truth of this hypothesis should be investigated.

The result from the present study reveals that IL-6 increased the expression of RANKL in HDP cells. This finding may lead to the implication that increasing IL-6 level in dental pulp, following inflammatory process (Hosoya and Matsushima, 1996; Matsushima et al, 1998; O'Boskey and Panagakos, 1998), may affect on mineralized tissue resorption (root resorption) due to the imbalance of RANKL/OPG system. Moreover, the combination of IL-6 and sIL-6R act as antagonistic effect on RANKL and OPG expression in protein level. The antagonistic effect of sIL-6R may perform as inhibitors of odontoclastogenesis in dental pulp by disturbing the balance of RANKL/OPG ratio.

Conclusion

IL-6 can increase RANKL expression in both mRNA and protein level but no effect on OPG and M-CSF. However, when adding sIL-6R in the experiment, the result show sIL-6R can up-regulate RANKL expression only in mRNA level. When analyze in protein level, the result indicate that combination of IL-6 and sIL-6R down-regulate RANKL and OPG protein in dose dependent manner.

Further studies

1. To study the effect of HDP cells stimulated by IL-6 on osteoclastogenesis both differentiation and activation.
2. To study mechanism of sIL-6R as a down-regulator on RANKL and OPG protein synthesis.

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