


รายงานวิจัยฉบับสมบูรณ์

โครงการความแตกต่างในความสามารถของของเซลล์สร้างกระดูกที่ได้จากกระดูกขากรรไกรและกระดูกสะโพกในการเกื้อหนุนการเกิดเซลล์ทำลายกระดูก

โดย

นางสาววรรณกร ศรีอาจ

เดือนกรกฎาคม ปี2561 ที่เสร็จโครงการ

สัญญาเลขที่ MRG5580139

รายงานวิจัยฉบับสมบูรณ์

โครงการความแตกต่างในความสามารถของของเซลล์สร้างกระดูกที่ได้จากกระดูกขากรรไกรและกระดูกสะโพกในการเกื้อหนุนการเกิดเซลล์ทำลายกระดูก

ผู้วิจัย

นางสาววรรณกร ศรีอาจ

สังกัด

คณะทันตแพทยศาสตร์

จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

Abstract

Project Code : MRG5580139

(รหัสโครงการ)

Project Title : โครงการความแตกต่างในความสามารถของของเซลล์สร้างกระดูกที่ได้(ชื่อโครงการ) จากกระดูกขากรรไกรและกระดูกสะโพกในการเกื้อหนุนการเกิดเซลล์ ทำลายกระดูก

Investigator : นางสาววรรณกร ศรีอาจ

(ชื่อนักวิจัย) คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

E-mail Address : w.sriarj@gmail.com

Project Period : 2 ปี

(ระยะเวลาโครงการ)

Abstract

Cytokines play important roles in bone remodeling by modulating the balance between bone formation and resorption. In mouse model, Interleukin-4(IL- 4) has been shown to inhibit osteoclast formation. However, the effect of IL-4 on human osteoblast is still unknown. Our study showed osteoblast-liked cells expressed both IL-4 receptor and STAT6, the main intermediate signaling molecules in the IL-4 pathway. The results prompt us to investigate the influence of IL-4 on osteoclastogenesis by determining the expression of RANKL/OPG in human osteoblast-like cells. Mandible-derived osteoblast-liked cells were established. The expression of RANKL/OPG mRNA was investigated using real-time RT-PCR. The results indicated IL-4 suppressed the expression of RANKL/OPG dose-dependently. The mechanism of IL-4- modulated RANKL/OPG expression was studied by means of inhibitors including STAT6 inhibitor (AS1517499), NF-kB inhibitor and PI3K inhibitor. The addition of STAT6 inhibitor as well as NF-kB inhibitor, but not PI3K inhibitor could abolish the effect of IL-4. Interestingly, addition of $CoCl_2$, an activator of HIF-1α, abolished the inductive effect of IL-4 on OPG expression indicating the influence of oxygen level. These findings suggested the role of IL-4 on osteoclastogenesis by suppressing RANKL/OPG ratio via STAT6 and NF-kB pathways and might be influenced by the oxygen level in the tissue.

Keywords : Interleukin-4, Osteoblasts, Osteoclastogenesis, RANKL/OPG expression

Introduction

The bone remodeling is the process of bone turnover, resulting from the action of osteoblasts and osteoclasts in response to microfracture, hormone, etc. In physiological condition, the formation and the resorption of bone are balanced. Any factors that interfered with this balance will resulted in pathological condition or disease; periodontal disease, osteoporosis, for example.

Osteoclasts are multinucleated giant cells directly responsible for bone resorption. The two major osteoblast-derived factors that play a pivotal role in the regulation of osteoclastogenesis are receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG). RANKL is expressed on the plasma membrane of osteoblasts/stromal cells, and binds to receptor activator of nuclear factor kappa-B (RANK) expressed on osteoclast progenitor cells, inducing osteoclast differentiation and stimulates osteoclast function (Fuller, Wong et al. 1998). In contrast, OPG acts as a decoy receptor for RANKL and inhibits osteoclastogenesis by blocking the formation of RANK/RANKL complex (Matsuzaki, Udagawa et al. 1998, Shiotani, Takami et al. 2002). Therefore, osteoblasts regulate osteoclast differentiation and activation through the balance between RANKL and OPG expression levels. From clinical evidence, an increase of the physiologic RANKL/OPG ratio has been correlated with pathologic bone resorption, as seen in osteoporosis and periodontal disease. It is reported that postmenopausal women, who have estrogen deprivation, undergo osteoporosis due to an increase in the RANKL/OPG ratio, which consequently stimulates osteoclast formation and bone resorption (Giner, Rios et al. 2009).

The osteoblast lineage composes of osteoblast, osteocyte and bone lining cells. Unlike osteoclast, osteoblast is originated from mesenchymal cells. Both osteoblasts and bone lining cells will align along the bone surfaces and connect with osteocytes through the canalliculi, a tiny tubule that connect all the lacunae together. When bone is loaded with force, the change of fluid flow in canalliculi is perceived by osteocyte, causing the release of certain signaling molecules. The signals generate from osteocytes will trigger either bone formation or resorption depending on the amount and

type of forces. Therefore, osteoblasts and bone lining cells in concert with osteocytes play an important role in bone remodeling by regulating the osteoclastogenesis (Katagiri and Takahashi 2002).

Previous study showed that cytokines play important roles in bone remodeling by modulating the balance between bone formation and resorption. In mouse model, IL-4 has been shown to inhibit osteoclast formation (Shioi, Teitelbaum et al. 1991, Bizzarri, Shioi et al. 1994). However, the effect of IL-4 on human osteoblast is still unknown. Our study showed human osteoblast-liked cells expressed both IL-4 receptor and STAT6, the main intermediate signaling molecules in the IL-4 pathway (Abu-Amer 2001). This prompts us to investigate the influence of IL-4 on the expression of RANKL and OPG, two key molecules that regulate osteoclastogenenesis in human osteoblast-like cells. In addition, the influence of oxygen level on the effect of IL-4 will also be examined.

The information obtained from this study will further our understanding of the bone resorption process and aid in developing alternative treatments for pathologic bone resorption in the future.

Objectives

This study aimed to investigate the effect of IL-4 on RANKL/OPG expression in human osteoblast-liked cells

Materials and Methods

Sample collection

Human mandibular osteoblasts (hMOBs) were obtained as previously described (Khonsuphap, Pavasant et al. 2017). The protocol was approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University, Thailand. After obtaining informed consent, mandibular alveolar bone was collected from tooth extraction sites of healthy patients aged 25–35 years at the Department of Surgery, Faculty of Dentistry, Chulalongkorn University. Only alveolar bone from extraction sites without infection and inflammation was collected for this study. The alveolar bone was kept in a sterile tube

containing culture medium, which was composed of 1000 mg/L glucose Dulbecco's Modified Eagle's Medium (Gibco, BRL, Carlsbad, CA) containing 15% (v/v) fetal bovine serum (HyClone, Thermo Scientific, Logan, UT), 2 mM L-glutamine (Gibco), 100 units/ml Penicillin (Gibco), 100 μg/ml Streptomycin (Gibco), and 5 μg/ml Amphotericin B (Gibco).

Osteoblast culture

The obtained alveolar bone was washed with sterile phosphate buffered saline extensively to wash out the blood and any soft tissue was removed using a surgical blade. The pieces of bone were seeded in 35 mm culture dishes (Corning, New York, NY). The explants were cultured in culture medium and maintained in 37°C with 5 % CO_2 and 100% humidity incubator.

The medium were changed every other day until the cells migrate out to culture dishes until cells reach confluency. The subculture was done with 1:3 ratio. Cells were used from passage 3-8 and the experiment were performed with cells established from at least 3 lines from 3 patients. (The age of the patients was limited to 25 year-old maximum)

Osteogenic Differentiation of Bone Cell Culture

To examine the osteogenic differentiation, the cells were seeded at a density of 25,000 cells/wells in a 24-well-plate and maintained in an osteogenic medium [growth medium supplemented with ascorbic acid (50 mg/ml), dexamethasone (100 nM), and b-glycerophosphate (10 mM)]. In the test wells, basic FGF (20 ng/ml) was added in the culture medium, untreated wells served as controls. The medium was changed every 48 h. Alkaline phosphatase, osteoblast marker gene expression, and calcium deposition were investigated using the methods described below.

In Vitro Mineralization Assay

hMOBs were fixed with cold methanol for 10 min, washed with deionized water, and stained with 1% Alizarin Red S(Sigma-Aldrich) solution for 3 minutes at room

temperature on a shaker. Excess stain was removed with deionized water, and the images were obtained.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

hMOBs were seeded in 12-well plates at a density of $1x10^5$ cells/well and incubated in culture media. The cells were treated with or without 1ng/ml of interleukin 4 (IL-4). To mimic hypoxia condition, hMOBs were cultured with 200μM of CoCl2. Total RNA was extracted with RiboEx™ lysis reagent (Gene all Biotechnology Co., Ltd, Seou l, Korea) accordingto the manufacturer's instructions. One microgram of mRNA from each sample was reverse transcribed to cDNA using Improm-II™ (Promega). Subsequently, qRT -PCR was performed using the miniOptic on Real-Time PCR Detection System (Bio-Rad, Singapore) with the FastStart Essential DNA Green Master kit (Roche Diagn ostic s, Indianapolis, IN). The PCR protocol was: denaturation at 94°C for 10 s, annealing at 60°C for 10 s, and extens ion at 72°C for 10 s for 45 cycles. The The oligonucleotide sequences of the primers used in this study are shown in Table 1. Bio-Rad CFC Manager3.1 (Bio-Rad) was used to determine gene expression. The expression of ALP, RANKL, OPG, IL-6, VEGF and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were studied. Gene expression was normalized to the GAPDH. For hypoxic condition, 18S was used as internal control. The data is shown as gene expression relative to control

TABLE 1. RT-PCR Primer Sequences

Gene	Forward primer Reverse primer
ALP	F: CGAGATACAAGCACTCCCACTTC R: CTGTTCAGCTCGTACTGCATGTC
OPG	F: TCAAGCAGGAGTGCAATCG R: AGAATGCCTCCTCACACAGG
RANKL	F: ATACCCTGATGAAAGGAGGA R: GGGGCTCAATCTATATCTCG
IL-6	F: CGCCCCACACAGACAGCCAC R: AGCTTCGTCAGCAGGCTGGC
VEGF	F: CAAGGCCAGCACATAGGAGA R: GGTGGGTGTGTCTACAGGAA
GAPDH	F: TGAAGGTCGGAGTCAACGGAT R: TCACACCCATGACGAACATGG
18S	F: GGCGTCCCCCAACTTCTTA R: GGGCATCACAGACCTGTTATT

Results

Osteoblast obtained from the trabecular bone of mandible was cultured in growth medium as describe previously. Cells were characterized by examining morphology and osteogenic differentiation. Fig. 1 showed the morphology of osteoblast-like cells derived from mandible (a). When cells were cultured in osteogenic medium for 14 days, in vitro calcification could be observed determining by ALP (b) and alizarin red S staining (c).

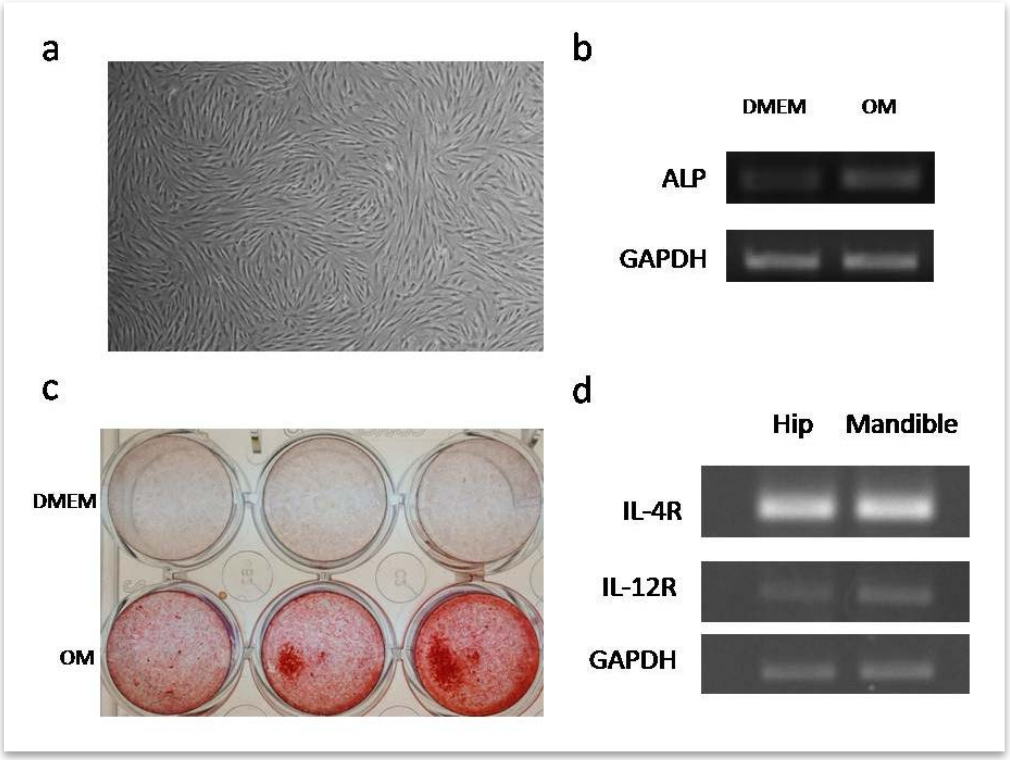

Figure 1 Micrograph showed osteoblast-like cells derived from mandible in culture (a). The expression of *ALP* (b) and alizarin red S staining (c) were used to determined osteogenic differentiation potential. The expression of IL-4R and IL-12R were determined using RT-PCR (d).

Next, we investigated the expression of interested genes from mandible-derived osteoblasts-liked cells using PCR array. The results showed that osteoblasts expressed STAT6, the main intermediate signaling molecules in interleukin-4 (IL-4) pathways (figure 1d). It is very interesting to reveal that osteoblasts expressed these receptors. Indeed, IL-4R has been reported to involve the balance of immune system. Therefore,

this finding leads to the question whether and how these receptors function in bone biology. There are only few reports showed the role of IL-4 in bone metabolism (Kitaura et al., 2002; Fujii et al., 2012). However, the exact function of these two cytokines in bone is still unclear and needs further clarification.

For the present study, the effect of IL-4 on the expression of RANKL/OPG was first examined. Because IL-4 involved the immune system, we hypothesized that this cytokines might implicate in the control of the expression of RANKL and OPG, the key factors in osteoclastogenesis.

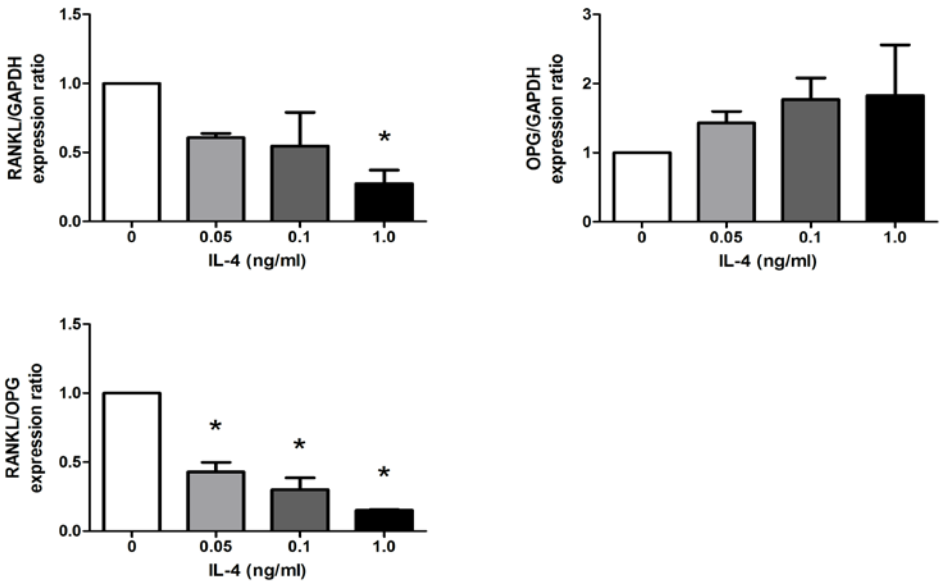

Figure 2 Exogenous IL-4 suppressed the RANKL/OPG expression ratio in a dose dependent manner. GAPDH was used as internal control. (*$p < 0.05$)

As shown in Fig. 2, the addition of IL-4 suppressed the RANKL/OPG expression ratio dose-dependently corresponds with the previous report (Fujii, Kitaura et al. 2012). The reduction of RANKL/OPG ratio should affect the formation of osteoclast. However, the results need further clarification.

The detailed mechanism of IL-4-modulated the RANKL/OPG expression in osteoblasts was studies by means of inhibitor. Since the main signaling pathway of IL-4 involved STAT6, the STAT6 inhibitor (STAT6i) was first selected for the study. The

optimal concentration of STAT6i inhibitor was chosen according to the preliminary study. In the present study, osteoblast-like cells were treated with the concentration of 40nM and 80nM of STAT6i respectively in the presence or absence of 1ng/ml of IL-4. The expression of RANKL/OPG was determined as shown in Fig.3. The results showed STAT6i could rescue the effect of IL-4 decreased RANKL/OPG expression in osteoblast-like cells from mandible.

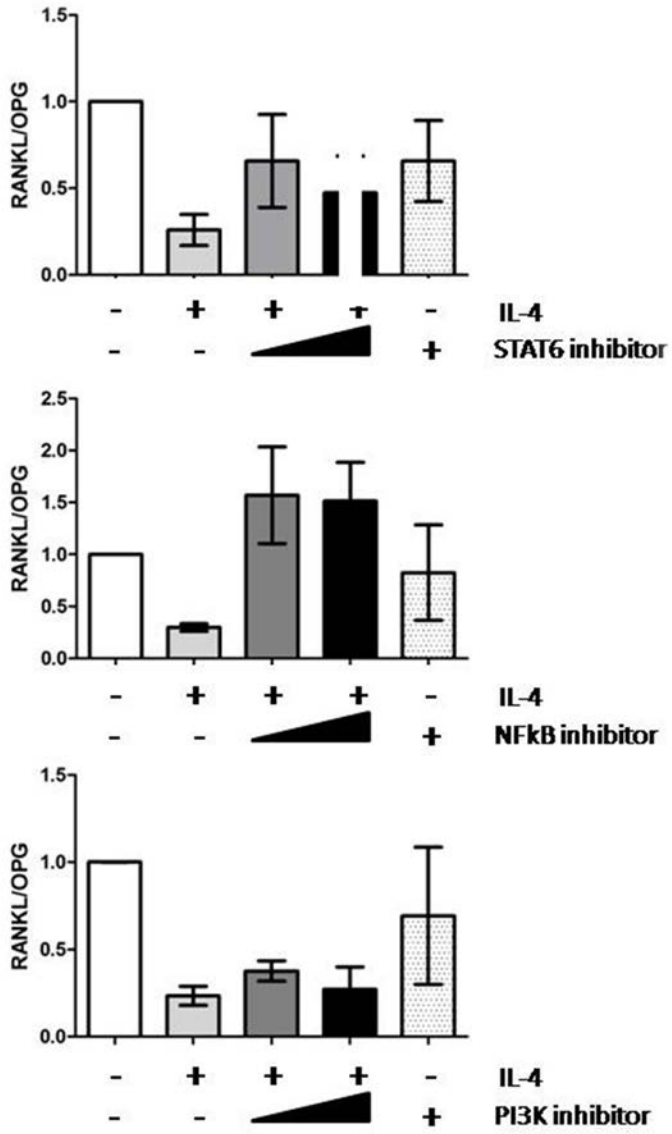

Figure 3 The expression of RANKL/OPG was determined by real-time RT-PCR. The addition of STAT6 inhibitor as well as NF-kB inhibitor, but not PI3K inhibitor could abolish the effect of IL-4.The level of GAPDH was used as internal control.

From clinical evidences, the oxygen level could modulate pathological mechanism in several diseases such as periodontitis, arthritis, or cancer. In the present study, the level of RANKL/OPG mRNA expression was increased in hypoxic condition (fig.4a). Apigenin, the inhibitor of CoCl2, was used to determine the role of oxygen level on the expression of RANKL/OPG. The results showed apigenin can reverse the effect of CoCl2 (fig. 4b).

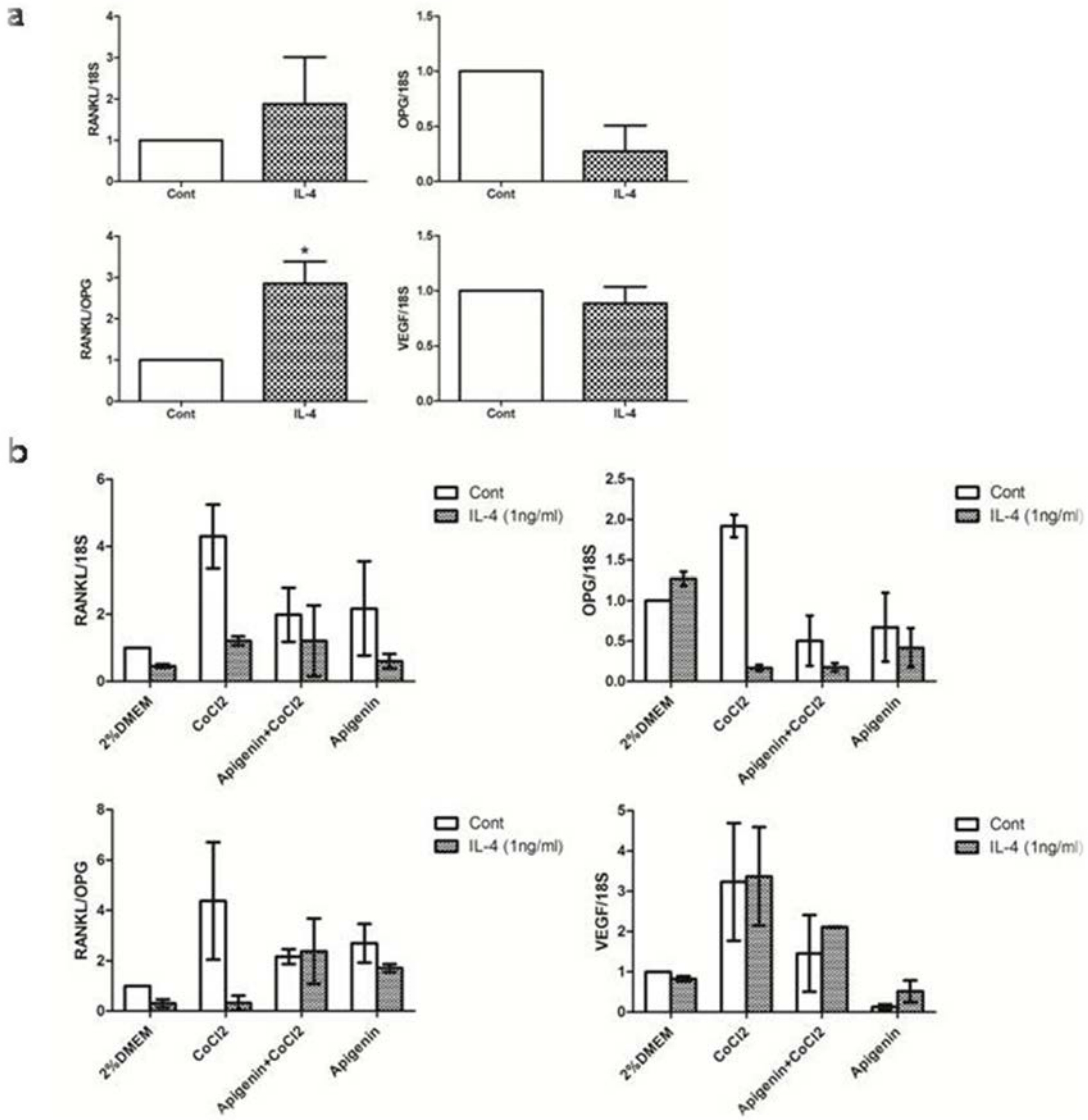

Figure 4 The effect of oxygen level on RANKL/OPG expression. The addition of CoCl2 (200μM) was used to mimics hypoxia. (a) Hypoxic condition promotes the expression of RANKL, while suppresses OPG, consequently the expression of RANKL/OPG was increased. (b) Apigenin, the inhibitor of CoCl2, was used to determine the role of oxygen level on the expression of RANKL/OPG. The expression of VEGF was used as a positive control.

Discussions and Conclusions

The role of IL-4 in bone metabolism was studied extensively. In mouse model, Il-4 was shown to play a role in bone remodeling by inhibiting bone resorption. The detailed mechanism of IL-4-modulated the RANKL/OPG expression in osteoblasts, which is important molecules in bone remodeling, was studies by means of inhibitor. We found that the addition of STAT6 inhibitor as well as NF-kB inhibitor, but not PI3K inhibitor could abolish the effect of IL-4. These findings suggested that IL-4 might be modulate the RANKL/OPG expression via STAT6 and NF-kB.

However, the influence of hypoxia in the function of Il-4 is less known. Hypoxia could modulate pathological mechanism in several diseases such as periodontitis, arthritis, or cancer. The results indicated IL-4 suppressed the expression of RANKL/OPG via STAT6 and NF-kB. Interestingly, addition of $CoCl_2$, an activator of HIF-1α, abolished the inductive effect of IL-4 on OPG expression indicating the influence of oxygen level. The results suggested the role of IL-4 on osteoclastogenesis might be influenced by the oxygen level in the tissue. The findings suggest the new insight to look at the bone remodeling and destruction. However, the detailed mechanisms were still needed to be clarified.

Acknowledgements

This work was supported by the Thailand Research Fund (TRF) and the Grants for Development of New Faculty Staff (to WS), Chulalongkorn University

References

1. Abu-Amer, Y. (2001). "IL-4 abrogates osteoclastogenesis through STAT6-dependent inhibition of NF-kappaB." J Clin Invest **107**(11): 1375-1385.
2. Bizzarri, C., A. Shioi, S. L. Teitelbaum, J. Ohara, V. A. Harwalkar, J. M. Erdmann, D. L. Lacey and R. Civitelli (1994). "Interleukin-4 inhibits bone resorption and acutely increases cytosolic Ca2+ in murine osteoclasts." J Biol Chem **269**(19): 13817-13824.
3. Fujii, T., H. Kitaura, K. Kimura, Z. W. Hakami and T. Takano-Yamamoto (2012). "IL-4 inhibits TNF-alpha-mediated osteoclast formation by inhibition of RANKL expression in TNF-alpha-activated stromal cells and direct inhibition of TNF-alpha-activated osteoclast precursors via a T-cell-independent mechanism in vivo." Bone **51**(4): 771-780.
4. Fuller, K., B. Wong, S. Fox, Y. Choi and T. J. Chambers (1998). "TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts." J Exp Med **188**(5): 997-1001.
5. Giner, M., M. A. Rios, M. A. Montoya, M. A. Vazquez, L. Naji and R. Perez-Cano (2009). "RANKL/OPG in primary cultures of osteoblasts from post-menopausal women. Differences between osteoporotic hip fractures and osteoarthritis." J Steroid Biochem Mol Biol **113**(1-2): 46-51.
6. Katagiri, T. and N. Takahashi (2002). "Regulatory mechanisms of osteoblast and osteoclast differentiation." Oral Dis **8**(3): 147-159.
7. Khonsuphap, P., P. Pavasant, R. A. Irwandi, C. Leethanakul and A. Vacharaksa (2017). "Epithelial Cells Secrete Interferon-gamma Which Suppresses Expression of Receptor Activator of Nuclear Factor Kappa-B Ligand in Human Mandibular Osteoblast-Like Cells." J Periodontol **88**(3): e65-e74.
8. Matsuzaki, K., N. Udagawa, N. Takahashi, K. Yamaguchi, H. Yasuda, N. Shima, T. Morinaga, Y. Toyama, Y. Yabe, K. Higashio and T. Suda (1998). "Osteoclast differentiation factor (ODF) induces osteoclast-like cell formation in human peripheral blood mononuclear cell cultures." Biochem Biophys Res Commun **246**(1): 199-204.
9. Shioi, A., S. L. Teitelbaum, F. P. Ross, H. G. Welgus, H. Suzuki, J. Ohara and D. L. Lacey (1991). "Interleukin 4 inhibits murine osteoclast formation in vitro." J Cell Biochem **47**(3): 272-277.
10. Shiotani, A., M. Takami, K. Itoh, Y. Shibasaki and T. Sasaki (2002). "Regulation of osteoclast differentiation and function by receptor activator of NFkB ligand and osteoprotegerin." Anat Rec **268**(2): 137-146.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. **ยังไม่มีผลงานตีพิมพ์**