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Research paper

# Interleukin-17A induces cathepsin K and MMP-9 expression in osteoclasts via celecoxib-blocked prostaglandin $E_2$ in osteoblasts

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ABSTRACT

Interleukin-17 (IL-17) is a cytokine secreted primarily by $T_H$-17 cells that can stimulate the development of osteoclasts (osteoclastogenesis) in the presence of osteoblasts. IL-17, through osteoblasts, has indirect effects on the expression of bone resorption-related enzymes in osteoclasts, which have not been well clarified. Here, using MC3T3-E1 cells and RAW264.7 cells as osteoblasts and osteoclast precursors, we aimed to clarify these effects of IL-17A. MC3T3-E1 cells were cultured in the presence or absence of IL-17A for 72 h and the conditioned media collected (in the presence of soluble receptor activator of NF-κB ligand) and used to culture RAW264.7 cells. To assess osteoclast differentiation, adherent cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). Our analyses demonstrated that the number of TRAP-positive multinucleated cells increases after 3 days of culture in conditioned medium from IL-17A-treated cells compared to untreated controls. In addition, we observed that the levels of cathepsin K and MMP-9 increase in the conditioned medium from IL-17A-treated cells, whereas CA II expression levels remain unaffected. $PGE_2$ production from MC3T3-E1 cells increased in the presence of IL-17A. Celecoxib, a specific inhibitor of cyclooxygenase-2 (COX-2), blocked both the IL-17A-stimulated increase in TRAP-positive multinucleated cells and the expression of cathepsin K and MMP-9. Furthermore, when MC3T3-E1 cells were transformed with small interfering RNA to silence COX-2 expression before IL-17A treatment, the resulting conditioned medium was less effective at inducing cathepsin K and MMP-9 expression in RAW264.7 cells. These results suggest that IL-17A induces the differentiation and function of osteoclasts via celecoxib-blocked prostaglandin, mainly $PGE_2$, in osteoblasts.



## 1. Introduction

In the normal skeleton, there exists a closely coordinated process of balanced osteoclast-mediated bone resorption and osteoblast-mediated bone formation that counteract and contribute to the constant remodeling of bone tissue. When this balance is disrupted and favors resorption, inflammation-mediated bone diseases including chronic periodontitis, rheumatoid arthritis, and osteoarthritis arise. This imbalance is caused by the increase of various cytokines in the inflammatory tissue [1].

Osteoblasts are involved in osteoclast differentiation. Osteoclast formation requires the presence of RANKL (receptor activator of nuclear factor κβ ligand) and M-CSF (macrophage colony-stimulating factor). These membrane-bound proteins are produced by neighboring stromal cells and osteoblasts, thus requiring direct contact between these cells and osteoclast precursors [2]. The expression of RANKL in bone marrow stromal cells or osteoblasts is enhanced by 1α,25-dihydroxyvitamin $D_3$, parathormone, prostaglandin $E_2$ ($PGE_2$), and interleukin (IL)-11 [3–5]. M-CSF expression in bone marrow

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stromal cells or osteoblasts is enhanced by $PGE_2$, whereas the expression of osteoprotegerin (OPG), a decoy receptor for RANKL, decreases [3,4]. In addition, the expression of M-CSF is reportedly enhanced via $PGE_2$ produced by nicotine and lipopolysaccharide-stimulated osteoblasts [6].

Osteoclasts are large specially differentiated multinuclear cells of a monocyte/macrophage lineage, which decompose bone matrix such as hydroxyapatite and type I collagen by producing various proteinases and hydrogen ions ($H^+$). At a site of active bone resorption, osteoclasts form a specialized cell membrane, the "ruffled border," which touches the surface of the bone tissue. Mature osteoclasts secrete hydrogen ions and proteinases such as cathepsin K and matrix metalloproteinase (MMP)-9 from this ruffled border. Hydrogen ions are produced via carbonic anhydrase II (CA II) in the cytoplasm and are secreted extracellularly by $H^+$-ATPases [7]. The expression of cathepsin K, MMP-9, and CA II in RAW264.7 cells has been reported to be induced by RANKL, but not M-CSF, in the presence of IL-1α [8].

IL-17 is produced exclusively by activated T cells [9] or neutrophils [10], and plays an important role in numerous autoimmune and inflammatory diseases [11,12]. Several studies have indicated that IL-17 is a pro-inflammatory cytokine crucial for osteoclastic bone resorption in the presence of osteoblasts [13–15]. IL-17 has been shown to promote osteoclast differentiation indirectly through the induction of IL-1, tumor necrosis factor-α, and RANKL expression. Blocking IL-17 function with neutralizing antibodies reduces synovial T-cell infiltration and bone erosion in the collagen-induced arthritis model and blocks osteoclast formation in bone marrow cell/osteoblast co-culture [16,17].

Recently, we examined the direct effects of IL-17A on the differentiation of osteoclast precursors into osteoclasts and on the function of osteoclasts using RAW264.7 cells as osteoclast precursors. The findings indicated that the differentiation is suppressed at high concentrations of IL-17A, and that IL-17A suppresses the hydrolysis of matrix proteins during bone resorption by decreasing the production of cathepsin K and MMP-9 in mature osteoclasts [18]. However, the details of the indirect effects of IL-17A via osteoblasts on the expression levels of these bone resorption-related enzymes of mature osteoclasts were not clarified.

Here, we aimed to elucidate the indirect effects of IL-17A using MC3T3-E1 cells and RAW264.7 cells as osteoblasts and osteoclast precursors. MC3T3-E1 cells were cultured in the presence or absence of IL-17A and/or celecoxib (a cyclooxygenase (COX)-2 inhibitor), and the collected medium was diluted and used as a conditioned medium in the presence of soluble RANKL. Our analyses demonstrated that in cells treated with conditioned medium, the number of multinucleated cells and the expression of both cathepsin K and MMP-9 increase, whereas CA II expression remains unaffected. In addition, $PGE_2$ production from MC3T3-E1 cells increases in the presence of IL-17A. When celecoxib was added, both the increase in multinucleated cells and the expression of cathepsin K and MMP-9 were blocked. Furthermore, when MC3T3-E1 cells were transformed with small interfering RNA (siRNA) to silence COX-2 expression before IL-17A treatment, the resulting conditioned medium was less effective at inducing cathepsin K and MMP-9 expression in RAW264.7 cells. Thus, the current study indicates that IL-17A indirectly induces the differentiation and function of osteoclasts via a COX-2-dependent prostaglandin, mainly $PGE_2$, in osteoblasts.

## 2. Materials and methods

### 2.1. Cell culture

Mouse calvarial cell lines (MC3T3-E1) obtained from the RIKEN BioResource Center (Tsukuba, Japan) were used as osteoblasts. These cells were established from newborn mouse calvaria [19] and displayed a typical osteoblastic phenotype [4,20–22]. Murine monocyte/macrophage cell lines (RAW264.7) used as osteoclast precursors [23] were obtained commercially (Dainippon Pharmaceutical, Osaka, Japan). RAW264.7 cells differentiate into osteoclast-like cells in the presence of RANKL, and RANKL is indispensable to osteoclast differentiation of RAW264.7 cells [8,24]. All cells were maintained in α-minimal essential medium (α-MEM; Gibco BRL, Rockville, MD, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA) at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$.

### 2.2. IL-17A and/or celecoxib treatments

For treatments with IL-17A (R&D Systems, Minneapolis, MN, USA) and/or celecoxib (Astellas Pharma, Tokyo, Japan), MC3T3-E1 cells were seeded onto 100-mm cell culture dishes at a density of $2 \times 10^4$ cells $cm^{-2}$ and left overnight to settle. The cells were then cultured for up to 72 h in α-MEM containing 10% (v/v) FBS with 0, 0.1, 1.0, or 10 ng $mL^{-1}$ IL-17A [12,20–22,25,26] alone or in the presence of 10 μM celecoxib [27,28].

### 2.3. Conditioned medium

MC3T3-E1 cells were cultured in the presence or absence of 10 ng $mL^{-1}$ IL-17A and/or 10 μM celecoxib for 72 h. The cell culture medium was changed to α-MEM without IL-17A and/or celecoxib, and the cells cultured for a further 24 h. Each sample of culture medium collected was diluted to 30% and supplemented with 50 ng $mL^{-1}$ of soluble RANKL [6,8,29] (Wako Pure Chemical Industries, Osaka, Japan) except for "2.7. Observation of resorption pits" in materials and methods.

### 2.4. Tartrate-resistant acid phosphatase (TRAP) staining

RAW264.7 cells were plated into 96-well microplates at a density of $1.25 \times 10^4$ cells $cm^{-2}$ and left overnight to settle. Conditioned medium containing 50 ng $mL^{-1}$ of soluble RANKL was then added to the cells for up to 10 days. Cells were then fixed and stained on days 3, 5, 7, and 10 of culture using a TRAP staining kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The number of osteoclast-like cells per well was then counted (TRAP-positive cells with more than three nuclei were considered to be osteoclast-like). Each experiment was performed in triplicate.

### 2.5. Real-time PCR

RAW264.7 cells were plated into 6-well microplates at a density of $1.25 \times 10^4$ cells $cm^{-2}$ and cultured for up to 10 days in the conditioned medium containing 50 ng $mL^{-1}$ of soluble RANKL. Total RNA was isolated from the cultured cells on days 3, 5, 7, and 10 of culture using an RNeasy mini kit (Qiagen, Valencia, CA, USA). The mRNA was reverse transcribed into cDNA using an RNA PCR kit (GeneAmp; PerkinElmer, Branchburg, NJ, USA), and the resultant cDNA was subjected to real-time PCR using the SYBR Green I dye. The reactions were performed in 25 μL of a SYBR® premixed Ex Taq™ solution (Takara Bio) containing 20 μM sense and antisense primers (Table 1). The primers were designed using the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). The PCR assays were performed on a Smart Cycler (Cepheid, Sunnyvale, CA, USA) and analyzed using the Smart Cycler software. The PCR protocol for cathepsin K, MMP-9, and CA II consisted of 35 cycles at 95 °C for 5 s and 60 °C for 20 s. All real-time PCR experiments were performed in triplicate, and the specificity of the PCR products was verified by melting curve analysis. Calculated