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IL-17A inhibits osteoclast differentiation of RANKL-stimulated RAW264.7 cells by suppressing JNK phosphorylation and c-Fos expression

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Periodontitis is a chronic inflammatory disease characterized by alveolar bone resorption. Inflammation-mediated bone loss is a major cause of various bone diseases, such as chronic periodontitis, and is due to an imbalance in bone remodeling that favors resorption. This imbalance is caused by increased inflammatory cytokines. Interleukin-17A (IL-17A) is a proinflammatory cytokine that is mainly secreted by activated T cells. IL-17A stimulates osteoclastic bone resorption via osteoblasts by inducing the expression of the receptor activator of NF-κB ligand (RANKL). However, little is known about the direct effects of IL-17A on the osteoclast precursors.

We confirmed that IL-17A suppresses the osteoclast differentiation of RAW264.7 cells in the presence of RANKL in a dose-dependent manner. We also found that treatment with SP 600125, a specific inhibitor of c-Jun N-terminal kinase (JNK), significantly inhibits the TRAP activity of RAW264.7 cells, which were stimulated by RANKL. In addition, we found that IL-17 A reduces the phosphorylation of JNK and expressions of c-Fos, which were increased by RANKL stimulation. These results suggest that IL-17A-induces inhibition of JNK phosphorylation and that expression of c-Fos may be one of the factors that suppresses the differentiation of osteoclast precursors into osteoclasts. (J Osaka Dent Univ 2014 ; 48 : 117–123)

Key words : Interleukin-17A (IL-17A) ; Osteoclast differentiation ; RAW264.7 cells

INTRODUCTION

Periodontitis is an infection-driven chronic inflammatory disease characterized by periodontal pocket formation and alveolar bone resorption. It is one of the most common chronic inflammatory diseases in aged populations and affects almost 90% of the population.[1] A balance between bone resorption by osteoclasts and bone formation by osteoblasts determines the level of bone mass.[2] Inflammation-mediated bone loss is a major feature of various bone diseases, including chronic periodontitis, rheumatoid arthritis, and osteoarthritis, and is caused by an imbalance in bone remodeling that favors resorption. This imbalance is caused by increased cytokines and mediators in the inflamed tissue.[3] Inflammatory cytokines produced by immunoregulatory cells regulate the immune responses to periodontal bacteria and play a protective and/or destructive role in disease progression.[4] However, the mechanisms of periodontal bone resorption remain to be established.

Bone remodeling is a physiological process that involves bone formation and resorption. The two major cell types responsible for bone formation and resorption are osteoblasts and osteoclasts.[5] Bone homeostasis results from tightly regulated activities of bone-forming osteoblasts and bone-resorbing osteoclasts. Therefore, the balance between these two cell types is important for maintaining bone mass, and the disruption of this relationship leads to bone disorders such as osteoporosis, rheumatoid arthritis, and periodontal disorders.[6] Usually, these pathological diseases are characterized by over formation and/or activation of osteoclasts. Osteoclasts are multinucleated giant cells formed by the fusion of monocyte/macrophage precursors, which are derived from hema-

topoietic progenitors. Two soluble factors are essential cytokines for osteoclast development : macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor-κB (NF-κB) (RANK) ligand (RANKL).[7, 8] RANKL induces the signaling essential for precursor cells to differentiate into osteoclasts.[9] M-CSF provides the proliferation of osteoclast precursor cells, maintains their survival and stimulates the expression of RANK.[10, 11]

Binding of RANKL with its receptor RANK induces the activation of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), which is linked to the NF-κB and c-Jun N-terminal kinase (JNK) pathways.[12] In addition, by an unclear mechanism, RANKL induces c-Fos expression. Furthermore, calcium signaling initiated by RANKL and its costimulatory receptor is indispensable for the induction of nuclear factor of activated T cells (NFAT) c1, a master gene of osteoclast differentiation.[13] Previous studies have shown that the coupling of c-Jun and c-Fos with the NFAT family is important for transcriptional events during osteoclastogenesis.[14–16] The activation of mitogen-activated protein kinases (MAPKs) results in the phosphorylation of c-Jun and its association with c-Fos to form the essential activator protein-1 (AP-1) transcription factor also involved in NFATc1 induction.[17–19] NFATc1 then regulates the transcription of several target genes such as calcitonin receptor (CTR), tartrate resistant acid phosphatase (TRAP), matrix metalloproteinase 9 (MMP9) or cathepsin K, which lead to the formation of bone resorption pits during osteoclast differentiation.[20]

Interleukin-17A (IL-17A) is a proinflammatory cytokine that is mainly secreted by activated T cells.[21] However, it has recently been reported that IL-17A can also be produced by several other innate immune cell types, such as lymphoid tissue inducer cells, natural killer and natural killer T cells, macrophages and Paneth cells.[22] It has been reported that IL-17A induces RANKL production by osteoblasts.[23] In addition, recent evidence has indicated that IL-17A stimulates RANKL gene expression in osteoblasts and induces osteoclast differentiation in co-cultures of osteoblasts and bone marrow cells.[7] Thus, IL-17A is a crucial cytokine for osteoclastogenesis via the RANK-RANKL system.

However, the direct effects of IL-17A on the differentiation of osteoclasts and on the function of osteoclasts remains unclear. In this study, we attempted to clarify whether and how IL-17A affects RANKL-induced osteoclast differentiation in RAW264.7 cells as osteoclast precursors. Here, we report a putative inhibitory mechanism for RANKL-induced osteoclast formation by IL-17A in RAW264.7 cells.

MATERIALS AND METHODS

Cell culture

We used the murine monocyte/macrophage cell line RAW264.7 cells as osteoclast precursors that were obtained from DS Pharma Biomedical (Osaka, Japan). RAW264.7 cells were cultured in minimal essential medium alpha modification (α-MEM) (Wako Pure Chemicals, Osaka, Japan) containing 10% fetal bovine serum (FBS) (Nichirei Biosciences, Tokyo, Japan), 100 μg/mL penicillin, 100 μg/mL streptomycin and 2 mM L-Glutamine (Wako Pure Chemicals). RAW 264.7 cells were incubated at 37°C under 5% CO_2. Accutase (Innovative Cell Technologies, San Diago, CA, USA) was used to detach the cells.

TRAP activity assay

RAW264.7 cells were seeded onto a 96-well plate at a density of 3.0×10^3 cells/well and cultured for up to 3 days in α-MEM containing 10% FBS and IL-17A at 0, 5, 10 or 20 ng/mL in the presence of RANKL (10 ng /mL). In some studies, RAW264.7 cells were preincubated with the indicated concentration of SP600125 (JNK inhibitor) for 30 min at 37°C before incubation with RANKL at 10 ng/mL. Osteoclast formation was assessed by TRAP activity assay. The cells were fixed with 10% formalin solution in phosphate buffered saline (PBS) for 1 min and equal parts acetone/ethanol (Wako Pure Chemicals) for 30 sec. The cells were then allowed to react with 50 mM sodium citrate and 10 mM tartaric acid (pH 4.6) containing 5 mM p-nitrophenyl phosphate. The enzyme reactions were terminated with an equal volume of 0.1 N sodium hydroxide, and absorbance was measured at 405 nm with a SpectraMax M 5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Phosphorelation of JNK in RAW264.7 cells

RAW264.7 cells (2.0×10^6 cells/sample) were treated with the indicated concentration of IL-17A in the presence of RANKL (10 ng/mL) for 15 min. Cells were solubilized with lysis buffer containing 50 mM Tris-HCl at pH 7.6, 0.5% TX-100, 300 mM NaCl, 5 mM EDTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium orthovanadate by gentle rocking for 30 min at 4°C. Insoluble material was removed by centrifugation and the supernatants were subjected to SDS-PAGE for western blotting. Cell lysates were eluted by boiling in SDS-containing sample buffer and fractionated by SDS-PAGE (10% polyacrylamide gels).

Proteins were electrophoretically transferred to PVDF membranes. The membranes were blocked using 20% Blocking One (Nacalai, Kyoto, Japan) in 50 mM Tris-HCl at pH 7.5 and 150 mM NaCl overnight. Membranes were incubated for 1 h with anti-phospho JNK (Cell Signaling Technology, Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibody (Anti-mouse IgG, HRP-linked whole Ab sheep ; Merck Millipore, Darmstadt, Germany) was used at a 1 : 2000 dilution and immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore). The images were analyzed using VersaDoc 5000 (Bio-Rad, Hercules, CA, USA). The membranes were stripped and reprobed with anti-JNK (Cell Signaling Technology) antibody. The results revealed that equal amounts of JNK were created by lysates obtained from each sample.

Expression of c-Fos from RAW264.7 cells

RAW264.7 cells (2.0×10^5 cells/sample) were treated with the indicated concentrations of IL-17A in the presence of RANKL at 10 ng/mL for 6 h. In a similar way, cell lysates were used for western blotting. Membranes were incubated for 1 h with anti-c-Fos (Cell Signaling Technology). HRP-conjugated secondary antibody (Anti-rabbit IgG, HRP-linked whole Ab sheep ; Merck Millipore) was used at a 1 : 2000 dilution and immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP Substrate. The images were analyzed using VersaDoc 5000.

RESULTS

Effect of IL-17A on RANKL-induced TRAP activity of RAW264.7 cells

To evaluate how IL-17A may control osteoclast physiology, we investigated its action on the osteoclast precursors RAW264.7 cells. TRAP is a well-known enzyme that is widely acccepted as a histochemical marker of osteoclasts. Its action can be significantly elevated by RANKL stimulation.[24] Initially, we investigated the effect of IL-17A on TRAP activity of RANKL-induced osteoclast differentiation in RAW 264.7 cells. RAW264.7 cells were cultured in the absence or presence of RANKL with the indicated concentration of IL-17A. TRAP activity was examined after 3 days stimulation. RAW264.7 cells were previously reported to differentiate into osteoclast-like TRAP-positive multinuclear cells on stimulation with RANKL. The enzymatic TRAP activity measured at the end of the differentiation process was increased by RANKL stimulation in RAW264.7 cells. When IL-17 A was present in the culture with RANKL, it decreased the RANKL-induced TRAP activity of RAW264.7 cells (Fig. 1). These results confirmed the potential of IL-17 A to suppress osteoclast differentiation in RAW264.7 cells.

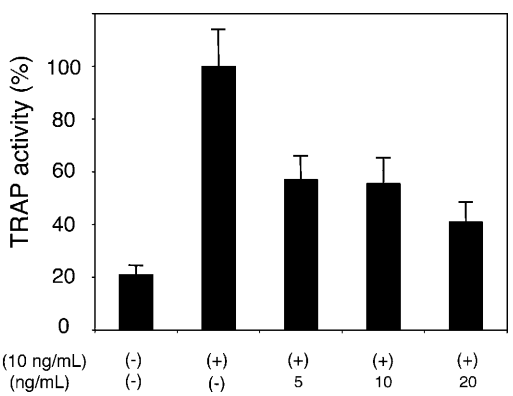

Fig. 1 Effect of IL-17A on RANKL-induced TRAP activity of RAW264.7 cells. RAW264.7 cells were seeded onto a 96-well plate at a density of 3.0×10^3 cells/well and cultured for up to 3 days in α-MEM containing 10% FBS and IL-17 at 0, 5, 10 or 20 ng/mL in the presence of RANKL at 10 ng/mL. TRAP activity was assessed by measuring absorbance at 405 nm. Values are expressed as the mean and standard deviation of triplicate experiments.

JNK plays a role in RANKL-induced osteoclastogenesis

MAPKs (mainly including ERK, JNK and p38 MAPK) are located at the downstream of the TRAF6 signalling complexes, and play an important role in RANKL-induced osteoclast differentiation by triggering a cascade reaction and up-regulating expressions of the essential transcription factors c-Fos and NFATc 1. In this study we focused on JNK among the MAPK. We investigated the effect of the JNK signaling pathway on RANKL-induced osteoclast differentiation. RAW264.7 cells were cultured with RANKL in the absence or presence of SP600125, a specific inhibitor of JNK. Consistent with previous results, SP 600125 treatment significantly inhibited the TRAP activity of RAW264.7 cells, which was stimulated by RANKL, compared with non-treatment of RAW264.7 cells. Furthermore, inhibition of TRAP activity of RAW 264.7 cells after SP600125 treatment occurred in a concentration-dependent manner (Fig. 2). It has been shown that JNK participate in the regulation of RANKL-induced osteoclast differentiation and that RANKL-induced activation of early signaling pathways is important for osteoclast differentiation.[25]

IL-17A inhibited RANKL-induced phosphorylation of JNK in RAW264.7 cells

RANKL is known to activate MAPKs (ERK, JNK and p38), which play important roles in the differentiation and formation of osteoclasts from osteoclast precursor cells. To determine the intracellular mechanism underlying the inhibition of osteoclast differentiation in the presence of IL-17A, we assessed the effects of IL-17A on RANKL-induced phosphorylation of JNK in RAW264.7 cells by immunoblotting. The JNK activation states were determined by immunoblotting using antibodies specifically directed against the phosphorylated forms of JNK, compared to data obtained with antibodies directed against the unphosphorylated states of the JNK. RANKL stimulation induced a marked phosphorylation of JNK compared with the situation of unstimulated RAW264.7 cells. The phosphorylation of JNK stimulated with RANKL was inhibited by 100 ng/mL IL-17A (Fig. 3 upper panel). To ensure that equal amounts of JNK were obtained from the lysates, the membranes were stripped and reprobed with anti-JNK antibody. The results revealed that equal amounts of JNK were created by lysates obtained from each sample (Fig. 3 lower panel).

IL-17A inhibited RANKL-induced c-Fos expression in RAW264.7 cells

Binding of RANKL to RANK activates several transcription factors that are responsible for promoting osteoclastic gene expression. At the final stage of osteoclast differentiation, NFATc1 cooperates with AP-1 to induce osteoclast-specific genes such as TRAP and

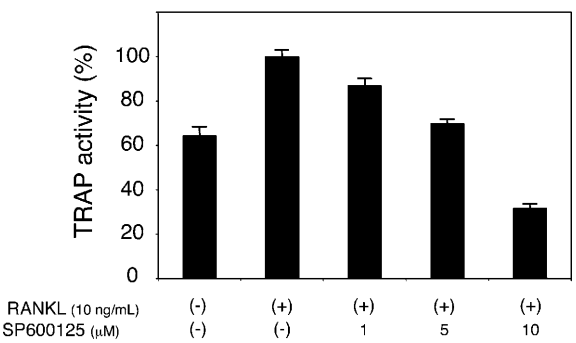

Fig. 2 Inhibition of SP600125 (JNK inhibitor) on RANKL-induced TRAP activity in RAW264.7 cells.RAW264.7 cells were seeded onto a 96-well plate at a density of 3.0×10^3 cells /well. Cells were preincubated with the indicated concentration of SP600125 (JNK inhibitor) for 30 min at 37℃ before incubation with RANKL at 10 ng/mL and cultured for up to 3 days in α-MEM containing 10% FBS. TRAP activity was assessed by measuring absorbance at 405 nm. Values are expressed as the mean and standard deviation of triplicate experiments.

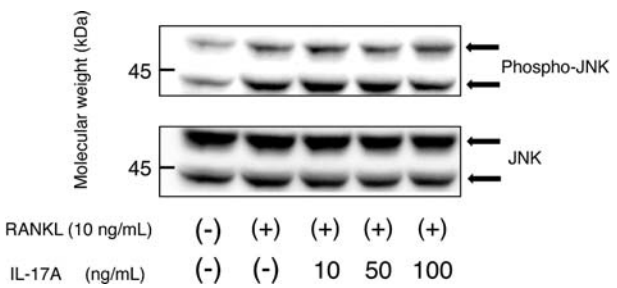

Fig. 3 IL-17A inhibited RANKL-induced phosphorylation of JNK in RAW264.7 cells. RAW264.7 cells (2.0×10^6 cells/sample) were treated with the indicated concentration of IL-17A in the presence of RANKL at 10 ng/mL for 15 min. Western blot analysis for phospho-JNK was performed. The phosphorylation of JNK stimulated with RANKL was inhibited by 100 ng/mL IL-17A (upper panel). To ensure that equal amounts of JNK were obtained from the lysates, the membranes were stripped and reprobed with anti-JNK antibody. Equal amounts of JNK were applied from lysates obtained from each sample (lower panel).

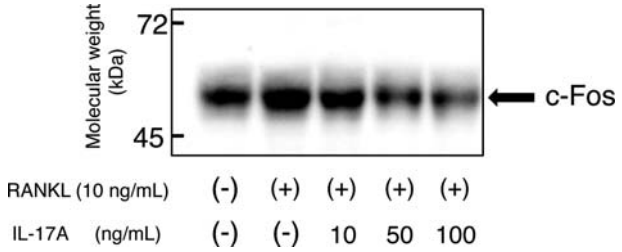

Fig. 4 IL-17A inhibited RANKL-induced c-Fos expression in RAW264.7 cells. RAW264.7 cells (2.0×10^5 cells/sample) were treated with the indicated concentration of IL-17A in the presence of RANKL at 10 ng/mL for 6 h. Western blot analysis for c-Fos expression was performed. IL-17A suppressed c-Fos expression in a dose-dependent manner.

calcitonin receptor. c-Fos plays an important role in RANKL-induced NFATc1 expression by forming AP-1 complexes with c-Jun.[26] RANKL has been shown to elevate the levels of c-Fos in osteoclast precursor cells.[27] Therefore, we examined the effect IL-17A had on RANKL-induced expression of c-Fos in RAW 264.7 cells by western blot analysis. RAW264.7 cells were cultured in the presence or absence of RANKL with the indicated concentration of IL-17A for 6 h. After incubation, the protein levels of c-Fos were determined by western blot analysis. As shown in Fig.4, RANKL stimulation increased the expression of c-Fos in RAW264.7 cells. Whereas RANKL augmented the levels of c-Fos, IL-17A suppressed c-Fos expression in a dose-dependent manner (Fig. 4).

DISCUSSION

Excessive RANKL signaling cascade enhances osteoclast differentiation and bone resorption activity, which ultimately causes bone-destructive diseases. Thus, the inhibition of osteoclast differentiation and/or its function may be an effective approach to the treatment of pathological bone loss. In this study, we found that IL-17A could inhibit RANKL-induced osteoclast differentiation in RAW264.7 cells. However, the precise intracellular mechanisms of the inhibitory action of IL-17A were not clearly identified.

The IL-17 cytokine family is a recently discovered group of cytokines. IL-17A, the original member of this family, was first identified in 1995.[28] The human IL-17 A gene product is a protein of 150 amino acids with a molecular weight of 15 kDa, and is secreted as a disulfide linked homodimer of 30–35 kDa glycoprotein.[29] The other members, IL-17 B to IL-17 F, were subsequently identified based on their homology to IL-17.[30] The IL-17 cytokine family, IL-17A to IL-17 F, appear to be critical players in host defence responses and inflammatory diseases. Considerable data support the role of these proteins in innate and adaptive immunity.[31] Several studies have indicated that IL-17A is a proinflammatory cytokine crucial for osteoclastic bone resorption in the presence of osteoblasts.[32] IL-17A has been shown to promote osteoclast differentiation indirectly through the induction of IL-1, TNF-α, and RANKL expression.[33]

While IL-17A is expressed by several leukocytes, such as T-cells and neutrophils, its receptor is expressed in all tissues examined to date.[34] IL-17 receptor families consist of five subtypes : IL-17 RA, IL-17 RB, IL-17 RC, IL-17 RD and IL-17 RE.[29] They share partial sequence homology to IL-17 RA, which is a single-pass transmembrane protein of approximately 130 kDa.[29] It has a long cytoplasmic tail with approximately 500 amino acids not found in any other cytokine receptor family, suggesting that they belong to a unique cytokine receptor family.[35] Members of the IL-17 family are also homodimeric and have been shown to bind and signal through both homodimeric and heteromeric counterstructures.[36] IL-17 RA and IL-17 RC form a heterodimer for mediating the signals of IL-17A and IL-17 F.[37] We confirmed the expression of IL-17 RC on the RAW264.7 cells by FACS analysis (data not shown). However, the direct effects of IL-17 A on the differentiation of osteoclast precursors into osteoclasts and on the function of osteoclasts remains unclear. We found direct effects of IL-17A on the osteoclast differentiation of RAW264.7 cells.

MAPKs have been implicated as key regulators of various cellular responses, including cell proliferation, apoptosis, differentiation and migration.[38] It has been shown that three well-known MAPKs, ERK, JNK, and p38, are activated in the RANKL signaling in osteoclast precursor cells[39] and RAW264.7 cells.[40] The treatment of cells with p38 or JNK specific inhibitors decreased RANKL-induced osteoclastogenesis, suggesting that p38 and JNK play an important role in osteoclast formation.[14, 40] Here, we found that SP

600125 could significantly reduce TRAP activity in RANKL-induced RAW264.7 cells. This result supports the notion that the activation of JNK plays an important role in RANKL-induced osteoclastogenesis. Furthermore, IL 17A has been shown to suppress the TRAP activity in RAW264.7 cells induced by RANKL stimulation. In our study, IL-17A down-regulated the phosphorylation of JNK in RANKL-stimulated RAW 264.7 cells. These results suggest that the inhibition of RANKL-induced osteoclast differentiation stimulated by IL-17A may be involved in the JNK signaling pathway.

NFATc1, in association with AP-1, a complex of c-Jun and c-Fos,[14, 15] is known to regulate the transcription of the genes involved in osteoclast differentiation.[41] The activation of MAPKs results in the phosphorylation of c-Jun and its association with c-Fos to form the essential AP-1 transcription factor also involved in NFATc1 induction.[17, 18] NFATc1 was one of the key transcription factors following RANKL stimulation, and regulated many osteoclast-specific genes, such as cathepsin K, matrix metalloproteinase 9 (MMP9) and TRAP.[41] Moreover, NFATc1 was shown to be required for in vivo osteoclastogenesis.[20, 23] In addition, RANKL-induced NFATc1 expression is abrogated in c-Fos-deficient mice,[41] indicating that NFATc 1 is downstream of c-Fos during osteoclast differentiation.[42] From these findings, NFATc1 is considered a master transcription factor for osteoclastogenesis.[26] c-Fos plays an important role in RANKL-induced NFATc1 expression by forming AP-1 complexes with c-Jun.[26] RANKL has been shown to elevates the levels of c-Fos in osteoclast precursor cells.[11, 27, 28] In this study, we confirmed that RANKL elevate the levels of c-Fos in RAW264.7 cells and that IL-17A significantly inhibits RANKL-induced c-Fos, suggesting that c-Fos is important for the IL-17A mediated inhibitory effect on osteoclast formation.

Taken together, we found that IL-17A inhibited the RANKL-induced osteoclast differentiation in part by attenuating the RANKL-induced phosphorylation of JNK, and by reducing the RANKL-induced expressions of c-Fos. Our findings provide new insights into mechanisms of IL-17A-induced inhibition of osteoclastogenesis. Therefore, we think that IL-17A might possibly be used in the development of a therapeutic drug for diseases that destroy bone.

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