Original

RANKL Induces IL-18 Binding Protein Expression in RAW264.7 Cells

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Abstract: The receptor activator of NF-κB (RANK) ligand (RANKL) is a cytokine that is essential for osteoclast development, whereas interleukin (IL)-18 suppresses osteoclastogenesis by increasing granulocyte-macrophage colony-stimulating factor (GM-CSF) production in T-cells. In the present study, we examined the effect of RANKL on the expression of IL-18 and IL-18 binding protein (IL-18BP), a natural inhibitor of IL-18, in RAW264.7 cells. We also examined the effect of conditioned medium derived from RAW264.7 cells on IL-18-induced GM-CSF expression in CD4+ T-cells isolated from the spleens of C57BL/6J mice. mRNA expression of IL-18 was significantly suppressed, whereas that of IL-18BP was significantly increased in RANKL-treated RAW264.7 cells compared with untreated cells. RANKL also increased the expression of IL-18BP protein in culture supernatants of RAW264.7 cells. GM-CSF protein expression in CD4+ T-cells stimulated with IL-18 was suppressed by the addition of conditioned medium derived from RANKL-treated RAW264.7 cells. These results suggest that RANKL suppresses the stimulatory effect of IL-18 on GM-CSF expression in CD4+ T-cells via enhancing the production of IL-18BP in RAW264.7 cells.

Key words: GM-CSF, IL-18, IL-18 binding protein, RANKL

Introduction

Osteoclast differentiation is controlled by cellular signals and cytokines1. Inflammation-mediated bone loss is a major feature of various bone diseases, including chronic periodontitis, rheumatoid arthritis and osteoarthritis; in these diseases, inflammatory tissue is a major source of a broad range of cytokines that are involved in osteoclast differentiation and activation. The receptor activator of NF-κB (RANK) ligand (RANKL) is a cytokine that is essential for osteoclast development.2,3 Osteoclastogenesis is strongly induced when membrane-anchored or soluble RANKL binds to RANK on osteoclast precursor cells.4,5 In addition, pro-inflammatory cytokines, including tumor necrosis factor-α, interleukin (IL)-1, IL-6, and IL-17, induce the differentiation of mature osteoclasts from osteoclast precursor cells via promoting RANKL expression in synovial fibroblasts, T lymphocytes and osteoblasts.6,8,9 However, IL-6 and IL-17 also play a role analogous to anti-osteoclastogenic factor when pro-inflammatory cytokines directly act on osteoclast precursor cells. IL-6 inhibits osteoclast differentiation by suppressing RANK signaling in osteoclast progenitors.10 We previously reported that the effect of IL-17 conflicts with the direct action on osteoclast precursors and indirect action through osteoblasts.8,10 Thus, the differentiation of osteoclast precursors into osteoclasts is suppressed at high concentrations of IL-17 in the presence of RANKL.9 On the other hand, IL-17 induces the differentiation of osteoclast precursors into osteoclasts by increasing RANKL expression and decreasing that of osteoprotegerin, a physiological inhibitor of RANKL, in osteoblasts.8,10

IL-18 is also known to inhibit and promote osteoclastogenesis. In an in vitro co-culture system using osteoblasts and spleen cells, IL-18 suppresses osteoclast formation by enabling T-cells to promote the expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), a differentiation factor for dendritic cells rather than osteoclasts.11,13 However, several reports have indicated that IL-18 plays a destructive role in rheumatoid arthritis.14 IL-18 stimulates osteoclast formation via upregulation of RANKL production in T-cells derived from synovitis in rheumatoid arthritis.15,16

IL-18 binding protein (IL-18BP) functions as a decoy receptor that prevents the interaction between IL-18 and the IL-18 receptor; therefore, the biological effect of IL-18 is regulated by IL-
Osteoclast cultures and tartrate-resistant acid phosphatase (TRAP) staining

The murine monocyte/macrophage cell line RAW264.7 was purchased from Dainippon Pharmaceutical (Osaka, Japan). Cells were seeded onto 96-well or 6-well flat-bottomed culture plates at a density of 5 × 10⁴ cells/cm². After overnight incubation, cells were cultured for up to 5 days in α-Minimal Essential Medium (α-MEM) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 1% (v/v) penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 0 or 50 ng/ml soluble RANKL (Wako Pure Chemical, Osaka, Japan); RANKL is an osteoclast differentiation factor that is involved in the process of osteoclast differentiation. Conditioned medium derived from RANKL-treated RAW264.7 cells was collected from the supernatants after 3 days. The conditioned medium was collected from RAW264.7 cells cultured with or without 50 ng/ml RANKL for 3 days. The conditioned medium was then changed to α-MEM without RANKL and FBS. Cells were cultured for an additional 24 h, and each culture medium sample was diluted to 30%.

CD4⁺ T cell isolation

C57BL/6J mice were purchased from Sankyo Labo (Tokyo, Japan) to purify CD4⁺ T cells. The experiments were performed in accordance with the Institutional Guidelines in Care and Use of Experimental Animals and were approved by the Animal Experimentation Committee of Nihon University School of Dentistry (Approval number: AP11D001). CD4⁺ T cells were isolated from the spleen using antibodies against CD4 coupled to magnetic beads (magnetic-activated cell sorting [MACS], Miltenyi Biotec, Auburn, CA, USA). CD4⁺ T cells were stimulated with plate-bound anti-CD3 (10 μg/ml) and soluble anti-CD28 (2 μg/ml) monoclonal antibodies (BioLegend, San Diego, CA, USA), and were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco, Carlsbad, CA, USA), 1% (v/v) penicillin/streptomycin (Sigma-Aldrich), 50 mM 2-mercaptoethanol (β-ME; Sigma-Aldrich), 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) (Sigma-Aldrich), and 50 U/ml IL-2 (R&D Systems, Minneapolis, MN, USA).

IL-18-induced GM-CSF expression in CD4⁺ T cells

CD4⁺ T cells were seeded at 1 × 10⁵ cells/cm² in 96-well flat-bottomed culture plates before collecting the supernatants. Cells were stimulated with 0 (control), 1 and 10 ng/ml IL-18 (Medical & Biological Laboratories, Nagoya, Japan) in the presence of the conditioned medium, which was collected from RANKL-treated or untreated RAW264.7 cells. The cells were cultured at 37 °C in a humidified incubator in a 5% CO₂ atmosphere for 24 h.

Real-time reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA was isolated from cultured cells on days 3 and 5 using NucleoSpin RNA (Takara Bio, Shiga, Japan). The isolated RNA was then treated with DNase. The amount of DNase-treated RNA was measured using a NanoDrop 1000 (ND-1000; Thermo Fisher Scientific, Wilmington, DE, USA), and was converted into complementary DNA (cDNA) using an RNA PCR Kit (Takara Bio). The cDNA (0.2 μg/2 μl) was subjected to real-time PCR using SYBR Green I dye. The reactions were performed in 25 μl of an SYBR premixed Ex Taq solution (Takara Bio) containing 20 μM sense and anti-sense primers (Table 1). PCR assays were performed using a Smart Cycler system (Cepheid, Sunnyvale, CA, USA) and analyzed using Smart Cycler software. The PCR protocol for IL-18 BP and IL-18 consisted of 30 cycles at 95 °C for 3 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.

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Figure 1. Effect of RANKL on TRAP staining in RAW264.7 cells. RAW264.7 cells cultured with 50 ng/ml soluble RANKL were stained using a TRAP staining kit on days 3 (a) and 5 (b) of culture. RAW264.7 cells cultured without RANKL were also stained on days 3 (c) and 5 (d) of culture. (Bar=100 μm).

Figure 2. Effect of RANKL on mRNA expression of IL-18BP in RAW264.7 cells. IL-18BP mRNA expression was determined by real-time RT-PCR on days 3 and 5 of culture. Each bar indicates the mean ± SD of three separate experiments; *p < 0.05, **p < 0.01, RANKL treatment vs. control.

Figure 3. Effect of RANKL on mRNA expression of IL-18 in RAW264.7 cells. IL-18 mRNA expression was determined by real-time RT-PCR on days 3 and 5 of culture. Each bar indicates the mean ± SD of three separate experiments; *p < 0.05, **p < 0.01, RANKL treatment vs. control.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

The culture medium from RAW264.7 cells was collected and dialyzed using a PD-10 column (GE Healthcare Bio-Science, Piscataway, NJ, USA). Next, the dialyzed culture medium was subjected to 10% SDS-PAGE (gel size: 8.3 cm × 6.5 cm × 0.75 mm) with a discontinuous Tris-glycine buffer system. Samples containing 20 μg of extracellular proteins were dissolved in 10 μl
sample buffer containing 1 % SDS, 2 M urea, 15 mg/ml dithiothreitol, and bromophenol blue, and then heated at 95 °C for 5 min prior to loading. Proteins were resolved by SDS-PAGE, transferred to a polyvinylidene fluoride membrane using a semidry transfer apparatus, and probed with the indicated antibodies: polyclonal goat IgG antibodies against mouse IL-18BP (Acris Antibodies, San Diego, CA USA), followed by the appropriate biotin-conjugated secondary rabbit anti-goat IgG (Chemicon International, Temecula, CA, USA). Membranes were then labeled with streptavidin-horseradish peroxidase (HRP) and visualized using a commercial chemiluminescence kit (GE Healthcare Bio-Science).

**Enzyme-linked immunosorbent assay (ELISA)**

The amount of GM-CSF in the culture medium derived from CD4+ T cells was determined using a commercially available ELISA kit (BioLegend) according to the manufacturer’s instructions. Assays were performed in triplicate, and the data were converted to pg GM-CSF/ ml.

**Statistical analysis**

All experiments were performed in triplicate. Each value represents the mean ± standard deviation (SD) of triplicate experiments. Significant differences (p < 0.05) were determined using Student’s t-test or one-way analysis of variance (ANOVA) followed by the Bonferroni modification of Student’s t-test.

**Results**

**Effect of RANKL on TRAP staining and expression of IL-18BP and IL-18 in RAW264.7 cells**

We first investigated the appearance of TRAP-positive multinucleated cells in the absence and presence of RANKL. We confirmed TRAP-positive multinucleated cells on days 3 and 5 of culture in the presence of RANKL, and their size on day 5 was larger than that on day 3 (Fig. 1a and b). We did not confirm TRAP-positive multinucleated cells on days 3 and 5 of culture in the absence of RANKL (Fig. 1c and d).

We next sought to examine the effect of RANKL on mRNA expression of IL-18BP and IL-18. IL-18BP mRNA expression was significantly increased in cells cultured with RANKL compared with those without RANKL on days 3 and 5 of culture (Fig. 2). However, IL-18 mRNA expression was significantly suppressed on days 3 and 5 by the addition of RANKL (Fig. 3).

**Effect of conditioned medium derived from RAW264.7 cells on IL-18-induced GM-CSF expression in CD4+ T cells.**

Before examining conditioned medium, we confirmed whether the stimulatory effects of RANKL on IL-18BP expression were observed at the extracellular protein level. Protein levels of IL-18BP in the culture supernatants derived from RANKL-treated RAW264.7 cells were markedly higher than those in the culture supernatants derived from untreated RAW264.7 cells (Fig. 4). We next measured GM-CSF protein expression in the culture supernatants after stimulating CD4+ T cells with IL-18 in the presence of conditioned medium derived from untreated RAW264.7 cells. GM-CSF expression was significantly higher in IL-18-stimulated CD4+ T cells (1 or 10 ng/ml) than in unstimulated control cells when CD4+ T cells were cultured with conditioned medium derived from RANKL-stimulated RAW264.7 cells.
Discussion

In the present study, RANKL induced mRNA expression of IL-18BP in RAW264.7 cells, and its effect was slightly diminished on day 5 compared with that on day 3 after stimulation of RANKL. However, we observed TRAP-positive multinucleated cells only in the presence of RANKL, and the size of TRAP-positive multinucleated cells was larger on day 5 than on day 3 of culture. We previously reported that the number of TRAP-positive multinucleated cells decreased gradually after day 5 of culture when RAW264.7 cells were cultured in the presence of RANKL for up to 10 days. These findings indicate that RANKL induces the expression of IL-18BP mRNA at the early stage of RANKL-induced osteoclast differentiation.

Previous studies have reported that IL-18BP is secreted from human monocytes, endothelial cells and macrophages, or mouse cardiomyocytes into culture supernatants. In the present study, we confirmed IL-18BP in the culture supernatants, and the expression levels of IL-18BP were markedly higher in the supernatants derived from RANKL-treated RAW264.7 cells than in untreated control cells. Foster et al. reported that lipopolysaccharide (LPS) of Porphyromonas gingivalis, a Gram-negative bacterial species involved in alveolar bone resorption in chronic adult periodontitis, induced both IL-18 and IL-18BP secretion in THP-1 human monocytes. They also reported that when THP-1 cells were cultured with LPS and an anti-IL-18BP antibody, the antibody increased the concentration of unbound IL-18 in the supernatants; thus, IL-18BP in the culture supernatant can directly bind to IL-18, and the anti-IL-18BP antibody disrupted the binding of IL-18 and IL-18BP. In the present study, IL-18BP mRNA expression was increased, whereas IL-18 mRNA expression was decreased, when RAW264.7 cells were treated with RANKL. Therefore, we speculate that the culture supernatants derived from RANKL-treated RAW264.7 cells contain sufficient IL-18BP that can neutralize IL-18. Based on previous studies that reported the stimulatory effect of IL-18 on GM-CSF expression in CD4+ T cells, we hypothesized that the free IL-18BP in culture supernatants derived from RANKL-treated RAW264.7 cells could suppress IL-18-induced GM-CSF production in CD4+ T cells. To verify this hypothesis, we stimulated CD4+ T cells with IL-18 in the presence of conditioned medium derived from RANKL-treated or untreated RAW264.7 cells and examined GM-CSF protein expression in the culture supernatants of CD4+ T cells. As expected, IL-18 did not induce GM-CSF expression in CD4+ T cells in the presence of conditioned medium derived from RANKL-treated RAW264.7 cells, whereas GM-CSF expression was induced by IL-18 in the presence of conditioned medium derived from untreated RAW264.7 cells. These results indicate that IL-18BP derived from RANKL-treated RAW264.7 may block the binding of IL-18 to IL-18 receptor in CD4+ T cells.

An increase in the level of IL-18 has been observed in the serum or pathological tissue in individuals with rheumatoid arthritis or periodontitis, both of which are well-recognized as inflammatory diseases with bone destruction, through RANKL-induced osteoclast formation. The results in our present study may help explain how RANKL, IL-18 and IL-18BP are involved in the cell-cell interactions between osteoclast precursor cells and CD4+ T cells in these bone-destructive disorders. In the inflammatory environment where RANKL is dominant, IL-18 cannot induce the production of GM-CSF in CD4+ T cells, because RANKL stimulates IL-18BP production in the early stage of osteoclast differentiation from osteoclast precursor cells. Consequently, IL-18 may not interfere with RANKL-induced osteoclastogenesis in bone-destructive disorders.

In conclusion, the results of the present study suggest that RANKL suppresses the stimulatory effect of IL-18 on GM-CSF expression in CD4+ T cells via enhancing the production of IL-18BP in RAW264.7 cells.

Acknowledgments

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Conflict of Interest

The authors have declared that no COI exists.

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