IL-6 trans-signalling directly induces RANKL on fibroblast-like synovial cells and is involved in RANKL induction by TNF-α and IL-17

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Introduction

Articular bone erosion is a characteristic feature of RA. As a pathogenic mechanism of bone destruction, osteoclasts activated by inflammatory cytokines are thought to be responsible for focal bone erosion. Tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells, namely osteoclasts, are often seen in synovia at sites of cartilage destruction in RA patients [1, 2].

RANK ligand (RANKL) is an essential factor for osteoclastogenesis [3, 4]; it stimulates precursor cells of the myeloid lineage to differentiate into osteoclasts by binding to its signalling receptor, RANK [5, 6]. RANKL also stimulates osteoclast migration, fusion, activation and survival, so it acts at all stages of osteoclast generation and activity. Several studies have shown that RANKL is expressed at sites of bone erosion in RA synovial membranes [2, 7].

Excess production of inflammatory cytokines such as IL-1, IL-6, IL-17 and TNF-α is observed in SF in RA patients [8–10]. Most of these cytokines are reported to stimulate osteoclast generation by inducing RANKL expression in bone marrow stroma cells or osteoblasts, which support osteoclast differentiation [10–12]. It was not clear, however, as to which cytokine is most important for inducing RANKL expression and thereby inducing osteoclast formation in RA synovium.

To answer this question, the present study investigated the effects of inflammatory cytokines on RA fibroblasts by investigating RANKL mRNA expression and RANKL protein production in the presence of various cytokines. The signalling pathway for RANKL induction was also investigated.

Materials and methods

Reagents and cells

Tocilizumab (a humanized anti-human IL-6 receptor antibody), recombinant human IL-6 and soluble IL-6 receptor (sIL-6R) were prepared in our laboratories. Recombinant human TNF-α, IL-17 and IL-1β were purchased from R&D systems, Minneapolis, MN, USA.

In this study, three different clones of RA-FLS were used. Two clones of human RA-FLS were purchased from Cell Applications (San Diego, CA, USA) and one clone of RA-FLS was isolated from RA synovium. Synovium was obtained from RA patients who underwent knee replacement surgery with informed consent. RA-FLS was isolated from synovium by enzyme treatment. They were cultured using synoviocyte growth medium (Cell Applications).

RAW 264.7 (RAW) cell was purchased from DS Pharma Biomedical Co. Ltd. (Osaka, Japan) and cultured using α-MEM supplemented with 10% fetal bovine serum.

IL-6 in supernatants was measured by ELISA kit for human IL-6 (Biosource, Camarillo, CA, USA).

Quantitative real-time PCR for RANKL mRNA expression

RA-FLS (4 × 10⁶ cells/2 ml well) were cultured in six-well plates for 2–3 days. After confluence, cells were stimulated by cytokines with or without sIL-6R for 24 h. After collecting supernatants, total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA, USA). Synthesis of cDNA was performed using an Omniscript® RT kit (Qiagen) with random 9 primer (TaKaRa, Shiga, Japan) according to the manufacturer’s protocol. Quantitative real-time PCR was performed by running a TaqMan gene expression assay (Applied Biosystems, Foster City, CA, USA), targeting human RANKL and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), on an ABI PRISM 7000 system (Applied Biosystems) according to the manufacturer’s protocol.
Proliferation assay
RA-FLS, (3 × 10⁵ cells/0.2 ml/well) were cultured with various cytokines in 96-well flat bottom plates for 3 days. This study was performed under sub-confluent condition. Proliferation assay was performed using the Cell Proliferation ELISA system (GE Healthcare, Buckinghamshire, UK) according to manufacturer’s instruction. Briefly, BrdU was added for the last 3 h, and its uptake was detected with anti-BrdU antibody. Substrate was added to elicit a colorimetric reaction, and absorbance was measured using a microplate reader.

Western blotting for RANKL protein
RA-FLS were cultured with cytokines with and without sIL-6R or sIL-6R plus tocilizumab for 48 h, washed with PBS and lysed using a total protein extraction kit (Millipore, Billerica, MA, USA) according to the manufacturer’s protocol. Protein concentrations were determined using a BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Cell lysates (10 μg protein) were separated by 15% SDS–PAGE and transferred to a PVDF membrane (Millipore). After blocking with 3% BSA/TBS overnight at 4°C, the lysate was incubated with mouse anti-human RANKL monoclonal antibody (R&D systems) diluted 1:250 or β-actin (Sigma, St Louis, MO, USA) diluted 1:1000 in 1% BSA/TBS for 2 h, and then with alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) for 1 h. Binding was visualized using phosphatase substrate.

IF microscopy
RA-FLS were cultured with cytokines on chamber slides for 48 h. After washing twice with PBS, the monolayers were fixed with 1% formaldehyde in PBS for 10 min at room temperature, washed and then incubated in 0.5% BSA/PBS with 0.05% Tween-20 for 45 min at room temperature for blocking. The monolayers were then incubated with RANK-Fc (250 ng/ml, R&D systems) in 0.5% BSA/PBS for 1 h at room temperature. In the study, to see the effect of osteoprotegerin (OPG), OPG (1250 ng/ml, R&D systems) was added with RANK-Fc. After washing five times, rabbit anti-RANK antibody (diluted 1:50 in 0.5% BSA/PBS, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added for 1 h at room temperature. After washing five times, the cells were incubated with 0.5% BSA/PBS containing goat anti-rabbit IgG (H+L) labelled with Alexa Fluor 568 (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. The cells were washed with 0.5% BSA/PBS and PBS five times, respectively and incubated with DAPI solution for 15 min at room temperature. After washing twice, about two drops of 90% glycerol were added to the slide, and the slide was examined using a Digital Eclipse C1 confocal laser-scanning IF microscope (Nikon, Tokyo, Japan).

NFATc1 mRNA induction by co-cultured RA-FLS and RAW cells
RA-FLS were cultured with cytokines in 96-well flat bottom plates for 24 h and then RA-FLS were co-cultured with 1 × 10⁵ cells/well RAW cells in α-MEM supplemented with 10% fetal bovine serum for 3 days. After 3 days, extracting mRNA and synthesizing cDNA were performed by using methods mentioned above. Quantitative real-time PCR was performed by using mouse NFATc1 and mouse GAPDH according to the manufacturer’s protocol.

Inhibition of signal transducer and activator of transcription and mitogen-activated protein kinase signalling
Parthenolide (PAR) (Sigma), a phosphorylation and signal transducer and activator of transcription (STAT) activation inhibitor [13], and PD98059 (PD) (Sigma), a mitogen-activated protein MAPK inhibitor [14], were dissolved in DMSO and diluted in medium to give a DMSO concentration of 0.5% (w/v). RA-FLS were cultured with 50 μM PAR or 25 μM PD for 2 h after serum deprivation for 24 h, as previously described [15]. IL-6 and sIL-6R were then added and the cells were cultured for 24 h. STAT3 and ERK phosphorylation was confirmed by Cell-Based Stat3 (Tyr705) and ERK1/2 ELISA kits (Ray Biotech, Norcross, GA, USA), respectively, and RANKL mRNA expression was measured by real-time PCR as described above.

Statistical analysis
All experiments were repeated at least twice to confirm the reproducibility and all three RA-FLS were tested in each experiment. The statistical significance of the differences was analysed using a software package (Statistical Analysis System: SAS Institute Japan, Tokyo, Japan). Unpaired t-test was used for the statistical analysis. The significance level for each analysis was set as 5%.

Results

RANKL mRNA induction on RA-FLS by cytokines
RA-FLS were cultured with IL-6 (100 ng/ml), IL-6 (1, 10, 100 ng/ml) + sIL-6R (100 ng/ml), TNF-α (1, 10, 100 ng/ml), IL-17 (1, 10, 100 ng/ml) or IL-1β (5 ng/ml) for 24 h and then RANKL expression was analysed by real-time PCR. RANKL mRNA expression was induced by IL-6 + sIL-6R, but not IL-6 alone, and by IL-1β (Fig. 1A). However, TNF-α or IL-17 did not induce RANKL mRNA expression, even if the concentration of 100 ng/ml was used (Fig. 1A shows the case of 100 ng/ml).

![Fig. 1. RANKL mRNA expression on RA-FLS by cytokines. (A) RA-FLS were cultured with IL-6, IL-6 + sIL-6R, TNF-α (100 ng/ml), IL-17 (100 ng/ml) and IL-1β for 24 h. After culture, relative expression of RANKL mRNA was measured by real-time PCR. (B) Time course of RANKL mRNA expression by IL-6+sIL-6R. RA-FLS were cultured with IL-6+sIL-6R for 6, 24 and 48 h. After culture, relative expression of RANKL mRNA was measured by real-time PCR. Each column and vertical line indicates mean and s.o. of triplicate cultures. The data indicate a representative of the experiment using three RA-FLS.](image-url)
RANKL mRNA induction by IL-6/sIL-6R stimulation was detectable at 6 h and reached maximum at 48 h after stimulation (Fig. 1B). However, TNF-α and IL-17 never induced RANKL at any periods.

Responsiveness of RA-FLS to TNF-α and IL-17
We checked the responsiveness of RA-FLS to TNF-α and IL-17, because these cytokines did not induce RANKL expression. The proliferation and IL-6 production by TNF-α and IL-17 were examined (Fig. 2). TNF-α and IL-17 at a concentration of 10 ng/ml induced the proliferation of RA-FLS and the production of IL-6, demonstrating that RA-FLS used were responsive to TNF-α and IL-17. Moreover, IL-6 + sIL-6R induced cell proliferation and IL-6 production. IL-1β also induced IL-6 production.

Induction of RANKL mRNA by cytokines and sIL-6R
Since TNF-α and IL-17 strongly induced IL-6 production, we hypothesized that TNF-α and IL-17 would induce RANKL mRNA expression in the presence of sIL-6R. On investigation, we found this to be the case (Fig. 3). Moreover, tocilizumab (100 μg/ml) inhibited induction of RANKL mRNA expression by IL-6 + sIL-6R, TNF-α + sIL-6R and by IL-17 + sIL-6R.

Production of RANKL protein in RA-FLS
Western blotting clearly showed that RANKL protein production correlated with RANKL mRNA expression (Fig. 4A). With immunostaining, strong RANKL staining was seen on RA-FLS incubated with IL-6 + sIL-6R (Fig. 4Bb). There was no staining when cells were cultured with tocilizumab in the presence of IL-6 and sIL-6R (Fig. 4Bc) or when the RANK homologue, OPG, was added with RANK-Fc (Fig. 4Bd).

**Fig. 2.** Responsiveness of RA-FLS to TNF-α and IL-17. (A) RA-FLS were cultured with cytokines for 3 days. After culture, cell proliferation was measured. (B) IL-6 + sIL-6R protein by real-time PCR after incubation with cytokines for 24 h. (C) IL-6 protein in supernatant was measured by ELISA. Concentrations: IL-6 and sIL-6R were 100 ng/ml, TNF-α and IL-17 were 10 ng/ml, and IL-1β was 5 ng/ml. Each column and vertical line indicates mean and S.D. of triplicate culture. The data indicate a representative of the experiment using three RA-FLS.

**Fig. 3.** Induction of RANKL mRNA by cytokines and sIL-6R. RA-FLS were co-cultured with RAW cells with or without IL-6 + sIL-6R for 3 days. After culture, total RNA was extracted and NFATc1 mRNA was measured. NFATc1 mRNA expression was induced by IL-6 + sIL-6R and was completely inhibited by the addition of tocilizumab (Fig. 5).

**Fig. 4.** RANKL protein production in RA-FLS. (A) RA-FLS were cultured for 48 h with cytokines with and without sIL-6R or sIL-6R + tocilizumab (100 μg/ml). RANKL mRNA was determined by real-time PCR. Concentrations of cytokines were as in Fig. 2. The data indicate a representative of the experiment using three RA-FLS.

**Fig. 5.** RANKL protein production in RA-FLS. (A) Protein was detected by western blotting using anti-RANKL monoclonal antibody. (B) β-Actin.

**Fig. 6.** RANKL protein production in RA-FLS. (A) RA-FLS were co-cultured with RAW cells with or without IL-6 + sIL-6R for 3 days. After culture, total RNA was extracted and NFATc1 mRNA was measured. NFATc1 mRNA expression was induced by IL-6 + sIL-6R and was completely inhibited by the addition of tocilizumab (Fig. 5).
Janus kinase/STAT pathway is involved in RANKL expression

Stimulation with IL-6/sIL-6R-induced STAT3 and ERK1/2 phosphorylation, and induction of these phosphorylations was completely inhibited by PAR and PD, respectively (Fig. 6A). The inhibition of STAT3 and ERK1/2 phosphorylation was not due to decreased expression of STAT and ERK, however, because PAR and PD did not cause a decrease in STAT or ERK protein content (data not shown). IL-6/sIL-6R complex-induced RANKL expression was completely inhibited by PAR, but was not affected by PD (Fig. 6B).

Discussion

In the present study, we examined that the influence of inflammatory cytokines on RANKL expression in RA-FLS. RANKL expression was induced by IL-6 + sIL-6R and by IL-1β. The fact that IL-6 did not induce RANKL without sIL-6R indicates that RA-FLS lack membrane-bound IL-6R (mIL-6R). This is the first report describing that IL-6/sIL-6R complex induces RANKL expression in RA-FLS.

It was reported that TNF-α induced RANKL in RA-FLS after incubation for 24 h [16]. However, we showed that TNF-α and IL-17 did not induce RANKL expression in our study. We also showed that TNF-α and IL-17 induced cell proliferation and IL-6 production from RA-FLS, clearly showing that the RA-FLS used were responsive to TNF-α and IL-17. The results of proliferation and IL-6 production regarding IL-6 and TNF-α are similar with our previous data [17]. Although we examined the effects of both incubation period and concentration of TNF-α or IL-17, no RANKL mRNA induction was observed at any timings or concentrations. We also performed the experiment using the same type of cells that Kubota et al. [16] used, synovial cells freshly isolated from RA patients, but the results were similar to those we obtained using purchased RA-FLS. The reason for the discrepancy between our results and those of others is unclear, but it is possible that the differences, such as drug therapy or disease activity of patients from whom synovium was collected affect the responsiveness.

IL-1β directly induced RANKL expression in RA-FLS. Since IL-1β is also a potent IL-6 inducer (Fig. 2), it is likely that IL-1β + sIL-6R and IL-1β synergistically induces RANKL. Moreover, it is reported that TNF-α and IL-17 induced IL-1 production [18, 19]. However, TNF-α or IL-17 might not induce IL-1 in RA-FLS, because they did not induce RANKL expression.
inthis study. It is thought that IL-1 induced by TNF-\(\alpha\) or IL-17 from cells other than RA-FLS participates in RANKL induction. In fact, the IL-1 receptor antagonist anakinra significantly inhibited the joint destruction (assessed by Sharp score); nevertheless it showed very weak improvement of signs and symptom of RA (assessed by ACR core set) [20]. The inhibition of IL-1-induced RANKL expression by anakinra is considered to be involved in this beneficial effect.

\(\text{sIL-6R}\) is present in blood of healthy subjects and RA patients, and the concentration is comparable between healthy subjects and RA patients. In contrast, higher concentration of \(\text{sIL-6R}\) is detectable in SF of RA patients compared with OA patients [21, 22]. These facts strongly suggest that \(\text{sIL-6R}\) is produced locally in the joints, probably inflamed synovium. Infiltrated inflammatory cells, such as monocytes and lymphocytes, into synovium are considered to be a source of \(\text{sIL-6R}\), because synovial fibroblasts themselves do not express IL-6R as we showed previously [17] and here in this study. Two mechanisms of \(\text{sIL-6R}\) production in inflamed joints are perceived: (i) \(\text{mIL-6R}\) was cleaved by TNF-\(\alpha\)-converting enzyme, which is activated by TNF-\(\alpha\) and IL-1 [23, 24]; and (ii) \(\text{sIL-6R}\) is produced by alternative splicing [25]. However, what kind of stimulation is involved in this production is still unclear.

IL-6, TNF-\(\alpha\) and IL-17 induced RANKL expression in the presence of \(\text{sIL-6R}\). IL-6 exerts its biological activities through two membrane molecules, a ligand-binding IL-6R and a non-ligand-binding signal transducer gp130. After the binding of IL-6 to \(\text{mIL-6R}\), IL-6/IL-6R complex associates with gp130 and then the signal is transmitted into the cell. In addition, \(\text{sIL-6R}\) can associate with gp130 in the presence of IL-6 and transmit the signal through gp130 [26]. From these lines of evidence, it is suggested that \(\text{sIL-6R}\) formed a complex with IL-6 that had been induced by TNF-\(\alpha\) or IL-17, and that the resulting IL-6/\(\text{sIL-6R}\) complex induced RANKL expression.

Western blotting and immunostaining clearly showed that RANKL protein production. There was no staining when tocilizumab was also added or when the RANK homologue, OPG, was added with RANK-Fc, indicating that \(\text{sIL-6R}\) was necessary for RANKL production and that OPG blocked the binding of RANKL to RANK, respectively. In addition, we showed that co-culture of synovial fibroblasts and RAW cells efficiently induced the expression of NFATc1 which is a main regulator of osteoclast differentiation [27] in the presence of IL-6 and \(\text{sIL-6R}\). These results demonstrated that RANKL induction by IL-6/\(\text{sIL-6R}\) was functional.

In IL-6 trans-signalling, IL-6/IL-6R complex forms and associates with membrane-bound gp130 and the resulting aggregation activates janus kinase-1 (JAK-1), JAK-2 and TYK-2. STAT-3 is recruited to phosphorylated tyrosine residues in the YXXQ motif of gp130, where it is activated and dimerized, and then enters the nucleus and regulates gene expression [28, 29]. In addition, Src homology 2 domain-bearing protein tyrosine phosphatase is recruited to a phosphorylated tyrosine residue (Y759) in gp130, leading to the activation of the ras-MAPK pathway [30]. In fact, we showed that stimulation with IL-6/\(\text{sIL-6R}\) induced STAT3 and ERK1/2 phosphorylation and that induction of these phosphorylations was completely inhibited by PAR and PD, respectively. IL-6/\(\text{sIL-6R}\) complex-induced RANKL expression was completely inhibited by PAR, but was not affected by PD. Taken together, these findings demonstrate that RANKL induction by IL-6/\(\text{sIL-6R}\) complex is mediated by the JAK/STAT pathway, not the MAPK pathway.

The treatment of RA patients with tocilizumab prevents joint destruction and improves symptoms [31]. Tocilizumab blocks IL-6 trans-signalling, so the mechanism by which it inhibits joint destruction may be that, by blocking IL-6 trans-signalling, it inhibits osteoclast formation in synovia by down-regulating RANKL expression on synovial cells.

We summarized the results in this study (Fig. 7). IL-6/\(\text{sIL-6R}\) complex directly induced RANKL expression in RA-FLS and it is essential for RANKL induction by TNF-\(\alpha\) and IL-17. Moreover, RANKL induction by IL-6/\(\text{sIL-6R}\) complex is mediated by the JAK/STAT signalling pathway, but not the MAPK signalling pathway.

**Rheumatology key messages**

- IL-6 trans-signalling is needed to induce RANKL expression in synovial cells.
- RANKL expression by IL-6 trans-signalling involves osteoclast formation in synovium of RA patients.

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