Leptin stimulates interleukin-6 production via janus kinase 2/signal transducer and activator of transcription 3 in rheumatoid synovial fibroblasts

S. Muraoka¹, N. Kusunoki¹, H. Takahashi², K. Tsuchiya², S. Kawai¹

¹Division of Rheumatology, Department of Internal Medicine; ²Department of Orthopaedic Surgery
Toho University School of Medicine, Tokyo, Japan.

Abstract

Objectives
The aim of this study was to determine the influence of leptin on the production of proinflammatory cytokines by rheumatoid synovial fibroblasts (RSFs).

Methods
Synovial tissue was obtained from patients with rheumatoid arthritis (RA). Leptin receptor mRNAs were detected by reverse transcription-polymerase chain reaction (RT-PCR). Productions of mRNA and protein of interleukin (IL)-1β, tumour necrosis factor-α (TNF-α), and IL-6 in the culture medium were detected by real-time PCR and ELISA kit, respectively. Small interfering RNA (siRNA) was transfected into RSF to down-regulate the expression of leptin receptor. Effects of inhibitors of janus kinase 2 (JAK2), phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) on IL-6 production were evaluated. Phosphorylation of signal transducer and activator of transcription 3 (STAT3) in RSF were determined by Western blot analysis.

Results
We detected leptin receptor mRNAs in RSFs. Expression of IL-1β and IL-6 mRNA was enhanced in a concentration-dependent manner by addition of leptin to RSFs. IL-6 secretion by RSFs showed an increase after leptin stimulation. Leptin-induced production of IL-6 by RSFs was decreased after exposure to siRNA targeting leptin receptor (Ob-Rb). A JAK2 inhibitor, but not PI3K and MAPK inhibitors, decreased leptin-induced IL-6 production. Enhanced phosphorylation of STAT3 was observed in RSFs after stimulation by leptin.

Conclusion
Leptin may be one of the proinflammatory cytokines that up-regulates IL-6 production in RSFs via activation of JAK2/STAT3. Leptin and JAK/STAT pathway may represent a new alternative therapeutic target in the treatment of RA.

Key words
leptin, rheumatoid arthritis, IL-6, JAK2, STAT3
Introduction

Adipose tissue is a structural component of many organs and a site for energy storage. In addition, recent studies have demonstrated that the major cellular component of adipose tissue, the adipocyte, has the ability to synthesise and release physiologically active molecules such as leptin, adiponectin, and resistin, as well as cytokines like interleukin (IL)-6 and tumour necrosis factor-α (TNF-α) (1). These molecules are called adipokines or adipocytokines. Some adipokines may have a central role in the regulation of insulin resistance (2), as well as being involved in many aspects of inflammation and immunity (3). Leptin is the product of the ob gene, and is a 16-kDa non-glycosylated peptide hormone synthesised almost exclusively by adipocytes that regulates appetite and energy expenditure centrally at the hypothalamic level (4). It is also suggested that leptin may contribute to inflammation and auto-immunity (5). This is not explained by gene background such as polymorphism of LEP rs2167270 (19 G>A) (6).

Rheumatoid arthritis (RA) is characterised by extensive inflammation and proliferation of the synovium that affects multiple joints. Since proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, play a central role in the pathophysiological mechanisms of RA, novel methods of neutralising these cytokines with monoclonal antibodies or soluble receptors have recently been developed as new treatments for this disease (7). Although blockade of the above-mentioned cytokines is beneficial, it is not curative and the effect is only partial, with many patients failing to respond. Therefore, it seems possible that other proinflammatory cytokines may also contribute to inflammation in RA. We previously reported that adiponectin (one of the adipokines) stimulates the production of IL-8 (8) and prostaglandin E2 (9) by rheumatoid synovial fibroblasts (RSFs). We also reported that the serum levels of leptin and adiponectin were elevated in patients with RA (10). Moreover, leptin levels are increased in synovial fluid of RA patients (11). These findings suggest that some adipokines may contribute to synovial inflammation in RA. Accordingly, we examined the direct effects of leptin on cultured RSFs in the present study.

Materials and methods

Materials

Recombinant human leptin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in 15 mM HCl and 7.5 mM NaOH at a pH of approximately 5.2, in accordance with the manufacturer’s instructions. Recombinant IL-6 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA) and was dissolved in sterile phosphate-buffered saline (PBS) containing 0.1% (volume/volume) bovine serum albumin to prepare stock solutions. Mouse anti-human IL-6 antibody was obtained from R&D Systems, Inc. Rabbit anti-human signal transducer and activator of transcription (STAT) 3 polyclonal antibody and rabbit anti-human phosphorylated STAT (phospho-STAT) 3 (Tyr705) polyclonal antibody were sourced from Cell Signalling Technology (Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA), ECL Western blotting detection reagent was purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK), and polyvinylidene difluoride membranes (Immobilon-P) were obtained from Millipore Corp. (Billerica, MA, USA). AG490 (2-cyano-3-[3,4-dihydroxyphenyl]-N-[phenylmethyl]-2-propenamide), a janus kinase (JAK) 2 inhibitor came from Merck KGaA (Darmstadt, Germany), while LY294002 (2-[4-morpholinyl]-8-phenyl-4H-1-benzopyran-4-one), a phosphatidylinositol 3-kinase (PI3K) inhibitor and PD98059 (2-[2-amino-3-methoxyphenyl]-4H-1-benzopyran-4-one), a mitogen-activated protein kinase (MAPK) inhibitor for extracellular signal-regulated kinase (ERK) were from Sigma-Aldrich. RPMI 1640 medium, penicillin/streptomycin solution, fetal bovine serum (FBS), and 0.25% trypsin/EDTA were sourced from Invitrogen Corp. (Carlsbad, CA, USA). PBS was purchased from Takara Shuzo.
Cell culture
RSFs were prepared from synovial tissue as described previously (12). RA tissue specimens were obtained from patients undergoing total knee replacement who fulfilled the revised criteria (13) for the classification of RA. The protocol for this study was approved by the Toho University Ethics Committee (approval number: 19021), and all patients gave written consent to the use of their tissue for the research. Synovial tissue was digested for 2 hours with 0.25% (weight/volume) bacterial collagenase (ImmunoBiological Laboratories, Gunma, Japan) and then was suspended in RPMI 1640 medium with 10% (v/v) FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The cells were incubated at 37°C under 5% CO₂ for several days, after which nonadherent cells were removed. Fibroblast-like adherent cells from the third or fourth passages were used as RSFs at a concentration of 2.5×10⁵ cells/75 cm² flask.

Reverse transcription – polymerase chain reaction (RT-PCR)
Cells were seeded in culture medium containing 10% (v/v) FBS, and total RNA was extracted with an RNeasy mini kit (Qiagen GmbH., Hilden, Germany), in accordance with the manufacturer’s instructions. Reverse transcription was performed with a SuperScript first-strand synthesis system for RT-PCR (Invitrogen Corp.) with the manufacturer’s recommendations (Applied Biosystems, Foster City, CA, USA). Cells were cultured under various conditions in medium containing 1% (v/v) FBS, after which extraction of total RNA and synthesis of cDNA were performed as described above. Specific probes for IL-6, IL-1β, and TNF-α, real-time PCR was performed using real-time TaqMan technology with a Sequence Detection System model 7000 according to the manufacturer’s recommendations (Applied Biosystems). The amplified complementary DNA (cDNA) fragments were resolved by electrophoresis on 2% (w/v) agarose gel, and were detected under ultraviolet light using an LAS-3000 (Fujifilm Corp. Tokyo, Japan) after the gel was stained with ethidium bromide.

Real-time PCR
To semi-quantitatively evaluate the expression of messenger RNA (mRNA) for IL-6, IL-1β, and TNF-α, real-time PCR was performed using real-time TaqMan technology with a Sequence Detection System model 7000 according to the manufacturer’s recommendations. Specific probes for IL-6, IL-1β, and TNF-α were obtained from TaqMan Gene Expression Assay (Applied Biosystems), with the ID numbers of the products being Hs99999032_m1 for IL-6, Hs99999029_m1 for IL-1β and Hs00174128_m1 for TNF-α. The threshold cycle was calculated from a standard curve and expression of the target mRNA was normalised for the expression of β-actin mRNA.

Western blot analysis
Cells were cultured under various conditions at a density of 5×10⁴/cm² in medium containing 1% (v/v) FBS. Then cell lysates were adjusted to 10 μg containing 0.1% (v/v) Tween 20 (TBST) and 5% (v/v) skim milk, the primary antibody (anti-human STAT3 antibody or anti-human phospho-STAT3 antibody) was added at a dilution of 1:1000 in TBST, and incubation was done for 18 hours at 4°C. After the membranes had been washed with TBST, the secondary antibody (HRP-conjugated goat anti-rabbit antibody) was added at a dilution of 1:10,000 in TBST and incubation was performed for 1 hour. After further washing with TBST, protein bands were detected with an enhanced ECL Western blotting detection reagent (GE Healthcare UK Ltd.) using LAS-3000 (Fujifilm Corp.).

Measurement of cytokines in the culture medium
Cells were seeded in 24-well plastic plates (1×10⁵/well) and cultured for 24 hours under various conditions in medium containing 1% (v/v) FBS under an atmosphere of 5% CO₂. Then the concentrations of IL-6, IL-1β, and TNF-α in the medium were measured with an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s recommendations (Invitrogen Corp.). Experiments using RSFs were done in triplicate wells, and the concentrations of IL-6, IL-1β, and TNF-α were measured in triplicate.

RNA interference assay with Ob-Rb
An RNA interference assay was performed to assess the effect on RSFs of down-regulating Ob-Rb expression. Small interfering RNA (siRNA) for Ob-Rb (Stealth™ RNAi) and negative control siRNA were purchased from Invitrogen Corp. For gene knockdown
Leptin stimulates IL-6 production / S. Muraoka et al.

experiments, RSFs were plated in 10 cm plastic dishes (3×10^5/dish) in RPMI 1640 medium with 10% (v/v) FBS and cultured for 18 hours. Then the medium was changed to serum-free RPMI 1640 medium, and the cells were transfected with siRNA (10 pmol/ml) for Ob-Rb or with control siRNA (10 pmol/ml) using Lipofectamine™ RNAiMAX (Invitrogen Corp.) according to the manufacturer’s recommendations. After 72 hours, the cells were replated into 35-mm plastic dishes for PCR or into 96-well plastic plates for measurement of IL-6 by ELISA.

**Results**

**Effect of leptin on production of inflammatory cytokines by RSFs**

To determine whether leptin increased the production of IL-1β, IL-6, and TNF-α by RSFs, real-time PCR was performed. The results showed that leptin significantly increased the expression of IL-1β and IL-6 mRNA by RSFs in a concentration-dependent manner (Fig. 1). In contrast, expression of TNF-α mRNA was not increased by leptin (data not shown). To confirm the production of IL-1β and IL-6 proteins, we measured the concentrations of these cytokines in the culture medium of RSFs incubated with leptin (Fig. 2). We found that leptin significantly increased IL-6 production by RSFs in a concentration-dependent manner. In contrast, concentrations of IL-1β and TNF-α were not detectable ever after stimulation with leptin.

**Effect of siRNA for leptin receptor on IL-6 production by RSFs**

mRNAs for both leptin receptors (Ob-Rb and Ob-Re) were expressed by cells from 3 patients with RA (data not shown). RSFs were transfected with siRNA targeting Ob-Rb (the leptin receptor) or with negative control siRNA, and then expression of Ob-Rb and Ob-Re mRNA was detected by RT-PCR. This showed that Ob-Rb mRNA expression by RSFs was decreased after exposure to the siRNA for Ob-Rb (Fig. 3A). When cells were seeded in 96-well plates and incubated with leptin for 18 hours, IL-6 production by RSFs transfected with the siRNA targeting Ob-Rb was significantly lower than that by RSFs transfected with negative control siRNA (Fig. 3B).

**Effect of leptin on STAT3 phosphorylation in RSFs**

We then examined more details of the signal transduction involved in these effects of leptin. To determine whether leptin induced STAT3 phosphorylation in RSFs, Western blotting was performed. This revealed that leptin increased STAT3 phosphorylation in a concentration-dependent manner (Fig. 4A). To investigate whether phosphorylation of STAT3 was related to the induction of IL-6 production by leptin, RSFs were
Leptin stimulates IL-6 production / S. Muraoka et al.

Leptin stimulates IL-6 production / S. Muraoka et al.

Incubated with an anti-IL-6 antibody (Fig. 4B). Phosphorylation of STAT3 in response to leptin was not inhibited by addition of the anti-IL-6 antibody, but STAT3 phosphorylation in response to IL-6 was inhibited by the antibody.

Effects of signalling pathway inhibitors on leptin-induced IL-6 production by RSFs

We examined the effects of inhibitors of major signalling pathways on leptin-induced IL-6 upregulation in RSFs. As a result, leptin-induced IL-6 production was significantly inhibited by addition of AG490, a JAK2 inhibitor (Fig. 5), but not by LY294002, a PI3K inhibitor (Fig. 6A) or PD98059, a MAPK inhibitor for ERK (Fig. 6B). These findings suggested that leptin induces IL-6 production in RSFs via the JAK2/STAT3 pathway.

Discussion

In the present study, we demonstrated that leptin induced the expression of IL-6 mRNA and protein in RSFs via the JAK2/STAT3 pathway. This finding is supported by data obtained in leptin-deficient ob/ob mice by Busso et al. (14), who reported that leptin-deficient mice were partly protected against antigen-induced arthritis, showing less synovial tissue proliferation and a weaker humoral response to the injected antigen. Moreover, Sugioka et al. (15) reported that acquired leptin resistance by high-fat feeding reduces inflammation from collagen antibody-induced arthritis in mice.

Harigai et al. (16) reported that TNF-α induced IL-6 production by synovial fibroblasts in a dose dependent manner. On the other hand, the present study showed that leptin stimulated IL-6 production. Gonzalez-Gay et al. (17) reported that leptin concentration was not changed by administration of anti-TNF-α-blocker infliximab. Therefore, TNF-α-induced IL-6 production might not be mediated by leptin.

IL-6 is a pleiotropic cytokine that is overexpressed in the synovial tissue of RA patients, who have elevated concentrations of IL-6 in both serum and synovial fluid (18). IL-6 influences the function of neutrophils, T cells, B cells, monocytes, and osteoclasts. It is a major inducer of the hepatic acute phase response, which is also a key feature of RA that is correlated with disease activity and joint destruction. Thus, IL-6 is thought to play a pivotal role in RA. Tocilizumab is a humanised anti-IL-6 receptor monoclonal antibody that has shown efficacy for treating RA in clinical trials (19). The average levels of IL-6 in serum and synovial fluid of
Leptin stimulates IL-6 production / S. Muraoka et al.

RA patients were 14 pg/mL and 4 ng/mL, respectively (20). In our study, 20–283 ng/mL of IL-6 were produced by 25–300 nM of leptin (Fig. 2). Thus, our present results suggest a contribution of leptin to the pathogenesis of RA via its influence on IL-6.

Although IL-1β mRNA in RSFs was increased by leptin, IL-1β protein was not detectable in culture medium of RSFs. In general, synovial fibroblasts are not the principal sources of inflammatory mediators in synovium (21). This might be one of the reasons of the discrepancy between changes in mRNA and protein of IL-1β in our study.

Six isoforms of the leptin receptor have been identified (22). The Ob-Re isoform is a soluble receptor that lacks the transmembrane and cytoplasmic domains, while Ob-Rb is a long form that has an intracellular signalling domain and is thought to be involved in intracellular signalling. In the present study, we found that both Ob-Rb and Ob-Re mRNAs were expressed by RSFs. In addition, the response of IL-6 to leptin was reduced when RSFs were transfected with siRNA targeting Ob-Rb. Therefore, the induction of IL-6 production by leptin was mediated by Ob-Rb.

It is known that JAK/STAT pathway is activated by leptin in several kinds of human cells, that is hepatocellular carcinoma (23), peripheral blood mononuclear cells (24), colorectal adenoma (25). However, these reports have not shown upregulation of IL-6 by leptin. In addition, this is the first report that leptin stimulates IL-6 production via JAK2/STAT3 in RSFs. Although leptin has been shown to stimulate IL-6 production in human osteoarthritic cartilage (26), this was mediated by the nuclear factor κB and MAPK pathway rather than the JAK2/STAT3 pathway. Since Migita et al. (27) reported that IL-6 induced acute-phase serum amyloid A genes via JAK2/STAT3 activation in RSFs, we determined whether STAT3 phosphorylation was affected by the leptin-induced upregulation of IL-6. Phosphorylation of STAT3 in response to leptin was not inhibited by the anti-IL-6 antibody, suggesting that STAT3 phosphorylation might be due to a direct effect of leptin on RSFs.

A previous study demonstrated that leptin activated two signalling pathways (PI3K and MAPK) in RSFs and human peripheral blood mononuclear cells (28, 29). Therefore, we investigated the effect of LY294002 (a PI3K inhibitor) and PD98059 (a MAPK inhibitor for ERK) on RSFs incubated with leptin. As a result, leptin-induced

**Fig. 5.** Effect of a JAK2 inhibitor on IL-6 production by RSFs. Interleukin (IL)-6 level in culture medium of rheumatoid synovial fibroblasts incubated for 18 hours with/without leptin and AG490. The IL-6 concentration in the culture medium was measured by enzyme-linked immunosorbent assay. Leptin-induced IL-6 production was significantly inhibited by addition of AG490, a janus kinase 2 inhibitor. Bars show the mean and SEM (n=3). *p<0.05; **p<0.01. Significance was evaluated by one-way analysis of variance with Bonferroni’s post hoc test.

**Fig. 6.** Effect of signalling pathway inhibitors on IL-6 production by RSFs. (A, B) Interleukin (IL)-6 level in culture medium of rheumatoid synovial fibroblasts incubated for 18 hours with/without leptin, LY294002, and PD98059. The IL-6 concentration in the culture medium was measured by enzyme-linked immunosorbent assay. Leptin-induced IL-6 production was not inhibited by LY294002, a phosphatidylinositol 3-kinase inhibitor (A) or PD98059, a mitogen-activated protein kinase inhibitor for extracellular signal-regulated kinase (B). Bars show the mean and SEM (n=3). *p<0.05; **p<0.01. Significance was evaluated by one-way analysis of variance with Bonferroni’s post hoc test.
IL-6 production was not mediated by signalling of PI3K and/or MAPK. The serum leptin level in RA patients was reported to be in the 1–30 nM range (10), so the concentration of leptin used in this study was higher, but it might be possible that leptin stimulates a vicious cycle of inflammation by a paracrine effect in the articular cavity (30). Further studies will be necessary to confirm the mechanism by which leptin influences RA.

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