Secreted frizzled-related protein 5 suppresses inflammatory response in rheumatoid arthritis fibroblast-like synoviocytes through down-regulation of c-Jun N-terminal kinase

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Abstract

Objective. This study was performed to investigate the effect of secreted frizzled-related protein 5 (Sfrp5), a novel anti-inflammatory adipokine that competes with the frizzled proteins for Wnt binding, on inflammatory response and the c-Jun N-terminal kinase (JNK) signalling pathway in RA.

Methods. Expression of Sfrp5 mRNA in peripheral blood mononuclear cells (PBMCs) and fibroblast-like synoviocytes (FLSs) from patients with RA and OA was determined using real-time quantitative PCR (qPCR). Sfrp5 RNA interference (RNAi) plasmids were transfected to abrogate Sfrp5 expression in RA FLSs, and adenovirus containing the Sfrp5 transcript was delivered into RA FLSs to strengthen Sfrp5 expression. Levels of pro-inflammatory genes and their protein products were determined using real-time qPCR and ELISA in RA FLSs. Production of mitogen-activated protein kinase kinase 7 (MKK-7), JNK and c-Jun were assessed by Western blot analysis.

Results. Expression of Sfrp5 mRNA was decreased in PMBCs and FLSs from patients with RA compared with patients with OA. Gene expression and production of IL-1β, IL-6, chemokine ligand 2 (CCL-2), CCL-7, cyclooxygenase 2 and MMP-9 were markedly increased in Sfrp5 RNAi plasmid-transfected RA FLSs, while transfection with adenoviral vectors encoding Sfrp5 induced reductions in those levels. Phosphorylated forms of MKK-7, JNK and c-Jun were increased by Sfrp5 RNAi plasmids and were decreased by adenoviral vectors encoding Sfrp5.

Conclusion. Sfrp5 suppressed the inflammatory response and down-regulated JNK signalling in RA FLSs. These findings provide evidence for the anti-inflammatory effect of Sfrp5 in RA.

Key words: rheumatoid arthritis, fibroblast-like synoviocytes, secreted frizzled-related protein 5, Wnt signalling, c-Jun N-terminal kinase.

Introduction

RA is characterized by polyarticular synovitis and extensive joint destruction. Various inflammatory cells including CD4+ T cells, B cells and macrophages infiltrate the synovium and the tissue undergoes extensive proliferation due to the increased number of fibroblast-like synoviocytes (FLSs). This proliferating synovial lining forms a pannus that invades the articular cartilage and bone tissue, leading to irreversible joint destruction. This process is mediated by various pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, chemokines, MMPs and cyclooxygenase 2 (COX-2) [1, 2]. Expression of these pro-inflammatory mediators is regulated by activation of transcription factors, including nuclear factor kappa B (NF-κB), activation protein 1 (AP-1) and mitogen-activated protein kinases (MAPKs) [3–7].

Wingless (Wnt) proteins are secreted signalling factors that belong to a large family of structurally related
proteins, while the frizzled (Fz) family includes membrane-integrated proteins that function as receptors for transduction of the Wnt signal into the cytoplasm [8, 9]. It has been documented that Wnt/Fz proteins play significant roles in embryonic development, haematopoiesis, synaptogenesis and mammary development [10–16]. Previous reports have shown that several Wnt and Fz proteins are expressed in the synovial tissue of patients with RA. Sen et al. [17, 18] reported that Wnt5a/Fz5 proteins are overexpressed in RA FLSs and are involved in IL-6 and IL-15 production in these cells. The activity of Wnt is highly controlled by negative extracellular regulators, such as the soluble factor Dickkopf-1 (DKK1) and secreted Fz-related proteins (Sfrps) [19, 20]. Of these, Sfrp5, a recently identified adipokine that belongs to a family of five secreted glycoproteins, has been reported to modulate Wnt–Fz interactions [21, 22]. Sfrp5 contains a cysteine-rich domain (CRD) that is homologous to that of Wnt receptor Fz proteins. It has been shown that Sfrp5 competes with Fz proteins for Wnt binding via this CRD, thereby interfering with Wnt signalling [20, 23]. Sfrp5 has also been implicated in metabolic homeostasis, in that it contributes to the resolution of insulin resistance and obesity-linked metabolic disorders through Wnt inhibition [24, 25]. Recently it was reported that Sfrp5 exerts anti-inflammatory effects. One study found that the expression level of Sfrp5 is inversely correlated with that of MMPs [26]. Additionally, Sfrp5 reduces the accumulation of activated macrophages in adipose tissue and suppresses the expression of pro-inflammatory cytokines such as TNF-α and IL-6 by inhibiting phosphorylation of c-Jun N-terminal kinase (JNK) [27].

Given these previous observations, it would be reasonable to assume that Sfrp5 may have an anti-inflammatory role in the pathogenesis of RA, which is one of the most common inflammatory autoimmune diseases. Until now, however, no studies have demonstrated a role for Sfrp5 in RA. We hypothesized that Sfrp5 would affect the expression and production of pro-inflammatory mediators in patients with RA, and thus we investigated whether expression of Sfrp5 is different in FLSs from patients with RA vs those with OA. Additionally we sought to determine whether Sfrp5 levels correlate with those of various pro-inflammatory mediators in RA FLSs.

Materials and methods

Patients and samples

Synovial tissue was obtained during total knee replacement surgery or arthroscopic synovectomy from five patients with RA (four women, one man, aged 57–68 years) who fulfilled the 1987 revised ACR classification criteria [28], as well as from six OA patients (five women, one man, aged 57–70 years) who fulfilled the 1986 ACR classification criteria [29]. At the time of surgery, fasting blood samples were collected from those patients and additional blood samples were obtained from another five RA patients and four OA patients. The study and the use of human samples were approved by the Ethics Committee of Gangnam Severance Hospital, Seoul, South Korea, and written informed consent was obtained from all patients.

Isolation and culture of cells

FLSs were isolated by enzymic dispersion of synovial tissue obtained from five patients with RA and six patients with OA. Once the connective tissue and fat were removed, synovial tissues were digested with 4 mg/ml collagenase II (Sigma, St Louis, MO, USA) in serum-free DMEM for at least 4 h at 37°C. Cell suspensions were passed through a nylon mesh and FLSs were then collected by centrifugation at 800g for 5 min and resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). Harvested cells were cultured in 75 cm² culture flasks (Costar, Cambridge, MA, USA) with DMEM supplemented with 1% penicillin/streptomycin and 10% FBS at 37°C in a humidified atmosphere of 5% CO₂. When the cells had grown to confluence, they were detached with 0.25% trypsin, split at a ratio of 1:3 and re-cultured in DMEM under the same conditions. Cells obtained from the fourth through sixth passages were used in this study.

Blood samples from 10 patients with RA and 10 patients with OA were used for the experiment. Immediately after blood samples were obtained, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by density gradient centrifugation using the Ficoll-Hypaque method (GE Healthcare, Waukesha, WI, USA). After centrifugation at 2000g for 20 min, the buffy coat was carefully collected and washed twice with PBS. Purified cells were cultured in RPMI 1640 supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated FBS.

RNA interference

After treatment of RA FLSs with 10 ng/ml TNF-α for 24 h where indicated, Sfrp5 RNA interference (RNAi) plasmids were transfected to suppress its expression in RA FLSs, while non-silencing RNAi plasmid was used as the control. The target sequence of Sfrp5 small interfering RNA (siRNA) was 5′-AAGGAAGAATAAGGAGATGAAGTT-3′, corresponding to 1022–1044 of the human Sfrp5 complementary DNA (cDNA) sequence (GenBank: NM 003015). RA FLSs were adjusted to a density of 2 × 10⁴ and were transfected with Sfrp5 siRNA or control siRNA using Lipofectamine 2000 (Bioneer, Alameda, CA, USA) according to the manufacturer’s protocol. To establish FLSs that stably expressed Sfrp5, cells were trypsinized and plated at low density. The efficacy of siRNA was confirmed at 48 h post-transfection by RT-PCR analysis using the appropriate primers.

Construction of expression plasmids and transient transfection

Adenoviral vector containing cDNA of wild-type Sfrp5 (pcDNA3.1 Sfrp5) or the empty vector was transiently transfected into RA FLSs to enhance the expression of Sfrp5. The full-length pcDNA3.1 (Bioneer) Sfrp5 vector
was made by cloning the full-length PCR product of Sfrp5 with PFU DNA polymerase (Bioneer). All plasmid sequences were confirmed by DNA sequencing. For transient transfection experiments, RA FLSs were plated in a 24-well plate at a density of 2 × 10^5 for 24 h prior to transfection. Lipofectamine 2000 (Bioneer) was used to perform transfection with 2.0 mg pcDNA3.1 Sfrp5 vector or 2.0 mg pcDNA3.1 empty vector according to the manufacturer’s instructions.

**Real-time quantitative PCR**

Total RNA from was isolated from FLSs using TRIzol and DNase I (Invitrogen, Carlsbad, CA, USA) and concentrations were determined using a NanoDrop (R&D Systems, Minneapolis, MN, USA). Total RNA was reverse transcribed using oligo(dT). Real-time quantitative PCR (qPCR) was performed using 1 μl of complementary DNA (cDNA) per well, TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA) and 250 nM each of sense and antisense primers. All primers used for qPCR to detect IL-1β, IL-6, chemokine ligand 2 (CCL-2), CCL-7, COX-2 and MMP-9 were purchased from Integrated DNA Technologies (Applied Biosystems). Results were evaluated using the ΔΔCT method and the calculated number of copies was normalized to the number of β-actin mRNA copies in the same sample.

**ELISA**

The concentration of IL-1β, IL-6, CCL-2, CCL-7, COX-2 and MMP-9 in the culture supernatants of RA FLSs was determined using commercially available ELISA kits (R&D Systems) according to the manufacturer’s protocol. Briefly, for the measurement of IL-1β, 100 μl of culture supernatant of RA FLSs and assay diluent were placed in each well of a microwell plate that was coated with a monoclonal antibody against IL-1β and incubated for 2 h at room temperature on a horizontal orbital microplate shaker at 500 g. Each well was aspirated and washed four times with wash buffer prior to the addition of 200 μl of conjugate. Plates were then incubated with shaking for 2 h at room temperature and each well was washed four times prior to the addition of 200 μl of substrate solution, which was prepared with equal amounts of stabilized hydrogen peroxide (H₂O₂) and tetramethylbenzidine. Following incubation for 30 min at room temperature, the reaction was quenched by addition of 100 μl of stop solution (2 N H₂SO₄) and absorbance was read using microplate reader (SpectraMax 340; Molecular Devices Co., Sunnyvale, CA, USA) at a wavelength of 450 nm. Intra- and interassay coefficients of variation were <8% for all tests.

**Western blot analysis**

RA FLSs (70–80% confluency) were plated on Matrigel-coated Petri dishes and cultured overnight. Supernatants were collected and concentrated with Microcon YM-10 columns (Millipore, Bedford, MA, USA). The same amount of total protein (5 μg) was loaded onto 3–8% SDS-polyacrylamide gels (Invitrogen) and electrophoresed under reducing conditions. Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). Membranes were blocked with 5% non-fat milk overnight at 4°C and subsequently incubated with each primary antibody, including anti-mitogen-activated protein kinase kinase 7 (Mkk-7), anti-phosphorylated JNK and anti-c-Jun in PBS with 0.1% Tween 20 for 1 h at room temperature. Horseradish peroxidase-conjugated goat anti-mouse IgG was used as a secondary antibody and was incubated for 1 h at room temperature. Immunoreactive protein bands were detected by enhanced chemiluminescence (ECL Plus; Amersham Biosciences, Piscataway, NJ, USA) and visualized using Kodak X-OMAT film. Quantification of the bands from RA FLSs (n = 5) was performed by densitometric analysis using Scion image software (Epson GT-X700, Tokyo, Japan).

### Statistical analysis

Results are expressed as the mean (s.e.m.). Each result reported represents the mean of at least three experiments performed on different days. Differences were assessed by one-way and two-way repeated-measures analysis of variance (ANOVA), followed by the Bonferroni post hoc test for comparison of multiple groups or a Mann–Whitney U-test for comparison of the arthritis index medians. P-values <0.05 were considered to be statistically significant.

### Results

**Sfrp5 expression in PBMCs and FLSs from RA and OA patients**

Expression of Sfrp5 mRNA in PBMCs and FLSs from patients with RA or OA was determined using real-time quantitative RT-PCR. As shown in Fig. 1, expression of Sfrp5 mRNA expression in OA.

**Fig. 1** Comparison of Sfrp5 expression in PBMCs and FLSs from RA and OA patients

The analysis was performed by real-time quantitative RT-PCR in PBMCs and FLSs of patients with RA or OA. Data are shown as mean (s.e.m.) of three independent experiments. *P < 0.05 vs Sfrp5 mRNA expression in OA. Sfrp5: secreted frizzled-related protein 5; PBMCs: peripheral blood mononuclear cells. FLSs: fibroblast-like synoviocytes.
Sfrp5 mRNA in RA FLSs was significantly decreased compared with OA FLSs; expression of Sfrp5 mRNA in OA FLSs was 63-fold higher than in RA FLSs. Although no statistical significance was found, mRNA expression of Sfrp5 in RA PBMCs was also decreased compared with OA PBMCs.

**Effect of Sfrp5 regulation on pro-inflammatory mediators in RA FLSs**

To investigate the effect of Sfrp5 regulation on inflammatory response in RA FLSs, expression of IL-1β, IL-6, CCL-2, CCL-7, COX-2 and MMP-9 mRNA was measured by real-time qPCR in RA FLSs transfected with Sfrp5 RNAi plasmid or pcDNA3.1 Sfrp5 vector after treatment with TNF-α. As shown in Fig. 2, expression of IL-1β, IL-6, CCL-2, CCL-7, MMP-9 and COX-2 mRNA compared with that of β-actin was augmented in RA FLSs by TNF-α stimulation. This TNF-α-stimulated expression of IL-1β, IL-6, CCL-2, CCL-7, COX-2 and MMP-9 mRNA was further increased in RA FLSs transfected with the Sfrp5 RNAi plasmid compared with the non-silencing RNAi plasmid. In contrast, TNF-α-stimulated expression of those pro-inflammatory genes was markedly decreased in RA FLSs transfected with the pcDNA3.1 Sfrp5 vector compared with those transfected with control vector.

We next performed ELISA to determine the effect of Sfrp5 regulation on pro-inflammatory mediator production. Transfection of RA FLSs with the Sfrp5 RNAi plasmid significantly increased the concentrations of pro-inflammatory mediators in culture supernatants of RA FLSs, and transfection with the pcDNA3.1 Sfrp5 vector decreased those levels (Fig. 3).

**Effect of Sfrp5 regulation on the JNK signalling pathway**

We assessed the effect of Sfrp5 on the JNK-mediated signalling pathway in RA FLSs. Phosphorylated forms of MKK-7, JNK and c-Jun were measured in RA FLSs transfected with the Sfrp5 RNAi plasmid or pcDNA3.1 Sfrp5 vector using western blotting. As shown in Fig. 4, phosphorylated MKK-7, JNK and c-Jun levels were increased in RA FLSs transfected with the Sfrp5 RNAi plasmid compared with the non-silencing RNAi plasmid. In contrast, phosphorylated MKK-7, JNK and c-Jun levels were decreased upon transfection of RA FLSs with the pcDNA3.1 Sfrp5 vector. Densitometric quantification of
bands from five RA FLSs showed that all of these changes had statistical significance.

**Discussion**

The evidence accumulated to date shows that the Wnt signalling pathway plays a critical role in the pathogenesis of RA. The expression of β-catenin, a downstream component of the Wnt pathway, was significantly higher in the synovium of patients with RA than those with OA or traumatic arthritis [30]. Expression of β-catenin was augmented by the activation of the canonical Wnt signalling pathway, and activation of β-catenin signalling subsequently contributed to the activation of RA FLSs [31]. A recent report by Hardy et al. [32] showed that DKK1, a Wnt antagonist, was secreted by RA FLSs in response to inflammatory insult, and the authors proposed that this protein may be a key regulator of the osteoblast-osteoclast axis that is imbalanced in patients with RA.

Several reports have also shown that Wnt5a-mediated signalling contributes to the induction of pro-inflammatory cytokines, chemokines and other inflammatory molecules in patients with RA. Sen et al. [17, 18] reported that Wnt5a-mediated production of cytokines and chemokines was at least partly induced by NF-κB, which was supported by the fact that treatment of RA FLSs with anti-Fz5 antiserum reduced the expression of cytokines and chemokines. Taken together, these findings suggest that the Wnt5a-mediated Wnt signalling pathway participates in promoting pro-inflammatory cytokine and chemokine production in RA FLSs.

In the present study we found the presence of Sfrp5 in PBMCs and FLSs from patients with RA and OA. Previously Ijiri et al. [33] determined the expressions...
of Sfrps in OA and RA synovial tissues, and their semi-quantitative RT-PCR analysis showed negative expressions of Sfrp5 in both. Although the expression levels of Sfrp5 in FLSs were much lower than those in PBMCs, our real-time quantitative RT-PCR analysis showed that Sfrp5 mRNA expressions were also detected in FLSs from patients with RA or OA. Although the reason for the difference between the data from Ijiri et al. [33] and ours is not well understood, data from the present study provides evidence for Sfrp5 expression in articular synovium.

In the present study we also found that the expression of Sfrp5 mRNA was markedly lower in both FLSs and PBMCs in patients with RA compared with OA and that expression of various pro-inflammatory genes such as TNF-α, IL-1β, IL-6, CCL-2, CCL-7, COX-2 and MMP-9 was enhanced by down-regulation of Sfrp5 and suppressed by overexpression of Sfrp5 in RA FLSs. These findings in patients with RA correspond to previous observations in patients and animal models of other diseases such as cancer and obesity. Decreased Sfrp5 gene expression enhanced the production of MMPs and contributed to the migration and metastasis of gastric cancer cells [34–36]. Additionally, methylation of the Sfrp5 promoter has been shown to augment the progression of ovarian [37] and breast cancer [38]. Circulating Sfrp5 levels were also lower in patients with impaired glucose tolerance or type 2 diabetes mellitus [39] and inversely correlated with markers for obesity [40]. Another study found that Sfrp5 was decreased in mice with genetic or dietary obesity and the administration of Sfrp5 improved both metabolic function and insulin resistance [27]. Like these previous observations, our data indicate that as a negative regulator of Wnt signalling, Sfrp5 is down-regulated in patients with RA and has anti-inflammatory effects in RA FLSs. To our knowledge, this is the first study to demonstrate down-regulation of the Sfrp5 gene and to associate this with levels of inflammatory mediators in patients with RA, and our data provide evidence for the anti-inflammatory effect of Sfrp5 in RA.

Sfrp5 can antagonize Wnt5a signalling, which contributes to the production of several pro-inflammatory cytokines and chemokines in patients with RA [17, 18]. On the other hand, up-regulated Wnt signalling has been shown to suppress the expression of Sfrp5 mRNA [24]. This negative feedback mechanism, in which a reduction in Sfrp5 activates Wnt signalling, can result in further enhancement of Wnt signalling as Sfrp5 expression decreases. A recent report by Ouchi et al. [27] showed that Sfrp5 restored metabolic dysfunction in obese mice through suppression of several inflammatory genes. In that study, the anti-inflammatory effects of Sfrp5 were mediated by the down-regulation of Wnt5a-JNK signalling, suggesting that the Sfrp5–JNK regulatory axis represents a potential target for control of obesity-linked abnormalities in glucose homeostasis. Similarly, we found that JNK, a downstream target of non-canonical Wnt signalling, was up-regulated by Sfrp5 RNAi plasmid transfection in RA FLSs compared with non-silencing RNAi plasmid transfection. Moreover, we found that the phosphorylated form of MKK-7, the upstream kinase that regulates JNK activity by controlling phosphorylation of the tyrosine and threonine residues in the TPY (Thr-Pro-Tyr) motif, and the phosphorylating activity of the JNK substrate c-Jun were all decreased. In contrast, when RA FLSs were transfected with the Sfrp5 vector, MKK-7, JNK and c-Jun phosphorylation were all decreased. These results indicate that down-regulation of Sfrp5 enhanced Wnt5a-induced JNK activation and the subsequent production of pro-inflammatory mediators in RA FLSs, and that overexpression of Sfrp5 in RA FLSs reversed the activated
non-canonical Wnt signalling and inhibited the expression of pro-inflammatory mediators.

In conclusion, the present study showed significant effects of Sfrp5 regulation on the inflammatory response in FLSs from patients with RA as well as the down-regulation of JNK signalling. Although the exact mechanisms underlying the anti-inflammatory effects of Sfrp5 in RA remain to be further clarified, our findings indicate a novel role for Sfrp5 as a negative regulator in rheumatoid inflammation and support its potential role in the treatment of RA.

**Rheumatology key messages**

- Expression of secreted frizzled-related protein 5 (Sfrp5) is decreased in RA compared with OA.
- Sfrp5 suppressed gene expression and production of pro-inflammatory mediators in RA fibroblast-like synoviocytes.
- Sfrp5 down-regulated c-Jun N-terminal kinase signalling in RA fibroblast-like synoviocytes.

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**Disclosure statement:** The authors have declared no conflicts of interest.

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