The effect of caveolin-1 knockdown on interleukin-1β-induced chemokine (C-C motif) ligand 2 expression in synovial fluid-derived fibroblast-like synoviocytes from patients with rheumatoid arthritis


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Abstract

Background. Rheumatoid arthritis (RA) is a chronic autoimmune disease leading to destructive changes in peripheral joints and their irreversible deformity. The influx of chemoattractant-mediated inflammatory cells to the joints is one of the main features of RA.

Objectives. The aim of this study was to investigate the effect of a knockdown of caveolin-1 (CAV1), a known regulator of multiple cell signaling pathways, on chemokine (C-C motif) ligand 2/monocyte chemoattractant protein-1 (CCL2/MCP-1) expression in synovial fluid-derived fibroblast-like synoviocytes (sfd-FLSs) obtained from patients with RA.

Material and methods. Primary cell cultures of sfd-FLSs were established from RA synovial fluids. Cells were transiently transfected with small interfering RNA (siRNA) specific for CAV1, and then incubated with interleukin (IL)-1β to induce CCL2 expression. The expression levels of CAV1 and CCL2 were assessed at transcript level, using quantitative polymerase chain reaction (qPCR) and at protein level by enzyme-linked immunosorbent assay (ELISA) and western blotting analysis.

Results. A transient CAV1 knockdown in sfd-FLSs resulted in a decrease in the IL-1β-induced CCL2 mRNA expression level vs non-transfected cells and cells transfected with non-targeting siRNA. The concentration of secreted CCL2 was not affected significantly.

Conclusions. Our study demonstrates that CCL2 expression in sfd-FLSs is CAV1-dependent, but only at transcript level. As the function of CAV1 has not been unequivocally determined, more studies are needed to confirm the role of CAV1 in inflammatory processes related to RA.

Key words: rheumatoid arthritis, caveolin-1, C-C chemokine ligand 2/monocyte chemoattractant protein-1, synoviocytes
**Introduction**

Rheumatoid arthritis (RA) is a chronic autoimmune disease resulting in destructive changes in peripheral joints, and subsequently in their irreversible deformity. Rheumatoid arthritis pathogenesis is extremely complex and, despite the identification of many compelling contributory factors, it has not been fully explained.\(^1\)

In RA, cells constituting the intimal lining layer of the synovium proliferate in an uncontrolled manner and form pannus tissue.\(^2\) There are 2 predominant cell types participating in synovial hyperplasia. Type A synoviocytes are terminally differentiated cells with little capacity to proliferate and uneven distribution in the synovial membrane; they activate synoviocytes type B, also called fibroblast-like synoviocytes (FLSs), by producing pro-inflammatory cytokines, chemokines and growth factors.\(^2,3\) Fibroblast-like synoviocytes represent a specialized type of cells and their implication in RA pathogenesis is considered in terms of 2 aspects: destructive changes in joints and chronic inflammation.\(^2–4\) Fibroblast-like synoviocytes present in pannus tissue secrete matrix metalloproteinases, cathepsins and aggrecanases, destroying cartilage which, in consequence, leads to bone resorption.\(^6\) Fibroblast-like synoviocytes promote the influx of chemoattractant-mediated inflammatory cells into the joint cavity in response to the joint milieu, factors such as interleukin (IL)-1β or tumor necrosis factor alpha (TNFα), which is one of the main features of RA.\(^6,7–8\) Secreted C-C chemokine ligand 2 (CCL2) chiefly induces monocytes migrating into the joints, where they differentiate into exudate macrophages, but it can also affect T cells, natural killer cells and basophils.\(^5\) Activated monocytes/macrophages play an important role in the maintenance of inflammation in RA by producing pro-inflammatory cytokines and mediators responsible for the development of synovitis. Therefore, the inhibition of their multi-step migration, driven by a chemoattractant gradient toward the sites of inflammation, is of therapeutic value.\(^2,9\)

Together, the processes in joints perpetuate RA development by recruiting and retaining inflammatory cells, pannus hyperplasia and bone damage. It is believed that effective therapy to restore the balance between pro- and anti-inflammatory cytokines should aim not only at individual cytokines but also at signaling molecules responsible for their production.\(^10\) Thus, the key issue is to find a crucial molecular process which would enable us to control the course of RA. Caveolin-1 (CAV1), expressed in 2 isoforms (α and β), is one of the major structural components of caveolae and has a number of signaling functions.\(^11,12\)

This protein contains a scaffolding domain in the position 82–101, which, by binding to signaling molecules (e.g., Src kinases, endothelial nitric oxide synthase 3, G protein α subunits, or protein kinase C), is able to negatively regulate cell signaling.\(^11,13–15\) On the other hand, CAV1α has the ability to positively regulate signal transduction intracellularly through phosphorylation on tyrosine-14 (Tyr14), which can happen in response to cellular stress, growth factors or stimulation with hormones.\(^16,17\)

Regarding inflammatory disorders, CAV1 could either prevent or induce inflammation, depending on cellular context.\(^11,18–21\) In this study, we decided to investigate the impact of CAV1 on CCL2 expression in synovial fluid-derived fibroblast-like synoviocytes (sfd-FLSs) obtained from patients with RA.

**Material and methods**

**Patients**

The protocol for this study was approved by the Bioethics Committee of Poznan University of Medical Sciences (Poland). Synovial fluids were aspirated for therapeutic reasons from inflammatory knee joint effusions from 3 RA patients (2 women/1 man; age: 48–52 years; disease duration: 1.5–20.5 years) fulfilling both the 1987 American College of Rheumatology (ACR)\(^22\) and the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR)\(^23\) classification criteria. Written informed consent was obtained from every patient before any study procedure was carried out.

**Cell culture**

Primary cell cultures of sfd-FLSs, which could be an alternative to FLSs derived from tissues after surgery,\(^24\) were established from RA synovial fluids based on the procedure described by Scanu et al.\(^25\) To summarize briefly, fluids were collected in tubes containing ethylenediaminetetraacetic acid (EDTA), diluted twice with sterile phosphate-buffered saline (PBS) and centrifuged at 1500 rpm for 10 min at room temperature. Pellets were suspended in a complete Dulbecco’s Modified Eagle’s medium (DMEM) (Sigma, St. Louis, USA) with 10% fetal bovine serum (Biochrom AG, Berlin, Germany) and antibiotic-antimycotic containing 10000 units/mL of penicillin G, 10 mg/mL of streptomycin sulfate and 25 μg/mL of amphotericin B (ABAM; Sigma). The cells were placed into T-25 flasks and incubated under standard conditions (37°C, 5% CO₂ in a humidified atmosphere). After 24 h, non-adherent cells were washed out and fresh DMEM was added to the cells. The medium was changed twice a week. Cells from passages 3–10 were used for the study. Synovial fluid-derived fibroblast-like synoviocytes were checked for the presence of macrophages by flow cytometry. The cells were stained with the following antibodies: phycoerythrin-conjugated CD14 (clone MφP9, 345785; BD, Franklin Lakes, USA), allophycocyanin/Cy7-conjugated CD45 (clone 2D-1, 348815; BD) and allophycocyanin-conjugated CD163 (clone 215927, FAB1607A; R&D Systems, Minneapolis, USA) for 20 min. Next, 500 μL of fluorescence-activated cell sorting (FACS)-lysing solution (BD) was added in a proportion
of 1:10 and incubated for 10 min, protected from light and at room temperature. The cells were washed twice with PBS by centrifugation at 1200 g for 4 min, suspended in 500 µL of PBS, and then subjected to flow cytometry on FACS Canto II (BD). A total of 30,000 events per sample were acquired. The results were analyzed with FACSDiva software (BD Biosciences, Franklin Lakes, USA). Cultured cells did not show markers characteristic for macrophages.

A dose-response study of IL-1β on CCL2 expression in sfd-FLSs

As FLSs lose their primary phenotype with increasing passage number and become quiescent by passage 3, they require exposure to IL-1β to restore their ability to produce chemokines. To determine the most effective IL-1β dose inducing CCL2 expression, FLSs were seeded on 6-well plates (1 × 10^5 cells/well) and serum starved for 24 h. After that, the cells were washed with PBS and treated with different doses of IL-1β (Sigma) (0.1 ng/mL, 1 ng/mL, 5 ng/mL, and 10 ng/mL) diluted in serum-free Opti-Mem Essential Medium (MEM) (Gibco; Life Technologies, Carlsbad, USA) or left untreated. The conditioned media were collected and frozen immediately after a 2-h incubation. The cells were washed with PBS, lysed with TRIzol (Sigma) and stored at –70°C for further RNA extraction.

Optimization of transfection conditions

Transfection conditions were optimized for 1 × 10^5 cells/well with 0.2% and 0.4% v/v concentrations of Lipofectamine2000 (Life Technologies) by using BLOCK-iT™ Fluorescent Oligo (Life Technologies, Carlsbad, USA) and Opti-MEM. Small interfering RNA (siRNA) uptake was assessed under a fluorescent microscope. Intensive fluorescence was visible at a minimal final concentration of labeled siRNA (10 nM) and 0.2% v/v Lipofectamine2000, which indicated high transfection efficiency (not shown). Such conditions were tested for CAV1-siRNA and control siRNA at final concentrations of 10 nM, 50 nM and 100 nM. All the transfections were performed according to the protocols provided by the manufacturer. Cell viability was assessed by the trypan blue exclusion test (Sigma).

Experiment protocol

The cells were seeded on 6-well plates (1 × 10^5 cells/well) in antibiotic- and serum-free DMEM 24 h before transfection. Next, they were washed with Opti-MEM and transiently transfected with siRNA specific for CAV1 or control siRNA (at a final concentration of 50 nM) (sc-29241 and sc-37007; Santa Cruz Biotechnology, Dallas, USA) in 2.5 mL of Opti-MEM by using 0.2% v/v Lipofectamine2000, or left untreated. Additionally, to check transfection efficiency, each time the cells were transfected with fluorescein labeled siRNAs BLOCK-iT™ and the uptake of siRNA was assessed under a fluorescent microscope. Based on information from the previous report and preliminary stages of this study, the cells were re-transfected after 48 h to counteract excessive CAV1 expression under the influence of IL-1β activity. Next, they were stimulated for 2 h with 1 ng/mL IL-1β diluted in Opti-MEM to induce CCL2 expression. Simultaneously, control incubations with non-transfected cells and without IL-1β induction were conducted. After incubation, supernatants were collected and frozen immediately at –70°C. The cells were washed with cold PBS and lysed using TRIzol for RNA extraction or an radioimmunoprecipitation assay (RIPA) buffer (Sigma) for protein isolation. Three independent experiments were conducted on cells obtained from 3 RA synovial fluids.

Total RNA extraction and cDNA synthesis

Total cellular RNA extraction was performed using TRIzol. The concentration and purity of RNA were measured using a NanoDrop1000 spectrophotometer (Thermo Scientific, Waltham, USA). Total RNA in a dose of 0.5 µg was reverse-transcribed into complementary DNA (cDNA) using a QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) in accordance with the protocol provided by the manufacturer.

Quantitative real-time polymerase chain reaction

The primers used in this study (Table 1) were synthesized by DNA Sequencing and Oligonucleotides Synthesis Laboratory (Institute of Biochemistry and Biophysics, the Polish Academy of Sciences, Warszawa, Poland;
Quantitative polymerase chain reactions (qPCRs) were carried out on a Corbett Rotor-Gene 6000 with Rotor-Gene 6000 Series Software 1.7 (Corbett Life Science, Sydney, Australia), employing a QuantiFast SYBR® Green PCR Kit (Qiagen). To determine the relative mRNA levels of the genes studied, standard curves were generated using a mix of randomly pooled cDNA samples in six 0.6-fold dilution series of cDNA. The thermal cycling conditions were as follows: denaturation at 95°C for 5 min, followed by 45 cycles of 95°C for 5 s and 60°C for 30 s and melting curve creation: 65–97°C. The levels of CCL2 and CAV1 mRNA were normalized to porphobilinogen deaminase (PBGD) mRNA levels. The results are expressed in arbitrary units as a ratio of the studied gene level to reference gene level. The reactions were done in triplicate.

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting analysis**

Equal amounts of protein (20 µg) were boiled for 10 min in Laemmli buffer, and then separated in 12% sodium dodecyl sulfate (SDS) polyacrylamide gel. Proteins from gel were transferred onto a polyvinylidene difluoride (PVDF) membrane using the Mini Semi-dry Blotting System (EPS-BIO, Chennai, India). The blots were cut into 2 pieces so as to identify CAV1 (~22 kDa) and β-actin (~43 kDa), and blocked with 5% w/v skimmed milk dissolved in Tris-buffered saline with Tween 20 (TBS-T). Pieces with lower-molecular-weight proteins were probed with a primary antibody for CAV1 (AP16383PU-N; Acris Antibodies, Herford, Germany) diluted 1:5000 in 2% skimmed milk in TBS-T for 2 h. Afterwards, this was washed with TBS-T and incubated for 1 h with a horseradish peroxidase (HRP)-conjugated secondary donkey anti-goat antibody (sc-2033; Santa Cruz Biotechnology) diluted 1:20000 in 2% skimmed milk in TBS-T. The second piece of membrane was incubated with a HRP-conjugated polyclonal anti-β-actin antibody (sc-1616; Santa Cruz Biotechnology). The proteins were visualized as bands using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, USA) by exposing the membrane to X-ray film. The proteins fold change was quantified using ImageJ software 1.47v (NIH, USA). The band intensities for CAV1 were normalized to β-actin as a loading control. The CAV/β-actin ratio for the control siRNA was assumed to be 1.

**Enzyme-linked immunosorbent assay**

Concentrations of secreted CCL2 were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit for recombinant human CCL2/monocyte chemoattractant protein-1 (MCP-1) (R&D) in accordance with the manufacturer’s protocol. The absorbance was measured with an ELISA plate reader ELx800 (Bio-Tek Instruments, Inc., Winooski, USA) using KC Junior analysis software (Bio-Tek Instruments, Inc.). The mean minimum detectable dose of CCL2 was 1.7 pg/mL.

**Statistical analysis**

The calculations were carried out with Microsoft Excel 2010 and GraphPad Prism v. 6.0 (GraphPad Software, La Jolla, USA). The data was analyzed by a two-way analysis of variance (ANOVA) followed by Šidák’s test, a one-way ANOVA followed by Tukey’s honest significant difference (HSD) test or, when compared to a single control, by Dunnett’s test. The results are expressed as means ± standard deviation (SD). The differences were considered to be statistically significant at p < 0.05.

**Results**

**IL-1β induces CCL2 expression in sfd-FLSs, but not in a concentration-dependent manner**

A statistically significant increase in CCL2 expression both at transcript and protein level was seen after a 2-h incubation. Interleukin-1β at a concentration of 1 ng/mL was used for further incubations. The data revealed a non-linear relationship between an increasing IL-1β concentration and CCL2 expression in sfd-FLSs (Fig. 1).

![Image](image-url)
CAV1 knockdown in sfd-FLSs impairs CCL2 transcription

Transfection with siRNA did not affect sfd-FLS viability. As CAV1 is a long-lived protein, transcript level did not correspond with protein level after transfections. For further transfections, 50 nM siRNA was chosen (Fig. 2). A longer exposure (for 1 min) of the PVDF membrane blot to X-ray film revealed both CAV1 isoforms with predominant β isoform.

The CAV1 level was transiently knocked down by about 70% in comparison with cells transfected with control siRNA.
siRNA or non-transfected cells. Incubation with IL-1β caused an increase in CAV1 expression, which is in line with the previous report (Fig. 3A,3B). Transcription of CCL2 in cells transfected with CAV1-siRNA was significantly suppressed (approx. 2-fold) in comparison with non-transfected cells and cells transfected with control siRNA (Fig. 3C). The CAV1 knockdown caused no significant change in the concentration of secreted CCL2 in comparison with cells transfected with control siRNA and non-transfected cells (Fig. 3D).

**Discussion**

Many chemoattractants overproduced by cells residing in the synovium enhance leukocyte migration into the joints, which is the main characteristic of RA. The activation of FLSs is believed to be the key process promoting inflammation and joint damage in RA. In this study, we checked whether the knockdown of CAV1, a regulator of intracellular signal transduction, affects CCL2 expression in sfd-FLSs obtained from RA synovial fluid. Our results showed that under the study conditions, the CAV1 knockdown significantly impaired IL-1β-induced CCL2 expression, but only at transcript level. To date, there have been no similar reports on associations between CAV1 and chemoattractants in cells important for RA development.

Majkova et al. showed that the CAV1 knockdown almost completely prevented CCL2 expression induced by 3,3′,4,4′-tetrachlorobiphenyl (PCB77) in endothelial cells. These results were also confirmed in vivo on LDL-R–/–/Cav1–/– mice. A similar expression pattern was observed at both the transcript and protein level for IL-6 and TNF-α. Thereby, it was additionally proven that CAV1 could be an important activator of inflammation. Based on molecular studies on the promoter region of CCL2, some cis-regulatory elements and trans-acting factors involved in CCL2 expression were identified. It was shown that 2 NF-κB-binding sites located 2.6 kb from the transcription initiation site are pivotal to CCL2 expression in response to stimuli such as IL-1β and TNF-α. This fact together with the study by Garreau et al. and studies mentioned above point to the importance of CAV1 in NF-κB activation, which could partially explain the inhibition of CCL2 expression at transcript level in sfd-FLSs with diminished CAV1 expression.

Studies showing the pro-inflammatory character of CAV1 are not consistent with the results presented by Gardner et al. In a study on the hepatotoxic activity of paracetamol, they revealed an elevated expression of CCL2 in the liver obtained from Cav1–/– mice. In this case, this was linked to the “positive” effect of monocyte influx, leading to repairing processes mediated by macrophages with an anti-inflammatory phenotype.

Looking at all the research conducted on animal models, it can be concluded that CCL2 expression is CAV1-dependent, but only to a certain extent. Our results suggest that CCL2 secretion by sfd-FLSs obtained from RA patients could be regulated at the posttranslational level. It would be worth seeing if the disproportions between CCL2 transcript and protein levels could be a result of the activity of some regulators controlled by the particular CAV1 isoform. This is due to the fact that only α isoform has a complete sequence and undergoes phosphorylation on Tyr14, which facilitates binding both inductors and effector proteins in signaling pathways. Kogo et al. suggested that the ratio of the 2 isoforms may vary depending on the cell type. We found that CAV1β (lacking N-terminal 31 amino acids) is predominant in sfd-FLSs. Therefore, it seems that the functional diversity of both isoforms should be considered when interpreting the role of CAV1 in a specific molecular context.

As some stimuli may act differently on various cell types, CCL2 expression could also vary among different cells and for different kinds of stimuli, and, as was shown in our study, the secreted CCL2 level does not always correspond with its transcript level, especially in cells with an impaired CAV1 expression. Here, it should be also noted that in vivo, the final level of a particular cytokine is very often an effect of complex, reciprocal synergistic and antagonistic associations between other cytokines. Additionally, understanding epigenetic mechanisms (DNA methylation and histone modifications as well as regulation by small non-coding RNAs) can be helpful in current studies on the pathogenesis of RA. A recent study by Li et al. presented that targeting CAV1 by restoring the
microRNA-192 levels in FLSs led to their apoptosis and the inhibition of cell proliferation, and possibly by regulating the expression of other relevant genes in a direct (chemokine (C-X-C motif) ligand 2; source: miRDB-an online database (http://www.mirdb.org/cgi-bin/search.cgi?searchType=miRNA&searchBox=hsa-miR-192-5p&full=1) or indirect way. To summarize, the normalization of pathological processes in R A, preferably by aiming at one pivotal factor, is highly desirable but, so far, unattainable.

Conclusions

Our results show a significant role of CAV1 in CCL2 transcription induction in RA sfd-FLSs under the influence of IL-1β. However, processes involving CAV1 still require more profound research into a broader spectrum of cytokines in auto-inflammatory disorders.

References
