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# CCN4 induces vascular cell adhesion molecule-1 expression in human synovial fibroblasts and promotes monocyte adhesion

Ju-Fang Liu [a,1], Sheng-Mou Hou [b,1], Chun-Hao Tsai [c,d], Chun-Yin Huang [d,e], Chin-Jung Hsu [d], Chih-Hsin Tang [f,g,*]

[a] Central Laboratory, Shin-Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan
[b] Department of Orthopedic Surgery, Shin-Kong Wo Ho-Su Memorial Hospital, Taipei, Taiwan
[c] Department of Orthopaedic Surgery, China Medical University Hospital, Taichung, Taiwan
[d] Graduate Institute of Clinical Medical Science, China Medical University, Taichung, Taiwan
[e] Department of Orthopaedic Surgery, China Medical University Beigang Hospital, Yun-Lin County, Taiwan
[f] Department of Pharmacology, School of Medicine, China Medical University, Taichung, Taiwan
[g] Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan

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ABSTRACT

CCN4 is a cysteine-rich protein that belongs to the Cyr61, CTGF, Nov family of matricellular proteins. Here, we investigated the intracellular signaling pathways involved in CCN4-induced vascular cell adhesion molecule-1 expression in human osteoarthritis synovial fibroblasts. Stimulation of OASFs with CCN4 induced VCAM-1 expression. CCN4-induced VCAM-1 expression was attenuated by αvβ5 or α6β1 integrin antibody, Syk inhibitor, PKCδ inhibitor (rottlerin), JNK inhibitor (SP600125), and AP-1 inhibitors (curcumin and tanshinone). Stimulation of cells with CCN4 increased Syk, PKCδ, and JNK activation. Treatment of OASFs with CCN4 also increased c-Jun phosphorylation, AP-1-luciferase activity, and c-Jun binding to the AP-1 element in the VCAM-1 promoter. Moreover, up-regulation of VCAM-1 increased the adhesion of monocytes to OASF monolayers, and this adhesion was attenuated by transfection with a VCAM-1 siRNA. Our results suggest that CCN4 increases VCAM-1 expression in human OASFs via the Syk, PKCδ, JNK, c-Jun, and AP-1 signaling pathways. The CCN4-induced VCAM-1 expression promoted monocyte adhesion to human OASFs.


## 1. Introduction

Osteoarthritis (OA) is a chronic joint disorder characterized by slow progressive degeneration of articular cartilage, subchondral bone alteration, and variable secondary synovial inflammation. In response to macrophage-derived proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α), OA synovial fibroblasts (OASFs) produce chemokines that promote inflammation, neovascularization, and cartilage degradation via activation of matrix-degrading enzymes such as matrix metalloproteinases (MMPs) [1,2]. Although the pathogenesis of the disease remains elusive, accumulating evidence indicates that mononuclear cell migration plays an important role in the perpetuation of inflammation in the synovium [3,4]. Mononuclear cell adhesion and infiltration into inflammatory sites are regulated by adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) [5,6].

Cell adhesion molecules are transmembrane glycoproteins that mediate cell–cell and cell–extracellular matrix interactions. VCAM-1 emerged as a highly significant predictor of the risk of OA [7,8]. VCAM-1 has been shown to be upregulated in the synovial lining of OA patients by immunohistochemical staining, and in cultured human OASFs by western blotting [7,8]. A reduction in the levels of VCAM-1 in synovial fluid may suppress the inflammatory response in knee OA [9]. Therefore, VCAM-1 is involved in the process of mononuclear cell infiltration into the synovium, leading to the initiation and progression of the disease. However, the molecular mechanisms by which cytokines induce VCAM-1 expression in human OASFs remain unclear.

CCN4 belongs to the CCN family of matricellular proteins, which also includes cysteine-rich 61 (Cyr61/CCN1), connective tissue growth factor (CTGF/CCN2), nephroblastoma overexpressed (NOV/CCN3), WISP-1/CCN4, WISP-2/CCN5, and WISP-3/CCN6 (which is highly expressed in skeletal tissues) [10]. CCN4 acts in an autocrine manner to accelerate cell growth, induce morphological transformation, increase saturation density, and promote tumorigenesis [11]. CCN4 also promotes osteoblastic differentiation [12]. A recent study showed that CCN4 expression was increased in the synovium and cartilage of mice with experimental OA [10]. Significantly, recombinant CCN4 elicited the release of MMPs

* Corresponding author at: Department of Pharmacology, School of Medicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung, Taiwan. Tel.: +886 4 22052121 7726; fax: +886 4 22053764.
*E-mail address:* chtang@mail.cmu.edu.tw (C.-H. Tang).
[1] These authors contributed equally to this work.

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and aggrecanase from macrophages and chondrocytes, which reduced OA progression [10].

Although the roles of cytokines and adhesion molecules in polymorphonuclear cell adhesion to endothelial cells have been well described, little is known about the mechanisms underlying the interaction between monocytes and human OASFs. Previous studies have shown that CCN4 plays an important role in OA pathogenesis [10]. In the present study, we investigated the intracellular signaling pathways involved in CCN4-induced VCAM-1 expression in human OASFs. The results show that CCN4 activates the integrin receptor and elicits the activation of the Syk, PKCδ, JNK, and AP-1 signaling pathways, leading to the upregulation of VCAM-1 expression. The increased VCAM-1 expression correlated with enhanced adhesion of monocytes to CCN4-stimulated OASFs.

## 2. Materials and methods

### 2.1. Materials

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for Syk, PKCδ, JNK, p-JNK, ERK, p-ERK, p38, p-p38, c-Jun, p-c-Jun, and β-actin, and siRNAs against Syk, PKCδ, and c-Jun were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody specific for Syk phosphorylated at $Thr^{874}$ and PKCδ phosphorylated at $Tyr^{331}$ was purchased from Cell Signaling and Neuroscience (Danvers, MA, USA). Mouse monoclonal antibodies specific for α5β1, αvβ3, αvβ5, and α6β1 integrins were purchased from Chemicon (Temecula, CA, USA). Syk inhibitor, rottlerin, GF109203X, Ro320432, SP600125, SB203580, U0126, PD98059, curcumin, and tanshinone were purchased from Calbiochem (San Diego, CA, USA). Recombinant human CCN4 was purchased from R&D Systems (Minneapolis, MN, USA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Cell cultures

The study was approved by the local ethics committee, and informed written consent was obtained from the subjects. Human synovial fibroblasts (SFs) were isolated by collagenase treatment of synovial tissues obtained from knee replacement surgeries of 38 patients with OA and 18 samples of normal synovial tissues obtained at arthroscopy from trauma/joint derangements. Synovial fluid concentrations of CCN4 were measured with an enzyme-linked immunosorbent assay (ELISA), according to the protocol provided by the manufacturer (Human CCN4 ELISA kit; R&D Systems). OASFs were isolated, cultured, and characterized as previously described [13,14]. Experiments were performed using cells from passages 3–6.

THP-1, a human leukemia cell line of monocyte/macrophage lineage, was obtained from American Type Culture Collection (Manassas, VA, USA) and grown in RPMI-1640 medium containing 10% fetal bovine serum.

### 2.3. Quantitative real-time PCR

Total RNA was extracted from OASFs using a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA and oligo(dT) primer [15,16]. Quantitative real-time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, Foster City, CA). cDNA templates (2 μl) were added to a 25-μl reaction along with sequence-specific primers and Taqman® probes. All the target gene primers and probes were commercially purchased (VCAM-1; ID, Hs01003370_m1). β-actin (ID, Hs99999903_m1) was used as the internal control (Applied Biosystems). The qPCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The cycling conditions comprised 10-min polymerase activation at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The threshold was set above the non-template control background and within the linear phase of the target gene amplification to calculate the cycle number at which the transcript was detected (denoted CT).

### 2.4. Western blot analysis

Cellular lysates were prepared as described previously [17,18]. Proteins were resolved on SDS-PAGE and transferred to immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies against PKCδ, VCAM-1, or JNK (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with donkey anti-rabbit peroxidase-conjugated secondary antibody (1:3000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence with Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and ImageQuant software (Supplemental data Fig. S1–6; Molecular Dynamics, Sunnyvale, CA, USA).

### 2.5. Transfection and reporter gene assay

Human synovial fibroblasts were co-transfected with 0.8 μg AP-1-luciferase plasmid, 0.4 μg β-galactosidase expression vector. Fibroblasts were grown to 80% confluent in 12 well plates and were transfected the following day with Lipofectamine 2000 (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then applied to cells. After 24 h transfection, cells were then incubated with the indicated agents. After further 24 h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector.

### 2.6. Flow cytometry

Human synovial fibroblasts were plated in six-well dishes. The cells were then washed with PBS and detached with trypsin at 37 °C. Cells were fixed for 10 min in PBS containing 1% paraformaldehyde. After being rinsed in PBS, the cells were incubated with mouse anti-human antibody against VCAM-1 (1:100) for 1 h at 4 °C. Cells were then washed again and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse secondary IgG (1:100; Leinco Tec. Inc., St. Louis, MO, USA) for 45 min and analyzed by flow cytometry using FACS Calibur and CellQuest software (BD Biosciences).

### 2.7. Syk kinase activity assay

Syk activity was assessed using a Syk Kinase Activity Assay Kit according to the manufacturer's instructions (Assay Designs, MI). The Syk activity kit is based on a solid-phase ELISA that uses a specific synthetic peptide as a substrate for Syk and a polyclonal antibody that recognizes the phosphorylated form of the substrate.

### 2.8. Cell adhesion assay

THP-1 cells were labeled with BCECF-AM (10 μM) at 37 °C for 1 h in RPMI-1640 medium and subsequently washed by centrifugation. OASFs grown on glass coverslips were incubated with CCN4 for 6 h. Confluent CCN4-treated OASFs were incubated with THP-1 cells