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

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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

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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

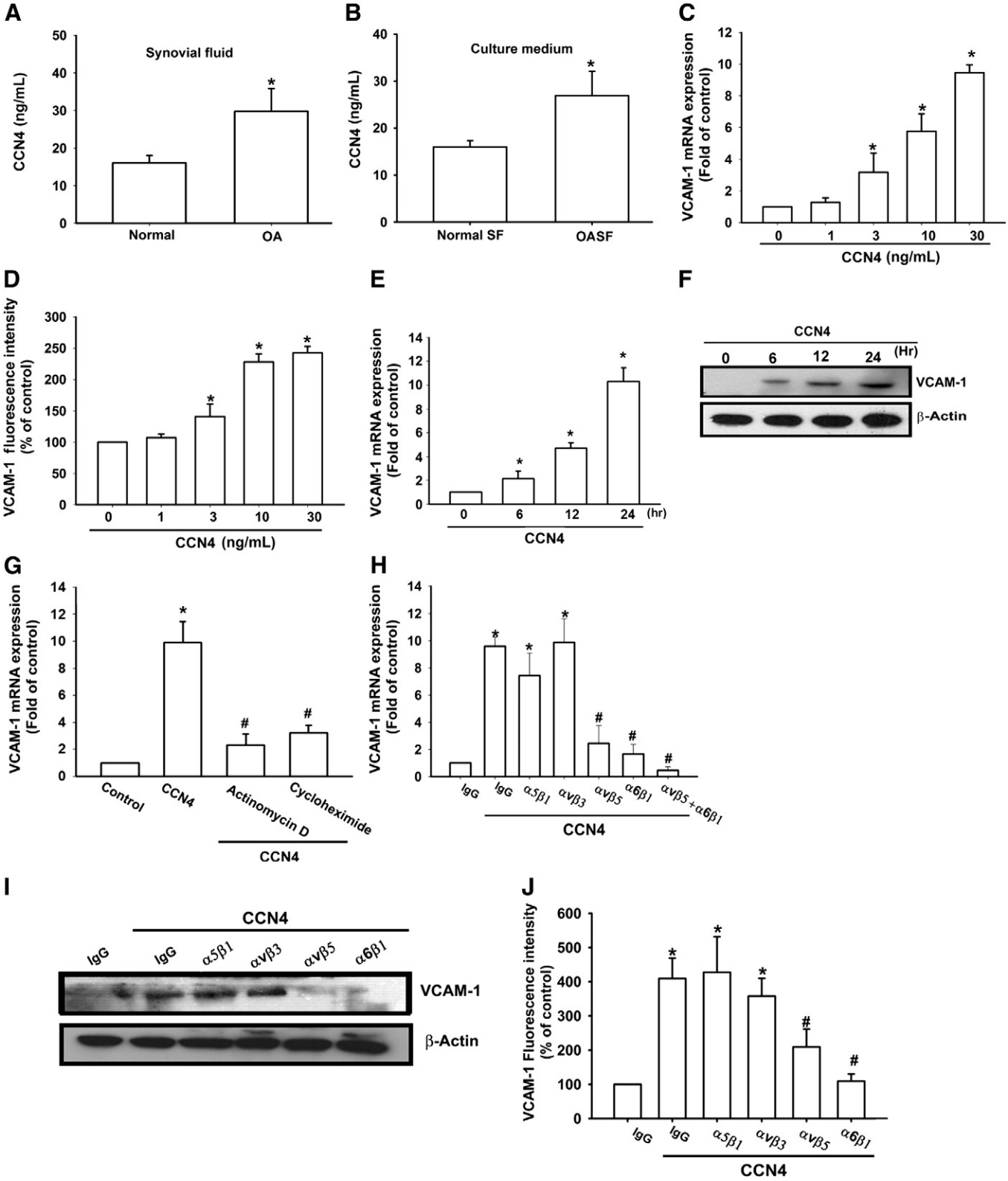

Fig. 1. CCN4 increases VCAM-1 expression through αvβ5/α6β1 integrin receptor. (A) Synovial fluid was obtained from normal (n = 10) or osteoarthritis patients (n = 15) and examined with ELISA for the expression of CCN4. (B) Human synovial fibroblasts were cultured for 48 h, and media were collected to measure CCN4. (C–F) OASFs were incubated with various concentrations of CCN4 for 24 h or with CCN4 (30 ng/ml) for 6, 12, or 24 h. The mRNA, cell surface, and protein expression of VCAM-1 were examined by qPCR, flow cytometry, and Western blotting. (G) OASFs were pretreated for 30 min with actinomycin D or cycloheximide followed by stimulation with CCN4 for 24 h, and VCAM-1 expression was examined by qPCR. (H–J) OASFs were pretreated for 30 min with α5β1, αvβ3, αvβ5, or α6β1 integrin antibody (3 μg/ml) followed by stimulation with CCN4 for 24 h, and VCAM-1 expression was examined by qPCR, flow cytometry, and Western blotting. Results are expressed as the mean ± S.E. *: $p<0.05$ as compared with basal level. #: $p<0.05$ as compared with CCN4-treated group.

(2×10^6 cells/ml) at 37 °C for 1 h. Non-adherent THP-1 cells were removed and gently washed with PBS. The number of adherent THP-1 cells was counted in four randomly chosen fields per well at 200× high power using a fluorescent microscope.

2.9. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation analysis was performed as described previously [19]. DNA was immunoprecipitated with an anti-

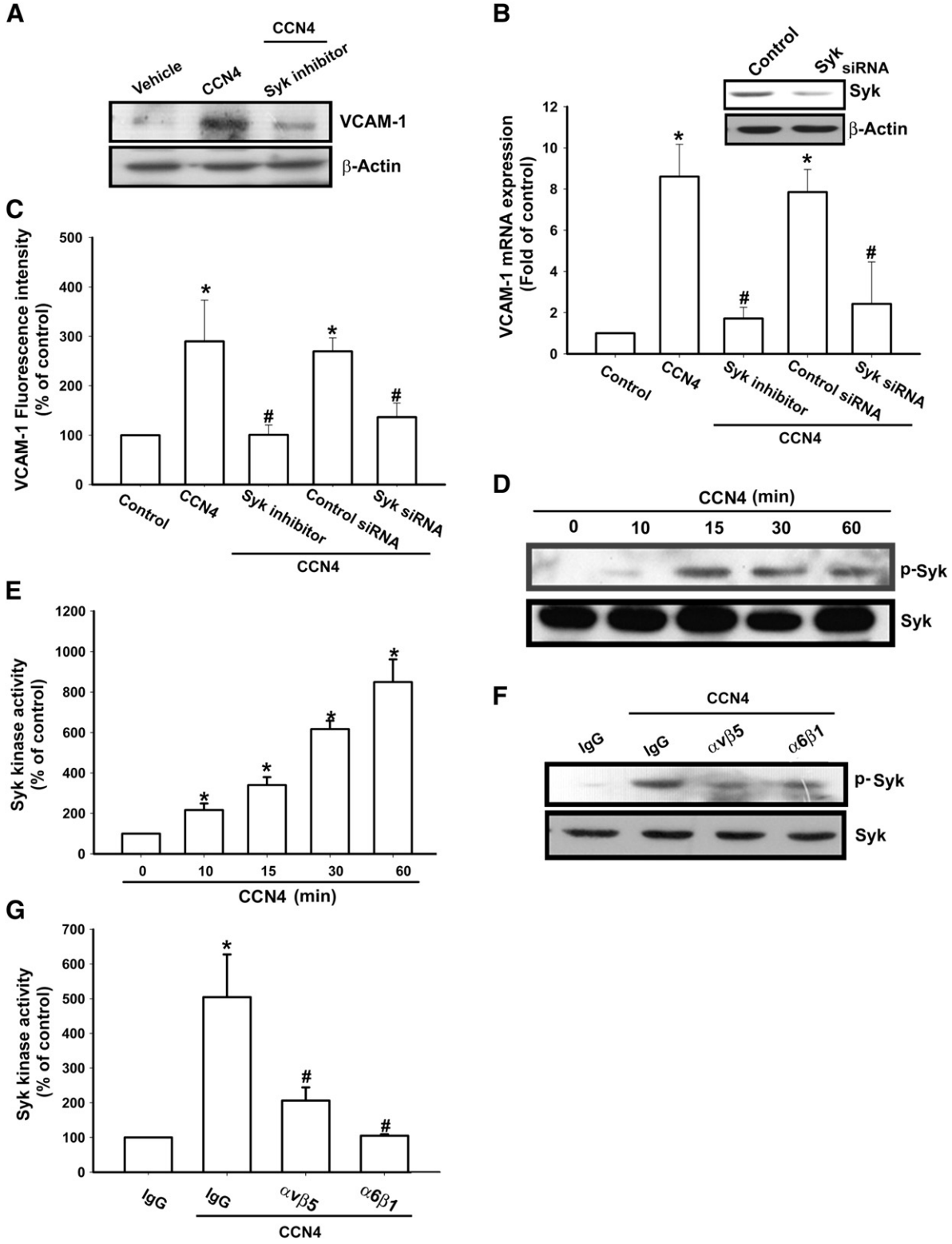

Fig. 2. Syk is involved in CCN4-induced VCAM-1 expression. (A–C) OASFs were pretreated for 30 min with Syk inhibitor (10 μM) or transfected with Syk siRNA for 24 h followed by stimulation with CCN4 for 24 h, and VCAM-1 expression was examined by qPCR, flow cytometry, and Western blotting. (D–G) OASFs were incubated with CCN4 for indicated time intervals or pretreated with αvβ5 or α6β1 mAb for 30 min before incubation with CCN4 for 15 min. The Syk phosphorylation and activity were determined by Western blotting and Syk kinase assay kit. Results are expressed as the mean ± S.E. *: $p<0.05$ as compared with basal level. #: $p<0.05$ as compared with CCN4-treated group.

c-Jun antibody and purified. The DNA was then extracted with phenol-chloroform. The purified DNA pellet was subjected to PCR, and PCR products were resolved using 1.5% agarose gel electrophoresis and visualized with UV light. The forward and reverse primers (5′-CGGTTAAATCTCACAGCCCA-3′ and 5′-TTCTCTTACAAGAGAAAGGA-3′, respectively) were specifically designed from the VCAM-1 promoter region (−403 to −30) [20].

2.10. Statistical analysis

Data are expressed as mean ± S.E. Statistical analyses were performed using the Student's *t*-test. Statistical comparisons of more than 2 groups were performed using one-way analysis of variance (ANOVA) with Bonferroni's post-hoc test. In all cases, $p<0.05$ was considered significant.

3. Results

3.1. CCN4 induces VCAM-1 expression in human synovial fibroblasts

CCN4 has been shown to play an important role in OA pathogenesis [21]. First, we examined human synovial tissues for the expression of CCN4 using ELISA. CCN4 concentrations in synovial fluid were significantly higher in patients with OA than in controls (Fig. 1A). The medium from OASFs exhibited significant levels of CCN4, which were higher than that of the medium from normal SFs (Fig. 1B). Next, we directly applied CCN4 to OASFs and examined the expression of VCAM-1 (an important regulator that promotes monocyte adhesion to endothelial cells). Treatment of OASFs with CCN4 (1–30 ng/ml) for 24 h induced VCAM-1 mRNA and cell surface VCAM-1 expression in a concentration-dependent manner, as determined by qPCR and flow cytometry (Fig. 1C and D). In addition, CCN4 also increased VCAM-1 mRNA and protein expression in a time-dependent manner (Fig. 1E&F). These data indicate that CCN4 increases VCAM-1 expression in human OASFs. To determine whether CCN4-induced VCAM-1 expression is dependent on de novo protein synthesis, the cells were pretreated with actinomycin D or cycloheximide and then incubated with CCN4. Treatment with actinomycin D or cycloheximide reduced CCN4-mediated VCAM-1 expression (Fig. 1G), suggesting that CCN4-induced VCAM-1 expression is mediated via the de novo protein synthesis through transcription and translation.

Integrins are well-known receptors for CCN proteins [22]. Therefore, we hypothesized that the integrin receptor signaling pathway may be involved in CCN4-induced VCAM-1 expression, and sought to identify the integrin receptor subtypes that may be involved in the CCN4-induced increase in VCAM-1 expression. We found that αvβ5 and α6β1 integrin receptor-specific mAbs significantly blocked the CCN4-induced increase in VCAM-1 expression in human OASFs,

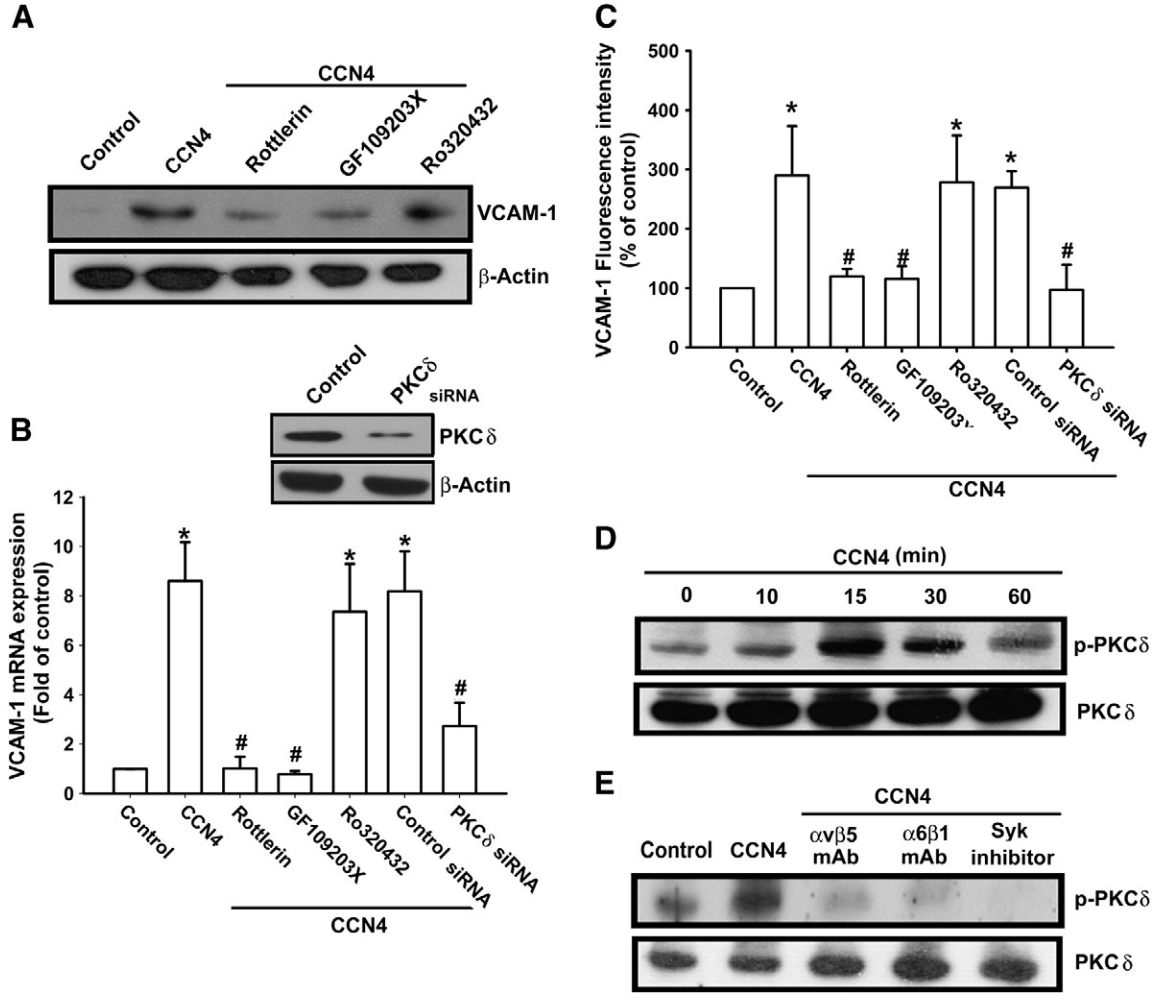

Fig. 3. PKCδ is involved in CCN4-induced VCAM-1 expression in synovial fibroblasts. (A–C) OASFs were pretreated for 30 min with GF109203X (3 μM), rottlerin (10 μM), and Ro320432 (10 μM) or transfected with PKCδ siRNA for 24 h followed stimulated with CCN4 for 24 h, and VCAM-1 expression was examined by qPCR, flow cytometry, and Western blotting. OASFs were incubated with CCN4 for indicated time intervals (D) or pretreated with αvβ5 mAb, α6β1 mAb, or Syk inhibitor for 30 min before incubation with CCN4 for 15 min (E), and PKCδ phosphorylation was determined by Western blotting. Results are expressed as the mean ± S.E. *: $p<0.05$ as compared with basal level. #: $p<0.05$ as compared with CCN4-treated group.

whereas α5β1 and αvβ3 integrin receptor-specific mAbs and the control IgG did not have this effect (Fig. 1H–J). A combination of αvβ5 and α6β1 integrin mAbs completely abolished CCN4-induced VCAM-1 expression (Fig. 1H). These data suggest that CCN4-induced VCAM-1 expression may occur via the activation of αvβ5 and α6β1 integrin receptors.

3.2. Syk is involved in the potentiating action of CCN4

Previous studies have demonstrated that integrin-mediated adhesion involves Syk phosphorylation [23,24]. To determine whether Syk was involved in CCN4-induced VCAM-1 expression, we pretreated OASFs with a Syk inhibitor (10 μM) for 30 min. The Syk inhibitor antagonized the potentiating effect of CCN4 (Fig. 2A–C). Next, we specifically inhibited Syk expression using an siRNA; the transfection of OASFs with a Syk siRNA reduced Syk expression and inhibited the CCN4-induced increase in VCAM-1 production (Fig. 2B and C). To further determine whether Syk played a crucial role in CCN4-induced VCAM-1 production, we directly measured Syk phosphorylation in response to CCN4. CCN4 treatment induced Syk phosphorylation in a time-dependent manner in OASFs (Fig. 2D). In addition, Syk kinase activity in OASFs was also increased in a time-dependent manner by CCN4 treatment (Fig. 2E). Furthermore, pretreatment of OASFs for 30 min with αvβ5 and α6β1 integrin mAbs inhibited the CCN4-induced increase in Syk phosphorylation and kinase activity (Fig. 2F and G). Based on these results, it appears that CCN4 acts through αvβ5/α6β1 integrin to enhance VCAM-1 expression in OASFs via Syk activation.

3.3. PKCδ and JNK signaling pathways are involved in CCN4-induced VCAM-1 expression

A Syk-dependent PKCδ pathway has been shown to be involved in TNF-α-induced gene expression in SFs [25]. Therefore, we examined whether PKCδ was involved in CCN4-induced VCAM-1 expression. Pretreatment of cells with PKC inhibitor (GF109203X, 3 μM) and PKCδ inhibitor (rottlerin, 10 μM) but not PKCα inhibitor (Ro320432, 10 μM) reduced CCN4-induced VCAM-1 expression (Fig. 3A–C). Furthermore, transfection of OASFs with a PKCδ siRNA specifically reduced PKCδ expression and inhibited the CCN4-induced increase in VCAM-1 expression (Fig. 3B and C). We then measured PKCδ phosphorylation in response to CCN4. As shown in Fig. 3D, treatment of OASFs with CCN4 resulted in a time-dependent phosphorylation of PKCδ. Pretreatment of cells with the αvβ5 and α6β1 mAbs or the Syk inhibitor blocked the CCN4-induced PKCδ phosphorylation (Fig. 3E). Taken together, these results indicate that an integrin receptor-, Syk-, and PKCδ-dependent pathway is involved in CCN4-induced VCAM-1 expression.

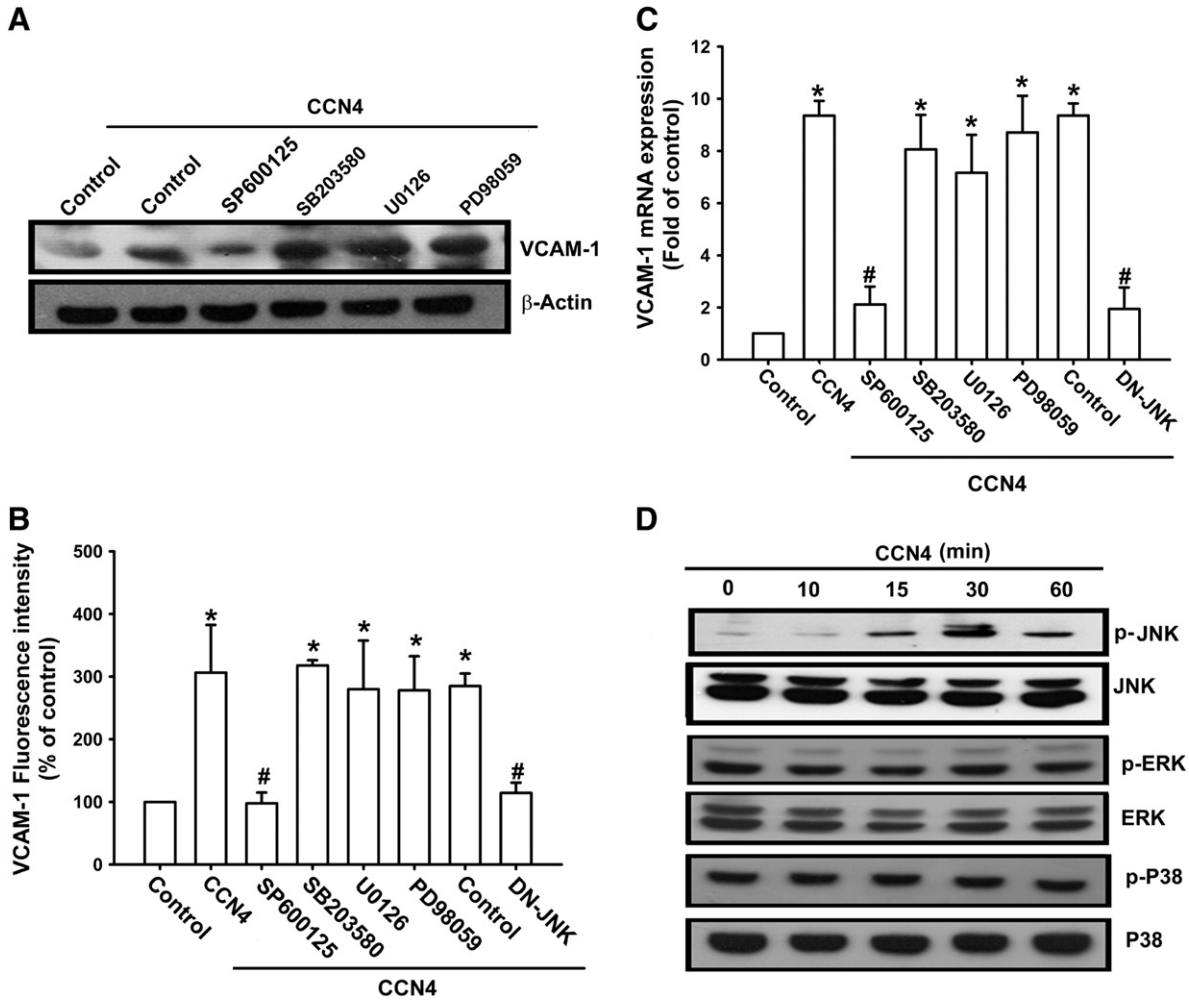

Fig. 4. JNK is involved in CCN4-induced VCAM-1 expression in synovial fibroblasts. (A–C) OASFs were pretreated for 30 min with SP600125 (3 μM), SB203580 (10 μM), U0126 (10 μM), and PD98059 (10 μM) or transfected with JNK mutant for 24 h followed stimulated with CCN4 for 24 h, and VCAM-1 expression was examined by qPCR, flow cytometry, and Western blotting. (D) OASFs were incubated with CCN4 for indicated time intervals, and ERK, p38, and JNK phosphorylation was determined by Western blotting. Results are expressed as the mean ± S.E. *: $p<0.05$ as compared with basal level. #: $p<0.05$ as compared with CCN4-treated group.

MAPK has been reported to mediate CCN4-induced cellular functions [26]. Therefore, we examined whether CCN4 stimulation enhanced MAPK activation in human OASFs. Pretreatment of cells for 30 min with a JNK inhibitor (SP600125) but not p38 inhibitor (SB203580) or ERK inhibitor (U0126 and PD98059) reduced CCN4-induced VCAM-1 expression (Fig. 4A–C). Furthermore, stimulation of OASFs with CCN4 induced the phosphorylation of JNK, but not ERK and p38, in a time-dependent manner (Fig. 4D). Based on these results, it appears that JNK activation is involved in CCN4-mediated VCAM-1 expression in human OASFs. CCN4 acts through a signaling pathway involving the αvβ5/α6β1 integrin receptor, Syk, PKCδ, and JNK to enhance VCAM-1 expression in human OASFs.

3.4. Involvement of AP-1 in CCN4-induced VCAM-1 expression

AP-1 is a transcription factor that plays a crucial role in immune and inflammatory responses. The VCAM-1 promoter contains binding sites for AP-1 [20,27]. Therefore, we examined the effect of CCN4 on AP-1 transcriptional activation. Pretreatment of cells for 30 min with AP-1 inhibitor (curcumin and tanshinone) inhibited CCN4-induced VCAM-1 expression (Fig. 5A–C). AP-1 activation was further evaluated by analyzing the phosphorylation and translocation of c-Jun as well as by chromatin immunoprecipitation assay. Stimulation of cells with CCN4 increased c-Jun phosphorylation and nuclear translocation (Fig. 5D).

The in vivo recruitment of c-Jun to the VCAM-1 promoter (−403 to −30) was assessed using chromatin immunoprecipitation assay [20]. In vivo binding of c-Jun to the AP-1 element of the VCAM-1 promoter was observed after CCN4 stimulation (Fig. 6A). The CCN4-induced binding of c-Jun to the AP-1 element was attenuated by Syk inhibitor, rottlerin, SP600125, but not Ro320432 (Fig. 6A). In addition, pretreatment of cells with αvβ5 or α6β1 integrin mAb, Syk inhibitor, GF109203X, and rottlerin also reduced CCN4-induced c-Jun phosphorylation (Fig. 6B). To directly determine whether AP-1 was activated after CCN4 treatment, OASFs were transiently transfected with AP-1 luciferase as an indicator of AP-1 activation. As shown in Fig. 6C, 24-h CCN4 treatment increased AP-1-luciferase activity in a dose-dependent manner. In addition, Syk inhibitor, GF109203X, rottlerin, curcumin, and tanshinone antagonized the CCN4-induced AP-1 luciferase activity (Fig. 6C). Co-transfection of cells with Syk and PKCδ siRNAs or the JNK mutant also reduced CCN4-induced AP-1 luciferase activity (Fig. 6C). These results indicate that CCN4-induced VCAM-1 expression is mediated through the αvβ5/α6β1 integrin, Syk, PKCδ, JNK, and AP-1 pathways in human OASFs.

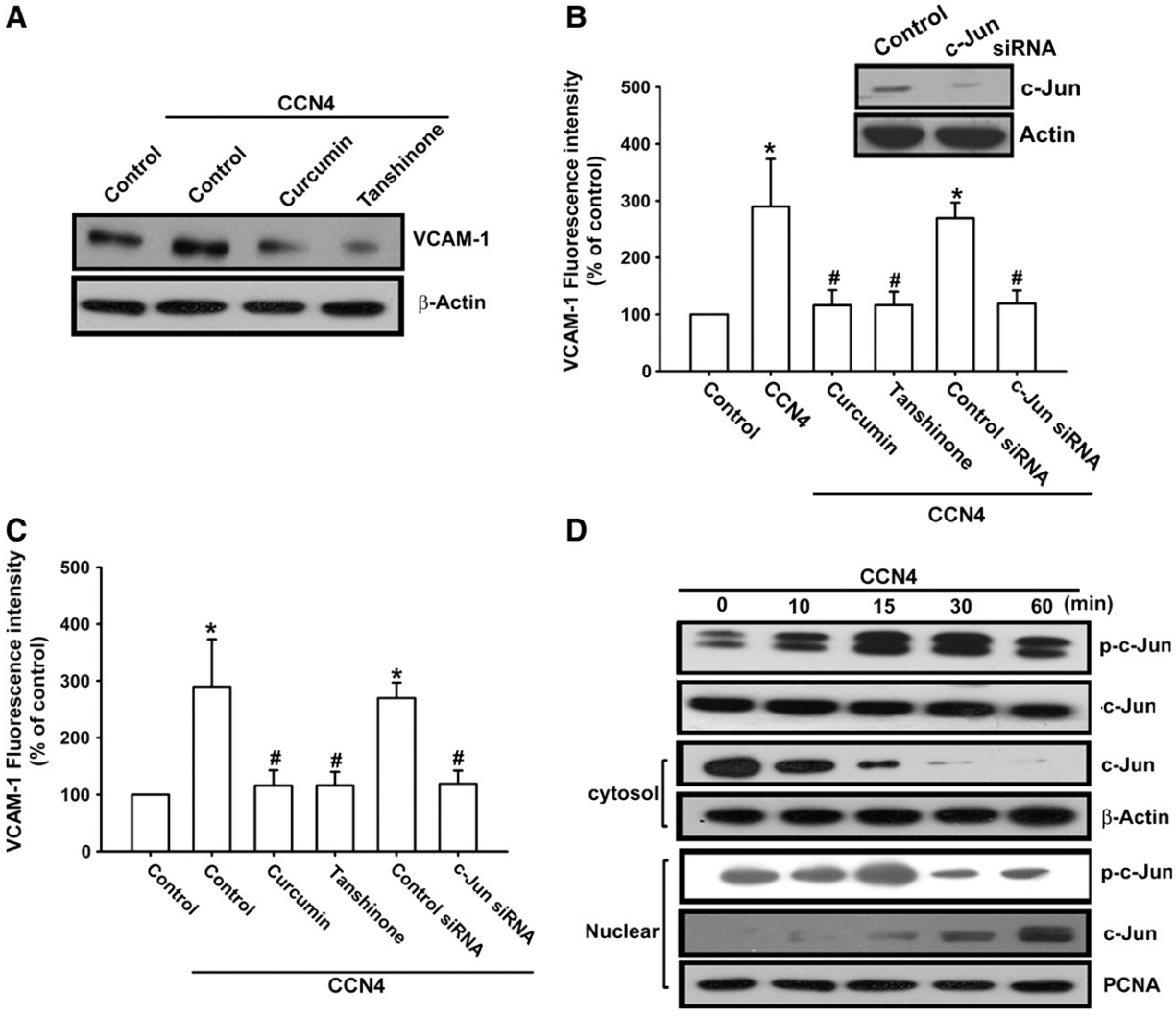

Fig. 5. AP-1 is involved in the potentiation of VCAM-1 expression by CCN4. (A–C) OASFs were pretreated for 30 min with curcumin (3 μM) and tanshinone (5 μM) or transfected with c-Jun siRNA for 24 h then stimulated with CCN4 for 24 h, and VCAM-1 expression was examined by qPCR, flow cytometry, and Western blotting. (D) OASFs were incubated with CCN4 for indicated time intervals, and c-Jun expression in cytosol and nucleus and c-Jun phosphorylation was determined by Western blotting. Results are expressed as the mean ± S.E. *: $p<0.05$ as compared with basal level. #: $p<0.05$ as compared with CCN4-treated group.

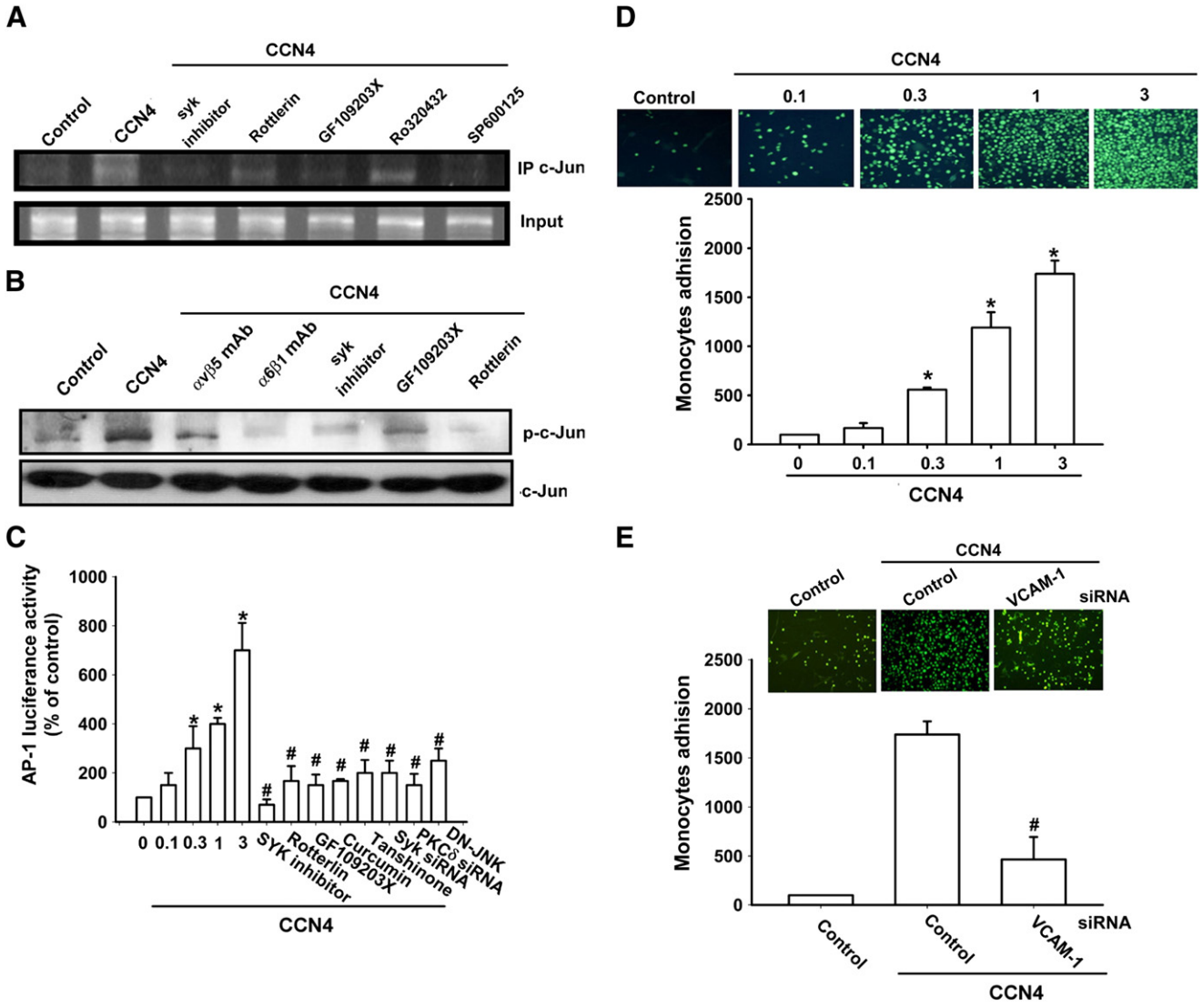

Fig. 6. CCN4 induced AP-1 activation through integrin/Syk/PKCδ and JNK pathways. (A) OASFs were pretreated with αvβ5 mAb, α6β1 mAb, Syk inhibitor, GF109203X, and rottlerin for 30 min then stimulated with CCN4 for 120 min, the chromatin immunoprecipitation assay was then performed. (B) OASFs were pretreated with Syk inhibitor, GF109203X, rottlerin, Ro320432, and SP600125 for 30 min then stimulated with CCN4 for 30 min, and p–c-Jun expression was determined by Western blotting. (C) OASFs were pretreated with Syk inhibitor, GF109203X, rottlerin, curcumin, and tanshinone for 30 min or transfected with Syk and PKCδ siRNA or JNK mutant before exposure to CCN4. AP-1 luciferase activity was measured, and the results were normalized to the β-galactosidase activity and expressed as the mean ± S.E. for three independent experiments performed in triplicate. (D&E) OASFs were incubated with various concentrations of CCN4 for 24 h or transfected with VCAM-1 siRNA for 24 h then stimulated with CCN4 for 24 h. THP-1 cells labeled with BCECF-AM were added to OASFs for 6 h, and then the THP-1 cell adherence was measured by fluorescence microscopy. Results are expressed as the mean ± S.E. *: $p<0.05$ as compared with basal level. #: $p<0.05$ as compared with CCN4-treated group.

3.5. CCN4-induced VCAM-1 expression promotes monocyte adhesion

To further determine the functional significance of VCAM-1 expression in OASFs, we examined monocyte adhesion to these cells after treatment with CCN4. The adhesion assay was carried out using THP-1 cells as a monocyte model. CCN4 dose-dependently enhanced the adhesiveness between OASFs and THP-1 cells (Fig. 6D). We next examined whether VCAM-1 induced monocytes to adhere to OASF monolayers. Transfection of OASFs with VCAM-1 siRNA significantly inhibited monocyte adhesion (Fig. 6E). These results indicate that CCN4 increases VCAM-1 expression in OASFs and promotes the adhesion of monocytes to OASFs.

4. Discussion

OA is a heterogeneous group of conditions associated with defective integrity of the articular cartilage as well as related changes in the underlying bone. The chronic inflammatory process is mediated by a complex cytokine network. The factors responsible for initiating the degradation and loss of articular tissues remain unknown. Although the pathogenesis of the disease remains elusive, upregulation of adhesion molecules on the surface of the synovial lining may play a key role in recruitment and infiltration of monocytes into sites of inflammation in OA [28]. Here, we identified VCAM-1 as a target protein for the CCN4 signaling pathway that regulates the cell inflammatory response. We also showed that potentiation of VCAM-1 by CCN4 requires activation of the integrin receptor, Syk, PKCδ, JNK, and AP-1 signaling pathways and promotes monocyte adhesion to OASFs.

CCN4 is known to activate integrin, including α5β1, αvβ3, αvβ5, and α6β1 [29,30]. However, we demonstrated that αvβ5 and α6β1 integrin but not α5β1 and αvβ3 integrin receptor were required for CCN4-induced VCAM-1 expression. Treatment cells with αvβ5 and α6β1 integrin mAb inhibited CCN4-induced VCAM-1 expression, but α5β1 and αvβ3 integrin mAb failed to inhibit CCN4-induced

VCAM-1 expression. These data suggest that αvβ5 and α6β1 integrin are involved in CCN4-induced VCAM-1 expression and release from synovial fibroblasts.

It has been reported that Syk can be activated by integrin adhesion receptors [31]. In addition, the interaction of integrins with other intracellular proteins such as tyrosine kinase Syk, which binds to the β3 integrin cytoplasmic tail leading to regulate osteoclast function and the capacity of the mature resorptive cell to resorb bone [22]. We demonstrated that Syk inhibitor antagonized the CCN4 mediated potentiation of VCAM-1 expression, suggesting that Syk activation is an obligatory event in CCN4-induced VCAM-1 expression in these cells. In addition, transfection of OASFs with Syk siRNA reduced CCN4 mediated potentiation of VCAM-1 expression from synovial fibroblasts. These data suggest that the Syk pathway is required for CCN4-induced VCAM-1 expression.

Several isoforms of PKC have been characterized at the molecular level and have been found to mediate several cellular molecular responses [32]. We demonstrated that the PKC inhibitor GF109203X antagonized the CCN4-mediated potentiation of VCAM-1 expression, suggesting that PKC activation is an obligatory event in CCN4-induced VCAM-1 expression in these cells. In addition, rottlerin but not Ro320432 also inhibited CCN4-induced VCAM-1 expression. However, current report indicated that rottlerin is not a specific PKCδ inhibitor but inhibits may other targets [33]. Therefore, we used PKCδ siRNA to confirm PKCδ function in OASFs. We found that PKCδ siRNA inhibited the enhancement of VCAM-1 expression. Incubation of synovial fibroblasts with CCN4 also increased PKCδ phosphorylation. On the other hand, αvβ5 and α6β1 mAb or Syk inhibitor blocked the CCN4-induced PKCδ phosphorylation. These data suggest that the αvβ5/α6β1 integrin and PKCδ pathways are required for CCN4-induced VCAM-1 expression.

There are several binding sites for a number of transcription factors including NF-κB, Sp-1, and AP-1 in the 5′ region of the VCAM-1 gene [34]. Recent studies of the VCAM-1 promoter have demonstrated that VCAM-1 induction by several transcription factors occurs in a highly stimulus-specific or cell-specific manner [35]. The results of our current study show that AP-1 activation contributes to CCN4-induced VCAM-1 expression in synovial fibroblasts. Pretreatment of cells with an AP-1 inhibitor curcumin or tanshinone reduced CCN4-increased VCAM-1 expression. Therefore, the AP-1 binding site is likely to be the most important site for CCN4-induced VCAM-1 production. The AP-1 sequence binds to members of the Jun and Fos families of transcription factors. These nuclear proteins interact with the AP-1 site as Jun homodimers or Jun-Fos heterodimers formed by protein dimerization through their leucine zipper motifs [36]. The results of our study show that CCN4 induced c-Jun phosphorylation. In addition, c-Jun siRNA abolished CCN4-induced VCAM-1 expression in OASFs. Therefore, c-Jun activation mediates by CCN4-increased VCAM-1 expression. Furthermore, CCN4 increased the binding of c-Jun to the AP-1 element within the VCAM-1 promoter, as shown by a chromatin immunoprecipitation assay. Binding of c-Jun to the AP-1 element was attenuated by Syk inhibitor, rottlerin, and SP600125. Using transient transfection with AP-1-luciferase as an indicator of AP-1 activity, we also found that CCN4 induced an increase in AP-1 activity. In addition, Syk inhibitor, GF109203X, rottlerin, curcumin, and tanshinone reduced CCN4-increased AP-1 promoter activity. These results indicate that the CCN4 may act through the αvβ5/α6β1 integrin, Syk, PKCδ, JNK, and AP-1 pathways to induce VCAM-1 production in human OASFs.

In conclusion, the signaling pathway involved in CCN4-induced VCAM-1 expression in human SFs was investigated. CCN4 increased VCAM-1 production by binding to the αvβ5/α6β1 integrin receptor and activating Syk, PKCδ, and JNK, which enhances AP-1 binding and transactivation of VCAM-1 expression. The CCN4-induced VCAM-1 expression promoted monocyte adhesion to human OASFs. These findings provide a better understanding of the mechanisms of OA pathogenesis.

Conflict of interest statement

All authors have no financial or personal relationships with other people or organizations that could inappropriately influence their work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2012.12.023.

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