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Simvastatin Inhibits Cytokine-Stimulated Cyr61 Expression in Osteoblastic Cells

A Therapeutic Benefit for Arthritis

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Objective. **To examine the effects of proinflammatory cytokines on Cyr61 expression in osteoblastic cells and the modulatory action of simvastatin, to assess the role of CREB in Cyr61 induction, and to investigate the relationship of osteoblastic expression of Cyr61 to disease progression in experimental arthritis.**

Methods. **Cyr61 expression and CREB phosphorylation at serine 133 were examined by Western blotting. Promoter activity of *Cyr61* was assessed by luciferase assay with promoter deletion/mutagenesis and forced expression/gene silencing of CREB. Interaction between CREB and the *Cyr61* promoter was evaluated by electrophoretic mobility shift assay and chromatin immunoprecipitation. CCL2 expression was examined by Northern blotting and enzyme-linked immunosorbent assay. In rats with collagen-induced arthritis (CIA), osteoblastic expression of Cyr61 was examined by immunohistochemistry, and disease progression was assessed by clinical, radiographic, and histologic examination.**

Results. **In primary human osteoblasts and U2OS cells, Cyr61 expression stimulated by tumor necrosis factor α, interleukin-1β (IL-1β), oncostatin M (OSM), and other IL-6–family cytokines was suppressed by simvastatin. In U2OS cells, simvastatin inhibited OSM-induced CREB phosphorylation and CREB–DNA binding. Knockdown of CREB by short hairpin RNA reduced Cyr61 synthesis. OSM-induced *Cyr61* promoter activation was dependent on CRE–CREB interaction and inhibited by simvastatin. Cyr61 enhanced CCL2 expression by U2OS cells. Intraarticular injection of simvastatin inhibited CIA progression and diminished the number of Cyr61+ osteoblasts and infiltrating macrophages.**

Conclusion. **Simvastatin inhibited cytokine-stimulated Cyr61 expression in osteoblastic cells and suppressed disease progression and osteoblastic expression of Cyr61 in inflammatory arthritis. This finding indicates that simvastatin may have potential as a therapeutic agent for inflammatory arthritis.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease leading to destruction of joints. Cytokines play a fundamental role in the processes that cause inflammation and articular destruction in RA (1). Besides tumor necrosis factor α (TNFα) and interleukin-1 (IL-1)–family cytokines, there is increasing evidence that IL-6 family members and signaling pathways downstream of the common gp130 receptor subunit are important in the pathogenesis of both murine and human inflammatory arthritis (2). Oncostatin M (OSM) is a member of the IL-6 family, and many studies have demonstrated that it has a stimulatory role in the progression of RA (3,4). In a previous study, we demonstrated that OSM induced strong expression of CCL2, a potent chemoattractant for monocyte/macrophages, in human osteoblastic cells, at both the messenger RNA

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(mRNA) and protein levels (5), supporting the notion that OSM and osteoblasts have an important role in the mediation of inflammatory bone diseases.

The cysteine-rich protein Cyr61 (also known as CCN1) belongs to the CCN protein family, which includes 6 members, CCN1–CCN6 (6). Once synthesized, CCN proteins are secreted, associate with cell surface or extracellular matrix through binding to integrins or heparan sulfate proteoglycans, and serve as matricellular signaling molecules (6). Functionally, Cyr61 has been shown to regulate angiogenesis and cell proliferation, adhesion, migration, and differentiation (6) and is important for wound healing (7) and embryo development (8).

However, previous studies have also implicated Cyr61 in the pathogenesis of inflammatory diseases, such as atherosclerosis (9), inflammatory cardiomyopathy (10), and Graves' ophthalmopathy (11). In a rat model of bacteria-induced apical periodontitis, we found that osteoblastic expression of Cyr61 correlates with the severity of inflammation-associated bone loss (12). A complementary DNA microarray analysis of B cells from monozygotic twins revealed significantly higher expression of Cyr61 in twins with RA compared with their healthy cotwins, and that study also showed increased immunoreactivity of Cyr61 in synovial tissue of RA patients (13). Recently, Zhang et al (14) found that Cyr61 plays a critical role in IL-17–mediated proliferation of fibroblast-like synoviocytes in RA. Nevertheless, whether Cyr61 is involved in inflammatory signaling of osteoblasts in RA remains to be elucidated.

The potential benefits of hydroxymethylglutaryl-coenzyme A (HMG-CoA) inhibitor (statin) therapy in patients with RA have been recognized recently (15). Beyond the cholesterol-lowering properties of statins arising from inhibition of HMG-CoA conversion to mevalonate, numerous studies indicate that these agents have broader effects, including alteration of inflammatory pathways (16). In particular, recent studies showed that simvastatin suppresses the secretion of proinflammatory cytokines from TNFα-stimulated rheumatoid synoviocytes (17,18), and lipophilic statins, including simvastatin, at high concentrations induce apoptosis of synoviocytes (19,20). However, conflicting results have been obtained from studies using animal models of RA. While Leung et al (21) showed that intraperitoneal simvastatin was effective in alleviating collagen-induced arthritis (CIA) in mice, these findings were not replicated in a later study (22). More recently, Funk and colleagues (23) demonstrated a bone-protective effect of subcutaneous simvastatin in rats with streptococcal cell wall–induced arthritis. Different routes of drug administration and different pharmacokinetics of different statins may be the factors responsible for the discrepancies between studies (23). Obviously, additional studies are needed to clarify the in vivo effects of statins on the development of RA.

In the present study, we explored the effects of proinflammatory cytokines on the expression of Cyr61 in osteoblastic cells. Since the promoter of either the human (24) or the mouse (25) *Cyr61* gene contains several potential binding sites for CREB and CREB is essential for signal transduction of inflammatory cytokines (26,27), we further tested the hypothesis that Cyr61 expression in osteoblastic cells might be regulated by CREB, using OSM as an example. The modulating effect of simvastatin on the process was investigated since statins are able to inhibit CREB signaling in various cell types (28,29). In a rat model of CIA, the therapeutic effect of locally delivered simvastatin and the relationship of osteoblastic expression of Cyr61 to disease progression were examined.

MATERIALS AND METHODS

Materials. Recombinant human TNFα, IL-1β, IL-6, leukemia inhibitory factor (LIF), and OSM were obtained from PeproTech. Recombinant human Cyr61 was purchased from Abnova. Anti-human/rat Cyr61 antibody and anti-CREB antibodies for supershift assay (sc-186X and sc-200X) were from Santa Cruz Biotechnology. Anti-CREB antibody for Western blot analysis and immunoprecipitation, mevalonate, geranylgeranyl pyrophosphate, farnesyl pyrophosphate, and the tetrazolium salt MTT were from Sigma-Aldrich. Anti–phospho-CREB (Ser-133) antibody was from Upstate Biotechnology. Anti-CD68 antibody was from Serotec. Lentivirus expression plasmid with short hairpin RNA (shRNA) construct targeting CREB was from OpenBiosystems. The human CCL2 enzyme-linked immunosorbent assay (ELISA) kit was from Bender MedSystems. Inactivated *Mycobacterium tuberculosis* and Freund's incomplete adjuvant (IFA) were from Difco. The plasmid pcDNA3.1-VP16-CREB was provided by Dr. A. Barco (Columbia University, New York, NY).

Preparation of simvastatin. The active form of simvastatin was prepared according to a previously described protocol (23), with a few modifications. Briefly, 25 mg simvastatin (Merck) was dissolved in 0.2 ml ethanol (95–100%), followed by addition of 0.3 ml NaOH. After heating at 50°C for 2 hours, the solution was neutralized to pH 7.2 with 1*N* HCl and brought to a 1-ml volume with normal saline. The final concentration of the stock solution was 4 mg/ml.

Cell culture. Primary cultures of human bone marrow–derived osteoblasts were established from alveolar bone explants as previously described (30). Cells were maintained in induction media for the duration of the study, without serum starvation. We also used U2OS cells, a human osteosarcoma cell line with a characteristic osteoblastic phenotype. U2OS

cells were made quiescent in serum-free media for 48 hours before treatment.

Assessment of Cyr61 expression and CREB phosphorylation at serine 133, CCL2 expression, and cell growth. The expression of Cyr61 and phosphorylation of CREB at serine 133 were examined by Western blotting. CCL2 expression was measured by Northern blotting and ELISA. The effects of simvastatin and Cyr61 on cell growth were assessed by MTT assay. Experiments were performed as previously described (31).

Lentiviral shRNA. Gene silencing was performed using lentiviral shRNA (31). Recombinant lentiviruses were produced by cotransfecting 293FT cells with shRNA-transferring plasmids and package plasmids, using the calcium phosphate method. Cell culture supernatants containing lentivirus were harvested 48 hours after transfection, filtered through a 0.45-μm filter, and added to U2OS cells in the presence of Polybrene. Twenty-four hours later, the efficiency of transduction was assessed by flow cytometric detection of green fluorescent protein expression. Stably transduced cells were selected using puromycin.

Luciferase assay. The pGL2-Cyr61 P1-Luc construct containing a 972-bp fragment of the 5′-flanking region of the Cyr61 gene starting from −169 bp upstream of the ATG start codon (A = +1) (24) was kindly provided by Dr. N. Schütze (University of Wurzburg, Wurzburg, Germany). Two deletion constructs (P2 and P3) were generated by polymerase chain reaction (PCR) using 2 forward primers and an identical reverse primer (P2 forward 5′-CAACTACCATCACCACCATCACG-3′; P3 forward 5′-AATGGAGCCAGGGGAGGCG-3′; P2 and P3 reverse 5′-CCCTCCGCGCCTTCTCC-3′). All constructs were confirmed by DNA sequencing. U2OS cells were cotransfected with pGL2-Cyr61-Luc and pRL-TK (thymidine kinase promoter–(*Renilla* luciferase reporter plasmid; Promega) using Arrest-In reagent (OpenBiosystems). After treatment, firefly luciferase activity was detected and normalized by RL-TK activity as described previously (31). Relative activity was expressed as the mean ± SD from 3 independent experiments.

Site-directed mutagenesis. Mutations were made using the QuickChange Site-Directed Mutagenesis protocol according to the specifications of the manufacturer (Stratagene). The putative CRE at *Cyr61* P3 was changed from -CGTCA- to -tGTCA- (Mut1) and -gccCA- (Mut2). Constructs were fully sequenced in both directions to confirm successful mutagenesis before use.

Electrophoretic mobility shift assay (EMSA). Preparation of nuclear extracts and EMSA were performed as previously described (31). Nuclear proteins were incubated with biotin-labeled oligonucleotide probe derived from the *Cyr61* promoter region at P3 (5′-AGAGCCGA<u>CGTCA</u>CTGCAACACGC-3′ [putative CRE underlined]). For supershift, antibodies against CREB were added to the incubation mixture for 30 minutes on ice and then for 30 minutes at room temperature before electrophoresis.

Chromatin immunoprecipitation (ChIP) assay. ChIP was performed as described previously (30), using an assay kit from Upstate Biotechnology. Briefly, the DNA–protein complex from treated U2OS cells was crosslinked with 1% formaldehyde for 10 minutes at room temperature. After washing with phosphate buffered saline, cells were pelleted and resuspended in sodium dodecyl sulfate lysis buffer. The lysates were then subjected to sonication, dilution, and incubation with a salmon sperm DNA–protein A agarose 50% slurry for 60 minutes at 4°C. The supernatant was incubated with anti-CREB antibody at 4°C overnight. Immunocomplexes were collected with a salmon sperm DNA–protein A agarose mixture. The bound DNA was recovered by phenol–chloroform–ethanol precipitation and used as a template for PCR to identify the region of the human *Cyr61* promoter flanking the CRE at P3. The primer sequences were as follows: (−376) 5′-AATGGAGCCAGGGGAGGCG-3′ and 5′-CCCTCCGCGCCTTCTCC-3′ (−169).

Animal model of CIA. CIA was induced in 20 male Sprague-Dawley rats weighing 220–250 gm, as described previously (31). The experimental protocol was approved by the Laboratory Animal Center, College of Medicine, National Taiwan University, and the animals were maintained according to the *Guide to Management and Use of Experimental Animals* (National Science Council, Taiwan). Bovine type II collagen (CII; Chondrex) in 4 mg/ml acetic acid was emulsified in an equal volume of Freund's complete adjuvant (CFA) containing 2 mg/ml inactivated *M tuberculosis* in IFA). On day 1, CIA was elicited by intradermal injection, into 5 sites (2 on the tail base, 3 on the back), of a total of 500 μg bovine CII in 500 μl CFA. On day 7, booster injections were administered to 3 sites (1 on the tail base, 2 on the back) with a total of 300 μg bovine CII in 300 μl IFA. Starting on day 0, rats were administered intraarticular injections (0.5 ml/kg) of simvastatin (0.5 mg/ml) in the right ankle joint and normal saline in the left ankle joint every 5 days, until they were killed on day 21.

Clinical assessment. The rats were examined every other day by an investigator who was blinded with regard to the treatment protocol. Ankle joints were evaluated using a 0–4 scale in which grade 0 = no swelling or erythema, grade 1 = slight swelling and/or erythema, grade 2 = low to moderate edema, grade 3 = pronounced edema with limited joint usage, and grade 4 = excess edema with joint rigidity (32).

Radiographic assessment. Ankle joints were placed in position on X-Omat TL high-resolution specimen-imaging film (Eastman Kodak) and radiographed with an x-ray system from Faxitron (model 43855A). Images were shot at 26 kV for 10 seconds. Using a semiquantitative scale (0–4), erosive changes were analyzed according to the degree of bony destruction/erosions, with 1 point each assigned for erosion in the tibia, the calcaneus, the talus, and the small tarsal bones. Thus, the maximum possible score was 4, i.e., erosions in the tibia, calcaneus, talus, and any 1 or more of the small tarsal bones (32).

Histologic examination and immunohistochemistry. The ankle joints were fixed, decalcified, embedded in paraffin, sectioned, and examined histopathologically. Histopathologic features were evaluated using a scale of severity ranging from 1 to 4, where grade 1 = hyperplasia of the synovial membrane and presence of polymorphonuclear infiltrates, grade 2 = pannus and fibrous tissue formation and focal subchondral bone erosion, grade 3 = articular cartilage destruction and bone erosion, and grade 4 = extensive articular cartilage destruction and bone erosion (31). Immunohistochemical staining was performed with antibodies against rat Cyr61 and CD68 (macrophage marker). For each animal, quantitative

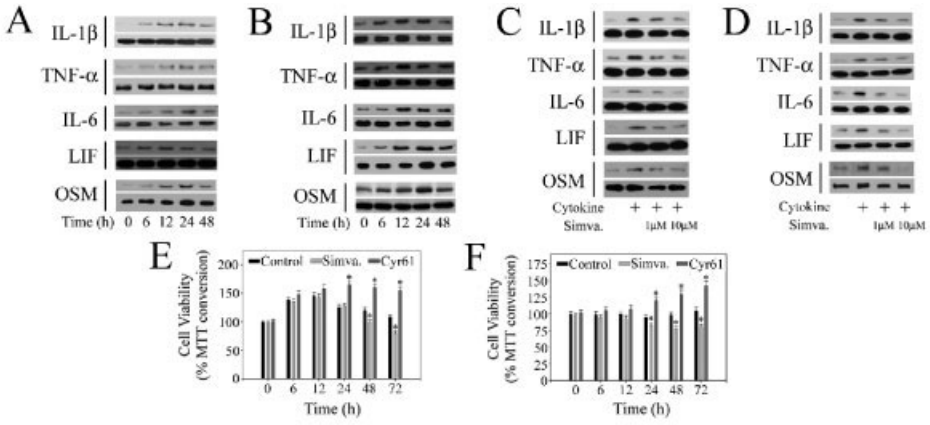

Figure 1. Effect of simvastatin (Simva.) on cytokine-stimulated Cyr61 expression, and effect of simvastatin and Cyr61 on the growth of osteoblasts and U2OS cells. **A** and **B,** Human bone marrow–derived osteoblasts (**A**) and U2OS cells (**B**) were incubated with interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), IL-6, leukemia inhibitory factor (LIF), or oncostatin M (OSM) (all at 10 ng/ml) for various lengths of time. **C** and **D,** Human bone marrow–derived osteoblasts (**C**) and U2OS cells (**D**) were incubated for 24 hours with each of the cytokines in combination with simvastatin (1 μM or 10 μM, added 3 hours before the addition of cytokines). Cyr61 levels were assayed by Western blotting. For each cytokine in **A–D,** Cyr61 and β-actin are shown in the upper and lower blots, respectively. **E** and **F,** The effects of simvastatin (10 μM) and exogenous Cyr61 (200 ng/ml) on the growth of human bone marrow–derived osteoblasts (**E**) and U2OS cells (**F**) were examined by MTT assay. Values are the mean ± SD of 3 independent experiments. $* = P < 0.05$ versus control.

analysis was performed on the 3 sections with the strongest inflammatory reactions. The field in each section exhibiting the highest osteolytic activity was selected and examined microscopically at 400× magnification. The number of osteoblasts lining the bone surface and the number of Cyr61+ osteoblasts were counted. The data were converted to the percentage of Cyr61+ osteoblasts lining the bone surface.

Statistical analysis. Data were assessed by analysis of variance for multiple comparisons and then by Fisher's protected least significant difference test. *P* values less than 0.05 were considered significant.

RESULTS

Simvastatin inhibits cytokine-stimulated Cyr61 expression in human bone marrow–derived osteoblasts and U2OS cells. Western blot analysis showed that IL-1β, TNFα, IL-6, LIF, and OSM stimulated Cyr61 expression in human bone marrow–derived osteoblasts (Figure 1A) and U2OS cells (Figure 1B). The stimulatory effects were time dependent and usually peaked at 12–24 hours in both cell types. Simvastatin attenuated cytokine-enhanced Cyr61 expression in a dose-dependent manner in both types of osteoblastic cells (Figures 1C and D). MTT assay revealed that exogenous Cyr61 enhanced the growth of both cell types, whereas simvastatin suppressed their proliferation (Figures 1E and F). The 2 cell types showed a similar response to the cytokines tested, but U2OS appeared to be a better producer of Cyr61 and exhibits more stable biologic behavior. Therefore, we used U2OS cells to study the influence of CREB signaling on Cyr61 expression.

CREB is required for OSM-enhanced Cyr61 expression, and activation of CREB is inhibited by simvastatin. To examine the role of CREB in Cyr61 induction, we assessed the effect of IL-6–family cytokines on CREB activation. Western blot analysis demonstrated that IL-6, LIF, and OSM phosphorylated CREB at serine 133, in a time-dependent manner (Figure 2A). Simvastatin suppressed the OSM-induced CREB phosphorylation, and the inhibitory effect was reversed by mevalonate and geranylgeranyl pyrophosphate, but not by farnesyl pyrophosphate (Figure 2B). Gene silencing experiments showed that shRNA targeting CREB successfully suppressed its expression (Figure 2C). Knockdown of CREB significantly attenuated OSM-enhanced Cyr61 synthesis, and additional treatment with simvastatin further repressed Cyr61 induction in U2OS cells (Figure 2D).

Transcriptional regulation of the *Cyr61* gene by OSM is dependent on CRE and CREB. Previous studies showed that the promoter of the human *Cyr61* gene

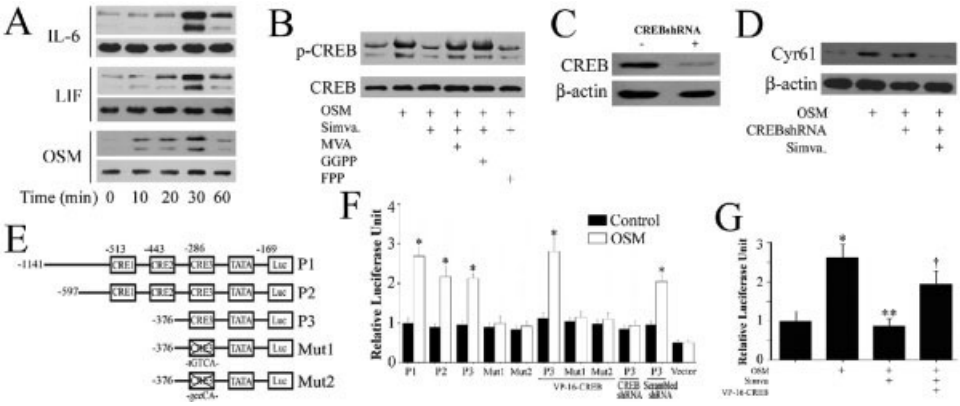

Figure 2. Role of CREB in OSM-stimulated Cyr61 expression and gene regulation, and inhibitory effect of simvastatin. **A,** U2OS cells were incubated with cytokines (all at 10 ng/ml) for various lengths of time. Phospho-CREB and total CREB are shown in the upper and lower blots, respectively. **B,** Cells were treated with OSM (10 ng/ml) for 30 minutes, in combination with 10 μM simvastatin (added 3 hours before the addition of OSM), with or without mevalonate (MVA; 100 μM), geranylgeranyl pyrophosphate (GGPP; 15 μM), or farnesyl pyrophosphate (FPP; 15 μM). Immunoblotting against phospho (Ser-133)–CREB was performed. **C,** U2OS cells were transduced with CREB short hairpin RNA (shRNA). The gene silencing effect was confirmed by Western blotting. **D,** Cells transduced with CREB shRNA were stimulated for 24 hours with OSM with or without simvastatin, and immunoblotting against Cyr61 was performed. **E,** Schematic diagram showing the putative CREs on the *Cyr61* promoter. Mut1 and Mut2 are mutations of the CRE3 at P3. **F,** U2OS cells were transfected with pGL2-*Cyr61*-promoter-Luc and treated with OSM for 30 minutes. Cells were cotransfected with pcDNA3.1-VP16-CREB for forced expression of CREB. **G,** U2OS cells transfected with pGL2-P1-Luc were incubated with OSM for 30 minutes, with or without simvastatin and pcDNA3.1-VP16-CREB. Promoter activities were determined by luciferase assay. Values in **F** and **G** are the mean ± SD of 3 independent experiments. $* = P < 0.05$ versus control; $** = P < 0.05$ versus OSM alone; $\dagger = P < 0.05$ versus OSM plus simvastatin. See Figure 1 for other definitions.

contains several CREs (24). To investigate the role of CREs in transcriptional regulation of Cyr61, activation of *Cyr61*-promoter-Luc reporter constructs was analyzed (Figure 2E). OSM significantly increased the luciferase activity of the P1 (−169 to −1141) reporter construct of the *Cyr61* gene. Deletion to position −597 (P2) moderately attenuated OSM-stimulated luciferase expression, but further deletion to −376 (P3) with the preservation of CRE3 resulted in no additional decrease of promoter activity. Mutations of the CRE3 at P3 completely abolished the OSM response (Figure 2F). The constitutively active pcDNA3.1-VP16-CREB raised the activity of the wild-type promoter but not that of the mutants (Figure 2F). Furthermore, shRNA targeting CREB suppressed promoter activation by OSM (Figure 2F).

Simvastatin inhibits OSM-stimulated promoter activation. Luciferase reporter assays showed that simvastatin inhibited promoter activation stimulated by OSM (Figure 2G). The constitutively active pcDNA3.1-VP16-CREB reversed this suppressive effect to a large extent (Figure 2G), implying that blockade of CREB activation is important for promoter attenuation by simvastatin.

OSM increases CREB–DNA binding, and simvastatin inhibits recruitment of CREB to the *Cyr61* promoter. To examine the binding between CREB and the consensus CRE sequence, nuclear proteins were incubated with biotin-labeled oligonucleotide probes. The results showed that OSM treatment caused an increase of binding between CREB and oligonucleotides corresponding to the CRE3 region of the *Cyr61* promoter (Figure 3A). Simvastatin significantly inhibited the formation of CREB–DNA complexes, and supershifts were clearly seen when anti-CREB antibodies were introduced (Figure 3B). Consistent with the observations from EMSA, ChIP assay demonstrated that OSM enhanced the occupancy of CREB on the *Cyr61* promoter of the CRE3 region, and simvastatin attenuated the action of OSM (Figure 3C).

Cyr61 enhances CCL2 expression in osteoblastic cells. To investigate the possible role of Cyr61 in mediating inflammation, we examined its effect on the ex-

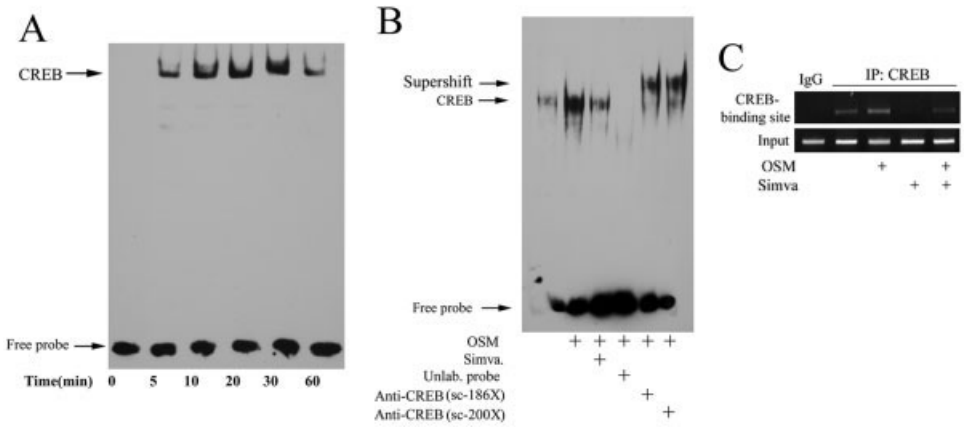

Figure 3. Role of OSM in CREB–DNA binding, and effect of simvastatin on CREB recruitment. **A** and **B,** U2OS cells were treated with OSM (10 ng/ml) for various lengths of time (**A**) or for 30 minutes with or without addition of 10 μM simvastatin (3 hours before OSM treatment), 50-fold unlabeled (Unlab.) probe, or anti-CREB antibodies (sc-186X and sc-200X) (**B**). Nuclear extracts were analyzed by electrophoretic mobility shift assay using a biotin-labeled probe corresponding to the region of CRE3 at position −286 upstream of the ATG start codon (A = +1) of the human *Cyr61* gene. **C,** Cells were subjected to chromatin immunoprecipitation with CREB antibody. Immunoprecipitates (IP) were analyzed by polymerase chain reaction using primers specific for CRE3. Equal input DNA was assessed. See Figure 1 for other definitions.

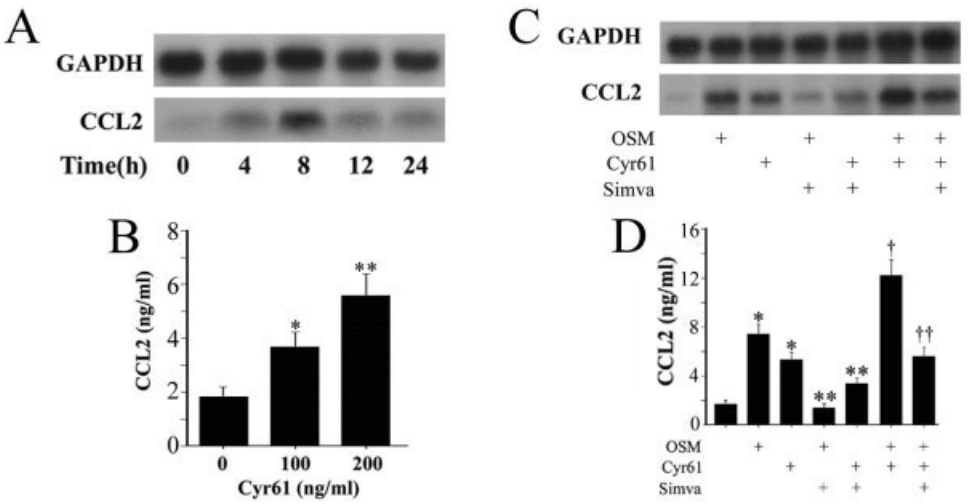

Figure 4. Effect of Cyr61 on CCL2 expression, and inhibitory effect of simvastatin. **A,** U2OS cells were incubated with Cyr61 (200 ng/ml) for various lengths of time, and CCL2 mRNA levels were assayed by Northern blotting. **B,** Cells were treated for 12 hours with Cyr61 (100 ng/ml or 200 ng/nl), and the amount of CCL2 released into the culture medium was quantified by enzyme-linked immunosorbent assay. Values are the mean ± SD of 3 independent experiments. * = $P < 0.05$ versus control; ** = $P < 0.05$ versus 100 ng/ml Cyr61. **C** and **D,** Cells were incubated with OSM (10 ng/ml) for 8 hours (**C**) or 12 hours (**D**) with or without simvastatin (10 μM) pretreatment and with or without addition of Cyr61 (200 ng/ml), and CCL2 mRNA levels (**C**) and the amount of CCL2 released into the culture medium (**D**) were assayed. Values are the mean ± SD of 3 independent experiments. * = $P < 0.05$ versus control; ** = $P < 0.05$ versus the respective stimulant (OSM or Cyr61) alone; † = $P < 0.05$ versus OSM alone and versus Cyr61 alone; †† $P < 0.05$ versus OSM plus simvastatin. See Figure 1 for definitions.

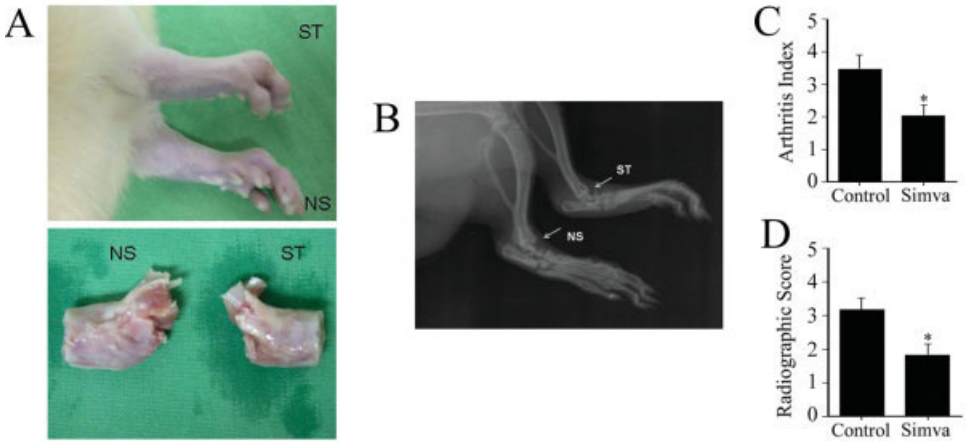

Figure 5. Effects of simvastatin in collagen-induced arthritis (CIA). CIA was elicited in 20 rats. Intraarticular injections of simvastatin (ST, Simva; 0.25 mg/kg) and normal saline (NS) were administered to the right and left ankle joints, respectively, every 5 days until the rats were killed. **A,** Feet and dissected ankles of a representative animal. **B,** Radiographic findings in a representative animal. **C** and **D,** Clinical (**C**) and radiographic (**D**) scores of arthritis severity. Values are the mean ± SD. $* = P < 0.05$ versus control.

pression of CCL2 in U2OS cells. Northern blot analysis showed that Cyr61 enhanced the transcription of CCL2 mRNA in a time-dependent manner (Figure 4A). ELISA revealed an increase of CCL2 secretion into the culture medium after Cyr61 treatment (Figure 4B). A synergistic stimulatory effect on CCL2 expression was noted when OSM and Cyr61 were added together (Figures 4C and D). Simvastatin significantly attenuated CCL2 expression stimulated by OSM or Cyr61. Moreover, the suppressive effect of simvastatin on CCL2

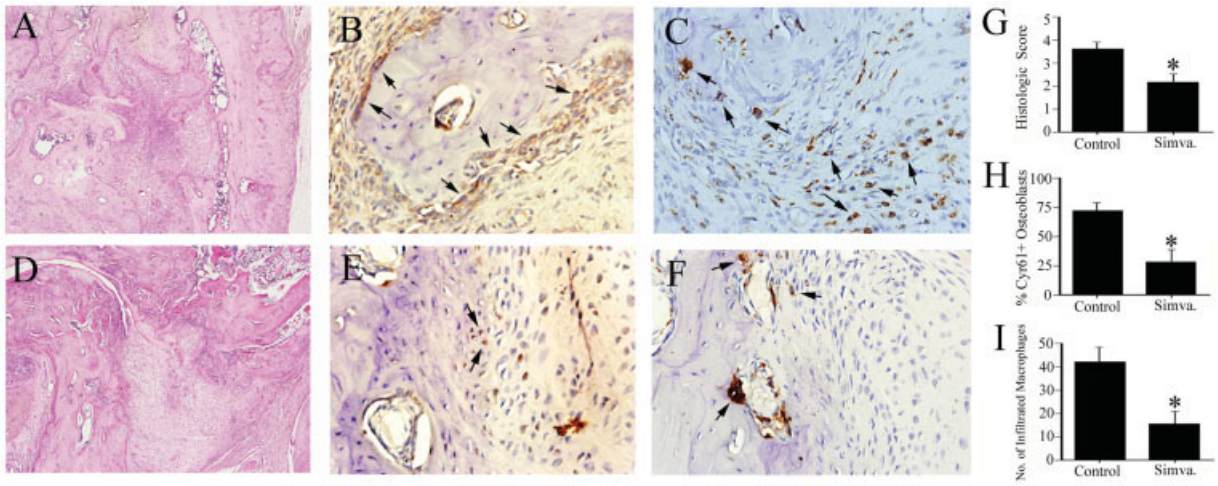

Figure 6. Histologic findings in the simvastatin (Simva.)–treated and control ankle joints. **A–C,** In control joints, extensive erosion of bone by pannus and heavy infiltration of inflammatory cells (**A**), marked expression of Cyr61 in osteoblasts (**arrows**) overlying the osteolytic areas (**B**), and numerous CD68+ macrophages and osteoclasts (**arrows**) adjacent to resorption lacunae (**C**) are seen. **D–F,** In simvastatin-treated joints, there is much less cartilage/bone erosion and inflammatory cell infiltration (**D**), and numbers of Cyr61+ osteoblasts (**E**) and CD68+ macrophages (**F**) are decreased. Original magnification × 40 in **A** and **D** (hematoxylin and eosin stained); × 400 in **B, C, E,** and **F** (avidin–biotin–peroxidase stained). **G–I,** The severity of arthritis was quantified by determination of the histologic score (**G**), the percentage of Cyr61+ osteoblasts (**H**), and the number of infiltrated macrophages (**I**). Values are the mean ± SD. $* = P < 0.05$ versus control.

induction by OSM was reverted by addition of exogenous Cyr61 (Figures 4C and D).

Intraarticular injection of simvastatin inhibits CIA progression and osteoblastic expression of Cyr61. To examine the relationship of Cyr61 expression in osteoblasts to arthritis development, a rat CIA model was used. No fluctuations in the body weight of the rats were observed during the course of the experiment. Clinical signs of arthritis appeared by day 12–13 after the initial immunization and gradually worsened. On day 21, swelling and erythema were marked in the control left ankle joints, whereas intraarticular administration of simvastatin reduced the clinical signs of arthritis on the right side (Figure 5A). At the end of the experiment, the arthritis score in the simvastatin-treated joints was significantly reduced compared with that in the control joints (mean ± SD 2.0 ± 0.3 and 3.4 ± 0.4, respectively; $P < 0.05$) (Figure 5C). Radiographic examination revealed marked erosions of the articular surface in most of the control ankles. In the simvastatin-treated joints, erosions of the small tarsal bones were frequently found, but the tibia, talus, and calcaneus were involved only occasionally (Figure 5B). Semiquantitative analysis of arthritis progression by radiography revealed elevated scores in the control joints, whereas administration of simvastatin reduced radiographic bone destruction (3.2 ± 0.3 and 1.8 ± 0.3, respectively; $P < 0.05$) (Figure 5D).

In the control joints, histopathologic examination revealed extensive pannus formation associated with irregular bone resorption and inflammatory cell infiltration (Figure 6A). Immunohistochemistry analysis demonstrated marked Cyr61 expression in osteoblasts lining the bone surfaces (Figure 6B). Cyr61 staining in the infiltrated mononuclear round cells and multinucleated osteoclasts was also noted. Prominent CD68 signals were observed in the macrophages infiltrating the osteolytic areas (Figure 6C). In contrast, simvastatin markedly diminished bone destruction, as evidenced by the preservation of joint space, substantially reduced cartilage and bone erosion, and decreased inflammatory cell infiltration (Figure 6D). Moreover, simvastatin reduced the number of Cyr61+ osteoblasts (Figure 6E) and recruitment of macrophages (Figure 6F). The mean ± SD histopathologic score in the control joints and the simvastatin-treated joints was 3.6 ± 0.3 and 2.2 ± 0.3, respectively ($P < 0.05$) (Figure 6G). A significant difference between the control and experimental joints in the percentage of Cyr61-positive osteoblasts was found (72.1 ± 6.6% and 28.7 ± 10.3%, respectively; $P < 0.05$) (Figure 6H). Compared with control joints, the number of infiltrating macrophages was also reduced in simvastatin-treated joints (42.2 ± 6.5 and 15.2 ± 5.2, respectively; $P < 0.05$) (Figure 6I).

DISCUSSION

In this study, we demonstrated that proinflammatory cytokines stimulate the expression of Cyr61 in human bone marrow–derived osteoblasts and U2OS cells, and simvastatin suppresses these effects in both cell types. Exogenous Cyr61 enhanced the growth of both types of osteoblastic cells, whereas simvastatin suppressed the proliferation. Our findings are in accordance with those of previous studies which showed that Cyr61 enhanced the proliferation of osteoblasts (33) and statins stimulated differentiation but induced growth arrest in osteoblasts (34). Since the responses were similar in the 2 cell types and U2OS cells have more stable biologic behavior and higher transfection efficiency, they were used to study the associated signaling pathway. We found that in U2OS cells, IL-6–family cytokines increased the phosphorylation of CREB at serine 133. Moreover, OSM recruited CREB to the *Cyr61* promoter and enhanced promoter activity. These findings indicate that CREB may play a pivotal role in Cyr61 up-regulation by proinflammatory cytokines, at least by those in the IL-6 family.

Information regarding the molecular mechanisms involved in regulation of the *Cyr61* gene is somewhat limited. Bioinformatics analysis of the human *Cyr61* promoter has demonstrated 3 CRE sites, at positions −513 (CRE1), −443 (CRE2), and −286 (CRE3) upstream of the ATG start codon (24,35). However, the role of CREB in regulating Cyr61 gene expression appears to be tissue specific. In melanoma cells, CREB is a negative regulator of Cyr61 expression (36), while in smooth muscle cells it is a positive regulator (35). In the present study, using deletion analysis, we found that CRE1 and CRE2 were not required for OSM-induced promoter activation. Mutation of CRE3 suppressed the promoter response to OSM, even when CREB was overexpressed. Knockdown of CREB expression by shRNA abolished promoter activation by OSM (Figure 2F). EMSA and ChIP assay confirmed that CRE3 functioned as a CREB binding element in osteoblastic cells (Figure 3). No binding was detected in EMSA when oligonucleotide probes derived from the CRE1 or CRE2 region were used (results not shown). Similarly, ChIP assay using primers flanking CRE1 and CRE2 did not reveal binding between CREB and the *Cyr61* promoter (results not shown).

Taken together, the findings of the present study

demonstrate that transcriptional activation of Cyr61 by OSM is CREB dependent. Of the 3 putative CREs at the *Cyr61* promoter, only the one at position −286 (CRE3) is essential for promoter activation, which is similar to findings in studies of sphingosine-stimulated calf smooth muscle cells (35). Nevertheless, deletion of the region distal to CREs resulted in a moderate decrease of promoter activity, implying that other transcription factors are also required for OSM-induced Cyr61 synthesis.

Given the central role of angiogenesis in the pathogenesis of inflammatory diseases such as RA, it is likely that Cyr61, a well-established angiogenic factor (6), is involved. Furthermore, we showed that Cyr61 up-regulated CCL2 secretion by osteoblastic cells, and a synergistic effect was observed when OSM and Cyr61 were added together. CCL2 is a potent chemoattractant for monocyte/macrophages, and its expression has been detected in pathologic conditions associated with macrophage aggregation, including RA (31). Results of the present study support the notion that excessive amounts of Cyr61 may be pathogenic. Numerous studies have confirmed that statins are able to influence the expression of inflammatory cytokines and other secreted mediators (37), but their regulatory actions on CCN-family proteins have seldom been investigated. In the present study, we demonstrated that simvastatin suppressed cytokine-stimulated Cyr61 expression. Although other signaling pathways may be involved since simvastatin exerted additional effects after knockdown of CREB (Figure 2D), diminished CREB activity played a major role in the inhibitory action of simvastatin, at least with regard to inhibition of the effects of OSM (Figures 2D and G).

We also showed that CREB phosphorylation suppressed by simvastatin was restored by mevalonate or geranylgeranyl pyrophosphate, but not by farnesyl pyrophosphate (Figure 2B). Because Rho-family proteins are typically geranylgeranylated and Ras-family proteins are farnesylated (16), our findings suggest that simvastatin may inhibit OSM-stimulated CREB activation predominantly by interfering with Rho-like protein activity. This observation is consistent with the findings of Crespo et al (29), who demonstrated that inhibition of the isoprenylation of geranylgeranylated proteins by simvastatin was key to the interference of CREB activation induced by low-density lipoproteins in smooth muscle cells. Interestingly, simvastatin also suppressed Cyr61-stimulated CCL2 synthesis (Figures 4C and D), implying that it may interfere with signaling downstream of Cyr61. Further investigation is needed to clarify the mechanism by which simvastatin blocks the action of Cyr61.

A recent study using microarray analysis of B cell transcripts from disease-discordant monozygotic twins revealed an association between RA and increased expression of Cyr61 (13). Immunohistochemistry analysis further showed increased staining for Cyr61 on synovial lining cells and macrophages from synovial tissue of RA patients as compared with that from osteoarthritis patients or healthy individuals (13). In the present study, to assess the role of osteoblastic Cyr61 in the pathogenesis of inflammatory arthritis, a murine CIA model was used. Consistent with findings of the in vitro experiments, the results of the CIA study showed that Cyr61 expression in osteoblasts was correlated with disease activity. Intraarticular injection of simvastatin alleviated arthritis, inhibited osteoblastic expression of Cyr61, and diminished the aggregation of CD68+ macrophages around Cyr61+ osteoblasts. Although Cyr61 has been shown to inhibit osteoclastogenesis in monocyte cultures, possibly by interfering with costimulatory pathways such as immunoreceptor tyrosine–based activation motif–dependent signals (38), results of another study showed that osteoblasts may compensate for these defects (39). In any case, our findings suggest that there is a connection between osteoblastic Cyr61 and the progression of arthritis, further supporting the notion of an etiologic role of Cyr61 in the pathogenesis of RA.

The present study provides the first reported evidence that intraarticular injection of statins is a promising therapy for RA. Previous investigations have shown that statins have a wide range of effects on cells and tissues involved in inflammation (37); however, animal studies on the therapeutic effects of systemically delivered statins in inflammatory arthritis have yielded conflicting results (21–23). Although the statin doses used in the mouse models (up to 40 mg/kg per day [21,22], equivalent to a daily human dose of 3.25 mg/kg [40]) were higher than those used in standard therapy in humans (0.1–1.0 mg/kg per day) (41), the amount of drug reaching the joints may be low since statins are designed to act primarily in the liver (42). The disparity in the results of animal studies may be due to liver-specific pharmacokinetics and poor distribution of statins to bone and joints. Nevertheless, in addition to suppression of proinflammatory signaling, lipophilic statins in high concentrations induce apoptosis of rheumatoid synoviocytes and are considered more effective for RA treatment (19,20).

Escalating the dosage of statins for arthritis treatment may not be practical, because this would increase

the risk of adverse effects (41). Local administration of statins may reconcile this contradiction, by increasing the concentration of drug in the microenvironment of the joint and minimizing systemic toxicities. Recently, using a rabbit model of osteoarthritis, Akasaki et al (43) also demonstrated a therapeutic effect of intraarticular mevastatin on disease progression.

In conclusion, we have demonstrated that proinflammatory cytokines stimulate Cyr61 expression in osteoblastic cells and that this action is inhibited by simvastatin. The CREB-dependent pathway is essential for cytokine-induced up-regulation of Cyr61, at least in the case of IL-6–family cytokines. Cyr61 expression in osteoblasts correlates with disease activity in inflammatory arthritis. Our data also provide proof-of-principle that intraarticularly injected simvastatin is effective for the treatment of inflammatory arthritis.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Lin had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Kok, Chang, Lin.

Acquisition of data. Kok, Hou, Hong, Liang, Chang, Hsiao, Lin.

Analysis and interpretation of data. Kok, Hong, Wang, Yang, Lai, Lin.

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