Novel Role of Pin1 Induction in Type II Collagen-Mediated Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation in joints through leukocyte sequestration and destruction of cartilage and bone. Inflammatory mediators such as PGs and proinflammatory cytokines contribute to RA progress. Pin1, a peptidyl prolyl isomerase, plays important pathophysiological roles in several diseases, including cancer and neurodegeneration. We found that both Pin1 and cyclooxygenase-2 (COX-2) were highly expressed in ankle tissues of type II collagen-induced RA mice. HTB-94 cells overexpressing Pin1 and primary cultured human chondrocytes showed increased basal expression of proinflammatory proteins (COX-2, inducible NO synthase, TNF-α, and IL-1β). Site-directed mutagenesis revealed that Pin1-mediated transcriptional activation of COX-2 was coordinately regulated by NF-κB, CREB, and C/EBP. Gel shift, reporter gene, and Western blot analyses confirmed that NF-κB, CREB, and C/EBP were consistently activated in chondrocytes overexpressing Pin1. Treatment of RA mice with juglone, a chemical inhibitor of Pin1, significantly reduced RA progress and COX-2 expression in the ankle tissues. Moreover, juglone dose dependently decreased the basal COX-2 expression in primary cultured chondrocytes from RA patients. These results demonstrate that Pin1 induction during RA progress stimulates proinflammatory protein expression by activating NF-κB, CREB, and C/EBP, and suggest that Pin1 is a potential therapeutic target of RA.


Materials and Methods

**Materials**

Both 5-bromo-4-chloro-3-indolylphosphate and NBT were supplied by Life Technologies. Anti-Pin1, COX-2, C/EBPα, C/EBPβ, CREB, c-Jun, c-Fos, JunD, Fraf1, tubulin, GFP, and p65 Abs were obtained from Santa Cruz Biotechnology. Abs that recognized phosphorylated or total ERK, p38 kinase and JNK were obtained from Cell Signaling Technology. HRP-conjugated donkey anti-rabbit and alkaline phosphatase-conjugated donkey anti-mouse IgGs were purchased from Jackson ImmunResearch Laboratories. The reagents used for molecular studies were primarily obtained from Sigma-Aldrich.

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Cell culture
HTB-94 cells and primary cultured human chondrocytes were obtained from ATCC and Y. Park (College of Medicine, Chonnam National University, Gwangju, South Korea), respectively, and cultured at 37°C in 5% CO2/95% air in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

CII-induced arthritis and juglone treatment
The institutional animal care and utilization committee of Chosun University approved all the animal procedures used in this study. Male DBA/1J mice (Joong-Ang Experimental Animals), age 8 wk, were used. Bovine CII was dissolved in 0.1 M acetic acid and emulsified in an equal volume of CFA. The mice were immunized intradermally at the base of the tail with 100 μl emulsion containing 150 μg of CII. On day 21, mice were boosted intradermally with 100 μg of CII, and arthritis development was monitored for 10 days. Juglone was dissolved in solubilization solvent (PEG400, Tween 80, ethanol, and sterile water) and i.p. injected from day 22 every other day (4× injection).

Assessment of arthritis
Mice were sacrificed on day 10 after second CII booster. Before sacrificing, arthritis in each limb of CII-treated mice was clinically assessed by visual edema scoring from 0 to 3, as follows: 0, no swelling; 1, detectable swelling in one joint; 2, nonsevere swelling in two or more joints; 3, severe swelling in two or more joints (19). The left hind limbs, including paws and ankles, were dissected, fixed immediately for 12 h in 10% neutralizing formaldehyde, dehydrated in Calci-Clear Rapid (National Diagnostics) for 12 h, and embedded in paraffin. Tissue sections (4 μm) were mounted on common slides for staining with H&E. A certified pathologist scored samples in a blinded fashion. The data are expressed as mean chronic inflammation, fibrosis, articular cartilage damage, synovial proliferation, bone damage, and ankylosis scores. All scores were semiquantitatively indexed based on a scale of 0–3. Radiographs were done using Softex CMBW-2 (Softex).

Immunohistochemistry
A universal immunoenzymic polymer method was used for immunostaining. Sections (4 μm) were cut from formalin-fixed, paraffin-embedded tissue blocks, mounted on polylysine-coated slides, dewaxed in xylene, and rehydrated through a graded ethanol series. After deparaffinization, Ag retrieval treatment was performed at 121°C for 15 min in 10 mM sodium citrate buffer (pH 6.0), and then was treated with 3% hydrogen peroxide in methanol solution for 20 min to quench endogenous peroxidase activity. To block intrinsic avidin-biotin binding, the tissue slides were treated with avidin-biotin blocking kit reagents (Vectastain Elite ABC kit; Vector Laboratories) for 15 min. Anti-Pin1 and anti-COX-2 Abs were used as the primary Abs. The final products were visualized using the 3,3-diaminobenzidine tetrahydrochloride detection system (DakoCytomation). All experiments were performed in duplicate.

Cell culture
HTB-94 cells and primary cultured human chondrocytes (passage 6) were obtained from ATCC and K. Lee (Chonnam National University, Gwangju, South Korea), respectively. Both the cell types were cultured at 37°C in 5% CO2/95% air in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. For all experiments, cells were grown to 80–90% confluency and subjected to no more than 15 passages.

Construction of Pin1 retroviral plasmid and infections
HTB-94 cells and primary cultured human chondrocytes stably expressing Pin1 were established using murine stem cell virus (MSCV)-GFP retroviral vector, and Phoenix cells (a packaging cell line) were transfected with MSCV-GFP (control) or MSCV-Pin1-GFP (Pin1 overexpression) plasmid. Supernatants containing amphotropic replication-incompetent retroviruses were collected and then stored at −80°C until required. Twenty percent confluent HTB-94 cells and chondrocytes obtained from osteoarthritic patients were multiply infected (12 times) with retrovirus particles. Intensities of infection were monitored by GFP fluorescence and Western blot analysis using Pin1 Ab.

Preparation of nuclear extract and Western blot analysis
Cells were removed using a cell scraper and centrifuged at 2500 × g for 5 min at 4°C. The cells were then swollen with 100 μl of lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet-P40, 1 mM DTT, and 0.5 mM PMSF). Tubes were vortexed to disrupt cell membranes, and samples were incubated for 10 min on ice and then centrifuged for 5 min at 4°C. Pellets containing crude nuclei were resuspended in 100 μl of extraction buffer (20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF), incubated for 30 min on ice, and centrifuged at 15,800 × g for 10 min; the supernatants containing the nuclear extracts were collected and stored at −80°C until required. SDS-PAGE and immunoblot analyses were performed, as described previously (21). Cell lysates were fractionated by 10% gel electrophoresis, and electrophoretically transferred to nitrocellulose membranes. The membranes were subsequently incubated with primary Ab, and then with alkaline phosphatase- or HRP-conjugated secondary Abs. Finally, the membranes were developed using either 5-bromo-4-chloro-3-indolylphosphate and NBT or an ECL chemiluminescence detection kit.

Gel shift assay
dsDNA probes (2 pmol/μl) for the consensus sequences of AP-1 (5’-CGTCTGATGATGATCACCGAGA-3’) were used for gel shift analyses after end labeling the probe with [γ-32P]ATP and T4 polynucleotide kinase. The reaction mixture contained 2 μl of 5× binding buffer with 20% glycerol, 5 mM MgCl2, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM DTT, 0.25 mg/ml poly(dI-dC), 50 mM Tris-Cl (pH 7.5), 10 μg of nuclear extracts, and sterile water to a total volume of 10 μl. Incubations were performed at room temperature for 20 min by adding 1 μl of probe (106 cpm) after a 10-min preincubation. The specificity of DNA/protein binding was determined through competition reactions using a 10-fold molar excess of unlabeled oligonucleotides. Samples were loaded onto 5% polyacrylamide gels at 100 V. After electrophoresis, the gels were removed, dried, and autoradiographed.

FIGURE 1. Pin1 induction in the ankle tissues from CII-induced arthritic mice. A, Immunohistochemistry analysis of Pin1 and COX-2 in the ankle tissues (×200). Tissue sections from the left hind ankles were used for the immunostaining. The brown color staining represents Pin1 and COX-2 expression. Positive immunohistochemical reaction was identified in lymphocytes (arrows), fibroblasts (open asterisks), and chondrocytes (closed asterisks) in the inflamed and destructed ankle joint. Counterstaining was performed by hematoxylin. B, Immunoblot of Pin1 and COX-2 in the ankle tissues from CII-induced arthritic mice. The left hind ankles were homogenized in PBS and centrifuged at 10,000 × g. The supernatants were subjected to immunoblotting. Each lane was loaded with 20 μg of the tissue lysates. Equal protein loading was verified using tubulin an internal standard. The results were confirmed by four separate experiments.
Construction of a COX-2 promoter-luciferase construct and reporter gene assays

To determine the transcriptional activity of the COX-2 gene, we used the pGL-COX-2-574 luciferase reporter gene. To construct the luciferase (LUC) reporter gene plasmid, COX-2-LUC(−574/55000), a DNA fragment containing −574 bp of 5′-flanking sequences and 113 bp of 5′-untranslated region from the human COX-2 gene, was first amplified by PCR using a human genomic clone as the template. The PCR fragment was then cloned into pGL3-Basic (Promega). Site-directed mutagenesis of NF-κB, cAMP response element (CRE)/AP-1, and NF-IL-6/CEBP binding sites was performed using a LAPCR in vitro mutagenesis kit (Takara Shuzo) (22). A total of 1 μg of the plasmid was transfected into the cells using Lipo-2000amine (Invitrogen) or Hilymax reagent (Dojindo Molecular Technologies), according to the manufacturer’s instructions. After 6 h, the transfection medium was replaced with the basal culture medium without serum and the cells were further incubated for 18 h. The luciferase activities in the cell lysates were then measured using a luminometer. The relative luciferase activity was calculated by normalizing the promoter-driven luciferase activity vs human Renilla luciferase or β-galactosidase.

RT-PCR

The total RNA was isolated using a total RNA isolation kit (RNAgents; Promega). The total RNA (1.0 μg) obtained from the cells was reverse transcribed using an oligo(dT) 18 mer as a primer and Moloney murine leukemia virus reverse transcriptase (Bioneer) to produce the cDNAs. PCR was performed using the selective primers for human TNF-α, IL-1β, IFN-γ, and S16 ribosomal protein genes. The PCRs were performed for 42 cycles using the following conditions: denaturation at 98°C for 10 s, annealing at 62°C and 50°C for IL-1β and TNF-α, respectively, for 0.5 min, and elongation at 72°C for 1 min. The band intensities of the amplified DNAs were compared after visualization using FLA-7000 (Fuji film).

ELISA

Commercial ELISA kits (Cayman Chemical and BD Biosciences) were used to determine PGE2 and IL-4 concentrations in culture medium, according to the manufacturer’s protocols.

Statistics

Paired Student’s t test was used to determine the significance of differences between treatment groups. Statistical significance was accepted for p values of <0.05.

Results

Pin1 induction in arthritic tissues

Pin1 contributes to degenerative disease in brain (13). To determine whether Pin1 is chronically expressed in arthritic lesions, we

FIGURE 2. Induction of proinflammatory mediators in Pin1-overexpressed chondrocytes. A, COX-2 induction in Pin1-overexpressed HTB-94 cells. A representative immunoblot shows COX-2 and Pin1 proteins in both GFP-HTB-94 and Pin1-HTB-94 cells. Each lane was loaded with 15 μg of protein. Equal protein loadings were verified using actin as an internal standard. Each lane represents different sample. The results were confirmed by two separate experiments. B, PGE2 and IL-4 production in Pin1-overexpressed HTB-94 cells. GFP-HTB-94 and Pin1-HTB-94 cells were incubated with serum-free medium for 24 h, and amounts of PGE2 and IL-4 in medium were determined using ELISA. The results shown represent the means ± SD of six (PGE2) and five (IL-4) different samples (significant as compared with GFP-HTB-94 cells; *, p < 0.05; **, p < 0.01). C, Induction of iNOS, TNF-α, IL-1β, and IFN-γ in Pin1-overexpressed HTB-94 cells. Left panel, iNOS was determined by Western blottings using specific Ab, and each lane was loaded with 15 μg of protein. Right panel, The mRNA expression levels of TNF-α, IL-1β, and IFN-γ were determined by RT-PCR analyses. S16 rRNA protein mRNA was determined as loading control. The results were confirmed by two separate experiments. D, Induction of COX-2 in Pin1-overexpressed primary cultured chondrocytes isolated from osteoarthritic patient. Each lane represents different sample. The results were confirmed by two separate experiments.
determined Pin1 levels by immunohistochemistry in the ankle tissues from CII-induced RA mice. Hind paw swelling and erythema were increased in all mice injected with CII (data not shown). RA tissues showed higher levels of Pin1 induction, mainly distributed in chondrocytes, lymphocytes, and fibroblasts (Fig. 1A, upper). COX-2 is highly expressed in human and animal arthritic tissues (6), and COX-2 staining showed similar patterns to Pin1 staining (Fig. 1A, lower). Western blot analysis also showed concomitant expression of COX-2 and Pin1 in RA tissue homogenates, but not in control (Fig. 1B).

**Role of Pin1 in proinflammatory protein expression**

To clarify the phenotypes of Pin1 overexpression in RA tissues, we established stably Pin1-overexpressing HTB-94 cells, a human chondrocyte cell line (Pin1-HTB-94) using retroviral infections. Pin1-HTB-94 cells had higher Pin1 levels than GFP-HTB-94 (GFP-overexpressing) cells (Fig. 2A). Western blot analysis showed that COX-2 expression was up-regulated in Pin1-HTB-94 cells (Fig. 2A). Production of PGE₂, a stable autacoid produced by COX-2, was 4.3-fold higher in Pin1-HTB-94 cells (Fig. 2B). Moreover, Pin1-HTB-94 cells showed higher protein or mRNA levels of iNOS, TNF-α, and IL-1β, representative proinflammatory enzymes and cytokines (Fig. 2C). It has been shown that Pin1 knockout mice show both Th1 and Th2 abnormalities (23). Hence, we additionally determined the mRNA expression of IFN-γ (Th1 dependent) and IL-4 production (Th2 dependent) in both GFP-HTB-94 and Pin1-HTB94 cells. Although IL-4 protein levels were slightly reduced (Fig. 2B), IFN-γ mRNA levels were enhanced in Pin1-overexpressing cells (Fig. 2C). These results were consistent with the previous reports showing Pin1 inhibition selectively suppressed IFN-γ expression in bronchoalveolar lavage cells (23, 24). We then established Pin1-overexpressing human primary chondrocytes using ankle tissues obtained during surgery in an osteoarthritic patient. Although primary chondrocytes showed basal levels of Pin1 and COX-2, stable Pin1 overexpression increased COX-2 expression as well (Fig. 2D).

**Pin1-dependent, simultaneous activation of NF-κB, CREB, and C/EBP is required for COX-2 expression**

Because COX-2-mediated PG production is a representative inflammation index in RA, we chose COX-2 gene expression as a model system for additional experiments. COX-2 expression is transcriptionally regulated by C/EBP, CREB, and NF-κB, either synergistically or independently (25, 26). We therefore transfected GFP-HTB-94 and Pin1-HTB-94 cells with either a wild-type COX-2 promoter-luciferase chimeric construct that contained the 574-bp 5′-flanking region of human COX-2 gene, a C/EBP mutant with an NF-IL-6 site (−132/−124) mutation, an NF-κB mutant (−223/−214), or a CRE/AP-1 mutant (−59/−53) (27). Wild-type COX-2 promoter activity was ~5-fold higher in Pin1-HTB-94 cells than GFP-HTB-94 cells (Fig. 3A). Mutants in each binding site (C/EBP, NF-κB, or CRE/AP-1) showed significantly lower Pin1-inducible reporter activity (Fig. 3A), particularly for NF-κB and CRE/AP-1 mutants (83 and 97% inhibition, respectively) (Fig. 3A). These results demonstrate that C/EBP, NF-κB, and CRE/AP-1 elements are all required for Pin1-mediated trans activation of COX-2.

The COX-2 promoter contains two NF-κB consensus sequences, and Pin1-inducible proinflammatory genes, including iNOS, IL-1β, and TNF-α, require NF-κB activation (28–30). The NF-κB minimal reporter activity and nuclear p65 levels were higher in Pin1 cells (Fig. 3B). Tosyl phenylalanyl chloromethyl ketone a specific NF-κB inhibitor, suppressed Pin1-mediated COX-2 expression (Fig. 3C). Pin1 selectively increased nuclear p65 sequestration by inhibiting p65 binding to I-κBα (31). Hence, Pin1-mediated induction of proinflammatory cytokines could result from p65/NF-κB activation.

![FIGURE 3. Role of NF-κB activation in Pin1-inducible COX-2 expression. A. Induction of luciferase activity by Pin1 overexpression in HTB-94 cells. Reporter activities in GFP-HTB-94 and Pin1-HTB-94 cells transiently transfected with pGL-COX-2-574, NF-κB mutant, C/EBP mutant, or CRE/AP-1 mutant construct were confirmed using a luminometer. Reporter gene activations were expressed as changes relative to human Renilla luciferase activity. The results shown represent the means ± SD of four separate samples (significant as compared with the pGL-COX-2-574-transfected GFP-HTB-94 cells; **, p < 0.01; significant as compared with the pGL-COX-2-574-transfected Pin1-HTB-94 cells; ##, p < 0.01). The results were confirmed by two separate experiments. B. NF-κB activation in Pin1-overexpressed chondrocytes. Upper panel. Nuclear levels of p65. Each lane represents different sample. Lower panel. NF-κB reporter gene analysis. HTB-94 cells were transfected with pNF-κB-Luc plasmid, and reporter gene analysis was performed, as described in the legend of A. Data represent the means ± SD of eight separate samples (significant as compared with GFP-HTB-94 cells; **, p < 0.01). The results were confirmed by two separate experiments. C. Role of MAPKs and NF-κB in COX-2 expression in Pin1-HTB-94 cells. Pin1-HTB-94 cells were incubated with PD98059 (an ERK inhibitor, 30 μM), SB203580 (a p38 kinase inhibitor, 20 μM), SP600125 (a JNK inhibitor, 20 μM), and Tosyl phenylalanyl chloromethyl ketone (a NF-κB inhibitor, 50 μM) for 36 h, and total cell lysates were subjected to immunoblotting.](http://www.jimmunol.org/ by guest on October 30, 2017)
FIGURE 4. Role of CREB and C/EBP activation in Pin1-inducible COX-2 expression. A, CREB activation in Pin1-overexpressed chondrocytes. Upper panel, Nuclear levels of CREB. Each lane represents different sample. Lower panel, CRE reporter gene analysis. HTB-94 cells were transfected with pCRE-Luc plasmid, and reporter gene analysis was performed, as described in the legend of Fig. 3A. Data represent the means ± SD of four separate samples (significant as compared with GFP-HTB-94 cells; **, p < 0.01). The results were confirmed by two separate experiments. B, C/EBP activation in Pin1-overexpressed chondrocytes. Upper panel, Nuclear levels of C/EBPα and C/EBPβ. Lower panel, C/EBP reporter gene analysis. HTB-94 cells were transfected with pC/EBP-Luc plasmid, and reporter gene analysis was performed, as described in the legend of Fig. 3A. Data represent the means ± SD of 10 separate samples (significant as compared with GFP-HTB-94 cells; **, p < 0.01). The results were confirmed by two separate experiments. C, AP-1 activation in Pin1-overexpressed chondrocytes. Left upper panel, Nuclear levels of c-Jun, JunB, JunD, c-Fos, and Fra1. Left lower panel, AP-1 reporter gene analysis. HTB-94 cells were transfected with pAP-1-Luc plasmid, and reporter gene analysis was performed, as described in the legend of Fig. 3A. Data represent the means ± SD of eight separate samples (significant as compared with GFP-HTB-94 cells; **, p < 0.01). The results were confirmed by two separate experiments. Right panel, AP-1 DNA-binding activity. Nuclear fractions were isolated from GFP-HTB-94 and Pin1-HTB-94 cells serum deprived for 18 h. All lanes contained 10 μg of nuclear extracts and radiolabeled putative AP-1 consensus sequence. D, Effects of c-Jun siRNA on the reporter activities of AP-1 and COX-2 promoter in Pin1-overexpressed chondrocytes. GFP-HTB-94 and Pin1-HTB-94 cells were cotransfected with pAP-1-luc or pGL-COX-2-574 reporter plasmid in combination with c-Jun siRNA (c-Jun siR, 20 pmol) or control siRNA (Con siR, 20 pmol). Data represent the means ± SD of three separate samples (significant as compared with GFP-HTB-94 cells, **, p < 0.01; significant as compared with control siRNA-transfected Pin1-HTB-94 cells, #, p < 0.05). The results were confirmed by two separate experiments. E, MAPK phosphorylation in Pin1-overexpressed chondrocytes. The activations of ERK, p38 kinase, and JNK were assessed by immunoblotting the phosphorylated forms of three MAPKs.
Because C/EBP and CRE/AP-1 elements regulate cytokine or UV B-inducible COX-2 gene transcription (32), we determined the activity of each transcription factor using minimal reporter genes. pCRE-Luc and pC/EBP-Luc activities were enhanced by 13.7- and 4.2-fold in Pin1-HTB-94 cells (Fig. 4, A and B). Nuclear levels of CREB were also sharply increased in Pin1-HTB-94 cells (Fig. 4, A and B), but C/EBP/H9251 and C/EBP/H9252 increased only marginally (Fig. 4, B). c-Jun and c-Fos are cis/trans isomerized by Pin1 to activate AP-1-dependent gene transcription after phosphorylation by MAPK (33, 34). c-Jun activation by platelet-derived growth factor or serum increases COX-2 protein levels via CRE/AP-1 (35). In this study, Pin1 increased AP-1 minimal reporter activity, AP-1-binding activity, and the nuclear distribution of c-Jun, c-Fos, and Fra1, but not nuclear levels of JunB and JunD (Fig. 4C). AP-1 reporter activity was almost completely suppressed by c-Jun small interfering RNA (siRNA). However, c-Jun siRNA failed to reduce wild-type COX-2 promoter activity, indicating that increased COX-2 expression by Pin1 may not be related to AP-1 (Fig. 4D). c-Jun/AP-1 phosphorylation and expression are controlled by MAPK (36), which also regulates COX-2 expression (37, 38). To study whether MAPK pathways are activated in Pin1 cells, we measured the phosphorylated form of each MAPK. Pin1 cells had higher levels of phosphorylated ERK, but not JNK or p38 kinase (Fig. 4E). However, incubation of Pin1 cells with specific MAPK inhibitors (PD98059, ERK inhibitor; SB203580, p38 kinase inhibitor; SP600125, JNK inhibitor) for 36 h did not change COX-2 protein levels (Fig. 3C). Thus,
NF-κB, C/EBP, and CREB, but not AP-1, affect Pin1-dependent COX-2 expression in chondrocytes.

Juglone inhibits RA progress in CII-inducible DBA/1J mice and suppresses COX-2 expression in human primary cultured RA chondrocytes

Next, we tested the effect of the Pin1 inhibitor, juglone, on CII-induced RA in DBA/1J mice. The compound covalently inacti- vates a cysteine residue in the active site of Pin1 isomerase (39). Intraperitoneal juglone injection was done every other day after a booster injection of CII and continued for 9 days (total four times). As shown in Fig. 5A, Softex x-ray images showed that CII-induced hind paw swelling was almost completely blocked in juglone-injected mice. Ankle edema score was also reduced in juglone-treated group (Table I). We further histopathologically evaluated ankle joints for RA grades by the severity of inflammation, fibrosis, damage to the articular cartilage and bone, and ankylosis after sacrificing the mice (Fig. 5B and Table I). Juglone treatment (1, 5, and 10 mg/kg) also significantly inhibited the histological damage and cumulative arthritis injury scores compared with the vehicle-treated CII-RA group (Table I). Moreover, juglone treatment reduced COX-2 expression in the RA tissues (Fig. 5C).

To determine whether Pin1 inhibition decreases proinflammatory mediators in human chondrocytes from RA patients, we determined COX-2 protein levels in primary cultured chondrocytes from a RA patient. Juglone treatment for 24 h dose dependently reduced basal COX-2 expression in these chondrocytes (Fig. 5D). These results imply that Pin1 could be a pharmacological target for RA.

Discussion

Although RA is a common inflammatory disease, the molecular pathogenesis of this disease is still unclear. Data presented in this study indicate that Pin1 expression increases in the lesion area of CII-mediated arthritis and plays a crucial role in the excess production of proinflammatory mediators, including PGs, NO, TNF-α, and IL-1β.

NF-κB, which forms a homo- or heterodimer complex, regulates inflammatory genes such as COX-2, iNOS, TNF-α, and IL-1β (40), and many phytochemicals that inhibit NF-κB can reduce RA in animal and human studies (41, 42). In this study, stable Pin1 overexpression caused both the sustained nuclear translocation of p65 and increased NF-κB transcription. Pin1 binds to the phosphorylated Th256-Pro of p65 and subsequently inhibits I-κBα binding, which increases the nuclear localization and the activity of NF-κB (31). We also revealed that COX-2 gene expression and its promoter activity were dependent on NF-κB activity. Thus, NF-κB activation in Pin1-overexpressed chondrocytes is obviously associated with the overwhelmed production of proinflammatory mediators during RA progress.

COX-2 and iNOS expression are also transcriptionally regulated by C/EBP and CREB as well as NF-κB, and these transcription factors may be synergistically or independently involved in the expression of this gene expression (26, 43, 44). In this study, CRE and C/EBPs were consistently activated by Pin1, and these transcription factors are active to increase COX-2 expression in Pin1-overexpressed chondrocytes. In silico analysis revealed that CREB variants contain Ser/Thr-Pro-rich segments and speculated that consensus phosphorylation sequences for Pin1 or Polo-like kinase were located in a highly conserved region of trans activation domains of C/EBP (45). Although there is still no report showing that transcriptional activity of CREB is dependent on Pin1, the isomerase may control the transcriptional activity of C/EBP and CREB.

Pin1 isomerizes phosphorylated c-Jun/c-Fos to activate AP-1 (33, 34). Pin1-overexpressed chondrocyte cell line showed higher nuclear levels of c-Jun, c-Fos, and Fra1, but not JunB and JunD. c-Jun siRNA blocked Pin1-induced AP-1 promoter activity, indicating that c-Jun/AP-1 activation is not essential for COX-2 expression. Several reports have shown that MAPKs, ERK, JNK, and p38 kinase regulate COX-2 expression through the regulation of NF-κB, C/EBP, or CREB (21, 37, 46).

Possible role of Pin1 induction in RA. Pin1 is overexpressed in chondrocytes, fibroblasts, and lymphocytes during RA development. The increased Pin1 activity persistently activates NF-κB, C/EBP, CREB, and c-Jun. The increased NF-κB, C/EBP, and CREB synergistically activate the transcription of COX-2 gene and finally produce PGs. Production of other proinflammatory mediators, including TNF-α, IL-1β, and NO, is also enhanced by Pin1 presumably by simultaneous activation of NF-κB, C/EBP, CREB, and c-Jun/AP-1. These findings suggest that Pin1 is a potential therapeutic target of RA.

**FIGURE 6.** Possible role of Pin1 induction in RA. Pin1 is overexpressed in chondrocytes, fibroblasts, and lymphocytes during RA development. The increased Pin1 activity persistently activates NF-κB, C/EBP, CREB, and c-Jun. The increased NF-κB, C/EBP, and CREB synergistically activate the transcription of COX-2 gene and finally produce PGs. Production of other proinflammatory mediators, including TNF-α, IL-1β, and NO, is also enhanced by Pin1 presumably by simultaneous activation of NF-κB, C/EBP, CREB, and c-Jun/AP-1. These findings suggest that Pin1 is a potential therapeutic target of RA.

**Table I. Effect of juglone treatments on CII-induced RA**

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<th>Group</th>
<th>Ankle Edema Score</th>
<th>Chronic Inflammation</th>
<th>Ankylosis</th>
<th>Fibrosis</th>
<th>Articular Cartilage Loss</th>
<th>Synovialis Proliferation</th>
<th>Bone Damage</th>
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<td>CII</td>
<td>2.11 ± 0.93</td>
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<td>1.89 ± 1.36</td>
<td>2.33 ± 0.87</td>
<td>2.00 ± 1.32</td>
<td>2.44 ± 1.13</td>
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<td>+ Juglone 1 mg/kg</td>
<td>1.29 ± 0.95</td>
<td>6 of 7</td>
<td>0.86 ± 1.21</td>
<td>1.29 ± 0.95*</td>
<td>0.71 ± 0.95*</td>
<td>1.00 ± 0.82**</td>
<td>0.71 ± 0.76*</td>
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<td>0.43 ± 0.53**</td>
<td>1.43 ± 0.79**</td>
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<td>+ Juglone 10 mg/kg</td>
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<td>0.75 ± 1.50</td>
<td>0.75 ± 0.96**</td>
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* Data represent the means ± SD of four to nine samples (significant as compared to CII-treated group; *, p < 0.05; **, p < 0.01).
Abnormal Pin1 activity contributes to the pathogenesis of various diseases, including cancer, Alzheimer’s disease, asthma, and allergies (24, 46, 47). We found in this study for the first time that Pin1 may also be a suitable target for RA. In fact, anti-cancer agents such as methotrexate and azathioprine are used for the treatment of RA (48). Pin1 inhibition has anti-cancer effects in experimental studies (49, 50), and recently, diverse chemical-based or peptide-based Pin1 inhibitors have been synthesized (51, 52). We found that multiple treatments of 10 mg/kg juglone caused severe toxic effects in mice, such as paralysis and even death (three among seven mice). A recent study also revealed that juglone inhibited postmitotic dephosphorylation and mitotic exit in a Pin1-independent manner, and consequently caused failure of mitotic spindle assembly (53). Because higher concentration ranges of juglone are cytotoxic, as evidenced by our animal study, we believe that juglone cannot be clinically used for RA. However, if more safe Pin1 inhibitors are available, the compounds could be applied for RA treatment.

Taken together, Pin1 is up-regulated in chondrocytes, lymphocytes, and fibroblasts of RA lesions in CIA-injected RA mice, and Pin1 overexpression induces proinflammatory proteins, including COX-2, iNOS, TNF-α, and IL-1β. Pin1-dependent COX-2 expression requires the simultaneous activation of NF-κB, p38 kinase, and ERK. Pin1-induced COX-2 expression is essential for regulation of mitosis.

Disclosures

The authors have no financial conflict of interest.

References


