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HMGB-1 Induces IL-6 Production in Human Synovial Fibroblasts Through c-Src, Akt and NF-κB Pathways

CHUN-HAN HOU,[1,2] YI-CHIN FONG,[3,4] AND CHIH-HSIN TANG[5,6]*

[1] Department of Orthopedic Surgery, National Taiwan University Hospital, Taipei, Taiwan

[2] Institute of Biomedical Engineering, College of Medicine and College of Engineering, National Taiwan University, Taipei, Taiwan

[3] School of Chinese Medicine, China Medical University, Taichung, Taiwan

[4] Department of Orthopaedics, China Medical University Hospital, Taichung, Taiwan

[5] Department of Pharmacology, School of Medicine, China Medical University and Hospital, Taichung Taiwan

[6] Graduate Institute of Basic Medical Science, China Medical University and Hospital, Taichung, Taiwan

High mobility group box chromosomal protein 1 (HMGB-1) is a widely studied, ubiquitous nuclear protein that is present in eukaryotic cells, and plays a crucial role in inflammatory response. However, the effects of HMGB-1 on human synovial fibroblasts are largely unknown. In this study, we investigated the intracellular signaling pathway involved in HMGB-1-induced IL-6 production in human synovial fibroblast cells. HMGB-1 caused concentration- and time-dependent increases in IL-6 production. HMGB-1-mediated IL-6 production was attenuated by receptor for advanced glycation end products (RAGE) monoclonal antibody (Ab) or siRNA. Pretreatment with c-Src inhibitor (PP2), Akt inhibitor and NF-κB inhibitor (pyrrolidine dithiocarbamate and L-1-tosylamido-2-phenylenylethyl chloromethyl ketone) also inhibited the potentiating action of HMGB-1. Stimulation of cells with HMGB-1 increased the c-Src and Akt phosphorylation. HMGB-1 increased the accumulation of p-p65 in the nucleus, as well as NF-κB luciferase activity. HMGB-1-mediated increase of NF-κB luciferase activity was inhibited by RAGE Ab, PP2 and Akt inhibitor or RAGE siRNA, or c-Src and Akt mutant. Our results suggest that HMGB-1-increased IL-6 production in human synovial fibroblasts via the RAGE receptor, c-Src, Akt, p65, and NF-κB signaling pathways.
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Osteoarthritis (OA) is a chronic joint disorder characterized by a slow progressive degeneration of articular cartilage, subchondral bone alteration, and variable secondary synovial inflammation. The exact etiology of OA is not well understood (Clouet et al., 2009). In response to the proinflammatory cytokines produced by macrophages, such as interleukin-1β and tumor necrosis factor-αTNF-α, osteoarthritis synovial fibroblasts (OASF) produce chemokines that promote inflammation, neovascularization, and cartilage degradation via activation of matrix-degrading enzymes, such as matrix metalloproteinases (MMPs) (Mor et al., 2005). Diagnosis of the disease and the progression of joint damage are mainly based on evaluation of clinical and radiological findings. Molecular markers can serve as promising indicators for OA evaluation because they can provide more direct information about the local inflammation, the alterations in joint tissues, and related bone and cartilage turnover (Wright et al., 2009).

IL-6 is a multifunctional cytokine that plays a central role in both innate and acquired immune responses. It is the predominant mediator of the acute phase response, an innate immune mechanism which is triggered by infection and inflammation (Graeve et al., 1993; Grimble, 1998). In addition to these roles in pathogen-specific inflammation and immunity, IL-6 levels are elevated in chronic inflammatory conditions, such as rheumatoid arthritis (RA) (Jones, 2005; Yokota et al., 2006). Several consensus sequences, including those for NF-κB, CREB, NF-IL-6, and AP-1 in the 5′-promoter region of the IL-6 gene, have been identified as regulatory sequences that induce IL-6 in response to various stimuli (Matsusaka et al., 1993; Grassl et al., 1999). NF-κB, a key transcription factor that regulates IL-6 expression, is a dimer of either transcription factor p65 or transcription factor p50. In a resting state, this dimer is associated with IκBs to retain NF-κB in the cytosol. IκB kinase, which is activated through stimulation by cytokines and bacterial products, phosphorylates IκBα at Ser (32) and Ser (36), and IκBα at Ser (19) and Ser (23), to produce ubiquitination of IκBα/β at lysine residues and degradation by the 26S proteasome (Chen et al., 1995; Maniatis, 1997).

High mobility group box chromosomal protein 1 (HMGB-1) is a widely studied, ubiquitous nuclear protein that is present in eukaryotic cells (Mosevitsky et al., 1989; Bustin et al., 1990). As a nuclear protein, HMGB-1 stabilizes nucleosomes and enables nicking of DNA, which facilitates gene transcription (Bustin, 1999). It has been identified as a mediator of endotoxin-induced lethality (Taniguchi et al., 2003) and a causative factor in arthritis (Pullerits et al., 2003), acting, at least in part, as a proinflammatory cytokine (Schmidt et al., 2001). Engagement of the receptor for advanced glycation end products (RAGE) by extracellular HMGB-1 triggers activation of proinflammatory signaling pathways (Schmidt et al., 2001; Sunahori et al., 2006), such as those resulting in elaboration of reactive oxygen

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*Correspondence to: Chih-Hsin Tang, Department of Pharmacology, School of Medicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung, Taiwan.
E-mail: chtang@mail.cmu.edu.tw

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intermediates and activation of NF-κB (Abeyama et al., 2000; Schmidt et al., 2001; Okamoto et al., 2008). Results of some studies have also suggested that the mode of cell injury might regulate the immune and inflammatory response, through an apoptosis-mediated anti-inflammatory mechanism and/or a necrosis-associated proinflammatory mechanism (Gallucci et al., 1999).

Previous studies have shown that HMGB-1 promotes inflammatory response (Taniguchi et al., 2003; Sunahori et al., 2006). Although a role for HMGB-1 in IL-6 induction has been implied for some cell types, the signaling pathway for HMGB-1 in IL-6 production in synovial fibroblasts has not been extensively studied. In the present study, we explored the intracellular signaling pathway involved in HMGB-1-induced IL-6 production in human synovial fibroblast cells. The results showed that HMGB-1 activates RAGE, c-Src, Akt, and NF-κB pathways, leading to up-regulation of IL-6 expression.

Materials and Methods

Materials

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for β-actin, c-Src, Akt, p-Akt, p65, p-p65, lamin B, and the small interfering RNAs (siRNAs) against RAGE, p65 and control for experiments using targeted siRNA transfection (each consists of a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal Ab-specific for c-Src phosphorylated at Tyr^{416} was purchased from Cell Signaling and Neuroscience (Danvers, MA). Mouse monoclonal Ab-specific for RAGE was purchased from R&D Systems (Minneapolis, MN). Pyrrolidine dithiocarbamate (PDTC), L-1-tosylamido-2-phenylenylethyl chloromethyl ketone (TPCK), PP2, and Akt inhibitor were purchased from Calbiochem (San Diego, CA). IL-6 enzyme immunoassay kit was purchased from Cayman Chemical (Ann Arbor, MI). The recombinant human HMGB-1 was purchased from PeproTech (Rocky Hill, NJ). The NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA). The c-Src dominant negative mutant was a gift from Dr. S. Parsons (University of Virginia Health System, Charlottesville, VA). The Akt (Akt K179A) dominant negative mutant was a gift from Dr. W.M. Fu (National Taiwan University, Taipei, Taiwan). The human IL-6 promoter construct pIL6-luc651(−651/+1), AP-1 site mutation (pIL6-luc651ΔAP1), NF-κB site mutation (pIL6-luc651ΔNF-κB), and C/EBP-β site mutation (pIL6-luc651ΔC/EBP-β) were gifts from

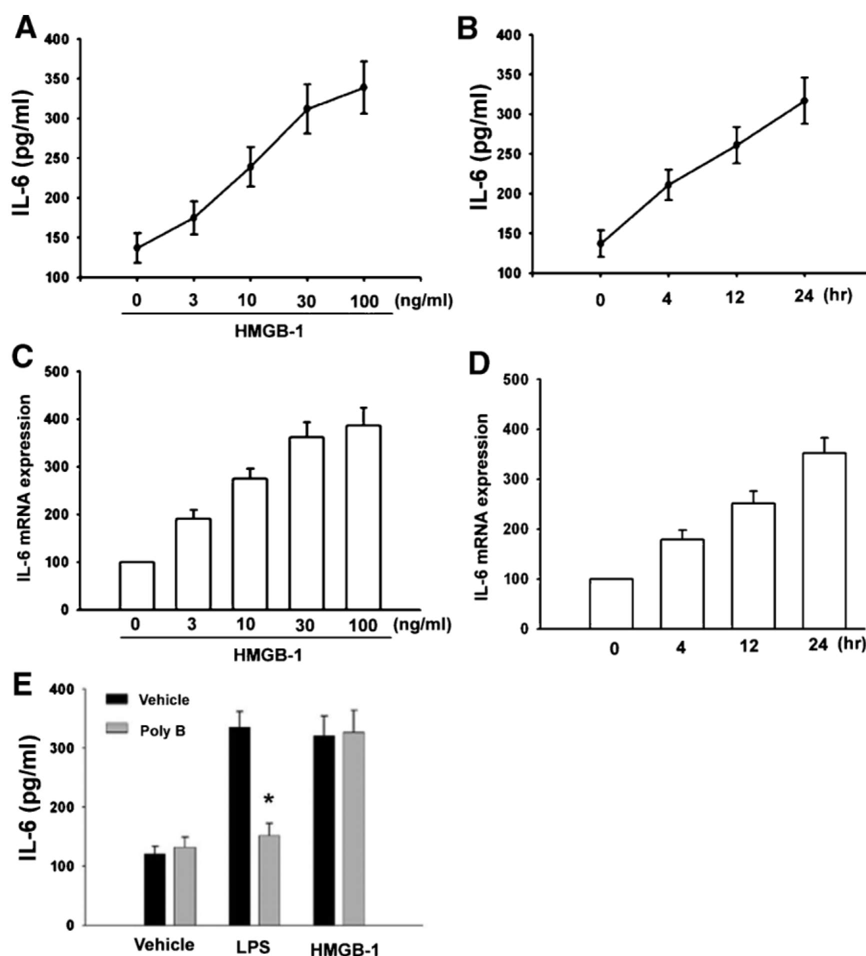

Fig. 1. Concentration- and time-dependent increases in IL-6 production by HMGB-1. OASF were incubated with various concentrations of HMGB-1 for 24 h (A) or with HMGB-1 (30 ng/ml) for 4, 12, or 24 h (B). Media were collected to measure IL-6. Results are expressed of four independent experiments performed in triplicate. OASF were incubated with various concentrations of HMGB-1 for 24 h (C) or with HMGB-1 (30 ng/ml) for 4, 12, or 24 h (D), the mRNA expression of IL-6 was examined by qPCR. E: OASF cells were pretreated with polymyxin B (poly B, 1 μM) for 30 min followed by stimulation with LPS (1 μM) or HMGB-1 (30 ng/ml) for 24 h. Media were collected to measure IL-6. Results are expressed of four independent experiments performed in triplicate. *$P < 0.05$ as compared with LPS or HMGB-1-treated group.

Dr. Oliver Eickelberg (Ludwig Maximilians University Munich, Munich, Germany). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

Cell cultures

Human synovial fibroblasts were isolated using collagenase treatment from synovial tissues obtained from knee replacement surgeries of 10 patients with OA after approval by the local ethics committee. Fresh synovial tissues were minced and digested in a solution of collagenase and DNase. Isolated fibroblasts were filtered through 70-μm nylon filters. The cells were grown on plastic cell culture dishes in 95% air–5% CO_2 with RPMI 1640 (Life Technologies, Grand Island, NY) that was supplemented with 20 mM HEPES and 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (pH adjusted to 7.6). Fibroblasts from passages four to nine were used for the experiments (Tang et al., 2007; Chiu et al., 2009). The cells from different patients were treated with HMGB-1 separately. All studies carried out on cells from least four patients. Results are expressed of four independent experiments (n = 4).

Primary cultures of human chondrocytes were isolated from articular cartilage as previously described (Hsu et al., 2007). Human articular chondrocytes were isolated from resected cartilage specimens obtained from undergoing primary total knee arthroplasty. Cartilage pieces were minced finely, and chondrocytes were isolated by sequential enzymatic digestion at 37°C with 0.1% hyaluronidase for 30 min and 0.2% collagenase for 1 h. Isolated chondrocytes were filtered through 70 μM nylon filters. The cells were grown on the plastic cell culture dishes in 95% air–5% CO_2 with Dulbecco's modified Eagle's medium (DMED; Gibco, Grand Island, NY) which was supplemented with 20 mM HEPES and 10% heat-inactivated FBS, 2-mM-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (pH adjusted to 7.6).

Measurements of IL-6 production

Human synovial fibroblasts were cultured in 24-well culture plates. After reaching confluence, cells were treated with HMGB-1, and then incubated in a humidified incubator at 37°C for 24 h. For examination of the downstream signaling pathways involved in HMGB-1 treatment, cells were pretreated with various inhibitors for 30 min before HMGB-1 (30 ng/ml) administration. After incubation, the medium was removed and stored at −80°C until assay. IL-6 in the medium was assayed using the IL-6 enzyme immunoassay kits, according to the procedure described by the manufacturer (Tang et al., 2007; Chiu et al., 2009).

Quantitative real-time PCR

Total RNA was extracted from synovial fibroblasts using a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA using oligo(dT) primer. The 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 per 25-μl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially (β-actin was used as internal control) (Applied Biosystems).

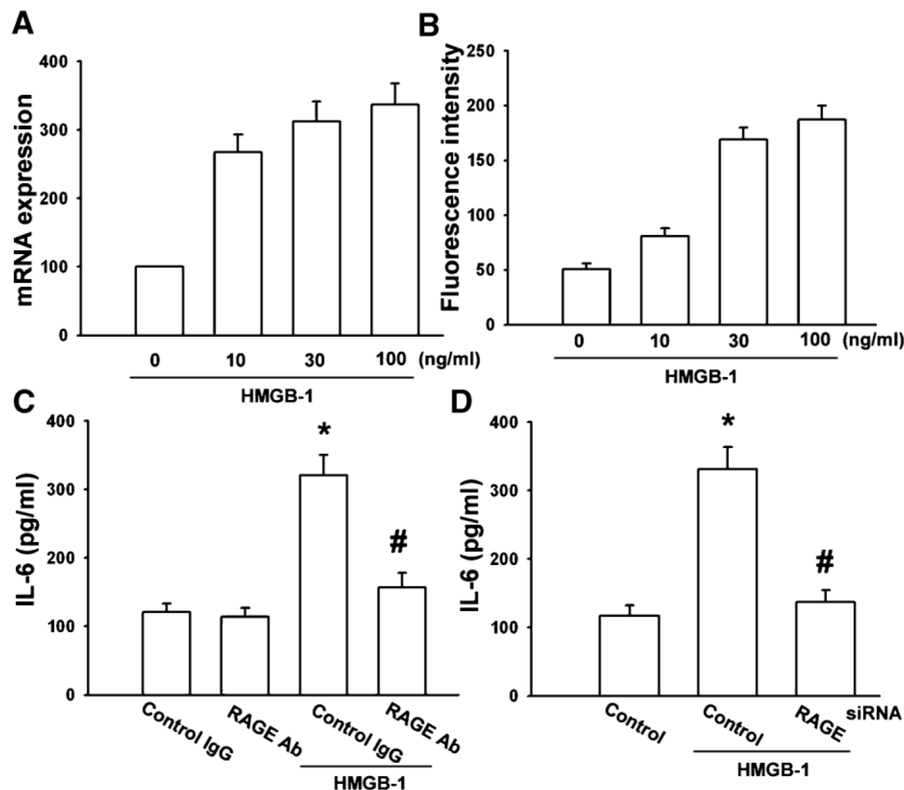

Fig. 2. Involvement of RAGE receptor in HMGB-1-mediated IL-6 production in synovial fibroblasts. A: OASF cells were incubated with HMGB-1 (30 ng/ml) for 24 h, and the mRNA expression of RAGE was examined by qPCR. B: OASF cells were incubated with HMGB-1 (30 ng/ml) for 24 h, and the cell surface RAGE expression was examined by flow cytometry. Cells were pretreated for 30 min with RAGE Ab (C) or transfected with RAGE siRNA (D) for 24 h followed by stimulation with HMGB-1 for 24 h. Media were collected to measure IL-6. Results are expressed of four independent experiments performed in triplicate. *$P < 0.05$ as compared with basal level. #$P < 0.05$ as compared with HMGB-1-treated group.

qPCR assays were carried out in triplicate on an StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 sec and 60°C for 60 sec. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T).

Flow cytometric analysis

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 Ab against RAGE (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) for 45 min and analyzed by flow cytometry using FACS Calibur and CellQuest software (BD Biosciences, San Jose, CA).

Western blot analysis

The cellular lysates were prepared as previously described (Tang et al., 2007; Chiu et al., 2009). Proteins were resolved on SDS-PAGE and transferred to immobilon polyvinyldifluoride membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies against Akt, p-Akt, c-Src or p-c-Src (1:1,000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary Ab (1:1,000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

Kinase activity assay

c-Src activity was assessed by c-Src Kinase Activity Assay Kit (Abnova, Taipei, Taiwan) according to manufacturer's instructions. Kinase activity kit is based on a solid-phase ELISA that uses a specific synthetic peptide as a substrate for c-Src and a polyclonal Ab that recognized the phosphorylated form of the substrate.

siRNA transfection

The siRNA against human RAGE and control siRNA were purchased from Santa Cruz Biotechnology. Cells were transfected with siRNAs (100 nM) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions (Tang et al., 2010b).

Transfection and reporter gene assay

Human synovial fibroblasts were co-transfected with 0.8 μg luciferase plasmid and 0.4 μg β-galactosidase expression vector.

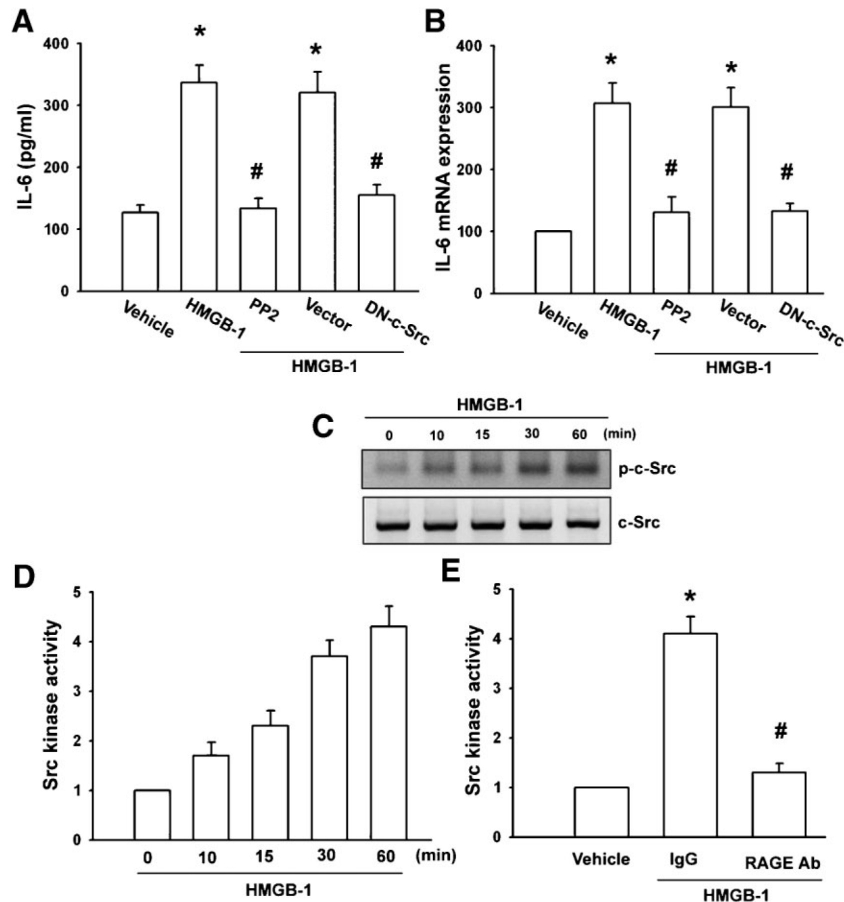

Fig. 3. c-Src is involved in HMGB-1-mediated IL-6 production in synovial fibroblasts. OASF cells were pretreated for 30 min with PP2 (3 μM) or transfected for 24 h with c-Src mutant followed by stimulation with HMGB-1 for 24 h. Total RNA and media were collected, and the expressions of IL-6 were analyzed by qPCR and ELISA (A,B). Cells were incubated with HMGB-1 for indicated time intervals, and c-Src phosphorylation was examined by Western blot (C). Cells were incubated with HMGB-1 for indicated time intervals (D) or pretreated 30 min with RAGE Ab for 30 min, followed by stimulation with HMGB-1 for 60 min, and c-Src kinase activity was determined by the c-Src kinase kit (E). *$P < 0.05$ as compared with control. #$P < 0.05$ as compared with HMGB-1-treated group.

OASF cells were grown to 80% confluence in 12 well plates and were transfected on the following day by Lipofectamine 2000 (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then applied to the cells. After 24 h transfection, the cells were incubated with the indicated agents. After a 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) 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.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation analysis was performed as previously described (Chiu et al., 2009). DNA immunoprecipitated by anti-p65 Ab was purified. The DNA was then extracted with phenol-chloroform. The purified DNA pellet was subjected to PCR. PCR products were then resolved by 1.5% agarose gel electrophoresis and visualized by UV light (Chiu et al., 2009).

Statistics

For statistical evaluation, Mann–Whitney *U*-test for non-Gaussian parameters and Student's *t* test for Gaussian parameters (including Bonferroni correction). The difference is significant if the *P* value is <0.05.

Results

HMGB-1 induces IL-6 production in human synovial fibroblasts

HMGB-1 has been involved in pathology of arthritis (Taniguchi et al., 2003; Steenvoorden et al., 2007). The typical pathology of OA includes chronic inflammation of the synovium, which is characterized by infiltrations of inflammatory cells and synovial hyperplasia, especially fibroblast-like synoviocytes. Therefore, we decided to use human synovial fibroblasts to investigate the signaling pathways of HMGB-1 in the production of IL-6, an inflammatory response gene. Treatment of OASF with HMGB-1 (3–100 ng/ml) for 24 h induced IL-6 production in a concentration-dependent manner (Fig. 1A), and this induction occurred in a time-dependent manner (Fig. 1B). After HMGB-1 (30 ng/ml) treatment for 24 h, the amount of IL-6 released had increased in OASF cells (Fig. 1B). In addition, stimulation of cells with HMGB-1 also increased mRNA expression of IL-6 concentration and time dependently (Fig. 1C,D). To further confirm this stimulation-specific mediation by HMGB-1 without lipopolysaccharide (LPS) contamination, we used polymyxin B, an LPS inhibitor. We found that polymyxin B (1 μM) completely inhibited LPS (1 μM)-induced IL-6 release. However, it had no effect on HMGB-1 (30 ng/ml)-induced IL-6 release in OASF cells (Fig. 1E).

Involvement of RAGE receptor in HMGB-1-mediated increase of IL-6 production

It has been reported that HMGB-1 exerts its effects through interaction with a specific RAGE receptor (Schmidt et al., 2001; Hou et al., 2002). Stimulation of OASF with HMGB-1 increased the mRNA and cell surface expression of RAGE (Fig. 2A,B), suggesting that the amplification loop strengthens the HMGB-1-RAGE-signaling pathway. In addition, treatment of OASF with HMGB-1 also increased protein level of RAGE in celluar and soluble RAGE (sRAGE) in supernatant (Supplementary Fig. 1A,B). Pretreatment of OASF cells with RAGE mAb-reduced HMGB-1-increaed IL-6 production (Fig. 2C). In addition, transfection of cells with RAGE siRNA antagonized HMGB-1-increased IL-6 expression (Fig. 2D). Therefore, an interaction between HMGB-1 and RAGE is very important for IL-6 production in human synovial fibroblasts.

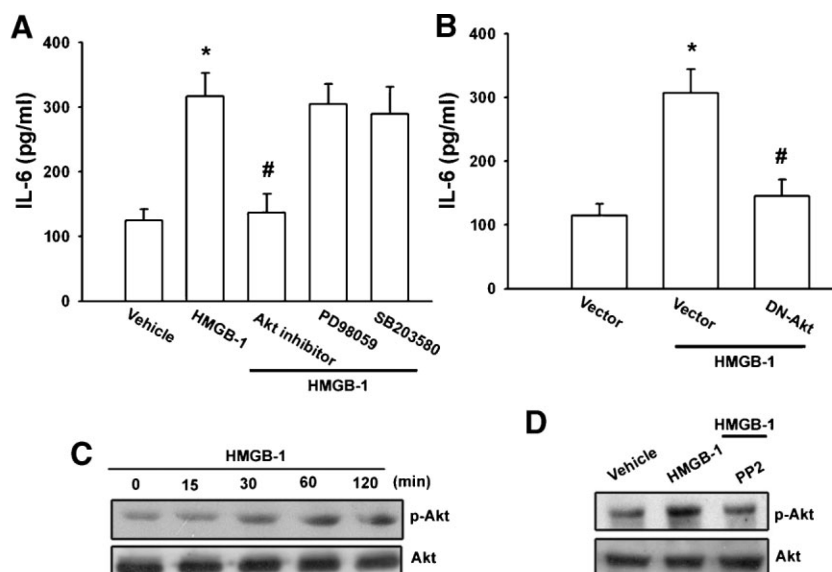

Fig. 4. Akt is involved in the potentiation of IL-6 production by HMGB-1. A,B: OASF cells were pretreated for 30 min with Akt inhibitor (10 μM), SB203580 (10 μM), and PD98059 (30 μM) or transfected for 24 h with Akt mutant followed by stimulation with HMGB-1 for 24 h. Media were collected, and the expressions of IL-6 was analyzed by ELISA. C: Cells were incubated with HMGB-1 for indicated time intervals, and Akt phosphorylation was examined by Western blot. D: Cells were pretreated with PP2 for 30 min then stimulated with HMGB-1 for 60 min, and Akt phosphorylation was examined by Western blot. *$P<0.05$ as compared with control. #$P<0.05$ as compared with HMGB-1-treated group.

Signaling pathway of c-Src and Akt are involved in the potentiating action of HMGB-1

It has been reported that c-Src activation regulates the IL-6 expression in synovial fibroblasts (Tang et al., 2010a). We then investigated the role of Src in mediating HMGB-1-induced IL-6 expression using the specific Src inhibitor PP2. As shown in Fig. 3A,B, HMGB-1-induced IL-6 expression was markedly attenuated by pretreatment of cells for 30 min with PP2 or transfected of cells for 24 h with c-Src mutant. The major phosphorylation site of c-Src at the Tyr^{416} residue results in activation from c-Src autophosphorylation (Roskoski, 2005). To directly confirm the crucial role of Src in IL-6 expression, we measured the level of Src phosphorylation at the Tyr^{416} in response to HMGB-1. As shown in Fig. 3C, treatment of fibroblasts with HMGB-1 resulted in a time-dependent phosphorylation of c-Src at Tyr^{416}. Next, we directly examined c-Src kinase activity in response to HMGB-1. Stimulation of cells with HMGB-1 also increased the kinase activity of c-Src time dependently (Fig. 3D). To determine the relationship among RAGE and c-Src in the HMGB-1-mediated signaling pathway, we found that pretreatment of cells for 30 min with RAGE Ab markedly inhibited the HMGB-1-induced c-Src kinase activity (Fig. 3E). Based on these results, it appears that HMGB-1 acts through RAGE receptor and c-Src-dependent signaling pathway to enhance IL-6 production in human synovial fibroblasts. Previously studies have shown that ERK, Akt, and p38 activation involved RAGE-dependent signaling (Chen et al., 2009; Liu et al., 2010). Pretreatment of cells with Akt inhibitor but not ERK inhibitor (PD98059) and p38 inhibitor (SB203580)-inhibited HMGB-1-increased IL-6 production (Fig. 4A). Therefore, Akt but not ERK and p38 involved in HMGB-1-mediated signaling in synovial fibroblasts. In addition, transfection of cells with Akt mutant also reduced HMGB-1-increased IL-6 production (Fig. 4B). We then directly measured Akt phosphorylation in response to HMGB-1. Stimulation of cells led to a significant increase of phosphorylation of Akt (Fig. 4C). Pretreatment of cells for 30 min with PP2 markedly

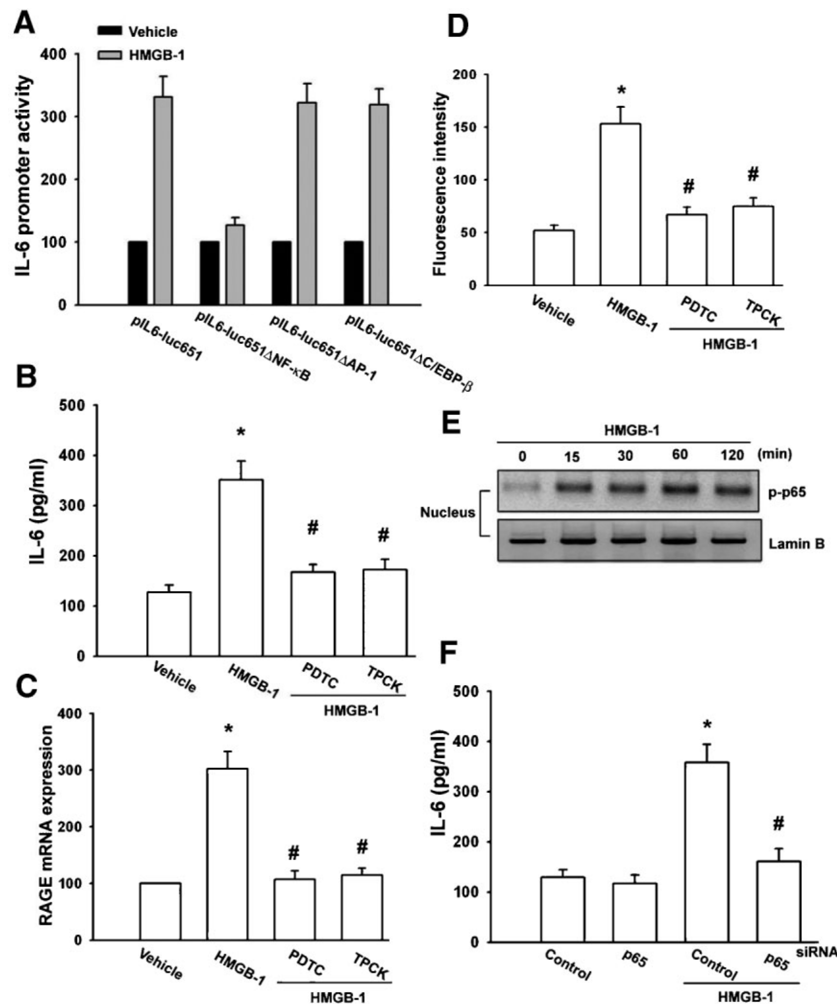

Fig. 5. NF-κB is involved in the potentiation of IL-6 production by HMGB-1. A: OASF cells were transfected with IL-6 luciferase plasmids before incubation with HMGB-1 for 24 h. Luciferase activity was then assayed. B: OASF cells were pretreated for 30 min with PDTC and TPCK followed by stimulation with HMGB-1 for 24 h. Media were collected to measure IL-6. C,D: OASF cells were pretreated for 30 min with PDTC and TPCK followed by stimulation with HMGB-1 for 24 h, the mRNA and the cell surface RAGE expression was examined by qPCR and flow cytometry. E: OASF cells were incubated with HMGB-1 for indicated time intervals, and p65 phosphorylation in nucleus was determined by Western blot. F: OASF cells were transfected with p65 or control siRNA for 24 h, and then stimulated with HMGB-1 for 24 h. Media were collected to measure IL-6. Media were collected to measure IL-6. $^{*}P < 0.05$ as compared with control. $^{\#}P < 0.05$ as compared with HMGB-1-treated group.

inhibited the HMGB-1-induced Akt phosphorylation (Fig. 4D). These results indicated that HMGB-1 acts through RAGE receptor, c-Src, and Akt-dependent signaling pathway to enhance IL-6 production.

Involvement of NF-κB in HMGB-1-induced IL-6 production

The promoter region of human IL-6 contains three known cis-regulatory elements including AP-1, C/EBP-β, and NF-κB-binding sites (Matsusaka et al., 1993; Grassl et al., 1999). Three different IL-6 promoter constructs containing mutations at NF-κB, AP-1, or C/EBP-β sites respectively were generated by site-directed mutagenesis. We found that HMGB-1-stimulated luciferase activity was abolished by NF-κB-binding site mutation, but not by AP-1 and C/EBP-β site mutations (Fig. 5A). The role of NF-κB was further established using the NF-κB inhibitor PDTC and showed that this inhibitor blocked the enhancement of IL-6 production induced by HMGB-1 (Fig. 5B). Furthermore, pretreatment of synovial fibroblasts with an IκB protease inhibitor TPCK (3 μM) also antagonized the potentiating action of IL-6 (Fig. 5B). Therefore, the NF-κB-binding site is more important than the AP-1 and C/EBP-β site in HMGB-1-induced IL-6 production. We further confirm whether HMGB-1-induced RAGE expression also through NF-κB. Pretreatment of cells with PDTC and TPCK-inhibited HMGB-1-induced RAGE expression (Fig. 5C,D). Therefore, NF-κB activation participate the amplification loop of HMGB-1-RAGE-signaling pathway.

RAGE/c-Src/Akt signaling pathway is involved in HMGB-1-mediated NF-κB activity and IL-6 expression

It has been reported that the NF-κB-binding site between −72 and −63 was important for the activation of the IL-6 gene (Matsusaka et al., 1993). NF-κB activation was further evaluated by analyzing the accumulation of phosphorylated p65 in the nucleus, as well as by the chromatin immunoprecipitation assay. Treatment of cells with HMGB-1 resulted in a marked accumulation of phosphorylated p65 in the nucleus (Fig. 5E). Transfection of cells with p65 siRNA suppressed HMGB-1-induced IL-6 expression (Fig. 5F). We next investigated whether p65 binds to the NF-κB element on the IL-6 promoter after HMGB-1 stimulation. The in vivo recruitment of p65 to the IL-6 promoter (−312 to −39) was assessed by the chromatin immunoprecipitation assay (Chiu et al., 2009). In vivo binding of p65 to the NF-κB element of the IL-6 promoter occurred after HMGB-1 stimulation (Fig. 6A). The binding of p65 to the NF-κB element by HMGB-1 was attenuated by the RAGE Ab and PP2 (Fig. 6A). To further confirm that the NF-κB element is involved in the action of HMGB-1-induced IL-6 expression, we performed transient transfection using the NF-κB promoter-luciferase constructs. Synovial fibroblasts incubated with HMGB-1 led to a 3.3-fold increase in NF-κB promoter activity. The increase of NF-κB activity by HMGB-1 was antagonized by RAGE Ab, PP2 and Akt inhibitor or RAGE and p65 siRNA, or c-Src and Akt mutant (Fig. 6B,C). Taken together, these data suggest that the activation of the RAGE, c-Src, Akt, p65, and NF-κB pathway is required for the HMGB-1-induced increase of IL-6 in human OASF cells.

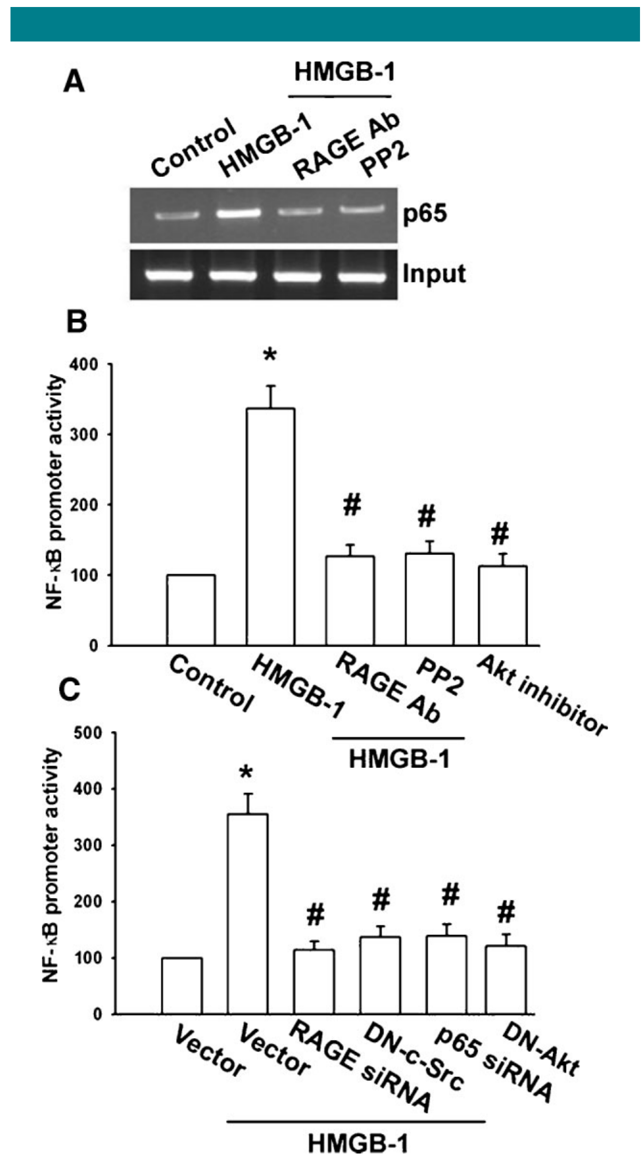

Fig. 6. RAGE/c-Src/Akt pathway is involved in HMGB-1-induced NF-κB activation. A: OASF cells were pretreated with RAGE Ab and PP2 then stimulated with HMGB-1 for 120 min, and the chromatin immunoprecipitation assay was then performed. Chromatin was immunoprecipitated with anti-p65. One percentage of the precipitated chromatin was assayed to verify equal loading (input). OASF cells were transfected with NF-κB-luciferase expression vector and then pretreated with RAGE Ab, PP2 and Akt inhibitor or cotransfeced with RAGE and p65 siRNA or c-Src and Akt mutant before incubation with HMGB-1 for 24 h (B,C). Luciferase activity was then assayed. $^{*}P < 0.05$ as compared with control. $^{\#}P < 0.05$ as compared with HMGB-1-treated group.

RAGE/c-Src/Akt/NF-κB pathway is involved in HMGB-1-induced MMP-3 expression in human chondrocytes

During OA, synovium may be involved in the induction of catabolic activities in the joint cartilage. Upon stimulation, chondrocytes in the joint cartilage release matrix-degradation enzymes, such as MMPs, which result in the destruction of cartilage (Pelletier et al., 2001). It has reported that activation of RAGE in OA leads to increased stimulation of chondrocytes (Steenvoorden et al., 2007). Whether the same signaling pathway involved in HMGB-1-mediated MMPs expression was further examined. Stimulation of chondrocytes with HMGB-1 increased MMP-3 but not MMP-2, -9, and -13 expressions (Fig. 7A). RAGE/c-Src/Akt and NF-κB inhibitors and mutants reduced HMGB-1-mediated MMP-3 expression (Fig. 7B,C). Treatment of chondrocytes also increased c-Src, Akt, and p65 phosphorylation (Fig. 7D). Therefore, the same signaling pathway is involved in HMGB-1-increased MMP-3 expression in human chondrocytes.

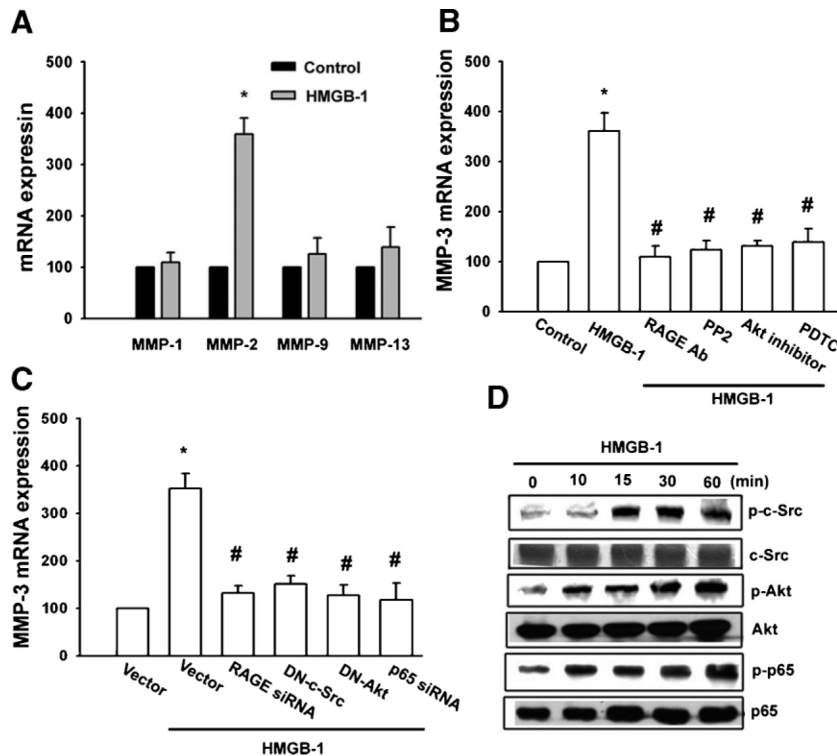

Fig. 7. RAGE/c-Src/Akt/NF-κB pathway is involved in HMGB-1-induced MMP-3 expression in human chondrocytes. A: Human primary chondrocytes were incubated with HMGB-1 for 24 h, the mRNA expression of MMPs was examined by qPCR. B,C: Human primary chondrocytes were pretreated with RAGE Ab, PP2, Akt inhibitor and PDTC or transfected with RAGE and p65 siRNA, or c-Src and Akt mutant before incubation with HMGB-1 for 24 h. The mRNA expression of MMP-3 was examined by qPCR. D: Human primary chondrocytes were incubated with HMGB-1 for indicated time intervals, and c-Src, Akt, and p65 phosphorylation was examined by Western blot. Results are expressed as the mean ± SE. $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with HMGB-1-treated group.

Discussion

Osteoarthritis is a heterogeneous group of conditions associated with defective integrity of articular cartilage, in addition to related changes in the underlying bone. The chronic inflammatory process is mediated through a complex cytokine network. It is not yet completely understood that all the factors are responsible for initiating the degradation and loss of the articular tissues. HMGB-1 has been involved in pathology of arthritis (Hou et al., 2002; Taniguchi et al., 2003). We further identified IL-6 as a target protein for the HMGB-1 signaling pathway that regulates the cell inflammatory response. We showed that potentiation of IL-6 by HMGB-1 requires activation of the RAGE receptor, c-Src, Akt, and NF-κB signaling pathways. These findings suggest that HMGB-1 acts as an inducer of inflammatory cytokines such as IL-6 and enhance the inflammatory response in OA. In addition, we also examine other inflammatory markers (sRAGE, IL-8, and MMP-1) after HMGB-1 stimulation in synovial fibroblasts. We found RAGE, c-Src, Akt and p65 mutant or siRNA reduced HMGB-1-induced sRAGE, IL-8 and MMP-1 expression (Supplementary Fig. S1). Therefore, this pathway may play important role in HMGB-1-mediated inflammatory responses.

RAGE plays important role in inducing invasion, cell cycle arrest, and proinflammatory change in synovial fibroblasts (Steenvoorden et al., 2007; Franke et al., 2009). The interaction of HMGB-1 with RAGE activates several intracellular signal transduction pathways (Schmidt et al., 2001). Stimulation of OASF cells with HMGB-1 increased the mRNA and protein expression of RAGE. In addition, HMGB-1 also increased cell surface RAGE expression in human synovial fibroblasts. These data suggesting that the amplification loop strengthens the HMGB-1-RAGE-signaling pathway. We further confirm that HMGB-1-induced RAGE expression through NF-κB activation. Therefore, NF-κB activation also participate the amplification loop of HMGB-1-RAGE-signaling pathway. Moreover, RAGE Ab or RAGE siRNA reduced HMGB-1-mediated IL-6 production. Therefore, an interaction between HMGB-1 and RAGE is very important for IL-6 production in human synovial fibroblasts.

Src, a tyrosine kinase, plays a critical role in the induction of chemokine transcription (Yeh et al., 2004). In human synovial fibroblasts, thrombin has reported to induce IL-6 expression through c-Src activation (Chiu et al., 2008). Therefore, we examined the potential role of c-Src in the signaling pathway HMGB-1-induced IL-6 expression. Treatment of cells with c-Src inhibitor PP2 or transfection of cells with c-Src mutant reduced HMGB-1-mediated IL-6 production. In addition, we also found that treatment of synovial fibroblasts with HMGB-1-induced increases in c-Src phosphorylation at Tyr^{416} and in c-Src kinase activity. These effects were inhibited by RAGE Ab indicating the involvement of RAGE-dependent c-Src activation in HMGB-1-mediated IL-6 induction. Taken together, our results provided evidence that HMGB-1 up-regulates IL-6 in human synovial fibroblasts via the RAGE/c-Src signaling pathway.

There are several binding sites for a number of transcription factors including NF-κB, CREB, NF-IL-6, and AP-1 box in the 5′-region of the IL-6 gene (Matsusaka et al., 1993; Grassl et al., 1999). Recent studies on the IL-6 promoter have demonstrated

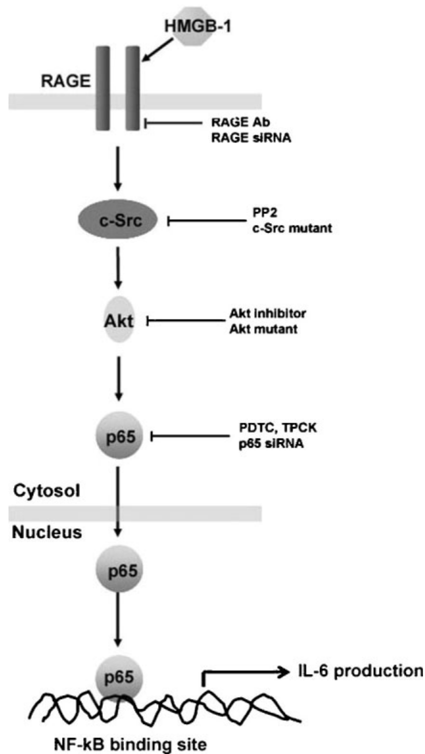

Fig. 8. Schematic presentation of the signaling pathways involved in HMGB-1-induced IL-6 expression of synovial fibroblasts. HMGB-1 and RAGE interaction activates c-Src and Akt pathways, which enhances binding of p65 to the NF-κB site, resulting in the transactivation of IL-6 expression.

that IL-6 induction by several transcription factors occurs in a highly stimulus-specific or cell-specific manner (Persson et al., 2005). The results of this study show that NF-κB activation contributes to HMGB-1-induced IL-6 production in synovial fibroblasts, and deletion of NF-κB site reduced HMGB-1-mediated IL-6 promoter activity. Pretreatment of cells with NF-κB inhibitor (PDTC and TPCK) also reduced HMGB-1-increased IL-6 production. Therefore, the NF-κB-binding site is most important in HMGB-1-induced IL-6 production. The NF-κB sequence binds to members of the p65 and p50 families of transcription factors. The results of this study show that HMGB-1-induced p65 nuclear accumulation. In addition, p65 siRNA abolished the HMGB-1-induced IL-6 production in OASF cells. Therefore, the p65 activation is mediated by HMGB-1-increased IL-6 expression. Furthermore, HMGB-1 increased the binding of p65 to the NF-κB element on the IL-6 promoter, as shown by chromatin immunoprecipitation assay. Binding of p65 to the NF-κB element was attenuated by RAGE Ab and PP2. Using transient transfection with NF-κB-luciferase as an indicator of NF-κB activity, we also found that HMGB-1-induced an increase in NF-κB activity. In addition, RAGE Ab, PP2 and Akt inhibitor or RAGE and p65 siRNA or c-Src and Akt mutant reduced HMGB-1-increased NF-κB promoter activity. These results indicate that HMGB-1 and RAGE interaction might act through the c-Src, Akt, p65, and NF-κB pathway to induce IL-6 activation in human OASF cells.

In conclusion, we explored the signaling pathway involved in HMGB-1-induced IL-6 production in human synovial fibroblasts. We found that HMGB-1 increases IL-6 production by binding to the RAGE receptor and activating c-Src and Akt which enhances binding of p65 to the NF-κB site and results in the transactivation of IL-6 production (Fig. 8). Based on our results, the same pathway also mediated the other inflammatory markers production in synovial fibroblasts and MMP-3 expression in chondroctyes. Therefore, this pathway is the common pathway in HMGB-1 mediated OA pathogenesis. Furthermore, the discovery of HMGB-1/RAGE-mediated signaling pathway helps us understand the mechanism of OA pathogenesis and may lead us to develop effective therapy in the future.

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