HMGB-1 induces cell motility and α5β1 integrin expression in human chondrosarcoma cells

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ABSTRACT

Chondrosarcoma is a type of highly malignant tumor with a potent capacity to invade locally and cause distant metastasis. 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 chondrosarcoma cells are largely unknown. In this study, we found that HMGB-1 increased the migration and the expression of α5β1 integrin in human chondrosarcoma cells. Transfection of cells with receptor for advanced glycation end products (RAGE) receptor siRNA reduced HMGB-1-induced cell migration and integrin expression. Activations of phosphatidylinositol 3-kinase (PI3K), Akt, and AP-1 pathways after HMGB-1 treatment were demonstrated, and HMGB-1-induced expression of integrin and migration activity was inhibited by the specific inhibitor and mutant of PI3K, Akt, and AP-1 cascades. Taken together, our results indicated that HMGB-1 enhances the migration of chondrosarcoma cells by increasing α5β1 integrin expression through the RAGE receptor/PI3K/Akt/c-Jun/AP-1 signal transduction pathway.

Running title: HMGB-1 induces cancer migration

Key Words: HMGB-1; Chondrosarcoma; Integrin; RAGE; Migration

1. INTRODUCTION

High mobility group box chromosomal protein 1 (HMGB)-1 is a widely studied, ubiquitous nuclear protein that is present in eukaryotic cells [1; 2]. As a nuclear protein, HMGB-1 stabilizes nucleosomes and enables nicking of DNA, which facilitates gene transcription [3]. It has been identified as a mediator of endotoxin-induced lethality [4] and a causative factor in arthritis [5], acting, at least in part, as a proinflammatory cytokine [6]. Engagement of the receptor for advanced glycation end products (RAGE) by extracellular HMGB-1 triggers activation of proinflammatory signaling pathways [6; 7], such as those resulting in elaboration of reactive oxygen intermediates and activation of AP-1 and NF-κB [6; 8; 9]. Recently, HMGB-1 also has been implicated in a variety of human cancers and promotes tumorigenesis [10; 11].

Chondrosarcoma is a malignant primary bone tumor with a poor response to currently-used chemotherapy or radiation treatment, making the management of chondrosarcomas a complicated challenge [12]. Clinically, surgical resection remains the primary mode of therapy for chondrosarcoma. Due to the absence of an effective adjuvant therapy, this mesenchymal malignancy has a poor prognosis and, therefore, it is important to explore a novel and adequate remedy [13].

Decades of scrutiny into the molecular bases of cancer have largely focused on what causes oncogenic transformation and the incipient emergence of tumors [14]. The invasion of tumor cells is a complex, multistage process. To facilitate cell motility, invading cells need to change the cell-cell adhesion properties, rearrange the extracellular matrix (ECM) environment, suppress anoikis and reorganize their cytoskeletons [15]. Integrins are a family of transmembrane adhesion receptors comprising 19 α and 8 β subunits that interact noncovalently to form up to 24 different heterodimeric receptors. The combination of different integrin subunits on the cell surface allows cells to recognize and respond to a variety of different ECM proteins including fibronectin, laminin, collagen, and vitronectin [16]. Because integrins are the primary receptors for cellular adhesion to ECM molecules, they act

as crucial transducers of bidirectional cell signaling, regulating cell survival, differentiation, proliferation, migration, and tissue remodeling [17]. Activation and elevated expression of integrin-coupled signaling effectors have been implicated in the induction of a wide variety of human cancers, including those of the breast, colon, prostate, and ovaries [18]. In addition, integrin has also been implicated in metastasis of lung, breast, bladder, and colon cancers [19; 20; 21].

Previous studies have shown that HMGB-1 modulates cell migration and invasion in human cancer cells [22; 23]. However, the effect of HMGB-1 on integrin expression and migration activity in human chondrosarcoma cells is mostly unknown. In this study, we explored whether HMGB-1 increased the migration and integrin expression of in human chondrosarcoma cells. In addition, RAGE receptor, phosphatidylinositol 3′-kinase (PI3K), Akt, c-Jun, and AP-1 signaling pathways may be involved in the increase of integrin expression and cell migration by HMGB-1.

2. MATERIALS AND METHODS

2.1 Materials:

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for β-actin, p85, p-p85, Akt, p-Akt, c-Jun, p-c-Jun, and the small interfering RNAs (siRNAs) against RAGE, α5 integrin, β1 integrin, c-Jun, 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). Mouse monoclonal antibody specific for RAGE was purchased from R&D Systems (Minneapolis, MN). Mouse monoclonal antibody specific for α5β1 integrin was purchased from Chemicon (Temecula, CA). Tanshinone IIA was purchased from BIOMOL (Butler Pike, PA). The recombinant human HMGB-1 was purchased from PeproTech (Rocky Hill, NJ, USA). The AP-1 luciferase plasmid was purchased from Stratagene (La Jolla, CA). The p85α and Akt (Akt K179A) dominant negative mutant

were gifts from Dr. W.M. Fu (National Taiwan University, Taipei, Taiwan). 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).

2.2 Cell culture

The human chondrosarcoma cell line (JJ012) was kindly provided by the laboratory of Dr. Sean P. Scully (University of Miami School of Medicine, Miami, FL, USA). The cells were cultured in Dulbecco's modified Eagle's medium/α-minimum essential medium supplemented with 10% fetal bovine serum and maintained at 37℃ in a humidified atmosphere of 5% CO_2.

2.3 Migration assay

The migration assay was performed by using Transwell (Costar, NY, USA; pore size, 8-μm) in 24-well dishes. Before migration assay, cells were pretreated for 30 min with different concentrations of inhibitors, including the Ly294002, Akt inhibitor, curcumin, tanshinone IIA or vehicle control (0.1% DMSO). Approximately 1×10^4 cells in 200 μl of serum-free medium were placed in the upper chamber, and 300 μl of the same medium containing HMGB-1 was placed in the lower chamber. The plates were incubated for 24 h at 37℃ in 5% CO_2, then cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. Each clone was plated in triplicate in each experiment, and each experiment was repeated at least three times. The number of invading cells in each experiment was adjusted by the cell viability assay to correct for proliferation effects of HMGB-1 treatment (corrected invading cell number = counted invading cell number/percentage of viable cells) [24].

2.4 Wound-healing migration assay

For the wound-healing migration assay, cells were seeded on 12-well plates at a density of 1 X 10^5 cells/well in culture medium. Twenty-four h after seeding, the confluent monolayer of the culture was scratched with a fine pipette tip, and migration was visualized under a microscope with magnification. The rate of wound closure was observed at the indicated times.

2.5 Flow cytometric analysis

Human chondrosarcoma cells were plated in six-well dishes. The cells were then washed with PBS and detached with trypsin at 37℃. Cells were fixed for 10 min in PBS containing 1% paraformaldehyde. After being rinsed in PBS, the cells were incubated with mouse anti-human antibody against α5β1 integrin (1:100) for 1 h at 4℃. Cells were then washed again and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary IgG (1:100; Leinco Tec. Inc., St. Louis, MO, USA) for 45 min and analyzed by flow cytometry using FACS Calibur and CellQuest software (BD Biosciences).

2.6 Quantitative real-time PCR

Total RNA was extracted from chondrosarcoma cells 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 [25; 26]. The quantitative real time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA). 2 μl cDNA templates 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, CA). qPCR assays were carried out in triplicate on an StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95 ℃ followed by 40 cycles at 95 ℃ for 15 s and 60 ℃ for 60 s. 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).

2.7 Western blot analysis

The cellular lysates were prepared as described previously [27; 28]. Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies against Akt, p-Akt, p85 or p-p85 (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

2.8 Transfection and reporter gene assay

Human chondrosarcoma cells were co-transfected with 0.8 μg luciferase plasmid and 0.4 μg β-galactosidase expression vector. 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, Madison, WI) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector [29].

2.9 Immunofluorocytochemistry

Cells were cultured in 12-mm coverslips. After treatment with HMGB-1, cells were fixed with 4% paraformaldehyde at room temperature. Thirty minutes later, 4% nonfat milk in PBS containing 0.5% Triton X-100 was added to the cells. The cells were then incubated with mouse anti-c-Jun (1:100) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:500; Leinco Technology Inc., St Louis, MO, USA) for 1 h, respectively. The FITC was detected using a Zeiss fluorescence microscope.

2.10 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation analysis was performed as described previously [30]. DNA immunoprecipitated by anti-c-Jun mAb 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. The primers 5'-GCGGGCTCAGAGTTCCAG-3' and 5'-CCGCTCTTCCCTGTCCTG-3' were utilized to amplify across the α5 integrin promoter region (–115 to +73) [31]. The primers 5'-ACGCAACTCACCAGGTTTTC-3' and 5'-CTAGGAGGAGGCGGAGGAT-3' were utilized to amplify across the β1 integrin promoter region (–1099 to –758) [32].

2.11 Statistics

The values given are means ± S.E.M. The significance of difference between the experimental groups and controls was assessed by Student's t test. The difference was significant if the *p* value was <0.05.

3. RESULTS

3.1 HMGB-1-directed chondrosarcoma cells migration involves α5β1 integrin up-regulation

HMGB-1 has been suggested to stimulate directional migration and invasion of human cancer cells [22; 23]. HMGB-1-triggered migration in chondrosarcoma cells was examined using the Transwell assay. HMGB-1 directed human chondrosarcoma cell (JJ012 cells) migration (Fig. 1A). However, treatment of JJ012 cells with HMGB-1 did not affect cell viability by using MTT assay (data not shown). We also measured the Transwell assay at a time point for 12 h. We found that HMGB-1 induced cell migration dose-dependently (data not shown). On the other hand, HMGB-1 also increased wound-healing activity in human chondrosarcoma cells (Fig. 1B). The invasion ability of chondrosarcoma cells through the Matrigel basement membrane matrix was increased by HMGB-1 stimulation (Fig. 1C). To further confirm this stimulation-specific mediation by HMGB-1 without LPS contamination, we used polymyxin B, an LPS inhibitor. We found that polymyxin B (1 μM) did not affect HMGB-1 induced cell migration (Fig. 1D). Previous studies have shown significant expression of integrin in human chondrosarcoma cells [33; 34]. We therefore, hypothesized that integrin may be involved in HMGB-1-directed chondrosarcoma cell migration. qPCR analysis showed that HMGB-1 induced α5 and β1 but not αv, α2, β3, and β5 integrin expression (Fig. 1E). To confirm this finding, expression of cell surface integrin in response to HMGB-1 was analyzed by flow cytometry. Treatment of JJ012 cells with HMGB-1 induced the cell surface expression of α5β1 integrin (Fig. 1F). Pretreatment of cells for 30 min with anti-α5β1 monoclonal antibody (mAb) (10 μg/ml) markedly inhibited the HMGB-1-induced cell migration (Fig. 1G). JJ012 cells were transfected with α5 or β1 integrin siRNA for 24 h, and the Western blot analysis showed that the expression of protein levels of α5 or β1 integrin was suppressed by transfection with α5 or β1 integrin siRNA, respectively (Fig. 1H). Transfection of cells with α5 or β1 integrin siRNA reduced the HMGB-1-increased cell migration (Fig. 1H). These data suggest that HMGB-1-induced cancer migration may occur via activation of α5β1 integrin.

3.2 Involvement of RAGE receptor in HMGB-1-mediated migration of

chondrosarcoma

It has been reported that HMGB-1 exerts its effects through interaction with a specific RAGE receptor [6; 35]. Therefore, we next examined whether RAGE was involved in HMGB-1-mediated cell migration in human chondrosarcoma cells. Transfection of cells with RAGE siRNA reduced RAGE expression (Fig. 2A). In addition, transfection of cells with RAGE siRNA or pretreatment of cells with RAGE antibody effectively inhibited the migration activity and α5β1 integrin expression of chondrosarcoma cells (Fig. 2A-C). These data suggest that HMGB-1/RAGE interactions play a key role in the migration and integrin expression of chondrosarcoma.

3.3 PI3K and Akt signaling pathways are involved in HMGB-1-mediated α5β1 integirn up-regulation and cell migration of chondrosarcoma cells

PI3K/Akt can be activated by a variety of growth factors, such as insulin and growth factors [36; 37; 38]. We examined whether HMGB-1 stimulation also enhanced PI3K activation. Stimulation of JJ012 cells led to a significant increase in phosphorylation of p85 (Fig. 3A). HMGB-1-induced migration and integrin expression of chondrosarcoma cells were greatly reduced by treatment with PI3K inhibitors Ly294002 and wortmannin (Fig. 3B-D). In addition, transfection of cells with p85 mutant also inhibited HMGB-1-induced migration and α5β1 integrin expression of chondrosarcoma cells (Fig. 3B-D). Ser473 residue phosphorylation of Akt by a PI3K-dependent signaling pathway causes enzymatic activation [39]. To examine the crucial role of PI3K/Akt in cancer migration and integrin up-regulation, we next determined Akt Ser473 phosphorylation in response to HMGB-1 treatment. As shown in Fig. 4A, treatment of JJ012 cells with HMGB-1 resulted in time-dependent phosphorylation of Akt Ser473. Pretreatment of cells with Akt inhibitor antagonized HMGB-1-induced migration and integrin expression of chondrosarcoma cells (Fig. 4B-D). In addition, the Akt mutant also reduced HMGB-1-mediated cell migration and integin up-regulation (Fig. 4B-D). Based on

these results, it appears that HMGB-1/RAGE axis acts through the PI3K and Akt-dependent signaling pathway to enhance α5β1 integrin expression and cell migration in human chondrosarcoma cells.

3.4 Involvement of AP-1 in HMGB-1-induced cell migration and integrin expression

It has been reported that AP-1 plays a critical role in cancer metastasis [40]. To examine the role of AP-1 binding site in HMGB-1-mediated integrin expression and cancer migration, the AP-1 inhibitors curcumin and tanshinone IIA were used. Pretreatment of cells with curcumin and tanshinone IIA reduced HMGB-1-induced cell migration and α5β1 integrin expression (Fig. 5A-C). c-Jun is major component of AP-1 transactivation. We next examine whether c-Jun is mediated the HMGB-1-induced cell migration and integrin expression. Transfection of cells with c-Jun siRNA suppressed the expression of c-Jun (Fig. 5A). HMGB-1-induced cell migration and α5β1 integrin expression was also inhibited by c-Jun siRNA but not by control siRNA (Fig. 5A-C). Treatment of cells with HMGB-1 resulted in a marked phosphorylation of c-Jun (Fig. 5D). Pretreatment of cells with Ly294002, wortmannin, or Akt inhibitor reduced HMGB-1-increaed c-Jun phosphorylation (Fig. 5E).

3.5 RAGE/PI3K/Akt signaling pathway is involved in HMGB-1-mediated AP-1 activity

AP-1 activation was further evaluated by analyzing chromatin immunoprecipitation assay, immunofluorocytochemistry as well as by the AP-1 luciferase assay. The *in vivo* recruitment of c-Jun to the α5 or β1 integrin promoter was assessed by the chromatin immunoprecipitation assay [30]. *In vivo* binding of c-Jun to the AP-1 element of the α5 or β1 integrin promoter occurred after HMGB-1 stimulation (Fig. 6A). The binding of c-Jun to the AP-1 element by HMGB-1 was attenuated by Ly294002, wortmannin, or Akt inhibitor (Fig. 6A). Stimulation of cells also increased c-Jun translocation into nucleus (Fig. 6B). Pretreatment of cells with

Ly294002, wortmannin, or Akt inhibitor also reduced HMGB-1-incrased c-Jun translocation (Fig. 6B). To directly determine AP-1 activation after HMGB-1 treatment, cells were transiently transfected with AP-1-luciferase as an indicator of AP-1 activation. As shown in Fig. 6C, HMGB-1 treatment of JJ012 cells for 24 h resulted in increased AP-1-luciferase activity. In addition, Ly294002, wortmannin, Akt inhibitor, curcumin, or tanshinone IIA antagonized the HMGB-1-induced AP-1-luciferase activity (Fig. 6C). Co-transfection of cells with p85 and Akt mutant or RAGE and c-Jun siRNA also reduced HMGB-1-increased AP-1-luciferase activity (Fig. 6D). Taken together, these data suggest that activation of RAGE, PI3K, Akt, and c-Jun are required for HMGB-1-induced AP-1 activation in human chondrosarcoma cells.

4. DISCUSSION

Unlike other mesenchymal malignancies, such as osteosarcoma and Ewing's sarcoma, which have seen dramatic increases in long-term survival with the advent of systemic chemotherapy, chondrosarcoma continues to have a poor prognosis due to the absence of an effective adjuvant therapy [41]. The metastatic potential for conventional chondrosarcomas correlates well with the histologic grade of the tumor. However, due to the relatively indolent growth rates of many low- and moderate-grade chondrosarcomas, ~15% of patients dying from metastatic disease do so >5 years after initial diagnosis [41]. Therefore, it is important to develop an effective adjuvant therapy for prevention of chondrosarcoma metastasis. We hypothesized that HMGB-1 would help to direct the metastasis of chondrosarcoma cells. We found that HMGB-1 increased the migration of chondrosarcoma cells. One of the mechanisms underlying HMGB-1-directed migration was transcriptional up-regulation of α5β1 integrin and activation of RAGE receptor, PI3K, Akt, c-Jun, and AP-1 pathways. In previously we found that HMGB-1 induces IL-6 production in human synovial fibroblasts [42]. Transfection of JJ012 cells with IL-6 siRNA did not affect HMGB-1-induced cell migration (data not shown). Therefore, IL-6 did not involve in HMGB-1 induces cell migration in human chondrosarcoma cells.

RAGE plays important role in inducing cell cycle arrest, proinflammatory change, and invasion [43; 44]. The interaction of HMGB-1 with RAGE activates several intracellular signal transduction pathways [6]. In this study, we found that RAGE siRNA reduced HMGB-1-induced cell migration. In addition, RAGE siRNA also blocked HMGB-1-incrased α5β1 integrin expression. Therefore, an interaction between HMGB-1 and RAGE is very important for cancer migration and α5β1 integrin expression in human chondrosarcoma cells.

Integrins link the ECM to intracellular cytoskeletal structures and signaling molecules and are implicated in the regulation of a number of cellular processes, including adhesion, signaling, motility, survival, gene expression, growth, and differentiation [45]. Here we found that HMGB-1 increased α5β1 integrin expression

by using flow cytometry analysis, which plays an important role during tumor metastasis. Furthermore, HMGB-1 also increased the mRNA levels of α5 and β1 integrins. In the present study, we used α5β1 integrin antibody to determine the role of α5β1 integrin and found that it inhibited HMGB-1-induced cancer migration, indicating the possible involvement of α5β1 integrin in HMGB-1-induced migration in chondrosarcoma cells. This was further confirmed by the result that the α5 or β1 integrin siRNA inhibited the enhancement of migration activity by HMGB-1, indicating the involvement of α5β1 integrin in HMGB-1-mediated induction of cancer migration.

A variety of growth factors stimulate cancer metastasis via signal-transduction pathways that converge to activate AP-1 complex of transcription factors [40]. The PI3K/Akt pathway is a major cascade mediating activation of the AP-1 signaling pathway in human cancer cells [40]. Phosphorylation of the p85 subunit is required for activation of the p110 catalytic subunit of PI3K [46]. We found HMGB-1-enhanced the p85 subunit phosphorylation in human chondrosarcoma cells. Pretreatment of cells with PI3K inhibitors Ly294002 or wortmannin antagonized an increase in migration and α5β1 integrin expression by HMGB-1 stimulation. This was further confirmed by the result that the dominant-negative mutant of p85 inhibited the enhancement of migration by HMGB-1. Moreover, we also found that HMGB-1 activated Akt Ser473 phosphorylation, while Akt inhibitor and Akt mutant inhibited HMGB-1-mediated cell migration and α5β1 integrin expression. Our data indicates that PI3K/Akt could play an important role in the expression of integrin and migration of human chondrosarcoma cells.

The AP-1 sequence binds to members of the Jun and Fos families of transcription factors. These nuclear proteins interact with the AP-1 site as Jun homodimers or Jun-Fos heterodimers formed by protein dimerization through their leucine zipper motifs. The results of this study show that HMGB-1 induced c-Jun phosphorylation. In addition, c-Jun siRNA abolished the HMGB-1-induced cell migration in chondrosarcoma cells. Furthermore, HMGB-1 also increased the binding of c-Jun to

the AP-1 element on the α5 and β1 integrin promoter, as shown by chromatin immunoprecipitation assay. The c-Jun phosphorylation and the binding of c-Jun to the AP-1 element as well as c-Jun translocation to nucleus were attenuated by Ly294002, wortmannin, or Akt inhibitor. These results indicate that HMGB-1 might act through the PI3K, Akt, c-Jun, and AP-1 pathway to induce α5β1 integrin expression and cell migration in human chondrosarcoma cells.

The prognosis of patients with chondrosarcoma distant metastasis is generally considered very poor; hence, preventing human chondrosarcoma metastasis is an important issue nowadays. Our study observes that HMGB-1 increases the activity of α5β1 integrin via the RAGE, PI3K, Akt, c-Jun, and AP-1-dependent pathway and enhances migration of human chondrosarcoma cells (Fig. 6E). Furthermore, the discovery of HMGB-1/RAGE-mediated signaling pathway helps us understand the mechanism of human chondrosarcoma metastasis and may lead us to develop effective therapy in the future.

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Conflict of Interest Statement

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

Abbreviations: HMGB-1, High mobility group box chromosomal protein 1; RAGE, receptor for advanced glycation end products; PI3K, phosphatidylinositol 3-kinase; ECM, extracellular matrix; siRNA, small interfering RNA; qPCR, quantitative real time PCR.

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FIGURE LEGENDS

Fig. 1 HMGB-1-directed migration of human chondrosarcoma cells involves up-regulation of α5β1 integrin

(A&C) JJ012 cells were incubated with HMGB-1 for 24 h, and *in vitro* migration and invasion was measured by Transwell after 24 hr. (B) Cells were treated with HMGB-1 for 24 h, and the wound-scratching assay was performed. (D) Cells were pretreated with polymyxin B (poly B, 1 μM) for 30 min followed by stimulation with HMGB-1 (50 ng/ml). The *in vitro* migration activity measured after 24 h. (E) JJ012 cells were incubated with HMGB-1 (50 ng/ml) for 24 h, and the mRNA levels of αv, α2, α5, β1, β3, or β5 integrin was determined using qPCR. (F) Cells were incubated with HMGB-1 (50 ng/ml) for 24 h, and the cell surface expression of α5β1 integrin was determined using flow cytometry. (G) Cells were pretreated with α5β1 monoclonal antibody (10 μg/ml) for 30 min followed by stimulation with HMGB-1. The *in vitro* migration activity measured after 24 h. (H) Cells were transfected with α5 or β1 integrin siRNA for 24 h followed by stimulation with HMGB-1, and *in vitro* migration was measured by Transwell. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with HMGB-1-treated group.

Fig. 2 Involvement of RAGE receptor in HMGB-1-directed migration of human chondrosarcoma.

(A; upper panel) Cells were transfected with RAGE or control siRNA for 24 h, and the RAGE expression was examined by Western blotting. (A-C) Cells were transfected with RAGE siRNA for 24 h or pretreated with RAGE antibody for 30 min followed by stimulation with HMGB-1, and *in vitro* migration and α5β1 integrin expression was measured by Transwell, qPCR, and flow cytometry. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with HMGB-1-treated group.

Fig. 3 PI3K is involved in HMGB-1-induced migration and integrin up-regulation in human chondrosarcoma cells.

(A) JJ012 cells were incubated with HMGB-1 (50 ng/ml) for indicated time intervals, and p-p85 was examined by Western blot analysis. (B-D) Cells were pretreated for 30 min with Ly294002 (10 μM) and wortmannin (1 μM) or transfected with dominant negative (DN) mutant of p85 for 24 h followed by stimulation with HMGB-1. The *in vitro* migration and integrin expression was examined by Transwell, qPCR, and flow cytometry analysis. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with HMGB-1-treated group.

Fig. 4 Akt is involved in HMGB-1-induced migration and integrin up-regulation in human chondrosarcoma cells.

(A) JJ012 cells were incubated with HMGB-1 (50 ng/ml) for indicated time intervals, and p-Akt was examined by Western blot analysis. (B-D) Cells were pretreated for 30 min with Akt inhibitor (10 μM) or transfected with dominant negative (DN) mutant of Akt for 24 h followed by stimulation with HMGB-1. The *in vitro* migration and integrin expression was examined by Transwell, qPCR, and flow cytometry analysis. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with HMGB-1-treated group.

Fig. 5 HMGB-1 induces cell migration and integrin up-regulation through AP-1.

(A-C) Cells were pretreated for 30 min with curcumin (10 μM) and tanshinone IIA (10 μM) or transfected with c-Jun siRNA for 24 h followed by stimulation with HMGB-1. The *in vitro* migration and integrin expression was examined by Transwell, qPCR, and flow cytometry analysis. (D) JJ012 cells were incubated with HMGB-1 (50 ng/ml) for indicated time intervals,

and p-c-Jun was examined by Western blot analysis. (E) Cells were pretreated for 30 min with Ly294002, wortmannin, and Akt inhibitor for 24 h followed by stimulation with HMGB-1 for 60 min, and p-c-Jun expression was examined by Western blotting. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with HMGB-1-treated group.

Fig. 6 RAGE/PI3K/Akt pathway is involved in HMGB-1-mediated AP-1 activation.

(A) JJ012 cells were pretreated with Ly294002, wortmannin, and Akt inhibitor for 30 min then stimulated with HMGB-1 for 120 min, and the chromatin immunoprecipitation assay was then performed. Chromatin was immunoprecipitated with anti-c-Jun. One percentage of the precipitated chromatin was assayed to verify equal loading (input). (B) JJ012 cells were pretreated with Ly294002, wortmannin, and Akt inhibitor for 30 min then stimulated with HMGB-1 for 120 min, and c-Jun immunofluorescence staining was examined. JJ012 cells were pretreated with Ly294002, wortmannin, Akt inhibitor, curcumin, and tanshinone IIA for 30 min (C) or transfected with mutant of p85 and Akt mutant or c-Jun and RAGE siRNA (D) before exposure to HMGB-1. AP-1 luciferase activity was measured, and the results were normalized to the β-galactosidase activity and expressed as the mean ± S.E. for three independent experiments performed in triplicate. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with HMGB-1-treated group. (E) Schematic presentation of the signaling pathways involved in HMGB-1-induced migration and α5β1 integrin expression of chondrosarcoma cells. HMGB-1 activates RAGE receptor, PI3K, and Akt pathways, which in turn induces AP-1 activation, which lead to α5β1 integrin expression and increases the migration of human chondrosarcoma cells.