IL-1β/HMGB1 signalling promotes the inflammatory cytokines release via TLR signalling in human intervertebral disc cells

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Synopsis

Inflammation and cytokines have been recognized to correlate with intervertebral disc (IVD) degeneration (IDD), via mediating the development of clinical signs and symptoms. However, the regulation mechanism remains unclear. We aimed at investigating the regulatory role of interleukin (IL)-1β and high mobility group box 1 (HMGB1) in the inflammatory response in human IVD cells, and then explored the signalling pathways mediating such regulatory effect. Firstly, the promotion to inflammatory cytokines in IVD cells was examined with ELISA method. And then western blot and real time quantitative PCR were performed to analyse the expression of toll-like receptors (TLRs), receptors for advanced glycation endproducts (RAGE) and NF-κB signalling markers in the IL-1-β or (and) HMGB1-treated IVD cells. Results demonstrated that either IL-1-β or HMGB1 promoted the release of the inflammatory cytokines such as prostaglandin E2 (PGE2), TNF-α, IL-6 and IL-8 in human IVD cells. And the expression of matrix metalloproteinases (MMPs) such as MMP-1, -3 and -9 was also additively up-regulated by IL-1-β and HMGB1. We also found such additive promotion to the expression of TLR-2, TLR-4 and RAGE, and the NF-κB signalling in intervertebral disc cells. In summary, our study demonstrated that IL-1-β and HMGB1 additively promotes the release of inflammatory cytokines and the expression of MMPs in human IVD cells. The TLRs and RAGE and the NF-κB signalling were also additively promoted by IL-1-β and HMGB1. Our study implied that the additive promotion by IL-1-β and HMGB1 to inflammatory cytokines and MMPs might aggravate the progression of IDD.

Key words: high mobility group box 1 (HMGB1), interleukin (IL)-1β, intervertebral disc degeneration (IDD).

INTRODUCTION

Intervertebral disc (IVD) degeneration (IDD) is clinically characteristic of chronic low back pain and the secondary symptoms and signs of disc herniation, spinal canal stenosis and spinal deformities [1]. And such spinal disorders lead the disability causes of in the workforce [2]. The aetiology of IDD is complicated with multiple factors such as aging, sustained stress, heredity, smoking, obesity and trauma [3–5]. However, the molecular mechanisms underlying IDD are largely unknown. IDD is mainly characterized in pathology of reduced (and abnormal) intervertebral disc cells, of broken-down extracellular matrix (ECM), and of promoted pro-inflammatory mediators [6,7], which then progressively cause IDD damage in structure and function. Matrix metalloproteinase (MMP) is widely considered to play a role in disc degeneration by degrading collagen and proteoglycans found in the matrix [8–10]. Studies reported that MMP expression was associated with the degradation of the matrix in IDD. However, the regulation on the MMP expression was not clear.

Interleukin (IL)-1 family is a group of cytokines (11 members), which is a key regulator in immune and inflammatory responses to infections or sterile insults [11,12]. The proinflammatory IL-1β is the most important cytokine, with a strong stimulation to the production of multiple proinflammatory mediators such as cytokines, chemokines and MMPs [12–14]. IL-1β expression has been reported to be significantly promoted in IDD tissues and cells [15], exerting regulation in multiple pathological processes of disc degeneration [16]. On the other side, the inhibition of IL-1β has been observed to promote ECM repair and block disc degeneration [17,18].

Abbreviations: ECM, extracellular matrix; FBS, fetal bovine serum; HAF, human annulus fibrosus cells; HMGB1, high mobility group box 1; IOD, integral optical density; IVD, intervertebral disc; MMP, matrix metalloproteinase; RAGE, receptors for advanced glycation endproducts; TIMP-1, tissue inhibitor of matrix metalloproteinase-1; TLR, toll-like receptor; TNF-α, tumour necrosis factor α.
High mobility group box 1 (HMGB1) is DNA-binding chromatin protein. Nuclear HMGB1 organizes DNA and regulates gene transcription [19], via interacting with nucleosomes, transcription factors and histones [20]. The nucleus-resided HMGB1 has been observed to be released to cytosol and even extracellular space upon inflammatory and other stimulation [21,22] to mediate inflammation in multiple injury models [23–25]. Recently, HMGB1 has been recognized as a potent proinflammatory mediator in degenerated human discs [26]. And it was markedly up-regulated in the IVD in a lipopolysaccharide (LPS)-induced rat IDD model [27]. Toll-like receptors (TLRs), such as TLR-2 and TLR-4 recognized HMGB1 and mediated the HMGB1-induced cytokine release [30]. Importantly, TLR signalling has been indicated to be active in IL-1/HMGB1-promoted inflammation [29]. In particular, TLRs, such as TLR-2 and TLR-4 recognized HMGB1 and mediated the HMGB1-induced cytokine release [30].

In the present study, we investigated the mediator role of IL-1β and HMGB1 in the inflammation in disc annulus fibrosus cells. In particular, we examined the additive effect of both cytokines in disc annulus cells in the production of such cytokines as prostaglandin E2 (PGE2), IL-6, IL-8 and tumour necrosis factor alpha (TNF-α) [31–33], which are promoted in intervertebral disc degeneration. In addition, we examined the possible involvement of TLRs, receptors for advanced glycation endproducts (RAGE) and nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) signalling in such additive effect between IL-1β and HMGB1.

**MATERIALS AND METHODS**

**Cells and reagents**

Human annulus fibrosus cells (HAFC, Cat. No. 4810) were purchased from the ScienCell Research Laboratories and were cultured under 5% CO₂ at 37°C in nucleus pulposus cell medium (NPCM, Cat. No. 4801) (ScienCell Research Laboratories), which were supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma–Aldrich). Human recombinant IL-1β (BD Biosciences) and human recombinant HMGB1 (Abnova) were dissolved in NPCM with 2% FBS. A final concentration of 0, 0.5, 1, 2 or 5 ng/ml IL-1β or a final concentration of 0, 20, 40 or 80 ng/ml HMGB1 was utilized to treat 85%-confluent HAFC for 0, 6, 12, 24 or 48 h. Then HAFC were collected with a cell scraper in ice-cold 1x PBS, and the cellular supernatant of treated cells was collected for the cytokine assay.

**ELISA analysis for supernatant levels of PGE2, TNF-α, IL-6 or IL-8**

The supernatant levels of PGE2, TNF-α, IL-6 or IL-8 were quantified with the ELISA kit for each cytokine (Excellbio) according to each kit’s guidance. Firstly, 100 μl serially-diluted standard samples or supernatant samples were added into the microplate and were incubated at 37°C for 1 h. Secondly, the plate was updated with 100 μl 1x antibody solution against PGE2, TNF-α, IL-6 or IL-8 in each well and was incubated at 37°C for another 1 h. Thirdly, the plate was updated with 100 μl horseradish peroxidase-linked secondary antibody for incubation at 37°C for 30 min. Four time-washing was performed in each well of the plate with 100 μl PBS with Tween 20 (PBST) before incubation. Finally, the plate was inoculated with 100 μl substrate at dark for 15 min; the specific binding optical density was assayed immediately at 450 nm with a spectrophotometer (Bio-Rad Laboratories).

**Real-time quantitative PCR analysis**

Total RNA was extracted from HAFC with TRIzol reagent (Life Technologies), was added with 1 μl SUPERase•in™ RNase Inhibitor (Thermo Scientific) and was assessed for quality by the OD260/OD280 ratio. Each RNA sample was quantified with One Step RT-PCT kit (Takara). And the RT-qPCR assay was performed for the reverse transcription firstly (at 42°C for 5 min, and then at 95°C for 10 s) and then for the PCR reaction (at 95°C for 5 s and at 60°C for 20 s, 40 cycles) using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The specificity of amplified products was determined by melting curve analysis. The relative expression levels of mRNA were calculated with the 2−ΔΔCt method [34] and were presented as the relative quantitative value normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used in the study were as follows: MMP-1 forward (5′-gag ttc cca car tgg aat aa-3′) and reverse (5′-tgct acc ctc gac ctt gc-3′); MMP-3 forward (5′- tgc cca aag atg ctg tgtt-3′) and reverse (5′-ttgc ggc tgc gcg ctc-3′); MMP-9 forward (5′-agg tgg tca cct cct ccc aga ct-3′); MMP-9 forward (5′-ggc agc aga cca gac ctt gc-3′); NF-κB forward (5′-tgc acc acc acc tgc tta gc-3′) and reverse (5′-tct gcg cag tga tgc tga-3′). The specificity of amplified products was determined by melting curve analysis. The relative expression levels of mRNA were calculated with the 2−ΔΔCt method [34] and were presented as the relative quantitative value normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used in the study were as follows: MMP-1 forward (5′-gag ttc cca car tgg aat aa-3′) and reverse (5′-tgct acc ctc gac ctt gc-3′); MMP-3 forward (5′- tgc cca aag atg ctg tgtt-3′) and reverse (5′-ttgc ggc tgc gcg ctc-3′); MMP-9 forward (5′-agg tgg tca cct cct ccc aga ct-3′); MMP-9 forward (5′-ggc agc aga cca gac ctt gc-3′); NF-κB forward (5′-tgc acc acc acc tgc tta gc-3′) and reverse (5′-tct gcg cag tga tgc tga-3′).

**Western blotting assay**

Cytosol and nuclear section of proteins were prepared with the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) in accordance with the manufacturer’s protocols, and were quantified by the BCA Protein Assay Reagent Kit (Pierce). Equal amounts (30 μg) of samples were separated by SDS/12% PAGE and then were electrotransferred to nitrocellulose membrane (Millipore). The membranes were
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Figure 1 Supernatant levels of PGE2, TNF-α, IL-6 and IL-8 in the disc annulus fibrosus cells which were treated with IL-1β or HMGB1

Disc annulus fibrosus cells were treated with 0, 0.5, 1, 2 or 5 ng/ml IL-1β or with 0, 20, 40 or 80 ng/ml HMGB1 for 24 h, then the supernatant levels of PGE2, TNF-α, IL-6 and IL-8 were examined with ELISA methods. (A and B) Supernatant levels of PGE2 and TNF-α (A) or IL-6 and IL-8 (B) in the IL-1β-treated disc annulus fibrosus cells. (C and D): Supernatant levels of PGE2 and TNF-α (C) or IL-6 and IL-8 (D) in the HMGB1-treated disc annulus fibrosus cells. Results are presented as mean ± S.E.M. for quartic independent experiments. P values were calculated by Student t test between two groups, post the ANOVA test for the homogeneity of variance. *P<0.05, **P<0.01, ***P<0.001 or ns: no significance.

RESULTS

IL-1β and HMGB1 additively promotes the inflammatory cytokines release in human intervertebral disc cells

In order to evaluate whether IL-1β and HMGB1 contribute to the inflammatory process in the degenerative human intervertebral disc, we investigated the effect of IL-1β and HMGB1 on human intervertebral disc cells in vitro. Firstly, intervertebral disc cells were incubated with 0, 0.5, 1, 2 or 5 ng/ml recombinant IL-1β

Statistical analysis

Quantitative data were presented as mean ± S.E.M. and was analysed with Student t test or the one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test. P values less than 0.05 were considered significantly. All statistics were performed using Prism (GraphPad Software, version 5).
Disc annulus fibrosus cells were treated with 2 ng/ml IL-1β or (and) with 40 ng/ml HMGB1 for 0, 6, 12, 24 or 48 h, then the supernatant levels of PGE2, TNF-α, IL-6 and IL-8 were examined. Results are presented as mean ± S.E.M. for quartic independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 or ns: no significance.

To elucidate whether there was an additive effect between IL-1β and HMGB1 on such cytokine promotion, we then treated the cells with 2 ng/ml IL-1β or (and) 40 ng/ml HMGB1 for 6, 12, 24 or 48 h for the supernatant cytokine assay. As indicated in Figure 2(A), the PGE2 was promoted from 12 to 48 h post treatment for 2 ng/ml IL-1β and from 24 to 48 h post treatment for 40 ng/ml HMGB1. And such promotion was more significant when cells were treated with both agents (*P < 0.001, Figure 2A), indicating an additive effect. And the promotion to TNF-α, IL-6 or IL-8 was also more significant by the combined treatment with IL-1β and HMGB1 than the treatment with either IL-1β or HMGB1 (*P < 0.05, **P < 0.01 or ***P < 0.001, Figures 2B and 2D). Therefore, IL-1β and HMGB1 additively promotes the inflammatory cytokines release in human intervertebral disc cells.

IL-1β and HMGB1 additively up-regulates the expression of MMPs in human intervertebral disc cells

We then examined the expression of MMPs in the intervertebral disc cells post the treatment with IL-1β or (and) HMGB1.
Involvement of TLR-4 and RAGE in the IL-1β- and HMGB1-induced CK release in human intervertebral disc cells

TLR signalling has been indicated to be active in IL-1/HMGB1-promoted inflammation [29]. In order to deduce the signalling pathway in the IL-1/HMGB1-promoted inflammatory cytokines release in human intervertebral disc cells, we then examined the expression of TLRs in the IL-1β/HMGB1-treated intervertebral disc cells. It was indicated that the expression of both TLR-2 and TLR-4 were markedly up-regulated by 2 or 5 ng/ml IL-1β (P < 0.05, P < 0.01 or P < 0.001, Figures 4A and 4B). However, the TLR-6 expression was not significantly regulated by the IL-1β treatment (Figure 4C). Figure 4(D) demonstrated that the RAGE mRNA level was also markedly up-regulated by 2 or 5 ng/ml IL-1β (P < 0.05 respectively). In addition, we examined the expression of TLR-4 and RAGE in the intervertebral disc cells, post the treatment with 40 ng/ml HMGB1. As indicated in Figures 4(E) and 4(F), the mRNA levels of both receptors were also significantly up-regulated by the HMGB1 treatment (P < 0.05 respectively). Moreover, there was also an additive effect in the TLR-4/RAGE promotion by IL-1β and HMGB1. The mRNA levels of both receptors were higher in the cells with combined treatment with IL-1β and HMGB1 (P < 0.05 or P < 0.01, Figures 4E and 4F). Therefore, both TLR4 and RAGE were additively promoted by IL-1β and HMGB1 in human intervertebral disc cells.

In addition, we knocked down TLR-4 in the in human intervertebral disc cells, and re-evaluated the IL-1β- and HMGB1-promoted release of inflammatory cytokines. As indicated in Figures 5(A)–5(C), the expression of TLR-4 in both mRNA (Figure 5A) and protein (Figures 5B and 5C) levels was markedly reduced in the siRNA-TLR-4 groups (P < 0.01 or P < 0.001, 30 or 60 nM). Figure 5(D) indicated that the promoted PGE2 release by both IL-1β (5 ng/ml) and HMGB1 (80 ng/ml) was significantly inhibited by the transfection with 60 nM siRNA-TLR-4 (P < 0.05 or P < 0.01). In addition, the IL-1β- and HMGB1-promoted release of TNF-α (P < 0.05 or P < 0.01, Figure 5E) and IL-6 (P < 0.05 respectively, Figure 5F) was also reduced in the siRNA-TLR-4 groups. However, such inhibition was not significant for IL-8.
Figure 4  Expression of TLRs and RAGE in mRNA levels in the IL-1\(\beta\)- or/and HMGB1-treated disc annulus fibrosus cells

Disc annulus fibrosus cells were treated with 0, 0.5, 1, 2 or 5 ng/ml IL-1\(\beta\) for 12 h, then the relative mRNA levels of TLR-2 (A), -4 (B), -6 (C) or RAGE (D) to GAPDH were examined with RT-qPCR method; (E and F) relative mRNA levels of TLR-2 and -4 (E), TLR-6 and RAGE (F) to GAPDH in the IL-1\(\beta\)- or (and) HMGB1-treated (for 12 h) disc annulus fibrosus cells. Results are presented as mean ± S.E.M. for quartic independent experiments. \(P\) values in A–D were calculated by the parametric ANOVA (Tukey–Kramer post hoc) test; and \(P\) value in E–F was calculated by Student \(t\) test. \(*P < 0.05\), \(**P < 0.01\), ***\(P < 0.001\) or ns: no significance.
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Figure 5: siRNA-mediated TLR-4 knockdown inhibited the IL-1β- and HMGB1-induced CK release

(A) TLR-4 mRNA level in the disc annulus fibrosus cells, which were transfected with 30 or 60 nM siRNA-Control or siRNA-TLR-4 for 24 h; (B and C) Western blot analysis of TLR-4 in protein level in the siRNA-Control- or siRNA-TLR-4-transfected disc annulus fibrosus cells for 24 h; (D–G) Supernatant levels of PGE2 (D), TNF-α (E), IL-6 (F) or IL-8 (G) in the IL-1β- (5 ng/ml for 24 h) or HMGB1- (80 ng/ml for 24 h) treated disc annulus fibrosus cells. Results are presented as mean ± S.E.M. for quartic independent experiments. P values were calculated by Student t test between two groups, post the ANOVA test for the homogeneity of variance. *P < 0.05, **P < 0.01 or ns: no significance.
**NF-κB signalling was additively promoted by IL-1β and HMGB1 in human intervertebral disc cells**

NF-κB signalling pathway has been indicated to involve in the IL-1β-mediated inflammatory response in osteoblast cells [35] and to be activated by HMGB1 by various types of cells [36,37]. To further identify the signalling pathway underlining IL-1β/HMGB1-promoted inflammatory cytokines release in human intervertebral disc cells, we then examined the activation of NF-κB signalling in the intervertebral disc cells post IL-1β/HMGB1 treatment. Figure 6(A) demonstrated that there was a significantly high NF-κB/p65 mRNA level in the IL-1β-treated or HMGB1-treated intervertebral disc cells (P < 0.05 or P < 0.01); whereas the IκBα mRNA was markedly reduced by the 40 ng/ml HMGB1 (P < 0.05). Moreover, the NF-κB/p65 mRNA up-regulation and the IκBα mRNA reduction were additively influenced by the treatment with both agents (2 ng/ml IL-1β and 40 ng/ml HMGB1) (P < 0.05 or P < 0.01). We then performed western blotting assay to evaluate the protein levels of NF-κB/p65 and IκBα in the IL-1β/HMGB1-treated intervertebral disc cells. As indicated in Figure 6(B), NF-κB/p65 protein level was also up-regulated by 2 ng/ml IL-1β or 40 ng/ml HMGB1 (P < 0.05 respectively), and such up-regulation was more significant by the combined treatment with both agents (P < 0.05 or P < 0.01, Figure 6C). And the IκBα reduction was also recognized in protein level in the IL-1β- or HMGB1-treated intervertebral disc cells, particularly, such reduction was also additive (P < 0.05 respectively). These data indicated that IL-1β and HMGB1 additively activated the NF-κB signalling pathway in intervertebral disc cells.

**DISCUSSION**

IL-1β is one member of IL-1 family, which is closely linked to innate inflammatory and immune responses [11]. And IL-1β is thought to be one of the most strong proinflammation stimulator, promoting the production of multiple proinflammatory mediators such as cytokines, chemokines and MMPs [12–14]. Particularly, IL-1β is indicated to involve in the multiple pathological processes of intervertebral disc degeneration [16]. HMGB1 has been observed to mediate inflammation in multiple injury models [23–25]. Recently, HMGB1 has also been recognized as a potent proinflammatory mediator in degenerated human discs [26]. In the present study, we found that either IL-1β or HMGB1 promoted the inflammatory cytokines release in human intervertebral disc cells, the secretion of PGE2, TNF-α, IL-6 and IL-8 was significantly up-regulated in the supernatant of the intervertebral disc cells post the IL-1β or HMGB1 treatment. And the expression of MMP-1, -3 and -9 in both mRNA and protein levels in the intervertebral disc cells was also up-regulated by the treatment with IL-1β or with HMGB1. Furthermore, there was an additive effect between IL-1β and HMGB1 on such cytokine and MMP promotion. The combined treatment with IL-1β and HMGB1 exerted more significant promotion to PGE2, TNF-α, IL-6 and IL-8. And MMP-1, MMP-3 and MMP-9 in both mRNA and protein levels were also additively up-regulated by both mediators.

Both TLRs and HMGB1 are expressed in isolated human intervertebral disc cells and in native intervertebral disc tissue [38,39]. The present study for the first time indicated that IL-1β and HMGB1 additively promoted the expression of TLR-2, TLR-4 and RAGE, which is another HMGB1 receptor [40] in human
intervertebral disc cells. It has been reported that the initiation of TLR signalling can cause the activation of NF-κB, and then result in the increased expression of proinflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α or MMPs in intervertebral discs [41]. We in the present study recognized the mediation of TLR-4 in the IL-1β- and HMGB1-induced CK release in human intervertebral disc cells. And the activation of NF-κB signalling was confirmed in the IL-1β/HMGB1-mediated additive promotion to inflammatory cytokines in intervertebral disc cells.

Up to now, various types of cytokines have been recognized to be implicated and to contribute to the progression of IDD. However, it is not clear whether there is interplay among these cytokines in such process. The present study for the firstly time recognized such kind of interplay between IL-1β and HMGB1 in intervertebral disc cells. The additive promotion by IL-1β and HMGB1 to inflammatory cytokines might aggravate the progression of IDD.

**CONCLUSION**

In summary, our study demonstrated that IL-1β and HMGB1 additively promotes the release of inflammatory cytokines and the expression of MMPs in human intervertebral disc cells. The receptors such as TLRs and RAGE and the NF-κB signalling were also additively promoted by IL-1β and HMGB1. Our study implied that the additive promotion by IL-1β and HMGB1 to inflammatory cytokines and MMPs might aggravate the progression of IDD.

**AUTHOR CONTRIBUTION**

Fang Fang and Dianming Jiang designed the study, performed the experiments, conceived of the study, drafted the manuscript and performed the statistical analysis. All authors read and approved the final manuscript.

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