Original Article

High-mobility group Box-1 regulates acute myocardial ischemia-induced injury through the toll-like receptor 4-related pathway

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Abstract: High-mobility group box-1 (HMGB1) is a nuclear protein released by necrotic cells as a result of its interactions with several receptors, including the receptor for advanced glycation end-products (RAGE) and members of the toll-like receptor family. HMGB1 has been implicated in autoimmune diseases and hepatic and intestinal ischemia/reperfusion (I/R) injury; however, its role in myocardial ischemia-induced injury remains unclear. In this study, isoproterenol (ISO) was used to establish a myocardial ischemia mouse model. Treating mice with recombinant HMGB1 (rHMGB1) worsened myocardial injury, whereas treating mice with antibodies that neutralized HMGB1 significantly reduced tissue damage. Interestingly, myocardial ischemia severity was not affected by rHMGB1 or HMGB1 antibody administration in toll-like receptor 4 (TLR4)-deficient mice (TLR4-/-), which demonstrated significantly reduced ischemia-induced cardiac tissue damage compared with wild-type (WT) mice. HMGB1 plays an important role in myocardial ischemia-induced injury by binding to TLR4, which results in proinflammatory pathway activation and enhanced myocardial injury. Therefore, blocking HMGB1 or TLR4 may represent a novel therapeutic strategy for treating myocardial ischemia-induced injury.

Keywords: High-mobility group box-1, toll-like receptor 4, myocardial ischemia, ISO, IL-6

Introduction

Myocardial ischemia-induced injury is a complex pathophysiological event that occurs when blood flow to the myocardium is obstructed. Myocardial blood flow obstruction deprives the heart of oxygen, disrupts myocardial energy metabolism, and increases the workload of the heart. The main mechanism underlying myocardial ischemia-induced injury is poorly understood; however, it is widely accepted that apoptotic cell death and inflammatory responses participate in the development of ischemia-induced heart damage [1]. HMGB1 is a novel inflammatory cytokine that has been shown to play an important role in myocardial ischemia-induced injury. A DNA-binding nuclear protein, HMGB1 is produced by virtually every cell type [2-4], is involved in stabilizing the nucleosome and enables DNA bending, which facilitates gene transcription.

Structurally, HMGB1 is composed of 215 amino acid and two DNA-binding domains, namely, HMG Box A and Box B, as well as a negatively charged tail comprising multiple short amino acid sequences. HMG Box B has been reported to recapitulate proinflammatory activity, and some sequence homology between the above-mentioned acidic tail and the receptor for RAGE has been described in previous studies [5].

HMGB1 can be passively released by necrotic cells and actively secreted by inflammatory cells, such as macrophages and natural killer cells, thereby triggering inflammation [4]. The release and effects of HMGB1 are attributable to its interactions with a series of receptors, including the RAGE, toll-like receptor (TLR)-2 and TLR-4 [6-9]. Recent clinical and experimental studies have suggested that acute myocardial ischemia may be caused by interactions between TLR-4 and HMGB1 [10, 11]. TLR4 is an important member of TLR family that can trig-
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The myeloid differentiation factor 88 (MyD-88)-dependent and MyD88-independent (also known as the TIR domain-containing adaptor inducing interferon (IFN)-β (TRIF)-dependent) signaling pathways are thought to be among the signaling pathways initiated by TLR4 and HMGB1 [12, 13]. The MyD88-dependent and MyD88-independent pathways both play critical roles in processes in which TLR4 stimulates the release of inflammatory cytokines. However, only a few studies have examined these pathways in ischemic heart disease. Furthermore, whether myocardial ischemia induces serious acute and chronic heart damage via these pathways has been not been determined [14]. In this study, we used a neutralizing anti-HMGB1 monoclonal antibody that interacts specifically with the C-terminal sequence of HMGB1, which is located within the abovementioned acidic tail, and TLR4-deficient (TLR4-/-) mice to determine the role of the HMGB1-TLR4 axis in the development of acute myocardial ischemia.

Materials and methods

Animals

Six-week-old C57BL/6 wild-type (WT) mice were purchased from Beijing HFK Bio-Technology Co. Ltd. (China), and TLR4-/- mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). All the mice were male and weighed between 15 and 20 g. They were housed in a specific pathogen-free facility and provided food and water ad libitum. The experiments were approved by the Institutional Animal Care and Use Committee of Shehezi Medical College, Shehezi University (Shehezi, China).

Based on the results of previous studies, we used 20 mg/kg isoproterenol (ISO) to induce myocardial ischemia in this study. Twenty-four C57BL/6 and Twenty-four TLR4-/- mice were randomly divided into the following four groups (n=6): a Control group, an ISO group, a recombinant HMGB1 group (rHMGB1 group), and an ISO+anti-HMGB1 group (anti-HMGB1 group). The C57BL/6 and TLR4-/- mice were intraperitoneally injected with isoproterenol. rHMGB1 (10 μg per mouse) was administered intraperitoneally 12 h before myocardial ischemic injury induction [15]. When indicated, neutralizing anti-HMGB1 antibodies (400 μg per mouse) were administered via intraperitoneal injection after ischemic injury induction [16].

Histologic evaluation

Parafformaldehyde-fixed, paraffin-embedded sections of myocardial tissue specimens were stained with hematoxylin and eosin and observed under a light microscope (<400 magnification).

Immunohistochemistry

Immunohistochemical studies were performed using immunoperoxidase methods. Paraffin-embedded sections were cut into 4-μm-thick sections and stained with antibodies against HMGB1 (Wuhan Service Biotechnology Co., LT, Wuhan, China) and TLR4 (Wuhan Service Biotechnology Co., LT, Wuhan, China).
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Western blotting

HMGB1 and TLR4 protein expression levels were determined by western blotting using a primary anti-mouse HMGB1 antibody and an anti-mouse TLR4 antibody. The protein concentration was determined using a BCA protein assay kit (cat. no. 23227; Thermo Fisher Scientific, Inc.). Protein extracted from tissue specimens was separated on 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gels and transferred to nitrocellulose membranes (Pierce, Rockford, IL), which were blocked with 5% nonfat milk in Tris buffered saline for 3 hr and then incubated with the indicated primary antibodies (0.2 Kg/mL) overnight at 4°C before being incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000) for 3 h. GAPDH was used as a loading control for between-sample comparisons.

Real-time PCR

Total RNA was extracted from the abovementioned cardiac muscle samples and purified using a Trizol Reagent Kit (Invitrogen, US), after which 1 µg of isolated RNA was reverse-transcribed into complementary DNA (cDNA) using a cDNA synthesis kit (TaKaRa, Japan), according to the manufacturer’s instructions. Real-time PCR was performed with an ABI Prism 7500 Sequence Detection System (PE Applied Biosystems) using a SYBR Green Real-time PCR Master Mix Kit (TaKaRa, Japan), according to the manufacturer’s instructions [17]. PCR comprised the following steps: pre-denaturation at 95°C for 15 s and combined annealing/extension at 60°C for 60 s. Semilog amplification curves were calculated by the comparative quantification method (2^ΔΔCT), and the β-actin housekeeping gene was used as an internal control. The primer sequences used in this study were as follows: HMGB1, forward primer 5'-CGG ATG CTT CTG TCA ACT TCT C-3' and reverse primer 5'-GTT TCT TCG CAA CAT CAC CAA T-3'; TLR4, forward primer 5'-TGA GGA CTG GGT GAG AAA TGA GC-3' and reverse primer 5'-CTG CCA TGT TTG AGC AAT CTC AT-3'; TNF-α, forward primer 5'-AAC TCC AGG CGG TGC CTA TG-3' and reverse primer 5'-TCC TCC ACT TGG TGG TTT GTG-3'; IL-6, forward primer 5'-CCC CAA TTT CCA ATG CTC TCC-3' and reverse primer 5'-CGC ACT AGG TTT GCC GAG TTA-3'; and β-actin, forward primer 5'-GCG ACT AGG TTT GCC GAG TTA-3' and reverse primer 5'-GCA TCA CTA GAA GCA C-3'.

Statistics

Data are presented as the mean ± SD. Differences were evaluated using one-way ANOVA for multiple comparisons followed by Student’s-Newman-Keuls’ post hoc test when necessary. All analyses were performed using SPSS 13.0 (SPSS, Chicago, IL), and a P value <0.05 was considered significant.

Results

Histopathological changes in heart tissue

The myocardial fibers of the tissue specimens from the WT and TLR4-/- mouse control groups...
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Figure 3. HMGB1 protein and mRNA expression levels were measured by real-time PCR (A) and western blotting (B) in WT and TLR4-/- mice. \(^{a}P<0.05\) compared with WT-control group. \(^{b}P<0.05\) compared with WT-ISO group. \(^{c}P<0.05\) compared with TLR4-/-control group. \(^{d}P<0.05\) vs TLR4-/-ISO group.

(Figure 1) were well organized, and the nuclei displayed normal staining. In contrast, the myocardial fibers of the tissue specimens from the WT mouse myocardial ischemia groups displayed partial rupture and lysis. Several red blood cells were present, and local inflammatory cell infiltration into the interstitium had occurred, as had pyknosis. The myocardial fibers of the tissue specimens from the TLR4-/- myocardial ischemia groups were swollen and ruptured, and slight interstitial edema was present.

**HMGB1 immunohistochemistry**

HMGB1 expression, which was indicated by the presence of diffuse brown-yellow staining in cardiac muscle cells, was observed in the myocardial tissue samples of all eight groups (Figure 2). The areas of HMGB1 expression in the myocardial tissue samples from the WT and TLR4-/- mouse myocardial ischemia groups were significantly larger than those in the myocardial tissue samples from the WT and TLR4-/- mouse control groups. HMGB1 protein expression levels in the myocardial tissues from the TLR4-/- myocardial ischemia groups were not significantly different from those from the TLR4-/- control group and were significantly decreased compared with those from the WT myocardial ischemia groups.

**HMGB1 and TLR4 mRNA and protein expression levels**

HMGB1 mRNA and protein levels were significantly increased in myocardial ischemia model mice compared with control mice. In both WT and TLR4-/- mice, HMGB1 mRNA and protein levels were significantly higher in the ISO and rHMGB1 groups than in the control group (Figure 3). The areas of HMGB1 expression in
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Figure 4. TLR4 levels in ischemic myocardial tissue samples from WT mice. A. TLR4 mRNA expression was assessed by real-time PCR. B. TLR4 protein expression was measured by western blotting. Original representative western blots are reported in the upper panel. The data from four independent experiments are shown and expressed as the mean ± SD. *P<0.05 compared with WT-control group. **P<0.05 compared with WT-ISO group.

Tissue necrosis factor-α (TNF-α) and interleukin (IL)-6 are key mediators of the pathophysiology of cardiac ischemic injury. TNF-α and IL-6 mRNA expression levels in the heart tissues of mice were analyzed using quantitative real-time PCR. TNF-α and IL-6 mRNA expression levels in WT myocardial ischemia mice were significantly increased compared with those in WT control mice. However, TNF-α and IL-6 mRNA expression levels in TLR4-/- myocardial ischemia mice were not different from those in TLR4-/- control mice (Figure 5).

Discussion

This study revealed that the HMGB1-TLR4 signaling pathway plays a crucial role in acute myocardial ischemia-induced
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Figure 5. Cardiac TNF-α and IL-6 mRNA levels in each group of WT and TLR4-/- mice are shown. The results were obtained by RT-PCR and expressed as relative increases in mRNA expression compared with control animals. **P<0.05 compared with WT-control group. ***P<0.05 compared with WT-ISO group.

injury. HMGB1 mediates inflammatory responses in a TLR4-dependent manner. That is, TLR4 promotes inflammatory responses, which aggravate cardiac injury. Our data indicate that endogenous HMGB1 is an early mediator of acute myocardial ischemia damage. HMGB1 expression levels were elevated after the successful establishment of a myocardial ischemia model. To analyze the molecular mechanisms underlying HMGB1 activation, we investigated the TLR4 signaling pathways known to be involved in HMGB1-dependent signaling and myocardial ischemia-induced injury.

In recent years, it has become apparent that HMGB1 is involved in tissue damage and infection and is passively released from necrotic cells to serve as an endogenous danger signal [4]. A highly conserved DNA binding protein, HMGB1 is also secreted extracellularly by activated macrophages and dendritic cells to produce cytokines, such as TNF-α, IL-1β, IL-6, and IL-17A. In the nucleus, HMGB1 participates in nucleosome construction and stability maintenance and also regulates gene transcription. Outside the nucleus, HMGB1 mediates inflammatory reactions and promotes cell differentiation and tumor growth. Native secreted HMGB1 also has a unique ability to self-amplify and prolong the inflammatory response [18]. HMGB1 performs the above functions through interactions with TLRs and the RAGE. TLRs, which can recognize molecules such as danger-associated molecular patterns, are a family of transmembrane proteins that can bind to a variety of microbial products and can also recognize endogenous ligands to mediate the secretion of cytokines and the generation of natural immune responses [19]. TLR4 is an important member of the TLR family that can trigger downstream signal-transduction pathways in response to tissue damage [20].

Activation of TLR4 results in activation of NF-κB, which plays a critical role in the release of inflammatory factors [21]. NF-κB is usually activated through two downstream pathways, namely, the MyD88-dependent and MyD88-independent pathways. The MyD88-dependent pathway causes the release of various inflammatory factors, such as IL-1, IL-6, IL-10, and TNF-α, mainly through the activation of NF-κB, while the MyD88-independent pathway depends mainly on the activation of IFN regulatory factor 3, followed by the expression of IFN-β and IFN-induced genes and the activation of transcription factors (NF-κB) that induce the synthesis and release of inflammatory factors.

Previous clinical and experimental studies have demonstrated that HMGB1 is involved in several diseases, including systemic lupus erythematosus, autoimmune diabetes, arthritis, sepsis, IR injury and cancer [22-25]. Recent studies have found that HMGB1 also plays a pathogen-
ic role in myocardial ischemia-induced injury [26, 27].

Some clinical studies have shown that higher peak serum HMGB1 levels were associated with cardiac rupture, pump failure and in-hospital cardiac death [28, 29]. Consistent with the findings of previous studies, we found that HMGB1 expression levels were significantly elevated in both WT and TLR4-knockout myocardial ischemia mice, as demonstrated by real-time PCR and western blotting.

However, recent studies have confirmed that extracellular HMGB1 is also associated with tissue repair and angiogenesis [30-32]. Kita-hara et al. reported that left ventricular remodeling and cardiac decompensation were significantly inhibited, survival rates were significantly increased, and capillary and arteriole formation was decreased, as demonstrated by immunohistochemistry, in transgenic mice overexpressing HMGB1 compared with control mice. Limana et al. also reported that direct injections of exogenous HMGB1 into infarcted myocardial tissue near infarcted areas increased myocardial cell numbers. All of these findings suggest that HMGB1 may be a predictor of adverse outcomes after some types of ischemic injury but may also be required for appropriate healing processes and for preserving the structural integrity of injured hearts.

To date, the positive and negative effects of HMGB1 in ischemic heart disease have not been elucidated. Specifically, the mechanisms by which extracellular HMGB1 regulates proinflammatory signaling pathways and promotes tissue repair are unclear. Differences in the results of previous studies have prevented the elucidation of these mechanisms; however, we may have gained new insights into the mechanisms underlying the effects of HMGB1 through analysis of the results of this study. Previous studies have demonstrated that neutralizing antibodies against HMGB1 ameliorate the inflammatory response and organ injury [33]. For example, Takashi et al. treated MI rats with neutralizing anti-HMGB1 antibodies subcutaneously for 7 days. TNF-α and IL-1β mRNA expression levels and the numbers of macrophages in the infarcted area were reduced in antibody-treated rats compared with control rats. Interestingly, HMGB1 blockade resulted in thinning and expansion of the infarct scar, as well as significant hypertrophy of the non-infarcted area.

Our experimental results were similar to those mentioned above. HMGB1 levels were significantly reduced in antibody-treated mice compared with rHMGB1 mice. In addition, there were no significant differences in HMGB1 expression levels between WT and TLR4-/- mice. TLR4 mRNA and protein levels were significantly up-regulated in WT rHMGB1 mice compared with WT ISO mice. TLR4 expression levels showed the same trend as HMGB1 expression levels. That is, as HMGB1 expression levels increased or decreased, TLR4 expression levels also increased or decreased. These findings indicate that HMGB1-induced inflammatory responses are mediated at least in part by TLR4. Our results also show that the levels of downstream inflammatory cytokines, including TNF-α and IL-6, in mice subjected to myocardial ischemic injury were significantly increased compared with those of control mice. Interestingly, the expression levels of TNF-α and IL-6 were not significantly increased in TLR4-/- myocardial ischemia mice compared with TLR4-/- control mice. Administering HMGB1 antibody prior to ischemia induction resulted in significant attenuation of inflammatory factor and HMGB1 mRNA and protein expression in treated mice compared with control mice. Based on these observations, we hypothesized that the HMGB1-TLR4 axis plays an important role in myocardial ischemia-induced damage.

In conclusion, our study has provided evidence that HMGB1 participates in myocardial ischemia-induced injury via a TLR4-related pathway, which plays an important role in the link between the innate and adaptive immune responses. As the precise molecular mechanisms underlying the effects of HMGB1-TLR4 signaling have not been completely elucidated, and the role of the HMGB1 in ischemic injury remains unclear, additional investigations are needed. A better understanding of these mechanisms may ultimately lead to the development of therapies aimed at preventing myocardial ischemia injury and facilitating immunologic tolerance by ameliorating the inflammatory response.

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Disclosure of conflict of interest

None.

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