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Targeting HMGB1 ameliorates cardiac fibrosis through restoring TLR2-mediated autophagy suppression in myocardial fibroblasts☆

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ABSTRACT

Background: Extracellular high-mobility group box 1 (HMGB1) has been identified as playing a critical role in the pathogenesis of tissue fibrosis. However, the underlying mechanism of its involvement in cardiac fibrosis is still not well-defined. Here, we aim to investigate whether toll-like receptor 2 (TLR2) contributes to the extracellular HMGB1-mediated development and progression of cardiac fibrosis.
Methods: A mouse model of cardiac fibrosis was induced by subcutaneous injection of isoproterenol (ISO). Glycyrrhizic acid (GA), an inhibitor of HMGB1 derived from natural products, was simultaneously administered by intraperitoneal injection. Echocardiography, H&E and Sirius red staining were used to evaluate cardiac function and fibrosis. The myocardial expression of autophagy-associated proteins was examined using immunoblotting. Cardiac fibroblasts were treated with different concentrations of HMGB1 to examine the expression levels of α-SMA, collagen I and autophagy markers. Interactions of HMGB1/TLR2 and α-SMA/p62 were examined by immunoprecipitation and immunofluorescence.
Results: ISO-treated mice showed characteristic cardiac fibrosis, increased expression and co-localization of HMGB1 and TLR2, as well as impaired autophagic signals in myocardial tissues, which could be prevented by silencing TLR2. Exogenous administration of HMGB1 blocked the autophagic flux in fibroblasts, which caused extensive accumulation of collagen I and α-SMA. In addition, cardiac fibrosis was alleviated by GA treatment through abrogating the interaction between HMGB1 and TLR2.
Conclusions: Our study suggests that the interaction between TLR2 and HMGB1 contributes to the pathogenesis of cardiac fibrosis via suppressing fibroblast autophagy, and that inhibiting HMGB1 with GA provides therapeutic benefits for the treatment of fibroproliferative heart diseases.

1. Introduction

Cardiac fibrosis is associated with several cardiovascular disorders, such as ischaemic and dilated cardiomyopathy, hypertrophic and diabetic cardiomyopathy, valvular cardiopathy, cardiac amyloidosis, and thalassemia, as well as heart transplantation [1–3]. With persistent heart oxidative stress and inflammatory damage, cardiac fibrosis may turn into a major determinant, resulting in myocardial remodeling and dysfunction, thereby predisposing individuals to heart failure and re-entry arrhythmias [4,5]. Molecular mechanisms involved in the development of cardiac fibrosis include inflammation, mitochondrial damage, myocardial apoptosis, collagen deposition, cardiac fibroblast proliferation and phenotype-conversion of fibroblasts to myofibroblasts [6–8]. However, the underlying mechanisms remain ambiguous, and no effective strategy has been established to prevent the progression of cardiac fibrosis.

High mobility group box protein 1 (HMGB1) is a highly conserved nuclear protein that functions as a divergent biological mediator inside and outside almost all eukaryotic cells [9]. In the nucleus, HMGB1 usually acts as a non-histone chromatin-binding protein to modulate gene transcription or to stabilize the structures of DNA and nucleosomes.

☆ All the authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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Upon cell injury, HMGB1 can be passively released from necrotic or damaged cells into the extracellular space, where it appears to be involved in the initiation of non-infective inflammation and regulation of apoptosis and autophagy [10]. A recent in vivo study revealed a diffuse myocardial expression of HMGB1 in the myocardium, which further exacerbated LV dysfunction and remodeling, and increased collagen deposition in diabetic mice. High glucose levels were shown to induce HMGB1 translocation and secretion in both cardiomyocytes and fibroblasts in vitro, which suggests that HMGB1 is an important mediator in fibrosis and a potential therapeutic target from a clinical perspective [11]. Accumulated evidence indicates that extracellular HMGB1 functions as a damage-associated molecular pattern (DAMP) molecule to stimulate PRRs, such as TLR2 and TLR4, on immune and resident cells to induce chronic inflammatory and immune responses [12]. The biological function of the activation of the HMGB1-TLR4 pathway is well-studied [13–15]. However, the interaction of HMGB1 and TLR2 is still poorly characterized to date.

Autophagy is a mechanism for degradation of dysfunctional cellular components inside cells [16]. Specifically, misfolded proteins and damaged organelles are engulfed by double-membrane-bound structures called autophagosomes, followed by their degradation in autolysosomes, the fused vesicles of autophagosomes and lysosomes. In basal conditions, autophagy is present at low levels in all cells and plays an important housekeeping role. Under stress conditions, such as starvation or hypoxia, autophagy is activated as a mechanism to promote cell survival by energy production and elimination of damaged organelles. However, excessive activation of autophagy triggers cell death. Basal levels of autophagy in the heart are important to preserve cardiomyocyte function and survival [17,18]. However, the role of stress-induced impairment of autophagy in cardiac diseases is quite complex [19,20]. The effect of autophagy, whether detrimental or protective, depends on the pathophysiological context [21]. The role of autophagy in cardiac fibrosis is yet to be established.

The implications of autophagy in the induction of a fibrotic response open a novel area for investigation of therapeutic targets against cardiac fibrosis and heart failure. We recently found that silencing TLR4 protects against isoproterenol (ISO)-induced cardiac fibrosis by modulation of autophagy [22]. We thus hypothesized that TLR2 signaling might also be involved in the pathogenesis of cardiac fibrosis. Our present study demonstrates that TLR2 activity critically participates in the pathogenesis of cardiac fibrosis by mediating extracellular HMGB1-dependent autophagy suppression in fibrotic heart tissues, and that targeting HMGB1 with glycyrrhizic acid has brilliant therapeutic potential against the development of cardiac fibrosis.

2. Methods

2.1. Materials

The mouse recombinant HMGB1 protein was purchased from Biolegend (San Diego, CA). The neutralizing mouse TLR2 mAb was obtained from R&D Systems (Minneapolis, MN). Anti-mouse α-SMA, Collagen I and HMGB-1 Abs were purchased from Abcam (Cambridge, MA). Anti-mouse GAPDH, p-mTOR and mTOR Abs were obtained from Cell Signaling Technology (Danvers, MA). Anti-mouse LC3 II/I and p62 were purchased from Sigma (St. Louis, MO). Alexa Fluor 488 and 647 Abs were obtained from Invitrogen (San Diego, CA). Other materials were purchased from commercial sources.

2.2. Ethics statement

All mice were housed in a facility with a 12-hour/12-hour light/dark cycle and given free access to water and standard rodent chow. The animal protocol was approved by the Institutional Animal Care and Use Committee at the Capital Medical University and conformed to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.3. Animal models and treatments

Eight-week-old male TLR2 knockout (KO) mice (C57BL/6J background) were obtained from the Model Animal Research Center at the Nanjing University, and male C57BL/6J (wild-type) mice were purchased from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The average weight was 20.5 g, and no significant differences were observed between the WT and the TLR2 KO mice. The mice were randomly divided into the following 6 groups: WT; WT with ISO; TLR2 KO; TLR2 KO with ISO; WT with glycyrrhizic acid (GA); WT pre-treated with GA and then ISO. The WT mice were treated with saline. Cardiac fibrosis was induced by daily subcutaneous injection of ISO in the shoulder (7 mg/kg, 3 times a day, for 15 days) [23]. In the GA group, intraperitoneal injection of GA (10 mg/kg/d for 15 days) was administered 1 h prior to ISO injection [24]. Fifteen days after the initial injection, echocardiography was performed for preliminary assessment of the cardiac fibrosis model before the mice were sacrificed. The mice were euthanized by an overdose of pentobarbital (i.p., 100 mg/kg) injection, and the hearts were harvested and sliced into several sections for the indicated analyses, as described below.

2.4. Animal echocardiography

Mice were anesthetized with intraperitoneal 4% chloral hydrate by weight, and the heart rate was controlled at approximately 500 bpm to acquire measurements under physiologically relevant conditions. Cardiac function was evaluated individually by estimation of the LV ejection fraction (LVEF), LV fractional shortening (LVFS), and the LV internal diastolic diameter (LVIDd), using a Vevo 770 high resolution imaging system (VisualSonics, Canada) with a 30-MHz image transducer [25].

2.5. Morphological and histological evaluation

The hearts were rapidly harvested and fixed with 4% paraformaldehyde and embedded in paraffin for histopathological examination. Tissue sections (3 μm thick) were stained with haematoxylin and eosin (H&E) or Sirius red, according to standard procedures. A Sirius red-positive area was measured in ten fields per slide ($n = 6$) and quantified using the Image-Pro Plus 6.0 software.

2.6. Cardiac fibroblasts isolation and culture

Adult mouse cardiac fibroblasts isolation was performed in a Langendorf apparatus as described previously with minor modifications [26]. Briefly, male C57BL/6J mouse was anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and heparinized (10,000 U/kg, i.p.). After dissected from the thorax, the heart was perfused with modified Krebs–Henseleit buffer for 5 min to wash out any residual blood, as well as an enzyme solution (0.7 mg/mL collagenase type II, 0.2 mg/mL hyaluronidase, 0.1% BSA and 25 mM $CaCl_2$) for 10 min to efficiently digest the extracellular matrix. Subsequently, $CaCl_2$ solution (100 mM) was added into the perfusion buffer for additional 5 min. Heart was then removed from the Langendorf apparatus and the atria, pericardium and any leftover fatty tissues were carefully removed with forces. The heart tissue was then mechanically dissociated and filtered into a single cell suspension. Gravity sedimentation is performed to remove cardiomyocytes, centrifuge the supernatant containing fibroblasts at 250g for 10 min. The cell pellet was resuspend in 8 mL of M199 medium with 10% FBS, and seeded on a cell culture dish. One hour later, the attached fibroblasts were washed with pre-warmed PBS for 3 times and then cultured in M199 medium, supplemented with 10% FBS and penicillin/streptomycin. All primary cells were passaged every 7 days.

2.7. Immunoblotting

Both cell and tissue proteins were extracted by homogenizing samples in 1 × RIPA buffer with 1 mmol/L phenyl methyl sulfonyl fluoride (PMSF) and protease inhibitor cocktail. Protein concentrations were determined using the Coomassie Plus reagent. The total protein (40 μg) was separated by SDS-PAGE and transferred on to PVDF membranes. The membranes were incubated overnight with indicated primary antibodies and then incubated with horseradish peroxidase-conjugated secondary antibodies, followed by signal detection with an enhanced chemiluminescence detection system (Amersham Bio-sciences, USA).

2.8. Co-immunoprecipitation

For the co-immunoprecipitation experiments, protein lysates were extracted from the heart or cell homogenates with 1 × RIPA buffer supplemented with 1 mmol/L PMSF and protease inhibitor cocktail. The p62 antibody was added to 500 μL of diluted lysates, and incubated overnight on a rotary wheel at 4 °C. Protein A/G PLUS agarose beads (Santa Cruz, CA) were added and incubated for 2 h on a rotary wheel at 4 °C. The agarose beads were washed 5 times with 1 × RIPA buffer, solubilized in 2 × SDS sample buffer, denatured at 95 °C for 10 min, and processed for immunoblotting.

2.9. Confocal microscopy

Frozen heart sections (5 μm thick) or cardiac fibroblasts cultured on glass cover slips were fixed and permeabilized for 10 min in 4% paraformaldehyde and 10 min in 0.2% Triton X-100. Nonspecific binding was blocked with 3% BSA for 45 min at room temperature (RT), and then the sections or cover slips were incubated with the indicated primary antibody (dilution 1:100) overnight at 4 °C. After washing twice with PBS, the sections or cover slips were incubated with the fluorescein-labelled secondary antibody (dilution 1:200) in dark for 1 h at 37 °C. The nuclei were stained with DAPI for 40 min at RT. Images were acquired using a confocal microscope (Leica Microsystems, USA) and analysed with the Leica confocal software.