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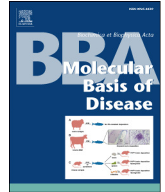

Toll-like receptor 3 plays a role in myocardial infarction and ischemia/reperfusion injury

Chen Lu [a,1], Danyang Ren [a,1], Xiaohui Wang [a], Tuanzhu Ha [a], Li Liu [b], Eric J. Lee [a], Jing Hu [a], John Kalbfleisch [c], Xiang Gao [d], Race Kao [a], David Williams [a], Chuanfu Li [a,*]

[a] Department of Surgery, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN 37614, USA
[b] Department of Geriatrics, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China
[c] Department of Biometry and Medical Computing, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN 37614, USA
[d] Animal Model Research Center, Nanjing University, Nanjing 210093, China

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ABSTRACT

Innate immune and inflammatory responses mediated by Toll like receptors (TLRs) have been implicated in myocardial ischemia/reperfusion (I/R) injury. This study examined the role of TLR3 in myocardial injury induced by two models, namely, myocardial infarction (MI) and I/R. First, we examined the role of TLR3 in MI. TLR3 deficient (TLR3$^{-/-}$) and wild type (WT) mice were subjected to MI induced by permanent ligation of the left anterior descending (LAD) coronary artery for 21 days. Cardiac function was measured by echocardiography. Next, we examined whether TLR3 contributes to myocardial I/R injury. TLR3$^{-/-}$ and WT mice were subjected to myocardial ischemia (45 min) followed by reperfusion for up to 3 days. Cardiac function and myocardial infarct size were examined. We also examined the effect of TLR3 deficiency on I/R-induced myocardial apoptosis and inflammatory cytokine production. TLR3$^{-/-}$ mice showed significant attenuation of cardiac dysfunction after MI or I/R. Myocardial infarct size and myocardial apoptosis induced by I/R injury were significantly attenuated in TLR3$^{-/-}$ mice. TLR3 deficiency increases Bcl2 levels and attenuates I/R-increased Fas, FasL, FADD, Bax and Bak levels in the myocardium. TLR3 deficiency also attenuates I/R-induced myocardial nuclear factor KappaB (NF-κB) binding activity, TNF-α and IL-1β production as well as I/R-induced infiltration of neutrophils and macrophages into the myocardium. TLR3 plays an important role in myocardial injury induced by MI or I/R. The mechanisms involve activation of apoptotic signaling and NF-κB binding activity. Modulation of TLR3 may be an effective approach for ameliorating heart injury in heart attack patients.

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1. Introduction

Cardiovascular disease is the number one killer in the United States [27]. Each year, an estimated 785,000 Americans will have a new coronary attack, 470,000 will have a recurrent attack and 195,000 Americans will have silent myocardial infarctions [27]. Despite extensive investigation, the cellular and molecular mechanisms that are involved in the initiation and progress of myocardial injury in response to ischemia/reperfusion (I/R) are still unclear.

Innate immune and inflammatory responses mediated by Toll-like receptors (TLRs) have been demonstrated to be involved in the pathophysiology of myocardial I/R injury [3,23]. TLRs are pattern recognition receptors that play an important role in the induction of innate immune and inflammatory responses [21,42]. TLR-mediated signaling predominately activates nuclear factor KappaB (NF-κB) which is an important transcription factor regulating the expression of genes associated with innate immunity and inflammatory responses as well as cell growth, cell survival, and cell death [21,42]. We and others have reported that TLR4 deficiency or modulation of TLR4 mediated signaling decreases myocardial injury following I/R [1,3,4,13,17,23].

TLR3 is located in intracellular endosomes and recognizes double-stranded RNA (dsRNA) and polyinosinic–polycytidylic acid (Poly I:C, a synthetic analog of dsRNA), resulting in induction of antiviral immune responses [20]. Recently, Cavassani et al. reported that TLR3 deficient (TLR3$^{-/-}$) mice showed an increased survival rate in cecal ligation and puncture induced sepsis [2]. We have shown that TLR3$^{-/-}$ mice exhibit protection against polymicrobial sepsis-induced cardiac dysfunction [10]. These data suggest that TLR3 plays an important role in cardiac function during sepsis. However, whether TLR3 contributes to myocardial injury induced by myocardial infarction or I/R has not been investigated. It is possible that TLR3 plays a role in myocardial ischemic injury by recognition of endogenous ligands, i.e. damage-

* Corresponding author at: Department of Surgery, East Tennessee State University, Campus Box 70575, Johnson City, TN 37614-0575, USA. Tel.: +1 423 439 6215; fax: +1 423 439 6259.
E-mail address: Li@etsu.edu (C. Li).
[1] The authors equally contributed to this work.

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associated molecular patterns (DAMPs) that are released during myocardial I/R injury.

We hypothesized that TLR3 contributes to myocardial injury by recognition of DAMPs during myocardial I/R. To evaluate our hypothesis, we examined the role of TLR3 in myocardial injury induced by either permanent ligation-induced myocardial infarction (MI) or ischemia/reperfusion (I/R) using TLR3 deficient ($TLR3^{-/-}$) mice. We observed that TLR3 deficiency significantly attenuates myocardial dysfunction induced by both models, i.e. MI and I/R. TLR3 deficiency also reduces infarct size and myocardial apoptosis after I/R injury. Our data indicate that TLR3 plays an important role in myocardial ischemic and I/R injury.

2. Materials and methods

2.1. Animals

TLR3 knockout mice ($TLR3^{-/-}$) and wild type (WT) genetic background control mice (C57BL/6) were obtained from Jackson Laboratory (Indianapolis, IN) [10]. The mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University (ETSU). The experiments outlined in this article conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication, 8th Edition, 2011). All aspects of the animal care and experimental protocols were approved by the ETSU Committee on Animal Care.

2.2. Models of myocardial infarction (MI) and ischemia/reperfusion (I/R) injury

Myocardial infarction was induced by permanent ligation of the left anterior descending (LAD) coronary artery as described previously [18]. Myocardial I/R injury was induced as described previously [11,13,17,38]. Briefly, $TLR3^{-/-}$ and age-matched WT male mice (26–28 g body weight) were anesthetized by 5.0% isoflurane inhalation, intubated and ventilated with room air using a rodent ventilator. Anesthesia was maintained by inhalation of 1.5% isoflurane driven by 100% oxygen flow. Body temperature was regulated at 37 °C by surface water heating. Following the skin incision, the hearts were exposed through a left thoracotomy in the fourth intercostal space. For induction of MI, the LAD coronary artery was permanently ligated with 8-0 silk ligature [18]. For induction of I/R injury, the LAD coronary artery was ligated with 8-0 silk ligature that was tied using a 'shoestring knot' over a 1 mm polyethylene tube (PE-10). After completion of 45 min of occlusion, the coronary artery was reperfused by pulling on the exteriorized suture to release the knot. Cardiac function was measured by echocardiography [26,38]. After completion of the experiments, the mice were euthanized by CO_2 inhalation and the hearts were harvested.

2.3. Evaluation of myocardial infarct size

Myocardial infarct size was evaluated by triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining as described previously [11,13,17,38]. Briefly, the hearts were perfused with saline on a Langendorff system to wash blood from the coronary vasculature. The LAD coronary artery was re-ligated at the previous site of ligation prior to staining with 1% Evans Blue in order to assess area at risk. Each heart was then sliced horizontally to yield five slices. The slices were incubated in 1% TTC for 15 min at 37 °C, fixed by immersion in 10% neutral buffered formalin. The area of infarction on both sides of each slice was determined by an image analyzer, corrected for the weight of each slice, and summed for each heart. Ratios of risk area vs. left ventricle area (RA/LV) and infarct area vs. risk area (IA/RA) were calculated and expressed as a percentage.

2.4. Echocardiography

Transthoracic two-dimensional M-mode echocardiogram and pulsed wave Doppler spectral tracings were obtained using a Toshiba Aplio 80 Imaging System (Toshiba Medical Systems, Tochigi, Japan) equipped with a 12-MHz linear transducer as described previously [26,38]. Percent fractional shortening (%FS) and percent ejection fraction (%EF) were calculated as described previously [26,38]. All measurements were made by one observer who was blinded with respect to the identity of the tracings. All data were collected from 10 cardiac cycles.

2.5. Western blot

Western blots were performed as described previously [11,13,17,38]. Briefly, the cellular proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ). The membranes were incubated with appropriate primary antibody anti-Fas (CD95), anti-FasL, anti-FADD, anti-vascular cell adhesion molecule-1 (VCAM-1), anti-intercellular adhesion molecule-1 (ICAM-1), anti-Bcl-2, anti-Bax, and anti-Bak (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and anti-phospho-IκBα (Cell Signaling Technology, Inc., Danvers, MA), respectively, followed by incubation with peroxidase-conjugated second antibodies (Cell Signaling Technology) and analysis by the ECL system (Amersham Pharmacia, Piscataway, NJ). To control for lane loading, the same membranes were probed with anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, Biodesign, Saco, Maine) after being washed with stripping buffer. The signals were quantified using the Syngene G: BOX gel imaging system (Syngene, USA, Frederick, MD).

2.6. In situ apoptosis assay

Myocardial apoptosis was examined as described previously [11,13,17,38] using the In Situ Cell Death Detection Kit, Fluorescein (Roche, USA). Briefly, the hearts were harvested and sliced cut horizontally. One slice was immersion-fixed in 4% buffered paraformaldehyde, embedded in paraffin and cut at 5 μm thick. The sections were incubated with the commercially prepared labeling mixture supplied by the manufacturer at 37 °C for 1 h. Nuclei of living and apoptotic cells were counterstained with Hoechst 33342 (Invitrogen). Three slides from each block were evaluated for percentage of apoptotic cells and four fields on each slide were examined at the border areas using a defined rectangular field area with 20× magnification. The numbers of apoptotic cardiac myocytes are presented as a percentage of total cells counted.

2.7. Caspase-activity

Caspase-3/7 and -8 activities in heart tissues were measured as described previously [19] using a Caspase-Glo assay kit (Promega).

2.8. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were isolated from heart samples as previously described [11,13,17,38] and NF-κB binding activity was measured using a LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific, Waltham, MA) according to the instructions of the manufacturer.

2.9. ELISA quantification of cytokines

The levels of inflammatory cytokines (TNF-α and IL-1β) in the serum were assessed by ELISA (PeproTech, Rocky Hill, NJ) according to the instructions provided by the manufacturer [10,24,38].

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