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Original article

Cardioprotection of recombinant human MG53 protein in a porcine model of ischemia and reperfusion injury

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ABSTRACT

Ischemic heart disease is a leading cause of death in human population and protection of myocardial infarction (MI) associated with ischemia–reperfusion (I/R) remains a challenge. MG53 is an essential component of the cell membrane repair machinery that protects injury to the myocardium. We investigated the therapeutic value of using the recombinant human MG53 (rhMG53) protein for treatment of MI. Using Langendorff perfusion of isolated mouse heart, we found that I/R caused injury to cardiomyocytes and release of endogenous MG53 into the extracellular solution. rhMG53 protein was applied to the perfusion solution concentrated at injury sites on cardiomyocytes to facilitate cardioprotection. With rodent models of I/R-induced MI, we established the *in vivo* dosing range for rhMG53 in cardioprotection. Using a porcine model of angioplasty-induced MI, the cardioprotective effect of rhMG53 was evaluated. Intravenous administration of rhMG53, either prior to or post-ischemia, reduced infarct size and troponin I release in the porcine model when examined at 24 h post-reperfusion. Echocardiogram and histological analyses revealed that the protective effects of rhMG53 observed following acute MI led to long-term improvement in cardiac structure and function in the porcine model when examined at 4 weeks post-operation. Our study supports the concept that rhMG53 could have potential therapeutic value for treatment of MI in human patients with ischemic heart diseases.

1. Introduction

Ischemic heart disease caused by coronary arteriosclerosis remains as the single largest cause of mortality in western countries and is increasingly common throughout the rest of the world. As a result of arteriosclerosis or cardiac surgery, blockade of blood flow leads to acute myocardial infarction (MI) that is associated with two types of myocardial damage, including ischemic injury induced by the initial loss of blood flow and reperfusion injury by the restoration of oxygenated blood flow [1–4]. Ischemia–reperfusion of the heart is thought to generate oxidative stress that opens the mitochondrial permeability transition pore leading to apoptosis; and lipid peroxidation leading to breakdown of the sarcolemmal membrane and cell necrosis [5–7]. While there are interventions available to reestablish coronary perfusion and to treat arrhythmias associated with MI, there are no effective treatments available to directly prevent or alleviate I/R-induced cardiomyocyte injury [8–13]. Elucidation of cardiac membrane repair mechanisms would provide insights into the etiology of myocardial remodeling and guide development of new strategies to reduce cardiomyocyte loss and minimize myocardial fibrosis.

Plasma membrane repair is of particular importance in the heart because cardiomyocytes are terminally differentiated cells with limited self-renewal capability [14]. Cardiomyocytes suffer transient membrane injuries under physiological conditions and can be exacerbated by various pathophysiological stresses [15]. In a recent series of studies,

Abbreviations: MG53, mitsugumin53 (also known as TRIM72); rhMG53, recombinant human MG53; MI, myocardial infarction; I/R, ischemia–reperfusion; TTC, triphenyltetrazolium chloride; CEMS, Chinese experimental miniature swine; LVEF, left ventricular ejection fraction; FS, fractional shortening; IVSs, systolic inter-ventricular septal thickness; LVPWs, left ventricular posterior wall thickness; NB-T, nitro blue tetrazolium; BS-ECG, body surface electrocardiogram; TUNEL, terminal dUTP nick end-labeling

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we discovered MG53, a tripartite motif (TRIM)-family protein, that is an essential component of the cell membrane repair machinery [16–18]. MG53 functions in vesicle trafficking and allows for nucleation of intracellular vesicles at sites of membrane disruption, and is uniquely positioned to protect against MI associated with ischemic heart diseases. Increased vulnerability to ischemia–reperfusion induced injury to the heart was observed in mouse with genetic ablation of MG53 [19,20]. AAV-mediated delivery of MG53 gene into animal models of muscular dystrophy and cardiomyopathy could rescue certain aspects of the defective muscle and heart function [21]. While overexpression of MG53 could improve membrane repair defects in certain disease conditions, the gene therapy-based approaches necessary to pursue this effort have disadvantages. In particular, since myocardial ischemia is an acute disease that requires immediate treatment, molecular manipulations that target expression of the intracellular MG53 protein may not be practical for treatment of MI.

We recently showed that disruption of the cell membrane leads to exposure of a signal to the external leaflet of the plasma membrane that can be detected by MG53, allowing recombinant MG53 protein to repair membrane damage when provided in the extracellular space [22]. Using several *in vivo* animal model studies, we found that intravenous delivery of the recombinant MG53 protein can repair membrane damage to skeletal muscle and lung epithelial cells and ameliorate the pathology associated with muscular dystrophy [22] and acute lung injury [23]. Here we show that recombinant human MG53 (rhMG53) protein has therapeutic value for treatment of MI involving I/R injury to the heart. We provide both *ex vivo* and *in vivo* data to suggest that application of rhMG53 either prior to ischemia or post-ischemia can protect injury to the myocardium in the porcine model of cardiac injury.

2. Methods

2.1. Langendorff perfusion of mouse hearts

Wild type mouse (C57BL6/J) hearts were subjected to global ischemia/reperfusion (I/R) during Langendorff perfusion. Hearts were perfused with Krebs buffer at a flow rate of 2 ml/min and allowed to equilibrate for 30 min before the Krebs buffer was supplemented with rhMG53 (40 μg/ml) or equimolar concentration of bovine serum albumin (BSA) as a control. Perfusion flow was ceased 5 min after the addition of protein and the heart was maintained in an ischemic state for 30 min. To induce I/R injury, the heart was reperfused for 60 min before it was removed from the apparatus and stained using triphenyltetrazolium chloride (TTC) to indicate infarct area using standard techniques [24]. In separate studies, rhMG53 was applied to the perfusate after the mouse heart had undergone 30 min of ischemia, in order to test the protective effect of rhMG53 against reperfusion-induced injury to the cardiomyocytes.

For immunohistochemistry studies, MBP-MG53 was used in perfusate in order to differentiate endogenous and exogenous MG53 during immunostaining. At the end of 60 min of reperfusion, the perfusion solution was changed from Kreb's solution containing MBP–MG53 to a solution containing FITC conjugated Annexin V (Annexin V-FITC) (BioLegend, Inc. San Diego, CA) and perfused for 1 more min. Then the hearts were fixed with perfusion of 4% paraformaldehyde for 10 min to remove unbinding Annexin V from the heart tissue. The hearts were longitudinally cut into half and embedded using optimal cutting temperature compound (OCT) for frozen sectioning. The slides were stained with antibody against MBP for confocal microscopy imaging of colocalization of Annexin V and MBP-MG53.

2.2. Purification of recombinant human MG53 protein

Purification of the recombinant human MG53 (rhMG53) protein has been described previously [22]. The present study employed two different forms of MG53 protein, MBP-MG53 and untagged rhMG53. Untagged rhMG53 was produced by cleavage of MBP from MBP-MG53 using thrombin digestion and separation of these two using gel filtration high pressure liquid chromatography. Untagged rhMG53 was lyophilized and stored at 4 °C as dry powder in a desiccator. The membrane protective activity of rhMG53 from each preparation was determined by our established micro-glass bead injury assay as described elsewhere [18,22].

2.3. Cardiomyocyte live cell imaging

Ventricular myocytes were enzymatically isolated from the hearts of adult male mice (12–14 weeks) following the protocol of Wang et al. [19]. The freshly isolated cardiomyocytes were plated onto coated Delta T dishes (Bioptechs inc. Butler, PA) with HEPES buffer containing (in mmol/L): 137 NaCl, 5.4 KCl, 20 HEPES, 1.8 $CaCl_2$, 15 D-glucose, 1.3 $MgSO_4$, 1.2 NaH_2PO_4, (pH 7.4). rhMG53 and BSA were conjugated with FITC (Lightning-Link® FITC, Innova Biosciences Ltd. Cambridge, UK) and added into dishes containing cardiomyocytes to a final concentration of 25 μg/ml. A Zeiss LSM780 confocal microscope was used for live cell imaging of the translocation of FITC-labeled rhMG53 or FITC-labeled BSA. The FITC signal was recorded at a rate of 3.13 s/frame.

2.4. Porcine model of angioplasty induced myocardial infarction

Chinese experimental miniature swine (CEMS), weighing 15 ± 2.5 kg, were provided by Beijing Experimental Animal Reproduction and Regulation Center (Grade II,Certificate No. Jing-030). All animal experiments in this study were performed in accordance with China Academy of Chinese Medical Sciences Guide for Laboratory Animals that conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. Experimental pigs underwent balloon inflation of the left anterior descending (LAD) coronary artery according to established methods [25,26] with minor modifications. Briefly, pigs were anesthetized with intravenous injection of pentobarbital (30 mg/kg) through a marginal ear vein. Animals were intubated and ventilated with a digital ventilator (SC-3, Shanghai Medical Equipment Factory), and continuously monitored for their reflexes, electrocardiography (ECG) and respiratory status. The right common carotid artery was surgically exposed, and an 8F sheath was placed in the carotid artery. Each animal was given a single dose of heparin (150 U/kg) *via* the arterial sheath. A coronary artery catheter was advanced to engage with the left main coronary artery under direct fluoroscopic imaging. A coronary angiogram was performed to define the anatomy of LAD. Angioplasty was accomplished by inflating a 2.5–3.5-mm balloon (1:1.2–1.3 balloon-to-artery ratio) to 8–10 atm in the LAD artery distal to the second diagonal branch. The balloon was deflated and withdrawn to allow reperfusion in distal LAD. Reperfusion was confirmed by ST-segment alterations on the ECG. The detailed operation procedures were illustrated in the Supplemental Movie S2. Administration of rhMG53 at different times of experimental interventions was achieved through the jugular vein. Whenever possible, experimental procedure and data analysis were conducted in a double-blinded manner.

2.5. Echocardiographic imaging in CEMS

During the ischemia/reperfusion surgery as well as different time points after surgery, echocardiograph was performed to evaluate cardiac morphology and function (Philips Medical System, Holland). Left ventricular ejection fraction (LVEF), fractional shortening (FS), systolic interventricular septal thickness (IVSs) and left ventricular posterior wall thickness (LVPWs) were evaluated to determine the global function of the left ventricle and the regional functional and structural changes of the myocardium. LVEF was determined from the apical two- and four-chamber views by using a modified Simpson's algorithm [27]. Regional wall thickness was measured at the end-systole (the end of T wave of

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