Journal of Molecular and Cellular Cardiology 77 (2014) 86–101

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

MMI-0100 inhibits cardiac fibrosis in myocardial infarction by direct actions on cardiomyocytes and fibroblasts via MK2 inhibition

Lei Xu [a], Cecelia C. Yates [b], Pamela Lockyer [c], Liang Xie [d], Ariana Bevilacqua [c], Jun He [c,e], Cynthia Lander [f], Cam Patterson [d,g,h,i], Monte Willis [c,i,*]

[a] Department of Cardiac Surgery, Shandong Provincial Hospital affiliated to Shandong University, PR China
[b] Department of Health Promotions and Development, School of Nursing, University of Pittsburgh, Pittsburgh, PA, USA
[c] Department of Pathology & Laboratory Medicine, University of North Carolina, Chapel Hill, NC, USA
[d] Department of Medicine, University of North Carolina, Chapel Hill, NC, USA
[e] General Hospital of Ningxia Medical University, Yichuan, Ningxia, PR China
[f] Moerae Matrix, 55 Madison Avenue Suite 400, Morristown, NJ 07960, USA
[g] Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC, USA
[h] Department of Pharmacology, University of North Carolina, Chapel Hill, NC, USA
[i] McAllister Heart Institute, University of North Carolina, Chapel Hill, NC, USA

ARTICLE INFO

Article history:
Received 26 March 2014
Received in revised form 7 September 2014
Accepted 10 September 2014
Available online 1 October 2014

Keywords:
Apoptosis
Cardiac ischemia
Fibrosis
MAPKAP kinase 2
MK2
MMI-0100

ABSTRACT

The cell-permeant peptide inhibitor of MAPKAP kinase 2 (MK2), MMI-0100, inhibits MK2 and downstream fibrosis and inflammation. Recent studies have demonstrated that MMI-0100 reduces intimal hyperplasia in a mouse vein graft model, pulmonary fibrosis in a murine bleomycin-induced model and development of adhesions in conjunction with abdominal surgery. MK2 is critical to the pathogenesis of ischemic heart injury as MK2$^{-/-}$ mice are resistant to ischemic remodeling. Therefore, we tested the hypothesis that inhibiting MK2 with MMI-0100 would protect the heart after acute myocardial infarction (AMI) in vivo. AMI was induced by placing a permanent LAD coronary ligation. When MMI-0100 peptide was given 30 min after permanent LAD coronary artery ligation, the resulting fibrosis was reduced/prevented ~50% at a 2 week time point, with a corresponding improvement in cardiac function and decrease in left ventricular dilation. In cultured cardiomyocytes and fibroblasts, MMI-0100 inhibited MK2 to reduce cardiomyocyte caspase 3/7 activity, while enhancing primary cardiac fibroblast caspase 3/7 activity, which may explain MMI-0100's salvage of cardiac function and anti-fibrotic effects in vivo. These findings suggest that therapeutic inhibition of MK2 after acute MI, using rationally-designed cell-permeant peptides, inhibits cardiac fibrosis and maintains cardiac function by mechanisms that involve inhibiting cardiomyocyte apoptosis, while enhancing primary cardiac fibroblast cell death.

1. Introduction

Ischemic heart disease is the most common cause of death in the world; in the United States alone, an estimated 785,000 people will have a myocardial infarction (MI) each year, approximately 1 per minute [1]. The adverse remodeling that occurs after MI contributes to the impaired function and heart failure that commonly develops post-MI. Interventional advances—largely early reperfusion therapies—have improved patient survival, but the adverse remodeling processes that lead to heart failure proceed unabated [2–4]. The size of the infarcted area, the infarcted wound healing, and chronic left ventricular (LV) remodeling determine the extent of the resulting heart failure [2–4]. To minimize the extent of heart failure after a large or recurrent MI, therapeutic strategies are needed to limit infarct wound healing in the early phase.

Use of rationally designed cell-permeant peptides that inhibit Mitogen Activated Protein Kinase Activated Protein Kinase II (MK2) activity and downstream fibrosis and inflammation is a unique approach. Recent studies have reported that the cell-permeant peptide MMI-0100 inhibits inflammation and fibrosis (intimal hyperplasia) in a mouse vein graft model [5], bleomycin-induced pulmonary fibrosis [6] and abdominal adhesions post-surgery [7]. These peptide drugs target the substrate-binding site of MK2, are carried into cells via cell-permeant domains and are rapidly taken up by macropinocytosis and targeted to endosomal compartments, where they are retained for up to 7 days [8]. MK2 is critical for both fibrosis and inflammation; therefore, MK2-driven processes central to the exuberant cardiac fibrosis and cytokine release that occur post-myocardial infarction remodeling represent an excellent therapeutic target.

* Corresponding author at: McAllister Heart Institute, Department of Pathology & Laboratory Medicine, University of North Carolina, 111 Mason Farm Road, MBRB 2340B, Chapel Hill, NC 27599, USA. Tel.: +1 919 843 1938; fax: +1 919 843 4585.
E-mail address: monte_willis@med.unc.edu (M. Willis).

http://dx.doi.org/10.1016/j.yjmcc.2014.09.011
0022-2828/

Myocyte death during lethal myocardial infarction, cardiac dysfunction, and fibrosis during post-MI remodeling and hypertrophy are associated with sustained activation of p38 [9–11]. Recent studies in MK2$^{-/-}$ mice have illustrated that MK2 acts downstream of p38 and is responsible for p38-induced heart failure [12]. Similarly, MK2$^{-/-}$ mice are resistant to ischemia reperfusion injury [13], indicating a critical role of MK2 in ischemic heart disease experimentally. Based on these recent findings, the present study tested the hypothesis that MMI-0100 therapy *post*-myocardial infarction would inhibit the extent of fibrosis in vivo. We demonstrated that MMI-0100 reduced fibrosis that developed after 2 weeks in a standard murine myocardial infarction model induced by permanent ligation of the left anterior descending (LAD) coronary artery. Since cardiomyocyte cell death, fibroblast differentiation to myofibroblasts and the secretion of a variety of extracellular matrix proteins, including collagen (resulting in fibrosis) are impacted by MK2, we determined whether MMI-0100 confers cardioprotective benefits by acting on both cell types independently in vitro. We found that MMI-0100 inhibits MK2 activity in both cardiac-derived cells (H9C2 and HL-1) and in primary rat cardiac fibroblasts, inhibiting cardiomyocyte caspase 3/7 activity, while enhancing fibroblast caspase 3/7 activity in vitro. These studies report for the first time that the cell-permeant peptide MMI-0100 can inhibit fibrosis associated with myocardial infarction, while illustrating mechanisms by which inhibition of MK2 in turn inhibits cardiomyocyte apoptosis and reduces fibrosis by direct effects on cardiac fibroblasts.

2. Materials and methods

2.1. Cell permeant peptide synthesis and delivery

The MMI-0100 peptide (YARAAARQARAKALARQLGVAA) was synthesized using standard Fmoc chemistry, as previously described [14]. MMI-0100 (MW = 2283.67 g/mol; Moerae Matrix, Inc.) was prepared and delivered daily intraperitoneally in PBS (50 μg/kg), as previously described [6]. In cell line studies, the peptide was dissolved in DMSO before adding to the cell media (final [0.5%] to target peptide intracellularly), as previously described [7], to give a final MMI-0100 concentration of 20 μM or 100 μM.

2.2. Animals and myocardial infarction (MI) model

Twelve week-old male C57BL/6 mice (25–30 g) were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in the University of North Carolina at Chapel Hill facilities for at least 7 days with free access to standard rodent food and water. Myocardial infarction was induced by permanent ligation of the left anterior descending (LAD) coronary artery as described previously [15,16]. Post-surgery, mice were immediately treated with lidocaine (6 mg/kg IM) and atropine (0.04–0.10 mg/kg IM) upon surgical closure, followed by lidocaine and atropine every 2–4 h for the first 24 h to prevent arrhythmias. Post-anesthesia, mice were given 0.1 mg/kg buprenorphine every 12 h for the first 48 h. Within the first hour post-MI, 50 μg/kg/day MMI-0100 peptide (or PBS control) was given intraperitoneally and repeated for a total of 14 days. In parallel, control groups underwent: 1) a sham operation that included every step except the coronary artery ligation; 2) daily MMI-0100 (50 μg/kg/day) intraperitoneally for 14 days. Cardiac function was measured by conscious echocardiography using a Vevo 2100 ultrasound biomicrscopy system (VisualSonics, Inc., Toronto, Canada) at baseline, 7, and 14 days, as previously described [17–19].

2.3. Histological analysis of fibrosis

Mice were euthanized by isoflurane and cervical dislocation at day 14, fixed in fresh 4% paraformaldehyde for 24 h, paraffin-embedded, processed, and stained with standard hematoxylin and eosin (H&E) and Masson's trichrome (MT). Starting at the ligation with fully faced tissue, 14–15 levels were cut on each block at 50 μm (one slide for H&E, one for MT, and 3 unstained; 50 μm skipped and then repeated). Controls were similarly cut starting at a comparable level. The area of fibrosis was analyzed in 3–4 blindly chosen hearts, each heart at 14–15 levels (point of ligation to apex), 3 sections at each level. Analysis of collagen was performed blinded to treatment on these 42–45 sections per heart. Slides were scanned using an Aperio ScanScope (Aperio Technologies, Vista, CA) and analyzed using Aperio ImageScope. The Algorithm Positive Pixel Count v9 was used to measure the Masson's trichrome staining of collagen (representing both fibrosis and collagen in extracellular matrix). Hue value (0.66) and hue width (0.1) were used analyzed the tissue outlined using the pen tool. Each section was analyzed and exported. The *N* positive/*N* total value (representing the % collagen of the entire section) was used to determine a weighted average for each slide.

2.4. Immunofluorescence staining of cardiac histological sections for vimentin, αSMA, TGF-β1 and TUNEL

Immunostaining was performed as described previously [20,21]. Cardiac sections adjacent to histological levels 1 and 7 (of 14-see histological analysis of fibrosis) were stained with antibodies against α-SMA (1:250, Abcam, Cambridge, MA), Vimentin (1:100, Santa Cruz, Dallas, TX), and TGF-β-1 (1:100, Abcam), or an irrelevant isotype mouse, rabbit or goat IgG (as a negative control) at 4 °C overnight. Slides were then incubated with Alexa Fluor 488-conjugated secondary antibodies and counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA).

Identification of apoptosis was determined in histological sections by identifying the presence of fragmented DNA by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using the Roche TUNEL in situ staining kit (Roche Molecular Biochemicals, Basel, Switzerland), according to the manufacturer's instructions. To detect DNA fragmentation associated with apoptosis, we used a fluorescence-based TUNEL followed by counterstaining with 4,6-diamidino-2-phenylindole (DAPI). Histological sections treated with a recombinant DNase I to allow TUNEL labeling of all nuclei were used as positive controls.

2.5. Cell culture of primary cardiac fibroblast cells and cardiomyocyte cell lines

The H9C2 is a myoblast cell line derived from rat myocardium obtained from ATCC® (CRL-1446, ATCC, Manassas, VA) and cultured according to the recommended protocols. Briefly, cells (p2) were maintained at 37 °C with 5% CO2 in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) and split at a ratio of 1:4 using 0.05% trypsin every 36 h. HL-1 cells were obtained from Dr. William Claycomb and cultured according to the published protocols [22,23]. Briefly, cells (p67) were cultured in Claycomb medium (JRH Biosciences, USA) supplemented with 10% fetal bovine serum (JRH Biosciences), 2 mM L-glutamine (Gibco, Grand Island, NY), 100 μM norepinephrine (Sigma, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) in flasks precoated with fibronectin and gelatin (Sigma), then incubated at 37 °C in 5% CO_2. Cells were split at a ratio of 1:4 using 0.05% trypsin every 48 h. Primary cardiac fibroblasts were obtained from 2–4-day-old Sprague Dawley® rats, according to previously described protocols (primary cardiomyocyte isolation kit, cat.#LK003300, Worthington Biochemical Corp., Lakewood, NJ) [24,25]. Harvested fibroblasts (p2) were seeded in 10 cm FALCON polystyrene dishes (BD Biosciences), and incubated for 45 min in DMEM with 10% fetal bovine serum and antibiotics. Cardiomyocytes that did not attach to the non-coated plates were rinsed away and the remaining fibroblasts were given fresh medium, grown to confluence, trypsinized (0.05%) and passaged twice before being used in experiments.