International Journal of Cardiology 167 (2013) 2546–2554

Contents lists available at ScienceDirect

International Journal of Cardiology

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

Thrombopoietin improved ventricular function and regulated remodeling genes in a rat model of myocardial infarction ☆

Kathy Yuen Yee Chan [b,1], Ligang Zhou [c,1], Ping Xiang [d,1], Karen Li [a,b,1], Pak Cheung Ng [b], Chi Chiu Wang [a,e], Ming Li [f], Nga Hin Pong [b], Liu Tu [g], Haiyan Deng [h], Carrie Ka Lai Kong [b], Rita Yn Tz Sung [a,b,*]

[a] Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong
[b] Department of Paediatrics, The Chinese University of Hong Kong, Hong Kong
[c] Neonatal Intensive Care Unit, Chongqing Health Center for Women and Children, Chongqing, China
[d] Department of Cardiology, Children's Hospital of Chongqing Medical University, Chongqing, China
[e] Department of Obstetrics & Gynaecology, The Chinese University of Hong Kong, Hong Kong
[f] Department of Physiology, The Chinese University of Hong Kong, Hong Kong
[g] Department of Physiology, Chongqing Medical University, Chongqing, China
[h] Department of Cardiology, Children's Hospital of Fudan University, Shanghai, China

ARTICLE INFO

Article history:
Received 8 November 2011
Received in revised form 3 May 2012
Accepted 9 June 2012
Available online 6 July 2012

Keywords:
Thrombopoietin
Myocardial infarction
Microarray profiling
Cardiac remodeling
Endothelial progenitor cells

ABSTRACT

Background: Thrombopoietin (TPO) protects against heart damages by doxorubicin-induced cardiomyopathy in animal models. We aimed to investigate the therapeutic efficacy of TPO for treatment of myocardial infarction (MI) in a rat model and explored the mechanisms in terms of the genome-wide transcriptional profile, TPO downstream protein signals, and bone marrow endothelial progenitor cells (EPCs).
Methods: Sprague–Dawley rats were divided into 3 groups: Sham-operated, MI (permanent ligation of the left coronary artery) and MI + TPO. Three doses of TPO were administered weekly for 2 weeks, and outcomes were assessed at 4 or 8 weeks post-injury.
Results and conclusions: TPO treatment significantly improved left ventricular function, hemodynamic parameters, myocardium morphology, neovascularization and infarct size. MI damage upregulated a large cohort of gene expressions in the infarct border zone, including those functioned in cytoskeleton organization, vascular and matrix remodeling, muscle development, cell cycling and ion transport. TPO treatment significantly reversed these modulations. While phosphorylation of janus kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3) and protein kinase B (AKT) was modified in MI animals, TPO treatment regulated phosphorylation of STAT3 and extracellular signal-regulated kinases (ERK), and bone morphogenetic protein 1 (BMP1) protein level. TPO also increased EPC colonies in the bone marrow of MI animals. Our data showed that TPO alleviated damages of heart tissues from MI insults, possibly mediated by multi-factorial mechanisms including suppression of over-reacted ventricular remodeling, regulation of TPO downstream signals and mobilization of endothelial progenitor cells. TPO could be developed for treatment of cardiac damages.

1. Introduction

Thrombopoietin (TPO) is an endogenous cytokine known to promote hematopoietic progenitor cells and the megakaryocytic/platelet lineage. TPO mimetics have recently been approved for treatment of thrombocytopenia [1–3]. In previous studies, we provided evidence that TPO reduced cardiac damage caused by acute or chronic treatments of doxorubicin in animal models [4,5]. In these animals, TPO significantly improved ventricular performance and ameliorated severe changes in gene expressions relevant for maintenance of heart functions, including apoptosis, blood vessel and matrix remodeling, cell division, ion channels and contractile proteins [4]. The protective mechanism might also involve phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and p42/p44 extracellular signal-regulated kinases (ERK 1/2) activation and bone marrow endothelial progenitor cell (EPC) induction [4]. Since these mechanisms are strongly implicated in heart damages caused by myocardial infarction (MI) in human and animal models [6–9], we pursued to investigate the efficacy of TPO treatment in a model of ischemic cardiomyopathy and the associated expression profile using a genome-wide microarray assay. Our results provided the first evidence that TPO improved

☆ Grant support: This project was supported by the Li Ka Shing Institute of Health Sciences Grant, The Chinese University of Hong Kong, and Earmarked Grant CUHK4521/05 M and Direct Grant 2008.1.070 from the Research Grant Council of Hong Kong.
* Corresponding author at: Department of Paediatrics, The Chinese University of Hong Kong, 6th Fl, Clinical Sciences Block, The Prince of Wales Hospital, Shatin, NT, Hong Kong. Tel.: +852 2632 7823; fax: +852 2636 0020.
E-mail address: yntzsung@cuhk.edu.hk (R.Y.T. Sung).
[1] These authors contributed equally to this manuscript.

0167-5273/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.ijcard.2012.06.038

ventricular morphology and functions post-MI, possibly mediated by down-regulation of excessive remodeling responses.

2. Materials and methods

2.1. Rat model of MI

The study was approved by the Animal Research Ethics Committee, The Chinese University of Hong Kong, Hong Kong and in compliance with the ethical policy of US National Institutes of Health (NIH A5613-01). Male Sprague–Dawley rats weighing 260–300 g (Laboratory Animal Services Centre, The Chinese University of Hong Kong) were allocated into 3 treatment groups: Sham-operated, MI and MI + TPO. Animals were subjected to left coronary artery ligation or sham surgery under anesthesia with single injection of i.p. ketamine (Alfasan, Woerden, Holland) and xylazine (Alfasan) at 75 mg/kg and 10 mg/kg, respectively. This dosage of anesthesia was adequate to prevent the animals from conscious activity and response to external stimuli. To MI + TPO animals, TPO (PeproTech, Rocky Hill, NJ) was administered at 10 μg/kg i.p. immediately after induction of MI, and 3 doses weekly for 2 weeks. The Sham-operated and MI animals were given saline under the same schedule. At week 4 or week 8, two-dimensional echocardiography and hemodynamic parameters [5,10] were measured on animals under anesthesia with i.p. ketamine (75 mg/kg) and xylazine (10 mg/kg). Animals were sacrificed after the echocardiography or hemodynamic procedure by cervical dislocation and under anesthesia. Heart tissues at the MI border zone were then harvested for morphology, capillary density, molecular and protein analyses. Endothelial progenitor cells in the bone marrow of the animals were also determined.

2.2. Heart function assessment by echocardiography

The left ventricular (LV) function of animals was evaluated at baseline (1 day before any treatment) and before sacrifice at week 4 or week 8 [4,5]. Transthoracic echocardiography was performed on animals under anesthesia, using Sonos 7500 (Philips Ultrasound, MA) with a 7.5–12 MHz probe. M-mode echocardiography of the LV was conducted at the papillary muscle level, as guided by two-dimensional short-axis images. Left ventricular end-diastolic dimensions (LVEDD) and left ventricular end-systolic dimensions (LVESD) were measured on M-mode tracings. The LV fractional shortening (% FS) was calculated as [(LVEDD − LVESD)/LVEDD] × 100. Cardiac output (CO) was calculated by the M-mode Teichholz formula.

2.3. Hemodynamic measurements

Animals were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg), and a microtip pressure transducer catheter (Millar Instruments, Houston, TX) was inserted into the left ventricle. Various hemodynamic parameters, including indices of contractility and relaxation, the maximal rates of increase and decrease in the left ventricular pressure (dp/dt + and dp/dt −) were recorded using the MacLab instrument (AD Instruments, NSW, Australia) [10].

2.4. Infarct size

Heart tissues were fixed in 4% formaldehyde, and 4 μm-thick paraffin sections were stained with Masson reagent (accustain trichrome) (Sigma) [11]. The infarct size was measured by the SPOT Advanced Software (Diagnostic Instruments, Sterling Heights, MI, USA) and presented as the average percentage of infarct epicardial length/epicardial circumference. Results are expressed as the average of 6 sections per heart.

2.5. Capillary density

Heart tissues were fixed in 4% paraformaldehyde. Five micrometer sections were stained with α-smooth muscle actin antibody (SMA, Chemicon, Millipore, Billerica, MA, USA) at 1:500 dilution overnight at 4 °C in a humidified chamber. The slides were then incubated with a secondary antibody-linked HRP polymer (Zymed, San Francisco, CA, USA) for 45 min. Signals were developed in DAB solution (Zymed, San Francisco, CA, USA). The sections were counterstained with Mayer hematoxylin. The control slide was processed with all reagents except the primary antibody. Arteriolar length density was calculated from 3 randomly selected fields at the MI border zone and septum, in a blinded manner [12].

2.6. Electronic microscopy

At week 4 post-MI, cardiac tissues were immersion-fixed overnight in 2.5% phosphate-buffered glutaraldehyde (pH 7.4), postfixed for 1 h with 1% osmium tetroxide, dehydrated through a graded ethanol series, and embedded in Epon medium. Ultrathin sections were stained with uranyl acetate and lead citrate. Ultrastructural changes of cardiomyocyte, myofibril arrangement and mitochondria were observed by transmission electron microscopy (CM120, Philips, The Netherlands).

2.7. Bone marrow EPC

Bone marrow mononucleated cells were cultured on fibronectin (Invitrogen, Carlsbad, CA) at 5×10^6 cells per mL IMDM, supplemented with murine growth factors (PeproTech): vascular endothelial growth factor, 20 ng/mL and basic-fibroblast growth factor, 5 ng/mL. After 48 h, non-adherent cells were transferred to fresh medium. Adherent (endothelial cell colony-forming unit, CFU-EC) and non-adherent cells (endothelial colony forming cells, ECFC) were further cultured for 7 days and colonies were counted under light microscopy as previously described [4].

2.8. RNA extraction, microarray and pathway analysis

Heart tissues collected at week 4 were homogenized in TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and purified using an RNeasy Mini kit (Qiagen, Valencia, CA) as previously described [4]. RNA samples were labeled using the Agilent Low Input Linear Amplification Labeling Kit (Agilent Technologies Inc., Palo Alto, CA). The Cy3-labeled cRNA (1.65 μg) was hybridized onto rat whole genome Agilent microarrays (4 × 44 K format, Agilent) for 17 h at 65 °C. The slides were scanned on an Agilent DNA Microarray Scanner (Agilent). The extracted feature intensities from each array were processed by the GeneSpring software version 10 (Silicon Genetics, San Carlos, CA) for normalization and $\log_2$ transformation. Per chip and per gene normalizations were done according to Agilent's recommendation using a 75th percentile shift and baseline transformation at the median of each array. Differentially expressed gene lists were generated with a two-fold cutoff between comparisons of SHAM versus MI and MI versus MI + TPO groups. Pathway analysis of differentially expressed genes including Agilent probe ID and log fold change values was performed using the MetaCore software (GeneGo, St. Joseph, MI). The gene ontology annotations of the differentially expressed genes were assigned using the web-based tool version 6 (http://david.abcc.ncifcrf.gov/) [13]. A link has been created to allow review of the microarray data GSE22489: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=lxothmwckakksni&acc=GSE22489.

2.9. Quantitative RT-PCR

The identified targets were validated in the same set of RNA samples and an independent cohort of samples was validated by quantitative RT-PCR (qPCR) using predesigned TaqMan assays and PDH (pyruvate dehydrogenase) as the endogenous control [4,14]. Amplification was performed for 40 cycles with denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min. The emission intensity was detected by the ABI real-time system 7300 (Applied Biosystems). Relative quantification values expressed as threshold cycle (Ct) were averaged and subsequently used to determine the relative expression ratios between cases.

2.10. Western-blot analysis

Frozen heart tissues harvested at 4 weeks post-MI were powdered in liquid nitrogen and suspended in radioimmunoprecipitation assay lysis buffer (Sigma Chemical Co, St. Louis, MO) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). The samples were homogenized and the lysates were centrifuged at 14,000 rpm for 15 min, and the supernatant was stored at − 80 °C. The protein concentration was measured by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin as a standard. Protein samples were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane (Amersham International, Buckinghamshire, England) for antibody binding. Specific antibodies against phospho-ERK, total ERK, phospho-AKT, total AKT, phospho-JAK2, total JAK2, phospho-STAT3, total STAT3 were products of Cell Signaling Technology Inc. (Boston, MA), whereas those against BMP-1 and biglycan were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The blots were visualized by means of chemiluminescence (ECL, Amersham), and the signals were quantified by densitometry. The membranes were reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which served as the loading control as described previously [15]. Expression levels are presented as the ratio of phospho-specific kinases to total kinases, or relative to those of GAPDH expression.

2.11. Statistical analysis

Survival rates of animals were compared by the log rank test. Heart and endothelial progenitor cell parameters were compared by the Mann–Whitney test. Statistical analysis of the microarray data were performed by GeneSpring software. One-way Analysis of Variance (ANOVA) test with a P value <0.01 was considered significant and the *Post-Hoc* analysis was performed using Student Newman–Keuls (SNK) test to reveal the difference between groups ($P<0.05$). Data with P values of <0.01 or expressions with a fold change of greater or less than 2 were further analyzed. mRNA and protein expression data were analyzed by the Mann–Whitney U test using SPSS version 17.0 (SPSS Inc, Chicago, IL). Data were presented as mean ± standard deviation (SD).

The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology.