Biomedicine & Pharmacotherapy 106 (2018) 303–308

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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

Effect of interleukin-6 on myocardial regeneration in mice after cardiac injury

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A R T I C L E I N F O

Keywords:
Interleukin-6
Mouse
Cardiac regeneration
Cardiomyocyte
Proliferation

A B S T R A C T

Our aim was to investigate the role of interleukin-6 (IL-6) in myocardial regeneration from mice after cardiac injury. The newborn mice were divided into the following 4 groups (16 in each group): sham group, model group, IL-6-/- group (IL-6 knockout) and IL-6 group (IL-6 overexpression). Electrocardiography was performed on all mice and found higher LVEDD, LVESD and IVST and lower LVEF and LVFS in the IL-6 group compared with the sham group. Using HE staining, severe myocardial injury combined with infarction and fibrosis were observed in the IL-6-/- group, while the damaged myocardial tissue was repaired to some extent in the IL-6 group. The expression of IL-6 in the IL-6 group were significantly up-regulated. BrdU immunofluorescence found that the IL-6-/- group had the least number of BrdU positive cells, while the IL-6 group had more BrdU positive cells than the model group and the IL-6-/- group. Expressions of IL-6, cyclinD1 and Bcl-2 in the IL-6 group were up-regulated compared with other groups. In conclusion, IL-6 overexpression could enhance cardiomyocyte proliferation and relevant protein expression in mice myocardium, thus promoting cardiac regeneration.

1. Introduction

Cardiomyocytes can die as one ages and diseases such as hypertension, heart disease and myocardial infarction can lead to massive apoptosis of this type of cell [1–3]. Currently, there is no effective method for treating severe cardiac diseases except for heart transplantation. However, the transplantation is hard to be carried due to its high cost and organ shortage [4,5]. Although some treatment methods have been developed to decrease the mortality of patients with cardiovascular disease, incidences of heart failure and myocardial infarction still remain quite high. Therefore, exploring an effective way for myocardial regeneration following cardiac injury has also become an important topic for medical workers and researchers.

Most studies have believed that cardiomyocyte, as a terminally differentiated cell, is hard to proliferate after cardiac tissue damage. When a person enters adolescence, cardiomyocyte can begin to resynthesize DNA, but loses the abilities of cytokinesis and karyokinesis [6]. A study has argued that mature heart tissues can still be regenerated at a certain rate, even though the rate is only about 1% and decreases with age; in addition, proliferation of cells located in the area where myocardial infarction occurs is even more difficult, hence it would be quite impossible to restore cardiac function through cardiomyocyte proliferation and regeneration [7]. However, in studies using lower animals, such as zebrafish for apical resection model, it was found that blood clots and fibrin deposition would first appear in apical area after cardiac injury, and cardiac regeneration would be achieved through epicardial activation and cardiomyocyte proliferation [8–10]. Also, some studies which explored the role of interleukin-6 (IL-6) in the regeneration of transplanted livers in mice have reported that exogenous IL-6 can promote liver regeneration in IL-6 knockout mice, which provides some useful guidance for the study of liver transplantation in mice [11,12]. However, the mechanism of IL-6 in the heart of newborn mice is still unclear. In order to elucidate the role of IL-6 in cardiac regeneration in newborn mice, we constructed apical resection mouse model, and explored the effect of IL-6 in promoting cardiomyocyte proliferation through IL-6 overexpression.

2. Materials and methods

2.1. Animal model construction

IL-6 knockout mice (normal B6 strain, IL-6-/-, B6. 129S2-116tmlKopf/J), purchased from Jackson Laboratory (JAX, USA), were used as study subjects. They were fed, mated and bred in the Animal Experiment Center of Fuwai Hospital. Seventy-five normal newborn mice and 25 IL-6 knockout newborn mice were selected for the study.

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https://doi.org/10.1016/j.biopha.2018.06.090
Received 24 May 2018; Accepted 14 June 2018
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Surgeries were operated around one day after their birth. Sex ratio couldn't be set as genders were unidentifiable in newborn mice. The animal experiments were conducted in compliance with *Declaration of Helsinki*.

Apical resection mouse model was constructed in both normal newborn mice (n = 50, randomly) and IL-6 knockout newborn mice (n = 25). The rest normal newborn mice (n = 25) were selected as control group (sham group). Baby mice (one day after birth) were placed on an ice bed for anesthesia for 3 min. Next, they were fixed on an operating table. Incision was made through skin from the 4th intercostal space on the left side and blunt dissection of subcutaneous tissue and muscles was performed using microsurgical scissors to allow access to pleura. A small amount of apical tissue was picked up using a microsurgical tweezer and cut with a microsurgical scissor. Criteria for a successful surgery were as follows: left ventricular cavity was opened for the excision; the weight of the excised myocardial tissue was around 15% of the total ventricle weight; a quick suturing using surgical suture 8/0 was performed to close the incision. Afterwards, the mice were rewarmed by exposure to thermal radiation for about 10 min. When animals were fully recovered from anesthesia and back to basic activities, they were put back to the cage where maternal mice were. Mice in the control group didn't receive any apical resection. After four weeks following the surgery, echocardiography was performed in mice using cardiac ultrasound system (SonoAce X8, Shanghai Jumu Medical Instruments Co., Ltd.). Model was constructed successfully if the left ventricular ejection fraction (LVEF) was ≤45%, otherwise, the mice were excluded from the study.

2.2. Plasmid construction

The fragment for IL-6 overexpression was designed based on the IL-6 mRNA sequence posted on Genebank website and RNA design principle and was synthesized by Shanghai Genechem Co., Ltd. All the fragments were analyzed by Bla*st*-test to ensure specificity. The linearized plasmids (pcDNA3.1 (-), Invitrogen, Germany) was cut with BamH I and Hind III (Invitrogen, USA) and the amplified target fragments were ligated into the plasmids overnight with T4 DNA ligase at 16 °C. The ligated products were then transfected into DH5α competent cells (CB101, Tiangen Biotech (Beijing) Co., Ltd., China) and the positive clones were selected and sequenced for verification.

2.3. Treatment

Mice were divided into 4 groups (16 in each group), which were sham group, model group (apical resection model was constructed in normal newborn mice), IL-6-/- group (apical resection model was constructed in IL-6 knockout mice) and IL-6 group (apical resection model was constructed in mice injected with IL-6 plasmid for inducing IL-6 overexpression). After anesthesia with 2% pentobarbital sodium, mice were placed on the operating table in a supine position, and received injections of 2 μL 0.7 μg/μL IL-6 plasmid solution through tail vein. Seven days after the injection, 8 mice were randomly picked from each group for a 12-hour fasting, followed by an anesthesia through intraperitoneal injection of 2% pentobarbital sodium (50 mg/kg). Afterwards, the cardiac apical part was taken from the mice in each group. The same procedure was conducted for the rest 8 mice in each group 14 days after the injection. All the apical samples were fixed in 10% neutral formalin solution for 24 h, followed by a dehydration through a gradient of alcohol and paraffin embedment. Remaining liquid nitrogen was kept for later use.

2.4. Echocardiography

All mice underwent mixed anesthesia by intraperitoneal injection of ketamine (50 mg/kg) and diazepam (5 mg/kg). The mice were strapped to wooden boards in a supine position and the following items were checked by color Doppler ultrasonic scanner (SSI-5000, Shandong Shukang Hentong Technology & Trading Co., Ltd.): left ventricular posterior wall dimension (LVPWD), interventricular septal thickness (IVST), left ventricular fractional shortening (LVFS), left ventricular ejection fraction (LVEF), left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD).

2.5. Hematoxylin and eosin (HE) staining

After 7 days' and 14 days' injection of plasmid, the paraffin-embedded cardiac apical samples in each group were sliced. and the thickness of serial sections was 5 μm. Sections were then flattened at 45 °C and picked up and dried at 60 °C for 1 h. Next, they were dewaxed in xylene and stained with HE (Beijing Solarbio Technology Co., Ltd., China) after hydration. Samples were dehydrated through a gradient of alcohol, cleared in xylene and sealed with neutral gum. The cardiomyocytes were observed under light microscope (XP-330, Shanghai Bingyu Optical Instrument Co. Ltd., China) for checking the damage and their repair.

2.6. Immunohistochemical staining

Some apical tissues were fixed in 10% neutral formalin solution for 24 h, followed by a dehydration through a gradient of alcohol and paraffin embedment for 12 h. Next, samples were cut into 5 sections at the thickness of 3–4 μm. The sections were dewaxed and hydrated in a regular way: dewaxed with xylene I and xylene II for 10 min respectively, hydrated with gradient alcohol 100%, 95%, 80%, and 70% for 2 min respectively and washed in phosphate buffer saline (PBS) for 5 min twice. Then, they were placed in 3% H_2O_2 for 10 min and washed again in PBS for 5 min twice. High pressure antigen retrieval was conducted for 90 s and samples were cooled at room temperature. Afterwards, they were washed again in PBS and incubated at 37 °C for 30 min after the addition of 5% bovine serum protein (BSA) for blocking. Samples were then reacted with 50 μL of rabbit anti-rat monoclonal antibody (IL-6; ab7737, Abcam, London, UK; 1:100) at 4 °C overnight, rinsed in PBS for 2 min, and incubated at 37 °C for 30 min after the addition of 50 μL biotinylated rat anti-goat immunoglobulin G (IgG; SF8-0.3, Solarbio, Beijing, China; 1:100). Then, the samples were added with SAB solution, stained with DAB and restained with hematoxylin for 5 min. After being rinsed in tap water for 10 min, samples were dehydrated, cleared and sealed for microscopic examination. PBS buffer was used in place of primary antibody for the negative control. Criteria for IL-6 protein positive cells were as follows: the positive cells showed a brownish color; positive expression rate = number of samples with positive expression/total number of samples * 100%; the experiment was repeated 3 times.

2.7. Detection of cardiomyocyte proliferation by BrdU immunofluorescence assay

Immunofluorescence assay was used to detect cardiomyocyte proliferation in mice. Five paraffin sections of cardiac apical sample were taken for antigen retrieval after dewaxing and dehydration with gradient alcohol. They were rinsed in 0.01 M PBST for 5 min 3 times and sealed with 2% BSA for 30 min at 37 °C in wet box. The dilution of fluorescent antibody (1:8) was added to the samples and the samples were placed in a wet box for 30 min at 37 °C. Afterwards, they were rinsed with 0.01 mol/L PBS (pH7.4) for 5 min 3 times and mounted with glycerol. Samples were then placed under a fluorescence microscope for observing. If the cell nucleus exhibited orange/red fluorescence, the cell could be considered as a positive cell. The experiment was repeated for 3 times.

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