



RESEARCH ARTICLE

EFFECT OF BONE MARROW STEM CELLS TRANSPLANTATION ON APOPTOSIS IN CARDIOMYOCYTES OF RATS AFTER ACUTE MYOCARDIAL INFARCTION

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ABSTRACT

Objective: To observe effects of transplanting allogenic bone marrow mono-nuclear cells on apoptosis in cardiomyocytes of rats after acute myocardial infarction.

Methods: 30- Healthy, male wistar rats were randomly divided into 2 groups, control and transplant group, n=15 each. As soon as myocardial infarction (MI) model was established, Dulbecco's Modified Eagle's Medium-low glucose (DMEM-LG) medium and BM-MNCs labeled with Bromodeoxyuridine (BrdU) were injected in the peri-infarct area in the control and transplant group respectively. After 4 weeks, apoptotic index (AI), expression of Bcl-2, Fas and FasL were detected.

Results: 4 weeks after transplantation, cardiomyocyte apoptotic index (AI) and expression of Fas and FasL decreased significantly ($P < 0.05$), while the expression of Bcl-2 increased significantly ($P < 0.05$) in the transplant group. BM-MNCs labeled with BrdU were observed around injected sites under immunohistochemical examination.

Conclusion: Allogenic BM-MNCs transplantation regulates the expression of Bcl-2, Fas, and FasL thereby inhibits cardiomyocytes apoptosis after AMI.

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INTRODUCTION

Myocardial infarction (MI) is a manifestation of coronary artery disease where coronary blood supply drastically reduced or interrupted. It is a lethal and emergency cardiovascular disease which is one of leading causes of disability and death in clinical practice (Kerr, 1994). Ventricular remodeling after myocardial infarction refers to neuro-hormonal and genetic regulatory mechanisms activated by inflammatory cytokines causing changes in quality of cell morphology and functions of myocardial cells (Majno *et al.*, 1995; Kerr *et al.*, 1972). Necrosis, apoptosis, myocardial fibrosis and scarring after acute myocardial infarction can be factors to reduce the function of the myocardial cells leading to heart failure. Many mode of treatment have been introduced for myocardial infarction. Regeneration of infarcted myocardial cells and inhibition of myocardial apoptosis has become the different mode of treatment for ischemic heart disease. This study was designed to investigate the influence of allogenic bone marrow mononuclear cells (BM-MNCs) transplantation on myocardial apoptosis in rats after acute myocardial infarction (AMI).

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MATERIALS AND METHODS

Grouping

30, healthy, male Wistar rats of 90 days with body mass of 250 ~ 300g from Henan Experimental Animal Center were randomly divided into two groups: the transplant group (15 rats) undergoing allogeneic BM-MNCs transplantation after AMI; control group (15 rats), injected DMEM-LG broth (US GIBCO BRL company) after AMI.

BM-MNCs separation

BM-MNCs separated from the Wistar, healthy male rats with body mass of 100 g ~ 150 g taken from Experimental Animal Center of Henan Province. After chloral hydrate intraperitoneal injection for anesthesia, femur and tibia were harvested. Pass with the right amount of DMEM-LG broth out of the bone marrow with a density of 1.077 lymphocyte separation medium bought from Tianjin TBD companies. After centrifugal separation at frequency of 2000 r/m, drawn out interface layer and then washed with Phosphate buffer saline (PBS) twice which sacrifices a small amount of cell count. Cell viability is evaluated by Trypan blue staining. Containing 10 μmol / L-bromo-azacytidine BrdU, 100 ml / L fetal bovine serum (Hangzhou Evergreen Company) the cells in DMEM

re-suspended in 5×10^6 cells / ml were seeded in culture flask with 50 ml / L CO₂ and cultured in a humidified incubator for 24 h and replacing the BrdU with fetal bovine serum of DMEM medium, regulating cell density of 1×10^8 cells/ ml, preserved in an ice tank for use.

Establishment of myocardial infarction and BM -MNC s model

After anesthesia by intra-peritoneal injection of chloral hydrate, incision at 4th intercostals space at the most thrilling point was made and exposed the heart. Myocardial infarction model was established by ligation of the left anterior descending coronary artery. Myocardial infarction was established in both transplant group and the control group. Sub epicardial injection of BM -MNC s [1×10^7 cells / (100 μ L/ point)] and broth (100 μ L/ point) a total of 4.00 points by micropipette at peri-infarct region. Observed the rats for 5 ~ 10 min, after retaining circulatory stability and cardiac rhythm, closed chest and continued feeding.

Calculation of Myocardial apoptosis index

Cardiac arrest was established by intravenous injection of 3ml (KCl 100 g / L), left atrium, right atrium and right ventricle were harvested after thoracotomy and flushed with PBS buffer and kept in formaldehyde solution 40g / L which later prepared paraffin-embedded sections. In situ labeling of apoptotic nuclei by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL method) using apoptosis detection kit instructions (Sino-American Biotechnology Company), using DAB color kit (Beijing Zhongshan Biotechnology Company), color hematoxylin counter stain with no Terminal deoxynucleotidyl transferase (TdT) enzyme were used as negative control. Each rat was observed in five slices, each slice count 10 high power field (x 400). The ratio of positive apoptotic cardiomyocyte nuclei to the total number of nuclei gives average value known as apoptosis index, AI).

Cardiac-related genes detected by Bcl-2, Fas, Fas-L immunohistochemistry kit (United States Santa Cruz company), respectively, a anti-Bcl-2, Fas, Fas L, Brd U monoclonal antibody (1B100 diluted), DAB color, hematoxylin. Use HM IAS-2000 color pathological image analysis system at 400 randomly selected 10x vision, Bcl-2, Fas, Fas L mean absorbance value immunohistochemical staining (A260 nm / A280 nm) were measured.

Statistical Methods

All data were expressed as mean \pm standard deviation (x \pm S). We used SPSS 16.0 statistical software for statistical analysis, comparison using analysis of variance, with P < 0. 05 as there are significant differences between the groups.

RESULTS

BM -MN Cs transplant morphology

Experimental group and the control group died one case each after the first day and second day respectively. The cause of death is unknown. Cell viability of BM -MNCs stained with Tryptan blue were greater than 90% while BrdU labeled BM -MNCs were more than 70%. BrdU positive nuclei were brown. 4 weeks after transplantation, BrdU positive BM -MNCs can

be seen in the myocardial infarction area. Main presence of BM -MNCs was found in the local injection site. A few focal BM -MNCs like growth of cell mass with disorderly growth were also found at peri-infarct zone and subendocardial region. Cell oval, uneven distribution, volume was significantly smaller than the normal myocardial cells; nuclei stained, small nuclear-cytoplasm ratio, abnormal morphology.

Cardiomyocyte apoptosis and related genes

TUNEL method showed normal myocardium nuclei blue, while positive apoptotic nuclei were brown myocardium (Figure 2A, 2B). BM -MNCs 4 weeks after transplantation, myocardial AI in experimental group was significantly lower than the control group (P < 0.05, Table 1); myocardial expression of Bcl-2, Fas and Fas L protein accumulates mainly in cytoplasm, brownish yellow; compared with the control group, the experimental group Bcl-2 protein was significantly increased (P < 0.05, Table 1, Figure 2C, 2D), value of Fas, Fas-L protein significantly lower in experimental group (P < 0.05, table 1).

Table 1. AI and related genetic test results in two groups of rats (n = 14, x \pm s)

Group	AI(%)	A _{Bcl-2}	A _{Fas}	A _{FasL}
Control	15.7 \pm 1.5	136 \pm 8	168 \pm 12	154 \pm 9
Experimental	8.0 \pm 1.6 ^a	179 \pm 15 ^a	105 \pm 10 ^a	111 \pm 14 ^a

Compared with the control group, ^aP<0.05

DISCUSSION

The common view on how cardiomyocytes die during or after myocardial infarction has altered in recent years. For a long time necrosis was regarded as the sole cause of cell death in myocardial infarction. However, recent studies indicate that apoptosis also plays an important role in the process of tissue damage subsequent to myocardial infarction. Although both necrosis and apoptosis result in the death of the cell, they differ in several morphological and cellular regulatory features. Necrosis is characterized by the rapid loss of cellular homeostasis, rapid swelling as a result of the accumulation of water and electrolytes, early plasma membrane rupture, and the disruption of cellular organelles. As a result of the membrane rupture and subsequent leakage of a broad array of cellular material, necrosis induces an inflammatory response (Kerr, 1994; Majno, 1995 and Kerr *et al.*, 1972). Apoptosis or programmed cell death is, unlike necrosis, a highly regulated and energy requiring process. Apoptosis is characterized by shrinkage of the cell and the nucleus. The nuclear chromatin is condensed into sharply delineated masses, and eventually breaks up. The cell then detaches from the surrounding tissue. At this stage, buds evaginate from its membrane, which eventually seal off to form membrane enclosed vesicles, called apoptotic bodies, containing condensed cellular organelles and nuclear fragments. These apoptotic bodies are either rapidly phagocytosed by neighboring cells or undergo degradation, which resembles necrosis in a process called secondary necrosis. However, apoptosis is generally considered not to trigger an inflammatory response (Saraste, 2000).

It was generally believed that cardio-myocytes are terminally differentiated cells and cannot undergo mitosis again. So, myocardial infarction can only be repaired to scar tissue causing heart failure. Pathological apoptosis increased after myocardial infarction and heart failure commences.

Myocardial apoptosis is programmed cell death mediated by a related gene as mentioned above, a major cause of ventricular remodeling after myocardial infarction. Later there is continued loss of myocardial contractility unit and heart function declined. At the same time, apoptosis and ventricular myocardium remodeling promote the development of heart failure (Getz, 2005 and Hansson, 2005). In recent years, numerous studies confirmed that (Daugherty, 2002; Khalou-laschet, 2006; Elhage *et al.*, 2013 and Naka Jim, 2002). The bone marrow stem cells transplantation into infarcted myocardium can locally differentiate into cardiomyocytes and endothelial cells, can increase the surrounding cells and expression of multiple cell activating factors which promotes local angiogenesis, increased capillary density and blood flow in the infarct area, reducing myocardial oxygen consumption, inhibit apoptosis of cardiomyocyte, reducing myocardial cell loss, increase the number of myocardial tissues effective to repair or replace damaged necrotic myocardium, restore myocardial contractility, improve heart function while reducing apoptosis in hypertrophic cardiomyopathy and reducing collagen formation. We observed BM-MNCs four weeks after transplantation at the myocardial infarction region. We found surviving BrdU positive BM-MNCs and improved heart function. Bcl-2 is an anti-apoptotic proteins localized to the outer membrane of mitochondria, where it plays an important role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins.

Apoptosis is decreased when Bcl-2 expression is increased while apoptosis is enhanced when Bcl-2 expression is decreased. Anti-apoptotic mechanism of Bcl-2 is related to mitochondrial function which inhibits mitochondrial pro-apoptotic proteins such as the release of cytochrome C, apoptosis-inducing factor, thereby preventing the occurrence of apoptosis (Getz, 2005). It is now widely recognized that Bcl-2 family inhibit apoptotic factor and promote the cell survival. We found that transplanted myocardial apoptotic index decreases, Bcl-2 protein levels increase hence myocardial apoptosis is inhibited. Fas is a part of the tumor necrosis factor receptor superfamily member 6 (Lichter, 1992; Inazawa, 1992) and type I transmembrane glycoprotein. FasL is a type I transmembrane glycoprotein that belongs to the tumor necrosis factor primarily expressed on activated T cells and B cells. Fas L in trimeric form and can induce Fas binding activity of sphingomyelin, decomposing sphingomyelin generating ceramide, following and trigger a series of biochemical reactions, mediated cardiomyocyte apoptosis. Changes in the expression of Bcl-2 family accompanied Fas and Fas L changes, it is speculated that Bcl-2 family is the regulator in apoptosis, Fas and Fas L is induced by Bcl-2 (Khalou-laschet, 2006).

Conclusion

4 weeks after transplantation, we found that, Fas-L protein level in transplantation group declined, so we believe that allogeneic BM-MNCs transplantation after myocardial infarction significantly inhibited apoptosis of cardiomyocytes. Inhibition of Fas and Fas-L gene as well as upregulation of Bcl-2 reduces myocardial cell apoptosis thereby protecting myocardium leading to improvement of cardiac function after AMI. But mechanism inhibiting myocardial apoptosis by migrated allogeneic BM-MNCs after AMI really remains unclear and needs further exploration.

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