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Allograftic bone marrow-derived mesenchymal stem cells transplanted into heart infarcted model of rabbit to renovate infarcted heart*

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Abstract: Objective: To investigate the directed transplantation of allograftic bone marrow-derived mesenchymal stem cells (MSCs) in myocardial infarcted (MI) model rabbits. Materials and Methods: Rabbits were divided into 3 groups, heart infarcted model with MSCs transplanted treatment (MSCs group, n=12), heart infarcted model with PBS injection (control group, n=20), sham operation with PBS injection (sham group, n=17). MSCs labelled by BrdUrd were injected into the MI area of the MSCs group. The same volume of PBS was injected into the MI area of the control group and sham group. The mortality, LVIDd, LVIDs and LVEF of the two groups were compared 4 weeks later. Tropomyosin inhibitory component (Tn I) and BrdUrd immunohistochemistry identified the engrafted cells 4 weeks after transplantation. Result: The mortality of the MSCs group was 16.7% (2/12), and remarkably lower than the control group's mortality [35% (7/20) (P<0.05)]. Among the animals that survived for 4 weeks, the LVIDd and LVIDs of the MSCs group after operation were 1.17±0.21 cm and 0.74±0.13 cm, and remarkably lower than those of the model group, which were 1.64±0.14 cm and 1.19±0.12 cm (P<0.05); the LVEF of the MSCs group after operation was 63±6%, and remarkably higher than that of the model group, which was 53±6% (P<0.05). Among the 10 cases of animals that survived for 4 weeks in the MSCs group, in 8 cases (80%), the transplanted cells survived in the non MI, MI region and its periphery, and even farther away; part of them differentiated into cardiomyocytes; in 7 cases (70%), the transplanted cells participated in the formation of blood vessel tissue in the MI region. Conclusion: Transplanted allograftic MSCs can survive and differentiate into cardiomyocytes, form the blood vessels in the MI region. MSCs transplantation could improve the heart function after MI.

Key words: Bone marrow-derived mesenchymal stem cells, Transplantation, Myocardial infarction
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INTRODUCTION

Myocardial infarction (MI) is one kind of high risk and dangerous disease in the cardiology department. Its damage is progressive (Mann, 1999). Modern clinical therapies for MI have focused traditionally on how to limit the infarct size. After the death and loss of normal cardiomyocytes, scar tissue formation and thinning of the MI region appear immediately. These changes could cause heart failure. Cell transplantation is a promising approach that offers the creation of new functional

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tissues to replace the damaged cardiomyocytes. The purpose of our study was to investigate the effect of allograftic bone marrow-derived mesenchymal stem cells (MSCs) transplantation in rabbit MI model, and cell survival, differentiation and integration, on left ventricular remodeling and function after extensive MI.

MATERIALS AND METHODS

Our study was performed in accordance with the guidelines of the Experimental Animal Care and Use Committee of Zhejiang University. The study lasted from Dec. 2001 to Mar. 2003.

Animal group

Forty-nine 1.76–3.58 kg adult male purebred New Zealand white rabbits provided by the Zhejiang Province Academy of Medical Sciences (No. of certificate of approval: Zhejiang Experimental Animal No. D00-002, approved in 2001). After echocardiography, the animals were divided into 3 groups: an MSCs group (12 cases) consisting of MI model with MSCs injected and then transplanted into the MI region; a control group (20 cases) consisting of MI model with cells-free PBS injected into the MI region; sham group (17 cases) consisting of sham operation animals with cells-free PBS injected into the same region.

Rabbit model of myocardial infarction

After male rabbits were anesthetized with 10 mg/kg pentothal sodium, the chest was opened along the median incision in order to avoid pneumothorax. After the left border branch artery was identified at the midpoint from the atrioventricular sulcus of the dissociative paries of the left ventricle to the apex of the heart (Podesser *et al*., 1997), a double ligation was made with silk thread (3/0) and after 45 min, the stitch was cut, and the coronary artery was opened (MacFarlane *et al*., 2000). An electrocardiograph monitor (HP, Zoll-21) was used to record ST segment change. When the ischemia region became pale and the ST segment changed, this indicated the ligation was effective (Ohno *et al*., 1998; Cohen *et al*., 1999). For the sham operation group, a double threading was made under the left border branch artery without ligation. The rest of the steps are the same as those for MSCs group and control operation group.

Labelling of cells

The MSCs cells were additionally tested for CD34, CD45, CD29, CD166 and CD117. The results were CD34 (+) 2.0%, CD45 (+) 1.5%, CD29 (+) 99.9%, CD166 (+) 78.8% and CD117 (+) 77.4%, which conformed with MSCs standard (Pittenger *et al*., 1999; Le *et al*., 2003). For further identification of the transplanted cells in the MI region, representative samples of cultured cells were labelled with 5-bromodexoyuridine (BrdUrd, sigma). DMEM culture medium containing 0.1 mmol/L BrdUrd was used to cultivate the cells for 48 h before transplantation (Tomita *et al*., 1999). A culture flask was randomly drawn out for the same lot of transplantation cells. Two pieces of cell tablets were used for Cardio troponin I (cTn I) and BrdUrd antibody immunohistochemical examination respectively. An MI model was set up, using pancreatin for cell digestion, and repeatedly rinsed and centrifuged 5 times to remove BrdUrd. The cell density was adjusted to 1×10^7 ml^{-1} by PBS.

Injected transplantation of MSCs

The artery was ligated for 45 min and then opened to identify the infarcted area visually on the basis of the surface turning pale. The MSCs group rabbits selected were given 3.3×10^6 MSCs in the MI region by 3 injections of 100 μl using a 27-gauge needle. The transplant region extended from the ligation of the artery to the apex, both sides of the artery, 4 mm wide, below the epicardium (Min *et al*., 2002). For the control group and sham group, the same amount of cell-free PBS liquid was injected into the same part.

Echocardiograph to evaluate remodelling and contractility

The Echocardiograph was an HP Company SONOC-5500 (2–4 MHz). At the level of left ventricle papillary muscles on the long axis section,

M ultrasound was used to measure the left ventricle internal diastolic diameter (LVIDd) and the left ventricle internal systolic diameter (LVIDs). All measurements were averaged for three consecutive cardiac cycles, and were performed by an experienced technician who was blind to the treatment group (Pennock *et al*., 1997; Donald *et al*., 2001). The Teich formula was used to calculate the left ventricle ejection fraction (LVEF). Animals in all groups had Echocardiograph examination before, and 28 days after operation.

Histological and immunohistochemical examination

Four weeks after cell transplantation, the animals were sacrificed with an overdose of phenobarbital. Hearts were harvested, and processed for histological and immunohistochemical examination. 10% formalin was used to fix it. The whole left ventricle was embedded in paraffin. Thin 4 μm slices were taken at 2 mm intervals perpendicular to the long axis of the heart, with 6–7 slices for every heart specimen. cTn I antibody (mouse source IgG1, anti rabbit, Sigma) and BrdUrd antibody (mouse source IgG1, IGN) were made for double marking immunohistochemical examination. Diaminobenzidine tetrahydrochloride (DAB, Sigma) and AEC (Dako) were added to the antibodies to give them coloration for display. The examination procedures followed those in the literature (Cohen *et al*., 1999; Chen *et al*., 2001). After that, hematoxylin was used to color the nucleus. Presence of cells of BrdUrd positive nucleus. Presence of positive cTn I in the cytoplasm.

BrdUrd labelling rate of MSCs and cTn I immunohistochemical examination before implantation

After fixing 2 cell slices of every lot of transplant cells, they were treated with cTn I and BrdUrd antibody respectively; and then treated with PV600 Pic TureTM bi-antibody (sheep source IgA, Dako) and DAB coloration. Those bearing with yellow cell nucleus are BrdUrd labelled cells. Under ×400 microscope, 10 visual fields of cell nucleus were randomly drawn out for counting the cells and calculating the BrdUrd labelling rate (McGinley *et al*., 2000) according to the following formula. The cTn I was checked to confirm the absence of cTn I positive cells before the transplantation.

BrdUrd labelling rate (%)=number of yellow cell nucleus/total number of cell nucleus×100%

Immunosuppressor treatment

Starting 24 hours before operation, all experimental animals were given cyclosporine A liquid (Fujian China Kerui Medicine Co. Ltd) at dosage of 15 mg/kg·d until the experiment stopped (Wasan and Sivak, 2003).

Statistical analysis

All values are shown as means±*SD*. SPSS11.0 software was used for statistical analysis. The differences of mean values among all groups were compared by unpaired *t* test. The differences of death rates among all groups were compared by χ^2 test. All tests were two-tailed and significance of $P<0.05$ was considered acceptable.

RESULTS

Mortality

In 4 weeks, the mortality of the MSCs group was 16.7% (2/12), and remarkably lower than that of the control group [35% (7/20), $P<0.05$]. The mortality of those in the sham operation group was 11.8% (2/17), and remarkably lower than that of the MSCs group and control group ($P<0.05$).

Among the animals that survived for 4 weeks, the LVIDd and LVIDs of the MSCs group after operation and 1.17±0.21 cm and 0.74±0.13 cm respectively, and remarkably lower than those of the control group, which were 1.64±0.14 cm and 1.19±0.12 cm ($P<0.05$); the LVEF of the MSCs group after operation was 63±6%, remarkably higher than that of the control group [53±6% ($P<0.05$)]; the LVIDd and LVIDs of the sham operation group were remarkably lower than those of the MSCs group and control group respectively ($P<0.05$); the LVEF of the MSCs group after operation was remarkably higher than that of the

MSCs group and control group (P<0.05) (Table 1).

Histological and immunohistochemical examination

In the sham group, large MI scar region was not observed; BrdUrd (+) cell was not observed. For cardiomyocyte cell tissue in the non-MI region of the control group, the cell nucleus was dyed in blue, and the cytoplasm appeared brownish yellow; indicating cTn I (+), BrdUrd (−), BrdUrd (+) cells were absent. In the control group, the cell in MI scar, the cytoplasm was leuco, and the cell nucleus blue (Fig.1a); indicating cTn I (−) and BrdUrd (−), BrdUrd (+) cells were absent. For the 12 cases of specimens in the MSCs group, the results of immunity assortment of 4 cases were the same as that of the control group. The other 8 cases (66.7%) had BrdUrd (+) cells, which were distributed as follows: For cardiomyocyte cell tissue in the non-MI region, besides cTn I (+) and BrdUrd (−) cells, the obviously small number of cells with red nucleus and brownish yellow cytoplasm was indicative of the presence of cTn I (+) and BrdUrd (+) cells (Fig.1b). In the MI scar, cTn I (−), BrdUrd (+), cTn I (+), and BrdUrd (+) cells were observed (Fig.1c); most of the cTn I (+) and BrdUrd (+) cells distributed in the junction part of the MI region and the non-MI region (Fig.1c). In 7 of the 8 cases (87.5%), below the left ventricle epicardium was a large network of capillaries, arteries, veins and blood vessel endothelium in slice shape distribution. cTn I (−) and BrdUrd (+) cells in the smooth muscle cell could be observed (Fig.1d).

BrdUrd labelling rate of MSCs and cTn I immunohistochemical examination before implantation

There were 5 successful cell transplantations in this experiment. The BrdUrd average labelling rate was 43.3±12.5%, but that of cTn I (+) cell was not observed.

DISCUSSION

Stem cell transplantation treatment is the hotspot of organ transplantation and tissue study. MSCs has become the focus of stem cell application study due to its advantages of convenient withdrawal of materials (bone marrow), non-rejection of transplant from the body itself, no controversy on ethics, high clinical feasibility, etc.

MSCs has the common biological characteristics of stem cell. It has self-reproduction capacity and multi-potential differentiation capacity. Many experiments have proved that MSCs can differentiate into many kinds of tissues (of bone, cartilage, lipocyte, neurocyte, hematopoietic cell, muscle cell, etc.) (Schmitt *et al*., 2003; Wang *et al*., 2004; Lu *et al*., 2001). Can MSCs differentiate into cardiomyocyte? From the viewpoint of embryology, MSCs is very similar to cardiomyocyte, which derive from the mesoderm and have the same embryonic origin. There are also reports of invitro experiments using 5 azacytidine to induce differentiation into cardiomyocyte form MSCs (Shinji *et al*., 1999). Also, some experiments put forward that the hypothesis MSCs repairs of damaged heart may

Table 1 LVIDd, LVIDs and LVEF of all groups' animals having survived for 4 weeks

	Sham operation group (n=15)	Control group (n=13)	MSCs group (n=10)
Weight (kg)	2.26±0.27	2.20±0.33	2.27±0.26
LVIDd before operation (cm)	0.91±0.08	0.95±0.12	0.92±0.09
LVIDs before operation (cm)	0.52±0.09	0.56±0.10	0.52±0.09
LVEF before operation (%)	0.77±0.09	0.76±0.06	0.79±0.07
LVIDd after operation (cm)	0.96±0.05	1.64±0.14▲	1.17±0.21†
LVIDs after operation (cm)	0.54±0.06	1.19±0.12▲	0.74±0.13†
LVEF after operation (%)	0.77±0.09	0.53±0.06▲	0.63±0.06†

▲ Comparison of LVIDd, LVIDs and LVEF of control group with those of sham operation group, P<0.05; † Comparison of LVIDd, LVIDs and LVEF with those of control group after operation, P<0.05

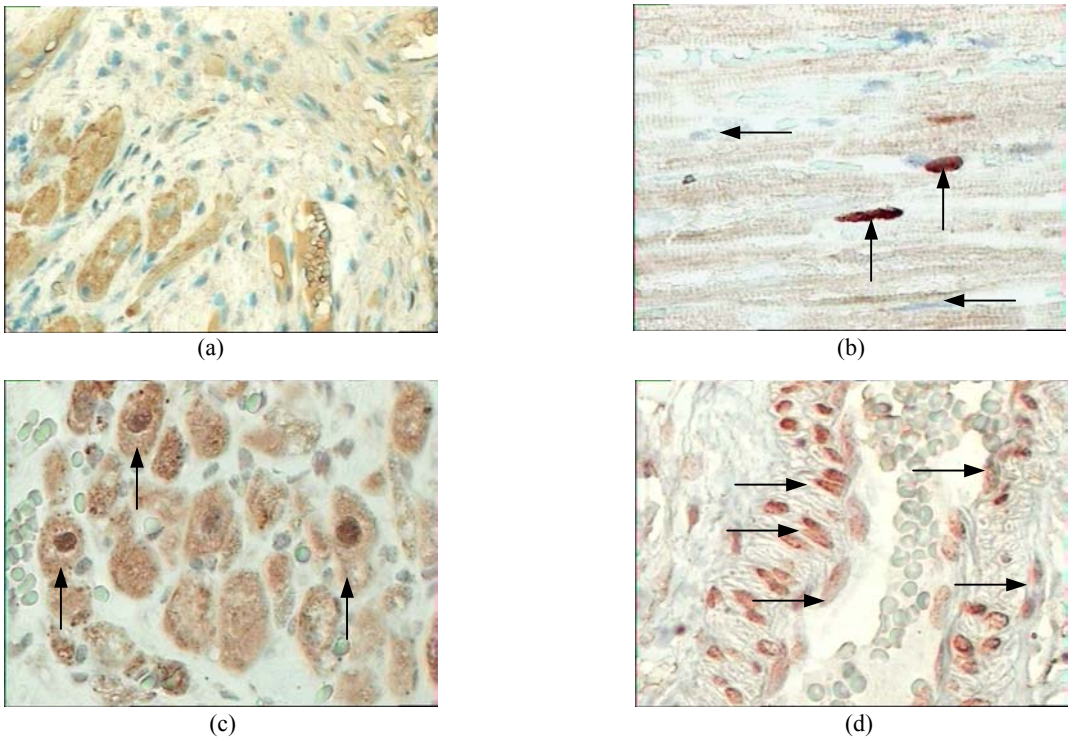

Fig.1 BrdUrdrd (+) cell was not observed in the control group (a, ×800). Cardiomyocyte cell in non-MI region of MSCs group (b, ×800), the cytoplasm is brownish yellow, the cell nucleus is blue (traverse arrow, b), cTn I (+), BrdUrd (–) cell; the cytoplasm was observed to be brownish yellow, the cell nucleus was fuscous (vertical arrow, b), cTn I (+) and BrdUrd (+) cell. In the periphery of MSCs group MI region (b, ×800), it was observed that the cytoplasm was brownish yellow, and the cell nucleus was fuscous (arrow, c), cTn I (+) and BrdUrd (+) cell. The cytoplasm of the blood vessel in the MSCs group (d, ×400), was observed to be not colored, and some cell nucleus were fuscous (arrow, d), cTn I (–) and BrdUrd (+) cell, which differentiate into blood vessel endothelium and smooth muscle cells

be in itself a kind of natural phenomenon. For instance, Federico and Konrad (2002) studied 8 cases of male patients who died of the transplantation of female heart into their body and found that: The weight of the transplanted heart increased obviously compared with that before the transplantation. Pathological examination showed that about 7%–10% of the coronary artery cells and capillary vessel cells of the transplanted heart had Y chromosomes; while in the ventricle muscle cells, about 12%–16% of them had Y chromosome. These experiments also showed that a kind of exo-heart cell can be transplanted into the heart and perform a repair function. This kind of cell may derive from MSCs, but these hypotheses need to be proved by more experiments.

The methods to send MSCs into damaged tissue can be divided as follows. Mobilizing the MSCs from bone marrow to the damaged tissue, transfusion from vein, transfusion from artery, and local injective transplantation. In this experiment, we used the method of MSCs MI region injective transplantation. The clinical significance lies in that when the coronary heart disease patient accepts coronary artery bypass operation, at the same time, he can accept from the body itself MSCs injective transplantation into those weak myocardial tissues so as to improve the heart blood supply. The experiment also proved that MSCs can differentiate in vivo into MI region cardiomyocytes with cTn I expression, which showed the existence of myoneme and the function of systoles. MSCs also participated in the formation of blood vessels, which include not only the capillaries but also the veins; and the formation the smooth muscle and blood vessel endothelium. These showed that MSCs has

strong differentiation function on blood vessel tissue. The LVIDd, LVIDs and LVEF of the heart of the MSCs group were remarkably better than those of control group, which showed that MSCs could improve the heart function after their transplant into the MI region of the heart. We consider that after transplantation, the process of MSCs differentiating into cardiomyocyte, blood vessel tissue and connective tissue has a cooperating role in improving the heart function.

In this experiment, it was observed that among MSCs existing in non-MI region, some differentiated into cardiomyocyte, but some did not. This showed that injective transplantation of MSCs has comparatively strong transfer capacity, and can differentiate into functional cells in normal tissue. The possible explanation for these phenomena is that: the chemotactic factor and adherence factor of MSCs expression help it to settle down in the tissue of damaged and undamaged region (Peter and Pamela, 1998), transfer to the tissue through the deformation of the cytoskeleton, exist at the site with appropriate physical chemical conditions, and differentiate into functional cells or not. Accordingly, the inflammatory mediators to (such as IL, TNF, IFN, necrotic flake of cardiomyocyte cell, and the disintegration product of ground substance) arisen from the inflammatory reaction of the damage region may also induce MSCs to differentiate into cardiomyocyte cells (Bittira and Kuang, 2002). The phenomena of cells' differentiation in the experiment distributed in the joint section of the MI region and non-MI region. Prompt: Conditions such as normal cardiomyocyte cell induction, comparatively abundant blood supply and appropriate inflammatory reaction, etc. may be advantageous for MSCs to differentiate into cardiomyocyte. In the MI region, there are many new blood vessels formed with MSCs participation; but few cardiomyocytes that differentiated from MSCs. So, in the region with intensive necrotic inflammatory reaction and comparatively insufficient blood supply, the tendency for MSCs differentiation to participate in the formation of new blood vessel is stronger.

BrdUrd labelling technique is one of the classical nucleus labelling techniques; and is now mostly used as a prelimary step in the transplantation of stem cell (Tomita *et al*., 1999). Compared with other labelling techniques, it has the following advantages: (1) Through the DNA reproduction before cytodiaeresis, BrdUrd integrates into the DNA of the nucleus. Therefore there is comparatively high labelling rate for fast multiplication of cell, with the rate usually being above 30% (Tomita *et al*., 1999). (2) BrdUrd labelling time is short, usually needing that for 1 cell multiplication. (3) Because it is DNA labelling and positioned at the cell nucleus, it can be distinguished from cytoplasm. Troponin is the structural protein of cardiomyocyte for systoles. cTn I is only expressed in cardiomyocyte, and is not expressed in the skeletal muscle. Therefore, cTn I is the antigen of cardiomyocyte specificity (Lang *et al*., 2002). This experiment used BrdUrd labelling transplant cell and sites at the cell nucleus, used cTn I as the specificity antigen of cardiomyocyte and sites at cytoplasm. This double labelling method achieved good results in this experiment.

To sum up the above, local injection transplanted MSCs can survive and settle down in the MI region, non-MI region, and their periphery, differentiate into cardiomyocyte, and can participate in the formation of new blood vessel in the MI region. Allograftic MSCs injective transplant in MI region can effectively improve the heart function after MI.

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