Effect of sphingosine-1-phosphate and myoblast transplantation on rat acute myocardial infarction

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ABSTRACT. In this study, we investigated the effects of sphingosine-1-phosphate (S1P) combined with myoblast transplantation on the treatment of acute myocardial infarction and provided a foundation for its clinical application. A rat model of acute myocardial infarction was established by ligating the anterior descending branch of the coronary artery. Serum-free media, myoblasts, myoblasts with S1P liposomes, or myoblasts with liposomes were then injected into the infarcted area. Apoptosis of the transplanted cells was assessed after 24 and 48 h, and changes in heart function and myocardial infarction area were assessed after 4 weeks. After transplantation of S1P into myoblasts, myocardial function was improved compared to that in the other groups. Specifically, the apoptosis of transplanted cells and the area of myocardial infarction decreased significantly (P < 0.01), while cardiac function significantly
improved (P < 0.01). The efficacy of S1P and myoblast transplantation on acute myocardial infarction was significantly better than that in the control group (i.e., injection of myoblasts and liposomes) and the serum-free medium group, demonstrating the feasibility of joint S1P and myoblast transplantation for treating myocardial infarction.

Key words: Acute myocardial infarction; Apoptosis; Cell transplantation; Myoblast; Sphingosine-1-phosphate

INTRODUCTION

Myocardial infarction causes heart failure and death in coronary heart disease patients by playing a key role in muscle cell death (Nadal-Ginard et al., 2003). Cell transplantation therapy can replace necrotized myocardial cells and increase cell number and contraction function to improve heart function; thus, it is a means for gradual treatment of heart disease (Barnett and van den Hoff, 2011). Treatment of myocardial infarction through autologous myoblast transplantation can result in the development of myoblasts into the myocardium in the myocardial infarcted microenvironment. Inclusion body formation is impaired by myocardial function, which is expected to improve heart function, quality of life, and the survival rate of patients (Hagège et al., 2003).

Compared to the use of traditional methods, the use of skeletal muscle myoblasts has been shown to be superior in the treatment of myocardial infarction. However, the use of myoblast transplantation is limited by the death of transplanted cells. Shortly after transplantation in an animal model of low-temperature freezing injury, 50% of the transplanted cells die (Zhang et al., 2001), thereby reducing the positive effects on cardiac function.

Sphingosine-1-phosphate (S1P) is a bioactive lipid metabolite secreted by a number of blood cells (platelets). This molecule regulates cell proliferation, regeneration, and migration, as well as the movement of intracellular calcium ions, the expression of adhesion molecules, monocyte activation, endothelial cell adhesion, and other biological effects. Recent studies have shown that S1P can significantly reduce the necrosis area caused by ischemia reperfusion injury in myocardial tissue (Egom et al., 2011). In myocardial infarction, aggregation and release of platelets can result in the generation of large amounts of S1P (Sano et al., 2002). S1P can reduce ischemia-reperfusion injury and inhibit myocardial cell apoptosis, and has a myocardial protective effect by activating the phosphatidylinositol 3 kinase-Akt signaling pathway (Del Re et al., 2008).

In this study, we combined skeletal muscle myoblast transplantation with S1P injection after establishing a model for acute myocardial infarction in rats. Serum-free media, myoblasts, myoblasts with S1P, and myoblasts with liposomes were injected into the infarcted area, and the mortality of transplanted cells, heart function, myocardial infarction area, and apoptosis of transplanted cells were assessed after 4 weeks. We determined the feasibility of combined S1P and skeletal muscle myoblast treatment of myocardial infarction.

MATERIAL AND METHODS

Materials and reagents

Healthy Sprague-Dawley rats weighing 250 ± 50 g were obtained from the Experimental
Animal Center of Jiujiang University. Several key reagents and their providers include the following: S1P (Enzo Biochem, Inc. Plymouth Meeting, New York, USA), phosphatidylcholine (Sigma, St. Louis, MO, USA), and in situ apoptosis detection kit (Promega, Madison, WI, USA).

The instruments and equipment used in this study included a confocal fluorescence microscope (TCS-SP, Leica, Wetzlar, Germany), an electric thermostatic drying oven (DHG-9203A, Shanghai Heng Technology Co. Ltd., Shanghai, China), an automatic high-pressure sterilizing pot (HVE-50, Hirayama Manufacturing Corp., Tokyo, Japan), a CO$_2$ constant temperature cell culture box (type SHELL/JB, Thermo Fisher Scientific Inc., Shanghai, China), an inverted phase contrast microscope (Olympus, Tokyo, Japan), a visible spectrophotometer (type 721, type 723, Shanghai Precision Instrument Co., Ltd., Shanghai, China), a desktop high-speed centrifuge (TGL-16G, Union Instruments, Jintan City, Jiangsu Province, China), a small animal ventilator (HX-100E, Chengdu Technology & Marker Co. Ltd., Chengdu City, Sichuan Province, China), a BL-420S physiological recorder (Chengdu Technology & Marker Co. Ltd., and a small animal operation instrument set.

Isolation and culture of skeletal muscle cells

The isolation and culture of skeletal muscle cells were performed as described by Blanco-Bose et al. (2001). Briefly, skeletal muscle samples were removed from rat thigh muscle, cut into 1-2-mm fragments, mixed with an enzyme digestion solution (2.4 U/mL dispase, 1% collagenase II, and 2.5 mM CaCl$_2$) and incubated at 37°C for 45 min. The growth media contained Dulbecco's modified Eagle's medium, 50% Ham F-10, 20% fetal bovine serum, 2.5 ng/mL basic fibroblast growth factor, 20 mM L-glutamine, and 1% penicillin and streptomycin. Cells were inoculated at 5 x 10$^5$ cells/mL on 6-cm Petri dishes coated with 0.1% poly L-lysine and cultured at 37°C in a 5% CO$_2$ incubator. Using the limited dilution and immunocytochemistry method, the staining-positive and single-cell clones were amplified and cultured.

Construction of the S1P liposome

The S1P liposome was constructed as described previously by Brailoiu et al. (2002). Briefly, 73 μL 1 mM S1P was dissolved in 140 mM KCl, pH 6.9, to prepare a solution of 8.7 x 10$^{-5}$ M 0.833 mL S1P. Next, 50 mg phosphatidylcholine was dissolved in 2.5 mL solution containing two ethers. The S1P/KCl solution and the phosphatidylcholine/two ether solution were mixed, and the resulting emulsion was vortexed for 5 min. This emulsion was then placed in a 20°C rotary evaporator to evaporate the organic solvent. The resulting solution was dialyzed against a Ringer's solution (110 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl$_2$, 2 mM Tris-HCl, pH 7.2, and 5.6 mM glucose) to remove unbound S1P. Control liposomes that did not contain S1P were prepared in the same manner as was used for the S1P liposomes. The concentration of S1P in the S1P liposome was estimated by using a thin-layer chromatography plate of indene three-ketone coupling reactions.

Preparation of rat model for acute myocardial infarction

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.4 mL/100 g) and fixed in the supine position. An electrode was then inserted into the subcutaneous tissue of the extremities, and their electrocardiogram was recorded. In addition, breathing was assisted...
with the aid of a gavage needle, orotracheal intubation, and a small animal ventilator (expiration/inspiration = 1:2, respiratory frequency was 70 breaths/min, and tidal volume of 15 mL). The skin on the left chest was disinfected with povidone iodine, and a horizontal skin incision was made on the left border of sternum (3-4 intercostal). A blunt separation of the muscular layer was made to gain access into the chest from the 3-4 intercostal space, and the pericardium was opened to fully expose the heart. The anterior descending branch of the coronary artery between the left atrial and pulmonary arterial cone was ligated using a 6-0 suture. The electrocardiogram displayed ST-segment elevation, indicating that the operation was successful.

**Determination of left ventricular function**

A physiological recorder was used to record the left ventricular systolic pressure (LVSP) and end-diastolic pressure (LVEDP). The pressure in the left ventricle was determined as the difference between the 2 measurements mentioned above [i.e., left ventricular developed pressure (LVDP) = LVSP - LVEDP]. LVSP and LVDP reflect the left ventricular systolic function, while LVEDP reflects left ventricular diastolic function. The function of the left ventricular cells was assessed before cell transplantation and 4 weeks after transplantation.

**Myocardial infarction area**

Ten rats were randomly selected from each group, and the myocardial infarction area was assessed 4 weeks after transplantation. Specifically, the animals were anesthetized as described above, and their hearts were excised. The left and right atria, as well as the right ventricles, were removed and the remaining left ventricle was flushed with phosphate-buffered saline. The left ventricle was divided into 5 parts parallel to the left ventricle long axis, fixed in 4% formalin, and then embedded with paraffin. Each section was sliced at a thickness of 10 μm and stained with 2,3,5-triphenyltetrazolium chloride. The percentage of myocardial infarct area to the total weight of left ventricular was then determined.

**Detection of apoptotic cells by in situ end-labeling (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL))**

Apoptotic cells were labeled using the TUNEL method. A confocal fluorescence microscope was used to detect green fluorescent protein-transfected myoblasts with an excitation peak centered at approximately 488 nm. A red fluorescent signal was detected at >620 nm for apoptotic nuclei, while a fluorescent signal for DAPI shifted its peak emission wavelength from 475 to 525 nm, with excitation at 360 nm, and showed normal staining of the nuclei. These signals were observed and counted using the single-blind method. At least 200 nuclei (10 random visual fields) were counted for each section (6 cards for each group) using a 40X objective lens. The apoptosis index was calculated as follows:

\[
\text{Apoptosis index} = \frac{\text{number of apoptotic cells}}{\text{total cell count}} \times 100\% \quad \text{(Equation 1)}
\]

**Experimental groups**

Rats were randomly divided into 4 groups: 1) control group, injected with cell culture fluid
into the infarct zone (N = 20), 2) myoblast transplantation group, (N = 20), 3) S1P liposomes (1 M) and myoblast transplantation group (N = 30), and 4) muscle cells transplanted into liposomes (N = 30).

**Statistical analysis**

All data were analyzed using the SPSS 11.5 software (SPSS, Inc., Chicago, IL, USA), and the statistical data from each group are reported as means ± standard deviation. Comparisons between groups were made by single-factor contrast analysis. P < 0.05 indicates that the difference was statistically significant.

**RESULTS**

**Identification of myoblast cells by immunocytochemistry in rats**

Myoblast cells positive for desmin immunocytochemical staining are shown in Figure 1.

![Figure 1. Immunocytochemical staining of myoblast cells. Visualization of desmin in the cytoplasm and nucleus with FITC staining; DAPI staining of the nucleus. DIC shows differential interference contrast imaging; MERGE shows overlap imaging.](image-url)
Left ventricular function before and after transplantation

The function of the left ventricle in the group that received only culture fluid (group 1) showed no difference in LVSP, LVDP, and LVEDP (P > 0.05; Table 1). The mean values of LVDP and coronary perfusion flow (CPF) in the group that received myoblast injection (group 2) were significantly higher than those of control group (i.e., group that received culture media; P < 0.01). Moreover, in the group that received both myoblasts and S1P liposomes (group 3), the mean values of LVDP and CPF were significantly higher than those in the group that received only myoblasts (group 2; P < 0.01). The mean values of LVDP and CPF showed no significant difference between the group that received myoblasts and liposomes (group 4) and the group that received only myoblasts (group 2; P > 0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart rate (bpm)</th>
<th>LVDP (mmHg)</th>
<th>CPF (mL/min)</th>
<th>Infarct size (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>213.01 ± 22.66</td>
<td>112.44 ± 4.37</td>
<td>10.35 ± 0.25</td>
<td>45.72 ± 1.20</td>
</tr>
<tr>
<td>2</td>
<td>225.86 ± 26.77*</td>
<td>132.01 ± 12.01*</td>
<td>11.69 ± 0.14*</td>
<td>38.80 ± 1.42*</td>
</tr>
<tr>
<td>3</td>
<td>212.12 ± 26.13</td>
<td>148.69 ± 3.16###</td>
<td>12.91 ± 0.26###</td>
<td>30.12 ± 1.91###</td>
</tr>
<tr>
<td>4</td>
<td>222.33 ± 29.25</td>
<td>130.01 ± 10.01###</td>
<td>11.47 ± 0.33###</td>
<td>37.60 ± 1.23###</td>
</tr>
</tbody>
</table>

Group 1 = control group (injected with serum-free media); Group 2 = injected with skeletal muscle myoblasts; Group 3 = injected with skeletal muscle myoblasts with liposomal S1P; Group 4 = injected with skeletal muscle myoblasts with liposomes. *P < 0.01 vs group 1, ###P < 0.01 vs group 2, $P > 0.05$ vs group 2.

Comparison of infarction area in each group before and after transplantation

Before transplantation, there was no significant difference between groups with respect to the infarction area (P > 0.05) (results not shown). Following transplantation, the mean infarction area decreased significantly when the rats received myoblasts (group 2), compared to that observed in the control group (group 1) and the pre-transplantation values (P < 0.01; Table 1). The mean LVDP and CPF in group 3 were clearly lower than those in group 2 (P < 0.01), while the mean LVDP, CPF, and other indicators showed no significant difference between group 4 and group 2 (P > 0.05).

Apoptosis of myoblasts after transplantation

The myoblasts were transfected with a green fluorescent protein-containing plasmid before transplantation. After 24 h, these myoblasts were transplanted into the rats with myocardial infarction. The apoptosis of transplanted cells in the group that received S1P liposomes decreased significantly at 24 and 48 h after transplantation compared to that observed in rats that received only liposomes (P < 0.01; Figure 2A, B and Figure 3).

DISCUSSION

With the development of in vitro cell culture, scientists have been attempting to utilize cultured cells to repair damaged myocardium, which may improve heart function. Current research has focused on the use of autologous cell transplantation, which avoids the issue of immune rejection as well as any ethical debates. In particular, autologous skeletal muscle
Effect of S1P and myoblasts on myocardial infarction

Figure 2. TUNEL-positive cardiomyocytes of all groups under a confocal fluorescence microscope. A. Graft cells are skeletal muscle myoblasts with liposomes and B. graft cells are skeletal muscle myoblasts with liposomal-S1P. (1) TUNEL-positive nuclei, (2) GFP-transfected myoblasts, (3) DAPI-stained nuclei, and (4) merged image of 1-3.

Figure 3. Myoblast AI at 24 and 48 h after transplantation with liposomes alone or S1P liposomes. TUNEL-positive myoblasts/total myoblasts x 100% (AI) at 24 and 48 h after transplantation. Comparisons between the groups were made with single-factor contrast analysis (**P < 0.01).
cell transplantation has progressed rapidly, and has even been examined in some clinical trials (Menasche et al., 2001; Povsic et al., 2011). However, there are several challenges to autologous skeletal muscle cell transplantation, including the limited supply of these muscle cells and difficulties in their ability to proliferate, differentiate, and form muscle tissue. Therefore, determining how to effectively inhibit apoptosis after transplantation may be essential for improving the efficacy of this treatment.

In this study, we established a model of acute myocardial infarction in rats. The infarcted area of these rats was injected with serum-free media, myoblasts, myoblasts with S1P liposomes, or myoblasts with liposomes. Apoptosis of the transplanted cells was then monitored 24 and 48 h after transplantation. The results showed that at both time points, the apoptosis of transplanted cells decreased when rats were injected with S1P liposomes compared with that observed in rats that received liposomes (Figure 2 and Figure 3). This suggests that S1P effectively inhibited myoblast apoptosis following transplantation. Test results after 4 weeks showed that cardiac function, myocardial infarction area, and other indicators of rats transplanted with myoblasts and S1P liposomes were significantly improved than those of the group that received myoblasts and liposomes (Table 1). Thus, myoblast transplantation with S1P liposomes effectively improved cardiac function and decreased apoptosis of transplanted cells.

Early reperfusion of myocardial tissue can cause apoptosis and even necrosis. Several animal experiments have confirmed that myocardial cell apoptosis plays an important role in lethal myocardial reperfusion injury (Zhao et al., 2000; Eefting et al., 2004). Therefore, mitigating apoptosis during early reperfusion may be an important factor in preventing significant myocardial reperfusion injury. S1P is mainly catalyzed by sphingosine kinase 1, which plays an important role in the process of ischemic preadaptation. The inhibition of sphingosine kinase 1 activity weakens its protective effect on the heart (Kennedy et al., 2009). S1P is mediated by a subfamily of G protein-coupled receptors encoded by endothelial differentiation genes (Edgs). In the myocardium, Edg-3 and Edg-5 receptors are expressed at high levels (Landeen et al., 2008). S1P can protect myocardial cells by binding to Edg-3 and Edg-5 receptors, which activates Akt and reduces ischemia/reperfusion injury (Means et al., 2007).

The results showed that S1P inhibited the apoptosis of transplanted cells. These results agree with those of a large number of studies demonstrating that S1P reduced ischemia/reperfusion injury and protected myocardial cells. This study provides a theoretical and experimental basis for the subsequent transplantation of skeletal myoblasts combined with exogenous S1P for treating ischemic diseases.

Conflicts of interest

The authors declare no conflict of interest.

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