Original Paper

Atorvastatin Improves Microenvironment to Enhance the Beneficial Effects of BMSCs Therapy in a Rabbit Model of Acute Myocardial Infarction

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Key Words

Atorvastatin • Bone marrow stem cells (BMSCs) • Transplantation • Acute myocardial infarction • Cardiac function • Apoptosis • Cell survival

Abstract

Background/Aims: To investigate the beneficial effects of atorvastatin added to the cell therapy with bone marrow-derived mesenchymal stromal cells (BMSCs) in a rabbit model of acute myocardial infarction (AMI). **Methods:** Rabbits were randomly divided into control group (n=10), bone marrow stem cells transplantation group (n=10), and BMSCs + atorvastatin group (n=10). AMI was established by ligating the left descending coronary artery. The left ventricular (LV) function was evaluated by echocardiography. H&E staining and Masson's Trichrome staining were performed to evaluate inflammatory cell infiltration and cardiac fibrosis. Immunohistochemistry and TUNEL were conducted to assess survival, differentiation, and apoptosis of transplanted cells and cardiomyocytes. **Results:** BMSCs decreased LV systolic and diastolic diameters and increased LV ejection fractions, LV fractional shortening, LV systolic pressure and LV end-diastolic pressure. Atorvastatin synergistically enhanced the BMSCs-induced improvements of ischemic cardiac dysfunction. Atorvastatin reduced inflammatory cell infiltration, cardiac fibrosis, and derangement of myocardial morphology/structure. Atorvastatin added a protective effect to cardiomyocytes against apoptotic cell death in infarct and peri-infarct areas, and also increased the survival rate of implanted BMSCs in acute myocardial ischemia. Atorvastatin also promoted cardiac differentiation of implanted BMSCs in infarct myocardium. **Conclusion:** Atorvastatin acts to improve the microenvironment both by synergistically enhancing the existing effects of BMSCs and by adding new therapeutic effects to BMSCs transplantation, and this combinational therapy is a superior cell/pharmacological therapeutic approach that merits future preclinical and clinical studies.

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Introduction

Acute myocardial infarction (AMI) is one of the major threats to human life and health; the pandemic continues to show an upward trend as the result of changing life styles and diets worldwide, despite the ever improvements of clinical treatment over the past decades. Though immediate intervention therapy may save the lives of many AMI patients by recovering the ischemic myocardium, this comes at the price of adverse cardiac remodeling and heart failure due to, at least partially, the fact that the lost cardiomyocytes during AMI cannot be regenerated [1]. The recent advances in stem cell therapy from both basic and clinical researches have made regeneration of damaged vascular and cardiac tissues possible and demonstrated promising outcomes in the treatment of myocardial infarction [2–6]. However, in the setting of AMI, the harsh micro-environment in the infarct zone, which is filled with inflammatory response, oxidative stress, neuroendocrine activation, etc., imposes a negative impact on the survival of implanted cells and execution of their differentiation and biological functions [7–14]. Thus, the optimal efficacy of stem cell therapy in AMI has been largely hampered by the poor survival and low differentiated rates of the implanted stem cells into the myocardium.

Recent studies have demonstrated that combined therapy with mesenchymal stem cells (MSCs) and atorvastatin, a blood cholesterol-lowering agent, produces synergistic beneficial effects in the treatment of AMI, despite that neither atorvastatin nor MSCs can individually improve ventricular function significantly [15–21]. These findings retrieved the declining interest in applying and investigating stem cell therapy of AMI, and stirred up new surges for searching better strategies to boost MSCs therapy: combined MSCs therapy and pharmacotherapy is one of these proof-in-principle approaches.

In this study, we sought to investigate comprehensively the beneficial effects of atorvastatin added to the cell therapy with bone marrow-derived mesenchymal stromal cells (BMSCs) in a rabbit model of AMI. To this end, we comparatively evaluated the changes of cardiac function, cardiac repair, cardiomyocyte apoptosis, and BMSCs survival and differentiation under various conditions in non-treated control, BMSCs-transplantation, and combined BMSCs transplantation and atorvastatin.

Materials and Methods

Rabbit model of acute myocardial infarction (AMI)

Thirty Japanese white rabbits (2.0±0.2 kg) were randomly divided into 3 groups (n=10 for each group): control group (Control group), bone marrow stem cell transplantation group (BMSCs group), BMSCs + atorvastatin group (BMSCs/Atorvastatin group). The animals were anesthetized by intraperitoneal injection of 3% sodium pentobarbital (40 mg/kg). Thoracotomy was performed and the left anterior descending coronary artery was ligated. Postoperative 12-lead ECG was recorded and the successful establishment of AMI model was verified by significant ST-segment elevation. A second thoracotomy was carried out one week after AMI. The animals of BMSCs and BMSCs/atorvastatin groups were injected with BMSCs (4×10⁷ cells/50 µl) into the peri-infarct zone, and the control group was injected with an equal volume of serum-free culture medium. For the BMSCs/atorvastatin group, atorvastatin (1.5 mg/kg/day) was given through the drinking water throughout the entire experimental period starting from 3 days prior to AMI.

Isolation and culturing of rabbit bone marrow stem cells (BMSCs)

The procedures for isolation and culturing of BMSCs were essentially the same as described in detail elsewhere [22]. Briefly, two days prior to AMI, 2 ml bone marrow was collected from femur under strictly sterile conditions, and diluted and dispersed by pipetting in phosphate buffered saline (PBS). The suspension was centrifuged at 1500 r/min at 4°C at room temperature (RT) for 25 min, and the resulting pellet was resuspended in PBS containing 2 mM EDTA, and separated in a Ficoll (1.073 g/ml) density gradient centrifugation (cell suspension: ficoll=1.5:1.0) at 500× g at RT for 30 min. The mononuclear fraction interphase was collected, washed twice in PBS/EDTA. The final pellet was resuspended in 12 ml Dulbeccos

Modified Eagle Medium (DMEM)/F12 cell culture medium (+10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin; 37°C, 5% CO₂) and seeded on gelatine-coated petri dishes. For the first 24 h cells were incubated with additional 10 µM 5-azacytidine (an inhibitor of DNA methyltransferase), then continued to be cultured for 4 days. Cell culture medium was changed every 48 h, and non-adherent hematopoietic cells were discarded.

Passaging was carried out at a confluence of >70%. The medium was removed and the cells were washed with serum-free medium. Trypsin (0.25%) was added into the flask for digestion. Trypsin was removed when the cell axons became retracted and rounded and serum-containing medium was added to terminate the digestion. The collected cell suspension was centrifuged and the supernatant was discarded. Cells were resuspended in culture medium 2×10⁵/ml in a 25 m² flask and an aliquot of medium was used for cell counting.

Immunocytochemistry characterization of BMSCs

The isolated and expanded BMSCs were fixed with 4% paraformaldehyde for 15 min and blocked in goat or rabbit serum for 1 h at 37°C. The cells were then incubated with various primary antibodies for 24 h at 4°C, followed by secondary antibodies (fluorescein isothiocyanate [FITC] or tetraethyl rhodamine isothiocyanate [TRITC] conjugated) for 1 h at 37°C. The primary antibodies for BMSC biomarker proteins included goat polyclonal anti-CD34, anti-CD44, anti-CD90, anti-CD166, and anti-BDNF, all purchased from Santa Cruz Biotech (CA, USA). Finally, nuclei were counterstained with Hoechst (1:1000) for 10 min at room temperature. The cells were visualized using a fluorescence microscope (Nikon 80i, Japan). Positive percentages of cells in five nonoverlapping fields of view (×200) of each slide were counted, and the average represented the positive rate of each marker.

Transplantation of BMSCs

Prior to transplantation, the BMSCs were labeled with DAPI (4,6-linked amidino-2-phenylindole dihydrochloride; 50 µg/ml) by incubating for 2 h and then washed with pre-cooled PBS three times. The cells were then digested with 0.25% trypsin, suspended at 4×10⁷ cells/50 µl in DMEM-F12 serum-free medium, and stored on ice until transplantation. Thoracotomy was performed to expose the heart in rabbits one week after AMI, and the prepared cells were intramuscularly injected into the peri-infarct myocardium. Intramuscular injection of penicillin (800 kunits/day) was carried out three days post surgery to prevent infection.

Echocardiography

Echocardiographic evaluation of cardiac function was conducted prior to AMI and four weeks after cell transplantation, using ACUSON Sequoia 512 Ultrasound Imaging system (USA) equipped with a cardiac probe (Model 7V3) and a 6 MHz transducer. Rabbits were fixed on four limbs in the supine position in a non-narcotic state. Two-dimensional M-mode imaging was taken in the parasternal long and short axis views apical two-chamber and four-chamber views, and left ventricular (LV) long and short axis views. LV end-diastolic diameter (Dd) and systolic diameter (Ds) were measured in the LV long axis view; LV ejection fraction (EF) was determined in the papillary muscle level short axis view; and LV shortening fraction was automatically calculated as: LVFS = [(Dd-Ds)/Dd] × 100%. All values were taken as the mean of triplicate measurements.

Hemodynamic detection

Four weeks after transplantation, the animals were fixed on the operation table and anesthetized by intraperitoneal injection of 3% sodium pentobarbital (40 mg/kg). The right common carotid artery was separated and cannulated with a 6F soft catheter to deliver a bolus of sodium heparin (500 IU/kg). The catheter was then advanced into LV. The catheter was connected to a 32-channel polygraph recorder through a transducer to record LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP) and other indicators of cardiac function.

Specimen processing

After measurements of cardiac function and hemodynamic parameters, chest was opened and heart was quickly excised and rinsed with cold saline. Then, the heart was cut along the short axis and chopped

Fig. 1. Immunocytochemical characterization of BMSCs at passage 2 stage. Immunoreactivities of BDNF and CD90 were detected by TRITC-labeled secondary antibody in red, and immunoreactivities of CD44 and CD166 were detected by FITC-labeled secondary antibody in green. Cell nuclei were counterstained with Hoechst in blue.

into ~1-cm chunks. The tissue blocks from infarct and border zones were separately cryo-sectioned and embedded in paraffin for later use.

Hematoxylin and eosin (H&E) staining and Masson's Trichrome staining

The paraffin-embedded sections were stained by H&E or by Masson's Trichrome dye. For Masson's Trichrome staining, collagen was in blue, muscle fibers in red, and nucleus in blue-black.

Immunohistochemistry

To determine the survival rate of transplanted cells and the differentiation of transplanted cells into cardiomyocytes and vascular cells, immunohistochemistry was carried out four weeks after cell transplantation. To this end, the frozen myocardial tissue preparations were cryo-cut into 5- μ m thick sections which were subject to DAPI staining to identify the transplanted BMSCs. Two sections were randomly selected from infarct and peri-infarct zones, respectively, for examination under a fluorescence microscope, and for each section, five fields were randomly selected for counting the number of cells labeled with DAPI. The survival rates of the transplanted cells were compared between BMSCs and BMSCs/Atorvastatin.

The sections were treated with primary antibodies to troponin T and VECFVIII to identify cardiac cells and vascular cells, respectively, with Cy3 (red fluorescence)-labeled antibody being used as the secondary antibody. The proportion of troponin T-positive cells (red; cardiac myocytes) to DAPI-positive cells (nuclei stained blue indicated the transplanted BMSCs) counted under a laser scanning confocal microscope was taken to indicate the differentiation rate to heart cells. Similarly, the proportion of VECFVIII-positive cells (red; vascular cells) to DAPI-positive cells was used to indicate the differentiation rate to vascular cells.

TUNEL staining

Apoptotic cells were detected from the tissue sections of the infarct and boarder zones, using TENEL staining methods. Detailed procedures were according to the manufacturer's instruction. For each section, apoptotic cells were counted from five randomly selected fields and percentage of apoptotic cells over the total number of cells counted was taken as the apoptosis index (AI).

Statistical analysis

Data are presented as mea $\pm$ SD. The group comparisons were performed by using ANOVA and *q* test. The intra-group differences of cell differentiation in animals subject to BMSCs transplantation were analyzed using the chi-square test. Two-tailed *P*<0.05 was considered statistically significant. All statistical analyses were performed using SPSS13.0 software.

Results

Characterization of BMSCs

Immunocytochemical staining indicated that nearly all cells were positive for BDNF (99.7%) and CD44 (99.2%), and most cells expressed CD166 (97.3%) and CD90 (96.5%)

Table 1. Comparisons of cardiac function measured by echocardiography and hemodynamic examination in a rabbit model of acute myocardial infarction (mean±SD)

Group (AMI)	Dd (cm)	Ds (cm)	LVFS (%)	LVEF (%)	LVSP	LVEDP
Control	1.39±0.17	1.05±0.12	24.57±1.43	48.67	97.3±7.4	-7.8±1.1
BMSCs	1.26±0.11*	0.92±0.09*	27.41±1.47*	59.14*	106.8±9.1*	-10.2±1.1*
BMSCs/Atorvastatin	1.15±0.08*#	0.81±0.07*#	29.52±1.51*#	67.32*#	113.4±11.7*#	-12.4±1.2*#

AMI: acute myocardial infarction; BMSCs: rabbit bone marrow stem cells; Dd: left ventricular end-diastolic diameter; Ds: left ventricular systolic diameter; LVFS: left ventricular fractional shortening; LVEF: left ventricular ejection fraction; LVSP: left ventricular systolic pressure; LVEDP: left ventricular end-diastolic pressure; *P<0.05 vs Control; #P<0.05 vs BMSCs.

Fig. 2. Reduction of cardiac fibrosis and inflammatory cell infiltration by atorvastatin in rabbit hearts. (A) Representative images of H&E staining and Masson's Trichrome staining (200× magnification) of sections from infarct and peri-infarct zones of a rabbit heart subject to acute myocardial infarction, showing the effects of BMSCs transplantation and combined atorvastatin and BMSCs on the inflammatory cell infiltration, cardiac fibrosis (blue), viable cardiomyocytes, and derangement of cardiac fibres. (B)

Mean data on the percent fibrotic tissues under varying conditions. The values were obtained from randomly selected 10 fields for each rabbit. Atorv: atorvastatin (1.5 mg/kg/day). *P<0.05 vs. MI; #P<0.05 vs. BMSCs; n=5 animals for each group. (C) Mean data on the percentage of inflammatory cell infiltration under varying conditions. The values were obtained from randomly selected 10 fields for each rabbit. *P<0.05 vs. MI; #P<0.05 vs. BMSCs alone; n=5 animals for each group.

(Fig. 1). All cells were negative for CD34. These data are in line with the characteristics of BMSCs [23, 24].

Atorvastatin enhances BMSCs-induced improvement of ischemic cardiac dysfunction

Four animals died during the course of the study period, with two in control group, one in the BMSCs group, and one in the BMSCs/Atorvastatin group. The causes of death were ventricular fibrillation and acute left ventricular failure.

BMSC transplantation significantly alleviated the AMI-induced enlargement of left ventricular end-diastolic diameter (Dd) and left ventricular systolic diameter (Ds; Table 1). On the contrary, BMSCs restored the diminished LVFS, LVEF, LVSP, and LVEDP in AMI. All these beneficial effects of BMSCs were markedly enhanced by atorvastatin treatment (Table 1).

Atorvastatin enhances BMSCs-induced repair of ischemic myocardial impairment

As depicted in Figure 2, H&E staining and Masson's Trichrome staining demonstrated profound inflammatory cell infiltration, severe cardiac fibrosis, sparse viable cardiomyocytes, and patchy distribution of lesions in the infarcted area in untreated control group. BMSCs

Fig. 3. Cytoprotection against cardiomyocyte apoptosis by atorvastatin in rabbit hearts. (A) Typical examples of TUNEL staining of defragmentated nuclei (brown; 200× magnification) showing the effects BMSCs transplantation and combined atorvastatin and BMSCs on apoptotic cells of cardiomyocytes in infarct and peri-infarct areas of a rabbit heart of acute myocardial infarction. (B) Mean data of % TUNEL positive cells. Note that atorvastatin reduced the number of cardiomyocyte apoptosis/TUNEL positive cells in acute myocardial ischemia. ^aP<0.05 vs. BMSCs alone; n=5 for each group.

transplantation diminished fibrosis and inflammatory cell infiltration, and increased viable cardiac cells. Atorvastatin treatment produced synergetic effects with BMSCs transplantation in extenuating the structural impairment, inflammatory response and cell death. For example, the percent fibrotic tissue was $68.7 \pm 5.3\%$ in MI, $51.6 \pm 3.8\%$ in the BMSCs group, and $37.3 \pm 3.1\%$ in the BMSCs/Atorvastatin group ($P < 0.05$; F-test and $P < 0.05$ between the BMSCs/Atorvastatin and BMSCs groups) (Fig. 2B). The percentage of inflammatory cell infiltration was $26.5 \pm 2.3\%$ in the MI group, $18.6 \pm 2.2\%$ in the BMSCs group, and $9.5 \pm 1.3\%$ in the BMSCs/Atorvastatin group ($P < 0.05$; F-test and $P < 0.05$ between the BMSCs/Atorvastatin and BMSCs groups) (Fig. 2C).

Atorvastatin diminishes cardiomyocyte apoptosis in acute myocardial ischemia

TUNEL staining revealed that BMSCs transplantation alone did not significantly alter apoptotic cell death of cardiomyocytes in infarcted and peri-infarct areas (Fig. 3), as indicated by the nearly identical AI values in the BMSCs group ($19.2 \pm 5.4\%$; $P > 0.05$) and the Control group ($20.1 \pm 5.8\%$). Combinational therapy with atorvastatin administration and BMSCs transplantation, however, significantly reduced AI to $9.7 \pm 1.8\%$, a ~50% reduction of apoptotic cells.

Atorvastatin enhances survival of implanted BMSCs in acute myocardial ischemia

Atorvastatin significantly preserved the survival of BMSCs in the infarcted area, as determined by the counts of DAPI-positive cells under a fluorescence microscope (358.7 ± 88.3 /high power field (HPF) for BMSCs/atorvastatin vs. 76.8 ± 20.9 /HPF for BMSCs alone; $P < 0.01$; Fig. 4).

Atorvastatin promotes cardiac differentiation of implanted BMSCs in infarct myocardium

Our results from immunohistochemistry experiments showed that some of the transplanted cells of the BMSCs group and BMSCs/Atorvastatin group expressed troponin T and VECFVIII (Fig. 4). The proportion of the troponin T-expressing transplanted cells after combination therapy with BMSCs and atorvastatin was significantly higher than that of BMSCs alone, indicating a higher rate of myocardial cell differentiation of transplanted cells in the presence of atorvastatin.

Fig. 4. Immunohistochemical assessment of implanted BMSCs (blue fluorescence; 200× magnification) (A & B), and differentiation to cardiac cells with troponin T staining (cTnT; red) (C), or to vascular cells with VECF VIII staining (vascular endothelial cell factor VIII; red) (D). Note that atorvastatin promoted the survival and myocardial differentiation of BMSCs, as indicated by the larger numbers of blue staining for BMSCs and red staining for cardiac cells, compared with BMSCs transplantation alone. * $P < 0.05$ vs. MI; ^o $P < 0.05$ vs. BMSCs alone; [‡] $P < 0.001$ vs. BMSCs alone; $n = 5$ animals for each group.

Discussion

Our data in this study revealed that atorvastatin offered significant beneficial effects on transplanted BMSCs and myocardium in the setting of AMI, as manifested by the following improvements. (1) Atorvastatin enhanced BMSCs-induced improvement of ischemic cardiac dysfunction, as reflected by the magnified restoration of the enlarged Ds and Dd, and the decreased LVFS, LVEF, LVSP, and LVEDP in AMI. (2) Atorvastatin enhanced BMSCs-induced cardiac repair of ischemic myocardial impairment, as indicated by the synergetic effects against inflammatory cell infiltration, cardiac fibrosis, and derangement of myocardial morphology/structure. (3) Atorvastatin added a protective effect to cardiomyocytes against apoptotic cell death in infarct and peri-infarct areas, an antiapoptotic property otherwise absent with BMSCs transplantation alone. (4) Atorvastatin enhanced the survival rate of implanted BMSCs in acute myocardial ischemia. And (5) atorvastatin promotes cardiac differentiation of implanted BMSCs in infarct myocardium. All these effects point to the ability of atorvastatin to improve the microenvironment so as to preserve implanted BMSCs and improve cardiomyocytes leading to amelioration of cardiac function and myocardial repair in AMI. Clearly, atorvastatin acts both by synergistically enhancing the existing effects of BMSCs and by adding new therapeutic components to BMSCs transplantation, and this combinational therapy is a superior cell/pharmacological therapeutic approach that merits future preclinical and clinical studies.

Although most of the animals and clinical trials have confirmed stem cell transplantation is safe for the treatment of cardiac disease, but the therapeutic efficacy has been found insignificant [8–14]. Studies have shown that stem cells transplanted into damaged tissue die because of apoptosis and perhaps other forms of cell death as well, mostly in a week after transplantation [25–27]. No more than 15% of the transplanted cells can survive for up to 12 weeks [21], which seriously restricted the potential of stem cells to repair damaged myocardium. Therefore, how to maximize the potential of stem cell transplantation in the treatment of AMI has become an urgent task for researchers and clinicians.

The causes for the substantial death of transplanted stem cells in the infarcted myocardium are likely multiple and intricate, but are not yet fully understood. Existing evidence suggests that ischemia and hypoxia, inflammation, apoptosis, oxidative stress, and neuroendocrine activation together create a grim and adverse microenvironment in the infarcted myocardium to threaten the survival of implanted stem cells [8–27]. Clearly,

maximizing the survival rate of transplanted cells is a critical step towards maximizing the potential of stem cell therapy for AMI. At present, there are two major strategies for promoting the survival rate of transplanted cells. The first approach is to boost up the ability of stem cells to tolerate ischemic insults by preconditioning or priming the cells prior to transplantation [28, 29], or by permanent or transient genetic engineering of stem cells to overexpress anti-inflammatory and anti-apoptotic genes [30, 31]. And the second approach is to improve the microenvironment by minimizing the harmful factors secreted into and around the area where stem cells are implanted, normally the infarct and peri-infarct zones, so as to relieve the insults to the implanted cells [32–39]. In this second scenario, implanted stem cells have better chance to survive and differentiate, so as to better execute their biological functions. By comparison, the first approach with genetic modification has the potential to bring about certain unwanted effects due to overexpression of foreign genes, which may limit its clinical applications. Thus, improving microenvironment for stem cell transplantation may be safer and more feasible.

Statins are one of the most commonly used agents in patients with coronary heart disease owing to their superior ability to reduce blood cholesterol levels [40]. Recent studies revealed that these agents exert a wide spectrum of cellular functions in addition to their lipid-lowering action, including anti-inflammatory, anti-apoptotic, anti-fibrotic, angiogenesis-promoting, and plaque-stabilizing effects [41–43]. These properties are well predicted to offer improvement of the microenvironment for implanted stem cells. The experimental results in the present study indeed provided several lines of evidence in support of this notion. Compared with BMSCs transplantation alone, the combined treatment with atorvastatin and BMSCs afforded synergistically beneficial effects on ischemic myocardial injury by AMI to retard the adverse ventricular remodeling and improve cardiac function.

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