Injection of Cardiac Stem Cells Prolongs the Survival of Cardiac Allograft Rats

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Abstract: Cardiac c-kit+ cells isolated from cardiac explant-derived cells modestly improve cardiac functions after myocardial infarction; however, their full potential has not yet been realized. The present study was undertaken to determine the isolation and culture of c-kit+ cardiac stem cells (CSCs), and the roles of myocardial injection of CSCs on the survival of rat cardiac allograft. Recipient Sprague-Dawley rats were transplanted with hearts from Wistar rats. In the *in vitro* experiment, c-kit+ cells were isolated from mouse heart fragment culture by magnetic cell sorting. CSCs expressed of cardiomyocyte specific protein cardiac troponin I, α smooth muscle actin and von Willebrand factor in conditioned culture. CSC injection increased graft survival of cardiac allograft rats. The effects of CSCs on increase in graft survival of cardiac allograft rats were blocked by stromal-derived factor-1 (SDF-1) knockdown. The expression of SDF-1 was increased after CSC injection into the cardiac of cardiac allograft rats. These results indicate that CSC injection into the cardiac prolongs graft survival of cardiac allograft rats. SDF-1 plays an important role in the effects of CSCs on the graft survival of cardiac allograft rats.

Key Indexing Terms: Cardiac stem cells; Cardiac allograft; Stromal-derived factor-1; Graft survival. **[Am J Med Sci 2015;349(1):67–71.]**

Cardiac transplantation has been established as an effective form of therapy for cardiac diseases.[1,2] Pediatric heart transplantation has excellent outcomes with survival beyond 20 years after transplant common, especially in the infant.[3] Stem cell transplantation has emerged as a promising therapeutic method, and donor-specific tolerance could be induced by injection of donor mesenchymal stem cells at the time of heart transplantation.[4]

For a long time, it was believed that cardiac tissue is terminally differentiated. However, cardiac stem cells (CSCs), progenitor cells in the cardiac, were isolated in rodents cardiac tissue.[5,6] CSCs have been classified according to biological markers such as c-kit+, side population and Islet-1+.[7,8] CSCs form cell lines that can be expanded in culture while keeping their progenitor state. Subsequently, they can differentiate in cardiovascular cell types, such as cardiomyocytes, endothelial cells, and smooth muscle cells.[9–11] Transplantation of CSCs aims to use and enlarge the heart's own regeneration capacity. After myocardial infarction (MI), CSCs and newly formed myocytes are found in the border of the infarct area, suggesting that the heart has some regeneration capacity.[12] The first phase 1 clinical trials using CSCs infusion were published, intracoronary infusion of autologous CSCs is effective in improving left ventricular systolic function and reducing infarct size in patients with heart failure after MI.[13] It has been demonstrated that intracoronary infusion of autologous CDCs after MI is safe, warranting the expansion of such therapy to phase 2 study.[14]

The stromal-derived factor-1 (SDF-1) is a 68-amino acid chemokine with a broad range of functions including lymphocyte and mononuclear cells trafficking to embryonic development, inflammatory sites, angiogenesis, and bone marrow stem cell homeostasis.[15,16] SDF-1 and its cellular receptor C-X-C chemokine receptor type 4 (CXCR4) are known as the most prominent stem cell chemotaxis. It has been shown that exogenously expressed vascular endothelial growth factor promoted myocardial repair at least in part through SDF-1α/CXCR4-mediated migration of CSCs.[17] The present study was designed to determine the isolation and culture and multiple differentiation of CSCs *in vitro*, and the effects of CSCs on the graft survival after cardiac transplantation.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (recipient, grade SPF; 250–300 g) and male Wistar rats (donor, grade SPF; 200–250 g) were purchased from Shanghai Laboratory Animal Centre (Chinese Academy of Sciences, Shanghai, China). All efforts were made to minimize the number of animals used and their suffering. Fifty-five rats were used in the experiment. The animals were housed in a temperature- and humidity-controlled room with a 12-hour on-off light cycle and given free access to food and water.

Isolation of c-kit+ CSCs

C-kit+ CSCs were isolated from neonatal BALB/c mice (3–5 days) according to previous methods.[7,18–20] Briefly, mouse hearts were removed and minced into small pieces, washed with phosphate-buffered solution, and digested 2 hours at 37°C with a mixture of 0.25% trypsin (Invitrogen, Carlsbad, CA) and 0.1% collagenase IV (Sigma, St. Louis, MO). Cells were cultured in Iscove's Modified Dubecco's Medium (IMDM) (HyClone, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 2 mmol/L of L-glutamine (Invitrogen) and 0.1 mmol/L of 2-mercaptoethanol (Sigma), 100 Units/mL penicillin G (Gibco), 100 mg/mL streptomycin (Gibco) at 37°C and 5% CO_2.

After 10 to 15 days, a layer of small, phase-bright cells above the adherent explants were collected by washing with 0.5 g/L trypsin-0.53 mmol/L EDTA. The cell suspension was filtered through a 40-μm cell strainer and isolated by magnetic cell sorting using anti-c-kit-coupled magnetic beads (Miltenyi Biotec, San Diego, CA).

Culture of C-kit+ CSCs

The purified c-kit+ cells were seeded at 0.5 to 2 × 10^5/mL in multiwell plates precoated with Dulbecco's modified Eagle's

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medium/Ham's F12 containing 10% FBS (Gibco), basic fibroblast growth factor (10 ng/mL; Sigma), insulin-transferrin-selenite (Sigma), epidermal growth factor (20 ng/mL; Sigma), stem cell factor (10 ng/mL; Sigma), human platelet-derived growth factor-BB (10 ng/mL; R&D, Minneapolis, MN), 2-mercaptoethanol (0.1 mM; Sigma), erythropoietin (2.5 U; Sigma) at 37°C and 5% CO_2.[21]

Heterotopic Cardiac Transplantation

The rat model of heterotopic cardiac transplantation was performed according to previous methods.[22,23] Briefly, rats were anesthetized with intraperitoneal injection of 2% sodium pentobarbital. To reduce the ischemic time, the recipient abdomen was opened before the donor heart was explanted. Immediately after explantation, the ascending aorta and pulmonary artery of the donor heart were side anastomosed to the recipient abdominal aorta and inferior vena cava. The donor heart was perfused with the recipient rat's blood and resumed contraction. Heart grafts were checked daily by palpation for signs of rejection. The day at which no cardiac contractions were palpable was regarded as graft rejection time and rejection was verified by direct inspection of the allograft by laparotomy.

Myocardial Injection

The rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal) and 1×10^5 c-kit+ CSCs in 50 mL saline were injected into the myocardium with a 30-G needle at 4 points. The sham-operated (Sham) rats were treated the same as the CSCs injection rats except without CSCs. Adeno-associated viral (AAV) vectors encoding short-hairpin RNAs targeting SDF-1 (AAV-shRNA-SDF-1) was purchased from Invitrogen. The myocardium was infected with AAV-SDF-1 at a multiplicity of infection of 10^4 as above. In addition, a scrambled oligonucleotide sequence was used for specificity control.

Multiple Differentiation of c-kit+ cells

The purified c-kit+ CSCs were identified by immunofluorescence using a monoclonal antibody for c-kit. To induce *in vitro* differentiation,[7] c-kit+ cells were seeded on 35-mm dishes (5×10^4 per dish) in Dulbecco's modified Eagle's medium supplement with 10% FBS, 1 mM dexamethasone (Sigma), 50 mg/mL ascorbic acid (Sigma), and 1 mM β-glycerophosphate (Sigma). The medium was changed twice a week. Immunofluorescence analysis was performed with primary antibodies specific for cardiac troponin I (cTn-I) (1:200; Santa Cruz, Dallas, TX), α-smooth muscle actin (1:200; Zymed Lab, Grand Island, NY) and von Willebrand factor (1:200; BD PharMingen, Sparks, MD). Goat anti-rabbit second antibody conjugated with fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate were used for immunocytochemistry staining. Staining was observed by a Leica fluorescence microscopy (Wetzlar, Germany).

SDF-1 quantitative real time-PCR

Total RNA was extracted from cultured cells using Trizol (Invitrogen). The cDNA was synthesized using a random primer from 1 μg total RNA with the RevertAid First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Fermentas). The following primers were used for qRT-PCR, sense: 5′-gctttgagtgactgggtt-3′, antisense: 5′-gtggcaagatgatggttt-3′, Polymerase chain reaction was performed following the manufacturer's instructions. The cycling conditions were 3 minutes at 94°C, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 60 seconds. Amplified DNA fragments were resolved by electrophoresis on 1% agarose gels containing ethidium bromide.

Immunohistochemistry was performed according to the standard protocol using rabbit polyclonal SDF-1 antibodies (Abcam, Cambridge, United Kingdom). The endogenous peroxidase activity was blocked with peroxidase block for 10 minutes, and the nonspecific antibody binding was blocked by bovine serum albumin in phosphate-buffered saline. The cardiac sections were incubated with SDF-1 antibodies at 4°C overnight. After washing, sections were incubated with biotinylated goat anti-rabbit immunoglobulin G for 10 minutes and then stained with diaminobenzidine according to the manufacturer's instructions. Sections were covered with mounting medium and SDF-1 immunoreactivity was observed under a light microscope (DP70; Olympus, Tokyo, Japan).

Western Blotting

SDF-1 protein expression was determined by Western blotting. Briefly, after process of electrophoresis and transmembrane, proteins on nitrocellulose membrane were probed with the primary antibody (1:500; Cell Signaling Technology, Inc, Beverly, MA) followed by incubation with the secondary antibodies (1:5000; Immunology Consultants Lab). The bands were visualized by enhanced chemiluminescence using electrogenerated chemiluminescent (Pierce Chemical, Milwaukee, WI) and captured on x-ray films. GAPDH (Bioworld Technology Inc, Louis Park, MN) protein was used as a loading control. The total SDF-1 protein level was normalized to the GAPDH protein level.

Statistical Analysis

Data were analyzed using SPSS 18.0 (IBM, Armonk, NY). Comparisons between 2 observations were assessed by Student's paired *t* test. One-way or 2-way analysis of variance was used followed by the Bonferroni test for *post hoc* analysis when multiple comparisons were made. All the data were expressed as mean ± standard error. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Culture of Explants and c-kit+ CSCs

Five days after the heart tissues culture, fibroblasts migrated from the adherent explants. Then, some small and round cells appeared (Figure 1A). After c-kit+ magnetic cell sorting isolation, these c-kit+ positive cells (green, Figure 1B) were seeded in CGM and gradually formed 3-dimensional spheres in 2 weeks (Figure 1C).

Multipotent Differentiation of c-kit+ CSCs

C-kit+ cells changed their shape in the conditioned culture. Confocal immunofluorescence analysis showed expression of smooth muscle-specific marker α-smooth muscle actin (red, Figure 2A), the endothelial specific marker von Willebrand factor (red, Figure 2B), and cardiomyocyte-specific protein cTn-I (green, Figure 2C).

Graft Survival

The mean graft survival in saline group was 4.6 days, CSCs treatment increased the graft survival to12.3 days. However, SDF-1 protein knockdown blocked the increase in the graft survival of CSCs injection (Figure 3).

SDF-1 Expression

CSCs injection increased the expression of SDF-1 mRNA, SDF-1 protein expression was also increased after CSCs injection in the heterotopic cardiac transplantation rats (Figure 4).