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Activation of cardiac progenitor cells through paracrine effects of mesenchymal stem cells

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ABSTRACT

Mesenchymal stem cells (MSC) transplantation has been proved to be promising strategy to treat the failing heart. The effect of MSC transplantation is thought to be mediated mainly in a paracrine manner. Recent reports have suggested that cardiac progenitor cells (CPC) reside in the heart. In this study, we investigated whether MSC had paracrine effects on CPC in vitro. CPC were isolated from the neonatal rat heart using an explant method. MSC were isolated from the adult rat bone marrow. MSC-derived conditioned medium promoted proliferation of CPC and inhibited apoptosis of CPC induced by hypoxia and serum starvation. Chemotaxis chamber assay demonstrated that MSC-derived conditioned medium enhanced migration of CPC. Furthermore, MSC-derived conditioned medium upregulated expression of cardiomyocyte-related genes in CPC such as β-myosin heavy chain (β-MHC) and atrial natriuretic peptide (ANP). In conclusion, MSC-derived conditioned medium had protective effects on CPC and enhanced their migration and differentiation.

Mesenchymal stem cells (MSC) transplantation has been proved to be promising strategy to treat ischemic heart disease [1–4]. We and others have demonstrated therapeutic potency of MSC transplantation for the treatment of cardiovascular disease [5]. The effect of MSC transplantation is thought to be mediated by the supply of cell protective, angiogenic and mitogenic factors, in addition to differentiation of transplanted MSC into specific cell types [6–8]. However, the underlying mechanisms of MSC therapy remain unclear.

Cardiomyocytes have been traditionally regarded as terminally differentiated cells that compensate for cardiac dysfunction through hypertrophy. However, recent reports suggested that multipotent cells reside in the adult heart and differentiate into smooth muscle cells, endothelial cells and cardiomyocytes [9–11]. Cardiac stem cells (CSC) transplantation has been shown to decrease the infarct size and improve cardiac performance in a rat model of myocardial infarction [9]. These findings suggest that CSC may play an important role in cardiac regeneration. However, some problems including isolation and expansion of CSC remain to be unresolved for clinical application of CSC transplantation. Thus, a novel strategy to activate endogenous CSC would be desirable for the treatment of heart failure. Recently, recombinant hepatocyte growth factor (rHGF) was reported to promote migration and survival of endogenous CSC [12].

We have shown that MSC secret a number of cytokines and growth factors including HGF, vascular endothelial growth factor (VEGF) and insulin-like growth-1 (IGF-1) [4–6]. Therefore, we hypothesized that transplanted MSC-derived cytokines might activate endogenous cardiac stem/progenitor cells, leading to improvement in cardiac function of the failing heart. Thus, the purpose of this study was to investigate whether transplanted MSC activate endogenous cardiac progenitor cells (CPC) by enhancement of proliferation, migration and differentiation of CPC in a paracine manner.

Materials and methods

Isolation and expansion of mesenchymal stem cells from rat bone marrow. All protocols were performed in accordance with the guidelines of the Animal Care Committee of the National Cardiovascular Center Research Institute, Japan. Isolation and expansion of MSC were performed according to previously described methods [2]. In brief, we used 6- to 8-week-old male Lewis rats (Japan SLC, Hamamatsu, Japan) and harvested their bone marrow by flushing the femoral and tibial cavities with phosphate-buffered saline

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(PBS). Bone marrow cells were cultured in a 10-cm dish with complete culture medium: α-minimal essential medium: αMEM (Invitrogen, Carsbad, CA), 10% fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (MP Biomedicals, Solon, OH). Non-adherent hematopoietic cells were removed, and the medium was replaced every 3–4 days. The adherent, spindle-shaped MSC population expanded to >5 × 10^7 cells within 4–5 passages after the cells were first plated.

Preparation of MSC-derived conditioned medium. MSC (1 × 10^6 cells) were plated in 10-cm dishes and cultured in complete culture medium for 2 days. The attached cells were washed three times with PBS and the medium was replaced with basal culture medium: αMEM, 100 U/mL penicillin, 100 μg/mL streptomycin, and after 48 h, conditioned medium was collected and centrifuged at 2000*g* for 10 min followed by filtering the supernatant through a 0.22-μm filtration unit (Millipore, Bedford, MA).

Isolation of CPC from neonatal rats. CPC were isolated from neonatal Lewis rats (Japan SLC), as reported previously with modification [11]. In brief, isolated myocardial tissue was cut into 1- to 2-mm^3 pieces and digested three times for 5 min at 37 °C with 0.2% trypsin (Invitrogen) and 0.1% collagenase II (Worthington Biomedical, Lakewood, NJ). These tissue pieces were washed with complete explant medium (Iscove's Modified Dulbecco's Medium, Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mmol/L 2-mercaptoethanol (Wako Pure Chemical Industries, Osaka, Japan). Then, the remaining tissue fragments were cultured as explants in complete explant medium at 37 °C under 5% CO_2. After 1–2 weeks, a layer of fibroblast-like cells was generated from adherent explants and phase-bright cells migrated over a layer of fibroblast-like cells. These phase-bright cells were collected by washing with PBS. Isolation of the phase-bright cells was performed twice at 3- to 5-day intervals from the same dish.

Reverse transcription-polymerase chain reaction. RT-PCR assay was performed according to a previously described method [13]. In brief, total RNA was extracted from CSC using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA was reverse-transcribed into cDNA using a QuantiTect reverse-transcription kit (Quiagen) according to the manufacturer's instructions. PCR amplification was performed in 50 μl containing 1 μl cDNA and 2.5 U Taq DNA polymerase (Takara, Otsu, Japan). The oligonucleotides used in RT-PCR analysis are listed in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA amplified from the same samples served as an internal control. PCR reaction mixtures were denatured at 95 °C for 5 min and cDNA templates amplified as follows: 35 cycles (21 cycles for GAPDH) of denaturation at 95 °C for 1 min, annealing at 55–66 °C for 45 s, and extension at 72 °C for 1 min. At the end of the cycling, the samples were incubated at 72 °C for 10 min.

Table 1
Primer pairs for RT-PCR

Primer	Sequence	Product size (bp)	Annealing temperature (°C)
ABCG2	5′-CAATGGGATCATGAAACCTG-3′ 5′-CAGGCTGATGAATGGAGAA-3′	536	58
c-Kit	5′-AGCAAGAGTTAACGATTCCGGAG-3′ 5′-CCAGAAAGGTGTAAGTGCCTCCT-3′	300	53
c-Met	5′-CAGTGATGATCTCAATGGGCAAT-3′ 5′-AATGCCCTCTTCCTATGACTTC-3′	725	60
CXCR4	5′-CAGAAGAAGCTGAGGAGCATGACA-3′ 5′-CTGATGAAGGCCAGGATGAGAACA-3′	197	55
Flt-1	5′-CATGGTCAGCTGCTGGGACACCGCG-3′ 5′-GACTCCCTGCATCACTAACAATAT-3′	400	62
IGF-1R	5′-ATTACGCACTGGTCATCTTC-3′ 5′-AAGCCATCTGAGTCACTGCT-3′	546	58
MEF-2c	5′-GGCCATGGTACACCGAGTACAACGAGC-3′ 5′-GGGGATCCCTGTGTTACCTGCATTGG-3′	401	62
GATA4	5′-CTGTCATCTCACTATGGGCA-3′ 5′-CCAAGTCCGAGCAGGAATTT-3′	275	60
ANP	5′-CCGAGACAGCAAACATCAGATCG-3′ 5′-CCGTGGTGCTGAAGTTTATTCGG-3′	762	58
β-MHC	5′-GCCAACACCAATGTCCAAGTTC-3′ 5′-TGCAAAGGCTCCAGGTCTGAGGGC-3′	205	66
GAPDH	5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′ 5′-CATGTAGGCCATGAGGTCCACCAC-3′	983	51

Cell proliferation assay. Cell proliferation assay was performed using CellTiter96 AQueous One solution cell proliferation assay (Promega, Madison, WI). Briefly, isolated CPC were plated on 96-well plates (5 × 10^3 cells per well), and cultured in basal culture medium ($n = 6$) and MSC-derived conditioned medium ($n = 6$) for 48 h. The cellular level of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), indicative of the mitochondrial function of living cells and cell viability, was measured with a CellTiter96 AQueous One Solution Kit (Promega, Madison, WI) and a Microplate Reader (490 nm, Bio-Rad, Hercules, CA).

Terminal dUTP nick-end labeling (TUNEL) assay. TUNEL assay was performed to evaluate apoptosis of cells induced by serum starvation and hypoxia. After incubation of CPC in basal culture medium or MSC-derived conditioned medium under hypoxia (1% O_2) for 12 h, CPC were fixed in 1% paraformaldehyde. TUNEL assay was performed according to the manufacturer's instructions (ApopTag Fluorescein kit, Chemicon, Temecula, CA). The cells were then mounted in medium containing 4′,6-diamidino-2-phenylindole (DAPI).

Caspase-3 activity assay. Isolated CPC were plated on 6-well plates (2 × 10^5 cells per well), and cultured in complete culture medium under normoxia (Control), in basal culture medium under hypoxia (1% O_2, 5% CO_2) ($n = 6$), or in MSC-derived conditioned medium under hypoxia ($n = 6$) for 12 h. Caspase-3 activity was measured using a CaspACE Assay System Colorimetric (Promega, Madison, WI) according to the manufacturer's instructions.

Western blot analysis. To identify the protein expression of phosphorylated Akt, Western blotting was performed with rabbit antibodies against phosphorylated Akt (Ser473) and Akt (Cell Signaling Technology, Danvers, MA). After CSC had been cultured with basal culture medium for 24 h, cell lysates were extracted with sample buffer. Then, 2 μg of protein was transferred into sample buffer, loaded on a 10% sodium dodecyl sulfate–polyacrylamide gel, and blotted onto a polyvinylidene fluoride membrane (Millipore). After being blocked for 120 min, the membrane was incubated with primary antibody at a dilution of 1:500. The membrane was incubated with peroxidase labeled with secondary antibody at a dilution of 1:2000. Positive protein bands were visualized with an ECL kit (GE Healthcare, Buckinghamshire, UK) and measured by densitometry.

Cell migration assay. Migration assay was performed using Chemotaxicell96 (Kurabo, Osaka, Japan) composed of a membrane with 5-μm pores. CPC (5 × 10^3 cells per well) suspended with 50 μl basal culture medium were cultured in basal culture medium in the upper chamber and incubated in basal culture medium ($n = 6$) or MSC-derived conditioned medium ($n = 6$) in the lower chamber for 12 h at 37 °C. The filter was removed from the plate, and the number of the cells that migrated to the lower chamber was manually counted under a microscope.

Differentiation of CPC into caridomyocytes. Isolated CSC were plated on 6-well plates (1 × 10^5 cells per well) and differentiation of CPC into cardiomyocytes was induced (1) by incubation in complete culture medium, (2) by treatment with 3 μM 5-azacytidine (5-AZA, Sigma, Louis, MO) for 24 h [14], and (3) by incubation in MSC-derived conditioned medium for 2 weeks. After induction of differentiation, total RNA was extracted using an RNeasy Mini Kit (Qiagen).