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Amniotic mesenchymal stem cells with robust chemotactic properties are effective in the treatment of a myocardial infarction model

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ABSTRACT

Background: We previously reported that amniotic mesenchymal stem cells (AMMs) possess high angiovasulogenic properties. In this study, we investigated the chemotactic abilities of AMMs for improved cardiac function and regenerative angiogenesis.
Methods: The expressions of chemotactic and angiogenic genes were determined by qRT-PCR. Myocardial infarction (MI) was induced in NOD/SCID mice and cells were directly transplanted into the border regions of ischemic heart tissue. Immunohistochemical analysis was also conducted.
Results: AMMs significantly expressed the representative chemotactic factor GCP-2, NAP-2 as well as angiogenic factor Hif-1a. AMMs also highly expressed the chemokine receptors CCR2, CCR3 and CCR5. AMM transplantation improved left ventricular function, capillary density, angiogenic cytokine levels, angiopoetin (Ang)-1 and vascular endothelial growth factor (VEGF-A) levels in affected tissue. Immunohistochemical assaying also revealed increased engraftment and endothelial phenotypes.
Conclusion: Our findings suggest that due to elevated survival and related chemotactic potential, AMMs are a promising stem cell source for the treatment of ischemic cardiovascular disease.

1. Introduction

Cardiovascular disease (CVD) remains a leading cause of mortality in western societies [1]. Myocardial infarction (MI), directly leading to heart dilation or cardiac failure, often results in significant disability. Despite the advancement of surgical technology related to the treatment of MI, there are still many challenges in repairing damaged heart tissue. Recently, stem cell transplantation has emerged as a novel potential therapy for the treatment of MI [2]. However, the low survival and transdifferentiation rates of transplanted stem cells in host tissues are regarded as substantial limitations, resulting in marginal therapeutic effects [3–6].

Human placenta-derived amnion is easy to obtain from discarded placenta and rich in multi-potent stem cells and other progenitor cells [7,8]. Human AMMs are highly angiogenic and were recently suggested to have potential for transdifferentiation into cell types of various tissues [9,10]. However, studies relating to the therapeutic capability and survival properties of AMMs in ischemic heart diseases are limited.

Chemokines are a small family of heparin-binding peptides, originally known for their immune surveillance and inflammation mediating properties, which exert influence on angiogenesis via recruitment of endothelial progenitors and pro-angiogenic inflammatory cells. Chemokines also activate chemokine receptors, playing an important role in the regulation of endothelial function through downstream signaling pathways. It has been reported that mesenchymal stem cells (MSCs) express the chemokine receptors CXCR1, CXCR2, and CCR2 [11]. However, the chemotactic properties of AMMs are not yet fully understood.

In this study we investigated the possibility of AMM-mediated improvement of cardiac function and compared them with human adipose-derived mesenchymal stem cells (ADMs) in an in-vivo MI model.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) and normal human dermal fibroblasts (HDFs) were purchased from the ATCC (Manassas, VA, USA). Human AMMs from five different donors were purchased from Thermo Scientific Inc. (Rockford, IL, USA). MSC characterization data and multilineage differentiation potentials were demonstrated by the manufacturer. HDFs, AMMs and ADMs were cultured in low-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco). HUVECs

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were cultured in the endothelial growth medium EGM-2 (Lonza Walkersville, MD, USA). All protocols involving human samples were approved by the Institutional Review Board of Dong-A University and the experiment conformed to the principles established by the Declaration of Helsinki.

2.2. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses

qRT-PCR assays were conducted as previously reported [12,13]. Total RNA was isolated from cells using RNA-stat reagent (Iso-Tex Diagnostics, Friendswood, TX, USA) according to the manufacturer's instructions before the extracted RNA was reverse-transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). The cDNA was synthesized using human specific primers and Taq polymerase (iNtRON Biotechnology, Sungnam, Korea). DNA levels were quantitatively assessed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). qRT-PCR cycling conditions were 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. After normalization to GAPDH, the relative expression levels of the target gene in the experimental samples was determined using the formula Rel Exp $= 2^{-\Delta CT}$ (fold difference), where $\Delta Ct =$ (Ct of target gene) − (Ct of control gene, GAPDH). The number of PCR cycles was measured using Lightcycler 3.5 software (Roche Molecular Biochemicals, Indianapolis, IN).

2.3. qRT-PCR and RT-PCR primers

Genes and catalogue numbers were as follows: GCP-2 (Hs00234140), NAP-2 (Hs00234007), MCP-1(Hs00234077), Hif-1a (Hs00153153); paired RT–PCR primers used:
5′-gaaggtggagaagctccctg/ttctttagccatgtggcctg-3′ for CCR2 (206 bp),
5′-atgactgtgagcggagcaag/ggaatgggatgtatctgccc-3′ for CCR3 (192 bp),
5′ gcaacatgctggtcatcctc/caaacacagcatggacgaca-3′ for CCR5 (279 bp),
5′-aaggcagaagcaacccaaat/tcaaggttcgtccgtgttgt-3′ for CXCR2 (145 bp),
5′ tggggcagcccagaacatca/gccgcctgcttcacacctt-3′ for GAPDH (198 bp). All primer/probe sets were purchased from Applied Biosystems.

.2.3.1. Chemotaxis assay

The chemotaxis assay was performed using the Transwell system purchased from Coring (NY, USA). Briefly, MCP-2 (R&D system, Minneapolis, MN, USA) at a concentration of 100 ng/ml was added to the lower chamber, and 1×10^5/well cells of each group were seeded into the upper 6 well chamber in serum-free DMEM. The transwell systems were then incubated for 12 h at 37 °C. The cells that migrated into the underside of the inserted membranes were quantified using five random separate fields.

.2.3.2. Adhesion assay

The adhesion assay was performed by modifying a previously reported method [14]. Cells (1×10^5/well) were seeded on 6 well plates pre-coated with 20 μg/well type I collagen (Sigma) in DMEM for 2 h at 37 °C and 5% CO_2. After 2 h, cells were gently washed three times with PBS and adherent cells were counted 5 random microscopic fields by independent blinded investigators.

2.4. Induction of myocardial infarction and cell transplantation

All procedures were approved by Dong-A University Medical School's institutional animal care and use committee (DIACUC). A total of 18 NOD/SCID mice (NODCB17-Prkdc^{scid}/J strain, The Jackson Laboratory Bar Harbor, Maine, USA) at 8–10 weeks of age, were randomly divided into four groups: MI + AMMs (n = 6), MI + ADMs (n = 6), MI + PBS (control, n = 6) and no MI + only surgery (Sham, n = 6). MI was induced by LAD ligation as previously described [17]. All animals were anesthetized by intraperitoneal injection of 3.5% chloral hydrate (1 ml/100 g), endotracheally intubated, and mechanically ventilated using the Inspira-Advanced Safety Ventilator (ASV, NP 55-7095, Harvard Corp, USA), which supplied 0.25-0.3 ml of room air 120 times per min. Each subject was placed in the right lateral posistionand the heart was exposed via a left thoracotomy. After removing the pericardium, the left anterior descending artery (LAD) was visualized using a stereomicroscope (Nissho Optical, Japan), and occluded with an 8.0 nylon suture (Tyco Healthcare, USA). Immediately following ligation, occlusion was confirmed by observation of the left ventricular pallor. An intra-myocardium injection of 40 μl media (control), $1x10^6$ AMMs or ADMs was delivered into 5 sites in the border zone of the infarcted region (apical lateral, apical anterior, basal anterior, mid anterior and mid lateral). The chest was closed, the lungs re-inflated, and the subject moved into the prone position until spontaneous breathing occurred. Before the procedure, AMMs and ADMs were labeled with 4 μM chloromethyl-benzamido-1,1′-dioctadecyl-3,3,3′3′-tetramethylindo-carbocyanine (Dil, Molecular Probes, USA) on 100 mm culture dishes with confluence levels of 1×10^6 stem cells for 15 min at 37 °C and 4 °C.

2.5. Measurement of cardiac function

Echocardiography was performed at 2 and 4 weeks post-cell transplantation in all 3 groups. Subjects were anesthetized with 3.5% chloral hydrate (1 ml/100 g). Echocardiography was performed using a commercially available echocardiography system (Sonos 4500, PHILIPS) with a 15-16 MHz small linear array transducer (hockey stick). All measurements were taken by an experienced cardiologist who was blinded to the study group. Left ventricular end systolic diameter (LVESD) and left ventricular end diastolic diameter (LVEDD) values were obtained by a two dimensional targeted M-mode view. Left ventricular ejection fraction (LVEF) was calculated automatically by the echocardiography system. We assessed the experimental data, in double-blinded fashion, after cell transplantation into the animal groups.

2.6. Histological examinations

Four weeks after stem cell injection, all subjects were euthanized, their hearts harvested and immediately cryofrozen using OCT Tissue-Tek medium (OCT compound; Miles, EIKhart, NJ, USA). The hearts were sliced transversely from the apex to the basal part of the left ventricle using a cryostat with 5 μm thicknesses (Leica RM 2145 microtome). For capillary density measurement, 5 frozen sections from each group of infarcted tissue were stained with biotinylated isolectin B4 (ILB4, 1:250; Vector Laboratory Inc, Burlingame, CA) primary antibodies, followed by streptavidin Alexa Fluor 488 (1:400; Invitrogen) secondary antibodies. Five fields from 5 tissue sections were randomly selected, and the number of capillaries in each field was counted. Images were recorded using a Zeiss LSM 510 laser-scanning confocal microscope (Goettingen, Germany). Masson's Trichrome staining was performed according to the procedure outlined by Schenk et al. [23], facilitating quantitative collagen analysis and enabling the measurement of infarction size. All histological sections were examined using digital scanning microscopy (Scanscope, Aperio, Vista, CA, USA). The infarct scar area and total area of the LV myocardium were traced manually on digital images and measured automatically by computer, following the process reported by Abbott et al. [24]. The infarct size, expressed as a percentage, was calculated by dividing the sum of all sections by the sum of LV areas from all sections and multiplying by 100. To examine apoptosis, TdT-mediated dUTP nick-end labeling (TUNEL) reaction was conducted using a fluorescein in situ cell death detection kit (Roche-Molecular) according to the manufacturer's instructions. Five fields from 5 tissue sections were randomly selected, and the number of apoptotic cells was counted in each field.

2.7. Western blot analysis

Western Blot analysis was conducted to measure Angiopoietin (Ang)-1, Ang-2, vascular endothelial growth factor (VEGF-A) and endothelial nitric oxide synthase (eNOS) expressed in the 3 groups of myocardium. Frozen tissue was homogenized in ice-cold lysis buffer [50 mM Tris (PH 8.0), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS) and 1% NP-40] containing a protease inhibitor cocktail (Sigma-Aldrich) using a Dounce homogenizer (Glas-Col., USA) on ice. Homogenate was briefly spun at 1000 *g* to pellet cell debris and protein concentrations were determined using a Bio-Rad protein assay. Homogenates were mixed with an equal volume of 5 × SDS sample buffer before being loaded onto a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Electrophoresis was followed by transfer to nitrocellulose membranes which were then incubated in blocking buffer (PBS containing 5% skim milk plus 0.1% Tween 20) for a minimum of 30 min. Membranes were probed with rabbit polyclonal anti VEGF-A (1:1000, Santa Cruz Biotechnology), rabbit polyclonal anti-eNOS (1:1000, Santa Cruz Biotechnology), goat polyclonal anti-Ang-1 (1:1000, Santa Cruz Biotechnology), goat polyclonal anti-Ang-2 (1:1000, Santa Cruz Biotechnology), anti-ß-actin (1:5000, Sigma) overnight at 4 °C. Membranes were incubated with secondary anti-rabbit HRP-linked IgG (1:2000, Cell Signaling) and anti-goat HRP-linked IgG (1:2000, Cell Signaling) for 1 h at room temperature, then developed with ECL chemiluminescent reagents, using a LAS 4000 imaging system (FUJIFILIM). Human specimens were probed with the following antibodies: IL-8, GCP-2 (R&D system) and ß-actin (Santa Cruz Biotechnology, Inc.).

2.8. Statistical analysis

All data was expressed as mean ± SD or SE. Statistical analysis was performed with the SPSS program package (SPSS version 12.0; SPSS, Chicago, IL, USA). Differences between the two groups were compared using a Student's t-test. Groups were compared using one-way analysis of variance (ANOVA) test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Characteristics of AMMs

The HUVECs (HUV), ADMs and AMMs were cultured and their morphological phenotypes examined. AMMs exhibited fibroblast-like morphology similar to ADMs when viewed under a phase contrast microscope (Fig. 1A). AMMs did not spontaneously differentiate and proliferated for more than 70 passages. Flow cytometrical results also revealed that AMMs minimally expressed haematopoietic cell markers (CD14, CD34 and CD45), but they highly expressed CD29, CD44, CD73, CD90, CD105 and CD166, demonstrating specific characteristics of MSCs (unpublished data).