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Gene manipulated peritoneal cell patch repairs infarcted myocardium

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Abstract

A gene manipulated cell patch using a homologous peritoneum substrate was developed and applied after myocardial infarction to repair scarred myocardium. We genetically engineered male rat mesenchymal stem cells (MSC) using adenoviral transduction to over-express CXCR4/green fluorescent protein (GFP) (MSC^{CXCR4}) or MSC^{Null} or siRNA targeting CXCR4 (MSC^{siRNA}). Gene expression was studied by real-time quantitative PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA). Cells were cultured on excised peritoneum for 9 days. Two weeks after left anterior descending (LAD) coronary artery ligation in female hearts, the peritoneum patch was applied over the scarred myocardium, cell side down. Efficacy of engraftment was determined by presence of GFP positive cells. One month after cell implantation, echocardiography was performed and hearts were harvested for histological analysis. Left ventricle (LV) fibrosis, LV anterior wall thickness (AWT) and blood vessel density at the margins of the graft were measured. There was significant up-regulation of the chemokines in the MSC^{CXCR4} group cultured under normoxic conditions when compared to the MSC^{Null} group and a further increase was observed after exposure to hypoxia. One month after cell transplantation with the peritoneum patch, substantial numbers of GFP-positive cells were observed in and around the infarcted myocardium in MSC^{CXCR4} group. LV AWT, LV fibrosis and LV function were significantly improved in the MSC^{CXCR4} group as compared to these same variables in the MSC^{Null} control. These salutary effects were absent in MSC^{siRNA} group. The gene manipulated MSC-seeded peritoneum patch promotes tissue nutrition (angiogenesis), reduces myocardial remodeling, and enhances heart function after myocardial infarction.

Keywords

MSC; CXCR4; Peritoneal; Tissue engineering; Myocardial infarction

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Disclosures None declared.

1. Introduction

Despite recent advances in pharmacological and surgical approaches to rescue injured myocardium, ischemic heart disease remains the leading cause of heart failure and death [1]. Both peritoneum and omentum have been reported clinically to promote wound healing and to stimulate revascularization of ischemic tissues [2,3]. Six decades ago, O'Shaughnessy [4] was first to report cardio-omentopexy procedures in which pedicled omental grafts were attached to the surface of the ischemic heart through the diaphragm in humans. Recently, Suzuki and colleagues have combined omentopexy with MSC transplantation as a novel technique to improve graft function through enhancement of angiogenesis and migration of blood vessels into the myocardial cell sheet [5]. Shimizu and coworkers have subsequently developed cell sheets using temperature-responsive culture dishes to generate three-dimensional myocardial tissue [6]. Still more recently, Nelson et al. [7] have reported major success in generating cardiomyocytes from induced pluripotent stem (iPS) cells. However, the mechanism(s) by which such cells enhance tissue regeneration remains unclear.

We have developed a method of gene manipulated MSC cultured on parietal peritoneum *ex vivo* that results in a cell patch for application to scarred myocardium. Stem cell transplantation represents the first realistic strategy for actually reversing the deleterious effects of what heretofore has been considered irreversible heart tissue loss following myocardial infarction. Such complementary features of this peritoneum-based stem cell patch putatively include enhanced tissue nutrition (via angiogenesis) and cardiomyocyte survival (anti-apoptosis) that in combination would reduce myocardial remodeling, limit infarction size and enhance heart function (via paracrine effects).

Stem cell transplantation for myocardial tissue regeneration in infarcted regions of the heart has been studied in various animal models. Cells used in cardiac cell patch have included endothelial cells, smooth muscle cells, hematopoietic stem cells, MSC, skeletal myoblasts, and both fetal and embryonic cardiomyocytes [8,9]. Currently, it is unclear which cell type is most effective for cardiac repair. However, both *in vivo* and *in vitro* evidence illustrate that MSC can differentiate into myriad non-hematopoietic cell types, including cardiac myocytes [10, 11]. Given their multipotency, low immunogenicity, and amenability to *in vitro* expansion and genetic modification, autologous bone marrow-derived MSC are particularly suitable for cell therapy. The most common methods of cell delivery for myocardial therapy are intravenously [12] or by direct intramyocardial injection into an infarcted area [13]. However, it is difficult to control grafted cell deposition using these methods [14,15]. To overcome these problems, homologous peritoneum with seeded MSC is a promising alternative approach to damaged heart tissue repair. We genetically engineered male rat MSC using *ex vivo* adenoviral transduction to overexpress CXCR4 on MSC and found that MSC^{CXCR4} released a number of cytokines and growth factors *in situ*. Therefore, we hypothesized that using the peritoneum as both cell culture substrate and cell sheet delivery vehicle, we would be able to achieve higher engraftment levels of MSC^{CXCR4} in the infarcted zone associated with enhanced blood vessel formation.

2. Methods

Experiments using animal subjects or animal-derived materials were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and University of Cincinnati Institutional Animal Care and Use Guidelines.

2.1. Experimental design

MSC originating from male rats in primary culture were randomly assigned to the following groups: (i) MSC with null vector (MSC^{Null}), (ii) MSC overexpressing CXCR4 (MSC^{CXCR4}),

or (iii) MSC with siRNA targeting CXCR4 (MSC^{siRNA}). To induce cytokine release and regulate the paracrine coupling of angiogenesis [16], MSC in each group were exposed to low glucose (1 g/L) DMEM and placed in a hypoxic incubator (Sanyo, O₂/CO₂ incubator-MCO-18M) for 8 h where oxygen was adjusted to 1.0% and CO₂ to 5%, then followed by 2-h reoxygenation, while other MSC were incubated at 37 °C in a humidified atmosphere of 95% room air:5% CO₂ as normoxic control.

Cytokine secretions from the respective MSC treatment groups were analyzed by qPCR and ELISA. *In vivo* study: A monolayer of MSC on the peritoneum substrate was confirmed after 9 days in culture. Female rats were randomly assigned to experimental groups, as follows: (i) Sham operated rats had a loose suture placed around LAD (Sham group), (ii) myocardial infarction alone (MI group) created by LAD ligation; (iii) MI plus peritoneum patch alone without MSC (MI+P group), (iv) MI plus confluent MSC^{Null} seeded peritoneum patch (MSC-P group), (v) MI plus confluent MSC^{CXCR4} seeded peritoneum patch (CXCR4-P group), or (vi) MI plus confluent MSC^{siRNA} seeded peritoneum patch (siRNA-P group). At 1 day before, or 1 week and 4 weeks after cell patch application, transthoracic echocardiography was performed. Animals were then euthanized for immunohistochemical staining of heart tissues.

2.2. MSC isolation, culture, and labeling

MSC were obtained from male Sprague-Dawley (SD) 8-week-old rats as described [17]. The confluent MSC in the seed cultures were removed from the flask by 0.25% trypsin (Sigma, St. Louis, USA). Passage 2–4 MSC were used in the study. The AdEasy™ Vector System (Qbiogene, Inc.) was used for regenerating recombinant adenovirus according to manufacturer's instructions. Recombinant adenovirus vector was generated and prepared as previously described [17]. In brief, the primers for qPCR containing *Bg*/II (5') and *Hind* III (3') linkers (in bold) were synthesized as follows: CXCR4 forward primer: 5'-CAGA AGA TCT GTT GCC ATG GAA CCG ATC-3', reverse primer: 5'-CAGA AAG CTT GGG TTA GCT GGA GTG-3'; siRNA-CXCR4 forward primer: 5'-CAC CGG ATC AGC ATC GAT TCC TTC ACG AAT GAA GGA ATC GAT GCT GAT CC-3', reverse primer: 5'-AAA AGG ATC AGC ATC GAT TCC TTC ATT CGT GAA GGA ATC GAT GCT GAT CC-3'. For transfections, MSC were seeded on six-well plates at 5×10³ cells/ml 24 h before treatment resulting in approximately 60% confluence at the time of transfection. MSC were transduced overnight using dilutions of concentrated virus equivalent to 1×10⁷ infectious units in non-FBS medium and MSC were assigned to the experimental groups. Since recombinant adenovirus expresses both green fluorescent protein (GFP) and CXCR4 [17], transfection efficiency of MSC with the viral system was evaluated by flow cytometry.

2.3. Western blot analysis for CXCR4

Western blot analysis was performed to determine CXCR4 expression in different treatment groups. Protein samples (30 µg of protein) were mixed with an equal volume of sample buffer (containing 2% SDS, 100 mM Tris, 0.2% bromophenol blue, 20% glycerol, and 200 mM DTT) and boiled for 15 min before loading into each well on 10% polyacrylamide gels (Precast Gels, ISC Bioexpress). These electrophoresed proteins were transferred from the gel to the nitrocellulose membranes (Bio-Rad). Equal loading and transfer of proteins was confirmed by Ponceau's red staining. The membranes were incubated for 60 min with 5% dry milk and Tris-buffered saline to block nonspecific binding sites. Membranes were immunoblotted overnight at 4 °C with antibodies against CXCR4 (1:3000, Cell Signaling) on a rocking platform overnight. After three 5-min washings, the membranes were incubated for an hour with HRP-conjugated secondary antibody, washed and developed with the ECL plus kit (Bio-Rad, USA).

2.4. Real-time qPCR for measurements of cytokine release

Levels of cytokines released from cells cultured in normoxic and hypoxic conditions were measured. The mRNA level of five cytokines (angiopoietin 1, an angiogenic factor; vascular endothelial growth factor (VEGF), insulin growth factor-1 alpha (**IGF-1 α**); eukaryotic initiation factor 4 E (eIF4E), a key player in the regulation of cell-cycle and differentiation; and cyclin A2 in cells) were examined by RT-PCR and qPCR. Total RNA was isolated by using Trizol reagent (Invitrogen), followed by DNase treatment and purification using RNeasy mini column kit (Qiagen). cDNA was synthesized using the SuperScript™ III RNase H-Reverse Transcriptase (Invitrogen) in a 20- μ l reaction mixture. An aliquot of the cDNA was amplified using Taq DNA polymerase (2.5 U) (Invitrogen) in the presence of sense and anti-sense primers (1 μ M). The sequences of PCR primers for angiopoietin 1, VEGF, IGF-1 α ; eIF4E, and cyclin A2 were as follows: Angiopoietin 1: 5'-GTGAGAGTGCGACAGAGCAG-3' and 5'-GTGGTTTGAAC CGCATTCT-3', VEGF: 5'-GCCCCATGAAGTGGTGAAGTT-3' and 5'-ACTCCAGGG CTTTCATCATTC-3', IGF-1 α : 5'-CAGTTCGTGTG TGGACCAAG-3' and 5'-TCAGCGGAGCACAGTACATC-3', eIF4E: 5'-CAAGCAAACCTTCGGTTG AT-3', 5'-CTCCCCGTTTGTCTTCTCA-3'; CyclinA2: 5'-ATGTCAACCCCGAAA AAGTG-3'; 5'-GGGACGTGCTCATCGTTTAT-3'. qPCR was carried out on the iQ5 real-time system (Bio-Rad) with the iQ SYBR Supermix (Bio-Rad). The expression of each target mRNA relative to GAPDH under experimental and control conditions was calculated based on the threshold cycle (C_T) as $r = 2^{-\Delta(\Delta C_T)}$, where $\Delta C_T = C_{T \text{ target}} - C_{T \text{ GAPDH}}$ and $\Delta(\Delta C_T) = \Delta C_{T \text{ experimental}} - \Delta C_{T \text{ control}}$.

ELISA for VEGF and IGF-1 α release: The concentrations of VEGF and IGF-1 α were measured in the supernatants obtained from MSC, MSC^{Null}, MSC^{CXCR4} and MSC^{siRNA} cultures, each grown under 10-h normoxic or 8-h hypoxic conditions followed by 2-h re-oxygenation using commercially available ELISA kit (R&D Systems for IGF-1 α and Calbiochem for VEGF) according to the manufacturer's instructions. All samples were plated in duplicate. Concentrations were calculated as picograms per milliliter and subsequently expressed as pg/10⁶ cells, based on previously obtained cell counts.

2.5. Surgical procedures for myocardial infarction and preparing peritoneum

A myocardial infarction (MI) model was developed in SD female rats (200–250 g), as previously described [18]. Briefly, isoflurane anesthesia was induced by spontaneous inhalation and maintained under general anesthesia with 1–2% isoflurane. The inhalation gas was a mixture of air and oxygen (total oxygen: 40%) and 2.4% isoflurane. The animals were mechanically ventilated using a rodent ventilator (Model 683, Harvard Apparatus, South Natick, MA) connected to a tracheal tube. Body temperature was maintained at 37 °C throughout the surgical procedure. The heart was exposed by left side limited thoracotomy and the left anterior descending artery (LAD) was ligated with a 6-0 polyester suture 1 mm from tip of the normally positioned left auricle. Before closing the thoracic cavity, positive endexpiratory pressure was applied to fully inflate the lungs, and then muscle layers and skin were closed separately. This model provides accurate and reproducible information about infarct size [16], MI in this model is reproducible to $\pm 5.7\%$ [17].

To harvest peritoneum, the abdominal cavity of additional animals was opened and abdominal contents were moved to the left side to expose the posterior peritoneum. This portion of peritoneum was specifically selected to avoid possible skeletal muscle transfer. A small incision was made in the posterior peritoneum and the parietal peritoneum was then detached along the surface of abdominal aorta. A small diameter ring was inserted behind the retroperitoneum under the detached portion of the peritoneum. This ring was then covered with a larger ring on the surface of peritoneum. After rinsing blood with PBS, the harvested segment of peritoneum (diameter=20 mm) with rings was placed in a cell culture dish, and then various

pretreated MSC (1×10^5) were seeded on the peritoneum in DMEM medium at 37 °C in a humidified atmosphere of 95% room air: 5% CO₂. Two weeks after MI, left thoracic cavity was re-opened. The cell-seeded side of the peritoneum was applied to the surface of LAD distribution overarching the MI area followed by chest closure (Fig. 1.4).

2.6. Immunohistochemical analysis

The immunohistochemical studies were performed on heart tissues at 4 weeks after the peritoneum MSC patch was applied to the area of LAD distribution. Heart tissue sections were harvested, fixed in 10% formalin, and sectioned at 5- μ m thickness. CD31 antibody (PECAM-1, Santa Cruz Biotechnology) was used to assess angiogenesis and capillary density and smooth muscle α -actin (SMA) antibody (M0951, DAKO) was used to evaluate vascular density, while 4', 6-diamino-2-phenylindole (DAPI, Sigma) was used to identify nuclei. Fluorescent imaging was performed with an Olympus BX41 microscope (Olympus America Inc., Melville, NY, USA) equipped with epifluorescence attachment and images were recorded using a digital camera with MagnaFire™ 2.1 software. FISH analysis of heart tissue samples was carried out using STAR*FISH Rat 12/Y Paints Protocol (CA-1631), as described previously [17]. Confocal images were obtained with Leitz DMRBE fluorescence microscope equipped with a TCS 4D confocal scanning attachments (Leica, Inc.).

2.7. Analysis of left ventricular (LV) fibrosis and anterior wall thickness (AWT)

Fixed heart tissues, embedded in paraffin, and LV cross-sections from mid-LV to apex stained with Masson-Trichrome were used to quantify fibrosis in the left ventricle in various treatment groups. An Olympus BX41 microscope equipped with CCD (Magna-Fire™, Olympus) camera captured LV area images on each slide. LV fibrosis area and total LV area of each image were measured using the Image-Pro-Plus (Media Cybernetics Inc., Carlsbad, CA, USA), and the fibrosis area as a percentage of the total LV area was calculated as (fibrosis area/total LV area) $\times 100$.

2.8. Cardiac function assessment by echocardiography

Echocardiography (iE33 Ultrasound System, Phillips) with a 15-MHz probe was performed 30 days after peritoneum cell patch placement by an expert, blinded to the study group or treatment, to assess systolic, diastolic dimensions and anterior wall thickness. Hearts were imaged in 2-D long-axis view at the level of the greatest LV diameter with animals under light general anesthesia. This view was used to position the M-mode cursor perpendicular to the LV anterior and posterior walls. LV end-diastolic and end-systolic diameters were measured from M-mode recordings using the leading-edge method. LV ejection fraction (EF) was calculated as: $EF (\%) = \frac{[left\ ventricular\ end-diastolic\ dimension\ (LVDd)^3 - left\ ventricular\ end\ systolic\ dimension\ (LVDs)^3]}{(LVDd)^3} \times 100$. Fractional shortening (FS) at the level of the MI and patch application was also determined as $[(LVDd - LVDs)/LVDd] \times 100$. All measurements were performed according to the American Society for echocardiology leading-edge technique, and averaged over three consecutive cardiac cycles.

3. Statistical analysis

Results were statistically analyzed with the use of the StatView 5.0 software package (Abacus Concepts Inc., Berkeley, CA). All values were expressed as mean $\pm$ S.E.M. Comparison between groups for echocardiographic longitudinal data was performed by two-way analysis of variance (ANOVA) for repeated measures. All resulting differences were analyzed by one-way ANOVA, followed by a Bonferroni/Dunn test or unpaired *t*-test. *p*-value ≤ 0.05 was considered statistically significant.

4. Results

4.1. In vitro study

4.1.1. CXCR4 promote the cytokine and growth factor release—Efficiency of siRNA-mediated CXCR4 knockdown in MSC was confirmed by Western blotting. CXCR4 expression was blocked by siRNA targeting CXCR4 (Figs. 1.1A and B). To test our hypothesis that over expression of CXCR4 in MSC will enhance cellular release of paracrine factors that contribute to stem cell homing, cell differentiation and proliferation, and angiomyogenesis, we examined the expression of angiogenic factors by qPCR (Fig. 1.2). Genes regulating angiopoietin 1, VEGF, IGF-1 α , eIF4E, and cyclin A2 expression were upregulated in the MSC^{CXCR4} *in vitro* at baseline normoxia ($*p \leq 0.05$ vs. MSC^{Null} under normoxia) and increased further after 8 h preconditioning exposure to hypoxia ($*p \leq 0.05$ vs. MSC^{Null}). These positive effects of CXCR4 were eliminated by siRNA targeting CXCR4, suggesting that CXCR4 plays a significant role in the gene expression process documented above. In addition, results from ELISA demonstrated that MSC^{CXCR4} produced significantly higher VEGF protein *in vitro* while under normoxic, immediately after 8 h hypoxic, and 2 h after re-oxygenation conditions and further increased after reoxygenation as compared with MSC^{Null} ($*p \leq 0.05$). This expression of VEGF was completely abolished in the MSC^{siRNA} group, which was similar to that of the MSC^{Null} (Fig. 1.3A), suggesting that CXCR4 overexpressing MSC play a significant role in the release of VEGF. Results for ELISA analysis of IGF-1 α were similar to that of VEGF, except in the normoxic condition, where there was no difference among the four groups (Fig. 1.3B).

4.2. In vivo study

4.2.1. In situ cell patch engraftment and angiomyogenesis—In vitro, the MSC began to proliferate at day 4 and continued until a confluent monolayer of cells developed on the peritoneal patch by day 9 when the patch was grafted seeded cell surface in contact with the anterior LV wall surface where the MI was previously created (Fig. 1.4).

We identified transferred MSC by simultaneous presence of both GFP and the Y-chromosome from genetically engineered male rat MSC previously treated with an ex vivo adenoviral transduction method for overexpressing CXCR4/GFP or Null/GFP alone. Four weeks after placing the peritoneum MSC patch *in situ*, newly formed blood vessels were identified in both the cell patch (cell seeded side) and LV infarct area (Fig. 2) in MSC^{CXCR4} group (data in other groups not shown). Some GFP positive cells identified by GFP antibody had formed new vessels in the cell seeded side of the cell patch (Fig. 2D, white arrows) and in the infarcted area (Fig. 2C, white arrows). This suggests to us that the MSC^{CXCR4} in the cell patch were orchestrating improved tissue nutrition (blood supply) from both sides of epicardium.

Four weeks after placement, the peritoneum MSC patch was tightly adhered to the surface of recipient heart (Figs. 3A and B). Large numbers of the GFP positive cells were mobilized into LV infarct area (Figs. 3C–F) in MSC^{CXCR4} group (data in other groups not shown), some of which had positive staining for sarcomeric α -actin, a marker for cardiomyocytes (Figs. 3D–F) and the unique Y-chromosome marker (Fig. 3H, white arrow). The number of GFP positive cells within the infarct region was significantly higher in MSC^{CXCR4} ($531 \pm 56/\text{mm}^2$) than in the MSC group ($217 \pm 30/\text{mm}^2$) ($*p \leq 0.05$). Notably, after treatment with siRNA, the number of GFP-labeled cells was significantly reduced to $154 \pm 23/\text{mm}^2$ (Fig. 3G), suggesting that CXCR4 plays an essential role for cell regeneration.

To verify whether CXCR4 played the significant role in angiogenesis in the infarcted area, we assessed angiogenesis by visualization of blood vessels positively stained for CD31 and smooth muscle α -actin (SMA) (Figs. 4A–F). Capillary density, as determined by positive staining for

CD31 (Fig. 4G), was not significant different between the MI ($3.4 \pm 0.4/\text{mm}^2$), MI+P ($4.7 \pm 0.5/\text{mm}^2$), or siRNA-P ($4.1 \pm 0.7/\text{mm}^2$) groups ($p > 0.05$), and was significantly higher in the CXCR4-P group ($14.8 \pm 1.9/\text{mm}^2$) as compared with the MSC-P group ($9.2 \pm 1.3/\text{mm}^2$) ($*p \leq 0.05$). A similar pattern was observed in vascular density as determined by positive staining for SMA (Fig. 4H). There was no significant difference in vascular density between MI ($3.5 \pm 0.3/\text{mm}^2$), MI+P ($3.8 \pm 0.4/\text{mm}^2$), or siRNA-P group ($2.2 \pm 0.3/\text{mm}^2$) groups ($p > 0.05$), but vascular density was significantly higher in the CXCR4-P group ($8.1 \pm 1.2/\text{mm}^2$) as compared to the MSC-P group ($5.7 \pm 0.6/\text{mm}^2$) ($*p \leq 0.05$).

4.2.2. Cell patch attenuated heart remodeling and fibrosis—Six weeks after MI, the pathological changes in fibrosis and LV AWT were assessed using Masson's Trichrome staining in the various patch treatment groups (Fig. 5A). The percentage of LV fibrosis was significantly reduced in MSC-P ($20.2 \pm 3.1\%$) and CXCR4-P ($16.1 \pm 3.9\%$) groups as compared with MI ($39.0 \pm 4.5\%$), or MI+P ($37.8 \pm 3.8\%$) groups, and was the lowest in CXCR4-P group when compared with other groups (Fig. 5B). LV AWT was significantly increased in MSC-P ($810 \pm 92 \mu\text{m}$) and CXCR4-P ($962 \pm 105 \mu\text{m}$) groups as compared with MI ($540 \pm 70 \mu\text{m}$), or MI+P ($580 \pm 80 \mu\text{m}$) groups and was the thickest in CXCR4-P group ($*p \leq 0.05$), while no remarkable changes were observed in thickness of the left ventricular septum in each group (Fig. 5C).

4.2.3. Assessment of heart function—Fig. 6 is an illustrative example of echocardiography performed on all subjects in the various treatment groups at different time points after cell patch implantation. There were no significant differences in left ventricular function as indicated by left ventricular end-diastolic (LVDd), end-systolic diameters (LVDs), left ventricular ejection fraction (EF), and fractional shortening (FS) in all groups measured at 1 day before and 1 week after cell patch treatment. At 4 weeks after MSC patch application, the MI+MSC-P group showed significant improvement in LVDs, FS, and EF over the MI and MI+P groups (Fig. 6, Table 1). The most significant changes were seen in the MI+CXCR4-P group. However, no significant changes were observed between 1 day before and 1 week after cell patch application. Values of LVDd, LVDs, FS, and EF in the MI+CXCR4-P group were not only improved significantly from the MI and MI+P groups, but also from the MI+MSC-P group (Fig. 6, Table 1). These remarkable findings in LV performance parameters achieved with CXCR4-P were consistent with the histological analysis and *in vitro* study and could indicate that LV remodeling occurred after MI and that application of the peritoneum MSC^{CXCR4} patch attenuated LV remodeling.

5. Discussion

Cell-based therapy appears to be a promising modality for myocardial repair of ischemic heart disease [11]. There are many methods of cell delivery in use today. These include intravascular injection and direct tissue injection into a region of infarcted myocardium. However, it is difficult to control the delivery of grafted cell populations to the target tissue using these methods. To overcome some of these issues and obstacles, the gene-manipulated cell-based peritoneum patch provides a supportive micro-environment for cell engraftment, migration, differentiation, and survival. The major findings of this study are: 1) CXCR4 overexpressing MSC (MSC^{CXCR4}) seeded on peritoneum promote angiogenesis when applied to the epicardial surface of minimally vascular, dyskinetic scar tissue of the left ventricle following MI; 2) MSC^{CXCR4} patch using homologous peritoneum appears to attenuate ventricular remodeling of post-myocardial infarction via increased anterior LV anterior wall thickness while to reducing LV collagen deposition and fibrosis.

Repair of infarcted LV myocardium by cell-based patching is currently limited by the inability of most substrate materials to cultivate local tissue replacement. Most tissue engineering

approaches are based on structural biodegradable scaffolds as an alternative to the extracellular matrix (ECM) [11]. Polymers, the largest class of engineered biomaterials used today for myocardial tissue reconstruction have many disadvantages, such as poor elasticity, poor attachment, biodegradation, poor ECM formation and biotoxicity, which collectively negate or severely limit their medical suitability. As early as 1937, omentopexy was used for treatment for ischemic heart disease [11]. However, the use of this material was abandoned because omentopexy failed to produce sufficient myocardial revascularization and was soon thereafter replaced by coronary artery bypass grafting (CABG) and coronary revascularization was subsequently also treated by percutaneous coronary angioplasty (PCTA). Suzuki and his colleagues have recently demonstrated that combination of omentopexy and MSC transplantation could supply a sufficient number of blood vessels directly to MSC, and thereby contribute to the survival of transplanted MSC leading to attenuate remodeling of the MI region [5]. Considering biological substrates for cell patches, a suitably sized great omentum limits its utility for wrapping myocardial scar formation in small animals, such as rats and mice, thus the reason we chose homologous peritoneum as the MSC culture substrate. In order to develop a new approach, we have in the current study addressed earlier limitations by making it possible to (i) manipulate genes so that progenitor cells release cytoprotective factors which exert beneficial effects on angiogenesis, (ii) enhance migration of progenitor cells into the scarred myocardium and promote new blood vessel formation, (iii) place the transplanted cells in a favorable microenvironment that favors cell survival and proliferation, and (iv) apply a homologous peritoneum patch to allow embedding MSC in the matrix to form a stronger, more adherent bond with the patch itself. In the current study for the first time, we report evidence on the feasibility of gene manipulated cell-based peritoneum patch to improve infarcted LV myocardium performance in a longitudinal study.

CXCR4, as the receptor for SDF-1 α , has been shown to express in cardiomyocytes and cardiac stem/progenitor cells [19]. To test our hypothesis that MSC^{CXCR4} release paracrine factors that contribute to cell survival, homing, proliferation and angiogenesis, we examined the expression of angiogenic and myocytogenic factors by qPCR using RNA extracted from MSC and MSC^{CXCR4} after exposure to normoxia or hypoxia. Our study demonstrated that several important angiogenic genes were significantly upregulated in the MSC^{CXCR4} at baseline and increased further after 8 h of exposure to hypoxia (* $p \leq 0.05$ vs. MSC^{Null}) (Fig. 1). Angiopoietin 1 and VEGF are efficacious angiogenic factors. IGF-1 α itself is a somewhat less efficacious inducer of angiogenesis as compared with other angiogenic growth factors such as VEGF. Importantly, the pro-angiogenic activity of IGF-1 α has been attributed to an associated VEGF upregulation [20]. Cyclin A2, a cell-cycle-perpetuating factor, is associated with cardiac regeneration after myocardial infarction and has been demonstrated to prevent heart failure [21]. eIF4E promotes cellular proliferation and can rescue cells from reacting to apoptotic stimuli via Akt signaling pathway [22]. Taken together, these four factors programmed to overexpress in MSCs convey salutary features guiding angiomyogenesis. These upregulated genes (angiopoietin 1, VEGF, IGF-1 α , eIF4E, and cyclin A2) may play an essential role in angiogenic process, cell homing, anti-apoptosis and inotropic actions [23,24]. In a previous study, we demonstrated that expression of CXCR4 on MSC is related to efficient SDF-1 α -induced migration *in vitro* [12]. SDF-1 α /CXCR4 interactions appear to be important for recruitment of progenitor cells to the heart after MI and can further increase homing in the presence, but not in the absence of injury [25] thus, genetically modified MSC provide cardiac protection consequent to a paracrine action. The purpose of this study was to investigate the putative attenuating effects of gene manipulated peritoneum cell patches seeded to confluence with MSC on LV dysfunction by promoting angiogenesis and myocyte differentiation in a rat model of MI.

In our experiments, increased CXCR4 expression by adenoviral gene delivery significantly enhanced vascularization in scarred myocardium (Fig. 4). To initiate tissue regeneration, the

cell patch must promote *in vivo* revascularization in concert with graft tissue integration into the host tissue. Although *in situ* differentiation of MSC into cardiomyocytes and other cell types constituting the cardiac tissue has been proposed [11,26,27], there is much debate over the frequency of this phenomenon [28]. MI triggers the release of various cytokines and chemokines that are involved in mediating the processes of post-infarction repair [29] and also conveys special emphasis on the role of CXCR4/SDF-1 α axis [30]. The SDF-1 α /CXCR4 axis has a crucial role in embryonic development [31] and its activation invokes stem cells mobilization, homing, and angiogenesis, signals transition and survival [32,33]. Neovascularization is believed to be critical to the recovery of damaged heart function. Our previous experiment demonstrated CXCR4 overexpressing MSC increase the expression of c-kit [17] which is linked to angiogenesis and further restoration of heart function [34]. Here, we corroborate this theory with an *in vivo* study at 4 weeks after confluent monolayer cell patch implantation (Fig. 2) when we documented that the engrafted patch supported newly formed vessels both on the cell patch itself and within the adjacent infarcted myocardium. Furthermore, the regulation of IGF-1 α in hypoxia, confirmed in our experiments, has been reported to increase cardiomyocyte contractility and to improve myocardial function both in normal and infarcted adult rat hearts [24]. We identified that clusters of GFP positive MSC^{CXCR4} had migrated into the infarcted area and these MSC had differentiated into cardiomyocytes as confirmed by the specific cardiomyocyte antibody marker, sarcomeric α -actin (Fig. 3). Therefore, the combination of MSC^{CXCR4} and peritoneum patch created a synergistic setting that promoted *in vivo* myogenesis in association with angiogenesis; critical for improving blood flow to supply nutrition to the damaged heart and to enhance ability restoration of heart function after MI.

Myocardial fibrosis, a hallmark of many cardiovascular diseases, involves the abnormal deposition of collagenous fibers in the interstitial space [35]. Our results demonstrated that fibrotic tissue formation was decreased and LV AWT was increased in CXCR4-P group and that these contributed to increased tissue mechanical strength preventing LV dilation. Although infarction size was not measured in this study, the fibrotic area in Masson's Trichrome staining is an index of comparative infarction size when applied to the same LV transect in hearts following LAD ligation at the same locus. We also found that LV function assessed by EF and LVDD indices with echocardiography was little improved acutely in the rat model with MI in the 1-week period following various MSC treatments. However, at 4 weeks, these same left ventricular contractile functional indices were significantly improved in CXCR4-P group as compared with MSC-P group (* $p \leq 0.05$). These favorable results can be explained by the following three events. First, cytokines or chemokines released by peritoneum MSC patch may provide a beneficial microenvironment for angiomyogenesis. Second, the graft provides mechanical reinforcement by forming a bridge across infarcted myocardium anchoring in viable myocardium at its perimeter and the subsequently regenerating epicardial tissue and reverse remodeling scar tissue to maintain and partially restore LV structure and elasticity. Finally, and perhaps most importantly, the cardioprotective effects induced by MSC^{CXCR4}, namely the angiomyogenic effects of CXCR4 facilitate the improvement of LV function. Thus, application of the MSC^{CXCR4}-based peritoneal patch over the scarred areas of myocardium achieves at least three outcomes: (1) nutrition from both outside the adjacent/adherent epicardium (new formed vessels in cell patch) and angiogenesis in the infarcted areas, (2) flattening of the aneurysm over the ischemic area and prevention of aneurysmal stretching of peri-ischemic myocardium, and (3) strengthening of the myocardial wall, and reduction of LV collagen deposition and prevention or reversal of further LV remodeling.

6. Conclusion

Grafting of a MSC^{CXCR4} peritoneum patch over scarred areas of myocardium subsequent to coronary artery ligation reduces or attenuates ventricular remodeling, enhances angiogenesis,

reduces fibrosis and improves heart function. This homologous cell patch offers an innovative approach for regenerative medicine in contrast to direct stem cell injection.

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Fig. 1. Efficiency of siRNA-mediated CXCR4 knockdown in MSC. Western blot shows the transfection efficiency of siRNA in MSC (1.1A). CXCR4 expression was knocked down by

siRNA targeting CXCR4 gene (1.1B). Values mean $\pm$ SEM, $n = 6$ for each group. $*p \leq 0.05$ vs. MSC group. CXCR4, MSC^{CXCR4}; Null, MSC^{Null}; siRNA, MSC^{siRNA}; 1.2: Quantitative data of MSC^{CXCR4} for enhancing expression of chemokines. Angiopoietin 1, an angiogenic factor; VEGF, vascular endothelial growth factor; IGF-1 α , insulin growth factor-1 α ; eIF4E, eukaryotic initiation factor 4E; cyclin A2, a cell-cycle-perpetuating. MSC, MSC^{Null}; CXCR4, MSC^{CXCR4}; siRNA, MSC^{siRNA}; $*p \leq 0.05$. All values expressed as mean $\pm$ SEM. $n = 6$ for each group. 1.3: ELISA results for release of VEGF (A) and IGF-1 α protein (B) in cell lysate samples from ordinary MSC (MSC), Null (MSC^{Null}), CXCR4 (MSC^{CXCR4}), siRNA (MSC^{siRNA}) groups under normoxic, immediately after 8-h hypoxic, and 2-h re-oxygenation conditions. $*p \leq 0.05$. All values expressed as mean $\pm$ SEM. $n = 6$ for each group. Fig. 1.4: Schematic depiction of experimental design. The posterior abdominal peritoneum was clipped by two overlapping plastic-rings (A); Cut peritoneum with ring was shifted to cell culture dish and MSC were seeded on the peritoneum patch (B); MSC at 4, 7, and 9 days after seeding on the peritoneum patch in the dish (C). GFP-expressing monolayered MSC expanded to confluence on the peritoneum patch at day 9 and ready to use (D). The cell patch was grafted to the surface scarred area of myocardium (E).

Fig. 2.

Characteristics of MSC^{CXCR4} in seeded peritoneal patch. Light microscopy showed that Masson's Trichrome staining for characteristics of MSC^{CXCR4} in seeded peritoneal patch applied to the epicardium of the scarred area at 4 weeks after cell patch implantation (A, B), (data in other groups not shown). Fluorescence microscopy demonstrated that newly formed blood vessels from infarcted areas (C) and inside of cell patch (D): In panel C, α -sarcomeric actin (red color) was used to identify myocytes, DAPI was used to identify all nuclei (blue color), and vessels derived from GFP positive MSC were identified by GFP antibody staining (green color). A = Original magnification $\times 100$. B and C = Original magnification $\times 200$; D = Original magnification $\times 400$.

Fig. 3. MSC^{CXCR4} migration and differentiation in situ at 4 weeks after patch graft. (A, B) shows immunofluorescent staining in CXCR4-P group (data in other groups not shown). Magnification $\times 100$. (C) A cluster of enhanced GFP positive MSC^{CXCR4} originating from peritoneum patch migrated and localized in the infarct area (green fluorescence, arrow). (D) Same section as C except α -sarcomeric actin staining for cardiac myocytes (red fluorescence). (E) Same section as C and D except for co-localization of α -sarcomeric actin, EGFP and DAPI. (F) Enlarged picture from E (magnification $\times 400$). C, D and E = Magnification $\times 200$. (G) Quantitative data showed that the number of GFP positive MSC was significantly higher in CXCR4-P group than any other groups. MI + MSC-P, MI with MSC^{null} seeded patch treatment; MI + CXCR4-P, MI with MSC^{CXCR4} seeded patch treatment; MI + siRNA-P, MI with MSC^{siRNA} seeded patch treatment. All values were expressed as mean $\pm$ SEM. $*p \leq 0.05$; $n = 6$ for each group. (H) Some of the cells in CXCR4-P group were differentiated into cardiomyocytes (green color) which were confirmed by FISH analysis for Y-chromosome nuclei (arrow, red point). H = Magnification $\times 630$.

Fig. 4.

Effects of MSC^{CXCR4} transplantation on angiogenesis in the infarct area. CXCR4-P group promotes vessel formation by significant expression of CD31 (green color; A, B, and C) and SMA (red color; D, E, and F) respectively in comparison with MSC-P and siRNA-P group, $*p \leq 0.05$ (magnification $\times 200$). Quantitative analysis of capillary density (anti-CD31 antibody staining; G) and vascular density (anti α -smooth muscle actin antibody staining; H) was determined by various treatments. MI indicates myocardial infarction alone; MI + P, MI with patch treatment alone; MI + MSC-P, MI with MSC^{null} seeded patch treatment; MI + CXCR4-P, MI with MSC^{CXCR4} seeded patch treatment; MI + siRNA-P, MI with MSC^{siRNA} seeded patch treatment. All values expressed as mean $\pm$ SEM. $*p \leq 0.05$; $n = 6$ for each group.

Fig. 5.

LV fibrosis and AWT in various treatment groups. At 4 weeks after cell patch treatment, left ventricular fibrosis and anterior wall thickness (AWT) were significantly attenuated and restoration of AWT was significantly increased in the MSC-P and CXCR4-P group as compared with other groups (A). AWT was thickest in CXCR4-P group ($*p \leq 0.05$).

Quantitative data were shown for LV fibrosis (B) and AWT (C). The scale bars indicate 1.0 mm. MI indicates myocardial infarction alone; MI + P, MI with patch treatment alone; MI + MSC-P, MI with MSC^{null} seeded patch treatment; MI + CXCR4-P, MI with MSC^{CXCR4} seeded patch treatment; MI + siRNA-P, MI with MSC^{siRNA} seeded patch treatment. All values expressed as mean $\pm$ SEM. $*p \leq 0.05$; $n = 12$ for each group.

Fig. 6.

Cardiac function assessment by echocardiography at 4 weeks after cell patch transplantation (A). M-mode echocardiograms are shown at 1 day before, 1 week and 4 weeks after cell patch implantation in various treatment groups (B). Sham indicates open-chest state without LAD occlusion; MI, myocardial infarction alone; MI + P, MI with patch treatment alone; MI + MSC-P, MI with MSC^{Null} seeded patch treatment; MI + CXCR4-P, MI with MSC^{CXCR4} seeded patch treatment; MI + siRNA-P, MI with MSC^{siRNA} seeded patch treatment. All values expressed as mean $\pm$ SEM. * $p \leq 0.05$ vs. MI, or MI + P, or MI + siRNA-P; # $p \leq 0.05$ vs. any other group; $n = 12$ for each group.

Table 1

Echocardiography data for rat heart function at 4 weeks post-treatment.

Parameter	LVDd (mm)	LVDs (mm)	LVES (%)	LVEF (%)
Sham	5.5 ± 0.24	2.4 ± 0.2	57 ± 2.9	92 ± 3.9
MI	7.6 ± 0.32	6.6 ± 0.29	18 ± 2.2	44 ± 2.8
MI + P	7.6 ± 0.32	6.4 ± 0.31	19 ± 3.1	46 ± 3.9
MI + MSC-P	7.2 ± 0.4	5.4 ± 0.31 [*]	24 ± 2.6 [*]	56 ± 3.2 [*]
MI + CXCR4-P	6.5 ± 0.29 [#]	4.5 ± 0.26 [#]	31 ± 2.8 [#]	67 ± 4.0 [#]
MI + siRNA-P	7.7 ± 0.21	6.5 ± 0.22	18 ± 2.1	44 ± 3.2

LVDd = left ventricle end diastolic dimension; LVDs = left ventricle end systolic dimension; LVEF = left ventricular ejection fraction; LVFS = left ventricle fractional shortening. All values were expressed as mean ± SEM.

^{*} $p \leq 0.05$ vs. MI, or MI + P, or MI + siRNA-P

[#] $p \leq 0.05$ vs. any other group; $n = 12$ for each group.