Correlation between Myocardial Regeneration and the Release of Cytokines in Ischemic Heart with Intramyocardial Implantation of Cardiomyogenic Mesenchymal Stem Cells

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Directed by Professor Byung-Chul Chang

The Doctoral Dissertation submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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This certifies that the Doctoral Dissertation of Young-Nam Youn is approved.

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< Acknowledgement >

This work was a consecutive process of trial and error. If there were no encouragements and guidance of my surroundings, I could have not finished this paper. During the time of experiments, I apprehended that only the passion and the endurance could produce the results.

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<Abstract>

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Mesenchymal stem cells (MSCs) represent a suitable source of autologous cells in cell therapy for the improvement of cardiac function, but its therapy has limitations due to the poor differentiation after cell transplantation. Recently, some reports demonstrate that paracrine factors generated from MSCs-implanted heart mediate endogenous regeneration, e.g. vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and basic fibroblast growth factor (b-FGF), which play an important role in ischemic tissues. Previously, we found cardiogenic differentiation system through protein kinase C (PKC) signal which activated by phorbol 12-myristate 13-acetate. This study was designed to investigate the correlation between myocardial regeneration and cytokine release of ischemic heart with intramyocardial

implantation of cardiogenic cells (CGCs). To examine the *in vivo* relevance of our *in vitro* findings, we studied the enhanced revelation of CGCs on LAD (Left anterior descending) ligated rat hearts. In the ligation group, the area of interstitial fibrosis was significantly larger (36.0±2.5%, versus 1.0±0.6% of the normal group, $p<0.05$). In contrast, transplantation of CGCs significantly decreased interstitial fibrosis to 8.5±3.2% when compared to the normal group ($p<0.05$). TUNEL-positive cells caused by ligation were significantly reduced in the CGCs-implanted group, compared with the ligation group and the MSCs-implanted group. The apoptotic indices were 32% in the ligation group, 13% in the MSCs-implanted group, and 6% in the CGCs-implanted group. In RT-PCR analysis for gene expression of cytokine molecules, the expression levels of HGF and VEGF in the CGCs-implanted group were higher (1.61 folds (HGF) and 1.33 folds (VEGF) of the level of the ligation group, respectively) and the expression levels of inflammation cytokines, such as IL-1β, IL-6 and TNF-α, were lower (0.94 folds (IL-1β), 0.80 folds (IL-6), and 0.71 folds (TNF-α) of the level of the ligation group, respectively). In conclusion, implantation of cardiogenic cell induced from MSCs and secreted cytokines proved useful in attenuating cardiac fibrosis and regenerating myocardium after myocardial infarction.

Key words: Mesenchymal stem cell, Protein kinase, Differentiation,

Regeneration, Cytokine

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I. INTRODUCTION

Myocardial infarction (MI) and the resulting loss of contractile heart muscle is a frequent cause of heart failure and death [1]. Myocardial ischemia induces a broad range of cell responses including loss of adhesion and cell death, depending on the cell type and the duration of the ischemic period [2]. In recent years, a number of human clinical trials have demonstrated improvement in indices of cardiac function following endothelial progenitor cell therapy in the setting of acute myocardial infarction [1,2]. Mammalian cardiomyocytes are terminally differentiated cells, which lose their proliferative potential shortly after birth, so that the adult heart is unable to replace dead or damaged cardiomyocytes after myocardial injury. The remaining myocytes in infarcted

tissues undergo progressive replacement by fibroblasts to form scar tissue. By few alternative sources of cardiomyocytes, recent attempts to repair infarcted hearts revealed that skeletal myoblasts or cardiomyocytes from fetal or neonatal mice replaced residence in cardiac tissue after injury. It is now clear that bone marrow stem cells can, when appropriately stimulated, differentiate into cardiac myocytes, endothelial cells, and vascular smooth muscle cells. Administration of bone marrow mesenchymal stem cells (MSCs) could improve cardiac function after acute MI. MSCs showed a capacity to proliferate in culture with an attached well-spread morphology and generated undifferentiated, stable phenotype by serial passage of cells. Transplantation of MSCs seems a promising concept for the treatment of myocardial infarction and heart failure. Previous studies suggest that MSCs survive and differentiate into new cardiomyocytes and endothelial cells, contributing to general improvement of the injured heart [3]. Although recent reports showed that the cardioprotective effect of MSCs may be mediated not only by their differentiation into cardiomyocyte-like cells and vascular cells, but also by their ability to supply large amounts of angiogenic, anti-apoptotic and mitogenic factors [4-6], the specific mechanism remains controversy and needs to be explored. Cardiac inflammation is associated with left ventricular remodeling and cardiac function after MI, while little is known about the anti-inflammation potential of implanted MSCs after acute MI [7]. These have been seriously challenged by others who found that myogenic or endothelial differentiation rarely occurs, and

cellular fusion between implanted MSCs and resident cells is also infrequent in MI tissues. Therefore paracrine action could be a plausible explanation. MSCs secrete various growth factors or cytokines, e.g. vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF), which play an important role in ischemic tissues [8-10, 12]. Even though the mechanisms of stem cell therapy appear to be far more complex, it has been suggested that stem cells protect cardiomyocytes from apoptotic cell death, release angiogenic ligands, induce proliferation of endogenous cardiomyocytes, and may recruit resident cardiac stem cells. In particular, it has been questioned whether and to what extent transplanted cells might acquire characteristics of functional cardiac muscle cells and improve the contractile force of damaged hearts.

In vitro IGF-I, oncostatin M, IL-1α, IL-6, and TNF-ß improved cardiomyocyte survival. Similar observations had been made for CT-1, IL-6, and TNF-a, which confer cytoprotective effects to adult cardiac myocytes, whereas others, such as insulin and IGF-I, stimulate the metabolism of cardiomyocytes. Interestingly, the same proinflammatory cytokines, such as granulocyte colony-stimulating factor, TNF-a, and IL-6, have profound effects on left ventricular contractility, although this effect strongly depends on the concentration of cytokines and exposition time of the heart. In addition to an improvement of cardiomyocyte survival, several kinds of the cytokines and growth factors have effects on macrophages and mesenchymal stem cells and modulate the inflammation reaction, interstitial matrix composition, scar

formation, and other processes related to cardiac remodeling, which might explain the reduced level of adverse remodeling after cardiac body engraftment. In this study, we are to investigate the correlation between myocardial regeneration and cytokine release of ischemic myocardium with intramyocardial implantation of cardiogenic cell modified from MSCs.

II. MATERIALS AND METHODS

1. Isolation and culture of MSCs

MSCs were isolated from the femurs and tibias of rats. Bone marrow-derived MSCs were collected from the aspirates of the femurs and tibias of 4-week-old male Sprague-Dawley rats (approximately 100 g) with 10 mL of MSC medium consisting of Dulbecco's modified Eagle's medium (DMEM)-low glucose, supplemented with 10% fetal bovine serum and 1% antibiotic-penicillin and streptomycin solution. Mononuclear cells recovered from the interface after centrifugation in Percoll were washed twice, resuspended in 10% fetal bovine serum (FBS)-DMEM, and plated in flasks at 1×10^6 cells per 100 cm^2. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 48 or 72 hrs, non-adherent cells were discarded, and the adherent cells were thoroughly washed twice with phosphate-buffered saline (PBS). Fresh complete medium was added and replaced every 3 or 4 days for approximately 10 days. To further purify the MSCs, the Isolex Magnetic Cell Selection System (Baxter Healthcare Corporation, Irvine, CA, http://www.baxter.com) was used. Briefly, cells were incubated with Dynabeads M-450 coated with anti-CD34 monoclonal antibody. A magnetic field was applied to the chamber and the CD34+ cell-bead complexes were separated magnetically from the remaining cell suspension with the CD34-negative fraction being further cultured. The cells were

harvested after incubation with 0.25% trypsin and 1 mM EDTA for 5 min at 37°C, replated in 1×10^5/100-cm^2 plates, and again grown for approximately 10 days.

2. MSCs characterization

Immunocytochemical characterization of MSCs was demonstrated below. Cells were cultured in 4 well side chamber, washed with PBS and incubated 1 % paraformaldehyde solution (Sigma, USA) for 10 minutes. PBS washing twice, then cells has permeated in 0.1 % Triton X-100 for 7 minutes. After PBS washing twice, they have blocked for 1 hours (blocking solution: 2 % bovine serum albumin, 10 % horse serum in PBS) and adhered with the following antibodies: CD34, CD71, CD90, CD105, and intracellular adhesion molecule (ICAM)-1. FITC-conjugated mouse, rabbit and goat (Jackson Immunoresearch Laboratories) were used as secondary antibodies. Then, they were detected confocal microscopy (Carl Zeiss, Germany).

MSCs were performed according to the procedure of fluorescence-activated cell sorting (FACS) staining described below. Briefly, cells were detached from the plate with 10 % trypsin-EDTA (Gibco BRL), washed in PBS and fixed in 70 % ethanol at 4 °C for 30 minutes with agitation. Cells were washed twice in PBS and resuspended at 2×10^6 cells/mL in blocking buffer (1 % BSA, 0.1 % FBS) containing the following antibodies: CD14, CD34, CD90, CD 105, ICAM-1 ; In the case of CD14, we used normal rabbit IgG as a negative isotype

control. After staining, cells were washed twice and then labeled with rabbit or mouse-FITC conjugated IgG for 20 minutes in the dark area. After two more washes, flow cytometric analysis was performed on a FACS Calibur system (Becton Dickinson, CA, USA) using CellQuest™ software (Becton Dickinson, USA) with 10,000 events recorded for each sample.

3. MSCs differentiation

MSCs were seeded in 60 ㎜ plate at 2×10^5 cells/mL. To induce differentiation, MSCs were treated with total concentration 1 μM of PMA at an interval of 3 days and changed fresh 10% FBS contained DMEM

4. Induction of myocardial infarction and transplantation

All experimental procedures for animal studies were approved by the Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine, and performed in accordance with the Committee's Guidelines and Regulations for Animal Care. Myocardial infarction was produced in male Sprague-Dawley rats (200±30 g) by surgical occlusion of the left anterior descending (LAD) coronary artery. After induction of anesthesia with ketamine (10 mg/kg) and xylazine (5 mg/kg), cutting the third and fourth ribs opened the chest, and the heart was exteriorized through the intercostal space. The left coronary artery was ligated 2-3 mm from its origin with a 6-0 prolene suture (Ethicon, Somerville, USA, http://www.ethicon.com). For

transplantation, cells were suspended in 30 μL of PBS (1×10^6 cells) and injected from the injured region to the border using a Hamilton syringe (Hamilton Co., Reno, USA, http://www.hamiltoncompany.com) with a 30-gauge needle 3 days after LAD ligation. Throughout the procedure, animals were ventilated with 95% O2 and 5% CO2 using a Harvard ventilator. Operative mortality was 10% within 48 hours. Six animals per group (ligation, MSCs, PMA-MSCs) were used for morphologic analysis after occlusion of left coronary artery. To label MSCs with DAPI for viable cells, sterile DAPI solution was added into the culture medium on the day of implantation at a final concentration of 50 μg/mL. The dye was allowed to remain in the culture dishes for 30 minutes. The cells were rinsed six times in PBS to remove all excess and unbound DAPI. Labeled cells were then detached with 0.25% (wt/vol.) trypsin and suspended in serum-free medium for grafting.

5. Determination of infarct size

TTC staining was used to assess myocardial tissue viability and determine myocardial infarct size. The tissue slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, St. Louis, USA, http://www.sigma.com) solution, pH 7.4, at 37°C for 20 minutes. Tissues were fixed in 10% PBS-buffered formalin overnight at 2~8°C. Hearts were sectioned transaxially at the mid-papillary level, and size of MI was evaluated as percentage of sectional area of infarcted tissue of the left ventricle to the sectional area of the

whole left ventricle. Both sides of each TTC-stained tissue slice were photographed with the digital camera.

6. Histologic determination of fibrosis area

Transplants were killed at several intervals after implantation, and their hearts were excised. The heart was perfusion-fixed with 10% (vol/vol) neutral buffered formaldehyde for 24 hours, transversely sectioned into four comparably thick sections around the mid portion of infarcted area, and embedded in paraffin by routine methods. Sections of 2 μm thicknesses were mounted on gelatin-coated glass slides to ensure different stains could be used on successive sections of tissue cut through the implantation area. Additionally, fibrosis was analyzed by Masson's trichrome staining. Interstitial fibrosis area was measured with MetaMorph software version 4.6 (Universal Imaging Corp., Downtown, USA, http://www.universal-imaging.com) from control (n=6), MI (n=6), MI+MSCs (n=6), MI+PMA-MSCs (n=6) groups and expressed as a percentage of the total left ventricle.

7. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

The TUNEL Assay was performed according to the instructions of the manufacturer (Chemicon International Inc. Temecula, USA, http://www.millipore.com). In brief, the excised heart tissues were fixed in 3.7%

buffered formaldehyde and embedded in paraffin and sectioned transversely at the infarcted area. The tissue sections, 5 μm thick, were deparaffinized, rehydrated and rinsed with PBS. A positive control sample was prepared from normal heart section by treating with DNase I (10 U/mL, 10 min at room temperature). The sections were pretreated with 3.0% H_2O_2, subjected to the reaction with TdT enzyme for 37°C for 1 hour and incubated digoxigenin-conjugated nucleotide substrate at 37°C for 30 minutes. Nuclei exhibiting DNA fragmentation were performed by 3,3-diamino benzidine (DAB) (Vector Laboratories, Burlingame, USA, http://www.vectorlab.com) for 5 minutes. The nuclei of apoptotic cardiomyocytes was stained dark brown. Lastly, the sections were counterstained with methyl green and then cover slipped. The sections were observed by light microscopy. 6 slices per group were prepared, 10 different regions were observed in each slice (× 400).

8. Detection of cytokines

Cytokines secreted by the cells used for transplantations were determined in the cell culture supernatants and cytokines from the cells of the border zone were measured using the rat cytokine array 3.1 (RayBiotech, Norcross, GA) according to the manufacturer's instructions. Briefly, for cell lysates and tissue lysates, it recommended using 1X cell lysis buffer to extract proteins from tissues. After extraction, we spined the sample down and saved the supernatant and the samples were dilutde with 2X cell lysis buffer with H_2O. We added 2

mL 1X blocking buffer and incubated at room temperature for 30 min to block membranes. The membranes were incubated with 1mL of sample at room temperature for 1 to 2 hours. Membranes were washed three times with Wash Buffer I and two times with Wash Buffer II at room temperature for 5 min per wash and incubated with biotin-conjugated antibodies at 4 °C overnight. Finally, the membranes were washed, incubated with HRP-conjugated streptavidin at room temperature for 1 h and with detection buffer for 1 min, and exposed to X-ray film for 40 s (Kodak, Inc.). The exposed films were digitized and the relative cytokine levels were compared after densitometry analysis (Scion NIH Image 1.63). The relative protein levels were obtained by subtracting the background staining and normalizing to the positive controls on the same membrane. Probing with the buffer that was devoid of protein samples did not produce positive staining except at the positive control spots coated with the biotinylated IgGs.

9. RT-PCR analysis

A. Isolation of total RNA

Total RNA was extracted by 500 μL/ 60 ㎜ plate Tri-reagent (Sigma, USA). 100 μL of chloroform was poured above Tri-reagent and a sample was voltexed about 10 seconds. Then, the sample was centrifuged at 12000 g, 4℃ and 15 minutes. 3 layers were appeared in the tube, transparency upper layer collected in new tubes. And poured 250 μL 2-propanol over the sample, and again the

voltex a sample about 30 seconds. Centrifugation was accomplished about 12000 g, 4℃ and 10 minutes. Left the pellet, supernatant was discarded and washed by 75% ethanol (Duksan, Korea)-mixed diethylpyrocarbonate (DEPC; Sigma) water. Centrifugation was also operated about 7500 g, 4℃ and 5 minutes. Dismissed the supernatant, pellet was dried on room temperature about 7 minutes. Finally, poured the 30 μL nuclease free water (NFW). The quality and quantity of the RNA was detected by OD260/OD280 with DU 640 spectrophotometer (Effendorf, Hambug, Germany)

B. cDNA synthesis

Complementary DNA (cDNA) was synthesized with RT-&GO™. Quantitative 1 μg total RNA was added to 1 μL anchored primer (dT)25V, 2 μL dithiothreitol (DTT) and NFW, totally 9 μL volume. To prevent secondary structures, incubated the mixture for 5 minutes at 70℃ and added 8 μL of RT-&GO™ mastermix. Sample was incubated the assay at 42℃ for 1 hour. At the conclusion, sample was inactivated the reverse transcriptase at 70℃ for 15 minutes. Alike isolation of total RNA, sample was detected by OD260/OD280 with DU 640 spectrophotometer.

C. PCR analysis

Quantitative 1 μg cDNA, each 10 pmol primer (forward and backward), 0.1

mM dNTP mixture, 1.25 U of Taq polymerase and 10 X reaction buffer were mixed with NFW, lastly total volume of 25 μL. PCR condition was fixing as fellow. A cycle of denaturing at 94℃ for 3 minutes followed by number of 35 cycles with denaturation at 94℃ for 30 seconds, annealing at 48℃ to 60℃ for 30 seconds, and elongation at 72℃ for 30 seconds. Then keep up 72℃ for 10 minutes. Primers were appeared Table 1. When PCR assay have finished, PCR product was separated by electrophoresis in a 1.2% agarose gel (Biorad, USA) and Gel-Doc (Biorad, USA) visualized after staining with ethidium bromide (EtBr; Sigma, USA).

Primer	Sequence
HGF	AGGCTCAGATTTGGTTTTACTGAAG CCAGCCGTAAATACTGCAAGTAGTC
VEGF	ACTGGACCCTGGCTTTACTG ACGCACTCCAGGGCTTCATC
TNF-alpha	GTAGCCCACGTCGTAGCAAA CCCTTCTCCAGCTGGGAGAC
IL-1beta	TGACCCATGTGAGCTGAAAG AGGGATTTTGTCGTTGCTTG
IL-6	GACTGATGTTGTTGACAGCCACTGC TAGCCACTCCTTCTGTGACTCTAACT

10. Statistical analysis

Data are expressed as means ±SE. Statistical analysis of two groups were

estimated by Student`s t-test. And examining from more than two groups was done by one-way ANOVA, using bonferroni test. $p < 0.05$ was considered significant.

III. RESULTS

1. Primary culture and characterization of MSCs

MSCs were first isolated from mixed cultures with hematopoietic cells based on their attachment on the culture plate. 3 days later after mixed cultures have seeded, tapped a plate, old culture medium was discarded, and fresh 10% FBS contained DMEM was poured into a plate. As time goes by, the cells were adherent, elongated and spindle-shaped, and they were yielded 3×10^6 cells within 2 weeks of culture with 95% purity. The MSCs maintained a fibroblastic morphology through repeated passages, and their identity was confirmed by immunocytochemistry and FACS analysis. Cultured MSCs expressed CD90, CD105, CD106, and ICAM. They expressed the hematopoietic marker CD34 (Fig. 1).

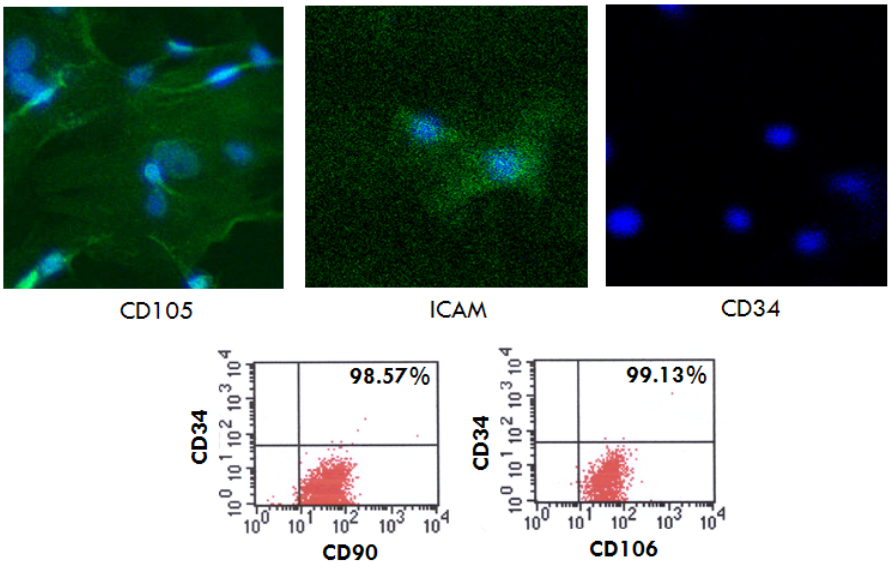

Figure 1. Characteristics of MSCs. Most adherent MSCs are practically fibroblastic in morphology and some polygonal cells were contained after a 6-week culture. Magnification, 100x. Cells were cultured from bone marrow after density fractionation and are shown at 10 days after plating. At 14 days, the MSCs were positive for CD90, CD105, CD106, and ICAM-1 and were negative for CD34 by flow cytometry.

2. Culture of MSCs differentiated with PMA

Primary culture MSCs were treated with 1 mM PMA (final concentration was 1 μM) according to the experimental group. Treatment of PMA was every 3 days. After 9 days in culture, MSCs adhered to the plastic surface and were spindle-shaped with one nucleus. MSCs were treated with 1 mM PMA (final concentration was 1 μM) according to the experimental group. The morphological differentiation from MSCs to myogenic-like cells evolved gradually after PMA induction. Normal BM MSCs were spindle-shaped until 9 days, but MSCs treated with PMA changed into a stick-like or branching-out appearance at 9 days. To examine where MHC or cTnT-expressing cells are located in differentiated MSCs, immunostaining was performed. Clearly, cardiogenic cells (CGCs) had significant morphological changes with positive immunocytochemical analysis for anti-MHC or -cTnT, but normal MSCs showed neither significant morphological changes nor cardiac-specific protein expression of MHC or cTnT during the 9 day period of observation with the same treatment (Fig. 2).

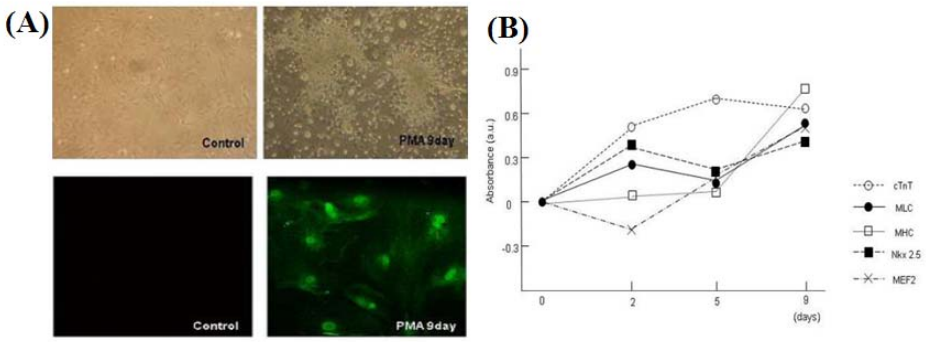

Figure 2. Effects of PMA on morphology of MSCs.

(A) Cellular morphologic alterations and immunochemistry staining. Micrographs of MSCs were taken after nine days. MSCs treated with PMA changed morphology to cardiomyocyte-like and fibroblast-like aggregated long-branches compared to their counterparts. Immunocytochemistry was detected for cardiac MHC and cTnT in MSCs treated with PMA. Representative photos of normal MSCs as positive control and MSCs treated with 1 μM PMA are shown (Magnification: X400). Cardiac MHC or cTnT visualized by FITC is located on the stress fibers.

(B) Changes of cardiac specific marker in MSCs treated with PMA. This change was detected by sandwich ELISA. Samples of protein were lysed to MSCs treated with PMA each harvest day (2, 5, and 9 days). Cardiac-specific markers (cTnT, MLC, MHC, Nkx 2.5 and MEF2) were elevated from two days to 9 days, especially day nine, which highly expressed in five markers.

3. Determination of infarct size

To examine the *in vivo* relevance of our *in vitro* findings, we studied the direct effects of enhanced adhesion of MSCs on LAD ligated rat hearts. The effect of CGCs on myocardial injury after infarction was evaluated by triphenyl tetrazolium chloride (TTC) and trichrome staining. We determined infarct size in the left ventricle (LV) using TTC staining (n=6 rat/group). In the PBS-injected rats, infarct size was 18% of the LV, comparable with normal rat. Injection of MSC resulted in a significant decrease in infarct size (4.5%), and injection of MSC- cardiomyogenic further reduced the infarct size (3%) (Fig. 3).

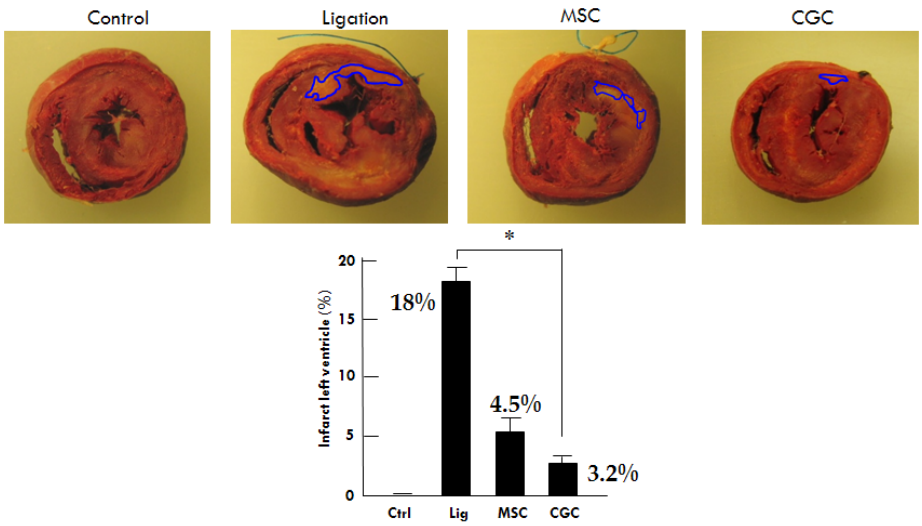

Figure 3. Extension of left ventricular myocardial infarction. Hearts were sacrificed after I (1h)/R (2wks). Photograph of a heart slice stained with triphenyltetrazolium chloride. Myocardial infarction was produced in Sprague-Dawley rats by surgical occlusion of the left coronary artery for 72 hr before sampling the tissue.

Ctrl, control group; *Lig*, ligation group; *MSC*, mesenchymal stem cells-implanted group; *CGC*, cardiogenic cells-implanted group

*$p<0.05$ vs. ligation group.

4. Determination of cardiac fibrosis area

To measure fibrosis area, rat heart was exposed through costal ribs and excised. The only ligated hearts showed significant interstitial fibrosis (36.0±2.5%) compared with the control hearts (1.0±0.6%). In contrast, transplantation of cardiomyogenic MSCs significantly decreased interstitial fibrosis to 8.5±3.2% (Fig. 4). Namely, the specimen of cardiomyogenic MSCs injected-ischemic myocardium showed decreased fibrosis in left ventricle and wall is thicker than ligation group.

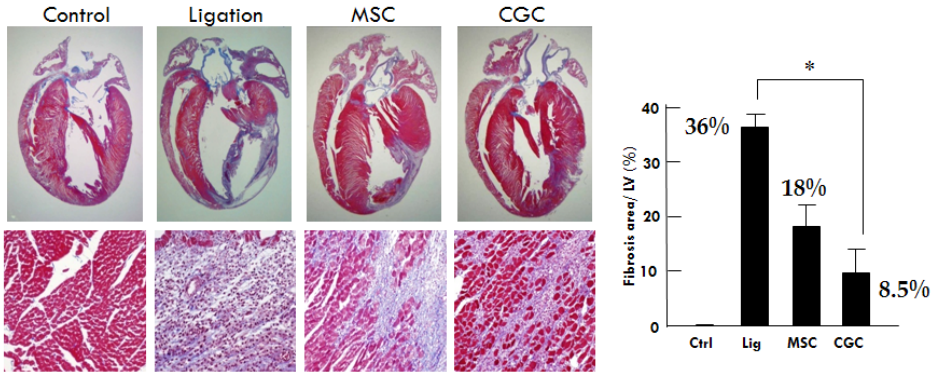

Figure 4. Histological analysis of infarcted hearts. Left panel shows representative Masson's trichrome images from histological sections (magnification: ×200) and right histogram shows less fibrosis (blue).

Ctrl, control group; *Lig*, ligation group; *MSC*, mesenchymal stem cells-implanted group; *CGC*, cardiogenic cells-implanted group

**p*<0.05 vs. ligation group.

5. Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) assay

The TUNEL Assay was performed according to the instructions of the manufacturer (Chemicon, CA, USA). The sections were observed by light microscopy. 6 slices per group were prepared, 10 different regions were observed in each slice (×200). TUNEL-positive cells caused by ligation were significantly reduced in CGCs-implanted group compared with ligation and MSCs-implanted group and the apoptotic indices were 32% in ligation group, 13% in MSCs-implanted group, and 6% in CGCs-implanted group. The incidence of TUNEL-positive myocardial cells caused by ligation was significantly reduced by 81.2% in the ligated hearts transplanted with cardiomyogenic MSCs compared with that of ligation group (Fig. 5).

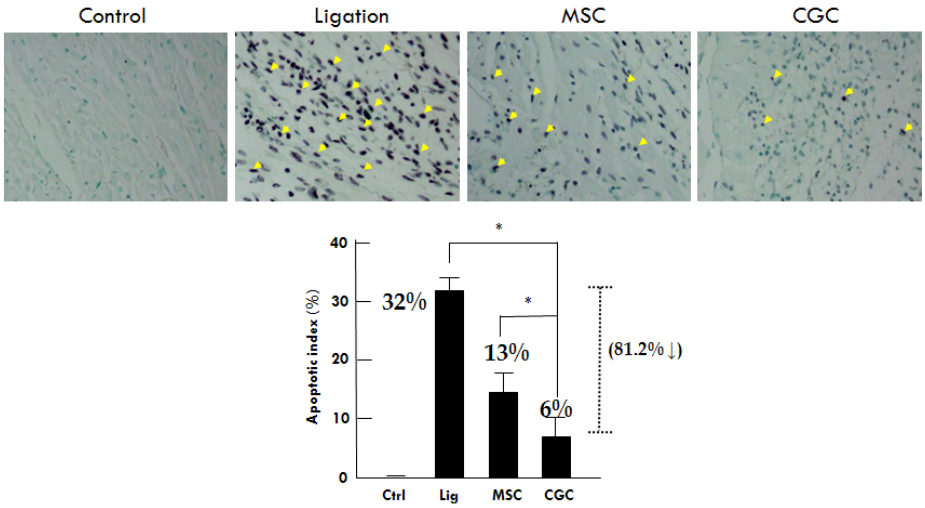

Figure 5. Histochemical characterization of myocardial apoptosis. Apoptosis assay in heart 1week after ligation. Up panel shows representative images of TUNEL staining (magnification: ×200). Staining for normal nuclei (green) was carried by methyl green and apoptotic nuclei were stained brown. Down panel shows summarized data for TUNEL staining with apoptotic indices.

Ctrl, control group; *Lig*, ligation group; *MSC*, mesenchymal stem cells-implanted group; *CGC*, cardiogenic cells-implanted group

**p*<0.05 vs. MSCs-implanted group and ligation group.

6. Rat cytokine array-based analysis with secretion of cytokines by MSCs and CGCs

Antibodies were chosen cardiac specific markers, and their bind was expression of CGCs. The levels of interleukin-1 alpha(IL-1α), beta(IL-1β), IL-6, interferon-gamma (IFN-γ), Tumor necrosis factor-alpha(TNF-α), and macrophage inflammatory protein-3 alpha (MIP-3α), the regulated upon activation, normal heart expressed and secreted cytokine, were measured in the sputum supernatants according to the respective manufacturers' protocols. When the cytokine levels were below the sensitivity, a level of '-'each cytokine was used for the comparisons of the levels, and '+' the level of the sensitivity was used for the correlations. Same sample was repeated 3 times, and their score was on the average.

In the CGC-implanted group, the inflammatory cytokines, such as IFN-ɣ, IL-1α, IL-1β, IL-6, TIMP, and TNF-α, were detected at lower level and VEGF at higher level (Fig. 6).

Cytokines	Control	Ligation	MSC	CGC
IFN-γ	+	++	+	-
IL-1α	+	+++	++	+
IL-1β	+	+++	++	+
IL-6	+	+++	++	+
MIP-3 α	+	+++	++	+
TIMP	+	+++	++	+
TNF- α	+	++	+	-
VEGF	-	+	++	+++

Figure 6. Expressions of secreted cytokines in heart by MSCs and CGCs. Levels of cytokines in CGCs transplanted with ischemic heart. The levels of cytokines in MSCs are quantitatively analyzed by Rat cytokine array from 6 separate experiments.

Control, control group; *Ligation*, ligation group; *MSC*, mesenchymal stem cells-implanted group; *CGC*, cardiogenic cells-implanted group

7. Attenuation of protein production and gene expression of angiogenesis and inflammation cytokines mRNA in cardiogenic cells

The expression levels of HGF and VEGF in CGCs-implanted group were higher (1.61 folds (HGF) and 1.33 folds (VEGF) of the level of the ligation group, respectively) and the expression levels of inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, were significantly lower (0.94 folds (IL-1β), 0.80 folds (IL-6), and 0.71 folds (TNF-α) of the level of the ligation group, respectively). (Fig. 7 and 8).

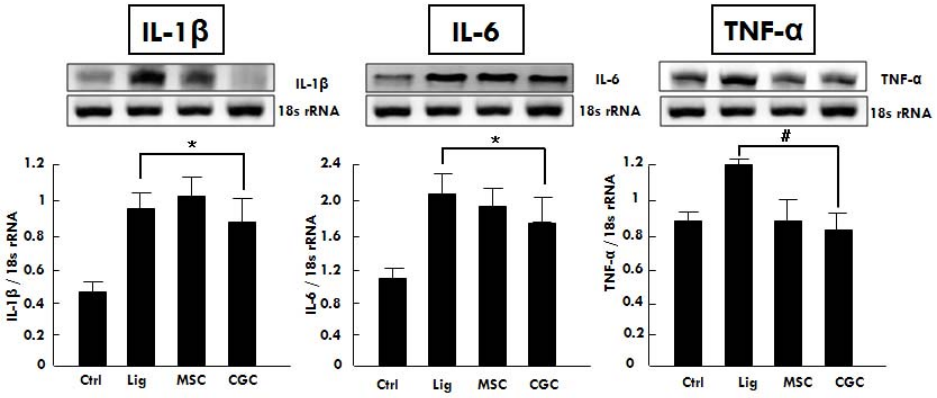

Figure 7. Expressions of inflammatory cytokines by MSCs and CGCs.

Expression levels of Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6) and Tumor necrosis factor-alpha (TNF-α) were estimated with RT-PCR analysis.

Ctrl, control group; *Lig*, ligation group; *MSC*, mesenchymal stem cells-implanted group; *CGC*, cardiogenic cells-implanted group

* $p<0.05$, # $p<0.001$ vs. ligation group.

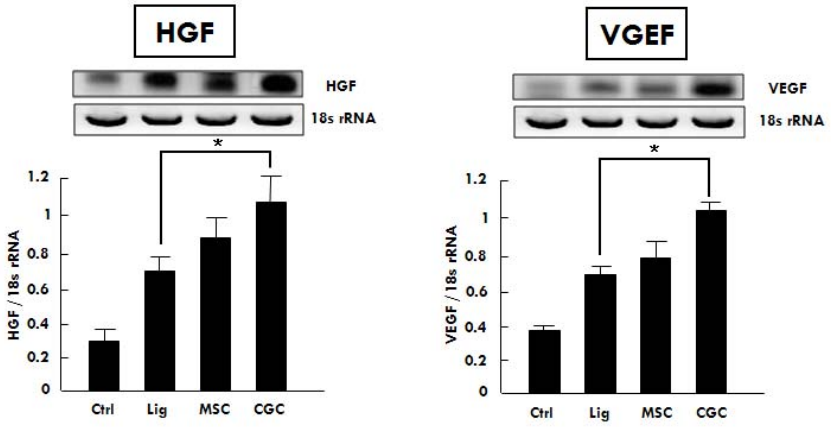

Figure 8. Expressions of angiogenetic cytokines by MSCs and CGCs. Expression levels hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) were estimated in ischemic heart by transplanted MSCs and CGCs.

Ctrl, control group; *Lig*, ligation group; *MSC*, mesenchymal stem cells-implanted group; *CGC*, cardiogenic cells-implanted group

*$p<0.05$ vs. ligation group.

IV. DISCUSSION

Cell implantation utilizing cardiac stem cells, embryonic stem cells, hematopoietic stem cells, skeletal myoblasts, and cardiac myocytes (adult, fetal, or neonatal myocytes) has been suggested as a promising clinical approach for restoration of cardiac infarction. Among these various candidate cell types, MSCs have a great advantage to generate functional cardiac myocytes in the infarcted myocardium because of the easy preparation and multi-lineage potential to differentiate to cartilage, bone, muscle, tendon, ligament and adipose tissue, both *in vivo* and *in vitro*, under appropriate culture conditions. Although MSCs represent a suitable source of autologous cells in cell therapy for improvement in cardiac function, MSCs therapy has limitations due to their poor viability after cell transplantation [6].

Many researchers suggest that MSCs can regenerate myogenic or cardiomyocyte-like cells in cardiac tissue [3]. These advances raise the prospect that damaged cardiac tissues might be repaired by administered adult human MSCs. Shake and colleagues focused on the therapeutic potential of cardioprotective effects of myocardiogenic differentiated MSC transplantation in the acute phase of myocarditis [5]. The major finding of this study is that MSCs have paracrine effects that differentiate into cardiomyocyte-like cells with PMA. This simple new model for differentiated MSCs may help clarify the cascade of transcriptional activation that regulates differentiation into

cardiomyogenic stem cells. Therefore, we concerned about paracrine effects by using secreted proteins from MSCs on ischemic myocytes [7, 11]. Previous studies showed that the injection of MSC culture media is a superior strategy for rescuing tissue damage caused by acute ischemic heart disease [8]. In this study suggests new evidence on the paracrine effect of cardiomyogenic MSCs. Previously, researchers experimented about 5'-azacytidine that MSCs were importantly used to differentiate into myocytes [12].

To determine whether CGCs had a heart like cell phenotype, we were accomplished cardiac-specific marker-related sandwich ELISA. The highest expression time of all antibodies (cTnT, MHC, MLC, Nkx 2.5 and MEF-2) was 9th days. And the morphology of the cells was the most similar to cardiac myocyte at 9th day. Especially, MHC expression was measured with immunocytochemistry. The specific MHC was found in the cytoplasm of the cells induced with PMA. Compared with normal MSCs, differentiated MSCs showed higher expression of MHC.

Adrenergic receptors are divided into 2 subtype, α-adrenergic and β-adrenergic receptors, and muscarinic receptors have 5 subtypes; M1 through M536. We explored α1A, α1B, α1D, β1 and β2-adrenergic receptor and M1 and M2 muscarinic receptor which reported other paper. Characteristics of receptors are as follow: critically implicated in modulating cardiac function, α1A-, α1B-, α1D- ; catecholamine induced increases in heart rate, conduction velocity and contractility, β1-, β2-adrenergic receptor; expressed in murine neonatal and

adult cardiomyocytes, M1, M2 muscarinic receptor. Surprisingly, when MSCs were treated with PMA for 9 days, the expression levels of specific markers for cardiomyocyte were significantly higher [9, 10, 18, 19].

We isolated cardiac-specific marker expression of CGCs using cytokine array-based analysis [9]. Antibodies were chosen cardiac specific markers, and their bind was expression of CGCs. Same sample was repeated 3 times, and their score was on the average. Expression of most part of specific marker was elevated about 2 days and peaked 9 days.

To examine the *in vivo*, we studied the direct effects of enhanced adhesion of MSCs on LAD ligated rat hearts [13-17, 20]. The effect of MSCs treated PMA on ischemic myocardial injury was evaluated by triphenyl tetrazolium chloride (TTC) and trichrome staining [21-24]. In the PBS-injected rats, infarct size was 18% of the LV, comparable with normal rat. Injection of MSC resulted in a significant decrease in infarct size (4.5%), and injection of MSC-PMA further reduced the infarct size (3.2%). The only ligated hearts showed significant interstitial fibrosis (36.0±2.5%) compared with the control hearts (1.0±0.6%). In contrast, transplantation of cardiomyogenic MSCs significantly decreased interstitial fibrosis to 8.5±3.2%. Namely, the specimen of cardiomyogenic MSCs injected-ischemic myocardium showed decreased fibrosis and the LV wall was thicker than ligation group. Apoptosis in fetal rat and mouse hearts has been demonstrated with the TdT-mediated dUTP nick end labeling (TUNEL) method, which stains in situ DNA breaks in individual nuclei in tissue sections

[25-28]. Although the TUNEL method has showed the lack of selectivity for apoptotic over non-apoptotic and caspase-independent cell death, it was used to identify the percentage of apoptotic cell in the cardiac tissue because apoptosis has a major role in cell deaths in myocardial infarction. The incidence of TUNEL-positive myocardial cells caused by ligation was significantly reduced by 81.2% in the ligated hearts transplanted with cardiomyogenic MSCs compared with that of ligation group. The mean microvessel count per field in the infarcted myocardium was significantly higher in the cardiomyogenic MSC group than in the sham and MSCs group (sham: 19.3±15.2; MSCs: 68.9±19.4; cardiomyogenic-MSCs: 129.1±23.5, $p<0.05$).

It has discussed key signaling factors, including cytokines, chemokines and growth factors, which are involved in orchestrating the stem cell driven repair process. It focused on signaling factors known for their patho-physiological healing process (TNF-a, IL-8, IL-10, HIF-1a, VEGF, G-CSF), signaling factors that are involved in cardiogenesis and neo-angiogenesis (VEGF, HGF) and inflammation (TNF-a, IL-1b, IL-6). The therapeutic application and capacity of secreted factors to modulate tissue repair after myocardial infarction relies on the intrinsic potency of factors and on the optimal localization and timing of a combination of signaling factors to stimulate stem cells in their niche to regenerate the infarcted heart. As a result, CGCs were more expressed at cardiogenesis cytokines (HGF, VEGF), as against depressed at inflammatory cytokine (TNF-a, IL-1b, IL-6). To assess the practical effect on cardiac function

by this cytokines, the echocardiographic study will be needed in the future.

The effect of cardiomyogenic MSC on ischemic heart functions might result from the induction of cytokine synthesis induced by hypoxic condition. It is postulated that therapy with cardiomyogenic MSCs and secreted cytokines could prove useful in attenuating cardiac remodeling and in cardiac disease.

V. CONCLUSION

Paracrine factors of Mesenchymal stem cells (MSCs) represent a suitable source of autologous cells in cell therapy for the improvement of cardiac function. We found cardiogenic differentiation system induced by PMA and PKC activation. This model may help clarify the cascade of transcriptional activation that regulates differentiation into cardiogenic cells (CGCs). This study was investigate to the correlation between myocardial regeneration and cytokine release of ischemic heart with intramyocardial implantation of CGCs. Ligated hearts showed significant interstitial fibrosis compared to the normal group. In contrast, transplantation of CGCs significantly decreased in interstitial fibrosis. TUNEL-positive cells to identify the percentage of apoptotic cell in the cardiac tissue caused by ligation were significantly reduced in CGC-implanted group compared with sham and MSC group. The therapeutic significance of cytokines secreted by CGCs could prove useful in attenuating cardiac fibrosis and regenerating myocardium after myocardial infarction. These results also contribute to a better therapeutic understanding on cardiac disease.

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Abstract (in Korean)

심근 유사 중간엽 줄기세포가 투여된 심근경색 심장에서 심근 재생과 싸이토카인 분비의 상관관계

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중간엽 줄기세포 (mesenchymal stem cell; MSC) 는 심장의 기능을 향상시키는데 기여하는 세포치료에서 이용될 수 있는 자가 세포로서 매우 적합한 것으로 알려져 있으나 MSC 는 이식 후 자가 분화능이 낮아 임상적 응용에 있어 제한점을 가지고 있다. 하지만 MSC 는 이식된 심장에서 허혈 심근재생에 중요한 역할을 하는 혈관내피 성장인자 (vascular endothelial growth factor; VEGF), 간세포 성장인자 (hepatocyte growth factor; HGF), 그리고 염기성 섬유모세포 성장인자 (basic fibroblast growth factor; b-FGF)등을 분비하여 내인성 심근재생을 유발하는 주변분비 작용 (paracrine effect)을 하는 것으로 보고되고 있다. 최근 연구에서, 단백질 카이네이즈 C (protein kinase C)의 신호전달 체계에 조작을 가하여 중간엽 줄기세포를 심인성 세포 (cardiogenic cells; CGC)로 분화시킬 수 있음이 밝혀졌다. 본 연구는

중간엽 줄기세포를 CGC 로 분화시킨 후 심근 경색 동물 모델에 심근 내에 주입하여 심근의 재생과 싸이토카인 분비의 연관성을 알아보고자 하였다. 먼저 100g 의 Sprague-Dawley 실험쥐로부터 MSCs 를 추출 및 배양한 후 단백질 카이네이즈 C 활성제인 Phorbol 12-myristate 13-acetate (PMA)를 투여하여 MSC 를 CGC 로 분화시켰다. 200g 의 Sprague-Dawley 실험쥐의 심장 좌전하행지를 결찰한 후 분화된 CGC 를 심근경색의 이행부위에 주입하였으며, 정상군, 결찰군, MSC 투여군, CGC 투여군 각각 6 마리를 대상으로 실험하였다. 결찰군에서 정상군에 비해 간질성 섬유화 (결찰군; 18.0±3.2%, 정상군; 1.0±0.6%)가 더 높음을 확인하였으며, CGC 투여군에서 유의하게 결찰군에 비해 간질성 섬유화 정도가 감소하였다 (2.5±1.0%, $p<0.05$). 허혈로 인해 발견되는 TUNEL 양성 세포를 측정하여 apopotic index 를 계산하였으며 결찰군 32%, MSC 투여군 13%, CGC 투여군 6%로 CGC 투여군에서 결찰군에 비해 81.2%의 감소효과가 있었다. 경색 이행부위에서 추출된 cytokine 분석에 있어서 MSC 투여군과 CGC 투여군에서 염증성 싸이토카인인 IFN-γ, IL-1α, IL-1β, IL-6, TNF- α 등이 낮게 나타난 반면 VEGF 와 같이 심장세포 재생에 기여하는 인자는 높게 나타났다. RT-PCR 을 통한 싸이토카인 생성 유전자 발현 검사에서 CGC 투여군에서 심장 재생관련 싸이토카인의 발현 정도는 결찰군에 비해 HGF 의 경

우 1.61 배, VEGF 1.33 배로 더 높게 나타났으며, 반면 염증성 싸이토카인의 경우 CGC 투여군의 발현은 결찰군에 비해 IL-1β 0.94 배, IL-6 0.80 배, 그리고 TNF-α 0.71 배로 더 낮게 발현되었다. 결론적으로 프로테인 카이네이즈 C 활성제에 의해 중간엽 줄기세포에서 분화된 심인성 세포에 의해 분비되는 싸이토카인은 심근 경색 후 심장의 섬유화를 감소시키고 심장을 재생 시키는 역할을 함을 알 수 있었다. 이러한 결과는 심장 질환의 치료 및 세포 치료에 있어서 더 나은 치료적 이해를 줄 수 있을 뿐 아니라 줄기 세포의 치료 방법을 개선 및 발전 시킬 수 있을 것으로 사료된다.

핵심되는 말: 중간엽 줄기 세포, 프로테인 카이네이즈, 분화, 재생, 싸이토카인