Protective effects for hypoxic cardiomyocytes by culture broth of mesenchymal stem cells

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Protective effects for hypoxic cardiomyocytes by culture broth of mesenchymal stem cells

Directed by Professor Yangsoo Jang

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ABSTRACT

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Ischemia is a major factor of cardiovascular diseases, which is chronically stunned and hibernating myocardium. It results in myocardial infarction (MI), left ventricular remodeling, and heart failure. As a clinical study, researchers have experimented with a variety of different cell types to induce functional improvement in the ischemia heart disease (IHD). In the cell types, mesenchymal stem cells (MSCs) are reported to have the capacity to survive cardiomyocytes. Especially, secreted proteins from MSCs have been recognized to recover cellular functions on IHD. So, we prepared hypoxia

conditioned media (HCM) from ischemic MSCs and found out that it contained several proteins by proteomic analysis. We investigated changes in the hypoxic cardiomyocytes survival rate in the presence of HCM, phosphorylation of ERK1/2, gene expression of Ca^{2+}-handling proteins, various ion channels, and ion exchangers. We also examined infarct size of heart by surgical occlusion of the left anterior descending (LAD) coronary artery. Survival rate of HCM-treated hypoxic cardiomyocytes was increased about 1.6-folds compared to hypoxic cardiomyocytes and phosphorylation of ERK1/2 was increased about 2.7-folds compared to hypoxic cardiomyocytes. In vivo, after heart ligation, infarct size of HCM-treated heart was decreased 22.3% in comparison with only ligated heart. We also observed significant induction and suppression in transcription levels of Ca^{2+}-handling proteins (calreticulin, calmodulin), ion channels (L-type $Ca2^{+}$-channel) and ion exchangers (SERCA2a, Phospholamban, $Na^{+}K^{+}$ATPase, Na^{+}/Ca^{+} exchanger) in HCM-treated cardiomyocytes. The altered levels of Ca^{2+} -related protein are nearly recovered from the effects of the hypoxia in the presence of HCM. These findings suggested that MSCs secrete paracrine-acting agents with cardio-protective and/or inotropic properties that can rescue hypoxic cardiomyocytes and improve ventricular function.

Key words: ischaemic heart disease, cardiomyocytes, mesenchymal stem cell, paracrine effect, hypoxia conditioned media

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I. INTRODUCTION

The main phenomenon of ischaemic Heart Disease (IHD) is the global or regional discrepancy between myocardial oxygen supply and demand, often manifested clinically as the pain of angina pectoris[1]. The blood vessels are narrowed or blocked due to the deposition of cholesterol plaques on their walls. This may eventually result in a portion of the heart being suddenly deprived of its blood supply leading to the death of that area of heart tissue, resulting in a heart attack[2]. It develops myocardial infarction (MI) and left

ventricular remodeling, heart failure, cardiac hypertrophy that is volume overload to the mass of infarcted myocardium[3].

Despite advances in medical and surgical treatment, there is an increasing burden of ischemic cardiovascular disease and heart failure. Over the past decade, a large number of preclinical and clinical studies have evaluated various biologic agents to treat these diseases. The formation of new blood vessels to perfuse ischemic myocardium and the generation of new myocardium to replace scar tissue represents the ultimate objective of biologic myocardial regenerative therapy. These therapies can be broadly classified into two categories[2]. The first is the use of various medications, using beta-blockers or calcium channel antagonists, and the second is the use of cell-based therapy. But, despite initial enthusiasm created by preclinical studies, clinical trials of medication have shown only modest symptomatic and functional improvements in patients with advanced coronary disease. Also, researchers have experimented with a variety of different cell types to induce functional improvement in the ischemic myocardium[2].

Recently, the established source for IHD is the bone marrow-derived mesenchymal stem cell (MSC)[4]. Different types of MSCs have been proposed as a potential source for cell therapy of acute myocardial infarction (MI)[5, 6, 7]. Given their multipotency, low immunogenicity, amenability to ex vivo

expansion, and genetic modification, autologous MSCs are particularly suitable for this purpose[8, 9, 10, 11]. Several groups have reported that intra-myocardial administration of MSCs reduces post-infarction ventricular remodeling and in some cases improves left ventricular function[12, 13, 14]. However, the mechanisms underlying these therapeutic effects have not been clearly defined. Although in situ differentiation of the MSCs into cardiomyocytes and other cell types constituting the cardiac tissue has been proposed by some groups[15, 16], there is much debate over the frequency of this phenomenon. Moreover, the low post-implantation viability of the injected cells may limit the capacity of this mechanism to result in meaningful cardiac regeneration[17]. On the other side about MSC, some hypothesis has been brought to attention. The first thing is gene therapy. Gene therapy is a rapidly developing and a promising therapy modality. Research beginning with the advances in molecular biology by the 1960s has begun progressing in 1990s with the achievement of clinical gene therapy. The investigators play an important role in transmission of genetic materials to the target cells and for this reason they must follow developments of high technology about gene therapy such as defining a gene, cloning, locating in a vector and detection of its expression[18].

We also investigated secreted proteins from MSCs on hypoxic

cardiomyocytes. The protein mediator becomes generally known that it influence multiple target cells in the area. This concept was called the paracrine effect. Generally, in paracrine signaling, cells secrete local protein mediator act only on cells in the immediate environment. In this case the target cell responds to a particular extracellular signal by means of specific proteins, called receptor, that bind the signaling molecule and initiate the response[19]. Accordingly, we prepared hypoxic conditioned media (HCM) from hypoxic MSCs and found out that it contained several proteins by proteomic analysis. But, we didn't clarify correct function of secreted proteins.

Ischemia in the heart causes cell to lose viability and disturbed calcium homeostasis. Therefore, we investigated the survival rate of hypoxic cardiomyocytes in the presence of HCM under hypoxia condition. Exposure of the cardiomyocytes to hypoxia with HCM increased cell survival rate compared with hypoxic cardiomyocytes, as measured by the trypan blue cell counting. Under pathological conditions of hypoxia, enhanced survival rate of cardiomyocytes was related to increased activation of ERK1/2. ERK1/2 is major member of the MAPK family. The phosphorylation of ERK1/2 decline is observed during ischemia in heart. But, ERK1/2 phosphorylation of hypoxic cardiomyocytes in the presence of HCM increased comparison to

ERK 1/2 of hypoxic cardiomyocytes.

There is also accumulating evidence that Ca^{2+}-induced Ca^{2+} release process that governs cardiac contractility is defective in cardiac hypertrophy, but the molecular mechanisms remain elusive[20]. Proteins involved in calcium removal from the cytosol were significantly altered in the hypoxia condition. SR-Ca^{2+}-ATPase, relevant for removal of calcium from the cytosol into the lumen of the sarcoplasmic reticulum, was decreased. Phospholamban, which inhibits the SR-Ca^{2+}-ATPase in the basal unphosphorylated state, was slightly decreased[21]. The sarcolemmal Na^{+}-Ca^{2+}-exchanger, relevant for transsarcolemmal calcium extrusion was increased in the hypoxia cardiomyocytes. Na^{+}-K^{+}-ATPase provides energy for calcium transfer at the external membrane level[22]. The activity of the Na^{+}-K^{+}-ATPase is reduced in the hypoxia cardiomyocyte. L-type Ca^{2+} channels are critically involved in excitation secretion coupling whereby membrane depolarization activate L-type Ca^{2+} channels, allowing extracellular Ca^{2+} entry and stimulation of exocytosis. The L-type Ca^{2+} channels were significantly lower in the hypoxia cardiomyocytes compared to controls. As Ca^{2+}-handling proteins, calreticulin and calmodulin are important regulators for ion homeostasis. While calmodulin is expressed interstitially, calreticulin is a calcium-binding chaperone of the sarcoplasmic reticulum[23]. Overexpression of calreticulin

leads to prevention of oxidant-induced $[Ca^{2+}]_I$ increase and results in cytoprotection. Similarly, generation of oxidative stress in the cardiomyocytes increased expression of the calreticulin gene, but treatment with HCM of MSCs decreased calreticulin expression levels. Previous studies have shown that expression level of calmodulin is reduced in human end-stage heart failure, and we found that calmodulin was decreased in hypoxia myocytes. Our results indicate that treatment with HCM of MSCs restores calmodulin expression levels[24].

In this study, we hypothesized that HCM of MSCs exert paracrine effect on the hypoxic cardiomyocytes by including biologically active factors. To prove our hypothesis, we tested the cyto-protective effect of HCM from cultured MSCs on isolated neonatal rat ventricular cardiomyocytes exposed to hypoxia in vitro and on rat hearts after experimental MI in vivo. We assessed the contraction characteristics of cardiomyocytes exposed to hypoxia in the presence of HCM. Most importantly, we determined the effect of HCM on ventricular performance in infracted rat hearts. Our results show that MSCs secrete proteins with cardio-protective and/or inotropic properties that can rescue hypoxic cardiomyocytes and improve ventricular function. This finding may represent an important breakthrough in understanding stem cell action in tissue protection and repair and in

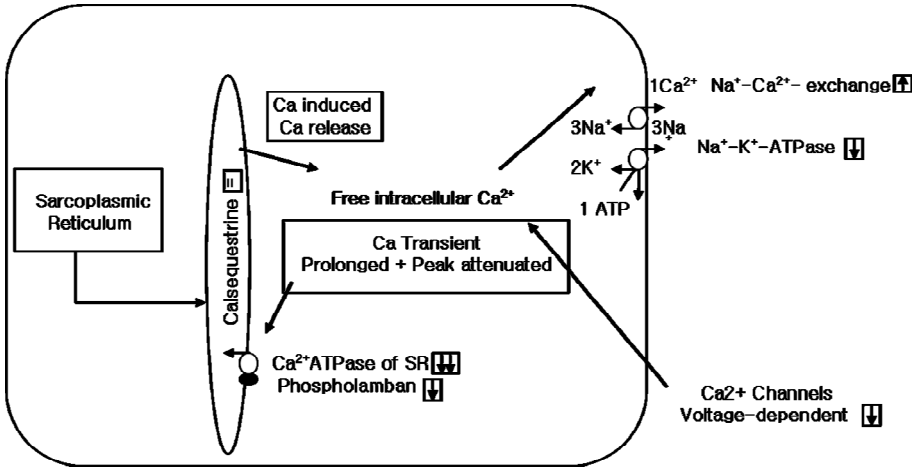

Figure 1. Calcium-regulating protein in hypoxic cardiomyocyte. Diagrammatic representation of cardiomyocyte internal and external membranes. Compensated cardiac hypertrophy, mainly experimental models; myocardial infarction, left ventricular remodeling heart failure, cardiac hypertrophy; ↑, increased activity (as compared with controls); ↓, decreased activity; =, activity unchanged; SR, sarcoplasmic reticulum.

developing effective molecular therapeutics.

II. MATERIALS AND METHODS

1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum, penicillin- streptomycine were from Gibco (Gibco BRL, Grand Island, NY, USA). Antibodies of Immunoblot analysis were used as follows: Monoclonal extracellular-signal related kinase 1 (ERK1) antibodies and anti-phospho ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horse-radish peroxidase-conjugated secondary antibodies to mouse or rabbit were obtained from Santa Cruz Biotechnology. Western blotting detection system was from Amersham Biosciences (Uppsala, Sweden).

2. Animals

Mesenchymal stem cells (MSCs) were isolated from the bone marrow of 4-week-old Sprague-Dawley male rats. Also, cardiomyocytes were isolated from the neonatal Sprague-Dawley rat 1~2days old. All animal procedures were carried out according to a protocol approved by the Yonsei University Animal Care Committee.

3. Cell culture

A. Mesenchymal stem cell

Rat bone-marrow MSCs were harvested from one-month-old (100~150g) male Sprague-Dawley rats. Following the rats' death by cervical dislocation, the tibia and femur were dissected, and whole bone-marrow plugs were flushed by means of an 18-gauge needle and 10-mL syringe loaded with Dulbecco's Modified Eagle's Medium-Low Glucose supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA). MSCs were centrifuged, and resuspended in serum-supplemented medium, loaded to Ficoll (Amersham, Uppsala, Sweden) density gradient centrifugation. After twice washing steps, MSCs were incubated in 7ml of fresh DMEM (Gibco BRL) by adding 10% FBS, 100U/ml penicillin/streptomycin (Gibco BRL). Cells were maintained in 95% air /5% CO_2 at 37℃ for 10-days, with fresh medium change every 3-4day. Upon reaching 80-90% confluence, primary MSCs were trypsinized (Gibco BRL), passaged at a density 5-7 x 10^5 cells/100mm culture plate [25, 26].

B. Neonatal rat ventricular cardiomyocytes

Cardiomyocytes were prepared from Sprague-Dawley neonatal rat hearts as previously described with minor modification. The isolated heart tissues were washed with Dulbecco's phosphate-buffered saline solution (pH 7.4 Gibco BRL) in order to deplete red blood cell. Using micro-dissecting scissors, hearts were minced until the pieces were approximately 0.5mm^3 and treated with 4ml of collagenase Ⅱ(1.4mg/ml, 270units/mg, Gibco BRL) for 5min at 37℃ humidified chamber. The supernatant was then removed and washed by 10% FBS DMEM. Cell pellets were obtained by centrifugation. The cells resuspended in equal volume of fresh medium containing 10% FBS. The remained tissue was treated with fresh collagenase Ⅱ solution for an additional 5min. The incubation procedure was repeated until the tissue was totally digested. The resulting supernatant was centrifuged at 2000rpm for 2min at room temperature. The cell pellet was resuspended in 5ml of cell culture medium and preplated in culture dish for at least 2hr at 37℃ in 5% CO_2 incubator. The adhered cells are fibroblast and non-adherent cells are cardiomyocytes. Unattached cardiomyocytes were replated on 100mm culture dish($5x10^5$cell/ml) and incubated in α-MEM supplemented with 10% FBS. Cells were then cultured in a CO_2 incubator at 37℃. To reduce fibroblast contamination, we used α-MEM with

0.1mM 5-bromo-2'-deoxyuridine (Brd-U) (Sigma, St. Louis, MO, USA) [27, 28].

4. Conditioned media generation

A. Hypoxia conditioned media

Conditioned media were generated to mesenchymal stem cells. 90% confluent second passage MSCs were fed with 10% FBS DMEM. The MSCs were placed in the hypoxic chamber and washed twice by degassed serum-free DMEM. After degassed fresh serum-free DMEM were exchanged, cells incubated for 12hr under hypoxia. The media was then collected and stored -70℃ for in vitro experiments. Hypoxic conditions were created by incubating the cells at 37℃ inside an anaerobic system (Technomart INC, Seoul, Korea) with an atmosphere of 5% CO_2, 5% H_2, 85% N_2. The oxygen level in the chamber was ~0.5%.

B. Normoxia conditioned media

The 90% confluent third passage MSCs were fed with 10% FBS DMEM. The MSCs were washing twice by serum-free DMEM in normoxic clean bench and exchanged for fresh serum-free DMEM. The cells were incubated for 12hr under 5% CO_2, 37℃ normoxic chamber. The media was then

collected and stored 4℃ for in vitro experiments.

5. Neonatal rat ventricular cardiomyocytes treatment

After overnight incubation, Neonatal rat ventricular cardiomyocytes were placed in hypoxic chamber and washing twice by fresh serum-free DMEM. Cardiomyocytes were incubated by hypoxic conditioned media or normoxic conditioned media in hypoxic chamber for 12hr. The cells were collected by disposable cell scraper (Greiner Bio-one, Boston, USA).

6. Cell counting by trypan blue stain

The standard growth medium of cardiomyocytes was replaced with serum free α-MEM, hypoxic conditioned media or normoxic conditioned media from MSCs; cardiomyocytes were subsequently exposed to hypoxia condition for 12hr. cardiomyocytes were digested with 0.25% trypsin for about 2 to 5 minutes at 37℃ until most of the cells were detached. The cells were washed by phosphate-buffered saline (PBS) (Gibco BRL, Grand Island, NY, USA). 0.5 ml of trypan blue solution was transferred to test tube and added to 0.3 ml of PBS, 0.2 ml of the cell suspension was mixed thoroughly. Allow to stand for 5 to 15 minutes. A small amount of trypan blue-cell suspension was transferred to both chambers of the hemocytometer by

carefully touching the edge of the cover-slip with the pipette tip and allowing each chamber to fill by capillary action. The dyed cell was counted in the 1mm center square and four 1mm corner squares.

7. Induction of myocardial infarction and injection of hypoxia conditioned media

Myocardial infarction was produced in male Sprague–Dawley rats (200 ± 30 g) by surgical occlusion of the left arterior descending coronary artery, according to previously described procedures. 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 5-0 prolene suture (ETHICON, UK) for 1week. For injection, 10ml of HCM were concentrated to 200μl (6mg/ml) and inpoured at the intraperitoneal injection (IP) one time a day for 1 week after LAD occlusion using a insulin syringe with a 30-gauge needle. Throughout the operation, animals were ventilated with 95% O_2 and 5% CO_2 using a Harvard ventilator.

8. Immunoblot analysis

Treated cardiomyocytes were collected in tube and centrifuged at 12000 rpm for 2 min at 4℃ temperature. Collected cells were solubilized in lysate buffer (Cell Signaling Technology, Beverly, MA), supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO). The homogenates were centrifuged and protein concentrations in the supernatants were determined by the BCA Protein Assay regent (Pierce Biotechnology, Rockford, IL). Equal amounts of protein were loaded onto and separated by 12% SDS–PAGE and transferred to membranes (Millipore, Bedford, MA, USA). The membranes were incubated in blocking buffer for 1 hr and incubated with antibodies to ERK1 (1:2000) (Santa Cruz), phospho-ERK1/2(1:500) (Santa Cruz) and Beta-actin (1:4000) (Sigma) overnight at 4℃. The membranes were washed and incubated in secondary antibodies (goat anti-rabbit or mouse HRP antibodies, 1:2000 dilution, Cell Signaling Technology). Immunocomplexes were visualized with enhanced chemiluminescence (ECL) regent. The band intensities were quantified using Phospho-Image System [29].

9. RT-PCR analysis

A. Isolation of total RNA

Total RNA was extracted by using 1 ml TRIzol reagent (Sigma, St. Louis, MO, USA). Total cellular RNA was extracted by the chloroform, isopropanol, DEPC-treated 75% ethanol procedure. The RNA was suspended in 30ml of DEPC-treated water. The quantity and quality of the isolated RNA was determined by OD260/OD280 with DU 640 spectrophotometer (Eppendorf, Hamburg, Germany).

B. RT-PCR assay

Complementary DNA (cDNA) was generated with Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instructions. 1μg of total RNA was reverse-transcribed in a 20 μL reaction mix containing 5 mmol/L MgCl2, 10 mmol/L Tris—HCl (pH9.0 at 25 ◦C), 50 mmol/L KCl, 0.1%Triton X-100, 1 mmol/L dNTP, 20 U of RNase inhibitor, 0.5 μg oligo-(dT)15 primer, 10U of reverse transcriptase for 15 min at 42℃and the reaction was terminated by heating at 99℃ for 5 min. To monitor cDNA synthesis, an aliquot of the RT reaction mixture was

subjected to PCR for GAPDH. The GAPDH primer sequences were as follows: 5-ACCACAGTCCATGCCATCAC-3 and 5-TCCACCACCCTGTTGCTG-3 (450bp). The PCR condition was a cycle of denaturing at 95℃ for 3 min followed by 35 cycles of denaturation at 95℃ for 1min, annealing at 49℃ for 1min, extension at 72℃ for 2min before a final extension at 72 ◦C for 10 min. The PCR of gene expression of ion channels, ion exchangers and Ca2+-handling proteins was performed as previously described with a little modification. The first round of PCR amplification was performed using 1μL of the cDNA from the reverse transcription. The PCR mix contained 10pmol/μl of each primer, together with 200mM Tris—HCl (pH8.8), 100mM KCl, 1.5 mmol/L $MgSO_4$, 1%Triton X-100, 0.1mM dNTP and 1.25U of Taq polymerase in a total volume of 25μL. PCR reactions were carried out in a thermal cycler using the following conditions: 95℃ for 3min, 95℃ for 1min and then followed by the individual conditions for each gene as outline in Table1. All RT-PCR products were separated by electrophoresis in a 1.2% agarose gel and visualized after staining with ethidium bromide [30].

Table 1. Used oligonucleotide primers in the RT-PCR

Gene	primer	bp	Cycler program
Calreticulin	Atgaccccacagatccag Gcataggcctcatcattggt	339	55℃, 60s/35 cyc.
Calmodulin	gaagcaggccagtcaaagc cgaatttggaagccaacact	348	55℃, 60s/35 cyc.
SERCA 2a	tccatc tgcctg tccat gcggttactccagtattg	196	55℃, 60s/35 cyc.
Na+-K+-ATPase	tgtgattctgg tga gaa cg aggacaggaaagcagcaaga	352	57℃, 60s/35 cyc.
NCX-1	tgtctgcgattgcttgtctc tcactcatctccaccagacg	364	55℃, 60s/35 cyc.
L-type Ca2+-channel	tgtcacggttgggtagtgaa ttgaggtggaagggacttg	346	55℃, 60s/35 cyc.
Phospholamban	gctgagctcccagacttcac gcgacagcttgtcacagaag	339	57℃, 60s/35 cyc.

10. Trichloroacetic acid protein precipitation

Trichloroacetic acid (TCA) precipitates the chilled products by adding 2 volumes of ice cold 20% TCA, vortex, and incubates from 30 min to several hours on ice. Spin out precipitate 15 min 4℃ in centrifugation, remove all supernatant, add 300ul acetone, and spin 5min at 4℃. The pellets dry in room temperature for 5-10 min. The samples were added 1x SDS-PAGE loading Buffer and loaded to 12% SDS-PAGE after heating at 65℃ for 3min.

11. Silver stain

After electrophoresis, remove the gel from the cassette and place into a tray containing appropriate volume of deionized water. The gel was soaked in this water for 10min. Deionized water was discarded and the gel was fixed in 40% methanol and 10% acetic acid solution for 60 min. After the solution was discarded, the fixed gel was incubated in 10% ethanol and 5% acetic acid solution for 30min and repeated twice. After the solution was discarded, the gel was added enough volume of the oxidizer for 10min with gentle rotation. The gel was washed out three times, 10min each time, in deionized water. Silver staining reagent was added in fixed gel and was shaked for 30 min to allow the silver ions to bind to proteins. After staining was complete, pour off the staining solution and rinsed the gel with a large volume of deionized water for 2min to remove the excess of unbound silver ions. The gel was shortly treated with the developing solution for 30 sec. The new portion of developing solution was added and protein image was developed by incubation the gel in 200 ml of developing solution for 2~5 min. The reaction can be stopped as soon as the desired intensity of the bands is reached. The reduction reaction of stained gel was stopped by 5 % acetic acid solution. Moist gels can be kept in 12 % acetic acid at 4℃ in sealed plastic bags or placed in the drying solution for 2 hr prior to vacuum drying.

III. RESULTS

1. Confirmation of HCM protein content

To verify whether hypoxic condition up-regulates the release of protein factors, components of medium were analyzed by SDS-PAGE through coomassie brilliant blue staining and silver staining. Double stained SDS-PAGE showed that several protein bands were produced much more intensive as time goes under hypoxic condition. However, we could not find detectable protein bands from normoxic condition media of MSCs. Therefore, there was no comparison band between HCM and NCM. Our data indicated that the secreted protein of incubated MSCs in hypoxia condition for 12hr was detected the highest density of other condition (Fig.2). To reify data for secreted protein patterns, we analyzed the media of MSCs exposed to hypoxia condition for 12hr. As a result, we found out various proteins and divided protein list into three classes; 1) HCM-only secreted proteins (Table 2), 2) HCM-high score proteins (Table 3), 3) HCM-only secreted protein as well as high score (Table 4).

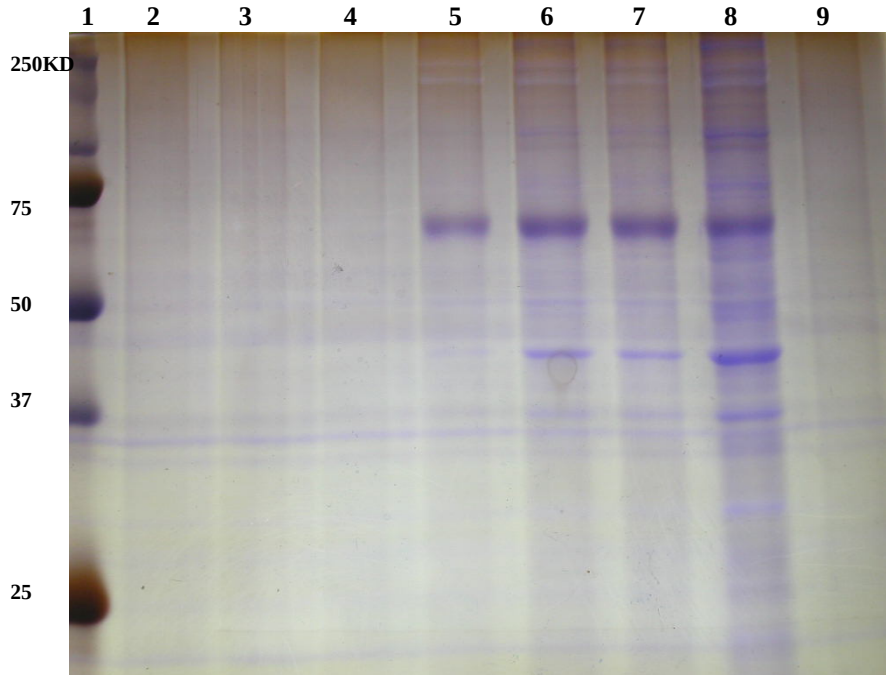

Figure 2. The band of stained gel indicated proteins of HCM and NCM. Each sample media is concentrated by trichloroacetic acid and stained by coomassie brilliant blue regent and silver staining regent. The lines are dual color protein standards marker (1), negative control (2-4), HCM; follow the time required hypoxic condition of MSCs (5-8), NCM; normoxic condition media of MSCs (9). The negative control lines are 5x loading dye (2), protein of normoxic serum free media (3) and degassing serum free media (4). The HCM lines were loaded protein sample incubated for 3 hr (5), 5 hr (6), 9 hr (7), and 12 hr (8) in hypoxic condition of MSCs. The NCM sample is media of incubated MSC in normoxic condition for 12 hr (9).

Table 2. HCM-only secreted proteins

Hypoxic CM(only)-secreted protein into EC	Score
52kD Ro/SSA autoantigen [Mus musculus]	10.14
annexin A2 [Mus musculus]	30.23
clusterin [Mus musculus]	30.20
follistatin-like 3 [Mus musculus]	20.12
high mobility group box 1 [Mus musculus]	48.20
lipocalin 2 [Mus musculus]	10.13
lysyl-tRNA synthetase [Mus musculus]	18.14
pre-B-cell colony-enhancing factor 1 [Mus musculus]	20.2
prohibitin [Mus musculus]	100.28

Table 3. HCM-high score proteins

High score in hypoxic CM-only (>50)	Score
adaptor protein complex AP-1, beta 1 subunit [Mus musculu]	444.2
adenylosuccinate synthetase 1 [Mus musculus]	600.33
ATP-binding cassette, sub-family A, member 4 [Mus musculus]	1148.30
capping protein (actin filament) muscle Z-line, alpha 2 [Mus musculus]	300.29
carbonyl reductase 1 [Mus musculus]	258.32
caspase 11 [Mus musculus]	270.31
chaperonin subunit 2 (beta) [Mus musculus]	258.28
chaperonin subunit 5 (epsilon) [Mus musculus]	250.33
creatine kinase, muscle [Mus musculus]	250.23
polymerase (RNA) II (DNA directed) polypeptide A [Mus musculus]	210.25
ribose 5-phosphate isomerase A [Mus musculus]	210.27
ribosomal protein S5 [Mus musculus]	208.30
ribosomal protein S8 [Mus musculus]	206.28
surfeit gene 6 [Mus musculus]	160.24
TAF6 RNA polymerase II, TATA box binding protein (TBP)-associated factor	158.29
thymoma viral proto-oncogene 1 [Mus musculus]	60.22
transcription factor AP-2, gamma [Mus musculus]	150.30
tripartite motif protein 27 [Mus musculus]	218.31
tripeptidyl peptidase II [Mus musculus]	140.27
tubulin, alpha 4 [Mus musculus]	140.25
tubulin, alpha 6 [Mus musculus]	140.25
ubiquitin specific protease 9, X chromosome [Mus musculus]	134.24
villin 1 [Mus musculus]	130.28
v-rel reticuloendotheliosis viral oncogene homolog A [Mus musculus]	226.27
zinc finger protein 92 [Mus musculus]	248.30

Table 4. HCM-only secreted protein as well as high score

Hypoxic CM (only)-all of secreted protein	Score
hepatoma-derived growth factor-related protein 2 [Mus musculus]	80.24
hydroxysteroid 11-beta dehydrogenase 2 [Mus musculus]	80.22
inhibin beta E [Mus musculus]	80.21
platelet-activating factor acetylhydrolase, isoform 1b, alpha1 subunit	100.30
pregnancy specific glycoprotein 17 [Mus musculus]	100.23
protein tyrosine phosphatase, non-receptor type substrate [Mus musculus]	346.30
recoverin [Mus musculus]	238.29
Rho-associated coiled-coil forming kinase 2 [Mus musculus]	210.30
secretogranin II [Mus musculus]	200.34
spermidine synthase [Mus musculus]	178.35
telomerase associated protein 1 [Mus musculus]	150.23
Tial1 cytotoxic granule-associated RNA binding protein-like 1 [Mus musculus]	148.35
transcription factor 2 [Mus musculus]	150.35
tripeptidyl peptidase I [Mus musculus]	50.28
ubiquitin-activating enzyme E1, Chr X [Mus musculus]	140.19
UDP-glucose dehydrogenase [Mus musculus]	138.32

2. Hypoxia conditioned media of MSCs protects cardiomyocytes exposed to hypoxia.

Present study hypothesized that the MSCs might release cyto-protective factors able to prevent cardiomyocytes loss. To test our hypothesis, we first studied the effects of HCM from cultured MSCs on the survival of cardiomyocytes subjected to hypoxia in vitro. The cardiomyocytes were exposed to hypoxic condition for 12 hours in degassing media, hypoxic conditioned media (HCM), or normoxic conditioned media (NCM) of MSCs. Also, cardiomyocytes maintained in control media (CTR-M) under normoxic conditions for 12 h were viably showed and exhibited their rhythmically contracting areas at 1 day after plating (Fig.3-A). Exposure of cardiomyocytes for 12 hours in hypoxia condition with degassing media resulted in 50% reduction in the number of survival cells (Fig.3-E). It is known that the transition of cardiomyocytes from the shape of typical volume to the shrinkaged shape coincides with ultra-structural alterations of either necrotic or apoptotic cell death (Fig.3-B)[37]. Exposure to NCM resulted in approximately 10% increase the survival number of cardiomyocytes comparison with hypoxia degassing media (Fig.3-D, E). In contrast, the HCM led to 1.6-folds increase in the number of cardiomyocytes compared with the hypoxia degassing media (Fig.3-E) and recovery of cell condition

(Fig.3-C). The results obtained that the presence of conditioned media of MSCs exposed to hypoxia were the most remarkable.

The activation of ERK1/2 plays an important role in mechanisms of cellular survival and proliferation through gene regulation. ERK1/2 is one of dual specificity kinases in MAPKs. We detected phosphorylation of ERK1/2 (42 and 44 kDa) by immunoblot assay. As shown in Fig. 4, the phosphorylation activities of ERK1/2 were decreased about 3.0-folds in hypoxic cardiomyocytes compared with normal cells. The effect of NCM in hypoxic cardiomyocytes scarcely indicated a visible change in ERK1/2 activity. But, treatment of hypoxia cells with HCM resulted in increase of 3.0-folds in ERK1/2 activities. The ERK1/2 activity of hypoxic cardiomyocytes in the presence of HCM is similar levels seen in normoxic control cells.

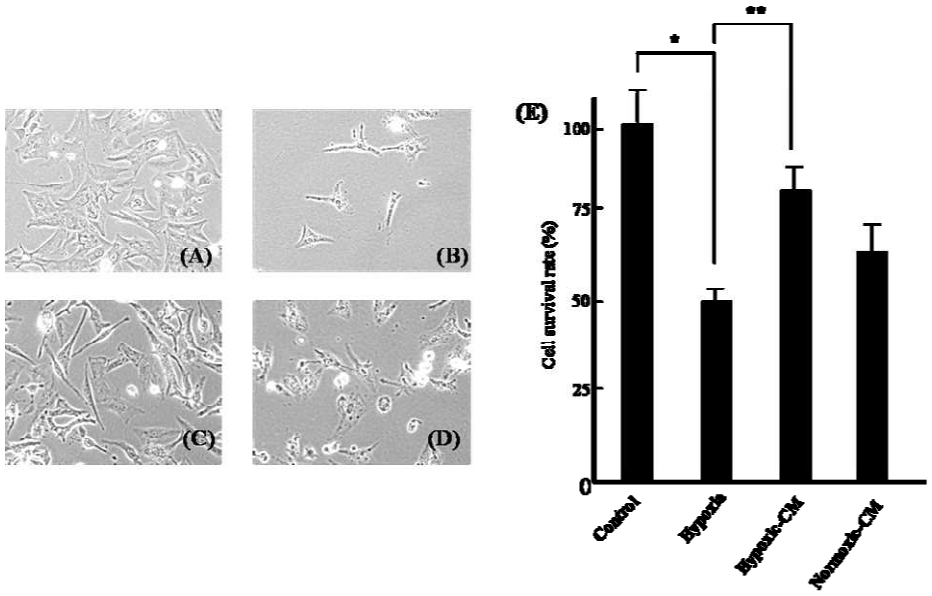

Figure 3. Effect of the MSC conditioned medium on survival of hypoxic-cardiomyocytes. (A) Representative microphotographs of cardiomyocytes grown in CTR-M under normoxic conditions for 12 hr. (B-D) Cardiomyocytes were incubated for 12 hr in hypoxia with CRT-M (B), HCM (C), and NCM (D). The each conditioned cardiomyocytes were washed twice by serum free DMEM and checked morphological character by microscopy. (E) Cardiomyocytes were plated in triplicate wells of 6 well plates at a density of $1*10^6$ per well, and put in a hypoxic and normoxic chamber for 12 hr. Cell viability was determined by trypan blue stain with hemocytometer chamber. (*$P < 0.01$ and **$P < 0.05$)

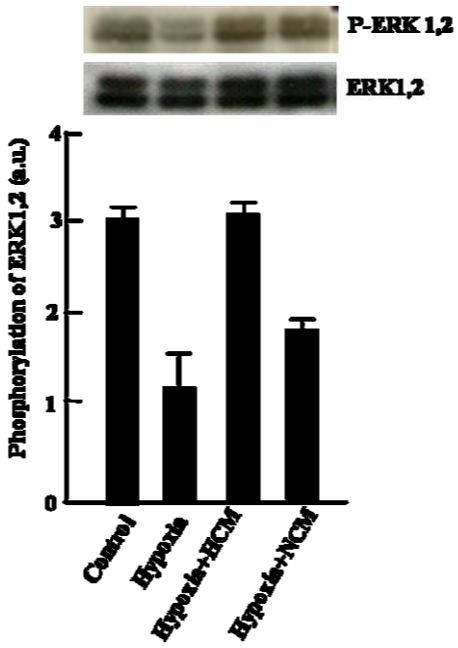

Figure 4. Effect of HCM on survival of hypoxic cardiomyocytes. Effect of HCM on activity of proteins related to survival of hypoxic cardiomyocytes. Immunoblot analysis of phosphorylation of ERK in cardiomyocytes exposed to a hypoxic chamber for 12 hr prior to exposure to HCM or NCM. At the indicated times, cells were harvested. Collected cells were solubilized in lysate buffer, supplemented with protease inhibitor cocktail. Equal amounts of protein were loaded onto and separated by 12% SDS–PAGE and transferred to membranes. Immunoblot analysis was repeated two times. Each signal was quantified by scanning densitometry.

3. Intra-myocardial injection with HCM of MSCs limits infarct size and improves ventricular function after acute MI

To examine the in vivo relevance of our in vitro findings, we studied the direct effects of the HCM on infarct size in LAD ligated rat hearts. On the basis of the in vitro results and to streamline the design of the in vivo experiments, we elected to use only media of MSCs exposed to hypoxia. Ligation of the LAD was performed in 8-week-old Sprague-Dawley male rats for infarcted myocardium. Concentrated HCM was inpoured at the intraperitoneal injection (IP) one time a day for 1 week after LAD occlusion. The effect of HCM on myocardial injury for 1 week after infarction was evaluated by trichrom staining (Fig.5). The infarction and fibrosis in the only ligated heart arose about 77.5% from left ventricle compared with other samples (Fig.5-B). In contrast, injection of concentrated HCM significantly limited infarct size about 22.3% of the ligated heart (Fig.5-C). These results were further confirmed by histopathological examination of stained sections.

In normal specimen, myocardium was well aligned and surrounded by myocardial fiber and little interstitial tissue (Fig.5-E). In contrast, section of ligated heart receiving no treatment indicated features unlike normal. The only ligated heart expressed a large increase of fibrous tissue and significant scarring occurs, and an area of relative thinning following the distribution of the occluded artery. Also, it was enlarged left ventricular cavity (Fig.5-F).

However, the specimen of HCM injected-ischemic myocardium decrease fibrosis in left ventricle and more thick than ligation control (Fig.5-G, H).

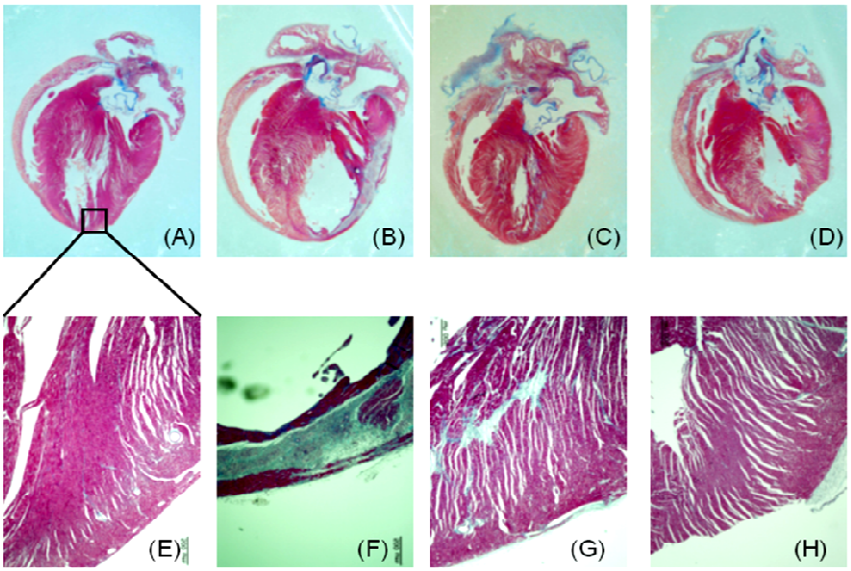

Figure 5. Effect of the concentrated HCM on infarct size. (A-H) The LAD of four hearts was ligated on an equal location. Concentrated HCM was inpoured at the intraperitoneal injection one time a day for 1 week after LAD occlusion. After 1 week, four hearts were isolated and cut lengthways. Then, the tissue samples were stained by trichrom staining. (A-D) Infarct size dimensions in the normal (A), ligated heart (B), and concentrated HCM-treated ligated heart (C, D) group respectively were observed wide difference between (B) and (C, D). (E-H) Left ventricle of each heart was magnified 100 times.

4. Effect of HCM on genes coding for Ca^{2+}-handling proteins of cardiomyocytes exposed to hypoxia in vitro

As Ca^{2+}-handling proteins, calreticulin and calmodulin are important regulators for ion homeostasis. While calmodulin is expressed interstitially, calreticulin is a calcium-binding chaperone of the sarcoplasmic reticulum. Compared with normoxic control cardiomyocytes, expression of the Ca^{2+}-handling protein carleticulin was increased about 13% in hypoxic cardiomyocytes. Treatment for the hypoxic cells with HCM induced a decrease in the expression of carleticulin. As shown in Figure 6, transcript patterns of the Ca^{2+}-handling protein calmodulin were totally different from carleticulin. Transcript levels of calmodulin decreased about 40% in hypoxic cells in normal cardiomyocytes, but the level of hypoxic cells treated with HCM was similar to normoxic control.

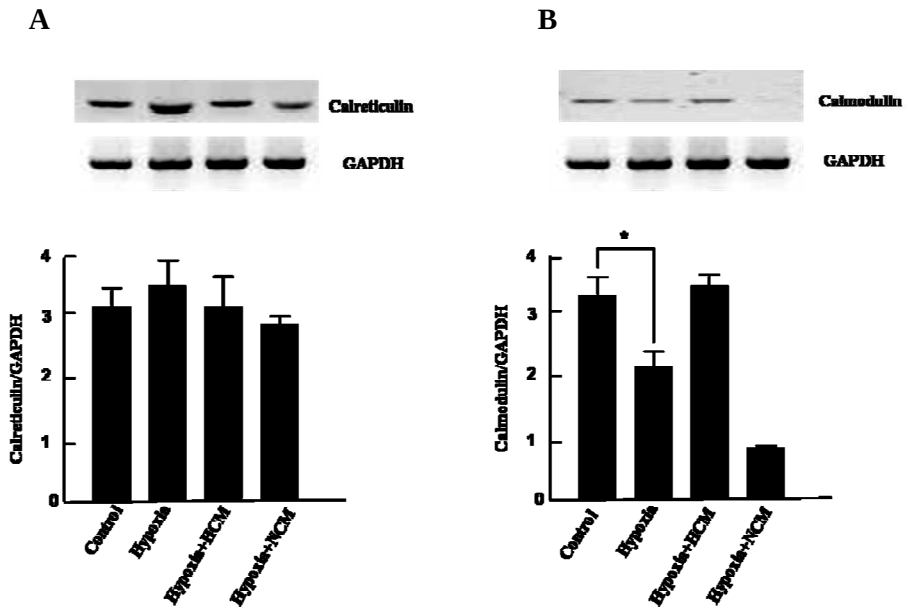

Figure 6. Effect of HCM on genes coding for Ca^{2+}-handling proteins of hypoxia cardiomyocytes. Cardiomyocytes treated with HCM or NCM was exposed to a hypoxic condition for 12 hr. The gene expression is (A) calreticulin and (B) calmodulin. The mRNA expression was established by separating amplification products by agarose gel electrophoresis and visualizing them by ethidium bromide staining. Each expression was quantified by scanning densitometer. Expression of the carleticulin was increased about 13% in hypoxic cardiomyocytes and calmodulin was decreased about 40% in hypoxic cells in normal cardiomyocytes. (*$P < 0.05$)

5. Effect of hypoxia conditioned media on genes coding for ion channels of cardiomyocytes exposed to hypoxia in vitro

Figure 7 displays representative examples of Ca^{2+} channel in hypoxic cardiomyocytes treated with or without HCM. Ca^{2+} entry through L-type Ca^{2+} channels into cardiaomyocytes is known to be the initiating event of the excitation–contraction coupling process. L-type Ca^{2+} channels are critically involved in excitation secretion coupling whereby membrane depolarization activate (open) L-type Ca^{2+} channels, allowing extracellular Ca^{2+} entry and stimulation of exocytosis. The density of L-type Ca^{2+} -channel was decresed about 53.3% in hypoxic condition compared with nomoxic control cells. After treatment of hypoxic cells with HCM, density of L-type Ca^{2+} -channel increased about 50%.

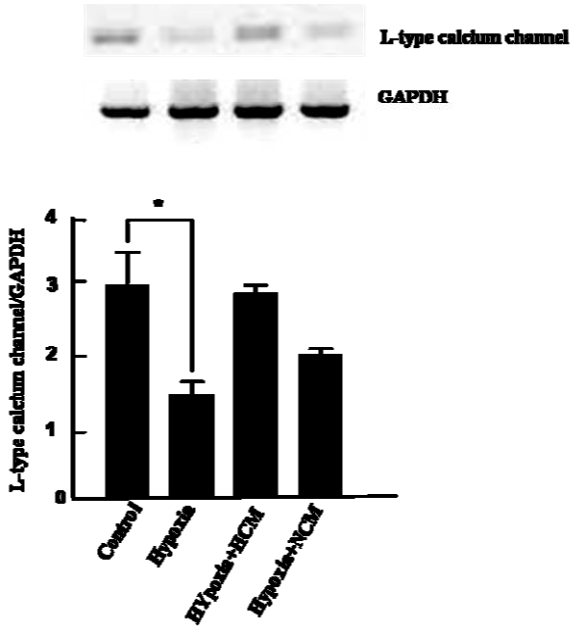

Figure 7. Effect of HCM on genes coding for ion channels of hypoxic cardiomyocytes. Cardiomyocytes treated with HCM or NCM was exposed to a hypoxic condition for 12 hr. The mRNA expression of genes was established by separating amplification products by agarose gel electrophoresis and visualizing them by ethidium bromide staining. Each expression was quantified by scanning densitometer. . The density of L-type Ca^{2+} -channel was decresed about 53.3% in hypoxic condition. After treatment of hypoxic cells with HCM, density of L-type Ca^{2+} -channel increased about 50%. ($^{*}P < 0.05$)

6. Effect of hypoxia conditioned media on genes coding for ion exchangers of cardiomyocytes exposed to hypoxia in vitro

The expression of ion exchangers differed from each other in hypoxic cardiomyocytes treated with or without HCM. Serca 2a gene expression in hypoxic cardiomyocytes treated with HCM increased about 73% compared with the absence of HCM and was the same as in normal cells. The transcript level of phospholamban was decresed about 47 % in hypoxic condition compared with nomoxic control cells. After treatment of hypoxic cells with HCM, level of phospholamban increased about 42%. Transcript levels of Na^+K^+ ATPase decreased about 36% in hypoxic cells compared with normoxic control, but the level of hypoxic cells treated with HCM was similar to normoxic control. Na^+/Ca^{2+} exchanger-1 transcription patterns formed a striking contrast to SERCA 2a. The treatment of hypoxic cardiomyocytes increased about 62.5% compared with normoxic control. But, the treated hypoxic cardiomyocytes with HCM decrease similar to normoxic control (Fig. 8).

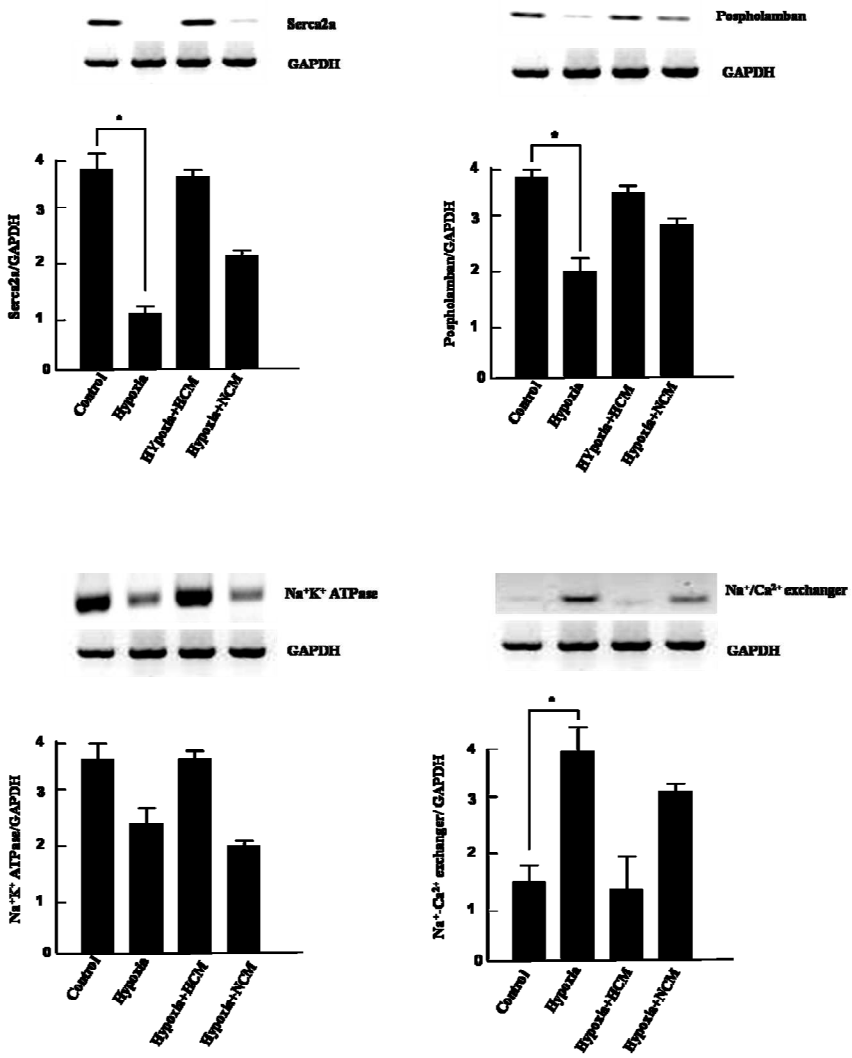

Figure 8. Effect of HCM on genes coding for ion exchangers of hypoxia cardiomyocytes. Cardiomyocytes treated with HCM or NCM was exposed to a hypoxic condition for 12 hr. The mRNA expression of genes was established by separating amplication products by agarose gel electrophoresis and visualizing them by ethidium bromide staining. Each expression was quantified by scanning densitometer. ($^{*}P < 0.05$)

IV. DISCUSSION

The different types of bone marrow-derived adult stem cells have been proposed as a potential source for cell therapy of acute myocardial infarction (MI) [5, 6, 7]. But, the mechanisms underlying these therapeutic effects have not been clearly defined and low post-implantation viability of the injected cells may limit the capacity of this mechanism to result in meaningful cardiac regeneration[15]. We concerned about paracrine effects by using secreted proteins from MSCs on ischemic myocytes. Previous studies showed that the injection of HCM is a superior strategy for rescuing tissue damage caused by acute IHD[16]. Here, we find out new evidence on the paracrine effect of action of HCM. Especially when exposed to hypoxia, MSCs released factors that exert beneficial effects on isolated cardiomyocytes in vitro (Fig. 2). Different hypoxia time of MSCs distinguished from density of protein band. The HCM was contained protein much more from MSCs as time goes by hypoxic condition. However, we could not detect protein band from NCM of MSCs. The proteins of the incubated MSCs in hypoxia condition for 12hr were detected the highest density of other condition (Fig. 2). To complement data for secreted protein patterns, we analyzed the media of MSCs exposed to hypoxia condition for 12hr. As a result, we found out various proteins and

divided protein list into three classes (Table 2, 3, 4). But, we didn't clarify correct function of secreted proteins.

The treatment of HCM in hypoxic cardiomyocytes improved cellular survival and reduced the damage caused by Ca^{2+} overload in hypoxic condition. We attempted to elucidate whether HCM has a stimulatory effect on survival of hypoxic cardiomyocytes (Fig. 3). Under pathological conditions of hypoxia, enhanced survival of neonatal rat ventricular cardiomyocytes was related to increase activation of ERK1/2 (Fig. 4). The ERK1/2, major members of the MAPK family, have been implicated in survival signaling in response to ischemia-reperfusion, oxidative stress, hypoxia and -adrenergic stimulation. In many systems, activation of ERK provides potent pro-survival signals (Fig. 4). Also, injection of HCM containing these putative substances significantly improves left ventricular condition in vivo. The infarct size in the ligation heart excessively arose from LV compared with other samples (Fig.5-B). In contrast, injection of concentrated HCM significantly limited infarct size of the LV (Fig.5-C). The in vivo data demonstrate that our observation on the cyto-protective properties of the HCM[21] and support the concept that the effect observed after injection of HCM into IHD are to a great extent attributable to paracrine protection and functional recovery of ischemic myocardium.

In general, myocardial ischemia initiates a range of cellular events that are initially mild and influenced progressive damage with increasing in ischemia condition. Ischemia depletes adenosine triphosphate (ATP), which inhibits ATP-driven Na^+-K^+ pumps. Increased $[H^+]_i$ enhances Na^+-H^+ exchange, leading to increased $[Na^+]_i$, and the associated augmentation of $[Ca^{2+}]_i$ via Na^+-Ca^{2+} exchange.

There is accumulating evidence that Ca^{2+}-induced Ca^{2+} release process governs cardiac contractility is defective in IHD, but the molecular mechanisms remain elusive. To examine the intermolecular aspects of Ca^{2+}-induced Ca^{2+} release process during IHD, we utilized RT-PCR to visualize the expression of variety cytoplasm Ca^{2+} channels in ischemic cardiomyocyte and recovery by transfusing HCM in ischemic myocyte [20]. Proteins involved in calcium removal from the cytosol were significantly altered in the IHD.

First, we evaluated the expression of genes coding for calcium handling proteins, cardiac ion channels and ion exchangers in hypoxic cardiomyocytes treated with or without HCM. As Ca^{2+}-handling proteins, calreticulin and calmodulin are important regulators for ion homeostasis. While calmodulin is expressed interstitially, calreticulin is a calcium-binding chaperone of the sarcoplasmic reticulum[23]. Overexpression of calreticulin

leads to induction of oxidant-induced $[Ca^{2+}]_I$ increase and results in apoptosis (Fig. 6). Similarly, generation of hypoxic stress in the cardiomyocytes increased expression of the calreticulin gene, but treatment with HCM decreased calreticulin expression levels. Previous studies have shown that expression level of calmodulin is reduced in human IHD, and we found that calmodulin was decreased in hypoxia myocytes. Our results indicate that treatment with HCM restores calmodulin expression levels [24] (Fig. 6).

Little is known about expression of various ion channels in hypoxic cardiomyocytes. L-type Ca^{2+} channels are critically involved in excitation secretion coupling whereby membrane depolarization activate (open) L-type Ca^{2+} channels, allowing extracellular Ca^{2+} entry and stimulation of exocytosis. The L-type Ca^{2+} channels were significantly lower in the hypoxia cardiomyocytes compared to controls but treatment of HCM restores expression levels (Fig. 7). Also, SR-Ca^{2+}-ATPase, relevant for removal of calcium from the cytosol into the lumen of the sarcoplasmic reticulum, was decreased in IHD. SERCA 2a expression in our experiments demonstrated that HCM was cardioprotective because decreased SERCA 2a expression levels in IHD returned to the same level as in normal cells with HCM. Phospholamban, which inhibits the SR-Ca^{2+}-ATPase in the basal

unphosphorylated state, was slightly decreased [22]. But, HCM treatment restored the level of Phospholamban. The sarcolemmal Na^{+}-Ca^{2+}-exchanger, relevant for transsarcolemmal calcium extrusion was increased in the hypoxia cardiomyocytes (Fig. 8). Na^{+}-K^{+}-ATPase provides energy for calcium transfer at the external membrane level. The activity of the Na^{+}-K^{+}-ATPase is reduced in the hypoxia cardiomyocyte. Alike, disturbed ion exchanger in IHD was recovered former condition by treatment of HCM.

In this study, we hypothesized that HCM of MSCs exert paracrine effect on the ischemic cardiomyocytes by including biologically active factors. To prove our hypothesis, we tested the cyto-protective effect of HCM from cultured MSCs on isolated neonatal rat ventricular cardiomyocytes exposed to hypoxia in vitro and on rat hearts after experimental MI in vivo. Also, we assessed the contraction characteristics of cardiomyocytes exposed to hypoxia in the presence of HCM. Our current results provide evidence that MSCs secrete paracrine-acting agents with cardio-protective and/or inotropic properties that can rescue ischemic cardiomyocytes and improve ventricular function.

Although beyond the scope of the current work, a proteomic and functional approach with the aim to elucidate all the factors and pathways involved in the therapeutic effects conferred by the conditioned medium is a high priority

considering the biological and therapeutic implications of our discovery.

Ⅴ. CONCLUSION

Our study suggests that HCM exert direct salutary effects on ischemic cardiomyocytes via paracrine mediators. The HCM was produced from MSCs exposed to hypoxia for 12hr. The therapeutic benefits of HCM appear to be attributable primarily to diffusible factors released by the cells that, acting in a paracrine fashion, limit infarct size, and improve cell survival rate. In other word, exposure of the cardiomyocytes to hypoxia with HCM increased cell proliferation rate compared with ischemia cardiomyocytes, as measured by the trypan blue cell counting. Also, ERK1/2 decline is observed during ischemia in heart. But, ERK 1/2 of ischemia cardiomyocytes in the presence of HCM increased comparison to ERK 1/2 of ischemia cardiomyocytes. Also, when injected into infarcted hearts, the HCM significantly limits infarct size and improves calcium channel function relative to controls.

The future identification of the exact nature and mechanism of action of the secreted factors may have important implications on the development of novel molecular therapies for the prevention of ischemic tissue damage.

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Ⅶ. ABSTRACT (in Korean)

간엽 줄기 세포의 배양액을 이용한 저 산소증 심근세포의 보호 효과

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이 선 주

허혈은 심근에 치명적인 재생 불가능한 손상을 일으키며, 이러한 경색된 부위의 심장 기능은 정상적으로 복원 되지 않는다. 심근 상처 조직 또는 손상된 심장에 줄기세포를 이식하는 방법은 심실의 기능을 복구 시킬 수 있는 치료법이 될 수 있다고 제안 되어왔고, 줄기 세포 중 성체줄기세포의 간엽 줄기 세포는 비 조혈 조직인 신경세포, 골격근육모세포, 심장 근육 세포 등 많은 조직으로 분화 될 수 있는 능력을 가지고 있다고 보고되었다. 이러한 특성을 이용하여 간엽 줄기 세포를 유사 심근 세포 (cardiac-like)로 분화시키기 위해 여러

연구가 수행되어 왔지만 여전히 미흡한 상태이며, 최근 들어서 줄기세포가 저산소증과 같은 특정상황에서 분비시키는 인자들에 의한 주변분비 효과가 저산소증 심근세포의 생육에 효과적으로 작용하여 심근의 기능을 개선시킨다는 새로운 이론이 밝혀지고 있다.

본 연구에서는 간엽 줄기 세포가 저 산소 성 상태에 있을 때 방출된 인자들을 함유한 배지(hypoxia conditioned media)를 이용하여 저 산소 증 심근 세포에 처리된 군과 처리 되지 않은 군을 비교하였다. 우선, 심근 세포에서 수축-이완의 기능을 조절하는데 중요하게 다루어지는 칼슘 통로의 발현 정도를 비교 함으로서 간엽 줄기 세포의 hypoxia conditioned media 가 저산소증 심근 세포의 칼슘 항상성을 향상시킴을 증명하였다. 또한 세포 분화와 관련된 신호 기작을 비교 대상으로 하여 hypoxia conditioned media 의 유무에 따른 저산소증 심근세포를 비교 분석한 결과, 간엽 줄기 세포로부터 분비된 인자가 저 산소 증 심근 세포의 분화를 활성화 시킨다는 결과를 얻을 수 있었다.

즉, 심근 세포에 허혈 상태가 유도 되었을 때 비정상적인 칼슘 통로 발현이 일어나며 세포 내 칼슘 항상성에 이상이 발생하여 과부하가 일어난다. 결국 심근 내 혈류 속도의 감소,

압력 과부하로 인한 심 부전 및 심 비대가 발생하게 되고, 이러한 현상에서 간엽 줄기 세포의 저 산소 성 상태의 배지를 투여하여 주변분비 효과를 유도, 이 는 심근 기능을 향상시키는 역할을 수행하였음을 밝혀 내었다.

핵심 되는 말: 골수 간엽 줄기 세포, 주변 분비 효과, 저 산소 성 심근세포