Role of reactive oxygen species in adhesion of mesenchymal stem cells

Minji Cha

Department of Medical Science

The Graduate School, Yonsei University

Role of reactive oxygen species in adhesion of mesenchymal stem cells

Directed by Professor Yangsoo Jang

The Master's Thesis submitted to the

Department of Medical Science,

the Graduate School of Yonsei University

in partial fulfillment of the requirements for the

degree of Master of Medical Science

Minji Cha

December 2008

This certifies that the Master's Thesis of Minji Cha is approved.

Thesis Supervisor: Yangsoo Jang

Thesis committee Member #1: Ki-Chul Hwang

Thesis committee Member #2: Dong-Wook Kim

The Graduate School

Yonsei University

December 2008

Acknowledgements

I would like to express my gratitude to all those who gave me the possibility to complete this thesis.

I especially want to thank my advisor, Dr. Yangsoo Jang and Dr. Dong-Wook Kim, for their guidance of the manuscript and deeply appreciate to Dr. Ki-Chul Hwang whose help, stimulating suggestion and encouragement helped me in all the time.
The generous support from Yonsei cardiovascular research institute is greatly appreciated. I want to thank Dr. Soyeon Lim, Woochul Chang, Byeong-Wook Song, Sun Ju Lee and Hye-Jung Kim for all their advice, support, interest and valuable hints from the heart. I also thank my colleagues Eunju Choi, Onju Ham and Chang-Yeon Lee for their efforts.

On a personal note I would like to thank my family and friends. In particular, I would like to express my sincere thanks to my father, mother and brother who constantly provided emotional support and took care of me in many aspects. I wish to thank my shining friends Ja-Young Choi, Bori Chun, Jueun Jeong, Eun-Hye Hwang, Youngkwan Seol, Won-Goo Lee, Sahng-Youn Hwang, members of HTTP from To-pyeong church of Seventh - day Adventist and companion of Chuncheon ACT, having gone through happiness and difficulty together.

Finally, I want to thank my lovely teachers, Mr. and Mrs. Lee, Mr. and Mrs. Shin, and respected Pastors for their prayers and supports.

Last but not least, I really thank my God. Must your name be exalted, honored, and glorified.

December, 2008

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ABSTRACT

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Stem cell therapy for repair of myocardial injury has inherent limitations due to the poor attachment of cells after cell transplantation. In ischemic surroundings after myocardial infarction (MI) and interaction of mesenchymal

stem cells (MSCs) with niche is associated with increased production of reactive oxygen species (ROS). The intracellular ROS plays a key role in the regulation of cell adhesion, migration, and proliferation. This study was designed to investigate the role of ROS on MSC - adhesion. After treatment of H_2O_2 (50 μM) on MSCs, detachment of MSCs was dose-dependently increased, but cell detachment was inhibited by pretreatment with the free radical scavenger, N-acetyl-L-cysteine (NAC, 2 mM). The H_2O_2 treated MSCs were detached on 3D-matrix gel about 2 - fold, compared with fibronectin coated plate. Intracellular ROS production was enhanced about 70.0% in H_2O_2-treated cells compared with non-treated cells and phosphorylation of FAK (77.1 ± 2.8%) and Src (62.2 ± 4.3%) were decreased. Expression level of paxillin and vinculin was reduced to 36.2 ± 4.8% and 29.6 ± 2.6%, respectively in H_2O_2-treated MSCs. Expression of integrin-related adhesion molecules was also depressed; integrin αV (60.0%), integrin β1 (79.4%), and fibronectin (32.5%) in MSCs treated with H_2O_2. The transcript level of Rac-1 was decreased about 75.4% in H_2O_2-treated cells compared with non-treated cells. These results indicate that ROS suppress the cell adhesion and downstream signaling molecules in the regulation of cell

adhesion. These results also contribute to a better understanding of the transplantation of MSCs on infarcted heart.

Key words: mesenchymal stem cells, reactive oxygen species

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I. INTRODUCTION

Ischemic heart disease - distinguished by reduced blood supply to the heart muscle - is the primary cause of death throughout the world[1]. Obstruction of coronary arteries leads to myocardial infarction (heart attack). 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].

A kind of various therapies for heart failure that addresses the fundamental problem of cardiomyocytes loss is cardiac transplantation. Cardiac transplantation has been emerging as a potentially novel therapy for regenerative medicine. New inventions on the regenerative potential of stem cells and progenitor cells for treating and preventing heart failure have transformed experimental research and led to an explosion in clinical investigation[2]. Clinical and basic studies of cell-based myocardial therapy developed at a rapid pace recently. While multiple studies have shown improvements in parameters of myocardial damage and function following mesenchymal stem cells (MSCs) delivery after myocardial infarction, the mechanisms underlying these phenomenon remain obscure[3,4]. MSCs transplantation has been proposed as a promising means for the repair and regeneration of cardiomyocytes and restoration of heart function[5-7].

Under appropriate stimulation, MSCs can be induced to differentiate into myocytes, adipocytes, osteoblasts, chondrocytes, tenocytes, and hematopoietic-supporting stroma[8]. Some researchers presumed several

mechanisms about the successful cardiac protection of MSCs after transplantation, including assist in matrix reorganization; differentiation of the engrafted MSCs into cardiomyocytes; fusion of the administered cells with the existing heart cells; stimulation of endogenous cardiac stem cell niches by injected cells[9-12]. However, one of the major challenges to successful stem cell therapy is the difficulty of cell survival and differentiation in the harsh microenvironment of diseased tissues or organs. Most of engrafted cells died within four days after grafting into injured hearts[3, 13].

Multiple mechanisms could contribute to the death of grafted cells, including hypoxic, nutrient deprivation, inflammatory environment after myocardial infarction, and loss of survival signal in cells. Although the therapeutic effect of MSCs transplantation, the cells are exposed to an extremely harsh, proapoptotic microenvironment in the infracted heart as they are transplanted to recipients. Self-renewal and differentiation of MSCs are regulated by interactions between them and the microenvironment which is designated as the niche. This may require prosurvival strategies to improve stem cell survival/number in the infarcted heart[14]. Although prosurvival strategies have been proven to be successful in vitro, they actually may not solve the

problems of poor adhesion of MSCs[15]. These results show that oxidative stress affects cell-matrix but not cell-cell adhesion as part of proximal cell response to ischemia. Recently, improving MSCs survival after transplantation with effective adhesion attracts much attention[16].

Left ventricular (LV) hypertrophy and dysfunction after myocardial infarction (MI) is associated with increased production of reactive oxygen species (ROS) and depressed antioxidant reserves, suggesting that oxidative stress might contribute to ventricular remodeling and development of heart failure[17]. ROS generated during prolonged ischemia is known to contribute to ischemic injury.

In cardiac cells, mitochondria are the major source of ROS, although intracellular NADPH oxidase, xanthine oxidase, monoamine oxidase, etc, may become important ROS sources under pathophysiological conditions[18]. ROS, such as superoxide (·O2-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH·), and others, are constantly produced during normal metabolism and in response to external stimuli. At various concentrations and following oxidative stress situations, ROS will damage proteins, lipids, and DNA and may induce apoptosis. The role of ROS in cell adhesion has not been studied.

Recent evidence suggests that disruption of integrin contact in fibroblasts can lead to cell detachment that is preceded by a rise in intracellular ROS levels[19]. In contrast, after stimulation by various external factors including growth factors, hormones and cytokines or cellular events such as adhesion, cells will produce low local amounts of ROS [19-21]. Experiments with isolated cardiac myocytes have demonstrated that hypoxia increases ROS production and implicate mitochondria as the major source. ROS are known to damage electron transport complexes, leading to further increases in ROS production, further respiratory dysfunction[18].

The members of the Rho family of small GTPases are key regulators of the actin cytoskeleton. Activation of Rac-1 is associated with increased cell adhesion, and a guanine nucleotide-exchange factor for Rac-1, Tiam-1, promotes cell-cell adhesion. In contrast, activation of Rac-1 is also associated with disassembly of cell contacts[22].

Integrin signaling involves a vast number of kinases, phosphatases, GTPases, and transcription factors or transcriptional modulators. It is highly related that altered intracellular ROS levels have an effect on integrin-mediated signaling and also an effect on cell adhesion, migration, differentiation, and survival[19, 23,

[24].

Loss of cell-extracellular matrix (ECM) contacts results in cell death by apoptosis, a phenomenon known as detachment induced apoptosis[25]. Focal adhesion sites are specific areas on the cell membrane where cells attach to ECM. They are complexes of structural and signaling proteins, anchoring actin filaments and microtubules to the plasma membrane where integrins locate[26]. Most integrin β-subunits interact with proteins, such as paxillin, talin, vinculin, and other focal adhesion proteins, which act as linkers between integrins and the actin cytoskeleton[27-29]. It has been known that key players in integrin-mediated signal transduction are a group of integrin-associated nonreceptor kinases, two of them being focal adhesion kinase (FAK) and integrin-linked kinase (ILK) [25, 29-31]. Integrins are essential for cell migration and invasion because they mediate adhesion of cells to the ECM and regulate intracellular signaling pathways that control cytoskeletal organization, force generation, and survival[32]. Activated integrins bind to the ECM, cluster at the binding site, and initiate focal adhesions by recruiting cytoplasmic proteins, such as FAK, Src, and paxillin[34]. Integrins also activate small GTPase, which in turn activates downstream effectors molecules, thereby leading to

rearrangement of actin stress fibers and activation of cell adhesion and spreading[35].

In this report, we demonstrate that detachment of MSCs results in a rise of ROS levels in Ischemic condition. To prove my hypothesis, it was tested the separative effect of ROS from MSCs exposed to H_2O_2 in vitro. Most importantly, it was determined the adherent characteristics of MSCs exposed to H_2O_2 in the presence of scavenger. These results show that ROS prevent MSCs with adhesion-related proteins that can prevent positive effects of MSCs after transplantation. This finding may represent an important breakthrough in various therapies for heart failure in stem cell transplantation.

II. MATERIALS AND METHODS

1. Metrials

A. Reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were from Gibco (Gibco BRL, Grand Island, USA). Antibodies of immunoblot analysis were used as follows: Focal adhesion kinases (FAK) and phospho-FAK were purchased from Santa Cruz Biotechnology (Santa Cruz, 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). For PCR, oligonucleotide synthesized from Bioneer (Bioneer, Korea), RNA was extracted by Chloroform and 2-Propanol (Sigma-Aldrich, USA), reverse transcription for cDNA synthesis used to RT-&GO^{TM} (MP biomedicals, Solon, USA), and polymerase chain reaction (PCR) experimented with Real-Hi DNA Polymerase, 10 mM dNTP mix and 10 X reaction buffer (RBC, Taiwan). N-acetyl-l-cysteine was purchased from Sigma (St. Louis, MO, USA).

B. Animals

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

2. Methods

A. Cell culture

(A) Isolation and primary culture MSCs

Isolation and primary culture of Rat bone-marrow MSCs from the femoral and tibial bones of donor rats were performed. After induction of anesthesia with 10 mg/kg Zoletil (Virbac Lab, France) and 5 mg/kg Rompun (Bayer, Korea) by intramuscular route was done, bones of rat leg were branched off. MSCs were harvested from one-month-old (100 ~ 150 g) 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 DMEM-low glucose supplemented with 10% FBS (Gibco BRL, USA). Flushed medium were

centrifuged at 1600 rpm, 5 minutes and resuspended in serum-supplemented medium, next loaded to 4 ml ficoll (Amersham biosciences, USA) density gradient centrifugation per 3 rats at 1600 rpm, 30 minutes. Mononuclear cells recovered from middle interface of ficoll-separated bone marrow and blood were washed twice and resuspended in PBS (Gibco BRL, USA). And they were suspended in fresh DMEM by adding 10% FBS, 100 U/ml penicillin/streptomycin and plated at 1 rat/100 mm plate. Cells were maintained at 37℃ humidified atmosphere containing 5% CO_2 chamber. After 72 hours, non-adherent cells were discarded, and fresh medium was added and replaced every 3 or 4 days for about 10 days. Upon attaining 80 ~ 90% measurement, primary MSCs were trypsinized (Gibco BRL, USA) and accompanied sub-culture.

(B) MSCs characterization

Immunocytochemical characterization of MSCs was demonstrated below. Cells were cultured in 4-well slide chamber, washed with PBS and incubated in 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 hour (blocking solution: 2% bovine serum albumin, 10% horse serum in PBS) and adhered with the following antibodies: CD34, CD71, CD90, CD106, and intracellular adhesion molecule (ICAM)-1. FITC-conjugated mouse, rabbit and goat (Jackson Immun. Lab, USA) 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, CD105, CD106, 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, USA) using CellQuest™ software (Becton Dickinson, USA) with 10,000 events recorded for each sample.

B. Cell viability

(A) Cell counting by trypan blue stain

Following the indicated periods of incubation with H_2O_2 dose-dependently, the percentage of cell death was determined by trypan blue exclusion assay. Briefly, the detached cells in culture media were harvested by centrifuging the media and the attached cells on culture dish were harvested by trypsinization and centrifugation. Then, the detached cells as well as the attached cells of each treatment were washed with PBS and pooled together for viability assay. Equal (100 μl) volumes of cell suspension and trypan blue solution were mixed together and the cells were counted using hemocytometer under light microscope.

C. Preparation of cardiac fibroblast-derived three-dimensional matrix (Cardiogel)

Cardiogel was prepared with a minor modification[37]. Briefly, 2 x 10^5 cells per 35 -mm dish were seeded and the medium changed every 48 hours until the matrix was denuded of cells. The medium was carefully aspirated and rinsed gently with PBS. Next, 1 ml of pre-warmed extraction buffer (0.5%

Triton X-100, 20 mM NH_4OH in PBS) was added, and the process of cell lysis was observed using an inverted microscope until no intact cells were visualized. The cellular debris was washed with PBS, and the matrices were incubated at 37°C for 30 minutes with 1 ml of DNase (10 units of DNase per milliliter of PBS) to minimize the DNA debris. The matrix coated plates were covered with a minimum of 3 ml of PBS containing 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml Fungizone for use afterward.

D. Assays for cell adhesion

Four-well plates (Nunc, USA) were coated with Fn (fibronectin; Sigma, USA) for 24 hours at 4℃ or cardiogel. Fn was dissolved in PBS (pH 7.4) to yield a final concentration of 10μg/ml, and a volume of 150 μl was added to the individual wells. The plates were then blocked with 10mg/ml BSA (Sigma, USA) in PBS for 1 hour at 37℃. As a Pro-antioxidant treatment, NAC 2 mM (sigma, USA) for 30 min during H_2O_2 treatment was used. As an oxidant treatment, H_2O_2 (Merck, USA) 50 μM for 30 min was used. Suspensions of $2x10^4$ viable MSCs were then added to each well and allowed to attach for 5, 15, 30, 60, 120 minutes at 37℃ and 5% CO_2. To determine MSC adhesion,

plates were carefully washed three times with PBS, and then four separate fields were photographed by phase contrast microscope. The number of attached cells was estimated by microscopic cell counting. Each experiment was performed in triplicate wells and repeated at least three times.

E. Measurement of ROS production

Intracellular ROS were measured by a fluorescent dye technique. MEFs cultured on glass coverslips were treated for 10 min with 10 μM 2',7'-dichlorofluorescin diacetate (H_2DCFDA; Molecular Probes, USA) in PBS. The probe H_2DCFDA (10 μM) enters the cell, and the acetate group on H_2DCFDA is cleaved by cellular esterases, trapping the non-fluorescent 2', 7'-dichlorofluorescin (DCFH) inside. Subsequent oxidation by reactive oxygen species yields the fluorescent product DCF. The coverslips were placed in the chamber, which was mounted on the stage of an inverted microscope (Axiovert; Carl Zeiss, German) equipped with a confocal laser-scanning system (Oz; Noran Instruments, Middleton, WI). The dye, when exposed to an excitation wavelength of 480 nm, emits light at 535 nm only when it has been oxidized. Labeled cells were examined using a luminescence

spectrophotometer for the oxidized dye. Fluorescence images were collected using a confocal microscope (Leica, Solms, Germany) by excitation with a 488 nm and emission greater than 500 nm with a long-pass barrier filter.

F. RT-PCR analysis

(A) Isolation of total RNA

Total RNA was extracted by 500 μl/ 60 mm plate Tri-reagent (Sigma, USA). Poured (100 μl) chloroform above Tri-reagent and voltexing a sample about 10 seconds. Then, sample was centrifuged at 12000 g, 4℃ for 15 minutes. 3 layers were appeared in the tube, supernatant 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, USA) 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 OD_{260}/OD_{280} with DU 640

spectrophotometer (Effendorf, Germany)

(B) cDNA synthesis

Complementary DNA (cDNA) was synthesized with RT-&GO™. Quantitative 1 μg total RNA was added to 1 μl anchored primer $(dT)_{25}V$, 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 OD_{260}/OD_{280} 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[27]. 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).

Table 1. PCR primers used in this study

Gene	primer	Size (bp)	Temp. (℃)
Integrin β1	gcc agt gtc acc tgg aaa at tcg tcc att ttc tcc tgt cc	344	48.6
Integrin αV	gga aca acg aag cct tag ca tga tca ggg ctt gaa act cc	508	44
Fibronectin	cct taa gcc ttc tgc tct gg cgg caa aag aaa gca gaa ct	300	48.4
GAPDH	acc aca gtc cat gcc atc ac tcc acc acc ctg ttg ctg	450	49

G. Immunocytochemistry

Immunocytochemistry methods were same as MSCs characterization. Primary antibody was MHC (1:50) and secondary antibody was FITC-conjugated goat-anti rabbit (1:500). Then, they have put the cover slide, and

were detected confocal microscopy (Carl Zeiss, Germany).

H. Western blot analysis

60 mm plate of 80~90% filled with MSCs treated with H_2O_2 was scrapped by scrapper (Sigma, USA) and centrifuged at 12000 rpm, 4℃ for 3 minutes. Gathered cells was washed in PBS and lysed in 1 X cell lysis buffer (10 X cell lysis buffer; Promega and 1 mM PMSF, Sigma, USA) in 4℃ for 20 minutes. After lysate was centrifuged at 12000 rpm, 4℃ and 10 minutes, supernatant have been collected. The protein concentration was detected by BCA protein Assay reagent (Pierce Biotechnology, Rockford, IL, USA). Like detected value, sample was prepared to same amount. With 5 X dye (0.225 M Tris·Cl, pH 6.8, 50 % glycerol, 5 % SDS, 0.05 % bromophenol blue, 0.25 M DTT), quantitative protein sample was denatured at 95~100℃ heat block for 5 minutes. Quickly, stick the sample in ice for 3 minutes. Then, it was loaded into 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and taken down separating (80 V, 30 minutes) and running (120 V, about 1 hour) gel. Protein of running gel was transferred into membrane (Millipore, Bedford, MA, USA), and it was blocked in blocking buffer (10%

skim milk in TBS-t; tween 20; Becton, MD, USA) for 1 hour. The membrane was incubated with primary antibodies to FAK, Rac-1, and Src, P38 MAPK, vinculin, paxillin, phospho-FAK, phospho-P38 MAPK, phospho-Src (1:1000 diluted in 2% BSA) and β-actin (1:4000) overnight at 4℃. Washed PBS-t 3 times, and then incubated in secondary antibodies to HRP-conjugated rabbit and mouse for 1 hour. And it was washed TBS-t 4 times and visualized with enhanced chemiluminescence (ECL; Amersham biosciences, USA) reagent. Specific band was quantified using Phospho-Image System (Kodak, Rochester, NY, USA).

I. Image analysis

Quantitative image analysis was performed with an image analysis software (ImageJ).

J. Statistical analysis

Results are expressed as mean ±SEM. Statistical analysis as performed by student's t-test. Relationships were considered statistically significant when p

value was less than 0.05

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 (figure 1, A) and FACS analysis (figure 1, B). Cultured MSCs expressed CD71, CD90, CD105, CD106, and ICAM-1. They expressed neither the hematopoietic marker CD34 nor CD14 (Figure 1).

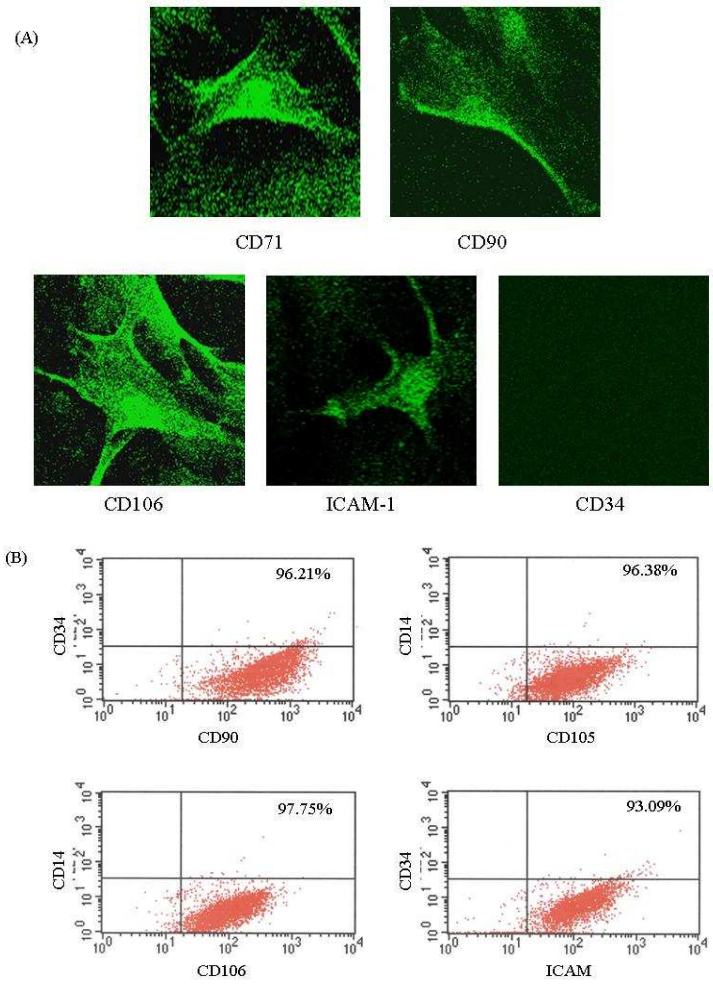

Figure 1. Characterization of isolated MSCs. MSCs were primarily cultured for 10 days. (A) MSCs transferred to a 4-well slide were executed before immunocytochemistry through a confocal microscopy was conducted. These cells were positive for CD71, CD90, CD106, and ICAM-1 but negative for CD34. (B) To confirm MSCs, cells were analyzed by CD14, CD34, CD90, CD105, CD106, and ICAM-1 by the FACS Caliber system. (Magnification: X400)

2. Detachment of MSCs by exogenous ROS

H_2O_2-induced cell death and the detach effect of MSCs causes cell death by necrosis and/or apoptosis in cultured MSCs[35]. We observed that cultured MSCs exposed to H_2O_2 under went dose-dependent cell detachment as monitored by trypan blue exclusion (Figure 2). To examine the effect of ROS on the H_2O_2-induced cell detach, MSCs were subjected to H_2O_2 for 48 hours. This led to a substantial decrease in adhesiveness.

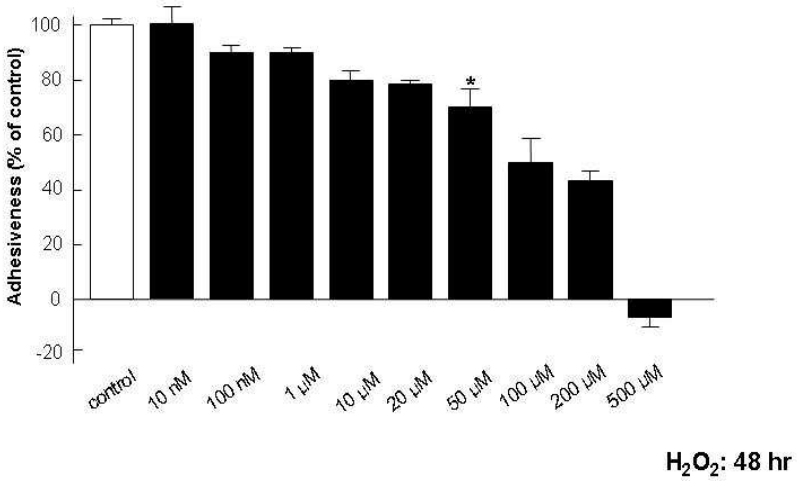

Figure 2. Effect of MSCs adhesion by dose dependent H_2O_2. Adhesive difference of cultured MSCs incubated under H_2O_2 treated conditions. MSCs were incubated in the incubator at 37°C for 48 hours. Cell viability was assessed by the trypan blue dye exclusion method. Data are averages of five independent experiments. * $P < 0.01$.

3. Rescue of cell detachment by scavenger in ROS-treated MSC

To examine the effect of ROS on the H_2O_2-induced cell detach, MSCs were pretreated with NAC and subjected to H_2O_2 for 48 hours. This led to a substantial increase in survival. As shown in Figure. 3, after H_2O_2 insult for 48 hours, the viability of MSCs in the NAC-treated group was increased, compared with the group without NAC. To examine the scavenging effect of NAC, MSCs were incubated with various concentrations of NAC for 30 min and exposed to H_2O_2. 2 mM NAC produced a significant increase in cell survival (Figure. 3). Since the best scavenging on appeared to be afforded by pretreatment with 2 mM NAC for 30 min. We used this pretreatment period in the following experiments.

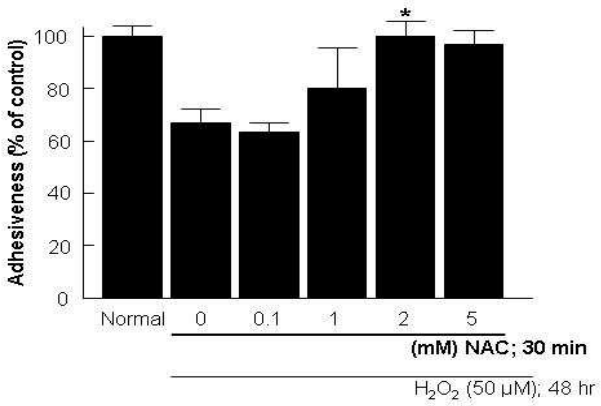

Figure 3. Effect of scavenger on adhesion of ROS-treated MSCs. MSCs were incubated with different concentrations (0−5 mM) of NAC for 30 min and subjected to H_2O_2. After 48 hours, adhesion rate was determined by trypan blue dye exclusion. The best scavenging on appeared to be afforded by pretreatment with 2 mM NAC. Data are averages of three independent experiments. * $P < 0.05$.

4. Effect of MSC adhesion on cardiogel by ROS

To determine the effect of the indicated substrates in adhesion of MSCs, it was performed quantitative adhesion assay with different culture condition. These distinctive in 3-D matrix adhesions differ in structure, localization, and function from classically described in 2-D adhesions. As shown in figure 4, the cell adhesion to cardiogel (3-D matrix) was significantly increased in normal MSCs as compared with adhesion to H_2O_2 treated MSCs. The adhesive difference was over doubling greater when prepared on cardiogel as compared with the Fn-coated plates (figure 5).

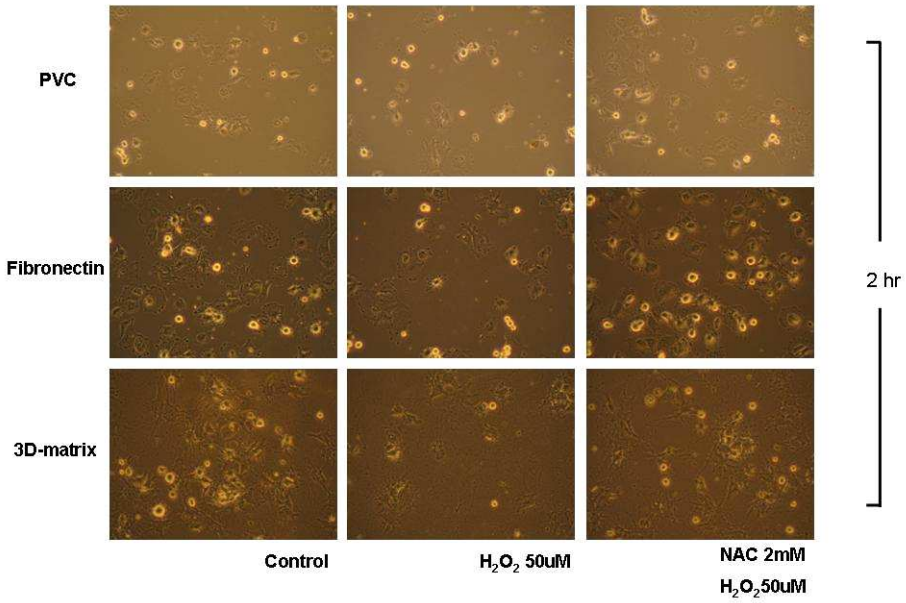

Figure 4. Adhesion rate of MSC on cardiogel by ROS. MSCs were plated on plastic, Fn-coated plate and cardiogel for 2 hours. Four separate fields were photographed by phase contrast microscope.

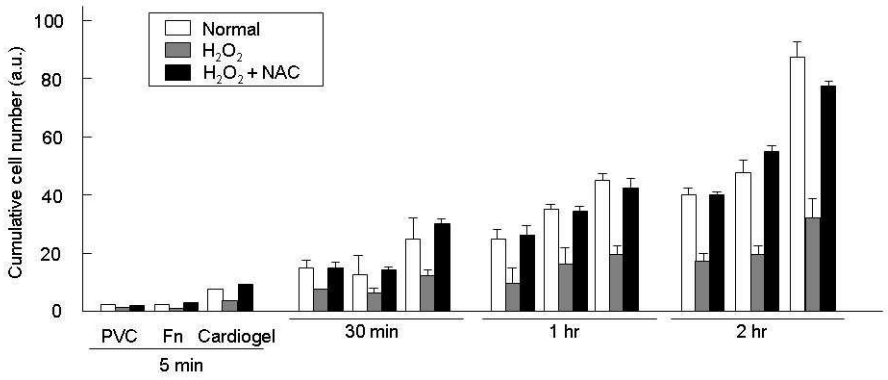

Figure 5. Time difference of MSC adhesion on cardiogel by ROS. The number of attached cells was estimated by microscopic cell counting. Above all, MSCs of 3D-matrix were best attached to the plate over 2 -fold as compared with the other plates. Data denote the means ±S.E.M. of 3 replicate measurements in three different cell cultures.

5. Expression of cell-matrix receptors on MSCs by ROS

To begin to address whether receptor mediated subtypes were involved in modulating the adhesion function of MSCs treated with H_2O_2, it was experimented below. Start from cell lysis with Tri-reagent, total RNA and cDNA was extracted. cDNA was measured by spectrophotometer. PCR with 1 μg cDNA was achieved in various temperature, DNA electrophoresis was accomplished, finally it was watched the expression level of receptor subtype mRNA. As a result, MSCs treated with H_2O_2 were more depressed than their counterpart 60.0% at Integrin αV and 32.5% at fibronectin. And the transcript level of Integrin β1 was decreased about 79.4% in H_2O_2 compared with control cells (Figure 6).

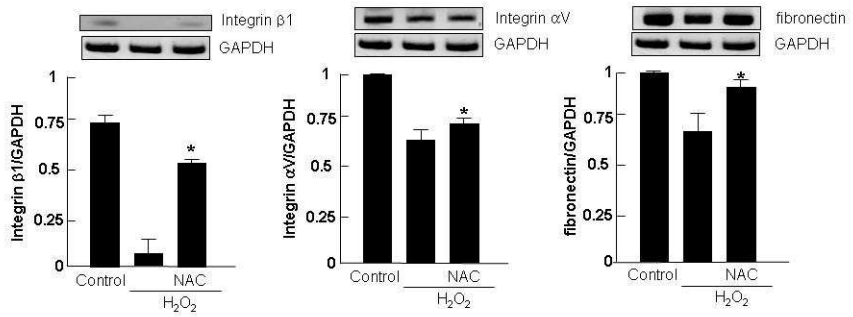

Figure 6. Effect of ROS on cell-matrix adhesion. The change of integrin-mediated cell adhesion in MSCs treated with H_2O_2. Each panel shows RT-PCR products for depression of integrin β1, integrin αV and fibronectin. Bar type of densitometry showed that MSCs treated with H_2O_2 were more depressed than their counterpart 60% at integrin αV and 32.5% at fibronectin were more depress than normal control. And the transcript level of integrin β1 was decreased about 79.4% in H_2O_2 compared with control cells. The mRNA expression of genes was established by DNA electrophoresis and visualized them by EtBr. Values are mean ± SEM. *P<0.01 vs controls.

6. Reactive oxygen species (ROS) production of H_2O_2-treated MSCs

Sustained activation of H_2O_2 was observed only in MSCs. When ROS production was examined using H_2DCFDA (Figure 7), marked ROS production was observed only in H_2O_2 treated MSCs. This result showed that H_2O_2 induced marked ROS production in MSCs. To elucidate the anti-oxidative stress activity of NAC, we first measured intracellular ROS levels in H_2O_2 exposure, using a cell-permeant indicator, H2DCFDA.

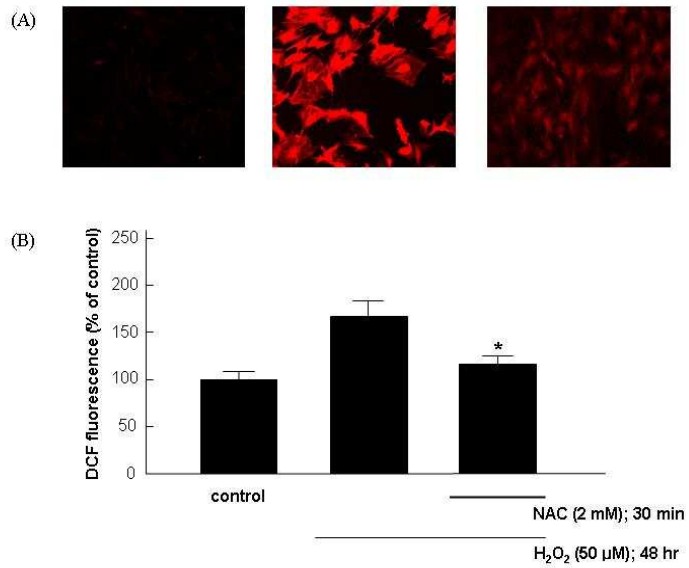

Figure 7. Measurement of intracellular ROS of H_2O_2-treated MSCs. MSCs were treated H_2O_2 with or without NAC and then incubated for 10 min with H_2DCFDA. Cell images were obtained with confocal laser microscope.

7. Expression of intracellular signals on H_2O_2-treated MSCs

The expression of intracellular differed from each other in H_2O_2 MSCs treated with or without NAC. The p-P38 MAPKinase expression in MSCs treated with H_2O_2 increased about 3 times compared with the pre-treatment of NAC and was the same as in control cells. The transcript level of Rac-1 was decreased 75.4% in H_2O_2 compared with control cells (Figure 8).

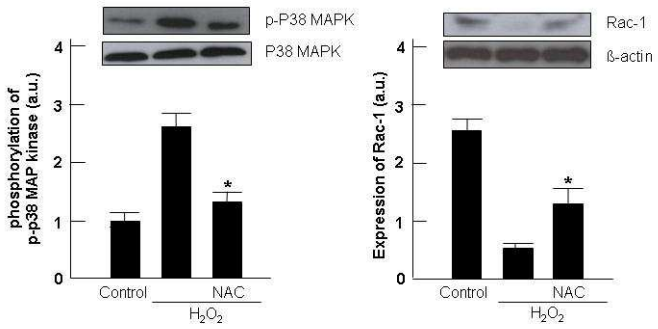

Figure 8. Effect of ROS on intracellular signaling of H_2O_2-treated MSCs. MSCs treated with or without NAC was exposed to a H_2O_2 for 48 hours. At the indicated times, cells were harvested. Equal amounts of protein were loaded onto and separated by 12% SDS–PAGE and transferred to membranes. Immunoblot analysis was repeated two times. Each signal was quantified by scanning densitometry. *P<0.01 vs controls.

8. Change in adhesion-related signals of MSCs treated ROS

The activation of Src, vinculin, FAK, and paxillin plays an important role in mechanisms of cellular activated integrin bind to the adhesion site and proliferation through gene regulation (figure 9). FAK, Src, paxillin, and vinculin are specificity adhesion site in cell matrix. It was detected phosphorylation of FAK (125 kDa), Src (60 kDa), paxillin (68 kDa) and vinculin (117 kDa) by immunoblot assay. As shown in Figure 9, the phosphorylation of FAK (77.1±2.8%), Src (62.2±4.3%), paxillin (36.2±4.8%) and vinculin (29.6±2.6%) was decreased in H_2O_2 MSCs compared with control cells.

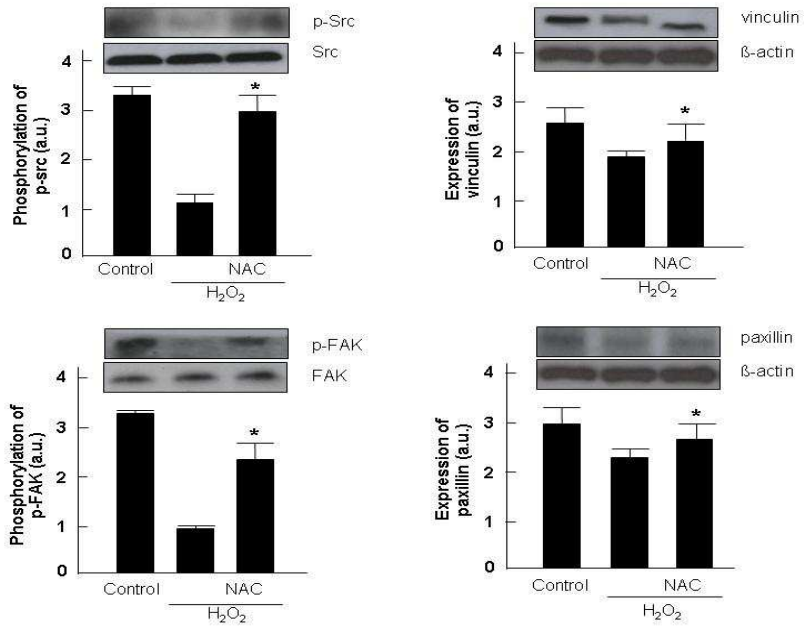

Figure 9. Effect of ROS on adhesion-related signals of H_2O_2-treated MSCs. Effect of H_2O_2 on activity of proteins related to cell adhesion of MSCs. Immunoblot analysis of phosphorylation of FAK, and expression of Src, paxillin, and vinculin in MSCs treated with or without NAC was exposed to a H_2O_2 for 48 hours. At the time, cells were harvested. Collected cells were solubilized in lysate buffer. Equal amounts of protein were loaded onto and separated by 12% SDS–PAGE and transferred to membranes. Immunoblot analysis was repeated 3 times. Each signal was quantified by scanning densitometry. *P<0.01 vs controls.

IV. DISCUSSION

In this study, ROS were reduced adhesiveness of MSCs into a matrix and spreading and migration of MSCs and reduces the assembly of focal adhesion complexes in vitro. In cell implantation studies for cardiac infarction, several cell types, including skeletal myoblasts[37-39] cardiac myocytes (adult, fetal, or neonatal myocytes) [40, 41], and embryonic stem cell-derived cardiac myocytes[42], were investigated, but each cell therapy appears to have clinical limitations. Autologous MSCs have a great advantage to generate functional cardiac myocytes in the infarcted myocardium because of the easy preparation from adult patients and immunologic safety. However, the frequency of MSCs engraftment was extremely low despite implanting large numbers of cells because of a poor rate of cell adhesion and survival[43]. Therefore, enhancement of cell adhesion and spreading should be one of the major aims in the development of cell transplantation techniques, including the therapeutic use of progenitor cells.

As shown in Figure 2, H_2O_2-induced cell death and the detach effect of MSCs causes cell death by necrosis and/or apoptosis in cultured MSCs[34]. It was observed that cultured MSCs exposed to H_2O_2 under went dose-

dependent cell detachment. To examine the effect of ROS on the H_2O_2-treated cell detach, MSCs were subjected to H_2O_2 for 48 hours. This led to a substantial decrease in adhesiveness. 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[44-47]. The absence of adhesion and spreading is probably the main cause of poor cell survival in cell transplantation[48, 49]. The causes of cell death are influenced by the ischemic environment, which is devoid of nutrients and oxygen, coupled with the loss of survival signals from inadequate interaction between cells and matrix[50].

NAC has antioxidant property [51] and as a sulf-hydryl donor, may contribute to the regeneration of endothelium-derived relaxing factor and glutathione[52]. Increasing evidence indicates that the action of NAC is pertinent to microcirculatory blood flow and tissue oxygenation. In the present report in which 48 hours of H_2O_2, the number of MSCs was elevated over that of the control with the effect being greatly reduced by NAC. This data demonstrate that NAC treatment exert an important protective effect against ROS (figure

3). Cell-matrix adhesions the cell surface structures that mediate cell interactions with ECM were composed of focal adhesions and other adhesive structures. Focal adhesions are integrin based structures that mediate strong cell-substrate adhesion and transmit information in a bidirectional manner between extracellular molecules and the cytoplasm[53-58]. The results indicate that expansion and adhesion of MSCs on 3-D matrix, cardiogel is greater than MSCs cultured on plate and fibronectin coated plate. The cell adhesion to cardiogel (3-D matrix) was significantly increased in normal MSCs as compared with adhesion to H_2O_2-treated MSCs. The adhesive difference was over doubling greater when prepared on cardiogel as compared with the Normal plates. These observations suggest that the basement membrane and ROS have an effect on the proliferation of MSCs. This study showed that ROS derived markedly decreased the growth rate and adhesion of MSCs on 3-D matrix (figure 5). The mechanism by which ROS stimulates MSCs adhesion has not been known but these results give a clue that adhesion between cells, -niche and extracellular matrix might be related to adhesion of MSCs.

The numbers of potential connections and the fact that some can be regulated

by phosphorylation or by the state of integrin aggregation or occupancy suggests the likelihood of variable compositions of adhesion complex. Direct binding interactions with integrin β1 subunits have been reported for talin, a-actinin, and FAK, and filamin binds to integrin β1 cytoplasmic domains[59]. Binding of calreticulin to integrin α subunits, and even of actin to integrin α cytoplasmic domain peptides, has been reported [60]. Integrins are essential for cell migration and invasion because they mediate adhesion of cells to the ECM and regulate intracellular signaling pathways that control cytoskeletal organization, force generation, and survival[61]. It explored α_1, α_2, αV, β_1 and integrin which reported other paper. Characteristics of receptors are as follow: critically implicated in modulating cardiac function, αV, β_1 (Figure 6). Integrin rate induced decreases in ROS, Surprisingly, when MSCs treated with H_2O_2 in 48 hours were compared with normal MSCs, detached MSCs were depression in the conclusion.

Oxidative stress, occurring as a consequence of increased intracellular levels of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), supposedly forms a common pathway leading to cell death in the conditions mentioned[62]. These results showed that H_2O_2 induced marked ROS production

in MSCs. To elucidate the anti-oxidative stress activity of NAC, it was first measured intracellular ROS levels in H_2O_2 exposure, using a cell-permeant indicator, H_2DCFDA.

Focal adhesion sites are specific areas on the cell membrane where cells attach to ECM. They are complexes of structural and signaling proteins, anchoring actin filaments and microtubules to the plasma membrane where integrins locate[28] Most integrin β-subunits interact with proteins, such as paxillin, talin, vinculin, and other focal adhesion proteins, which act as linkers between integrins and the actin cytoskeleton[29-31]. It has been known that key players in integrin-mediated signal transduction are a group of integrin-associated nonreceptor kinases, two of them being focal adhesion kinase (FAK) and integrin-linked kinase (ILK) [28, 32-34]. Activated integrins bind to the ECM, cluster at the binding site, and initiate focal adhesions by recruiting cytoplasmic proteins, such as focal adhesion kinase (FAK), Src, and paxillin[35]. Integrins also activate small GTPase, which in turn activates downstream effect molecules, thereby leading to rearrangement of actin stress fibers and activation of cell adhesion and spreading[36].

Cell migrations induced by these cytokines and growth factors were

mediated by important signal kinases such as MAPK, protein kinase C (PKC), FAK, Src, and PI3 kinase[59, 60, 61-65]. MAPK signaling was required for gene expression of β-integrin subunit and the cysteine proteases cathepsin induced by cell adhesion molecules[66]. One of the most promising mechanism for sustained MAPK signaling is the cross talking between major intracellular signals cascades of which integrin, PKC mediated-pathway are the most important. It is well established that integrin-mediated signal cascade can trigger sustained MAPK activation for a lot of cellular effects including proliferation, adhesion, migration and metastasis[67-69]. Firstly, two nonreceptor kinases Src and FAK are recruited and activated in the focal adhesion. FAK may autophosphorylate at Y397 for binding to a domain of Src followed by phosphorylation on multiple residues resulting in elevation of its kinase activity. Activated FAK/Src may phosphorylate the adaptor protein P130CAS and scaffold protein paxillin. Paxillin may be phosphorylated at tyrosine residue 31 and 118 between its N-terminal generating docking sites for P130CAS. As shown in Figure 10, the phosphorylation activities of FAK, Src, paxillin, and vinculin was decreased in H_2O_2 MSCs compared with control cells.

This may facilitate the recruitment of trigger activation of small GTPase Rac leading to cell adhesion and cell migration. The MAPK cascades are the most important down stream signaling of the FAK–Src–paxillin–CAS/CRK–Rac axis. The paxillin-associated CRK may trigger the Rac specific guanidine exchange factor (GEF) to activate Rac and its down stream[67]. As shown in figure 9, p-P38 MAPKinase expression in MSCs treated with H_2O_2 increased about triplet times compared with the pre-treatment of NAC and was the same as in control cells. The transcript level of Rac-1 was decreased about 75.4% in H_2O_2 compared with control cells.

In this study, MSCs were treated with ROS not adhered in response to treated with H_2O_2. By means of this system, it was demonstrated that cell detachment involves actin cytoskeleton reorganization, Rac-1 activation, expression of integrins. Moreover, it was conclude that MSCs was not sufficient to cell detachment, which also downstream signals disruption. These results show, for the first time, that oxidative stress affects cell−matrix but not cell−cell adhesion as part of proximal cell response to ROS.

V. CONCLUSION

This study suggests that ROS was involved in adhesion and downstream signaling between MSCs and their niche. MSCs were isolated from rat tibias and femurs, and characterized with MSCs positive and negative markers. The intracellular ROS in MSCs was produced by H_2O_2 for 48 hours. The MSCs pre-treated with NAC showed difference of adherent rate dose-dependently, compared with non-treated cells, as measured by the trypan blue cell counting. The MSCs treated with H_2O_2 were displayed cohesion difference of morphological pattern, depression in adhesion receptors and integrin-related proteins, and showed time-dependently segregate expression compared with normal MSCs.

In summary, the ROS had greater effects on the attachment of MSCs. In the future identification of mechanism of action of the adhesion factors may have important implications on the development of Stem cell therapy for repair of myocardial injury after cell transplantation.

REFERENCES

1. Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJ. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet 2006;367(9524):1747-57.
2. Segers VF, Lee RT. Stem-cell therapy for cardiac disease. Nature 2008;451(7181):937-42.
3. Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE. Cardiomyocyte grafting for cardiac repair: graft cell death and anti death strategies, J Mol Cell. Cardiol. 2001;33:907–21.
4. Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002;105:93-8.
5. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410(6829):701-5.
6. Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, et al. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet 2003;361(9351);45–6.

7. Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, et al. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomized controlled clinical trial. Lancet 2004; 364;141–8.
8. Shake JG, Gruber PJ, Baumgartner WA, Senechal G, Meyers J, Redmond JM, et al. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. Ann Thorac Surg. 2002;73:1919–25.
9. Graf T, Differentiation plasticity of hematopoietic cells. Blood 2002;99:3089–99.
10. Minguell JJ, Erices A. Mesenchymal stem cells and the treatment of cardiac disease. Exp Biol Med. 2006;231:39–49.
11. Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest. 1999;103(5):697-705.
12. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002;105:93–8.

13. Song H, Chang W, Lim S, Seo HS, Shim CY, Park S, et al. Tissue transglutaminase is essential for integrin-mediated survival of bone marrow-derived mesenchymal stem cells. Stem Cells 2007;25(6):1431-8.
14. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, et al. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res. 2004;95:911–21.
15. Wollert KC, Drexler H. Mesenchymal stem cells for myocardial infarction: promises and pitfalls. Circulation 2005;112:151-3.
16. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006;98:1076–84.
17. Shi RZ, Li QP. Improving outcome of transplanted mesenchymal stem cells for ischemic heart disease. Biochem Biophys Res Commun. 2008;376:247–50
18. Korge P, Ping P, Weiss JN. Reactive oxygen species production in energized cardiac mitochondria during hypoxia/reoxygenation: modulation by nitric oxide. Circ Res. 2008;103(8):873-80.
19. Chiarugi P, Buricchi F. Protein tyrosine phosphorylation and reversible oxidation: two cross-talking posttranslation modifications. Antioxid Redox

Signal. 2007;9:1–24.

20. Finkel T. Oxidant signals and oxidative stress. Curr. Opin. Cell Biol. 2003;15:247–54.

21. Fruehauf JP, Meyskens FL. Reactive oxygen species: a breath of life or death? Clin Cancer Res. 2008;13:789–94.

22. Lorenowicz MJ, Fernandez-Borja M, van Stalborch AM, van Sterkenburg MA, Hiemstra PS, Hordijk PL. Microtubule dynamics and Rac-1 signaling independently regulate barrier function in lung epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2007;293(5):1321-31.

23. Chiarugi P, Fiaschi T. Redox signalling in anchoragedependent cell growth. Cell Signal. 2007;19:672–82.

24. Burridge, K., Sastry, S.K., and Sallee, J.L. Regulation of cell adhesion by protein-tyrosine phosphatases I Cell-matrix adhesion. J Biol Chem. 2006;281:15593–6.

25. Kheradmand F, Werner E, Tremble P, Symons M, Werb Z. Role of Rac1 and oxygen radicals in collagenase-1 expression induced by cell shape change. Science 1998;280:898 –902.

26. Frisch SM and Screaton RA. Anoikis mechanisms. Curr Opin Cell Biol.

2001;13:555–62.

27. Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol. 2005;6:56–68.

28. Brown MC, Turner CE. Paxillin: adapting to change. Physiol Rev. 2004;84:1315–39.

29. Gilmore AP. Anoikis. Cell Death Differ 2005;12:1473–7.

30. Ziegler WH, Liddington RC, Critchley DR. The structure and regulation of vinculin. Trends Cell Biol. 2006;16:453–60.

31. Attwell S, Roskelley C, Dedhar S. The integrin-linked kinase (ILK) suppresses anoikis. Oncogene 2000;19:3811–5.

32. Parson JT. Focal adhesion kinase: the first ten years. J Cell Sci. 2003;116: 1409–16.

33. Legate KR, Montañez E, Kudlacek O, Fässler R. ILK, PINCH and parvin: the tIPP of integrin signalling. Nat Rev Mol Cell Biol. 2006;7:20–31.

34. Huang Q, Shen HM, Shui G, Wenk MR, Ong CN. Emodin inhibits tumor cell adhesion through disruption of the membrane lipid Raft-associated integrin signaling pathway. Cancer Res. 2006;66:5807–15.

35. Salsmann A, Schaffner-Reckinger E, Kabile F, Plancon S, Kieffer N. A new functional role of the fibrinogen RGD motif as the molecular switch that selectively triggers integrin aIIbh3-dependent RhoA activation during cell spreading. J Biol Chem. 2005;280:33610–19.
36. Cukierman E, Pankov R, Stevens DR. Taking cell-matrix adhesions to the third dimension. Science 2001;294:1708–12.
37. Lefer DJ, Granger DN. Oxidative stress and cardiac disease. Am J Med. 2000;109(4):315-23.
38. Taylor DA, Atkins BZ, Hungspreugs P, Jones TR, Reedy MC, Hutcheson KA, et al. Regenerating functional myocardium: Improved performance after skeletal myoblast transplantation. Nat Med. 1998;4:929 –33.
39. Reinecke H, Zhang M, Bartosek T, Murry CE. Survival, integration, and differentiation of cardiomyocyte grafts: A study in normal and injured rat hearts. Circulation 1999;100:193–202.
40. Lipsic E, van der Meer P, Henning RH, Suurmeijer AJ, Boddeus KM, van Veldhuisen DJ, et al. Timing of erythropoietin treatment for cardioprotection in ischemia/reperfusion. J C Pharm. 2004;44:473– 79.
41. Murry CE, Wiseman RW, Schwartz SM, Hauschka SD. Skeletal myoblast

transplantation for repair of myocardial necrosis. J Clin Invest. 1996;98:2512–23.

42. Li RK, Mickle DA, Weisel RD, Mohabeer MK, Zhang J, Rao V, et al. Natural history of fetal rat cardiomyocytes transplanted into adult rat myocardial scar tissue. Circulation 1997;96:179–86.

43. Watanabe E, Smith DM Jr, Delcarpio JB, Sun J, Smart FW, Van Meter CH Jr, et al. Cardiomyocyte transplantation in a porcine myocardial infarction model. Cell Transplant 1998;7:239–46.

44. Mangi AA, Noiseux N, Kong D, He H, Rezvani M, Ingwall JS, et al. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med. 2003;9:1195–201.

45. Wollert KC, Drexler H. Clinical application of stem cells for the heart. Circ Res. 2005;96:151–63.

46. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation 2001;104:1046–52.

47. Fuchs S, Baffour R, Zhou YF, Shou M, Pierre A, Tio FO, et al. Transendocardial delivery of autologous bone marrow enhances collateral perfusion and regional function in pigs with chronic experimental myocardium ischemia. J Am Coll Cardiol. 2001;37:1726–32.
48. Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res. 2004;94:678–85.
49. Guérette B, Skuk D, Célestin F, Huard C, Tardif F, Asselin I, et al. Prevention by anti-LFA-1 of acute myoblast death following transplantation. J Immunol. 1997;159:2522–31.
50. Aruoma OI, Halliwell B, Hoey BM. The antioxidant action of acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxyde, and hypochlorous acid. Free Radic Biol Med. 1989;6:593–7.
51. Harrison PM, Wendon YA, Gimson AES. Improvement by acetylcisteine of hemodinamics and oxygen transport in fulminant hepatic failure. N Engl J Med. 1991;324:1852–7.
52. Jockusch BM, Bubeck P, Giehl K, Kroemker M, Moschner J, Rothkegel M,

et al. The molecular architecture of focal adhesions. Annu Rev Cell Dev Biol. 1995;11:379-416.

53. Yamada KM, Geiger B. Molecular interactions in cell adhesion complexes. Curr Opin Cell Biol. 1997;9(1):76-85.

54. Chung JH, Im EK, Park HY, Kwon JH, Lee S, Oh J, Hwang KC, Lee JH, Jang Y. A novel uracil-DNA glycosylase family related to the helix-hairpin-helix DNA glycosylase superfamily. Nucleic Acids Res. 2003;31(8):2045-55.

55. Adam JC. Cell-matrix contact structures, Cell Mol Life sci. 2001;58:371

56. Hynes RO. Integrin: versatility, modulation and signaling in cell adhesion. Cell 1992;69:11.

57. Schwartz MA, Schaller MD, Ginsberg MH. Integrins: emerging paradigms of signal transduction. Annu Rev Cell Dev Biol. 1995;11:549-99.

58. Gao CF, Vande Woude GF. HGF/SF-Met signaling in tumor progression. Cell Research 2005;15(1): 49–51.

59. Bierie B, Moses HL. Tumour microenvironment: TGF beta: The molecular Jekyll and Hyde of cancer. Nature 2006;6(7):506–20.

60. Hood JD, Chere DA. Role of integrins in cell invasion and migration. Nat

Rev Cancer. 2002;2:91–100.

61. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci U.S.A. 1993;90:7915–22.

62. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, et al. Cell migration: Integrating signals from front to back. Science 2003;302:1704–9.

63. Kim H, Muller WJ. The role of the epidermal growth factor receptor family in mammary tumorigenesis and metastasis. Experimental Cell Research 1999;253(1):78–87.

64. Qiang YW, Walsh K, Yao L, Kedei N, Blumberg PM, Rubin JS, et al. Wnts induce migration and invasion of myeloma plasma cells. Blood 2005;106(5):1786–93.

65. Silletti S, Yebra M, Perez B, Cirulli V, McMahon M, Montgomery AM. Extracellular signal-regulated kinase (ERK)-dependent gene expression contributes to L1 cell adhesion molecule-dependent motility and invasion. J Biol Chem. 2004;279(28):28880–8.

66. Guo W, Giancotti FG. Integrin signalling during tumour progression.

Nature 2004;5(10):816-26.

67. Giancotti FG, Ruoslahti E. Integrin signaling. Science 1999;285(5430): 1028–32.

68. Mitra SK, Schlaepfer DD. Integrin-regulated FAK-Src signaling in normal and cancer cells. Curr Opin Cell Biol. 2000;18(5):516–23.

69. Hood JD, Cheresh DA. Role of integrins in cell invasion and migration. Nature Reviews 2000;2(2):91–100.

ABSTRACT (in Korean)

중간엽 줄기세포의 부착성에 미치는 활성 산소 종의 역할

<지도교수 장 양 수>

연세대학교 대학원 의과학과

차 민 지

심근 손상의 복원을 위한 줄기 세포 치료는 세포 -이식 후 세포의 적은 부착성에 의해 치료에 있어 제한을 받는다. 심근 경색 후 허혈은 활성 산소 종의 발생을 증가 시켜 중간엽 줄기 세포와 그 주변의 상호작용과 관련을 갖는다. 세포내의 활성 산소 종은 세포 부

착, 유입과 증식에 있어서 중요한 역할을 한다. 중간엽 줄기 세포와 그 주변의 상호 작용에서의 활성 산소 종의 역할을 확인하고자 하였다. 과산화수소의 처리 후, 중간엽 줄기세포는 과산화수소의 농도가 증가함에 따라 분리가 되나, 유리기 scavenger인 NAC(N-acetyl-L-cysteine, 2 mM)을 처리하였을 경우 세포의 분리가 저해되는 것을 확인할 수 있다. 섬유상 단백질이나 3차원 세포기질을 처리 한 배양 접시에 과산화수소를 50 μM 처리한 경우와 처리하지 않은 경우의 비교군 실험에서, 과산화수소의 처리가 이루어지지 않은 3차원 세포 기질의 배양접시에서 섬유상 단백질을 처리한 배양접시와 비교하였을 경우 두 배 가량 높은 양의 중간엽 줄기 세포의 부착 성이 보였다. 또, 과산화수소에 의해 세포 내 활성 산소 종의 생성이 70% 증가 하였다. FAK(Focal Adhesion Kinase) 인산화와 MAPK와 Src의 활성과 paxillin의 발현을 포함한 다른 하위 신호는 산화적 신호에 의해 의미 있게 감소하였다. 과산화수소를 처리한 중간엽 줄기세포의 발현은 FAK에서 77.1±2.8%, Src에서 62.2±4.3%, paxillin에서 36.2±4.8% 그리고 vinculin에서 29.6±2.6%, 각각 감소하였다. 또한, 세포 부착에서 주요한 물질인 integrin의 발현에서 대조 군과 비교 하여 과산화수소를 처리한 경

우, Integrin αV와 fibronectin의 발현은 60%, 32.5% 그리고 integrin β1은 79.4% 감소하였다. H_2O_2 처리 이후, 중간엽 줄기세포의 부착에서 integrin-유도 활성 산소 종 생성의 조절과 관련이 있는 Small GTPase Rac-1의 발현은 과산화수소 처리 군에서 대조 군과 비교 하였을 경우 75.4% 감소했다. 결과적으로 활성 산소 종은 세포부착의 조절과 주변 상호 조절에서 세포부착과 하위 신호물질을 저해하는 것을 나타내었다. 이러한 결과로 경색된 심장의 중간엽 줄기세포 이식에 있어 더 나은 결과를 가져 오게 될 방법을 발견하게 만들 수 있을 것을 기대된다.

핵심되는 말: 중간엽 줄기 세포, 활성 산소 종