Enhancement of mesenchymal stem cell adhesion by PKC activation

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The Graduate School, Yonsei University

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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

Choi, Eunju

December 2010

This certifies that the Master's Thesis

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December 2010

Acknowledgements

I am grateful my thesis supervisor professor Yangsoo Jang, for help and concern always. I thank to professor Ki-Chul Hwang for encouragement and support from experimental guidance to discussion and other committee member, professor Sahng Wook Park for your advice and concern.

The generous support from the Yonsei Cardiovascular Research Institute is greatly appreciated. I thank to Dr. Heesang Song, Dr. Eunmi Choi, Il-kwon Kim, Byeong-Wook Song, Min-Ji Cha, Onju Ham, Changyoun Lee, Se-yeon Lee, Jun-hee Park, Eunhye Lee, Sun Ju Lee, Woochul Chang, Soyeon Lim and Hye-Jung Kim for the your help.

On a personal note, I would like to thank my family and my friends. My father, mother and grandmother sincerely provided me of emotional support and took care of me. My brother Jung-Hwan and my relatives really appreciate their encouragement. I also would like to thank SIRIC Lab B members, include Ji-Won Choi, Hyun Ju Jeon and Hae Min Pyun. I wish to thank my friends Na-Rae, Song, So-Yong, Ji-Yong, two Eun-Yongs, Su-Jung (Hyn-Jung), Jung-Yi, Eun-Hee, Kung Ha, Chae-Won, Boon, Ji-Min.

Finally, I really thank to my God.

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ABSTRACT

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Emerging evidence suggests that cell therapy with bone marrow-derived mesenchymal stem cells (MSCs) has beneficial effects on the injured heart. However, decreased survival and/or adhesion of MSCs under ischemic

conditions limit the application of cell transplantation as a therapeutic modality. This study investigated a potential method for enhancing the adhesion ability of MSCs to improve their efficacy in the ischemic heart. Because protein kinase C (PKC) is a key factor in the regulation of cellular function, treatment of MSCs with PKC activators increased cell adhesion and spreading in a dose-dependent manner, and significantly prevented MSCs from detachment. When MSCs were treated with PKC inhibitor, adhesion of MSCs was slightly diminished, and detachment was also decreased compared to treatment with PKC activator. MSCs treated with both the PKC activator and inhibitor behaved similarly to normal controls. In the 3D matrix cardiogel, treatment with PKC activator increased the number of MSCs compared with normal controls or MSCs treated with PKC inhibitor. Expression of focal adhesion kinase and cytoskeleton-associated proteins, including paxillin, vinculin, and talin was clearly increased in PMA-treated MSCs by immunoblotting and immunocytochemistry. The

effect of PMA treatment on MSCs was validated in vivo. Following injection into rat hearts, the PMA-treated MSCs exhibited significant bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) fluorescence compared with control groups. Fibrosis and infarct size were reduced in rat hearts injected with PKC activator-treated MSCs compared with hearts injected with untreated MSCs. These results indicate that PKC activator is a potential target for niche manipulation to enhance adhesion of MSCs for cardiac regeneration.

Key words: mesenchymal stem cells, protein kinase C, adhesion, cardiac regeneration

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I. INTRODUCTION

Mesenchymal stem cells or stromal cells (MSCs) are a promising source of stem cells for regenerative therapy [1]. Stem cell therapy provides a potential treatment for myocardial infarction (MI) through myocardial regeneration caused by transplantation of MSC [2]. However, the effects of clinical

applications of MSCs have been confined to limited conditions and have been restricted by the poor survival of MSCs in vivo. To increase the survival rate, several studies have focused on methods of strengthening the cells [3].

One such method involves pretreatment of the stem cells to modify their cellular function [4]. Several kinds of pretreatment can influence cellular mechanisms [5]; for example, regulation of the protein kinase C (PKC) pathway is a recognized method of physiologically changing cells [6]. PKC is a family of kinases consisting of at least twelve different isoforms [7-8]. The conjunctiva contains at least eight of these isoforms and closely related PKCs [9-10]. The serine/threonine kinase PKC induces phosphorylation of focal adhesion kinase. And disengaged phosphorylation is transported with the energy to cell surface [11]. It is used to an actin binding protein localized within cortical actin structures at the cell membrane [12]. Adhesion to the matrix results in PKC-dependent phosphorylation from actin structure [13]. The PKC

activator phorbol 12-myristate 13-acetate (PMA) has significant effects in the cells and plays a role in cell adhesion related signaling [14]. PMA is a phorbol ester that is a well-known tumor suppressor [15]. Previous studies have clearly shown that cells treated with PMA show stronger survival than controls [2]. Since adhesion signaling mediates cell survival and apoptosis [16], it is likely that the cells treated with PMA associate through adhesion involving several kinds of focal adhesion molecules and stress fibers. An increase in adhesion molecules such as focal adhesion kinase (FAK) and Src is associated with a concomitant increase in tyrosine phosphorylation of cytoskeleton-associated protein such as paxillin, vinculin, and talin at the same site. It is already known that FAK localizes to focal adhesion sites in cells [17] and that phosphorylation of FAK is important for integrin-mediated cell attachment and spreading [18]. Integrins are a family of αβ heterodimetric transmembrane adhesion receptors that participate in cell-cell or cell-matrix interactions within the immune system [19-21]. They are cell surface receptors

for extracellular matrix molecules that play critical roles in a variety of biological process [22]; for example, they activate the cellular focal adhesion-related kinases and bind to the ECM molecules, and also regulate stress fibers. Several focal adhesion molecules have been identified, including FAK, Src, paxillin, talin, vinculin, α-actin, and tensin. An activated FAK/Src complex binds to potential substrates in the actin cytoskeleton such as pexillin, talin, and vinculin [23]. The relationship between adhesion signaling and the cytoskeleton has been investigated in several studies. [23] However, there are no studies on the adhesion of implanted MSCs that were pretreated with PKC activator in the heart infarct zone.

In this study, it was showed that treatment with PKC activator increased the MSC adhesion rate using an adhesion assay, detachment assay, and spreading assay. By immunoblotting and immunocytochemistry, we also observed FAK and integrin-related signaling that would increase the adhesion rate. To show enhanced adhesion of MSCs *in vivo*, it observed the

infarct and fibrosis area of rat hearts after myocardial infarction. In adition, BCECF-AM dye was used to show the location and number of injected modified MSCs.

II. MATERIALS AND METHODS

1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). BCECF-AM (2',7-bis(carboxyemethyl)-5,6-carboxyfluorescein) and PMA (phorbol 12-myristate 13-acetate) were obtained from Sigma (Sigma-Aldrich Inc, St Louis, USA). Rottlerin was obtained from calbiochem (EMD Biosciences Inc, Darmstadt, Germany). Antibodies to focal adhesion kinase (FAK), phospho-FAK, vinculin, and integrin α5βⅢ (α5β1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to paxillin and Rac-1 were purchased from Upstate (Chemicon International Inc. Temecula, CA, USA) and antibodies to talin were purchased from Abcam (Abcam Plc, CB, UK). Horseradish peroxidase-conjugated secondary antibodies to mouse or rabbit were obtained from

Santa Cruz Biotechnology. The western blotting detection system was from Amersham Biosciences (Amersham Pharmacia Biotech, Tokyo, Japan).

2. Isolation and culture of MSCs

Mesenchymal stem cells were purified as previously described. Briefly, bone marrow from femoral and tibial bones of four-week-old male Sprague-Dawley rats (approximately 100 g) was aspirated with 10 ml DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution. Mononuclear cells recovered after centrifugation in Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) were washed twice, resuspended in DMEM with 10% FBS, and plated in flasks at 1 x 10^6 cells per 100 cm^2. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 48 hours, non-adherent cells were discarded, and adherent cells were thoroughly washed twice with phosphate-buffered saline (PBS). Fresh complete medium was added and replaced every 3 to 4 days for approximately 10 days. The

characteristics of MSCs were demonstrated by immunophenotyping. To verify the nature of the cultured MSCs, cells were analyzed for various surface and intracellular markers. Cells were harvested, washed with PBS, and labeled with the following antibodies conjugated with fluorescein isothiocyanate (FITC): CD14, CD34, CD71, CD90, CD105, CD106, and ICAM-1. FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as secondary antibody and labeled cells were assayed by flow cytometry.

3. Assays for cell adhesion and spreading

Suspensions of 2 x 10^4 MSCs were added to each well of a six-well plate and allowed to attach for 1 hour at 37°C and 5% CO_2. Cells were treated with PKC activator and inhibitor as indicated. To determine MSC adhesion, cells were carefully washed three times with PBS, and then four separate fields were photographed with a phase contrast microscope and the cells were

counted. Each experiment was performed in triplicate wells and repeated at least three times. For spreading assays, MSCs were plated for 4 hours on four-well plates using the conditions of the adhesion experiments described above. To determine MSC spreading, plates were washed three times with PBS, fixed with 3% formaldehyde, stained with Coomassie blue, destained, and four separate fields were photographed using a phase contrast microscope.

4. Trypsinization assay

Cells were grown to 90% confluency on culture plates prior to trypsinization for 3 min at 37°C. Following by a washing step, the remaining adherent cells were incubated for 1 hour at 37°C and the cells were counted in several different areas of the plate.

5. Preparation of cardiac fibroblast-derived three- dimensional matrix

(cardiogel)

Cardiogel was prepared with a minor modification. Briefly, $2x10^5$ viable MSCs were added to each well of a four-well plate and allowed to attach for 1 hour at 37°C and 5% CO_2. The medium was changed every 48 hours until the matrix was denuded of cells. The matrix was carefully and gently rinsed with PBS. Next, 1ml 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 followed using an inverted microscope until no intact cells were observed. The plates were incubated at 37°C for 30 minutes with 1ml 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 3ml PBS containing 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml Fungizone for later use.

6. Immunoblot analysis

Cells were washed once in PBS and lysed in buffer (Cell Signaling Technology, Danvers, MA, USA) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2-EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined using the Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Proteins were separated in a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Chemicon International Inc, Temecula, CA, USA). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5% nonfat dried milk for 1 hour at room temperature, the membrane was washed twice with TBS-T and incubated with primary antibody for 1 hour at room temperature or overnight at 4°C. The membrane was washed three times with TBS-T for 10 min and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary

antibodies. After extensive washing, bands were detected by enhanced chemiluminescence reagent (Santa Cruz Biotechnology, CA, USA). Band intensities were quantified using the Photo-Image System (Molecular Dynamics, CA, USA).

7. Immunocytochemistry

Cells were grown on four-well plastic dishes, washed twice with PBS and then fixed with 4% paraformaldehyde in 0.5 ml PBS for 30 min at room temperature. The cells were washed again with PBS and permeabilized for 30 min in PBS containing 0.2% triton X-100. Next, the cells were blocked with 10% goat serum in PBS and incubated for 1 hour with primary antibodies against integrin α5, integrin α5β3, p-FAK, paxillin and vinculin. The MSCs were washed three times for 10 min with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and Texas red-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove,

PA, USA) as secondary antibodies for 1 hour. All images were produced using an excitation filter under reflected light fluorescence microscopy (Carl Zeiss microscope, NY, USA).

8. Induction of myocardial infarction

Experiments were conducted in accordance with the International Guide for the Care and Use of Laboratory Animals. Under general anesthesia, eight-week-old Sprague-Dawley male rats (approximately 250g) were intubated, and positive-pressure ventilation(180mL/min) was maintained with room air supplemented with oxygen (2L/min) using a Harvard ventilator. The heart was exposed through a 2-cm left lateral thoracotomy. The pericardium was incised and a 6-0 silk suture (Johnson &Johnson, New Brunswick, New Jersey, USA) was placed around the proximal portion of the left coronary artery, beneath the left atria appendage. Ligature ends were passed through a small length of plastic tube to form a snare. For coronary artery occlusion, the snare was

pressed onto the surface of the heart directly above the coronary artery, and a hemostat was applied to the snare. Ischemia was confirmed by the blanching of the myocardium and dyskinesis of the ischemic region. After 60 min of occlusion, the hemostat was removed and the snare was released for reperfusion, with the ligature left loose on the surface of the heart. Restoration of normal rubor indicated successful reperfusion. Before starting the experiment, MSCs were treated with PKC activator and/or inhibitor for 4 hours. During ligation of the rat heart, the MSCs were treated with BCECF-AM and injected into the heart after ligation. The wounds were sutured, and the thorax was closed under negative pressure. Rats were weaned from mechanical ventilation and returned to cages to recover. In sham-operated rats, the same procedure was executed without tightening the snare.

9. Histology and determination of the area of fibrosis

At 3days after implantation, animals were killed and their hearts were excised.

Each heart was perfusion- fixed with 10% (v/v) neutral-buffered formaldehyde for 24 hours, transversely sectioned into four comparably thick sections, and embedded in paraffin by routine methods. Sections of 5 μm thickness were mounted on gelatin-coated glass slides to ensure that different stains could be used on successive tissue sections cut through the implantation area. Fibrosis was analyzed by Masson's Trichrome staining. The interstitial fibrosis areas in the MI following injection with PMA-treated MSCs were measured using confocal microscopy (Carl Zeiss inc, NY, USA) and expressed as a percentage of the total LV.

10. Statistical analysis

All quantified data represent an average of at least triplicate samples. The error bars represent the standard deviation of the mean. Statistical significance was determined by Student's t-test, with $p<0.05$ considered significant.

III. RESULTS

1. Effects of PKC activation on adhesion of MSCs

Adherence of the PMA-treated MSCs was observed during 2-8 hours. At 4 hours, there was a difference in the adhesion of the DMSO-treated and PMA-treated MSCs. MSC adhesion was induced by PMA at a, concentration of 100 nM and gradually increased in a dose dependent manner (Fig. 1). The results clearly showed that the quantity of MSCs treated with PKC activator increased more than that of the control.

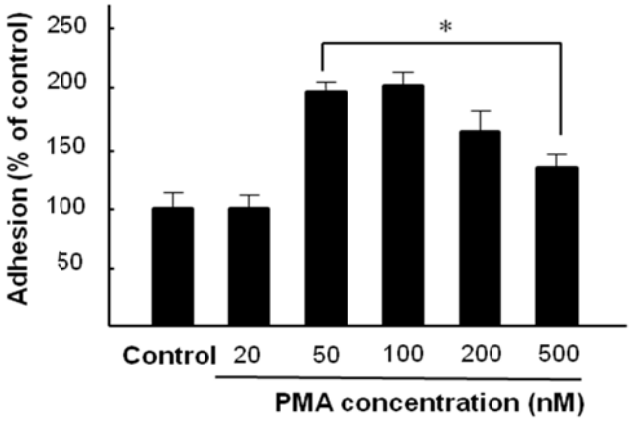


Figure 1. Changes in MSC adhesion induced by treatment with PMA. The adhesive properties of cultured MSCs treated with PKC activator. PKC activator was added when the cells were seeded and the plates were incubated for 4 hours under growth conditions at 37°C and 5% CO_2 . The plates were washed and cell viability of adherent cells was assessed by Trypan blue staining. *$P < 0.05$ vs. control.

2. Effect of PKC activation on cell spreading

In this study, MSCs were treated with a PKC activator and adhesion was measured by an adhesion assay and a spreading assay, since cell spreading is involved in cell adhesion and migration. The PKC activity of MSCs was increased by treatment with 100 nM PMA for 4 hours (Fig.2A). PMA also affected the number of cells and their capacity to change shape. To determine whether PMA modulates spreading, It was plated MSCs, stained the cells with coomassie brilliant blue and photographed them (Fig. 2B).

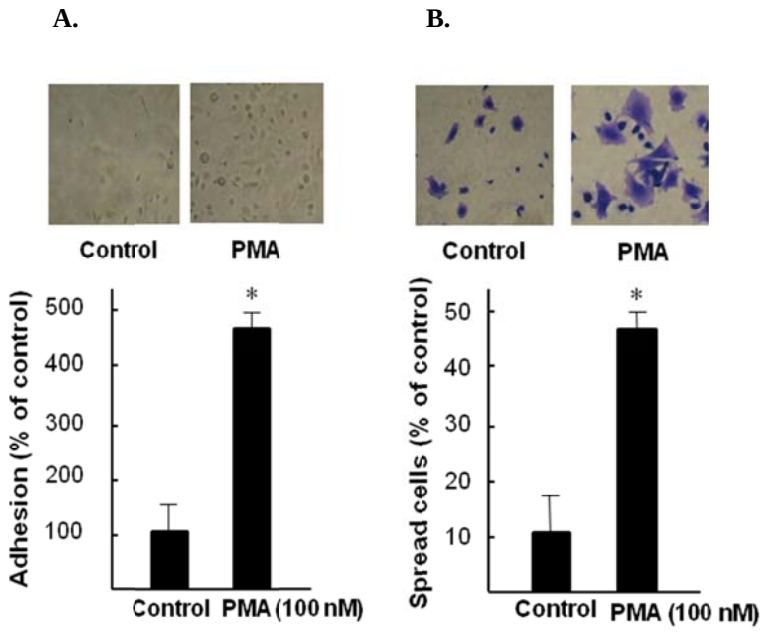


Figure 2. PKC activator increases adhesion and spreading rate of MSCs. (A) Representative microphotographs of MSCs-treated for 4 hours with PMA (100 nM) (right) or with DMSO (left). (B) Cell spreading morphology of MSCs treated with DMSO (left). *$P < 0.05$ vs. control.

3. Determination of MSCs viability

To examine the effect of PKC activator on adhesion rate, MSCs were plated for 1 day and treated with PKC activator PMA for 4 hours. Each plate was subjected to a trypsinization assay. The data confirmed that an increased adhesion rate led to increased cell survival. As shown in Figure 3, MSCs treated with PMA had an adhesion rate 48.5% than that of normal control and showed a reduced detachment ratio.

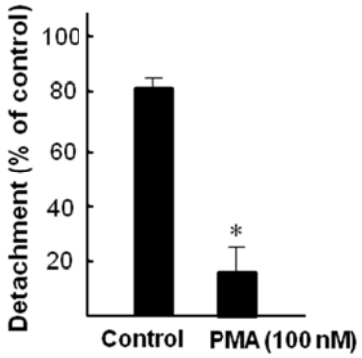
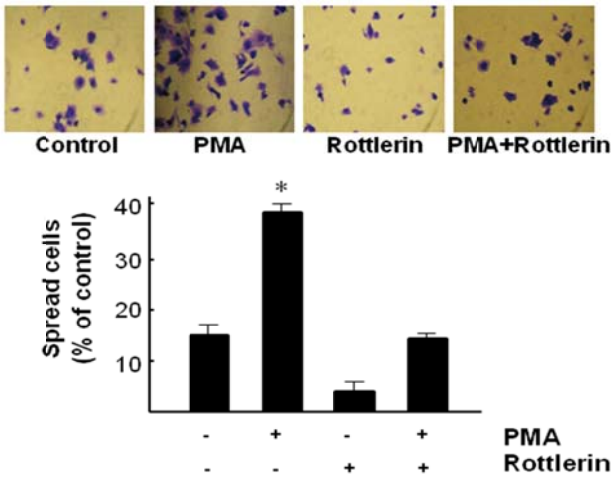


Figure 3. Improvement of detachment ratio of MSCs. The same number of MSCs was plated in each plate. The next day, PKC activators were added to each plate and a trypsinization assay was performed to measure the cell detachment rate. *$P < 0.05$ vs. control.

4. Changes in cell adhesion by PKC activation and inhibition on adhesion of MSCs

To confirm the effect of PKC activation and inhibition on the adhesion rate, It was performed a cell spreading assay. The results were similar to those reported above. The MSCs that were treated with PKC activator PMA showed increased adhesion compared with normal control, whereas MSCs treated with the PKC inhibitor Rottlerin showed decreased adhesion. MSCs treated with both activator and inhibitor exhibited adhesion rates similar to those of normal controls. In this study, we used two different PKC activators. These showed a similar trend, but resulted in different adhesion rate. The adhesion of MSCs that were treated with PMA was increased by 48.5% relative to normal control (Fig. 4A). The detachment assay showed the same trend as in Figure 3. MSCs treated with PMA had a detachment ratio 60% that of the normal control, whereas MSCs treated with both activator and inhibitor showed similar results to the normal control (Fig. 4B).

A.



B.

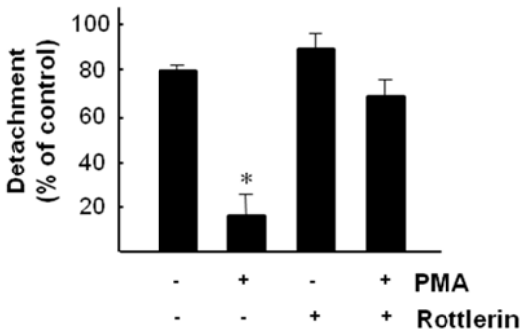


Figure 4. Changes in MSC adhesion induced by PKC activator and inhibitor. (A-B) Representative microphotographs of MSCs treated with PMA (100 nM) and Rottlerin (3 μM). (A) Cells were treated with DMSO, PMA or Rottlerin for 4 hours and (B) were measured detachment assay. When MSCs were treated with both PKC activator and inhibitor, the cells were treated in each plate with the PKC inhibitor before 10min, then with the activator. *$P < 0.05$ vs. control.

5. Changes in cardiogel following treatment with PKC activator and inhibitor

Cardiogel mimics the environment of myocardial cells using cardiac fibroblasts [24-25]. The distinctive 3-D matrix adhesions differ from classically described 2-D adhesions in structure, localization, and function. As shown in Figure 6, cell adhesion to the cardiogel 3-D matrix was significantly increased in PMA treated MSCs compared with MSCs treated with DMSO. It was compared cells grown for 2 hours and 16 hours. The difference in adhesion was over two times greater on cardiogel than on the fibronectin coated plates (Fig. 5A, B).

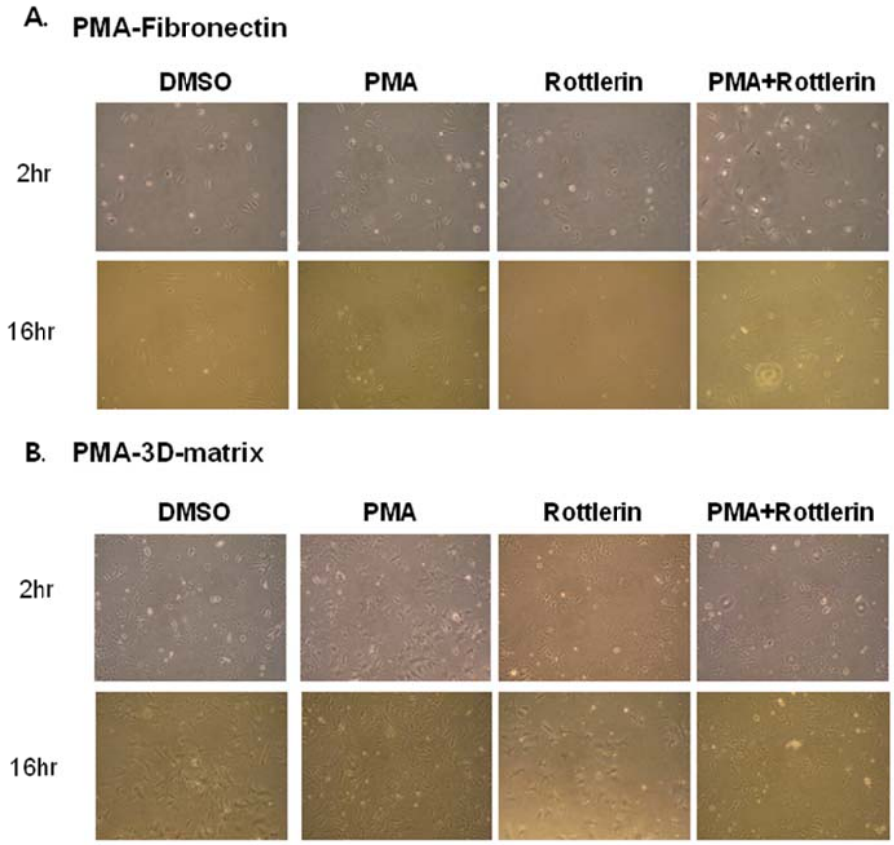


Figure 5. Adhesion rate of MSCs treated PKC activator or inhibitor on 2D and 3D-matrices. (A) Adhesion of MSCs in a 2D fibronectin (Fn) matrix. MSCs were plated on Fn-coated plates and incubated for 2 hours (upper panel) and 16hours (lower panel). (B) Adherence of MSCs in a 3D cardiogel matrix. Cells were grown on cardiogel for 2 hours (upper panel) and 16 hours (lower panel). Cells were treated with PKC activator and inhibitor at the time of plating. Several separate fields were photographed using a phase contrast microscope.

6. Changes in adhesion-related signaling by regulation of PKC activation

Previous studies showed that PMA induced up-regulation of PKC affected the ability of MSCs to adhere. PMA-responsive PKCs translocate from the cytosolic fractions during activation. To determine whether adhesion of MSCs causes an increased up-regulation of membrane- associated PKC, it was evaluated the expression of adhesion-related molecules in MSCs treated with PKC activators using immunoblot analysis. It was observed specific protein induction in MSCs treated with PKC regulator compared with control MSCs.

In particular, It was observed an increase in P-FAK in MSCs treated with PKC activators relative to control MSCs (Fig. 6A), in MSCs treated with PMA, the level of P-FAK was increased by 28.5% compared with normal controls. There was also an increase in expression levels of paxillin, talin and vinculin following PMA treatment: expression of paxillin was increased

by 46.2% (Fig. 6B), talin was increased by 25% (Fig. 6C), and vinculin was increased by 10% (Fig. 6D). However, expression of these proteins was decreased in MSCs treated with PKC inhibitor, and was similar to normal controls in cells treated with both PKC activator and inhibitor.

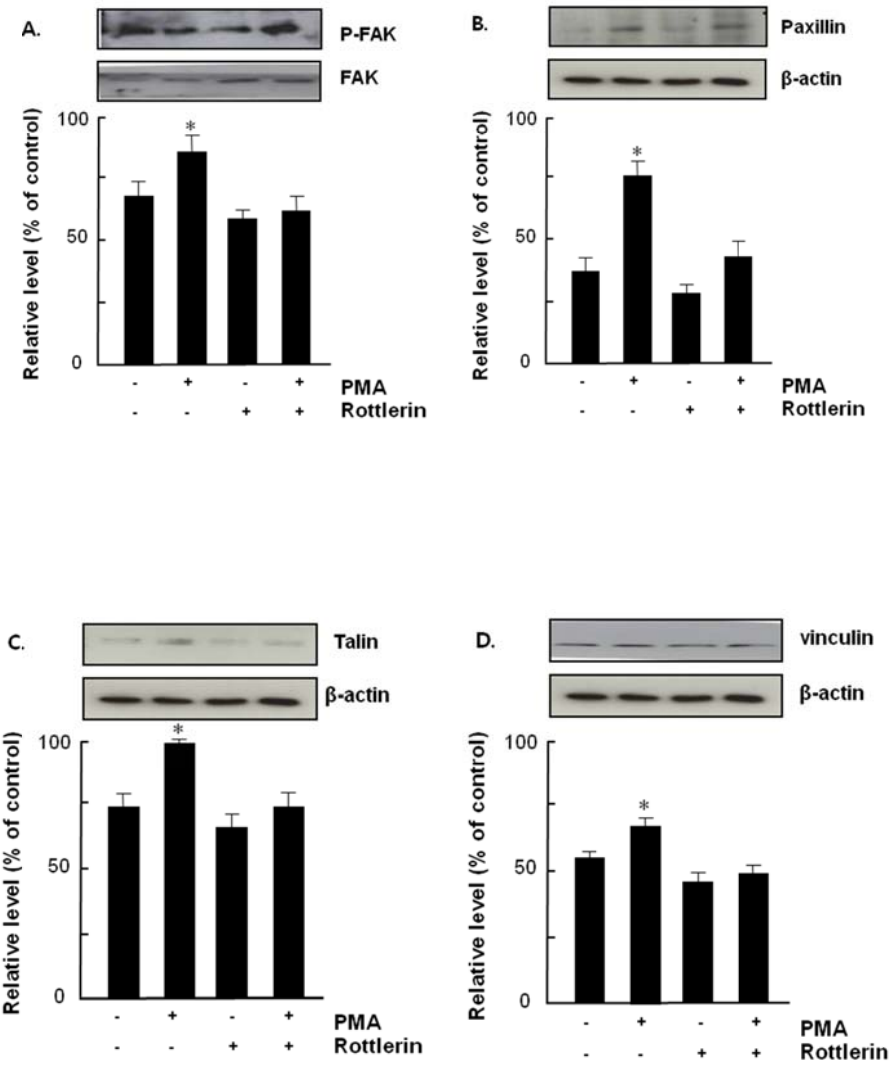

A.
P-FAK
FAK
Relative level (% of control)
100
50
0
*
- + - +
- - + +
PMA
Rottlerin
B.
Paxillin
β-actin
Relative level (% of control)
100
50
0
*
- + - +
- - + +
PMA
Rottlerin
C.
Talin
β-actin
Relative level (% of control)
100
50
0
*
- + - +
- - + +
PMA
Rottlerin
D.
vinculin
β-actin
Relative level (% of control)
100
50
0
*
- + - +
- - + +
PMA
Rottlerin

Figure 6. Expression of adhesion-related proteins in MSCs treated PKC activator. It was compared MSCs treated with PMA or Rottlerin with control cells treated with DMSO. Each panel shows western blot data for the expression of adhesion-related proteins. (A) MSCs treated with PMA expressed phosphorylated-FAK. Densitometric analysis was performed and the ratio of the expression level was calculated relative to that of the non-phosphorylated form. MSCs treated with PMA showed higher expression of paxillin (B), talin (C), vinculin (D) than the other groups. Densitometric analysis was performed and the ratio of the band intensity of each protein was calculated relative to that of β-actin. $*P < 0.05$ vs. control.

7. Altered expression of small GTPase-proteins in MSCs treated PKC regulators.

To investigate the activity of the Rho GTPases, we measured expression of Rac-1. The transcript level of Rac-1 was increased in MSCs treated with both PKC activators and decreased in cells treated with the inhibitor. The transcription level of Rac-1 was increased 53% in MSCs treated with PMA (Fig. 7). When MSCs were treated with both activator and inhibitor, Rac-1 expression was similar to that of control cells.

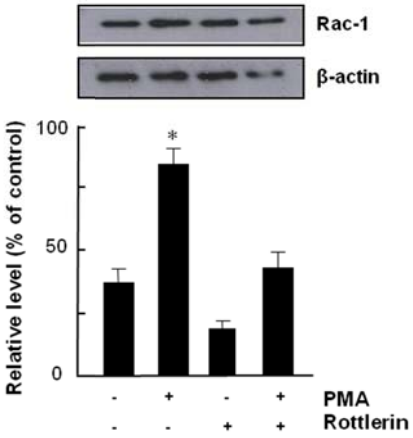


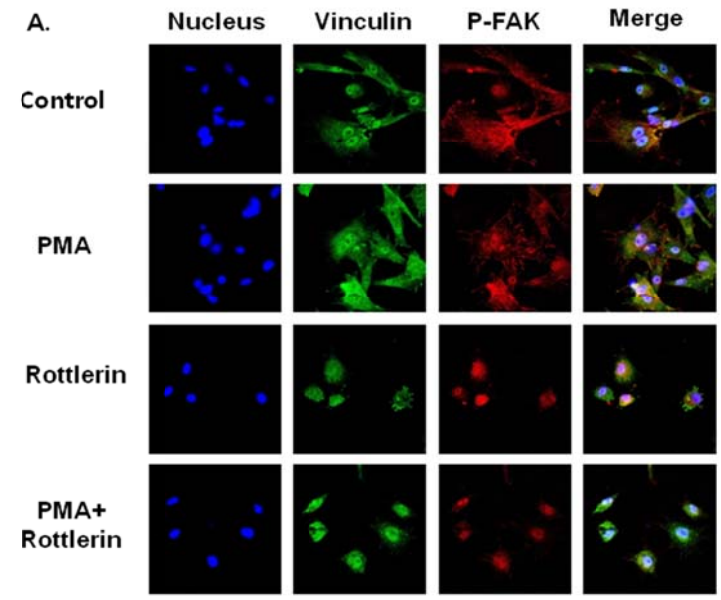
Figure 7. Expression of small-GTPase proteins in MSCs treated PKC activator. To confirm activity of the Rho GTPases, it was examined the expression of Rac-1. The transcript level of Rac-1 was increased in MSCs treated with both PKC activators, and decreased in MSCs treated with inhibitor. The transcription level of Rac-1 was increased 53% in MSCs treated with PMA. When MSCs were treated with both activator and inhibitor, the expression of Rac-1 was similar to that of control cells. *$P <$ 0.05 vs. control.

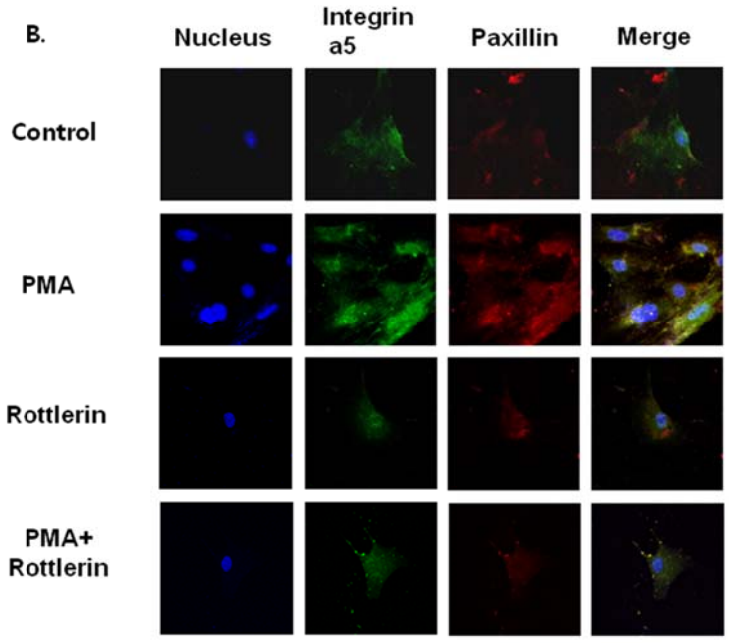
8. Expression both adhesion related molecules and stress fiber colocalization site on MSCs treated PKC regulators

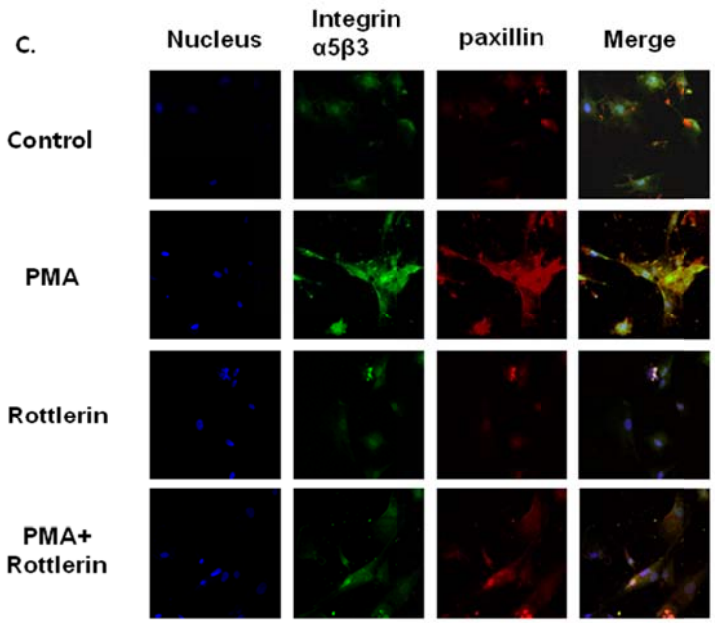
It next tested whether adhesion molecules co-localized within the cell using immunocytochemistry. Double-immunostaining showed co-localization of vinculin and P-FAK (Fig. 8A). It also showed co-localization of integrin α5 (α5β1) and paxillin (Fig. 8B), integrin α5β3 and paxilin (Fig. 8C), and integrin α5 (α5β1) and vinculin (Fig. 8D). MSCs treated with PMA exhibited more intense co-locaization than the control MSCs. The intensity of these sites in MSCs treated with the PKC inhibitor Rottlerin was lower than in control, whereas MSCs treated with both PKC activator and inhibitor showed similar signal intensity to the control.

During the first 3 hours of attachment, cells found their location and settled to adhere through actin stress fibers. Specific staining of several focal adhesion molecules and actin stress fibers was observed in the MSCs and

there was a distinct difference between control MSCs and MSCs treated with PMA.


A.
Nucleus
Vinculin
P-FAK
Merge
Control
PMA
Rottlerin
PMA+
Rottlerin


B.
Nucleus
Integrin
a5
Paxillin
Merge
Control
PMA
Rottlerin
PMA+
Rottlerin


C.
Nucleus
Integrin α5β3
paxillin
Merge
Control
PMA
Rottlerin
PMA+ Rottlerin

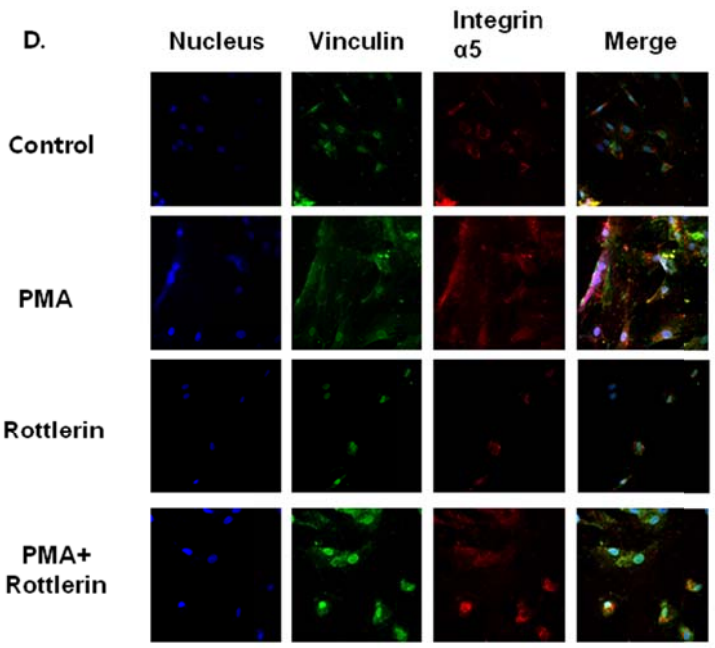

D.
Nucleus
Vinculin
Integrin α5
Merge
Control
PMA
Rottlerin
PMA+ Rottlerin

Figure 8. Intracellular localization of adhesion-related proteins in MSCs treated with PKC activator and inhibitor. (A) Immunofluorescence staining of vinculin and P-FAK. MSCs were stimulated with 100 nM PMA and 3 μM Rottlerin, fixed at each time point and stained with secondary anti-FITC, rhodamine, and DAPI. The localization of adhesion related -molecules and nuclei were observed using a confocal laser scanning microscope (LSM700, Carl Zeiss Inc, NY, USA). (B) Immunofluorescence staining of paxillin and integrin α5β1 showed that integrin α5 colocalized with paxillin in the cytosol. MSCs were stimulated, fixed at each time point and stained with secondary anti-FITC, rhodamine, and DAPI. The localization of adhesion related -molecules and nuclei were observed using a confocal laser scanning microscope LSM 700. (C) Immunofluorescence staining of paxillin and integrin α5β3. (D) Immunofluorescence staining of vinculin and Integrin α5.

9. Improvement of myocardial infarction by PKC activator-treated MSCs

This experiment showed the direct effects of injection of MSCs into the rat heart by measuring infarct size using Masson's trichrome staining. MSCs treated with PMA helped to rescue the infarct zone compared with the untreated myocardial infarction (MI) model. This observation was confirmed by comparing the fibrosis rate of the control MI model and the MI rats injected with PMA-treated MSCs (Fig. 9A, B) Using the fluorescence dye bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), It was observed that most of the cells treated with PKC activator were attached to the infarct border zone of the left ventricle. By Masson's trichrome staining, this study demonstrated that the PMA-treated MSCs reduced fibrosis and muscle damage. These results were further confirmed by histopathological examination of stained sections (Fig. 9C). The specimen of ischemic myocardium that was injected with PMA-treated MSCs showed decreased

fibrosis in the left ventricle compared with untreated specimen.

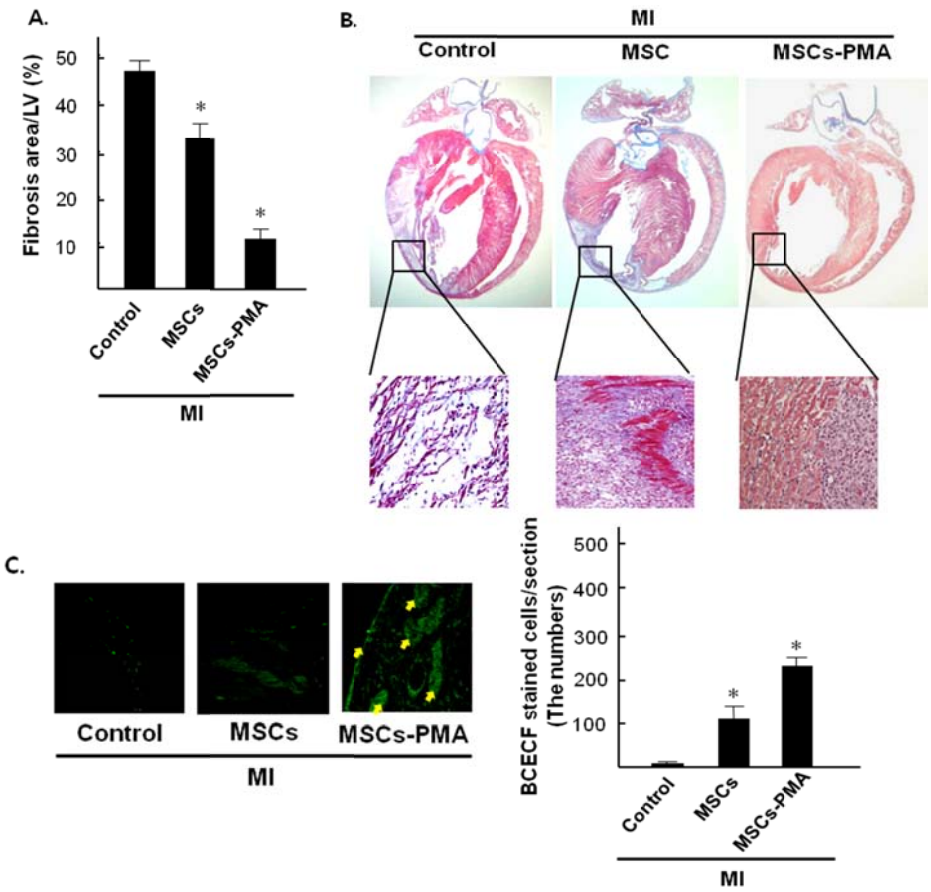


Figure 9. Implantation of MSCs treated with PKC activator into rat hearts. The heart LAD was ligated in a defined location and MSCs treated with PKC activators were injected into the heart. (A) Fibrosis in heart injected with MSCs treated with PMA (100 nM) (B) Left ventricle of each heart magnification: x40. (C) BCECF staining of heart sections. The areas marked by yellow arrows were confirmed by confocal microscopy. *P < 0.05 vs. control.

IV. DISCUSSION

In this study, it was investigated the modification of MSCs by pretreatment with PKC activator. Several studies using different systems have highlighted the importance of protein kinase C in integrin-mediated cell adhesion and spreading, as well as in cell migration, FAK phosphorylation and focal adhesion formation. Different approaches have been used to study the specific role of PKC in integrin signaling. PKC also appears to be a key intermediate between integrins and FAK signaling in muscle cells and other cell types. Several studies have indicated that PKC activation is required for FAK phosphorylation in cells. Although PKC and FAK co-localize at focal adhesion sites, the precise functional relationship between these two kinases is not known [6, 20, 22, 23, 26, 27].

This study provides evidence that key signaling small molecules can control factors downstream of the PKC pathway that may mediate cellular

changes. These results add to our understanding of the cellular components involved in the integrin-signaling cascade and the regulation of cellular adhesion attachment and spreading (Fig. 1-2).

To determine the importance of PKC activation in the adhesion process, it was studied MSCs treated with PKC activator and inhibitor. The adhesion rate of the cells treated with PKC activator was increased, whereas that of cells treated with PKC inhibitor was decreased, compared with the control MSCs. Moreover, when MSCs were treated with both activator and inhibitor, the adhesion rate was similar to that of controls, although the experiments did show minor differences (Fig. 3-4).

The extracellular matrix, or some of its components such as fibronectin and vitronectin, is known to be involved in cell growth and development [8]. For this reason, It was performed a 3 –D matrix experiment to demonstrate the functional effect of PKC activator on cells after culturing in *in vivo* conditions. Previous studies have shown that during in vivo cardiogenesis,

myofibrils are initially distributed in sparse, irregular myofibillar arrays, which gradually mature into parallel arrays of myofibrils and ultimately align into densely packed sarcomeres [6, 28]. In general, the adhesion rate in cardiogel was increased but in particular, cells treated with PKC activator showed a greater increase than the other samples (Fig. 5).

PMA is a nonphysiological activator of PKC. In previous reports, PMA has been shown to influence cell adhesiveness. Other studies report that ligand stimulation of tyrosine kinase growth-factor receptors can activate PKC in several cell types [29]. Therefore, it is possible that PKC activator will not only affect cell adhesion, but will also influence cell survival. Heidkamp et al reported increased cell survival downstream of activation of the MAPK cascades and demonstrated an increase in ERK1/2 activation in NRVMs after 8 hours of PKC epsilion (25moi) overexpression. Their studies examined ERK1/2 activation at 4, 8, 24 and 48 hours after PKC induction [30-31] and indicate that PKC signaling is related to cell survival. Because cell

survival is in turn related to cell adhesion, increased of cell survival is related to increased cell adhesion. Protein kinase C leves in the cell cytosol correlate with DAG-induced formation of FAK/Src signaling complexes important for regulating the adhesion machinery [26, 30, 31]. The integrin cytoplasmic domain plays a key role in focal contacts, and is involved in the activation of FAK. Integrins also bind the proteins paxillin and vinculin [28]. First, integrins in the extracellular matrix associated with paxillin and vinculin, then they crosslink actomyosin stress fibers and attach them to focal contacts [31]. Subsequently integrin clustering leads to association of the protein tyrosine-kinase Src and the adaptor protein p130as with the membrane, also associated with the focal contacts [18].

It was showed that modulation of the PKC signal in MSCs affected their adhesion. It was already known that PKC activation is significantly induced before FAK phosphorylation during adhesion [32-33]. Here, it was showed that activation of PKC by PMA induced increased adhesiveness of MSCs and

increased, protein synthesis of integrin (Fig 6).

In downstream signaling, RhoA is induced by PMA, suggesting that PMA plays a role upstream of the RhoA GTPase Rac in controlling cell adhesion [26]. Using immunoblotting it was showed that Rac-1expression was increased in the MSCs treated with PKC activator. In contrast, cells treated with the PKC inhibitor Rottlerin exhibited decreased Rac-1 expression [34].

RhoA GTPase has been shown to be one of the critical regulators of cell motility and cytoskeleton function. Previous studies, examined the role of, RhoA GTPase in the regulation of monocyte adhesion to vascular endothelium using monocytes pretreated with a 3-hydroxy-3-methylglutaryl coenzymeA reductase inhibitor or a statin [34]. Although a similar inhibitory action toward RhoA has been shown for statin, the mechanisms underlying the pyridines are not likely to inhibit intracellular cholesterol synthesis or the important intermediates required for the activation of small GTP proteins, including the RhoA GTPase Rac [34]. Instread of statin, it was used PKC

activator. Statin and PKC activator show similar activation of MSCs; they both trigger downstream activation of the Ras/MAP kinase and Rho/Rac pathways [16]. It has been demonstrated that Rho-family GTPases play important roles in the regulation of integrin clustering and the subsequent interaction of integin with focal adhesion molecules [35-36]. This explains the activation of the Rac-1 protein observed in PMA-treated MSCs (Fig. 7). Using immunofluorescence methods, it was demonstrated the co-localization of adhesion related molecules and stress fibers (Fig. 8). Moreover, vinculin and P-FAK showed co-localization indicating that it was received the phospho-energy to stress fibers (Fig. 8A). And stress fibers were regulated vinculin, paxillin and talin. Received the energy were transferred the fibers. It was grown up the adhesion. It was also showed co-localization of integrin and paxillin (Fig. 8B, 8C). MSCs treated with PMA showed increased co-localization compared with the control group, MSCs treated with inhibitor, or MSCs treated with both activator and inhibitor. It was confirmed integrin-

vinculin co-localization (Fig. 8D)

In our *in vivo* study, it was aimed to confirm survival of the modified MSCs that were treated with PKC activator in rat hearts. It was prepared slide sections, and examined the cell morphology and cell numbers by staining and microscopy. The staining dyes used were Masson's Trichrome stain and BCECF. BCECF-AM is commonly used to measure intracytoplasmic pH. To confirm the survival of modified MSCs in cardiomyocytes, it was injected cells that were treated with the fluorescent dye BCECF directly into the rat heart (Fig 9). By intracellular staining, it was possible to distinguish the MSCs in the rat heart [37-38].

V. CONCLUSION

In conclusion, activation of PKC induces cell adhesion, which in turn enhances PKC signaling and integrin signaling complexes, resulting in activation of FAK and its downstream pathways. Our findings might explain the relationship between activation of PKC by small molecule stimulation and cell adhesion as follows: formation of a complex of activated PKC/Integrin signal/FAK/Stress fibers may enhance cell adhesion through phosphorylation and activation of FAK. In an *in vivo* study, when the MSCs treated with PKC activator were injected into the MI rat heart, the cells showed effective adhesion in the border zone and association with myocytes. Targeted therapies that activate PKC in MSCs may improve the adhesion and survival of injected cells in the treatment of cardiac MI.

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Abstract (in Korea)

PKC활성화에 의한 골수유래 중간엽 줄기세포의 부착능 향상

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최 은 주

심근 손상의 복원을 위한 줄기 세포 치료는 세포 –이식 후 세포의 적은 부착성에 의해 치료에 있어 제한을 받는다. 본 연구에서는 중간엽 줄기세포의 임상적 이용을 증가시키는 방안을 연구하기 위하여 세포기능의 활성을 조절하는 매우 중요한 효소인 C형 단백질 인산화 효소(Protein Kinase C; PKC)의 활성화가 중간엽 줄기세포의 부착능에 미치는 연구를 진행했다. 세포의 부착과 확전은 활성

형 PKC, PKC 활성인자로 알려진 Phorbor easter (PMA)에 의하여 증가되는 반면, 세포의 분리는 PKC activator를 처리한 중간엽 줄기세포에서 유의하게 감소되었다. PKC 억제제인 Rottlerin을 PKC activator 와 같이 처리 하였을 때, PKC activator를 처리한 중간엽줄기세포의 부착 능력은 약간 줄어들었으며, 세포의 생존 능력 또한 PKC 억제제를 같이 처리 한 것에 비해, activator 를 처리한 줄기세포 쪽이 늘어나게 되었다. 세포 부착 능력을 확인 하기 위해 3D-matrix에 줄기세포를 배양하였다. PKC 활성제를 처리한 줄기세포의 수는 처리되지 않은 줄기세포와 PKC 억제제를 처리한 줄기세포에 비교하여 의미있게 증가하였다. Focal adhesion kinase (FAK)와 Paxillin, Vinculin, 과 Talin을 포함한 세포골격 연관 단백질들은 면역블롯검사와 면역세포화학 염색법을 통해 확인했다. 백서를 이용한 실험에서 좌심방 관상동맥을 묶음으로 생기는 심근허혈을 유도 한 후, 1 x 10^6 (20 μl PBS)의 중간엽 줄기세포를 이식했다. PKC activator를 처리한 중간엽 줄기세포를 이식한 경우,

이 그룹은 PKC activator를 처리하지 않은 그룹과 비교하여 허혈 심근에서 더 많은 숫자가 발견되었다. 이것은 형광 염색제인 bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)를 줄기세포에 염색을 시켜 현미경을 통해 육안으로 확인하였다. 따라서 섬유화와 경색 부위는 PKC 활성제를 처리한 줄기세포를 쥐의 심장에 주입했을 때, 중간엽 줄기세포만을 주입한 쥐의 심장보다 줄어들었다. 이러한 결과를 토대로 심근 재생과정에 중간엽 줄기세포의 부착능과 확전을 증가시키기 위하여 PKC의 활성이 중요한 요소로 작용하고 있음을 밝혔으며, 이는 허혈성 심장질환에서 중간엽 줄기세포의 세포치료 적용을 증가시키는 방법으로 유의성을 가질 것으로 사료된다.

핵심되는 말: 중간엽 줄기세포, 단백질 카이네이즈 C, 부착능, 심근 재생