Introduction of Integrin-Linked Kinase Gene into Mesenchymal Stem Cells for Prevention of Anoikis in Infarcted Myocardium

Suk-Won Song

Department of Medicine

The Graduate School, Yonsei University

Introduction of Integrin-Linked Kinase Gene into Mesenchymal Stem Cells for Prevention of Anoikis in Infarcted Myocardium

Directed by Professor Byung-Chul Chang

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

Suk-Won Song

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This certifies that the Doctoral Dissertation of Suk-Won Song is approved.

Thesis Supervisor: Byung-Chul Chang

Thesis Committee member#1: Yangsoo Jang

Thesis Committee member#2: Ki-Chul Hwang

Thesis Committee member#3: Myoung Hee Kim

Thesis Committee member#4: Seung Hwan Oh

The Graduate School

Yonsei University

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The last, I appreciate to my parents, my father-in-law, my mother-in-law, my wife, my lovely son, and daughter who encouraged me. I appreciate to God who blesses me.

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Mesenchymal stem cells (MSCs) represent a suitable source of autologous cells in cell therapy for the improvement of cardiac function, but MSCs therapy has limitations due to the poor viability of MSCs after cell transplantation. Integrin-mediated adhesion is a prerequisite for cell survival, and also a key factor for the differentiation of MSCs. As a novel anti-death strategy to improve cell survival in the infarcted heart, MSCs were genetically modified to over-express integrin-linked kinase (ILK). The survival rate of ILK-transfected

MSCs (ILK-MSCs) was augmented by about 1.5-fold. In addition, the phosphorylation of ERK1/2 and Akt in ILK-MSCs were increased by about 3- and 2-fold, respectively. Furthermore, ILK-MSCs resulted in increase of 2-fold in ratio of Bcl-2/Bax and inhibited caspase-3 activation, compared with hypoxic MSCs. Adhesion rate of ILK-MSCs also had a 30.0% increase on the cardiac fibroblast-derived 3D matrix. Moreover, ILK-MSCs showed higher retention by about 4-fold than MSCs in infarcted myocardium. *In vivo*, ILK-MSC transplanted rats had a 12.0% smaller infarct size than MSC-treated animals after ligation of left anterior descending coronary artery. Transplantation of ILK-MSCs not only led to a 15.5% decrease in the fibrotic heart area, but also significantly reduced the apoptotic positive index by about 17.0% compared with ligation only. The mean microvessel count per filed in the infarcted myocardium of ILK-MSCs group (129.1±23.5) was increased relative to sham group (19.3±15.2) and MSCs group (68.9±19.4). In conclusion, ILK-MSCs further assisted cell survival, proliferation and adhesion, and improved myocardial damage compared to MSC only after transplantation.

Key words: Integrin-linked kinase, Mesenchymal stem cell, Myocardial infarction

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I. INTRODUCTION

As a consequence of myocardial infarction, irreversible tissue damage and deficit of performance occurs to heart[1]. The remaining myocytes in infarcted tissues undergo progressive replacement by fibroblasts to form scar tissue and may lead to remodeling of the left ventricle as a result of increasing mechanical wall stress[2]. By alternative sources of cardiomyocytes, recent attempts to repair infarcted hearts revealed that myogenic cells from various stem cells replaced residence in cardiac tissue after injury[3]. Especially, mesenchymal stem cells (MSCs) have become one of the most potentiated cell sources for infarcted

myocardium, because MSCs are multipotent cells capable of differentiating into cardiac myocytes, endothelial cells, and vascular smooth muscle cells, both *in vivo* and *in vitro*, under appropriate culture conditions[4,5,6]. Although MSCs represent a suitable source of autologous cells in cell therapy for improvement in cardiac function, MSCs therapy has limitations due to their poor viability after cell transplantation[7].

This may require anti-death strategies to improve stem cell survival/number in the infarcted heart[8]. The absence of adhesion is probably the main cause of poor cell survival in cell transplantation. Cell adhesion to the extracellular matrix (ECM) is mediated by integrin, which control a variety of important processes including gene expression, proliferation, differentiation and even survival[9-13]. Integrin-linked kinase (ILK) is a 59-kDa Ser/Thr kinase that binds to the cytoplasmic domain of β-integrin and participates in the regulation of cell adhesion, growth, cell shape change, and ECM assembly as an upstream of Akt/PKB and MAP kinase[14-16].

In this study, ILK gene was transfected into MSCs to enhance adhesion after implantation and thereby was investigated the adhesion to 3D culture system derived from cardiac fibroblast and viability under hypoxic condition. Moreover, MSCs harboring ILK gene were examined in the infarcted myocardium whether implanted cells have changed physiologically and morphologically.

II. MATERIALS AND METHODS

1. Isolation and Culture of MSCs

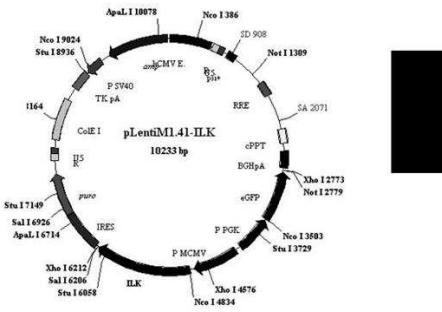
MSCs were isolated from the femoral and tibial bones of rats. Bone marrow-derived MSCs were collected from the aspirates of the femurs and tibias of 4-week-old male Sprague-Dawley rats (approximately 100 g) with 10 ml of MSC medium consisting of Dulbecco's modified Eagle's medium (DMEM)-low glucose,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

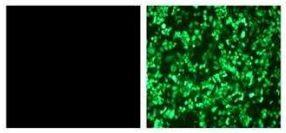
with the CD34-negative fraction being further cultured. The cells were harvested after incubation with 0.25% trypsin and 1 mM EDTA for 5 min at 37°C, replated in 1×10^5/100-cm^2 plates, and again grown for approximately 10 days.

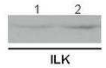
2. LentiV-mediated, Stable Genetic Mmodification of MSCs

For stable genetic modification, we used the lentiviral vector (LentiV) for transgene delivery. The lentiviral vector for expressing ILK was constructed by inserting the gene fragment into *Eco*RI- *Cla*I site of Lentiviral vector (Macrogen Inc., Seoul, Korea, http://www.macrogen.co.kr). The ILK(BC001554) cDNA clone was purchased from KOBIC (Korean Bioinformaion Center) and cDNA was amplified using a following primer set :Forward: 5'-gggaattcgccaccatggacgacatt-3', Reverse: 5'-attatcgattcctacttgtc -3'. The LentiM1.41 lentiviral vector was designed to produce interesting gene promoted from mCMV promoter and to express GFP from pgk promoter. The nucleotide sequences of the constructs were verified by sequencing analysis. The recombinant lentiviral vectors were purchase from Macrogen Inc. as described earlier. Briefly, three plasmids, a transfer vector, a VSV-G expression vector, and a *gag-pol* expression vector, were co-transfected into 293T cells at a 1:1:1 molar ratio by using LIPOFECTAMINE PLUS™ (Invitrogen, Carlsbad, USA, http://www.invitrogen.com). The culture supernatant containing viral

vector particles was harvested 48 hrs after transfection, clarified with a 0.45 μM membrane filter (Nalgene Labware, Rochester, USA, http://www.nalgenelabware.com), and stored at -70^{o}C deep-freezer immediately. Titers were determined by p24 ELISA or infection into HeLa cells. The eGFP expression of transduced cells was observed and photographed under fluorescence microscope; in routine preparation, the titer was approximately 1×10^{7} transduction unit (TU) per without further concentration. MSCs were transduced by adding purified LentiVs to cells with 8 μg/ml polybrene to facilitate transduction for at least 9 hrs (Fig. 1).







Lane 1: MSC cell
Lane 2: Lenti-ILK transduced MSC cell

Figure 1. Construction and transduction activity of ILK-lentiviral vector in MSCs. (A) Structure of ILK-Lentiviral vectors (B) Expression levels of cellular ILK were determined by immunofluorescence image and RT-PCR. Immunofluorescence image with green fluorescence indicated positive MSCs transfected with ILK. The mRNA expression of ILK was established by DNA electrophoresis and visualized by EtBr.

3. Immunoblot Analysis

Cells were washed once in PBS and lysed in a lysis buffer (Cell Signaling Technology, Beverly, USA, http://www. cellsignal.com) 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, USA, http://www.bio-rad.com). Proteins were separated in a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, USA, http://www.millipore.com). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5% nonfat dried milk for 1 hr at room temperature, the membrane was washed twice with TBS-T and incubated with primary antibody for 1 hr at room temperature or overnight at 4°C. The membrane was washed

three times with TBS-T for 10 min and then incubated for 1 hr at room temperature with horseradish peroxidase-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Santa Cruz, USA, http://www.scbt.com). The band intensities were quantified using NIH ImageJ version 1.34e software (http://www.rsbweb.nih.gov/ij/).

4. Cell Proliferation

Cellular proliferation was measured with the PreMix WST-1 Cell Proliferation Assay System (TAKARA BIO Inc., Shiga, Japan, http://www.takara-bio.com). This system enables the measurement of cell proliferation with a colorimetric assay, based on the cleavage of the slightly red tetrazolium salt (WST-1) by mitochondrial succinate-tetrazolium reductase in viable cells. An increase in enzyme activity leads to an increase in the production of formazan dye, and so the quantity of formazan dye is related directly to the number of metabolically active cells in the medium. Cells (2×10^4) were seeded into wells of a 96-well culture plate and incubated under hypoxic conditions after transduction of lentiviral vector. WST-1 cell proliferation reagent was added directly to the supernatant (10 μl/100 μl growth medium), and incubated at 37ºC for 3 hrs. The absorbance of the solubilized dark red formazan product was then determined at 450 nm.

5. Measurement of Caspase 3 Activity

Relative caspase 3 activity was determined using ApopTarget^{TM} Capase-3 Colorimetric Protease Assay, according to manufacturer's instructions (BioSource International Inc., Grand Island, USA, http://www. invitrogen.com). This assay is based on the generation of free DEVD-pNA chromophore when the provided substrate is cleaved by caspase-3. Upon cleavage of the substrate by Caspase-3, free pNA light absorbance can be quantified using a microplate reader at 405 nm. Briefly, the cultured MSCs (2×10^6) after different treatments were harvested in lysis buffer (1 M DTT), and cell extracts were centrifuged to eliminate cellular debris. Aliquots (50 µl) of the cell extracts were incubated at 37°C for 2 hrs in the presence of the chromophore substrate. Free DEVD-pNA is determined colometrically. The comparison of absorbance of pNA from apoptotic sample with uninduced control allows determination of the fold increase in Capase-3 activity.

6. Preparation of Cardiac Fibroblast-derived Three-dimensional Matrix (Cardiogel)

Cardiogel was prepared with a minor modification[17]. Briefly, 2×10^5 cells per 35-mm dish were seeded and the medium changed every 48 hrs until the matrix was denuded of cells. The medium was carefully aspirated and rinsed gently with PBS. Next, 1 ml of prewarmed 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 min 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.

7. Assay for Cell Adhesion

Cells were isolated by trypsinization and were washed once in DMEM with 10% FBS to stop trypsin activity and twice with serum-free DMEM to remove serum components. Suspensions of 2×10^4 viable MSCs were then added to each well and allowed to attach for 30 min at 37°C 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 using a hemacytometer. Each experiment was performed in triplicate wells and repeated at least three times.

8. Induction of Myocardial Infarction and Transplantation

All experimental procedures for animal studies were approved by the

Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine, and performed in accordance with the Committee's Guidelines and Regulations for Animal Care. Myocardial infarction was produced in male Sprague-Dawley rats (200±30 g) by surgical occlusion of the left anterior descending (LAD) coronary artery, according to previously described procedures[17]. Briefly, after induction of anesthesia with ketamine (10 mg/kg) and xylazine (5 mg/kg), cutting the third and fourth ribs opened the chest, and the heart was exteriorized through the intercostal space. The left coronary artery was ligated 2–3 mm from its origin with a 5-0 prolene suture (Ethicon, Somerville, USA, http://www.ethicon.com) for 3 days. For transplantation, cells were suspended in 10 μl of serum-free medium (1×10^6 cells) and injected from the injured region to the border using a Hamilton syringe (Hamilton Co., Reno, USA, http://www.hamiltoncompany.com) with a 30-gauge needle. Throughout the operation, animals were ventilated with 95% O_2 and 5% CO_2 using a Harvard ventilator. Operative mortality was 10% within 48 hrs. Six animals per group (ligation, MSCs, ILK-MSCs) were used for morphologic analysis after occlusion of left coronary artery. To label MSCs with DAPI for viable cells, sterile DAPI solution was added into the culture medium on the day of implantation at a final concentration of 50 μg/ml. The dye was allowed to remain in the culture dishes for 30 min. The cells were rinsed six times in PBS to remove all excess and unbound DAPI. Labeled cells

were then detached with 0.25% (wt/vol) trypsin and suspended in serum-free medium for grafting.

9. Histology and Determination of Fibrosis Area

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

10. Terminal Deoxynucleotidyl Transferase–mediated dUTP Nick-end Labeling (TUNEL) Assay

The TUNEL Assay was performed according to the instructions of the

manufacturer (Chemicon International Inc. Temecula, USA, http://www.millipore.com). In brief, the excised heart tissues were fixed in 3.7% buffered formaldehyde and embedded in paraffin. Tissue sections, 5 μm thick, were deparaffinized, rehydrated and rinsed with PBS. A positive control sample was prepared from normal heart section by treating with DNase I (10 U/ml, 10 min at room temperature). The sections were pretreated with 3.0% H_2O_2, subjected to the reaction with TdT enzyme for 37°C for 1 hr and incubated digoxigenin-conjugated nucleotide substrate at 37°C for 30 min. Nuclei exhibiting DNA fragmentation were performed by 3,3-diamino benzidine (DAB) (Vector Laboratories, Burlingame, USA, http://www.vectorlab.com) for 5 min. The nuclei of apoptotic cardiomyocytes was stained dark brown. Lastly, the sections were counterstained with methyl green and then cover slipped. The sections were observed by light microscopy. 6 slices per group were prepared, 10 different regions were observed in each slice (× 400).

11. Determination of Infarct Size

TTC staining was used to assess myocardial tissue viability and determine myocardial infarct size. The tissue slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, St. Louis, USA, http://www.sigma.com) solution, pH 7.4, at 37°C for 20 min. Tissues were fixed in 10% PBS-buffered formalin overnight at 2~8°C. Hearts were sectioned transaxially,

and size of MI was evaluated as percentage of sectional area of infarcted tissue of the left ventricle to the sectional area of the whole left ventricle. Both sides of each TTC-stained tissue slice were photographed with the digital camera.

12. Histological Analysis

Histological analysis was performed according to the instructions of the manufacturer (R.T.U VECTASTAIN Universal Quick Kit, Vector Laboratories). In brief, the excised heart tissues were fixed in 3.7% buffered formaldehyde and embedded in paraffin. Tissue sections, 5 μm thick, were deparaffinized, rehydrated and rinsed with PBS. Sodium citrate antigen retrieval was experimented with 10 mM sodium citrate (pH6.0) in microwave for 10 min. Sections were incubated in 3% H_2O_2 in order to quench endogenous peroxidase. Sample was blocked in 2.5% normal horse serum, and incubated in primary antibody. Biotinylated pan-specific universal secondary antibody and streptavidin/peroxidase complex reagent was treated with heart section. Using DAB substrate kit, heart section was stained with antibody. Counterstain was operated in 1% methyl green and dehydration was progressed with 100% N-butanol, ethanol and xylene.

13. Statistical Analysis

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

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

III. RESULTS

1. ILK Enhances Survival of MSCs in Hypoxic Condition

Western blot analysis was used to characterize the endogenous expression of ILK in MSCs under hypoxic conditions. Endogenous ILK expression in MSCs was slightly down-regulated during 6 hrs after hypoxic stress, and then it was decreased rapidly (Fig. 2A). To define the effect of ILK on the MSCs survival under hypoxic condition, cells were transfected with lenti-ILK at normal culture conditions. The plates were incubated under hypoxic conditions for 9 hrs. Then cell viability was determined by WST-1 assay. As shown in Fig. 2B, the survival rates of MSCs incubated in hypoxic condition was reduced of 50%, compared with MSCs cultured in normoxic condition. However, lenti-ILK transfected MSCs shows that reduction of survival rate was attenuated about 0.5-fold, compared with non-treated MSCs.

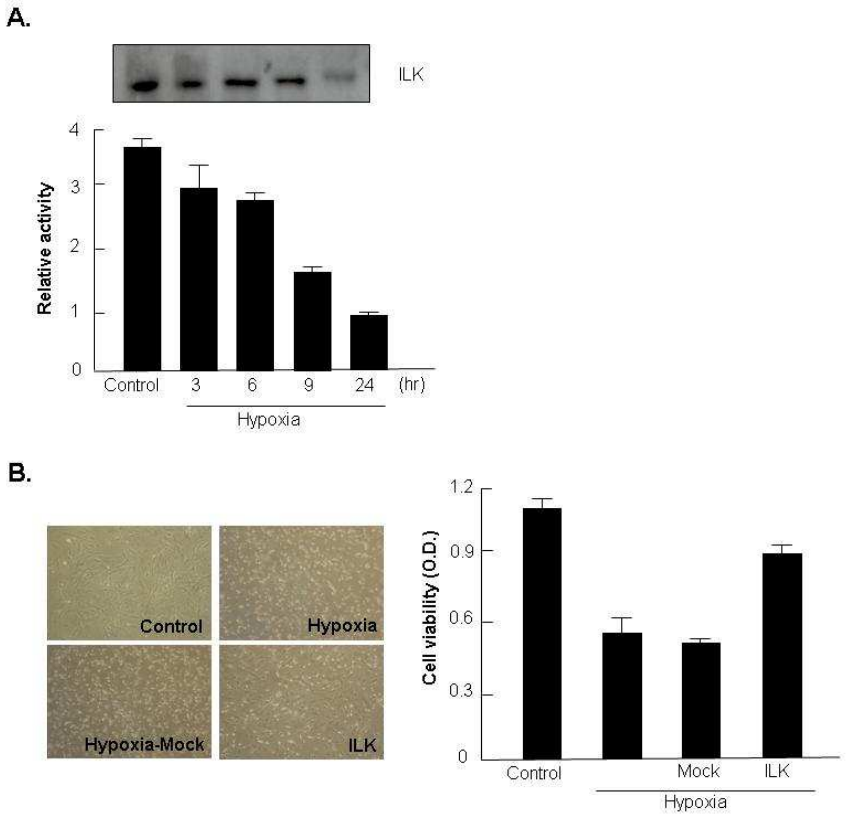


Figure 2. Effect of ILK expression on survival of MSCs under hypoxic condition. (A) Expression of ILK in MSCs under hypoxic condition. At the indicated times, cells were harvested. The expressions of each protein were monitored by SDS-PAGE followed by immunoblot analysis. The band intensities were quantified using the Photo-Image System and each point represented the mean value obtained in 3 experiments (p<0.001). (B) Representative microphotographs of MSCs, Lenti- (Mock) or Lenti-ILK-MSCs under hypoxic condition for 9 hrs. MSCs, Lenti- (Mock) or Lenti-ILK-MSCs (1.5×10^4) were seeded in 96-well culture plates and cultured for 24 hrs. After 24 hrs exposure to hypoxia and serum starvation (1% FBS), WST-1 reagent was

added to each well and incubated for 3 hrs at 37 ℃. Cell proliferation was measured by spectrophotometry (λ = 450 nm) ($p < 0.001$).

2. Effect of ILK on Survival Signaling of MSCs Under Hypoxic Condition

During ischemia, multiple changes such as cell adhesion and so on, contribute to weakening survival signaling molecules. To study the effect of ILK expression on intracellular survival signaling molecules level, we detected phosphorylation of ERK1/2 and Akt by immunoblot assay. Endogenous ILK expression in MSCs was down-regulated after hypoxic stress but ILK of MSCs transfected with lenti-ILK increased significantly by 2-fold compared with hypoxic control. As shown in Fig. 3, the phosphorylation activities of both ERK1/2 and Akt were dramatically decreased in hypoxic MSCs compared with normal cells. Hypoxic MSCs transfected with lenti-ILK led to increase of 3.0- and 2.0-folds in ERK1/2 and Akt activities respectively.

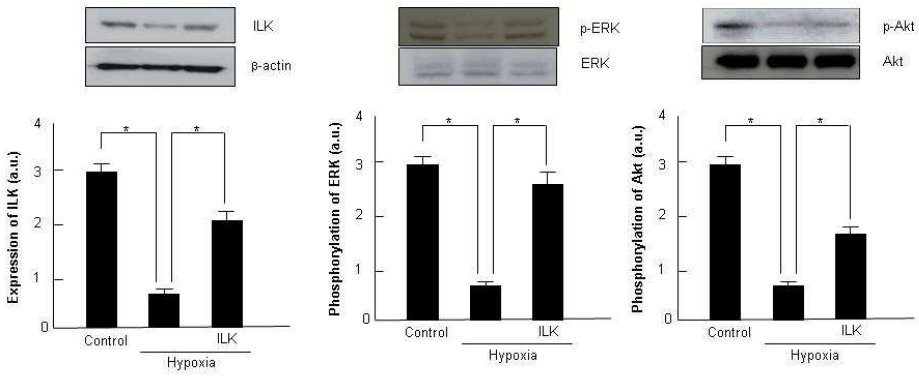


Figure 3. Effect of ILK on activity of proteins related to survival. Phosphorylation of ERK and Akt was enhanced at ILK-MSCs as compared to MSCs. MSCs or ILK-MSCs (1×10^6) were plated and incubated under hypoxic condition for 9 hrs. The phosphorylated states or total expressions of each protein were monitored by SDS-PAGE followed by immunoblot analysis with antibodies specific for the ILK, ERK1/2, p-ERK1/2, Akt, p-Akt and β-actin, respectively. The band intensities were quantified using the Photo-Image System and each point represented the mean value obtained in 3 experiments (*$p < 0.001$).

3. Effect of ILK on Hypoxia-induced MSCs Anoikis

To examine whether ILK expression of MSCs inhibits apoptosis in hypoxic condition, ratio of Bcl-2/Bax and caspase-3 activation were examined. Generally, PKB/Akt inhibits apoptosis through several pathways, resulting in

the downstream inhibition of the processing and activation of caspases. As shown in Fig. 4A, caspase-3 activities were increased for 3-folds after hypoxia treatment of cells compared with normal control. In contrast, ILK overexpression significantly reduced caspase-3 activity by 45% compared with hypoxic control. Hypoxia induced the decreased ratio of Bcl-2/Bax than normal condition but the transfection with lenti-ILK resulted in increase of 2-folds in ratio of Bcl-2/Bax compared with hypoxic condition (Fig. 4B).

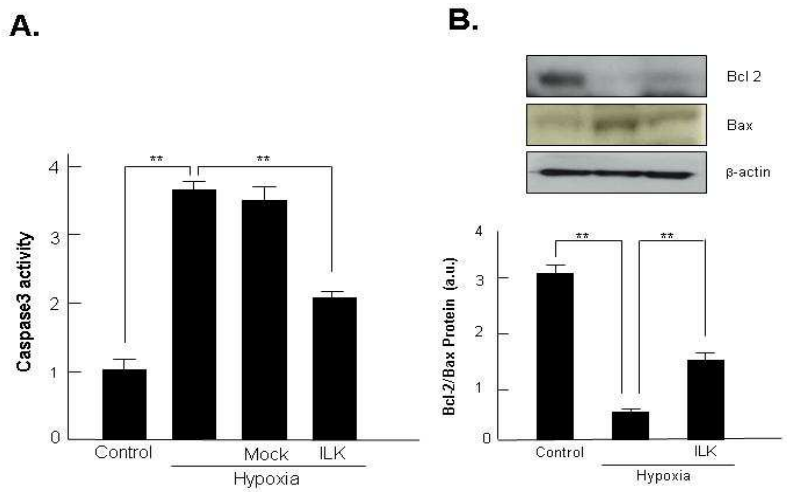


Figure 4. Effect of ILK on expression and activation of apoptosis-related proteins. (A) Relative caspase 3 activity was determined using the ApopTarget^{TM} Capase-3 Colorimetric Protease Assay. Data denote

means ± S.E.M. of 3 to 5 replicate measurements in three different cell cultures (**$p < 0.001$). (B) Expression of Bcl-2 and Bax was determined by immunoblot analysis in ILK-MSCs or MSCs under hypoxic condition for 9 hrs. At the indicated times, cells were harvested. The expressions of each protein were monitored by SDS-PAGE followed by immunoblot analysis with antibodies specific for the Bcl-2, Bax and β-actin, respectively. The band intensities were quantified using the Photo-Image System and each point represented the mean value obtained in 3 experiments (**$p < 0.01$).

4. ILK-mediated Adhesion of MSCs

To determine the effect of overexpressed ILK in adhesion of MSCs, we performed quantitative adhesion assays with ILK-MSCs. As shown in Fig. 5A, adhesion of MSCs to 3D culture system derived from cardiac fibroblast was enhanced approximately 30% by ILK introduction. To further evaluate the adhesive function of ILK in vivo, we transplanted ILK-MSCs into the border region between the infarcted area and the normal area. After 3 days, we dissected implants of infarcted myocardium by 2-μm thickness. ILK-MSCs were retained in approximately 800 cells per section compared with MSCs only in approximately 200 cells per section (Fig. 5B).

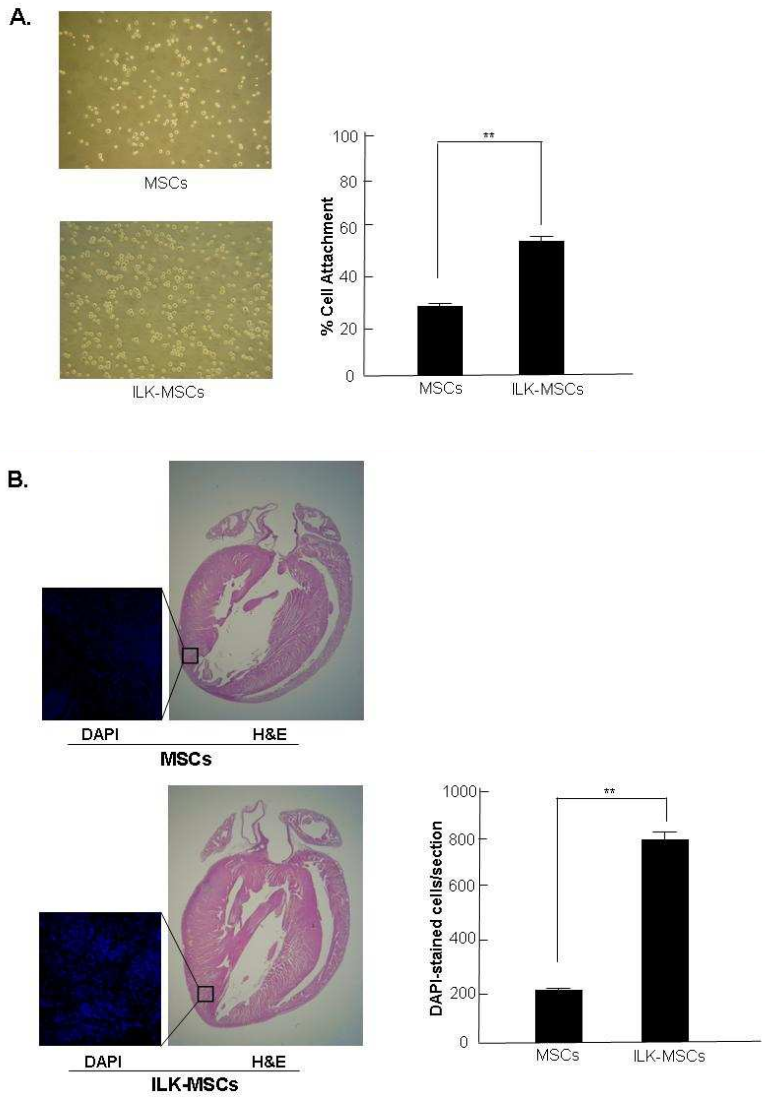


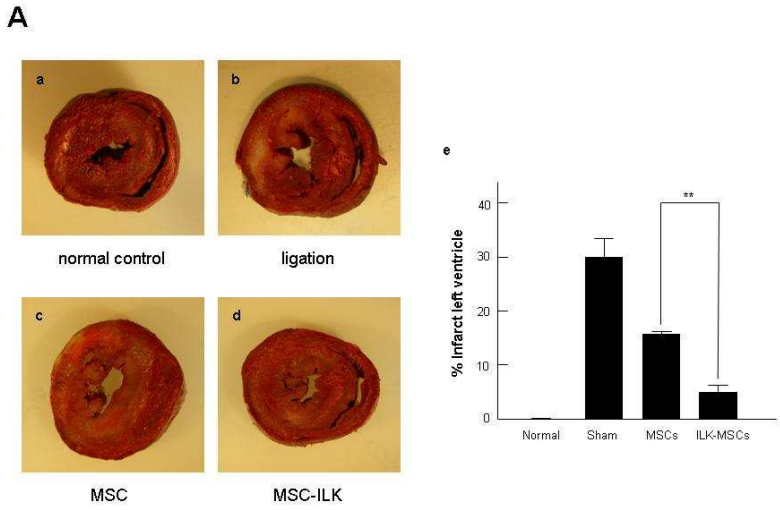
Figure 5. Effect of ILK on adhesion of MSCs. (A) ILK-MSCs were plated on cardiogel for 3 hrs. Data were expressed as percentage of supplement of 2 X 10^4 cells/well, which represented 100% and represented the mean value obtained in 3 experiments done in duplicate. The mean attachment values ± S.D. on

cardiogel were 6102 ± 98 in MSCs and 12541 ± 120 in ILK-MSCs (**$p < 0.01$). (B) Analysis of myocardial repair after implantation into infarcted myocardium. Engraftment was significantly improved with ILK overexpression at 3 days after injection of cells (1×10^6). (**$p < 0.01$)

5. Intramyocardial Injection of ILK-MSCs Limits Infarct Size and Improves Ventricular Function after Acute MI

To examine the in vivo relevance of our in vitro findings, we studied the direct effects of enhanced adhesion of MSCs on LAD ligated rat hearts. The effect of ILK-MSCs on myocardial injury after infarction was evaluated by triphenyl tetrazolium chloride (TTC) and trichrome staining. We determined infarct size in the left ventricle (LV) using TTC staining (n = 6 rat/group). In the PBS-injected rats, infarct size was 29% of the LV, comparable with normal rat. Injection of MSC resulted in a significant decrease in infarct size (17%), and injection of MSC-ILK further reduced the infarct size (5%) (Fig. 6A). The only ligated hearts showed significant interstitial fibrosis (18±3.2%) compared with the control hearts (1.0±0.6%). In contrast, transplantation of ILK-MSCs significantly decreased interstitial fibrosis to 2.5±1.0%. Namely, the specimen of ILK-MSCs injected-ischemic myocardium showed decreased fibrosis in left ventricle and wall is thicker than ligation control (Fig. 6B). A TUNEL assay was used to identify the percentage of apoptotic cell in the cardiac tissue because apoptosis has a major role in cell loss in myocardial infarction. The

incidence of TUNEL-positive myocardial cells caused by ligation was significantly reduced by 17% in the ligated hearts transplanted with ILK-MSCs compared with that of sharm and MSCs (Fig. 6C). The mean microvessel count per field in the infarcted myocardium was significantly higher in the ILK-MSC group than in the sham and MSCs group (Sham: 19.3±15.2; MSCs: 68.9±19.4; ILK-MSCs: 129.1±23.5) (Fig. 6D).



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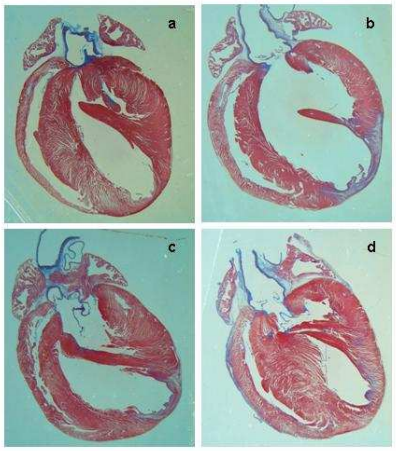
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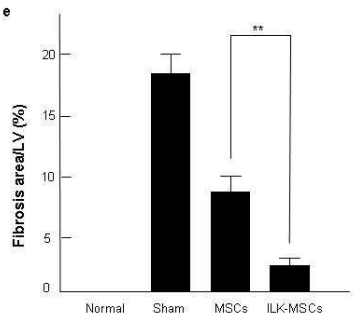
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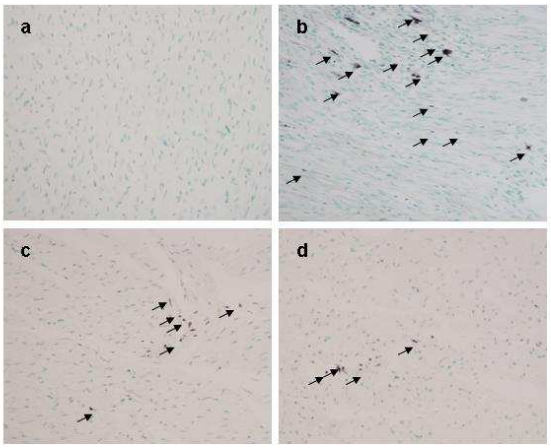
Fibrosis area/LV (%)
20
15
10
5
0
**
Normal
Sham
MSCs
ILK-MSCs

C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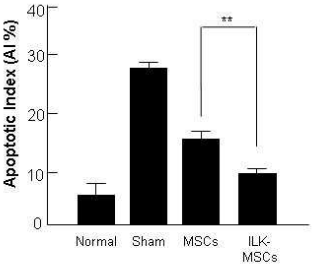
Apoptotic Index (AI %)
40
30
20
10
0
**
Normal
Sham
MSCs
ILK-MSCs

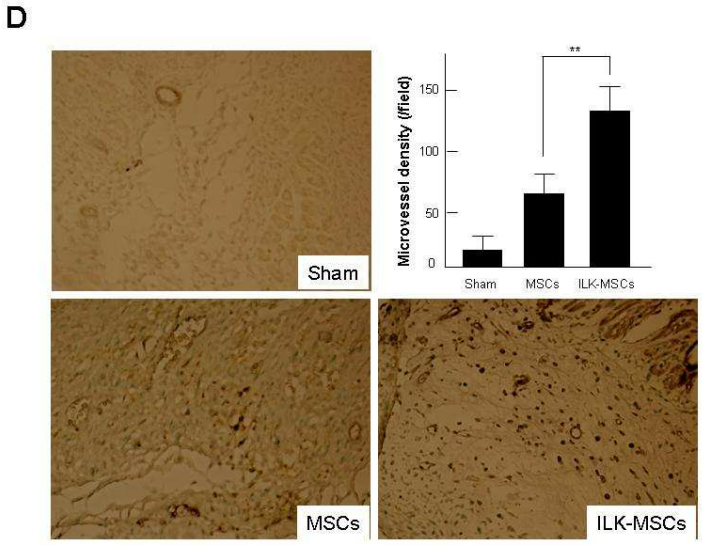

Figure 6. Effect of ILK-MSCs on infarct size, myocardial apoptosis and microvessel density. (A) Intramyocardial injection of MSC reduced left ventricular (LV) infarct size as assessed by TTC staining at 1 week post-MI. Cross sections showing greater infarct (yellow-white) area in the PBS-injected heart. Injection of MSC reduced the infarcted area, and MSC-ILK further decreased the area of necrotic tissue. Each value is the mean ± SEM of six independent experiments (**p < 0.01). (B) LAD of four hearts was ligated at equal locations. At 1 week after injection of cells, hearts were isolated and cut lengthways. The left panel shows representative Masson's trichrome images from histological sections, and the right histogram shows less fibrosis (blue) in normal, saline treated-ligated heart, MSCs-transplanted ligated heart and ILK-MSCs-transplanted ligated heart groups. Each value is the mean ± SEM of six independent experiments (**p < 0.01). (C) An apoptosis assay was performed in heart tissue 2 weeks after reperfusion. The left panel shows representative images of TUNEL staining (magnification: ×200). Staining for normal nuclei (green) was carried out using methyl green, and apoptotic nuclei

were stained brown. The right panel shows summarized data for TUNEL staining in normal, saline treated-ligated heart, MSCs-transplanted ligated heart and ILK-MSCs-transplanted ligated heart groups. Each value is the mean ± SEM of six independent experiments (**$p < 0.05$). (D) Quantitative analysis showed that microvessel density was significantly higher in ILK-MSCs groups than in MSC and sham groups (**$p < 0.01$).

IV. DISCUSSION

In this paper, it has established that the level of ILK expression affects cell adhesion and such adhesion related modification of mesenchymal stem cells (MSCs) with ILK enhances the survival rate of transplant-modified MSCs in infarcted myocardium by LAD ligation. Cell implantation utilizing cardiac stem cells[18], embryonic stem cells[19], hematopoietic stem cells[20], skeletal myoblasts[21-23], and cardiac myocytes (adult, fetal, or neonatal myocytes)[24, 25] has been suggested as a promising clinical approach for restoration of cardiac infarction. Among these various candidate cell types, MSCs have a great advantage to generate functional cardiac myocytes in the infarcted myocardium because of the easy preparation and multilineage potential to differentiate to cartilage, bone, muscle, tendon, ligament and adipose tissue[26, 27], both *in vivo* and *in vitro*, under appropriate culture conditions. Although MSCs represent a suitable source of autologous cells in cell therapy for improvement in cardiac function, MSCs therapy has limitations due to their poor viability after cell transplantation[7]. Thus, it is necessary to enhance MSCs against anoikis incurred from hypoxic condition through genetic modification in order to improve the efficacy of cell therapy. The absence of adhesion is probably the main cause of poor cell survival in cell transplantation, based on our previous study[17]. This study showed prevention of anoikis might enhance cell survival and adhesion

under hypoxic condtion (Fig. 2 and 5).

Cell-extracellular matrix (ECM) adhesion is crucial for control of cell behavior. Adhesion of cells to ECM stimulates signal transduction cascades including proliferation, migration, differentiation, and even survival[28-31]. Especially, anchorage-dependent cells depend upon interactions between integrins and the extracellular matrix for survival. Namely, the interaction of epithelial and endothelial cells with ECM inhibits default apoptotic pathways, which become activated if cell-ECM interactions are disrupted, and this type of suspension-induced apoptosis is termed anoikis[32, 33].

Integrins are a family of heterodimeric, transmembrane glycoproteins mediating cell-ECM interactions and connection between integrins and the ECM takes place at focal adhesions (FA). At the molecular level, most of the integrin-associated molecule in FA are multifunctional and serve as scaffolds for the attachment of enzymes such as kinases and phosphatases that modify and regulate these complex interactions. One of these enzymes is integrin-linked kinase (ILK) which interacts with the β1-subunit of integrin and plays a crucial role in integrin-mediated cell adhesion and signaling[34-36]. The kinase activity of ILK can be modulated by the interaction of cells with components of the extracellular matrix or by integrin clustering and growth factors. It binds to PINCH by way of its N-terminal ANK domain[37-39] and α- or β-Parvin, members of the CH-ILKBP/α-Parvin/actopaxin/affixin protein

family[40-47], by way of its C-terminal domain, resulting in the formation of a ternary complex within cells. Recent work has shown that ILK, PINCH, and Parvin are recruited to cell-ECM sites as a preassembled complex, indicating that the formation of the complex precedes the integrin-mediated cell adhesion and spreading. The assembly of the PINCH-ILK-Parvin complex is known to be regulated by two main signaling pathways, although other levels of regulation may be also involved. First, the complex formation is regulated by the protein kinase C (PKC) signaling pathway. Inhibition of PKC leads to down- regulation of the assembled complex, suggesting that the PINCH-ILK-Parvin complex is an important downstream target of the PKC pathway through which many cellular processes such as cell migration, spreading, and proliferation are regulated. Second, the formation of the PINCH–ILK–Parvin complex is regulated by the phosphatidylinositol-3 (PI-3) kinase pathway, because inhibition of PI3K pathway by small compound inhibitors or by overexpressing PTEN also results in the inhibition of the complex assembly.

ILK enhances phosphorylation of protein kinase B PKB/Akt, which plays a critical role in regulation of adhesion-mediated cell survival signals. Therefore, ILK functions as an important mediator in the signal transduction from the ECM, regulating adhesion-dependent cell progression and the apoptotic pathway (Fig. 3, 4 and 5).

The purpose of this in vitro study was to confirm the role of ILK on adhesion of

MSCs and the effect on cellular survival signals. Based on our observation, ILK-MSCs showed a significant increase in adhesion and viability under hypoxic condition (Fig. 2 and 5). In addition, we observed that activation of adhesion-mediated cell survival signals such as Akt and ERK was significantly increased (Fig. 3). We also confirmed an increase in Bcl-2/Bax ratio and inhibition of caspase-3 activation which are a major signal mediator of cell apoptosis (Fig. 4). According to histology, transplantation of ILK-MSCs resulted in a further decrease in infarct size and a further improvement of LV function in the MSC group. Moreover, the onset of microvessel density was closely correlated with repopulation of infracted area (Fig. 6). Finally, we suggest that genetic enhancement of ILK increases adhesion of MSCs on the ECM of infarcted myocardium, improving stem cell survival after implantation.

V. CONCLUSION

Although MSCs represent a suitable source of autologous cells in cell therapy for improvement in cardiac function, MSCs therapy has limitations due to their poor viability after cell transplantation. Adhesion mediated by integrin is a prerequisite for cell survival and also a key factor for the differentiation of MSCs. As a novel anti-death strategy to improve stem cell survival/number in the infarcted heart, we genetically engineered MSCs to overexpress integrin-linked kinase (ILK), with intention to enhance adhesion and ultimately cell survival after implantation. Genetic modification with ILK enhanced cell adhesion, survival rate and related signaling *in vitro*. ILK-MSCs further improved cardiac function of infarcted myocardium as compared to MSC transplantation alone. These findings suggested that introduction of ILK gene into MSC seems to be a gene modulaton and an effective therapeutic approach for infarcted myocardium.

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Abstract (in Korean)

허혈성 심근조직에서 Integrin-linked kinase 유전자에 의한 간엽줄기세포의 anoikis 억제

< 지도교수 장 병 철 >

연세대학교 대학원 의학과

송 석 원

간엽줄기세포는 심장기능을 향상시키기 위한 세포치료에서 자가이식이 가능한 세포로 알려져 있으나, 이식 후 생존률이 낮다는 문제점이 있다. Integrin에 의해 매개되는 생착은 세포 생존의 선행요건 일 뿐 아니라, 간엽줄기세포 분화의 중요한 요소로 알려져있다. 본 연구에서는 심근경색을 유발한 심장에 이식된 줄기세포의 생존을 향상시키기 위한 새로운 방법으로 세포이식 후 생착능을 향상시키기 위해 integrin-linked kinase (ILK) 유전자를 과발현시켰다. ILK가 함유된 간엽줄기세포는 대조군에 비해 생존률은 약 1.5배, ERK1/2와 Akt 의 인산화반응은 각각 약

3배 및 2배 증가하였다. 또한, ILK가 함유된 간엽줄기세포에서는 저산소 실험군과 비교하여 Bcl-2/Bax의 비가 2배 증가하였으며, caspase-3 활성이 억제되었다. ILK가 함유된 간엽줄기세포는 심장의 섬유아세포로 만든 3-D matrix와의 생착능도 약 30.0% 증가하였으며, 간엽줄기세포 만을 이식한 심근과 비교하여 4배 더 세포 유지를 시킬 수 있었다. 생체실험에서, ILK가 함유된 간엽줄기세포가 이식된 동물에서 좌전하행지 동맥 결찰 후 간엽줄기세포만을 이식한 경우와 비교했을 때 심근경색 범위가 12.0% 감소하였다. ILK가 함유된 간엽줄기세포를 이식한 경우 섬유화의 정도는 15.5% 감소하였을 뿐만 아니라, 세포사멸 비율 지표도 17.0% 정도 감소하였다. 그리고, 단위구획에서의 미세혈관 밀도는 ILK가 함유된 간엽줄기세포 (129.1±23.5)에서 대조군(19.3±15.2)과 간엽줄기세포 (68.9± 19.4)와 비교하여 상승하였다. 결론적으로, 간엽줄기세포만 이식한 경우에 비해 ILK가 함유된 간엽줄기세포에서는 세포 생존과 증식 및 부착능에 향상하여 심근손상을 개선시켰다.

핵심되는 말: integrin-linked kinase, 간엽줄기세포, 심근경색