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Integrin-Linked Kinase Is Required in Hypoxic Mesenchymal Stem Cells for Strengthening Cell Adhesion to Ischemic Myocardium

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ABSTRACT

Mesenchymal stem cells (MSCs) therapy has limitations due to the poor viability of MSCs after cell transplantation. Integrin-mediated adhesion is a prerequisite for cell survival. As a novel anti-death strategy to improve cell survival in the infarcted heart, MSCs were genetically modified to overexpress integrin-linked kinase (ILK). The survival rate of ILK-transfected MSCs (ILK-MSCs) was augmented by about 1.5-fold and the phosphorylation of ERK1/2 and Akt in ILK-MSCs were increased by about three and twofold, respectively. ILK-MSCs demonstrated an increase of twofold in the ratio of Bcl-2/Bax and inhibited caspase-3 activation, compared with hypoxic MSCs. The adhesion rate of ILK-MSCs also had a 32.2% increase on the cardiac fibroblast-derived three-dimensional matrix and ILK-MSCs showed higher retention by about fourfold compared to unmodified MSCs. Six animals per group were used for the in vivo experiments analyzed at 1 week after occlusion of the left coronary artery. ILK-MSC transplanted rats had a 12.0% ± 3.1% smaller infarct size than MSC-treated rats after ligation of left anterior descending coronary artery. Transplantation of ILK-MSCs not only led to a 16.0% ± 0.4% decrease in the fibrotic heart area, but also significantly reduced the apoptotic positive index by two-thirds when compared with ligation only. The mean microvessel count per field in the infarcted myocardium of ILK-MSCs group was increased relative to the sham group and MSCs group. In conclusion, the ILK gene transduction of MSCs further assisted cell survival and adhesion, and improved myocardial damage when compared with MSC only after transplantation. STEM CELLS *2009;27:1358–1365*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

As a consequence of myocardial infarction (MI), irreversible tissue damage and physiologic dysfunction occur in the heart [1]. The damaged myocytes in infarcted tissues undergo progressive replacement by fibroblasts to form scar tissue which may lead to remodeling of the left ventricle (LV) as a result of a rise in mechanical wall stress [2]. Recent attempts to repair infarcted hearts using alternative sources of cardiomyocytes revealed that myogenic cells from various stem cells replaced resident myocytes in cardiac tissue after injury [3]. In particular, mesenchymal stem cells (MSCs) have become one of the most potent cell sources for repairing 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 the appropriate culture conditions [4–6]. Although MSCs represent a suitable source of autologous cells for cell therapy focused on the improvement of cardiac function, MSC therapy has limitations due to the poor viability of MSCs after cell transplantation [7].

To overcome this limitation, antideath strategies may be required to improve stem cell survival/number in the infarcted heart [8]. The absence of adhesion is probably a main cause of poor cell survival in cell transplantation. Cell adhesion to the extracellular matrix (ECM) is mediated by integrins, which control a variety of important processes including gene

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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 upstream of the Akt/protein kinase B (PKB) and mitogen-activated protein (MAP) kinase pathways [14–16].

In this study, we hypothesized that MSCs that were genetically engineered to enhance adhesion would ultimately improve cell survival after implantation. To test this hypothesis, we transfected the ILK gene into MSCs (ILK-MSCs) and analyzed their adhesion under hypoxic conditions. Moreover, we implanted ILK-MSCs into infarcted myocardium and examined the resulting physiological and morphological changes in the implanted heart tissue.

Materials and Methods

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 femurs and tibias from 4-week-old male Sprague-Dawley rats (approximately 100 g) with 10 ml of MSC medium comprising Dulbecco's modified Eagle's medium (DMEM)-low glucose supplemented with 10% fetal bovine serum (FBS) and a 1% antibiotic-penicillin and streptomycin solution. Flushed media were centrifuged at 1,600 rpm, 5 minutes and resuspended in serum-supplemented medium, next loaded to Percoll density gradient centrifugation at 1,600 rpm, 30 minutes. Mononuclear cells were recovered from the middle interface after centrifugation in Percoll, were washed twice, resuspended in 10% FBS-DMEM, and plated in flasks at a density of 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 hours, 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, we used the Isolex Magnetic Cell Selection System (Baxter Healthcare Corporation, Irvine, CA, http://www.baxter.com). 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. The CD34-negative fraction was then cultured further. After incubation, the cells were harvested with 0.25% trypsin and 1 mM EDTA for 5 minutes at 37°C, replated in 1×10^5/100 cm^2 plates, and grown for approximately 10 days. The characteristics of MSCs were demonstrated by immunophenotyping. To verify the nature of cultured MSCs, cells were labeled against various surface and intracellular markers, and analyzed by flow cytometry. Cells were harvested, washed with PBS, and labeled with the following antibodies conjugated with fluorescein isothiocyanate (FITC) or Texas red: CD14, CD34, CD71, CD90, CD105, and ICAM-1. FITC-conjugated goat anti-mouse IgG and Texas red-conjugated goat anti-rabbit IgG from Jackson ImmunoResearch Laboratories (West Grove, PA, http://www.jacksonimmuno.com) were used as secondary antibodies. Labeled cells were assayed by flow cytometry and analyzed with CellQuest Pro Software (BectonDickinson, San Jose, CA, http://www.bd.com). MSC were lentivirally transduced at second passage and used for in vitro and in vivo experiments between passage 4 and 6.

Lentiviral Vector-Mediated Stable Genetic Modification of MSCs

For stable genetic modification, we used a lentiviral vector (LentiV) for transgene delivery. The LentiV harboring ILK gene with mCMV promoter and green fluorescent protein (GFP) gene with pgk promoter was constructed by inserting the gene fragment into the *EcoR* I- *Cla* I site of the LentiV (Marcrogen, Seoul, Korea, http://www.macrogen.co.kr) and was obtained commercially. The ILK (BC001554) cDNA clone was purchased from Korean Bioinformation Centor and the cDNA was amplified using the following primer set: Forward: 5′-gggaattcgccaccatggacgacatt-3′, Reverse: 5′-attatcgattcctacttgtc-3′. The nucleotide sequences of the constructs were verified by sequencing analysis. Passage-2 MSCs were plated at a concentration of 2×10^5 cells per well in 12-well plates. MSCs were transduced by adding purified LentiVs to the cells in the presence of 8 μg/ml of polybrene at MOIs of 50 in 250 μl of medium (to facilitate transduction). At approximately 20 hours after transduction, the virus-containing medium was removed and replaced with fresh MSC complete medium.

Immunoblot Analysis

Cells were washed once in PBS and lysed in lysis buffer (Cell Signaling Technology, Beverly, MA, 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, CA, http://www.bio-rad.com). Proteins were separated in a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, http://www.millipore.com). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-T; 0.1% Tween 20) and 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. Next, the membrane was washed three times with TBS-T for 10 minutes and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibodies. After extensive washing, the bands were detected with an enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com). The band intensities were quantified using a Photo-Image System (Molecular Dynamics, Sunnyvale, CA, http://www.mdyn.com). Expression and phosphorylation of proteins was normalized to β-actin and expression levels, respectively.

Cell Survival

Cell survival was measured with the PreMix WST-1 Cell Counting System (TAKARA BIO, Shiga, Japan, http://www.takara-bio.com). This system enables the measurement of cell survival with a colorimetric assay that is based on the cleavage of 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, thus the quantity of formazan dye is related directly to the number of metabolically active cells in the medium. Wells of a 96-well culture plate were seeded with 2×10^4 cells and incubated under hypoxic conditions after transduction of the LentiV. The WST-1 cell counting reagent was added directly to the supernatant (10 μl/100 μl growth medium) and incubated at 37°C for 3 hours. We then determined the absorbance of the solubilized dark red formazan product at 450 nm.

Measurement of Caspase-3 Activity

Relative caspase-3 activity was determined using the ApopTargetTM Capase-3 Colorimetric Protease Assay according to the manufacturer's instructions (BioSource International, Grand Island, NY, http://www. invitrogen.com). This assay is based on the generation of free Asp-Glu-Val-Asp (DEVD)-*p*-nitroanilide (pNA) chromophore when the provided substrate is cleaved by caspase-3. On cleavage of the substrate by Caspase-3, free pNA light absorbance at 405 nm can be quantified using a microplate reader. After different treatments, the cultured MSCs (2×10^6)

were harvested in lysis buffer (1 M dithiothreitol) and the cell extracts were centrifuged to eliminate cellular debris. Fifty microliter aliquots of the cell extracts were incubated at 37°C for 2 hours in the presence of the chromophore substrate. Free DEVD-pNA was then determined colometrically. By comparing the absorbance of pNA from apoptotic samples with uninduced controls, we could determine the fold increase in capase-3 activity. Data denote means ± SEM of two to three replicate measurements in three different cell cultures.

Preparation of Cardiac Fibroblast-Derived Three-Dimensional Matrix (Cardiogel)

Cardiogel was prepared with a minor modification [17]. Briefly, 2×10^5 cells (cardiac fibroblast) per 35-mm dish were seeded and the medium changed every 48 hours until the matrix prepared. About 4 days, cells were in full confluence. 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 minutes with 1 ml of DNase (10 units of DNase per milliliter of PBS) to minimize the DNA debris. For later use, 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.

Assay for Cell Adhesion

Assay for cell adhesion was achieved in compliance with our previous study [17]. ILK-MSCs or untransfected control MSCs were isolated by trypsinization, washed once in DMEM with 10% FBS to stop the trypsin activity, and then washed twice with serum-free DMEM to remove the serum components. Suspensions of 2×10^4 viable MSCs were then added to each well and allowed to attach for 30 minutes at 37°C under 5% CO_2. To quantify MSC adhesion, the 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 counting the number of cells using a hemacytometer under a microscope. Each experiment was performed in triplicate wells and repeated at least three times. Number of attached cells was calculated by subtracting the number of rinsed cells from number of seeded cells.

Induction of MI 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. MI was produced in male Sprague-Dawley rats (200 ± 30 g) by surgical occlusion of the left anterior descending coronary artery, according to previously described procedures [17]. Briefly, after induction of anesthesia with ketamine (10 mg/kg) and xylazine (5 mg/kg), the third and fourth ribs were cut to open 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, NJ, 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 at three injection sites into anterior and lateral aspects of the viable myocardium bordering the infarction with a Hamilton syringe (Hamilton, Reno, NV, http://www.hamiltoncompany.com) with a 30-gauge needle. Cells were kept on ice prior to injection for preventing cell damage. Throughout the operation, animals were ventilated with 95% O_2 and 5% CO_2 using a Harvard ventilator. The operative mortality was 10% within 48 hours. Six animals per group (ligation, MSCs, and ILK-MSCs) were used for morphologic analysis at 1 week after occlusion of the left coronary artery. To investigate the adhesion degree of cell containing ILK gene in vivo, we used six animals per group at 3 days after occlusion.

Histology and Determination of the Area of Fibrosis

At several intervals (3 or 7 days) 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 2 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. 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. The interstitial fibrosis areas were measured from control ($n = 6$), MI ($n = 6$), MI + MSCs ($n = 6$), MI + ILK − MSCs ($n = 6$) groups using MetaMorph software version 4.6 (Universal Imaging, Downingtown, PA, http://www.universal-imaging.com), and expressed as a percentage of the total LV.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed according to the manufacturer's instructions (Chemicon International, Temecula, CA, http://www.chemicon.com). In brief, 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 a normal heart section by treating the section with DNase I (10 U/ml, 10 minutes at room temperature). The sections were pretreated with 3.0% H_2O_2, subjected to terminal deoxynucleotidyl transferase enzyme for 37°C for 1 hour, and incubated digoxigenin-conjugated nucleotide substrate at 37°C for 30 minutes. Nuclei exhibiting DNA fragmentation were visualized by adding 3,3-diamino benzidine (DAB; Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) for 5 minutes. The nuclei of apoptotic cardiomyocytes were stained dark brown. Lastly, sections were counterstained with methyl green. A coverslip was place on top of each section and the sections were observed by light microscopy. For each group, six slices were prepared and 10 different regions were observed in each slice (×400)

Determination of Infarct Size

2,3,5-Triphenyltetrazolium chloride (TTC) staining was used to assess myocardial tissue viability and determine the size of the myocardial infarct. The tissue slices were incubated in a 1% TTC (Sigma, St. Louis, MO, http://www. sigma.com) solution, pH 7.4, at 37°C for 20 minutes. The tissues were fixed in 10% PBS-buffered formalin overnight at 2°C–8°C. The hearts were sectioned transaxially and the size of the MI was evaluated as a percentage of the sectional area of the infarcted tissue of the LV to the sectional area of the whole LV. Both sides of each TTC-stained tissue slice were photographed with a digital camera.

Determination of Capillary Density

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. Antigen retrieval was performed with 10 mM sodium citrate (pH 6.0) in microwave for 10 minutes. Sections were incubated in 3% H_2O_2 to quench endogenous peroxidase. Sample was blocked in 2.5% normal horse serum, and incubated in anti-CD31 antibody. Biotinylated pan-specific universal secondary antibody

and streptavidin/peroxidase complex reagent was used to treat the heart sections. Using the DAB substrate kit, the heart sections were stained with antibody. Counterstaining was performed with 1% methyl green and dehydration was progressed with 100% *N*-butanol, ethanol, and xylene before mounting in VectaMount Mounting Medium (Vector Laboratories). For quantification, capillaries were counted in 10 randomly chosen fields from two separate slides using the (20×) objective. The mean number of capillaries per field in the infarcted myocardium was used for statistical analysis.

Statistical Analysis

Data are expressed as means ± SE. Statistical comparisons between the two groups were performed using the Student's *t* test. In addition, a one-way analysis of variance using a Bonferroni test was used when comparing more than two groups. A p value $<.05$ was considered significant.

Results

Expression and Survival of ILK-MSCs in Hypoxic Condition

MSCs were first isolated from mixed culture with hematopoietic cells based on their attachment to the culture plate, and the isolated MSCs were further purified by exclusion using magnetic beads targeting the hematopoietic marker CD34, yielding 3×10^6 cells (95% purity) after 2 weeks of culture. Consistent with the previous report the cultured MSCs expressed CD71, CD90, CD105, CD106, and ICAM, but the hematopoietic marker CD34 and CD14 were not expressed (data not shown) [17]. Hypoxia facilitates disruption of tissue integrity, which leads to cellular anoikis. During the 24 hours after hypoxic stress, endogenous ILK expression in MSCs was time-dependently

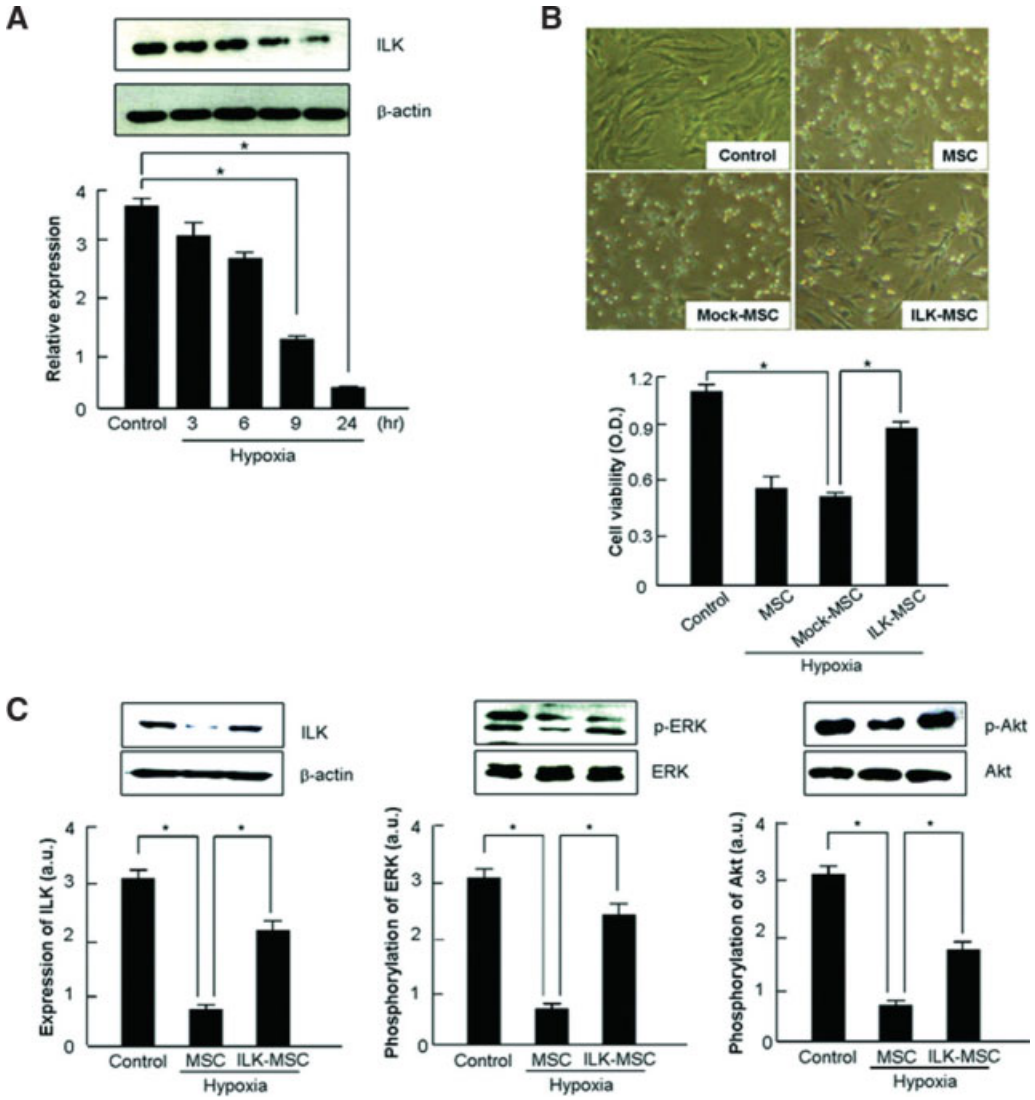

Figure 1. Expression and survival of ILK-MSCs in hypoxic condition. **(A):** Expression of ILK in MSCs under hypoxic conditions was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblot analysis (*, $p < .001$). **(B):** Cell proliferation under hypoxic conditions for 9 hours was measured by WST-1 Cell Proliferation Assay System ($\lambda = 570$ nm; *, $p < .001$). **(C):** Effects of ILK on the activity of survival-related proteins under hypoxic conditions were monitored by SDS-PAGE with antibodies specific for ILK, ERK1/2, p-ERK1/2, Akt, p-Akt, and β-actin (*, $p < .001$). Band intensities were quantified using the Photo-Image System; each point represents the mean value obtained from three experiments. Abbreviations: ILK-MSCs, integrin-linked kinase-transfected MSCs; MSCs, mesenchymal stem cells; p-Akt, phosphorylated Akt; p-ERK, phosphorylated ERK.

downregulated by Western blot analysis (Control vs. Hypoxia 9h: 3.62 ± 0.27 vs. One.41 ± 0.05, $p < .001$; Control vs. Hypoxia 24h: 3.62 ± 0.27 vs. 0.51 ± 0.01, $p < .001$; Fig. 1A). MSCs were transduced with LentiV harboring ILK gene and a significant dose-response was observed with increasing virus titer for MOI 1 to 50. Transduction efficiency was a maximum of 94% at MOIs of 50. To define the effect of ILK on MSC survival in hypoxic conditions, ILK-MSCs were incubated under hypoxic conditions for 9 hours and cell viability was then determined by a WST-1 assay. As shown in Figure 1B, ILK overexpression enhanced the survival rate of MSCs by about 50% compared with Mock-MSCs (Control vs. mock-MSC: 1.09 ± 0.05 vs. 0.49 ± 0.03, $p < .001$; ILK-MSC vs. mock-MSC: 0.86 ± 0.05 vs. 0.49 ± 0.03, $p < .001$). Hypoxic ILK-MSCs had a threefold increase in ERK1/2 activity (control vs. MSC: 3.11 ± 0.16 vs. 0.81 ± 0.10, $p < .001$; ILK-MSC vs. MSC: 2.41 ± 0.27 vs. 0.81 ± 0.10, $p < .001$) and a twofold increase in Akt activity compared with nontransformed hypoxic MSCs (control vs. MSC: 3.20 ± 0.22 vs. 0.79 ± 0.11, $p < .001$; ILK-MSC vs. MSC: 1.75 ± 0.21 vs. 0.79 ± 0.11, $p < .001$; Fig. 1C).

Effect of ILK on Hypoxia-Induced MSC Anoikis

To determine the effect of ILK overexpression on MSC adhesion, we performed quantitative adhesion assays with ILK-MSCs. Adhesion of MSCs to a three-dimensional culture system derived from cardiac fibroblasts was enhanced by twofold with the introduction of ILK (ILK-MSC vs. MSC: 62.7% ± 0.6% vs. 30.5% ± 0.5%, $p < .01$; Fig. 2A). To further evaluate the adhesive function of ILK in vivo, we transplanted ILK-MSCs into the border region of infarcted hearts. ILK-MSCs had an approximate retention rate of 800 cells per section, while the control MSCs had a retention rate of approximately 200 cells per section (ILK-MSC vs. MSC: 758 ± 40 vs. 218 ± 9, $p < .01$; Fig. 2B). To examine whether ILK expression of MSCs inhibits apoptosis in hypoxic conditions, we examined the ratio of Bcl-2/Bax as well as caspase-3 activation. As shown in Figure 2C, ILK overexpression significantly reduced the caspase-3 activity by 42.4% compared with Mock-MSCs (control vs. Mock-MSC: 1.03 ± 0.14 vs. Three.52 ± 0.18, $p < .001$; ILK-MSC vs. Mock-MSC:

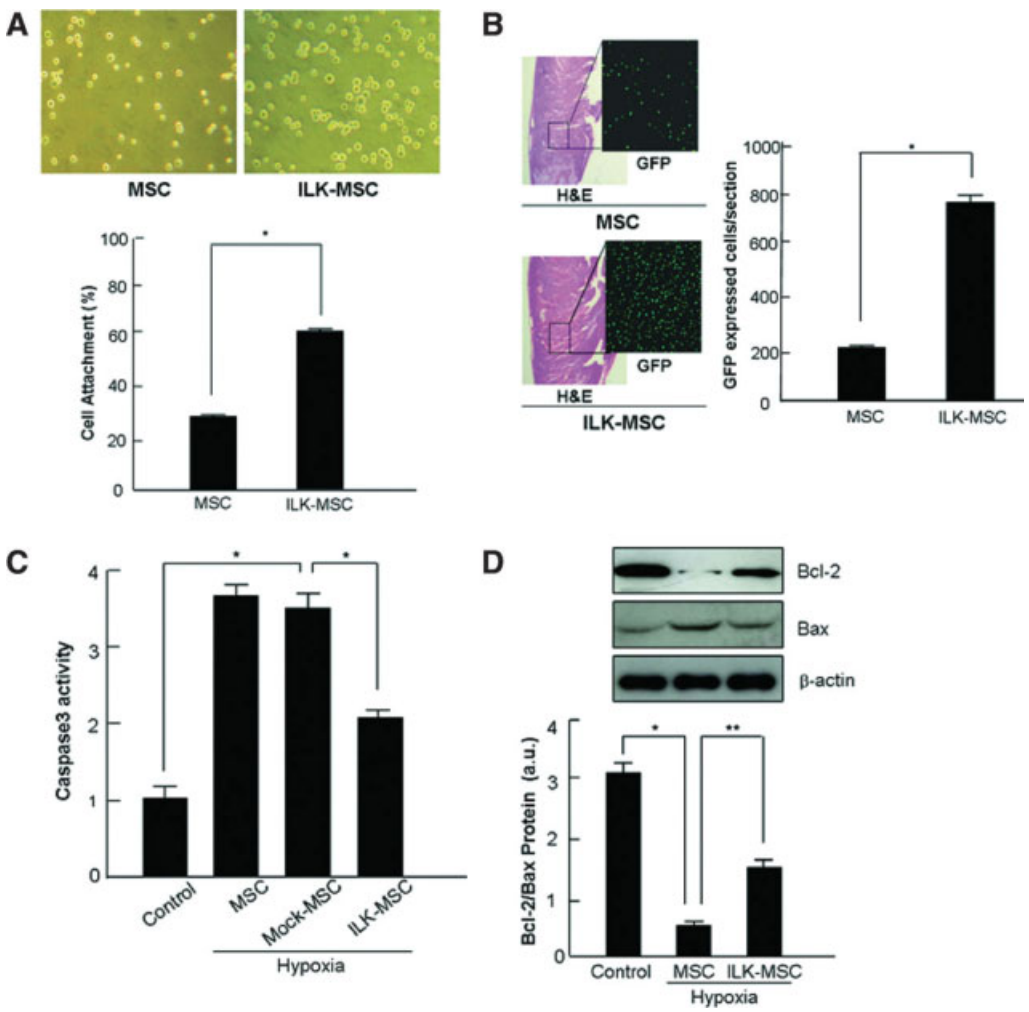

Figure 2. Effect of ILK on hypoxia-induced MSCs anoikis. **(A):** Effect of ILK on MSC adhesion was examined with a cardiogel for 3 hours. The mean attachment values ± SD on the cardiogel were 6,102 ± 98 in MSCs and 12,541 ± 120 in ILK-MSCs (*, $p < .01$). **(B):** Analysis of myocardial repair after implantation into an infarcted myocardium was operated with GFP expression. Engraftment was significantly improved with ILK overexpression 3 days after the cells were injected (1×10^6; *, $p < .01$). Each value is the mean ± SEM of six independent experiments. **(C):** Relative caspase-3 activity was determined using the ApopTarget Capase-3 Colorimetric Protease Assay (*, $p < .001$). **(D):** Expression of Bcl-2 and Bax were determined by immunoblot analysis in ILK-MSCs or MSCs that were incubated in hypoxic conditions for 9 hours (*, $p < .001$, **, $p < .01$). Abbreviations: GFP, green fluorescent protein; ILK-MSCs, integrin-linked kinase-transfected MSCs; MSCs, mesenchymal stem cells.

2.03 ± 0.08 vs. Three.52 ± 0.18, $p < .001$). The ILK-MSCs in the hypoxic condition induced an increased Bcl-2/Bax ratio of about twofold when compared with the control MSCs (control vs. MSC: 3.14 ± 0.18 vs. 0.59 ± 0.09, $p < .001$; ILK-MSC vs. MSC: 1.52 ± 0.15 vs. 0.59 ± 0.09, $p < .01$; Fig. 2D).

Effect of Transplantation of ILK-MSCs Following Acute MI

We determined the infarct sizes in the LV using TTC staining ($n = 6$ rat/group). Injection of MSCs resulted in a significant decrease in infarct size, and injection of MSC-ILKs further reduced the infarct size (Sham vs. MSC: 28.2% ± 5.5% vs. 17% ± 1.2%, $p < .05$; ILK-MSC vs. MSC: 5% ± 1.9% vs. 17% ± 1.2%, $p < .01$; Fig. 3A). Transplantation of ILK-MSCs significantly decreased interstitial fibrosis to 2.5% ± 1.0%. Namely, the specimens with ILK-MSC injected-ischemic myocardium showed decreased fibrosis in the LV and a thicker wall compared with the ligation controls and the MSC-injected hearts (Sham vs. ILK-MSC: 18.5% ± 1.4% vs. 2.5% ± 1.0%, $p < .01$; MSC vs. ILK-MSC: 8.3% ± 1.7% vs. 2.5% ± 1.0%, $p < .01$; Fig. 3B). The incidence of TUNEL-positive myocardial cells caused by ligation was reduced by two-thirds in the ligated hearts transplanted with ILK-MSCs when compared with sham and was one-third lower than MSC injected hearts (Sham vs. ILK-MSC: 28.3% ± 1.8% vs. 10.1% ± 0.2%, $p < .05$; MSC vs. ILK-MSC: 15.3% ± 0.7% vs. 10.1% ± 0.2%, $p < .05$; Fig. 3C). 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 vs. ILK-MSC: 19.3 ± 15.2 vs. 129.1 ± 23.5, $p < .01$; MSC vs. ILK-MSC: 68.9 ± 19.4 vs. 129.1 ± 23.5, $p < .05$; Fig. 3D).

Discussion

Cell implantation utilizing cardiac stem cells [18], embryonic stem cells [19], hematopoietic stem cells [20], MSCs [21–23], skeletal myoblasts [24–26], and cardiac myocytes (adult, fetal, or neonatal myocytes) [27, 28] has been suggested to be a promising clinical approach for restoration of myocardial function after cardiac infarction. Among these various candidate cell types, MSCs have a great advantage with respect to generating functional cardiac myocytes in the infarcted myocardium because of their ease of preparation and potential to differentiate into a cardiac lineage [29, 30] both in vivo and in vitro under appropriate culture conditions. In addition, the delivered MSCs have clearly improved the physiological functions of the infarcted heart by limiting infarct size and preserving at-risk regions [31, 32].

Although MSCs represent a suitable source of autologous cells in cell therapy for the improvement of cardiac function, MSC therapy has limitations due to the poor viability of MSCs after cell transplantation [7]. Thus, to improve the efficacy of cell therapy, it is necessary to protect MSCs from anoikis resulting from hypoxic conditions by genetic modification. Recently, in a new approach to enhance the viability of MSCs, they were transduced with the gene for the survival signal, Akt, prior to transplantation [33]. On the basis of our previous study, the absence of adhesion is probably the main cause of poor cell survival after cell transplantation [17].

The purpose of the in vitro section of our study was to confirm the role of ILK on MSC adhesion and the effect of ILK on cellular survival signals. On the basis of our observation, our data showed that hypoxic surroundings supressed expression of the ILK protein which interacts with the β1-subunit of integrin and plays a crucial role in integrin-mediated cell adhesion and

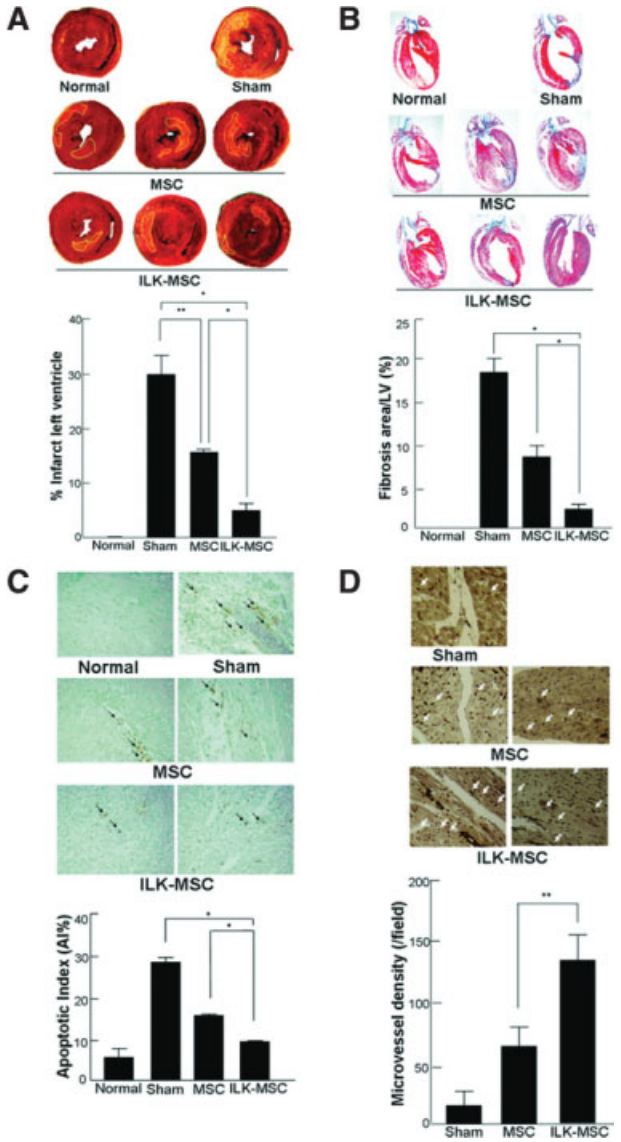

Figure 3. Effect of transplantation of ILK-MSCs following acute myocardial infarction (MI). **(A):** Intramyocardial injection of MSCs reduced the LV infarct size as assessed by 2,3,5-triphenyltetrazolium chloride staining at 1 week post-MI. ILK-MSCs further decreased the area of necrotic tissue (*, $p < .01$; **, $p < .05$). **(B):** The left panel shows representative Masson's trichrome images from histological sections and the right histogram shows fibrosis (blue) ratio of each heart at 1 week post-MI (*, $p < .01$). **(C):** An apoptosis assay was performed on heart tissue at 1 week after the ligation of LAD. The left panel shows representative images of the Terminal deoxynucleotidyl Transferase-mediated dUTP nick-end labeling staining (original magnification ×200). Staining for normal nuclei (green) was carried out using methyl green while apoptotic nuclei were stained brown. The right panel shows the summarized data from left panel (*, $p < .05$). **(D):** Quantitative analysis showed that microvessel density was significantly higher in ILK-MSCs groups than in MSC and sham groups at 1 week post-MI (*, $p < .01$, **, $p < .05$). The arrowheads indicate the representative CD31-positive microvessels. Brown: positively stained for CD31. Each value is the mean ± SEM of six independent experiments. Abbreviations: Abbreviations: ILK-MSCs, integrin-linked kinase-transfected MSCs; LV, left ventricle; MSCs, mesenchymal stem cells.

signaling [10, 11]. In addition, we observed that transfection with the ILK gene enhanced phosphorylation of ERK and Akt, proteins which plays a critical role in regulation of adhesion-mediated cell survival signals in hypoxic MSCs. We also confirmed an increase in the Bcl-2/Bax ratio and an inhibition of caspase-3 activation. These data suggested that ILK gene promoted cell adhesion in hypoxic condition, which avoided cell apoptosis. Cell-ECM adhesion is crucial for the control of cell behavior. Adhesion of cells to the ECM stimulates signal transduction cascades that are involved in proliferation, migration, differentiation, and even survival [12, 34–37]. In particular, anchorage-dependent cells depend on interactions between integrins and the ECM for survival. Namely, the interaction of mesenchymal as well as epithelial and endothelial cells with the ECM inhibits default apoptotic pathways, which become activated if cell-ECM interactions are disrupted. This type of suspension-induced apoptosis is termed anoikis [38, 39]. ILK enhances phosphorylation of PKB/Akt, which plays a critical role in regulation of adhesion-mediated cell survival signals. In addition, Danielle et al. reported that ILK was involved in regulation of anoikis in human MSCs [40]. Therefore, ILK functions as an important mediator in the signal transduction from the ECM, thus regulating adhesion-dependent cell signaling and the apoptotic pathway. According to our in vivo histological findings, transplantation of ILK-MSCs resulted in a further decrease in infarct size and a further improvement in LV function compared to the MSC group. Moreover, the improved microvessel density was closely correlated with the presence of ILK-MSCs in the infarcted area.

Conclusion

Although MSCs represent a suitable source of autologous cells for cell therapy targeted to improve cardiac function, MSC 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 antideath strategy to improve stem cell survival/number in the infarcted heart, we genetically engineered MSCs to overexpress ILK to enhance adhesion and ultimately increase cell survival after implantation. Genetic modification of MSCs with ILK enhanced cell adhesion, survival rates, and cell adhesion-related signaling in vitro. ILK-MSCs further improved the cardiac function of infarcted myocardium when compared with myocardium that had been transplanted with MSCs alone. These findings suggested that genetic modification of MSCs might be an effective therapeutic approach for treating infarcted myocardium.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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