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Tissue Transglutaminase Is Essential for Integrin-Mediated Survival of Bone Marrow-Derived Mesenchymal Stem Cells

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ABSTRACT

Autologous mesenchymal stem cell (MSC) transplantation therapy for repair of myocardial injury has inherent limitations due to the poor viability of the stem cells after cell transplantation. Adhesion is a prerequisite for cell survival and also a key factor for the differentiation of MSCs. As a novel prosurvival modification strategy, we genetically engineered MSCs to overexpress tissue transglutaminase (tTG), with intention to enhance adhesion and ultimately cell survival after implantation. tTG-transfected MSCs (tTG-MSCs) showed a 2.7-fold and greater than a twofold increase of tTG expression and surface tTG activity, respectively, leading to a 20% increased adhesion of MSCs on fibronectin (Fn). Spreading and migration of tTG-MSCs were increased 4.75% and 2.52%, respectively. Adhesion of tTG-MSCs on cardiogel, a cardiac fibroblast-derived three-dimensional matrix, showed a 33.1% increase. Downregulation of tTG by transfection of small interfering RNA specific to the tTG resulted in markedly decreased adhesion and spread of MSCs on Fn or cardiogel. tTG-MSCs on Fn significantly increased phosphorylation of focal adhesion related kinases FAK, Src, and PI3K. tTG-MSCs showed significant retention in infarcted myocardium by forming a focal adhesion complex and developed into cardiac myocyte-like cells by the expression of cardiac-specific proteins. Transplantation of 1×10^6 MSCs transduced with tTG into the ischemic rat myocardium restored normalized systolic and diastolic cardiac function. tTG-MSCs further restored cardiac function of infarcted myocardium as compared with MSC transplantation alone. These findings suggested that tTG may play an important role in integrin-mediated adhesion of MSCs in implanted tissues. STEM CELLS *2007;25:1431–1438*

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

INTRODUCTION

Under proper stimulation, mesenchymal stem cells (MSCs) can be induced to differentiate into myocytes, adipocytes, osteoblasts, chondrocytes, tenocytes, and hematopoietic-supporting stroma [1]. With recent reports that MSCs derived from bone marrow can differentiate into cardiac muscle in vitro [2] and in vivo [3], MSC autologous transplantation is a promising new therapeutic modality for the repair of myocardial infarctions and prevention of postinfarct congestive heart failure. However, in experimental models, poor viability of the transplanted cells is a major limiting factor of cell therapy. The survival rate of transplanted cells into an uninjured mouse heart was very low, 4 days post transplantation [3]. This may require prosurvival strategies to improve stem cell survival/number in the infarcted heart [4]. Although prosurvival strategies have been proven to be successful in vitro, they actually may not solve the problems of poor adhesion of MSCs.

Adhesion to structural glycoproteins of the extracellular matrix (ECM) is necessary for survival of the differentiated adherent cells in the cardiovascular system, including endothelial cells, smooth muscle cells, fibroblasts, and cardiac myocytes [5, 6] as well as differentiation of MSCs [7]. Adhesion of cells to the matrix, predominantly via the integrin molecules, generates an endogenous tensile stress within the cells, called tensegrity [8], and repression of apoptotic signals [9], whereas detachment has the opposite effect. This physiological cellular process may be necessary for MSC differentiation, survival, and growth. Recently, tissue transglutaminase (tTG) on the cell surface and in the ECM is reported to act as a coreceptor for fibronectin (Fn) in cell adhesion associated with integrin [10, 11]. In this study, as a novel prosurvival modification strategy, we genetically engineered MSCs to overexpress tTG, with intentions to enhance adhesion and ultimately cell survival after implantation.

MATERIALS AND METHODS

Isolation and Culture of MSCs

MSCs were isolated from the femoral and tibial bones of rats [12]. Bone marrow-derived MSCs were collected from the aspirates of the femurs and tibias of 4-week-old male Sprague-Dawley rats

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(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 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, 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− fraction being further cultured. The cells were harvested after incubation with 0.25% trypsin and 1 mM EDTA for 5 minutes at 37°C, replated in 1×10^5/100-cm^2 plates, and again 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 fluorescin isothiocyanate (FITC) or Texas Red: CD14, CD34, CD71, CD90, CD105, and intracellular adhesion molecule (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. In the case of CD14, we used normal rabbit IgG (SC-3888; Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) as a negative isotype control for flow cytometry. Labeled cells were assayed by flow cytometry and analyzed with CellQuest Pro software (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). To label viable MSCs with 4,6-diamidino-2-phenylindole (DAPI), sterile DAPI solution was added to the culture medium on the day of implantation at a final concentration of 50 μg/ml for 30 minutes. The cells were rinsed six times in PBS to remove unbound DAPI, detached with 0.25% (wt/vol) trypsin, and suspended in serum-free medium for grafting [13–15].

Transglutaminase Activity

Transglutaminase activity associated with the extracellular surface was measured by the incorporation of biotinylated cadaverine into Fn [16, 17]. For this assay, 2×10^5 cells per milliliter were plated into 96-well plates precoated with plasma Fn in 100 μl of complete DMEM medium without serum in the presence of 0.1 mM biotinylated cadaverine. As a negative control, Fn-coated 96-well plates were incubated with 100 μl of serum-free DMEM containing 0.1 mM biotinylated cadaverine alone. Cells were allowed to incubate for 1 hour at 37°C, and then they were washed twice with PBS, pH 7.4, containing 3 mM EDTA, in order to stop the reaction. A detergent solution (100 μl) consisting of 0.1% (wt/vol) deoxycholate in PBS, pH 7.4, containing 3 mM EDTA, was then added to each well and the mixture incubated with gentle shaking for 20 minutes. The supernatant was discarded and the remaining Fn layer washed three times with Tris-HCl, pH 7.4. Wells were then blocked with 3% (wt/vol) bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) in Tris-HCl buffer for 30 minutes at 37°C, washed with buffer, and then incorporated biotinylated cadaverine revealed with a 1/5,000 dilution of ExtrAvidin peroxidase conjugate (Sigma), which was incubated for 1 hour at 37°C using 3,3′,5,5,′-tetramethylbenzidine as a substrate. Color development was stopped by adding 50 μl of stop solution to each well. The resulting color was then read in an enzyme-linked immunosorbent assay plate reader at 450 nm.

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

Cardiogel was prepared with a minor modification [18]. Briefly, 2×10^5 cells per 35-mm dish were seeded and the medium changed every 48 hours until the matrix was denuded of cells. The medium was carefully aspirated and rinsed gently with PBS. Next, 1 ml of 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. 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.

Immunoblot Analysis

Protein-treated cells were washed once in PBS and lysed in a 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 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) 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 minutes and then incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence reagent (Santa Cruz Biotechnology). The band intensities were quantified using the Photo-Image System (Molecular Dynamics, Sunnyvale, CA, http://www.mdyn.com).

Assays for Cell Adhesion, Spreading, and Migration

Four-well plates (Nunc, Rochester, NY, http://www.nuncbrand.com) were coated with Fn (Sigma) for 24 hours at 4°C. Fn was dissolved in PBS (pH 7.4) to yield a final concentration of 10 μg/ml, and a volume of 150 μl was added to the individual wells. The plates were then blocked with 10 mg/ml BSA (Sigma) in PBS for 1 hour at 37°C. MSCs either transfected with tTG (tTG-MSCs) or not 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 minutes 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. For spreading assays, MSCs were plated for 3 hours on either Fn-coated or noncoated 4-well plates using the conditions of the adhesion experiments described above. Plates were washed with PBS, fixed with 3% formaldehyde, stained with Coomassie blue, destained, and photographed. To determine MSC spreading, plates were carefully washed three times with PBS, and then four separate fields were photographed by phase contrast microscope. Migration was assayed by a modification of the Boyden chamber method using microchemotaxis chambers and polycarbonate filters (Nunc) with a pore size of 8.0 μm. The filters were coated with 10 μg/ml Fn and placed between the chambers. MSCs were trypsinized and suspended at a concentration of 2×10^4 cells per milliliter in DMEM supplemented with 0.5% FBS. The MSC suspension (100 μl) was placed in the upper chamber, and 600 μl of DMEM containing 5 ng/ml basic fibroblast growth factor (bFGF) was placed in the lower

chamber. The chamber was incubated at 37°C and 5% CO_2 for 5 hours. The filter was then removed, and the cells on the upper side of the filter were scraped off with a cotton tip. The MSCs that migrated to the lower side of the filter were fixed in methanol and stained with hematoxylin. The number of migrated cells was estimated by microscopic cell counting using a hemacytometer. Experiments were performed in triplicate and were repeated at least three times.

Transfection and RNA Interference

Transient transfections of tTG cloned into the modified eukaryotic expression vector pMT2 were performed using Lipofectamine PLUS Reagent (Gibco-BRL, Gaithersburg, MD, http://www.gibcobrl.com) [19]. Briefly, MSCs (second passage) cultured in a 60-mm culture plate (3×10^5 cells per plate) were washed twice with serum-free DMEM. Lipofectamine PLUS Reagent was diluted with serum-free DMEM and combined with 5 μg of DNA for each plate. The DNA and Lipofectamine PLUS Reagent were added into each plate containing fresh medium on cells. After 12 hours of incubation in a CO_2 incubator at 37°C, the medium was exchanged with 10% FBS-DMEM. The cells were further incubated for 24 hours at 37°C. For function-blocking experiments, small interfering (si)RNA molecules were targeted at tTG mRNA. A 21-nucleotide sequence for siRNA was derived from the human tissue transglutaminase mRNA sequence (GenBank accession number GI: 42476286) and obtained from Ambion (Austin, TX, http://www.ambion.com). siRNA against tTG (sense, 5′-GGGUUACCGGAAU AUCAUCTT-3′; antisense, 5′-GAUGAUAUUCCGGUAACCCTT-3′)and silencer negative control siRNA were also designed and synthesized by Ambion. MSCs were transfected with si-tTG duplexes by using siPORT NeoFX (Ambion). Briefly, RNA duplex (10 nM final concentration) was incubated in serum-free DMEM containing 15 μl of siPORT NeoFX for 10 minutes. The complex was added to the empty 60-mm culture plate and overlaid with a MSC suspension (1×10^5 cells per plate) onto the culture plate wells containing transfection complexes. The transfected cells were incubated in normal cell culture conditions until ready to assay.

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 coronary artery, according to previously described procedures [20]. 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, 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 from the injured region to the border using a Hamilton syringe (Hamilton Co., Reno, NV, 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 hours. Eight animals per group (ligation, MSCs, mock-MSCs, tTG-MSCs) were used for morphologic analysis at 1 week after occlusion of left coronary artery. For functional studies, we used eight animals per group for echocardiography at 3 weeks after occlusion. 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 minutes. 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.

Histology and Immunohistochemistry

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 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 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. Other serial sections were analyzed with mouse anti-major histocompatibility complex (MHC), mouse anti-myosin light chain (MLC), mouse anti-cardiac troponin T (CTn T) obtained from Santa Cruz Biotechnology, rabbit anti-Ca^{2+} channel (Cav2.1) from Alomone Labs (Jerusalem, http://www.alomone.com), and rabbit anti-connexin 43 and rabbit anti-N-cadherin from Cell Signaling Technology. FITC-conjugated goat anti-rabbit IgG and Texas Red-conjugated goat anti-mouse IgG or mouse anti-goat IgG from Jackson Immunoresearch Laboratories were used as secondary antibodies. All images were made by using an excitation filter under reflected light fluorescence microscopy and transferred to a computer equipped with MetaMorph software version 4.6 (Universal Imaging Corp., Downtown, PA, http://www.universal-imaging.com).

Assessment of Cardiac Function

Transthoracic echocardiographic studies were performed by an experienced cardiologist who was blinded to the group to which the animals had been allocated at baseline (before left anterior descending coronary artery [LAD] ligation), immediately after LAD ligation (before treatment), and 3 weeks after cell transplantation (after treatment) on a GE Vivid Seven ultrasound machine (GE Healthcare, New York, http://www.gehealthcare.com/usen/index.html) with a 10.0 MHz transducer. The rats were anesthetized generally and placed in the left lateral decubitus position. The echo transducer was placed on the left hemithorax, and short axis views were recorded. Two-dimensional (2D) images were obtained at midpapillary level [21, 22]. The M-mode tracing of left ventricular (LV) contraction was also obtained at the same level as the short-axis view. LV end diastolic diameter (LVEDD) and LV end systolic diameter (LVESD) were measured with the M-mode tracing. Percent fractional shortening was determined as ([LVEDD − LVESD]/LVEDD) × 100 (%). LV end diastolic volume (LVEDV) was calculated as $7.0 \times \text{LVEDD}^3/(2.4 + \text{LVEDD})$, LV end systolic volume (LVESV) as $7.0 \times \text{LVESD}^3/(2.4 + \text{LVESD})$, and LV ejection fraction (EF) as EF (%) = (LVEDV − LVESV)/LVEDV × 100. Two images were obtained in each view, and each parameter was measured from three consecutive beats in each image. Six values of each parameter were measured, and the average was recorded. Echocardiograms were stored digitally and analyzed offline with the EchoPAC program with custom 2D strain rate imaging software. More than three images were obtained in the short axis view, and the parameters were measured from three consecutive beats in each image. For the quantitative analysis of regional LV systolic function, peak systolic circumferential strain and peak systolic radial strain were measured on six segments (anteroseptum, anterior, anterolateral, posterolateral, inferior, inferoseptum) of the mid-LV level in the parasternal short-axis view. For quantitative analysis of global LV systolic function, the average values of peak systolic circumferential and that of peak systolic radial strain of the six segments were calculated.

Statistical Analysis

All data were expressed as means ± SD. We considered p values less than .05 statistically significant (analysis of variance and Student's t test).

RESULTS

Isolation and Characteristics of MSCs and Transfection of tTG into MSCs

MSCs were first isolated from mixed cultures with hematopoietic cells based on their attachment on the culture plate. The

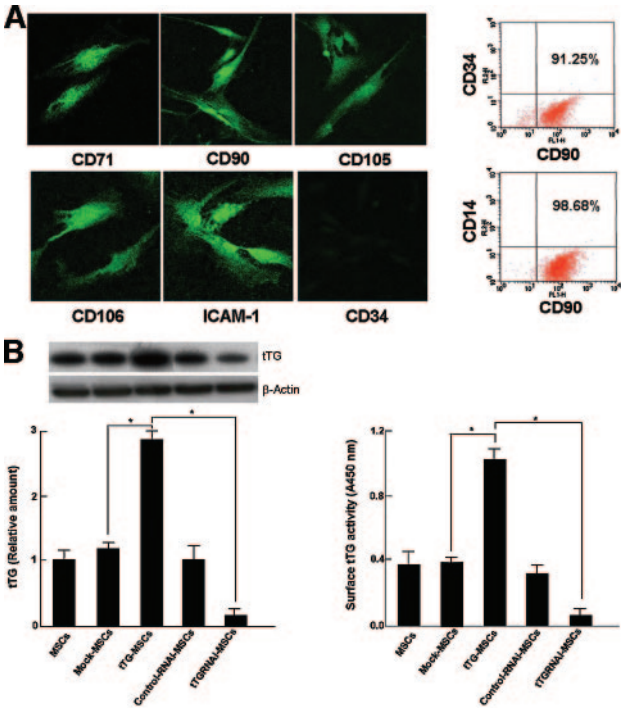

Figure 1. The characteristics of MSCs and expression of tTG. **(A):** Most adherent MSCs are practically fibroblastic in morphology, and some polygonal cells were contained after a 6-week culture. Magnification, ×100. Cells were cultured from bone marrow after density fractionation and are shown at 10 days after plating. At 14 days, the MSCs were positive for CD71, CD90, CD105, CD106, and intracellular adhesion molecule-1 and were negative for CD14 and CD34 by flow cytometry. **(B):** Expression levels of cellular tTG were determined by immunoblot with anti-tTG antibody, and relative amounts were estimated using densitometry. Activity of surface tTG was determined using cell-mediated incorporation of biotinylated cadaverine into fibronectin (Fn). MSCs were plated (2×10^5 cells per well) in complete Dulbecco's modified Eagle's medium without serum in the presence of 0.1 mM biotinylated cadaverine. Cells were allowed to incubate in the Fn-coated plates for 1 hour at 37°C, and the reaction was stopped by washing cells with phosphate-buffered saline containing 3 mM EDTA. Color development was determined by using an enzyme-linked immunosorbent assay plate reader set to 450 nm. Each point represents the mean value obtained in three experiments done in duplicate. *, $p < .05$. Abbreviations: tTG, tissue transglutaminase; tTG-MSCs, tissue transglutaminase-transfected MSCs; tTGRNAi, tissue transglutaminase RNA interference.

isolated MSCs were further purified using bead targeting the hematopoietic marker CD34, yielding 3×10^6 cells within 2 weeks of culture with 95% purity. The MSCs retained a fibroblastic morphology through repeated passages, and their identity was confirmed by immunocytochemistry and fluorescence-activated cell sorting (FACS) analysis. The cultured MSCs expressed CD71, CD90, CD105, CD106, and ICAM. They expressed neither the hematopoietic marker CD34 nor CD14 (Fig. 1A). The tTG gene was introduced into the MSCs using Lipofectamine PLUS, and cells harboring the tTG gene were collected by FACS to implant into infarcted myocardium. Overexpression of tTG led to a 2.7-fold increase in the cellular tTG content in the tTG-transfected MSCs as compared with the vector-transfected MSCs. The surface tTG activity also showed an approximate 2- to 2.5-fold increase in tTG-transfected MSCs (Fig. 1B). Introduction of siRNA molecules for tTG into MSCs significantly decreased both in protein level of tTG and in surface tTG activity.

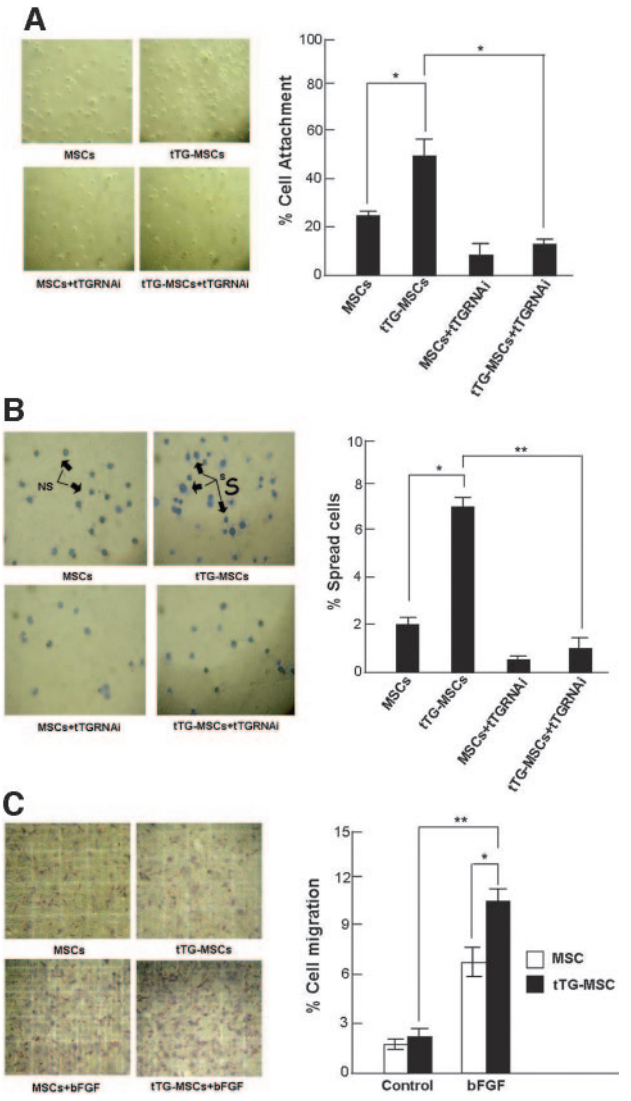

Figure 2. Tissue transglutaminase (tTG)-dependent adhesion, spreading, and migration of MSCs. Quantitative adhesion **(A)** and spreading assays **(B)** with MSCs and tTG-MSCs were measured on fibronectin (Fn)-coated plates for 30 minutes and 3 hours, respectively. Function blocking of tTG was conducted with transfection of small interfering RNA molecules specific for tTG mRNA. **(C):** Migration assays of MSCs and tTG-MSCs were conducted using a Boyden transwell chamber with Fn-coated transmembrane for 5 hours, and bFGF (5 ng/ml) was used as a migration stimulating chemoattractant. Data were expressed as percentage of supplement of 2×10^4 cells per well, which represented 100% and represented the mean value obtained in three experiments done in duplicate. The mean attachment values ± SD on Fn were 5,012 ± 52 in MSCs and 9,025 ± 28 in tTG-MSCs. The mean values of spread cells were 380 ± 19 in MSCs and 1,330 ± 38 in tTG-MSCs, and the mean values of migrated cells were 1,404 ± 61 in MSCs and 1,908 ± 35 in tTG-MSCs after addition of bFGF as a chemoattractant. *, $p < .05$; **, $p < .01$. Abbreviations: bFGF, basic fibroblast growth factor; NS, no spreading; S, spreading; tTG-MSCs, tissue transglutaminase-transfected MSCs; tTGRNAi, tissue transglutaminase RNA interference.

tTG-Mediated Adhesion, Spreading, and Migration of MSCs

To determine the effect of overexpressed tTG in adhesion of MSCs, we performed quantitative adhesion assays with tTG-MSCs. As shown in Figure 2A, adhesion of MSCs to Fn-coated

culture plastic surfaces was enhanced approximately 20% by tTG introduction. To further evaluate the adhesive function of tTG, we cointroduced siRNA molecules for tTG with or without the tTG vector into MSCs. We observed markedly decreased adhesion lower than that of controls in both MSCs and tTG-MSCs. More spreading cells appeared in the tTG-MSC cultures than in the control MSC cultures at the indicated time (typically to 4.75% of control values). Introduction of siRNA molecules for tTG into MSCs also significantly decreased the number of spreading cells to a control level (Fig. 2B). To test the involvement of surface tTG in migration of MSCs with bFGF as a chemoattractant, we used transmigration assays with Transwells (Corning Life Sciences, Acton, MA, http://www.corning.com/lifesciences) where the undersurface was coated with Fn. tTG-MSCs exhibited a 2.52% greater transmigration onto Fn-coated membrane than control MSCs (Fig. 2C).

tTG-Dependent Adhesion of MSCs on Cardiac Fibroblast-Derived Three-Dimensional Matrix (Cardiogel)

We further examined the adhesion of MSCs on cardiogel because three-dimensional (3D) matrix interactions display more biological activities relevant to living organisms in comparison to 2D surfaces coated with Fn. These distinctive in vivo 3D matrix adhesions differ in structure, localization, and function from classically described in vitro adhesions [18]. The cell adhesion to cardiogel was significantly increased in both cases of tTG-MSCs and control MSCs as compared with adhesion to flat plates in spite of Fn coating. The adhesive difference between MSCs and tTG-MSCs was over 33.1% greater when prepared on cardiogel as compared with the Fn-coated flat plates (Fig. 3A).

Effect of tTG Overexpression on the Adhesion-Related Signals in MSCs

It is well established that integrin activates the cellular focal adhesion-related kinases FAK, Src, and PI3K at the adhesive stage and acts, along with tTG protein, as a coreceptor of integrin. Therefore, we investigated the effect of overexpression of tTG in MSCs on the expression of the above-mentioned three proteins at the early adhesive stage. Overexpression of tTG significantly increased the phosphorylation of all three kinases, suggesting that transfection of tTG has a significant effect on the adhesive mechanisms of MSCs (Fig. 3B).

Immunohistochemistry and Histologic Analysis

To address the effect of tTG-MSCs in vivo, we transplanted tTG-MSCs labeled with DAPI 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. tTG-MSCs were retained in approximately 750 cells per section compared with MSCs only in approximately 280 cells per section (Fig. 4A). At 1 week after coronary ligation, the size of left ventricular infarct was recovered both in MSCs and tTG-MSCs compared with the control, but the degree of fibrosis of the infarct zone was higher in MSCs compared with tTG-MSCs (Fig. 4B). After 4 weeks, H&E staining of implants in infarcted myocardium showed alignment of tTG-MSCs with host cardiomyocytes in the DAPI-stained regions, indicating that DAPI-labeled donor cells were incorporated into the nonlabeled cell (host cardiomyocytes). To confirm that the implanted cells had formed cardiac myocyte-like cells, immunohistochemistry was performed and showed that the cardiac-specific markers CTn T, MHC, MLC, and Cav2.1 were detectable in the regions that were DAPI-stained. DAPI-stained tTG-MSCs also expressed connexin-43 and N-cadherin at the regions with the host cardiomyocytes (Fig. 4C).

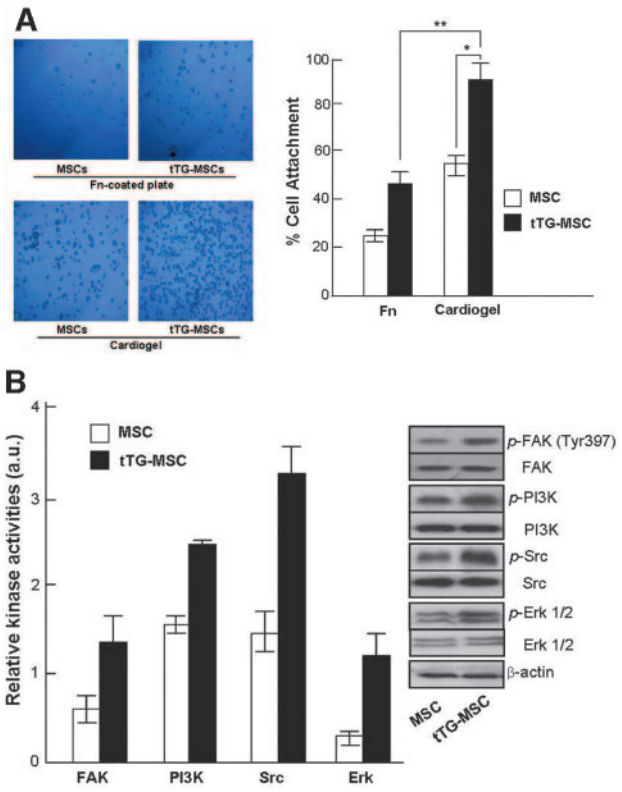

Figure 3. Tissue transglutaminase (tTG)-dependent adhesion of MSCs and integrin-mediated adhesion-related signals on cardiac fibroblast-derived three-dimensional matrix. **(A):** tTG-MSCs were plated on cardiogel for 3 hours. Function blocking of tTG was conducted with transfection of small interfering RNA molecules specific for tTG. Data were expressed as percentage of supplement of 2×10^4 cells per well, which represented 100% and represented the mean value obtained in three experiments done in duplicate. The mean attachment values ± SD on cardiogel were 11,200 ± 142 in MSCs and 17,825 ± 228 in tTG-MSCs. **(B):** Activation of these proteins was enhanced at the adhesive state of tTG-MSCs on Fn as compared with MSCs. MSCs or tTG-MSCs (1×10^6) were plated on Fn-coated 60 Ø plates. Activation levels and total levels of each protein were determined after plating and incubating for 3 hours. The phosphorylated states or total expressions of each protein were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblot analysis with antibodies specific for ERK1/2, FAK, p-FAK, PI3K, p-PI3K, Src, and p-Src. The band intensities were quantified using the Photo-Image System, and each point represented the mean value obtained in three experiments. *, $p < .05$; **, $p < .01$. Abbreviations: a.u., arbitrary unit; Fn, fibronectin; tTG-MSCs, tissue transglutaminase-transfected MSCs.

Evaluation of Cardiac Function

Cardiac dimensions and performance parameters were measured by transthoracic echocardiography. At baseline (i.e., after infarction and before cell transplantation), echocardiographic parameters were not different between the groups. As Figure 5 shows, transplantation of MSCs decreased cardiac dimension and improved systolic performance as compared with the control. Transplantation of tTG-MSCs resulted in a further decrease in the left ventricular chamber size (16.4% decrease in LV end diastolic diameter and 39.1% decrease in LV end diastolic volume) and a further improvement of LV systolic function (66.1% increase in fractional shortening and 46.4% increase in

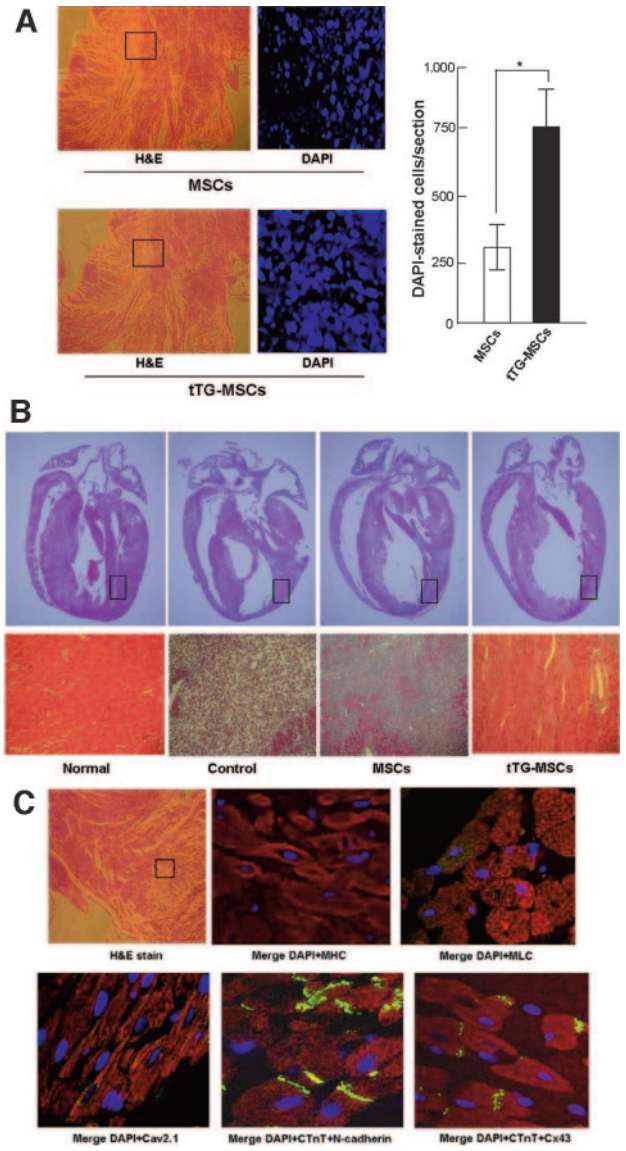

Figure 4. Analysis of myocardial repair after implantation into infarcted myocardium. **(A):** Engraftment was significantly improved with tissue transglutaminase (tTG) overexpression at 3 days after injection of DAPI-stained cells (1×10^6). **(B):** Masson's Trichrome stain of infracted region resulted in a blue coloration of the fibrotic areas and muscle tissue appeared orange/brown. **(C):** H&E staining demonstrates the infiltration of viable, mature cardiac myocytes from the border zone into the scar area and infiltrated viable cells lighted with DAPI at 4 weeks after implantation. Immunohistochemical staining showed the cardiac-specific markers CTn T, MHC, MLC, and Cav2.1 were expressed in the DAPI-labeled cells. Merged images of DAPI (blue) and double staining of sections for connexin-43 or N-cadherin (green) and Cav2.1 and CTn T (red) demonstrated that MSC-derived cardiac myocytes express connexin-43 and N-cadherin in the contact surface to the host myocytes. *, $p < .05$. Abbreviations: Cav2.1, rabbit anti-Ca^{2+} channel; CTn T, cardiac troponin T; Cx43, connexin-43; DAPI, 4,6-diamidino-2-phenylindole; MHC, major histocompatibility complex; MLC, myosin light chain; tTG-MSCs, tissue transglutaminase-transfected MSCs.

ejection fraction) as compared with the MSC group. The peak circumferential and radial strain in the infarct zone and global LV were improved in the MSC group as compared with the control. These parameters assessed by 2D strain rate imaging

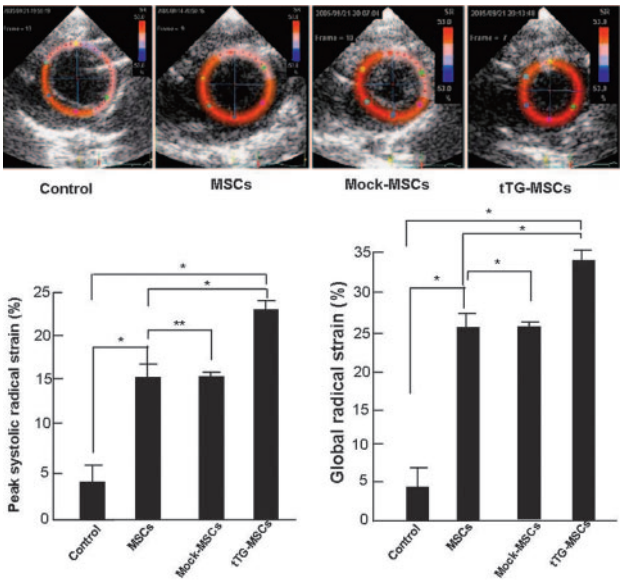

Figure 5. Cardiac function measured with two-dimensional strain imaging. Representative images for control, MSC, mock-MSC, and tTG-MSC groups. The M-mode tracing of left ventricular (LV) contraction was also obtained at the same level as the short-axis view. LV end diastolic diameter (LVEDD) and LV end systolic diameter (LVESD) were measured with the M-mode tracing. Percent fractional shortening was determined as ([LVEDD − LVESD]/LVEDD) × 100 (%). LV end diastolic volume (LVEDV) was calculated as $7.0 \times \text{LVEDD}^3/(2.4 + \text{LVEDD})$, LV end systolic volume (LVESV) as $7.0 \times \text{LVESD}^3/(2.4 + \text{LVESD})$, and LV ejection fraction (EF) as EF (%) = (LVEDV − LVESV)/LVEDV × 100. Abbreviation: tTG-MSCs, tissue transglutaminase-transfected MSCs.

were also further improved in the tTG-MSC group as compared with the MSC group (Table 1).

DISCUSSION

In this study, we have demonstrated that tTG enhances adhesiveness of MSCs into a matrix and spreading and migration of MSCs and enhances the assembly of focal adhesion complexes in vitro. Moreover, tTG-MSCs further restore cardiac function of infarcted myocardium as compared with MSC transplantation alone.

In cell implantation studies for cardiac infarction, several cell types, including skeletal myoblasts [23–25], cardiac myocytes (adult, fetal, or neonatal myocytes) [26, 27], and embryonic stem cell-derived cardiac myocytes [12], were investigated, but each cell therapy appears to have clinical limitations. Autologous MSCs have a great advantage to generate functional cardiac myocytes in the infarcted myocardium because of the easy preparation from adult patients and immunologic safety. However, the frequency of MSC engraftment was extremely low despite implanting large numbers of cells because of a poor rate of cell adhesion and survival. Recently, in a new approach to enhancing the viability of MSCs, they were exposed to the survival signal Akt in the early post-transplant period [28]. Even though the mechanisms of stem cell therapy appear to be far more complex, it has been suggested that stem cells protect cardiomyocytes from apoptotic cell death, release angiogenic ligands, induce proliferation of endogenous cardiomyocytes, and may recruit resident cardiac stem cells [29–32].

Table 1. Cardiac dimensions and performance parameter between MSCs and tTG-MSCs

Variables	Control ($n = 8$)	MSCs ($n = 8$)	Mock-MSCs ($n = 8$)	tTG-MSCs ($n = 8$)
LVEDD (mm)	7.21 ± 0.50	6.64 ± 0.48	6.72 ± 0.52[a]	5.62 ± 0.40[a,b,c]
LVESD (mm)	6.11 ± 0.38	5.28 ± 0.29[a]	5.35 ± 0.50[a]	3.71 ± 0.36[a,b,c]
FS (%)	14.63 ± 2.22	20.15 ± 2.90[a]	20.33 ± 1.95[a]	33.77 ± 2.84[a,b,c]
LVEDV (ml)	0.84 ± 0.07	0.66 ± 0.10[a]	0.69 ± 0.19[a]	0.42 ± 0.04[a,b,c]
LVESV (ml)	0.54 ± 0.08	0.35 ± 0.09[a]	0.37 ± 0.12[a]	0.13 ± 0.05[a,b,c]
LVEF (%)	35.5 ± 4.8	46.7 ± 5.3[a]	47.0 ± 3.7[a]	68.8 ± 3.7[a,b,c]
Peak S cir (%; infarct zone)	−1.90 ± 0.40	−4.79 ± 1.20[a]	−4.31 ± 1.72[a]	−8.62 ± 1.02[a,d,e]
Peak S rad (%; infarct zone)	3.91 ± 1.07	16.97 ± 1.97[a]	16.33 ± 1.40[a]	24.08 ± 2.67[a,b,c]
Global S cir (%)	−4.23 ± 1.63	−8.10 ± 2.31[a]	−7.64 ± 1.27[a]	−14.42 ± 1.18[a,d,e]
Global S rad (%)	5.58 ± 2.42	26.00 ± 3.81[a]	25.14 ± 3.65[a]	34.22 ± 3.55[a,d,e]

Values are given as mean ± SD.
[a] $p < .01$ vs control.
[b] $p < .05$ vs MSCs.
[c] $p < .05$ vs mock-MSCs.
[d] $p < .01$ vs MSCs.
[e] $p < .01$ vs mock-MSCs.
Abbreviations: FS, fractional shortening; LVEDD, left ventricular end diastolic diameter; LVEDV, left ventricular end diastolic volume; LVEF, left ventricular ejection fraction; LVESD, left ventricular end systolic diameter; LVESV, left ventricular end systolic volume; S cir, circumferential strain; S rad, radial strain; tTG-MSCs, tissue transglutaminase-transfected MSCs.

The absence of adhesion and spreading is probably the main cause of poor cell survival in cell transplantation [33, 34]. The causes of cell death are influenced by the ischemic environment, which is devoid of nutrients and oxygen, coupled with the loss of survival signals from inadequate interaction between cells and matrix [5]. Therefore, enhancement of cell adhesion and spreading should be one of the major aims in the development of cell transplantation techniques, including the therapeutic use of progenitor cells. Although the adhesion is mainly mediated by integrin $\alpha_v\beta_3$ and the β_1 family integrin $\alpha_5\beta_1$ [35], tTG enhances adhesion by acting as a bridge between integrins and Fn or by mediating the formation of ternary complexes where all three proteins interact with each other [11]. The purpose of this in vitro study was to confirm the role of tTG on adhesion, spreading, and migration of MSCs and the effect on cellular signals. Based on our observation, tTG-MSCs showed a significant increase in adhesion and spreading and a relatively minor increase in migration. In addition, we observed that phosphorylation of focal adhesion-related kinases including FAK, Src, and PI3K was significantly increased. We also confirmed an increase in the phosphorylation level of extracellular signal-regulated kinases, which are a major signal mediator of cell proliferation.

siRNA molecules were used against tTG to reduce tTG expression. In these function-blocking experiments, adhesion and spreading of MSCs were both markedly reduced. Our results show siRNA molecules for tTG can reduce the ability of MSCs to adhere and spread on Fn, suggesting that cell surface tTG may have an important cell-binding role on the ECM of infarcted myocardium after transplantation. According to transthoracic echocardiography, transplantation of tTG-MSCs resulted in a further decrease in LV chamber size and a further improvement of LV systolic function in the MSC group. Finally, we suggest that genetic modification of MSCs with tTG increases adhesiveness on the ECM of infarcted myocardium, overcoming the problem of cell death after implantation.

Conclusion

Autologous mesenchymal stem cell transplantation therapy for tissue repair has inherent limitations due to the poor viability of the stem cells after cell transplantation. Adhesion is a prerequisite for cell survival and also a key factor for the differentiation of MSCs. As a novel prosurvival modification strategy, we genetically engineered MSCs to overexpress tissue transglutaminase, with intention to enhance adhesion and ultimately cell survival after implantation. tTG-transfected MSCs enhanced cell adhesiveness, including spreading and migration into a matrix as well as enhancing the assembly of focal adhesion complexes in vitro. tTG-MSCs further restored cardiac function of infarcted myocardium as compared with MSC transplantation alone. These findings suggest that tTG may play an important role in integrin-mediated adhesion of MSCs in implanted tissues.

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Disclosure of Potential Conflicts of Interest

K.-C.H. owns stock in Yonsei University.

References

1 Minguell JJ, Erices A. Mesenchymal stem cells and the treatment of cardiac disease. Exp Biol Med 2006;231:39–49.
2 Makino S, Fukuda K, Miyoshi S et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 1999;103:697–705.
3 Toma C, Pittenger MF, Cahill KS et al. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002;105:93–98.

4 Zhang M, Methot D, Poppa V et al. Cardiomyocyte grafting for cardiac repair. Graft cell death and anti-death strategies. J Mol Cell Cardiol 2001;33:907–921.
5 Meredith JE Jr, Fazeli B, Schwartz MA. The extracellular matrix as a cell survival factor. Mol Biol Cell 1993;4:953–961.
6 Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 1994;124:619–626.
7 Gartner S, Kaplan HS. Long-term culture of human bone marrow cells. Proc Natl Acad Sci U S A 1980;77:4756–4759.
8 Ingber DE. Mechanical signaling and the cellular response to extracellular matrix in angiogenesis and cardiovascular physiology. Circ Res 2002;91:877–887.
9 Matter ML, Ruoslahti EA. Signaling pathway from the alpha5beta1 and alpha (v) beta3 integrins that elevates bcl-2 transcription. J Biol Chem 2001;276:27757–27763.
10 Gaudry CA, Verderio E, Jones RA et al. Tissue transglutaminase is an important player at the surface of human endothelial cells: Evidence for its externalization and its colocalization with the beta (1) integrin. Exp Cell Res 1999;252:104–113.
11 Akimov SS, Krylov D, Fleischman LF et al. Tissue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin. J Cell Biol 2000;148:825–838.
12 Watanabe E, Smith DM Jr, Delcarpio JB et al. Cardiomyocyte transplantation in a porcine myocardial infarction model. Cell Transplant 1998; 7:239–246.
13 Miyahara Y, Nagaya N, Kataoka M et al. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat Med 2006;12:459–465.
14 Kraitchman DL, Tatsumi M, Gilson WD et al. Dynamic imaging of allogenic mesenchymal stem cells trafficking to myocardial infarction. Circulation 2005;112:1451–1461.
15 Kraitchman DL, Heldman AW, Atalar E et al. In vivo magnetic imaging of mesenchymal stem cells in myocardial infarction. Circulation 2003; 107:2290–2293.
16 Jones RA, Nicholas B, Mian S et al. Reduced expression of tissue transglutaminase in a human endothelial cell line leads to change in cell spreading, cell adhesion and reduced polymerisation of fibronectin. J Cell Sci 1997;110:2461–2472.
17 Agah A, Kyriakides TR, Bornstein P. Proteolytic of cell-surface tissue transglutaminase by matrix metalloproteinase-2 contributes to the adhesive defect and matrix abnormalities in thrombospondin-2 null fibroblasts and mice. Am J Pathol 2005;167:81–88.
18 Cukierman E, Pankov R, Stevens DR. Taking cell-matrix adhesions to the third dimension. Science 2001;294:1708–1712.
19 Hwang KC, Gray CD, Sivasubramanian N et al. Interaction site of GTP binding Gh (Transglutaminase II) with phospholipase C. J Biol Chem 1995;270:27058–27062.
20 Kang SM, Lim S, Song H et al. Allopurinol modulates reactive oxygen species generation and Ca^{2+} overload in ischemia-reperfused heart and hypoxia-reoxygenated cardiomyocytes. Eur J Pharmacol 2006;535:212–219.
21 Langeland S, D'hooge J, Wouters PF et al. Experimental validation of a new ultrasound method for the simultaneous assessment of radial and longitudinal myocardial deformation independent of insonation angle. Circulation 2005;112:2157–2162.
22 Nagaya N, Kangawa K, Itoh T et al. Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation 2005;112:1128–1138.
23 Taylor DA, Atkins BZ, Hungspreugs P et al Regenerating functional myocardium: Improved performance after skeletal myoblast transplantation. Nat Med 1998;4:929–933.
24 Reinecke H, Zhang M, Bartosek T et al. Survival, integration, and differentiation of cardiomyocyte grafts: A study in normal and injured rat hearts. Circulation 1999;100:193–202.
25 Lipsic E, van der Meer P, Henning RH et al. Timing of erythropoietin treatment for cardioprotection in ischemia/reperfusion. J Cardiovasc Pharmacol 2004;44:473–479.
26 Murry CE, Wiseman RW, Schwartz SM et al. Skeletal myoblast transplantation for repair of myocardial necrosis. J Clin Invest 1996;98:2512–2523.
27 Li RK, Mickle DA, Weisel RD et al. Natural history of fetal rat cardiomyocytes transplanted into adult rat myocardial scar tissue. Circulation 1997;96(suppl 9):S179–S186.
28 Mangi AA, Noiseux N, Kong D et al. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med 2003;9:1195–1201.
29 Wollert KC, Drexler H. Clinical application of stem cells for the heart. Circ Res 2005;96:151–163.
30 Kamihata H, Matsubara H, Nishiue T et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation 2001;104:1046–1052.
31 Fuchs S, Baffour R, Zhou YF et al. Transendocardial delivery of autologous bone marrow enhances collateral perfusion and regional function in pigs with chronic experimental myocardium ischemia. J Am Coll Cardiol 2001;37:1726–1732.
32 Kinnaird T, Stabile E, Burnett MS et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res 2004;94:678–685.
33 Guerette B, Skuk D, Celestin F et al. Prevention by anti-LFA-1 of acute myoblast death following transplantation. J Immunol 1997;159: 2522–2531.
34 Zvibel I, Smets F, Soriano H. Anoikis: Roadblock to cell transplantation. Cell Transplant 2002;11:621–630.
35 Thomas FT, Contreras JL, Bilbao G et al. Anoikis, extracellular matrix, and apoptosis factors in isolated cell transplantation. Surgery 1999;126: 299–304.