Tissue Kallikrein-Modified Mesenchymal Stem Cells Provide Enhanced Protection Against Ischemic Cardiac Injury After Myocardial Infarction

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**Background:** Genetically modified mesenchymal stem cells (MSCs) are a promising approach to the treatment of cardiac injury after myocardial infarction (MI).

**Methods and Results:** Rat MSCs were transduced with adenovirus containing human tissue kallikrein (TK) gene (TK-MSCs), and they secreted human TK into culture medium. Cultured TK-MSCs were more resistant to hypoxia-induced apoptosis and exhibited reduced caspase-3 activity compared to control GFP-MSCs. The effect of TK-MSC injection on cardiac injury was evaluated in rats at 1 and 14 days after MI. At 1 day after MI, human TK expression in the myocardium was associated with improved cardiac function and decreased inflammatory cell accumulation, proinflammatory gene expression and apoptosis. The beneficial effect of TK-MSCs against apoptosis was verified in cultured cardiomyocytes, as TK-MSC-conditioned medium suppressed hypoxia-induced apoptosis and caspase-3 activity, and increased Akt phosphorylation. At 2 weeks after MI, TK-MSCs improved cardiac function, decreased infarct size, attenuated cardiac remodeling, and promoted neovascularization, as compared to GFP-MSCs. Furthermore, the TK-MSC-conditioned medium, containing elevated vascular endothelial growth factor levels, stimulated the proliferation, migration and tube formation of cultured human endothelial cells.

**Conclusions:** Our results indicate that TK-modified MSCs provide enhanced protection against cardiac injury, apoptosis and inflammation, and promote neovascularization after MI, leading to cardiac function improvement.

**Key Words:** Angiogenesis; Apoptosis; Inflammation; Remodeling

**C**hronic heart failure induced by myocardial infarction (MI) leads to a loss of cardiac tissue and impairs left ventricular function. Adult stem cells have been shown to provide cardiac protection, and mesenchymal stem cells (MSCs) are a promising strategy for repairing and regenerating heart cells, and restoring heart function after an ischemic insult because of their ability to home to sites of tissue injury and inflammation. In addition, MSCs exert paracrine actions that produce therapeutic effects, such as neovascularization and attenuation of cardiac remodeling. Genetically modified MSCs have been shown to augment the protective effects of MSCs. For example, genetic modifications with the antiapoptotic Akt gene or the antioxidant heme oxygenase (HO)-1 gene have been observed to enhance ischemic cardiac function and MSC viability, and decrease ventricular remodeling and apoptosis. Moreover, MSCs that overexpress the transcription factor GATA-4 are more resistant to oxidative stress, exhibit increased survival and promote angiogenesis in the heart after MI. Thus, utilization of a gene that exhibits antiapoptotic, antioxidant and proangiogenic properties would enhance the beneficial effects of MSCs against cardiac injury.

Tissue kallikrein (TK) is a serine proteinase that releases vasoactive kinin peptides from a low-molecular-weight kininogen substrate. Our previous studies showed that TK gene delivery improved cardiac function and reduced infarct size and apoptosis after acute MI, and that TK treatment prevented hypoxia-induced apoptosis of cultured cardiomyocytes. Moreover, TK suppressed cardiomyocyte apoptosis in vitro and in vivo by inhibiting oxidative stress and activating Akt-mediated signaling pathways. TK also attenuated cardiac inflammation, hypertrophy and fibrosis, and increased nitric oxide formation in rats after MI. We demonstrated that administration of TK-modified MSCs in the kidney protected against ischemia-induced renal injury by suppression of apoptosis and inflammation. Therefore, in the present study we investigated the potential beneficial effects MSCs genetically
modified with TK after acute and chronic MI.

**Methods**

**MSC Isolation**

Rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA). All procedures complied with the standard for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Resources, National Academy of Sciences). Animal study protocols were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina.

Rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and femurs and tibias were aseptically removed. Bone marrow (BM) was flushed from the shaft of the bone with low-glucose DMEM medium (Sigma, St. Louis, MO, USA) containing 5% fetal calf serum (FCS) plus penicillin/streptomycin (Invitrogen, Paisley, Scotland), and then filtered through a 100-μm sterile filter (Falcon, Bedford, MA, USA) to produce a single-cell suspension. MSCs were recovered from the BM by their tendency to adhere tightly to plastic culture dishes and were identified by positive immunostaining of vimentin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a-smooth muscle actin (α-SMA; Sigma).

**Generation of Genetically Modified MSCs**

Adenovirus harboring human tissue kallikrein cDNA (Ad. CMV-TK) or green fluorescent protein (GFP) gene (Ad.CMV-GFP) was generated as previously described. Cultured MSCs were transduced with Ad.CMV-TK (TK-MSCs) or Ad.CMV-GFP (GFP-MSCs) at multiplicity of infection of 50 for 2 h, followed by a second transduction at MOI of 100 for another 2 h. Expression of TK was identified by positive immunostaining and a specific enzyme-linked immunosorbent assay (ELISA).

**MSC-Conditioned Medium Preparation and Measurement of Vascular Endothelial Growth Factor (VEGF) Secretion**

Conditioned medium was generated as previously described. Isolated MSCs were transduced with Ad.CMV-GFP or Ad.CMV-TK as described. At 12 h after the second transduction, MSCs were incubated with low-glucose DMEM without FCS for 24 h. Medium was then collected and concentrated 5-fold by Centricon concentrators (Millipore, Billerica, MA, USA). VEGF levels were determined in concentrated medium by an ELISA kit (R&D Systems, Minneapolis, MN, USA).

**Detection of Apoptotic MSCs Induced by Hypoxia**

MSCs were seeded in 12-well plates and transduced with Ad.CMV-GFP or Ad.CMV-TK as described for 48 h. MSCs were then subjected to hypoxia for another 24 h in serum-free DMEM. Hypoxic conditions were created by incubating MSCs at 37°C inside an airtight Plexiglas chamber with a mixture of 5% CO₂ and 95% N₂. The oxygen level in the chamber was maintained at 0.5% and measured by an oxygen analyzer. Apoptotic MSCs were stained with Hoechst 33385, and caspase-3 activity in cells lysates was determined.

**MSC Injection After MI**

All animal procedures were performed with the approval of the Institutional Animal Care and Use Committee. Permanent ligation of the left descending coronary artery was induced in male Wistar rats weighing 250–300 g. Animals were divided into 2 groups for histological and biochemical analyses at 1 day and 14 days after MI. At 20 min after coronary artery ligation, GFP-MSCs or TK-MSCs (5×10⁶ cells) suspended in 200 μl saline were injected into 8 sites in the infarct border area of the left ventricle with a 30G needle (n=8 per group). MSCs were injected into rat hearts 4 days after transduction to attain maximal expression of human TK. Control animals underwent coronary ligation and saline injection (n=6). Animals were killed at 1 or 14 days after MSC injection. Expression of human TK in the myocardium was identified by immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR).

**Infarct Size and Cardiac Function Measurements**

Infarct size was quantified with planar morphometry by 2,3,5-triphenyltetrazolium chloride staining and Masson’s trichrome staining at 1 and 14 days after MI, respectively. For each slice, the area at risk and infarct area were delineated and calculated. Hemodynamic parameters were measured by right carotid artery catheter upon completion of infarction at 1 and 14 days after coronary ligation.

**Detection of Inflammation and Apoptosis at 1 Day After MI**

Monocytes/macrophages were identified by immunohistochemistry using primary anti-ED-1 antibody (Chemicon International, Temecula, CA, USA) and a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA), and counted in a double-blind fashion from 20 different fields of each section ×400 magnification. Infiltrated neutrophils were quantified in tissue sections stained with hematoxylin and eosin (H&E). In brief, neutrophils were counted under ×400 magnification from 20 different fields and distinguished from macrophages by size and a polymorphonucleus. DNA fragmentation was determined by terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay (Roche Applied Science, Indianapolis, IN, USA) in 4μm-thick paraffin-embedded sections. The ratio of TUNEL-positive cardiomyocytes to the total number of cardiomyocytes was calculated and expressed as a percentage. Caspase-3 activity was determined as a measurement of cellular apoptosis.

**In Vitro Experiments With MSC-Conditioned Medium on Cardiomyocyte Apoptosis**

Neonatal cardiomyocytes were isolated from 1–3-day-old Sprague-Dawley rats by 4 rounds of digestion using collagenase II ( Worthington, Lakewood, NJ, USA) and pancreatic (Sigma). Cardiomyocytes were collected after 1 h of differential plating for myofibroblasts. Cardiomyocyte origin was confirmed by immunocytochemical staining for α-sarcomeric actinin (Sigma). After overnight starvation, DMEM was replaced with 0.5 ml of serum-free DMEM or conditioned medium from either GFP-MSCs or TK-MSCs. Cardiomyocytes were placed in a hypoxic chamber for 24 h and apoptosis was then assessed.

**Hypertrophy, Fibrosis and Neovascularization at 2 Weeks After MI**

Cardiomyocytes were visualized by Gordon and Sweet’s silver staining. To determine cardiomyocyte size, 40 cardiomyocytes were chosen from random fields in each infarct border area. In each field, only cardiomyocytes positioned in the same orientation and with maximum diameters were selected for tracing with NIH Image software. Myocardial fibrosis was identified by Sirius red and collagen I (Chemicon International) staining in the infarct border area. The percentage of collagen density in noninfarcted left ventricles was analyzed from Sirius-red stained sections.
stained slides by Adobe PhotoShop software. Tissue sections were stained with antibodies to the angiogenic factors CD31 (PharMingen, San Jose, CA, USA) or α-SMA (Sigma). Random fields (n=30) around each infarct border area were selected and numbers of capillaries and arterioles were counted. Immunohistochemistry was performed using a Vectastain ABC kit (Vector Laboratories).

Real-Time PCR
Total RNA was extracted from the left ventricle using TRIzol reagent (Invitrogen) and reverse transcribed using a RT kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was carried out using the Taqman Gene Expression Assays Rn00562055_ml for tumor necrosis factor α (TNF-α), Rn00564227_ml for intercellular adhesion molecule (ICAM)-1 and Rn01456716_gl for monocyte chemoattractant protein (MCP)-1 on a 7300 RT-PCR system (Applied Biosystems). Gene expression was normalized by the housekeeping gene GAPDH (Mm99999915_gl) and quantified by Relative Quantification Software (Applied Biosystems).

Caspase-3 Activity Assay
In brief, cells were lysed in lysis buffer (50mmol/L HEPES, pH 7.4, 100mmol/L NaCl, 0.1% CHAPS, 1 mmol/L dithio-}

reitol, 0.1 mmol/L EDTA) on ice for 10 min, then centrifuged for collection of supernatant. Equal volumes of lysates were incubated with or without caspase-3 inhibitor in equal volumes of assay buffer (lysis buffer with 0.1% glycerol) at 37°C for 10 min. Freshly prepared colorimetric substrate was added to the mixtures for 30 min incubation. The samples were mixed and fluorescent intensity (380 nm excitation, 460 nm emission) was recorded at room temperature.

Proliferation, Migration and Tube Formation in Cultured Human Umbilical Vein Endothelial Cells (HUVECs)
HUVECs were obtained from Clonetics (San Diego, CA, USA) and cultured in endothelial growth medium-2 (EGM-2; Lonza, Walkersville, MD, USA). The cell proliferation assay was performed using CellTiter AQues One Solution (Promega, Madison, WI, USA). Briefly, HUVECs were seeded on 48-well plates (1×10^4 cells/well) and incubated with concentrated conditioned medium (0.5 ml). After 24-h incubation, the CellTiter solution was added into each well and absorbance at 490 nm was recorded 2 h later.

For determination of endothelial cell migration, HUVECs were seeded into 12-well plates and scratched with a 200-μl pipette tip. After washing with phosphate-buffered saline and replacing the serum-free medium with 0.1% FCS, concentrated
Figure 2. TK-MSC injection results in human tissue kallikrein (TK) expression and reduces inflammatory cell accumulation and proinflammatory gene expression in the infarct area at 1 day after myocardial infarction (MI). Human TK was detected in rat hearts injected with TK-MSCs, as determined by (A) immunohistochemical staining and (B) reverse transcription-polymerase chain reaction. Positive immunohistochemical staining is indicated by brown color. Original magnification, ×200. Reduced inflammation by TK-MSC administration was determined by (C) ED-1 immunohistochemical staining, (D) monocyte/macrophage quantification, (E) neutrophil quantification, and gene expression of (F) TNF-α, (G) ICAM-1, and (H) MCP-1. ED-1-positive cells are indicated by arrows. Original magnification, ×200. Data are mean±SEM (n=5–8). *P<0.05 vs. other MI groups; **P<0.05 vs. MI/Control group. MSC, mesenchymal stem cell.
Figure 3. TK-MSCs protect against cardiac cell apoptosis at 1 day after myocardial infarction (MI) and in vitro. TK-MSC administration reduced apoptosis in the infarct area at 1 day after MI, as determined by (A) TUNEL staining, (B) quantification of apoptotic cells, and (C) caspase-3 activity. Original magnification, ×200. Data are mean±SEM (n=5–8). *P<0.05 vs. other MI groups. Cultured cardiomyocytes treated with 0.5 ml of TK-MSC-conditioned medium exhibit higher tolerance to hypoxia-induced apoptosis, as evidenced by (D) Hoechst staining, (E) quantification of apoptotic cells, and (F) caspase-3 activity. Cells with nuclear condensation were recognized as being Hoechst positive. Apoptotic cells are indicated by arrows. (G) Conditioned medium from TK-MSCs (0.5ml) induced Akt phosphorylation in cultured cardiomyocytes, as determined by western blot. GAPDH was used as internal control. Data are mean±SEM of 3 experiments. *P<0.05 vs. other groups. MSC, mesenchymal stem cell; TK, tissue kallikrein.
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Table. Hemodynamic Parameters at 1 and 14 Days After MI

<table>
<thead>
<tr>
<th></th>
<th>MAP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>dP/dt max (mmHg/s)</th>
<th>dP/dt min (mmHg/s)</th>
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<tr>
<td>Sham</td>
<td>107.6±4.3</td>
<td>1.6±0.4</td>
<td>3,350±110</td>
<td>2,939±78</td>
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<td>MI (1 day)</td>
<td>104.8±3.0</td>
<td>8.0±1.3</td>
<td>2,693±80</td>
<td>1,871±332</td>
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<td>Control</td>
<td>103.3±3.3</td>
<td>4.1±1.3**</td>
<td>2,954±76**</td>
<td>2,549±102**</td>
</tr>
<tr>
<td>GFP-MSC</td>
<td>103.8±4.3</td>
<td>3.5±0.8**</td>
<td>2,981±86**</td>
<td>2,601±116**</td>
</tr>
<tr>
<td>TK-MSC</td>
<td>103.8±4.3</td>
<td>3.5±0.8**</td>
<td>2,981±86**</td>
<td>2,601±116**</td>
</tr>
<tr>
<td>MI (14 days)</td>
<td>69.9±3.8</td>
<td>13.9±1.6</td>
<td>2,405±97</td>
<td>1,847±70</td>
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<tr>
<td>Control</td>
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<td>1,808±84</td>
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<tr>
<td>GFP-MSC</td>
<td>86.1±4.9</td>
<td>6.7±1.6</td>
<td>3,165±133*</td>
<td>2,570±137*</td>
</tr>
<tr>
<td>TK-MSC</td>
<td>86.1±4.9</td>
<td>6.7±1.6</td>
<td>3,165±133*</td>
<td>2,570±137*</td>
</tr>
</tbody>
</table>

n=6–8, *P<0.05 vs. other MI groups; **P<0.05 vs. MI/Control.
MAP, mean arterial pressure; MI, myocardial infarction; LVEDP, left ventricular end-diastolic pressure; dP/dt max, maximum first derivative of pressure; dP/dt min, minimum first derivative of pressure.

Figure 4. TK-MSC injection reduces infarct size and restores left ventricular wall thickness at 2 weeks after myocardial infarction (MI). Infarct size visualized by (A) Masson’s trichrome staining and calculated as (B) the percentage of infarct area to area at risk. (C) LV wall thickness was increased after TK-MSC administration. Data are mean±SEM (n=6–8). *P<0.05 vs. other MI groups.

MSC, mesenchymal stem cell; TK, tissue kallikrein.

Conditioned medium (0.5 ml) was added to the wells. After 24h, cells were fixed and those migrating into the central region were quantified. Modified Boyden chambers (Sigma) were also used to measure EC migration. The lower surface of the chambers was coated with a solution of collagen I (10μg/ml) in phosphate-buffered saline for 1h at 37°C and blocked with 1% bovine serum albumin. Cells (2×10^4) were added in 200 μl of endothelial basal medium-2 (Lonza) to the upper chamber. Concentrated conditioned medium (0.5 ml) was added to the bottom chamber and incubated at 37°C for 16h. The upper surface of the membrane was wiped with a cotton tip to mechanically remove non-migratory cells. The migrating cells attached to the lower surface were fixed and stained. After washing, cells on the lower surface of the filter were counted from 6 random fields.

For determination of EC tube formation, growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA) was added to 24-well plates (200 μl/well). After polymerization at 37°C for 30 min, gels were overlaid with a total of 5×10^4 HU-VECs in concentrated conditioned medium. The cells were incubated for 12h and tube images were obtained using an inverted microscope.

Statistical Analysis
Data were analyzed by 1-way ANOVA followed by Newman-Keuls multiple comparison test. Unpaired 2-tailed Student’s
GAO L et al. identified by immunohistochemistry and RT-PCR in the TK-MSC group at 1 day after MI, but not in the sham, control or GFP-MSC MI groups (Figures 2A, B).

TK-MSCs Reduce Myocardial Inflammatory Cell Accumulation at 1 Day After MI
ED-1 immunostaining and quantitative analysis indicated that monocyte/macrophage numbers in the infarct area were significantly elevated in the control and GFP-MSC MI groups compared to the sham group, and markedly reduced after TK-MSC injection (Figures 2C, D). Similarly, TK-MSCs, but not GFP-MSCs, dramatically decreased the neutrophil accumulation induced by MI, as determined by quantitative analysis of H&E-stained sections (Figure 2E). Furthermore, real-time PCR indicated that TNF-α, ICAM-1 and MCP-1 gene expressions were significantly inhibited by TK-MSC injection (Figures 2F–H). Interestingly, a significant difference in TNF-α expression was observed between the GFP-MSC and TK-MSC groups, whereas ICAM-1 and MCP-1 mRNA levels were comparable in both groups.

TK-MSCs Reduce Myocardial Apoptosis at 1 Day After MI
High levels of apoptotic cells in the infarct area were identified by immunohistochemistry and RT-PCR in the TK-MSC group at 1 day after MI, but not in the sham, control or GFP-MSC MI groups (Figures 2A, B).

Results
Resistance of MSCs Expressing TK to Hypoxia-Induced Apoptosis
In cultured MSCs transduced with Ad.CMV-TK, the human TK levels in the medium were monitored for 3 weeks, with peak levels from days 5 to 8 (Figure 1A). After exposure to hypoxia for 24h, TK-MSCs had significantly fewer apoptotic cells compared to control MSCs and GFP-MSCs, as shown by representative staining (Figure 1B). Quantitative analysis verified this result (Figure 1C). Caspase-3 activity was also significantly decreased in TK-MSCs compared to the control MSCs and GFP-MSCs (Figure 1D).

Human TK Expression After TK-MSC Implantation in Rat Myocardium
To evaluate the effect of TK-MSCs on ischemia-induced cardiac damage, GFP-MSCs or TK-MSCs were injected into rat hearts 20min after MI induction. TK-MSC implantation was evident by expression of human TK in ischemic myocardium, identified by immunohistochemistry and RT-PCR in the TK-MSC group at 1 day after MI, but not in the sham, control or GFP-MSC MI groups (Figures 2A, B).

Figure 5. TK-MSC administration protects against cardiac remodeling in the peri-infarct area 2 weeks after myocardial infarction (MI). TK-MSC administration reduces cardiomyocyte hypertrophy and fibrosis, as determined by (A) silver staining, Sirius-red staining, and collagen I immunohistochemical staining. Original magnification, ×400 for silver staining, and ×200 for immunohistochemical and Sirius-red staining. Quantification of (B) cardiomyocyte size, and (C) collagen fraction volume. Data are mean±SEM (n=4–6). *P<0.05 vs. other MI groups; **P<0.05 vs. MI/Control group. MSC, mesenchymal stem cell; TK, tissue kallikrein.
Figure 6. TK-MSC administration promotes neovascularization at 2 weeks after myocardial infarction (MI) and in vitro. TK-MSC administration increases capillary and arteriole density in the peri-infarct area 2 weeks after MI, as determined by (A) CD31 and α-SMA immunohistochemical staining of capillaries and arterioles, respectively, and quantitative analysis of (B) capillary and (C) arteriole density. Original magnification, x200. Data are mean±SEM (n=4). *P<0.01 vs. other MI groups; **P<0.01 vs. MI/Control group. (D) MSCs transduced with Ad.CMV-TK secreted VEGF into the culture medium, as determined by ELISA. Cultured HUVECs treated with 0.5 ml of TK-MSC-conditioned medium increases endothelial cell proliferation (E), as well as their migration by (F) modified Boyden chamber and (G) scratch methods, and stimulates endothelial cell tube formation (H). Data are mean±SEM of 3 experiments. *P<0.01 vs. GFP-MSC group. ELISA, enzyme-linked immunosorbent assay; HUVEC, human umbilical vein endothelial cell; MSC, mesenchymal stem cell; SMA, smooth muscle actin; TK, tissue kallikrein.
groups at 1 day after MI (Figure 3A). TK-MSC administration, however, significantly reduced apoptotic cell death. Quantitative analysis verified these results (Figure 3B). Caspase-3 activity measurements further confirmed decreased numbers of myocardial apoptotic cells after TK-MSC injection (Figure 3C).

**TK-MSC-Conditioned Medium Reduces Hypoxia-Induced Apoptosis of Cultured Primary Cardiomyocytes**

Hoechst staining showed that conditioned medium from TK-MSCs significantly reduced the apoptosis of cultured cardiomyocytes after exposure to hypoxia for 24 h, whereas conditioned medium from GFP-MSCs had no protective effect (Figures 3D,E). Moreover, conditioned medium of TK-MSCs, but not GFP-MSCs, markedly decreased hypoxia-induced caspase-3 activity in cardiomyocytes (Figure 3F). The protective effects of TK-MSC-conditioned medium were associated with increased phosphorylation of Akt, as determined by western blot (Figure 3G).

**TK-MSC Injection on Infarct Size and Hemodynamic Parameters at 1 and 14 Days After MI**

At 1 day after MI, TK-MSC and GFP-MSC administration reduced infarct size to similar levels compared to the control MI group (TK-MSC: 23.1±3.9% and GFP-MSC: 21.6±4.8% vs. Control: 50.9±2.9%, P<0.05). Moreover, TK-MSCs and GFP-MSCs improved cardiac function in a comparable manner, as indicated by decreased left ventricular end-diastolic pressure (LVEDP) and increased dP/dt (Table). Mean arterial pressure (MAP) was not significantly different among all groups at 1 day after MI (Table). At 2 weeks after MI, rats in the TK-MSC group showed a significant reduction in infarct size and a marked increase in left ventricular wall thickness compared to rats receiving GFP-MSCs (Figure 4). Furthermore, TK-MSC injection significantly improved cardiac function as evidenced by reduced LVEDP and increased dP/dt, as compared to rats in the GFP-MSC group (Table). MAP was not significantly different among all groups at 2 weeks after MI (Table).

**TK-MSCs Attenuate Cardiac Remodeling at 2 Weeks After MI**

Silver-stained heart sections and cardiomyocyte area measurement showed that TK-MSCs reduced cardiomyocyte hypertrophy in the peri-infarct area, but GFP-MSC injection had no effect (Figures 5A,B). Moreover, analysis of both Sirius-red staining and collagen I immunohistochemistry in the peri-infarct region indicated that MSC administration decreased collagen deposition, and the effect was more pronounced in the TK-MSC group than in the GFP-MSC group (Figure 5A). Collagen fraction volume measurement of Sirius-red stained heart sections verified this result (Figure 5C).

**TK-MSCs Promote Neovascularization in the Myocardium at 2 Weeks After MI and Angiogenesis in Cultured ECs**

Administration of TK-MSCs increased both capillary and arteriolo density in the peri-infarct area, as determined by CD31 and α-SMA immunohistochemical staining, respectively, compared to rats in the GFP-MSC group (Figure 6A). Quantification of capillaries and arterioles confirmed this effect (Figures 6B,C). In vitro, transduction of ECs with Ad.CMV-TK resulted in higher levels of VEGF in the culture medium compared to the GFP-MSC group (Figure 6D). Proliferation of cultured ECs was increased by TK-MSC-conditioned medium compared to GFP-MSC-conditioned medium (Figure 6E). Conditioned medium from TK-MSCs significantly increased the migration of cultured ECs compared to conditioned medium from GFP-MSCs, as determined by modified Boyden chamber method (Figure 6F) and by scratch method (Figure 6G). Moreover, TK-MSC-conditioned medium promoted EC tube length compared to GFP-MSC-conditioned medium (Figure 6H).

**Discussion**

MSCs display many desirable traits for the treatment of ischemic cardiac diseases. However, their sensitivity to the hypoxic and inflammatory environment in ischemic tissue is problematic. In the setting of ischemic heart disease, genetic modification of MSCs has been found to improve their survival, enhance cardiac function and promote repair of the damaged region. The protective effects of Akt-modified MSCs are attributable to secreted paracrine factors such as VEGF. Similarly, cultured TK-MSCs secrete higher levels of VEGF in culture medium compared to control GFP-MSCs, as demonstrated in both the present study and a previous report. In addition, TK enhances VEGF expression in cultured ECs. Therefore, it is likely that the protective actions of TK-MSCs against ischemic cardiac damage are a result of both autocrine and paracrine mechanisms.

Cardiomyocyte apoptosis is a major contributor to heart injury, morbidity and mortality. We showed that cultured TK-MSCs are more resistant to hypoxia-induced apoptosis compared to GFP-MSCs. It is possible that the higher tolerance of TK-MSCs to hypoxic conditions allowed these cells to engraft in the infarct area in greater number than GFP-MSCs. TK-MSC implantation was evident by expression of human TK. TK-MSC administration reduced myocardial apoptosis and caspase-3 activity at 1 day after MI. In addition, TK-MSC culture medium prevented hypoxia-induced cardiomyocyte apoptosis in conjunction with increased Akt phosphorylation. TK and VEGF secreted from TK-MSCs may contribute to Akt activation, as Akt functions to promote cell survival in endothelial cells and cardiomyocytes. TK has been shown to improve cardiac function and myocardial blood flow after MI, whereas dominant-negative Akt abolished TK’s protective effect, indicating an Akt-mediated signaling event. Kallikrein gene delivery also inhibited cardiomyocyte apoptosis after ischemia-reperfusion via activation of Akt-GSK-3β and Akt-Bad-14-3-3 signaling pathways. Moreover, TK protein infusion prevented cardiomyocyte apoptosis in association with increased Akt and GSK-3β phosphorylation. TK’s protective effect against cardiomyocyte apoptosis is facilitated by constitutively active Akt, but blocked by dominant-negative Akt. In contrast to Akt, enzymes known as cathepsins are known to promote cellular apoptosis, which can lead to cardiovascular diseases. Cathepsins contribute to cardiac injury by targeting antiapoptotic molecules, whereas cathepsin inhibition exhibits cardiovascular protective actions. Although the role of cathepsins was not investigated in the current study, the potential effect of TK on regulating their activity would be an interesting topic of research. Taken together, these data indicate that TK, VEGF, and possibly other factors protect against cardiomyocyte apoptosis and lead to cell survival through Akt signaling cascades.

Ischemia induces the production of reactive oxygen species and a number of inflammatory molecules, such as TNF-α, ICAM-1 and MCP-1. Because inflammation is such an important contributor to heart damage after MI, numerous studies have focused on inhibiting the inflammatory response with the aim of reducing infarct size and apoptosis and improving cardiac repair. Our previous study showed that TK infusion reduced inflammation in the infarcted heart in conjunction
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with suppressed NF-κB activation and MCP-1 and VCAM-1 expressions. In the current study, administration of TK-MSC led to a significant decrease in neutrophil and monocyte/macrophage infiltration of the myocardium, in association with downregulation of TNF-α, ICAM-1 and MCP-1 at 1 day after MI. TK-MSC administration also resulted in reduced myocardial inflammatory cell accumulation at 2 weeks post-MI (data not shown).

MI-induced ventricular remodeling is characterized by progressive enlargement of the myocardium and deposition of extracellular matrix proteins. Capillary density is decreased in the noninfarcted region during MI-induced stress conditions. However, restoration of capillary density reduces cardiac remodeling because of the augmented oxygen and nutrient availability. Although capillaries distribute blood flow to individual cells, arteries provide bulk flow to tissues, thus having more importance in overall tissue health. In the current study, parallel changes in capillary density and arteriole density were not observed in the MI groups at 2 weeks after MI. The similar level of capillaries in the MI control and MI/GFP-MSC groups reflect the possibility that GFP-MSCs did not produce sufficient amounts of growth factors to stimulate angiogenesis, despite the observation that GFP-MSC-conditioned medium stimulated VEGF secretion in vitro. Rats in the MI/TK-MSC group had a comparable capillary density with the sham group, indicating probable stimulation of pre-existing capillary growth. Arteriole density was slightly increased in the MI control group, suggesting probable stimulation of pre-existing arteries. Administration of both GFP-MSCs and TK-MSCs facilitated arteriole enlargement, and perhaps collateral growth, possibly because of the implantation of MSCs in the larger vessels. We previously reported that kallikrein gene transfer attenuated cardiac remodeling and increased capillary density after MI. Our current findings are consistent with those studies, as TK-MSCs induced both angiogenesis and arteriogenesis in the heart and diminished myocyte hypertrophy and cardiac collagen accumulation. The in vivo pro-angiogenic effect of TK-MSCs was further verified in cultured ECs. Indeed, significant increases in EC proliferation, migration and tube formation occurred in association with elevated VEGF secretion from TK-MSCs. There was also a marked reduction of collagen deposition upon GFP-MSC administration compared to the control MI group. However, there was no significant difference between these 2 groups with regard to cardiomyocyte hypertrophy, infarct size or left ventricular wall thickness. Importantly, both cardiomyocyte size and collagen deposition were significantly reduced by TK-MSC administration compared to the GFP-MSC MI group.

TK has been shown to activate the matrix metalloproteinases (MMPs) pro-gelatinase and pro-collagenase. Thus, it is likely that TK secretion from TK-MSCs may be responsible for the further reduction in cardiac fibrosis via MMP activation. Taken together, these observations suggest that TK-MSCs secrete soluble factors to elicit protective actions against cardiac injury induced by MI.

Interestingly, administration of GFP-MSCs and TK-MSCs on the day after MI had the same effect on infarct size and cardiac function, yet TK-MSCs caused a significant reduction in myocardial apoptosis and inflammatory cell accumulation compared to control GFP-MSCs. Moreover, the degree of cardiac apoptosis and inflammation was similar between the control and GFP-MSC MI groups. This discrepancy between cardiac function data and histological observations 1 day post-MI might be explained by additional molecular mechanisms activated by TK secreted from TK-MSCs as opposed to cytokine secretion alone from GFP-MSCs. Modification of MSCs with the TK gene could provide enhanced cardiac protection possibly by TK’s ability to activate the kinin B2 receptor, either dependent or independent of kinin formation, thereby attenuating myocardial injury through Akt signaling and nitric oxide production. Although the contribution of kinins to the protective effects of TK-MSCs was not investigated in the present study, Koch et al showed that transgenic rats overexpressing TK have increased basal kinin levels that may be responsible for the improvement in cardiac function and remodeling in these rats after MI. Furthermore, while no difference in infarct size and cardiac function was observed between the TK-MSC and GFP-MSC MI groups 1 day after MI, the effects of TK-MSCs on inflammation and apoptosis may lead to improvement in these areas over a longer period of time. Indeed, at 2 weeks after MI induction, infarct healing, ventricular remodeling and cardiac function were vastly improved in rats receiving TK-MSCs compared to those in the control and GFP-MSC MI groups.

Conclusions

Our study demonstrates that TK-modified MSCs offer enhanced protection against cardiac damage after MI. Taken together with the results of in vitro studies, our findings indicate that TK-MSCs augment cardiac protection through TK’s pleiotropic effects on reducing hypoxia-induced apoptosis of MSCs and cardiomyocytes, inflammatory cell infiltration, inflammatory factor expression, myocardial hypertrophy and fibrosis. At the same time, TK-MSCs promoted neovascularization in vivo and in vitro through MSC-mediated paracrine mechanisms. TK-MSC administration provided enhanced advantages in promoting cardiomyocyte survival, inhibiting the process of ventricular remodeling, and improving cardiac function. Therefore, MSCs genetically-modified with human TK are a potential therapeutic for ischemic heart diseases.

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Disclosures

The authors declare no conflicts of interest.

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