Tissue Inhibitor of Matrix Metalloproteinase-3 or Vascular Endothelial Growth Factor Transfection of Aged Human Mesenchymal Stem Cells Enhances Cell Therapy after Myocardial Infarction

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Abstract

Mesenchymal stem cell (MSC) transplantation has been proposed as a potential therapeutic approach for ischemic heart disease, but the regenerative capacity of these cells decreases with age. In this study, we genetically engineered old human MSCs (O-hMSCs) with tissue inhibitor of matrix metalloproteinase-3 (TIMP3) and vascular endothelial growth factor (VEGF) and evaluated the effects on the efficacy of cell-based gene therapy in a rat myocardial infarction (MI) model. Cultured O-hMSCs were transfected with TIMP3 (O-TIMP3) or VEGF (O-VEGF) and compared with young hMSCs (Y-hMSCs) and non-transfected O-hMSCs for growth, clonogenic capacity, and differentiation potential. *In vivo*, rats were subjected to left coronary artery ligation with subsequent injection of Y-hMSCs, O-hMSCs, O-TIMP3, O-VEGF, or medium. Echocardiography was performed prior to and at 1, 2, and 4 weeks after MI. Myocardial levels of matrix metalloproteinase-2 (MMP2), MMP9, TIMP3, and VEGF were assessed at 1 week. Hemodynamics, morphology, and histology were measured at 4 weeks. *In vitro*, genetically modified O-hMSCs showed no changes in growth, colony formation, or multi-differentiation capacity. *In vivo*, transplantation with O-TIMP3, O-VEGF, or Y-hMSCs increased capillary density, preserved cardiac function, and reduced infarct size compared to O-hMSCs and medium control. O-TIMP3 and O-VEGF transplantation enhanced TIMP3 and VEGF expression, respectively, in the treated animals. O-hMSCs genetically modified with TIMP3 or VEGF can increase angiogenesis, prevent adverse matrix remodeling, and restore cardiac function to a degree similar to Y-hMSCs. This gene-modified cell therapy strategy may be a promising clinical treatment to rejuvenate stem cells in elderly patients.

Introduction

DESPITE GREAT IMPROVEMENT IN OUR understanding and treatment of ischemic heart disease, acute myocardial infarction (MI) remains a leading cause of mortality and morbidity, especially in the elderly.¹ Current treatments are unable to restore heart function or reduce ventricular matrix remodeling in the remote myocardium.

It has been demonstrated in several preclinical studies that cell therapy can reverse cardiac dilation and ventricular dysfunction and enhance angiogenesis after MI,²⁻⁴ but the results of initial clinical studies were disappointing,

especially in elderly patients.^{5,6} Notably, most preclinical studies were performed in young animals, whereas the clinical trials enrolled mostly aged patients with heart disease. Previous studies have demonstrated an age-related decrease in stem cell number,⁷ proliferation potential,⁸ regenerative capacity,⁹ and differentiation potential¹⁰ and an increase in senescence and apoptosis. We recently found that human mesenchymal stem cells (hMSCs) derived from old patients were less robust in growth and produced weaker functional benefits after implantation into infarcted hearts than cells from young donors.¹¹ Among the various cell types investigated for use in cell therapy, hMSCs are good

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candidates because of their self-renewal and multi-lineage potential.^{12,13} Moreover, hMSCs are desirable for autologous cell therapy because they are easy to harvest, isolate, culture, and expand *in vitro*.

Cell-based gene therapy permits the temporally and spatially regulated release of a gene product and has been shown to greatly improve the function of infarcted animal hearts compared to MSCs alone.^{14–17} Vascular endothelial growth factor (VEGF) is a critical factor in the regulation of angiogenesis,¹⁸ and transplantation of VEGF-engineered stem cells promoted angiogenesis and cardiomyocyte regeneration and improved heart function in a rat MI model.^{15,19,20} Tissue inhibitor of matrix metalloproteinase-3 (TIMP3) is highly expressed in the normal heart but is reduced in failing hearts in association with maladaptive myocardial remodeling.^{21–23} A main contributor to the adverse changes in ventricular dimensions and function that follow MI is a shift in the expression or activity of the degradative matrix metalloproteinase (MMP) enzymes relative to that of the TIMPs. Overexpressing TIMP3 in MSCs was a potentially effective approach to generate transient MMP inhibition and achieve functional recovery in TIMP3-deficient mice following MI.¹⁴

In this study, old hMSCs (O-hMSCs) were genetically modified to overexpress VEGF or TIMP3, with the aim of improving angiogenesis and regulating the MMP/TIMP balance in the remote myocardium, respectively. We proposed that transplantation of O-hMSCs overexpressing VEGF or TIMP3 would induce angiogenesis, prevent matrix degradation, and improve cardiac function following MI.

Materials and Methods

Experimental animals

Male Wistar rats (220±20 grams) were obtained from laboratory animal center of the Second Affiliated Hospital of Harbin Medical University (Harbin, China). All animal procedures were conducted in accordance with the *Guidelines for the Care and Use of Laboratory Animals* (NIH, revised 1996) and approved by the Animal Care Committee of the University Health Network.

Collection of bone marrow

Human bone marrow was collected as previously described.¹¹ Briefly, bone marrow aspirates were obtained from the sternum of patients undergoing cardiac surgery at the Second Affiliated Hospital of Harbin Medical University. All studies were done in accordance with university regulatory committees. “Young” (Y) bone marrow was collected from patients aged 1–5 years with congenital heart diseases, whereas “old” (O) bone marrow was obtained mainly from patients aged 50–70 years with valve diseases and coronary heart disease.

Isolation, culture, and identification of hMSCs

hMSCs were isolated from bone marrow aspirates by centrifugation with a Ficoll-Paque gradient (1.073 g/mL density; GE Healthcare), plated into 25-cm² culture flasks in Iscove modified Dulbecco medium (IMDM; Gibco) containing 10% fetal bovine serum (FBS; Biological Industries), and incubated at 37°C in a humidified 5% CO₂ atmosphere. Approximately 48 hr later, the non-adherent fraction was

removed. The medium was replaced every 3 days until adherent cells reached 80% confluence, and the cells were then passaged by 1:2. All experiments were done with cells in passages 3–5.

Cells at passage 3 were characterized by flow cytometry (FACSCalibur, Becton Dickinson) using antibodies against CD29 (FITC), CD90 (FITC), CD34 (FITC), CD105 (PE), CD133 (PE), and CD45 (PerCP) to identify hMSCs. Mouse isotype immunoglobulin G (IgG) antibodies were used as controls (BD Pharmingen). Data analysis was performed with CellQuest software (BD Biosciences).

Genetic modification of O-hMSCs

VEGF or TIMP3 was cloned into pIRES2-EGFP (pIRES2-EGFP/VEGF, pIRES2-EGFP/TIMP3; Clontech) as previously described.²⁴ Briefly, the plasmids were amplified in DH5 α *Escherichia coli* and purified using the EndoFree Plasmid Maxi Kit (Qiagen). One day before transfection, the O-hMSCs were trypsinized and plated (3×10⁵ cells/well) in a six-well plate according to the protocol of Roche FuGENE HD Transfection Reagent. In brief, 2.0 μ g of DNA (pIRES2-EGFP/VEGF or pIRES2-EGFP/TIMP3) and 5 μ L of FuGENE HD were separately diluted in 100 μ L of IMDM (without serum and antibiotics). The transfection mixture was mixed immediately, incubated for 15 min at room temperature, and added to the adherent MSCs with 1 mL of complete medium. Transfection was optimized according to the supplier's instructions by varying the amount of DNA and the volume of transfection reagent at a ratio between 3:2 and 8:2. Transfection efficiency was assessed 48 hr after transfection by confocal microscopy and flow cytometry. VEGF and TIMP3 mRNA levels were determined by RT-PCR at 0, 3, 7, 14, and 28 days after transfection. The primers were designed as follows: VEGF forward, 5'-GCACCCATGGCAGAAGG AGG-3', reverse, 5'-CCTTGGTGAGGTTTGATCCGCATA-3', 263 bp; TIMP3 forward, 5'-CTGCTGACAGGTCGCGTC-3', reverse, 5'-CAACCCAGGTGATACCGATAGT-3', 122 bp. VEGF (Santa Cruz Biotechnology) and TIMP3 (Abcam) protein levels were assessed by western blotting at 0, 3, 7, 14, and 28 days after gene transfection.

Differentiation potential of hMSCs in vitro

O-hMSCs transfected with VEGF (O-VEGF) or TIMP3 (O-TIMP3) were assessed for their multipotency by inducing their differentiation into adipocytes, osteocytes, and cardiomyocytes. Osteogenic differentiation was induced by culturing 70% confluent hMSCs for 3 weeks in IMDM, 10% FBS, 10⁻⁸ M dexamethasone, 20 mM β -glycerophosphate, and 50 μ M ascorbate-2-phosphate. Osteogenic differentiation was assessed by staining cells with Alizarin Red S.

Adipogenic differentiation was induced by plating cells at a density of 3×10³/cm² for 3 weeks in culture medium supplemented with 0.5 μ M isobutyl-methylxanthine (IBMX), 50 μ M indomethacin, and 0.5 μ M dexamethasone. Adipogenic differentiation was determined using Oil Red O stain as an indicator of intracellular lipid accumulation.

Cardiomyocyte differentiation was induced by culturing 70% confluent hMSCs for 24 hr in medium supplemented with 10 μ M 5-azacytidine. Conditioned medium was replaced with IMDM containing 10% FBS. Medium was changed every 3 days for 2 weeks. Cardiomyocyte differentiation was

verified by immunostaining with antibodies against desmin and cTnT, as described previously.^{25,26}

Cell growth characteristics

The hMSCs were serially subcultured under standard conditions to the third passage. Y-hMSCs, O-hMSCs, O-VEGF, and O-TIMP3 were trypsinized and seeded into 96-well plates at a starting density of 1,000 cells/well (three wells/group/time). Cell growth was evaluated with a Cell Counting Kit-8 (CKK-8; Dojindo, Japan) colorimetric assay at 1, 3, 5, 7, 9, and 11 days after plating.

Colony-forming unit fibroblast assay

To count the number of cell colonies, the cells were plated in six-well plates (100 cells/well) with MesenCult medium (Stem Cell Technologies). Two weeks later, the cells were washed with phosphate-buffered saline (PBS) and stained with 0.5% Crystal Violet (Sigma) in methanol for 5 min at room temperature. The plates were washed twice with PBS, and colonies containing 50 cells or more were counted.

MI model and cell transplantation

Beginning 3 days before cell transplantation, all rats were immunosuppressed with intraperitoneal cyclosporine A (5 mg/kg; Novartis Pharmaceuticals) each day until the end of the experiment. MI was performed as described previously.¹¹ Thirty minutes after coronary artery ligation, cells suspended in IMDM ($2 \times 10^6/100 \mu\text{L}$) were injected into one site in the center of the ischemic myocardium and into three sites in the border of the infarct region. Controls received MI and culture medium only (no cells). The incision was closed, and an antibiotic (150,000 U/mL penicillin G) was given intraperitoneally.

Echocardiography and hemodynamics

Echocardiography was performed prior to and at 1, 2, and 4 weeks after ligation ($n=6$ animals/group) with a 12-MHz transducer (Vivid 7, GE). The left ventricular end-diastolic dimension (LVEDD) and volume (LVEDV) and the left ventricular end-systolic dimension (LVESD) and volume (LVESV) were measured by two-dimensional and M-mode images. Left ventricular ejection fraction (%) and fractional shortening (%) were calculated automatically by the echocardiography system. All echocardiography data were averaged from at least three consecutive cardiac cycles.

Cardiac hemodynamics were measured at 4 weeks after ligation ($n=6$ animals/group). The right carotid artery was isolated and cannulated with a microtip catheter filled with heparinized (10 U/mL) saline solution, which was connected with an MLT0699 disposable pressure transducer (ADInstruments). Hemodynamic parameters were monitored and recorded by the PowerLab data acquisition system (ADInstruments).

RT-PCR

Hearts ($n=6$ animals/group) were harvested 7 days after cell implantation, frozen in liquid nitrogen, and powdered completely. RT-PCR was performed to detect tissue mRNA levels of TIMP3, MMP9, MMP2, and VEGF. Gene cDNA was

synthesized using the Reverse Transcription System (Promega) according to the manufacturer's protocol. The primers were as follows: TIMP3 forward, 5'-GCCGTTTATGGAGTTGAT-3', reverse, 5'-AGCATTGAGCAGGGTAGA-3', 280 bp; VEGF forward, 5'-CCCACGACAGAAGGAGAGCA-3', reverse, 5'-GCACACAGGACGGCTTGAA-3', 151 bp; MMP9 forward, 5'-GCACGGCAACGG AGA AGGC-3', reverse, 5'-CCGTCCG CTGGTACAGGAAGAG-3', 258 bp; MMP2 forward, 5'-AAG TCTGAAGAGTGTGAA GT-3', reverse, 5'-GTGAAGGAGA AGGCTGATT-3', 180 bp. RT-PCR products were resolved by 2% agarose gel electrophoresis and compared by density intensity. β -Actin served as an internal control.

MMP and TIMP3 activity

Hearts ($n=6$ animals/group) were harvested 7 days after cell implantation, frozen in liquid nitrogen, and powdered. Samples were treated with extraction buffer for 1 hr on ice for determination of MMP2, MMP9, and TIMP3 activities. The activity levels of MMP2 and MMP9 were estimated by gelatin zymography, and TIMP3 was evaluated by reverse-gelatin zymography, as described previously.^{21,27}

Morphometric assessment and immunohistochemical studies

At 4 weeks, the hearts were removed, and the left ventricle was distended at a ventricular pressure of 20 mmHg and fixed in 4% paraformaldehyde. After 48 hr, each heart was sectioned transversely (1-mm sections). The size of the infarct was defined as the ratio (percentage) of scar length to the entire left ventricular circumference, as described previously.¹⁶ The infarct size was measured in each section using Image-Pro Plus 6.0 software (Media Cybernetics Inc.) then summed and averaged.

To assess capillary density, left ventricular paraffin sections were immunostained with anti-CD31 antibody (Santa Cruz Biotechnology). Positively stained vessels were counted in 10 random fields within the peri-infarct zone at a magnification of $200\times$ in two sections per animal, in a blinded fashion.

To quantify the number of transplanted hMSCs in the heart, left ventricular paraffin sections were immunostained with an antibody against human mitochondria (Millipore). Positive cells were counted in the peri-infarct zone, infarct, and remote regions. The quantification of surviving cells was performed as described above for capillary density.

Collagen content and structure

To evaluate the collagen content, histological slides were stained with Masson's Trichrome. The infarcted area with an increase in collagen content (blue staining) was measured by computerized morphometry and expressed as a percentage of the total infarct area.²⁸ Five sections from apex to base were assessed and averaged.

Tumor necrosis factor- α level

Prior to and at 3, 7, and 14 days after ligation, tumor necrosis factor- α (TNF- α) levels were quantified in myocardial extracts by enzyme-linked immunosorbent assay (ELISA) using a commercial rat TNF- α ELISA Kit (Invitrogen) according to the manufacturer's protocol. Each sample at each time point was performed in triplicate and averaged.

Statistical analysis

All data are expressed as mean $\pm$ standard deviation (SD). Comparisons between groups were done by one-way analysis of variance (ANOVA) with least significant differences *post hoc* tests. Repeated-measures ANOVA compared the effects of cell treatment (medium, Y-hMSCs, O-hMSCs, O-VEGF, O-TIMP3) on echocardiographic variables. A value of $p < 0.05$ was considered statistically significant.

Results

Characterization and proliferation of the hMSCs

Flow cytometry analysis of expressed cell-surface antigens showed that the hMSCs were uniformly positive for CD29, CD90, and CD105 and negative for the leukocyte common antigen CD45 and hematopoietic lineage markers CD34 and CD133, regardless of age. The morphology of hMSCs from young donors (Y-hMSCs) and old donors (O-hMSCs) was

similar. The cells displayed a homogeneous spindle-shaped population in the initial passages, but there were increased numbers of enlarged O-hMSCs in later passages (data not shown).

The proliferative capacity of Y-hMSCs and O-hMSCs was significantly different (Fig. 1a,b). The Y-hMSCs proliferated more rapidly than O-hMSCs, with or without genetic modification. We used a colony-forming unit fibroblast (CFU-F) assay to estimate the colony-forming ability of the hMSCs from young and old donors (Fig. 1c,d). Significantly more colonies were observed with the Y-hMSCs than the O-hMSCs, regardless of whether they were genetically modified.

Overexpression of TIMP3 and VEGF in genetically modified O-hMSCs

The transfection efficiency of O-hMSCs was evaluated by green fluorescent protein (GFP) 48 hr after plasmid transfection (Fig. 2a). Fluorescence-activated cell sorting (FACS)

FIG. 1. Proliferation of young human mesenchymal stem cells (Y-hMSCs) and old human mesenchymal stem cells (O-hMSCs). **(a)** Photomicrographs of cultured hMSCs on day 3, 7, and 11 of the initial passage. Magnification, 100 $\times$. **(b)** The Cell Counting Kit-8 (CCK-8) colorimetric assay was performed on cells (passage 3) to assess proliferation. Growth curves showed a significant difference between Y-hMSCs and O-hMSCs (** $p < 0.01$) whether or not they were genetically modified (O-hMSCs transfected with vascular endothelial growth factor [O-VEGF] and tissue inhibitor of matrix metalloproteinase-3 [O-TIMP3]). **(c)** Cultured cells (passage 3, 100 cells/well) stained with Crystal Violet 2 weeks after plating (arrows indicate representative colonies). **(d)** Quantification of colonies revealed significantly more colony-forming units (CFUs) in the Y-hMSC cultures. (**) $p < 0.01$ vs. all other groups.

FIG. 2. Transfection of old human mesenchymal stem cells (O-hMSCs) with plasmid vectors. **(a)** Photomicrographs of O-hMSCs transfected with the tissue inhibitor of matrix metalloproteinase-3 (TIMP3) or vascular endothelial growth factor (VEGF) green fluorescent protein (GFP)-expressing vector. Magnification, 200 $\times$. **(b)** Flow cytometry assessed the transfection efficiency for O-hMSCs transfected with TIMP3 (14.5% $\pm$ 1.1%) and VEGF (13.3% $\pm$ 1.5%). **(c and d)** RT-PCR showed that TIMP3 and VEGF mRNA levels in O-hMSCs were highest 3 days after transfection. A significant overexpression of TIMP3 and VEGF in transfected O-hMSCs lasted for 7 days (**) $p < 0.01$ vs. prior to transfection. **(e and f)** Western blots showed that TIMP3 and VEGF protein levels in O-hMSCs were highest 3 days after transfection and lasted for 7 days. (**) $p < 0.01$ vs. prior to transfection. (β -Actin served as internal control; day 0 was prior to transfection).

FIG. 3. Genetically modified old human mesenchymal stem cells (O-hMSCs) enhanced cardiac function. **(a and b)** Analysis of ejection fraction **(a)** and fractional shortening **(b)** showed that, compared to the medium control and O-hMSC groups, Y-hMSC, and O-hMSCs transfected with vascular endothelial growth factor (O-VEGF) and tissue inhibitor of matrix metalloproteinase-3 (O-TIMP3) groups showed significant functional improvement. (**) $p < 0.01$. Day 0 was the day of myocardial infarction (MI) and cell transplantation. **(c and d)** Quantitative analysis of hemodynamic parameters dP/dt max **(c)** and dP/dt min **(d)** showed significant improvement in the O-hMSC, Y-hMSC, O-VEGF, and O-TIMP3 groups. (*) $p < 0.05$ vs. medium control; (#) $p < 0.05$ vs. O-hMSCs.

analysis (Fig. 2b) showed that $14.5\% \pm 1.1\%$ and $13.3\% \pm 1.5\%$ of O-hMSCs were transfected with TIMP3 (O-TIMP3) and VEGF (O-VEGF), respectively. To evaluate the expression level of TIMP3 and VEGF in the genetically modified O-hMSCs *in vitro*, RT-PCR (Fig. 2c,d) and western blotting (Fig. 2e,f) were performed at various times post-transfection. Significant overexpression of TIMP3 and VEGF was observed in the O-hMSCs at 3 and 7 days after transfection.

Overexpression of TIMP3 and VEGF did not affect the multipotency of the O-hMSCs. The cells were stained *in vitro* with markers for osteogenic, adipogenic, and myogenic differentiation, and both O-VEGF and O-TIMP3 cells maintained their multi-differentiation capacity (data not shown).

Transplantation of genetically modified O-hMSCs enhanced cardiac function

Echocardiography demonstrated that LVEsV and LVEDV improved more in the Y-hMSC, O-VEGF, and O-TIMP3 groups than in the O-hMSC and medium control groups following MI and cell transplantation. Ejection fraction and fractional shortening in the Y-hMSC, O-VEGF, and O-TIMP3 groups were significantly better than in the O-hMSC and medium control groups ($p < 0.01$; Fig. 3a,b). The O-hMSC and medium control groups did not differ in cardiac function, and neither did the Y-hMSC, O-VEGF, or O-TIMP3 groups.

FIG. 4. Vascular endothelial growth factor (VEGF), tissue inhibitor of matrix metalloproteinase-3 (TIMP3), matrix metalloproteinase (MMP) mRNA expression and MMP activity. **(a)** Expression of VEGF, TIMP3, MMP2, and MMP9 mRNA in the rat myocardium was evaluated by RT-PCR 1 week after MI and cell transplantation. β -Actin served as internal control. **(b)** VEGF mRNA levels were significantly higher in the young human mesenchymal stem cells (Y-hMSCs) and old (O-VEGF) groups. **(c)** TIMP3 mRNA levels were significantly higher in the Y-hMSC and O-TIMP3 groups. **(d and e)** MMP2 **(d)** and MMP9 **(e)** mRNA levels were significantly lower in the Y-hMSC and O-TIMP3 groups. **(f)** TIMP3 activity was evaluated using reverse zymography and was significantly higher in the Y-hMSC and O-TIMP3 groups. **(g)** MMP2 and MMP9 activities were evaluated using zymography, and both were significantly lower in the Y-hMSC and O-TIMP3 groups. (*) $p < 0.05$ vs. all other groups. C, Medium control; O, O-hMSCs; Y, Y-hMSCs; O-V, O-VEGF; O-T, O-TIMP3.

FIG. 5. Cell survival and capillary density increased with transplantation of genetically modified old human mesenchymal stem cells (O-hMSCs). **(a)** Photomicrographs of myocardial sections 4 weeks after myocardial infarction (MI) and cell transplantation. Scale bar, 200 μ m. The sections were stained with a human mitochondrial antibody (brown, indicated by arrows). **(b)** A greater number of transplanted cells was present in the infarcted myocardium of the young (Y)-hMSCs and O-hMSCs transfected with vascular endothelial growth factor (O-VEGF) and tissue inhibitor of matrix metalloproteinase-3 (O-TIMP3) groups. (**) $p < 0.01$ vs. O-hMSCs. **(c)** Peri-infarct zone stained with CD31 antibody (brown, indicated by arrows) 4 weeks after MI and cell transplantation. Scale bar, 200 μ m. **(d)** Capillary density was increased in the Y-hMSC, O-VEGF, and O-TIMP3 groups. (*) $p < 0.05$, (**) $p < 0.01$ vs. medium control and O-hMSCs.

Compared with the medium control group, all other groups showed significant improvement in the hemodynamic parameters of left ventricular function (dP/dt max and dP/dt min) 4 weeks after MI and cell transplantation ($p < 0.05$; Fig. 3c,d). Also, dP/dt max and dP/dt min were significantly greater in the Y-hMSC, O-VEGF, and O-TIMP3 groups than in the O-hMSC group ($p < 0.05$; Fig. 3c,d). There were no differences among the young and genetically modified groups.

VEGF, TIMP3, MMP mRNA expression, and MMP activity in vivo

To assess the potential paracrine effects of the genetically modified hMSCs, we examined VEGF mRNA expression in the myocardium 1 week after cell transplantation using RT-PCR (Fig. 4a). VEGF levels were significantly elevated in the Y-hMSC and O-VEGF groups (Fig. 4b). The high-level expression of VEGF in the Y-hMSCs and O-VEGF cells may provide cardioprotective and pro-angiogenic effects.

To identify myocardial matrix modulation, we quantified the balance between MMP and TIMP gene expression in the myocardium 1 week after cell transplantation. Using RT-PCR, we found that MMP2 and MMP9 expression was sig-

nificantly lower, whereas TIMP3 expression was significantly higher, in the Y-hMSC and O-TIMP3 groups (Fig. 4a,c-e). Consistent with the mRNA expression data, MMP2, MMP9, and TIMP3 activities also differed in the myocardial tissue (Fig. 4f,g). Zymography and reverse zymography demonstrated MMP2 and MMP9 activities were significantly lower and TIMP3 activity was significantly higher, respectively, in the Y-hMSC and O-TIMP3 groups. Upregulation of TIMP3 may inhibit degradation of the extracellular matrix in the infarcted heart.

Cell survival and capillary density increased with transplantation of genetically modified O-hMSCs

To identify the implanted hMSCs, we used an antibody against human mitochondrial protein that was species specific and did not cross-react with rat mitochondria. The number of hMSCs in the infarcted myocardium 4 weeks after cell transplantation was higher in the Y-hMSC, O-VEGF, and O-TIMP3 groups than in the O-hMSC group (Fig. 5a,b).

Capillary density in the peri-infarct zone was evaluated based on CD31 (PECAM-1) immunostaining 4 weeks after cell transplantation. We observed that capillary density was significantly increased in the Y-hMSC, O-VEGF, and O-

FIG. 6. Collagen content and infarct size decreased with transplantation of genetically modified old human mesenchymal stem cells (O-hMSCs). **(a)** Masson's Trichrome staining of the infarct zone 4 weeks after cell transplantation (blue collagen; red myocardium). Scale bar, 200 μ m. **(b)** Quantitative analysis of collagen content in the infarcted area showed less collagen in the young (Y)-hMSC and O-hMSCs transfected with vascular endothelial growth factor (O-VEGF) and tissue inhibitor of matrix metalloproteinase-3 (O-TIMP3) groups. vs. the medium control and O-hMSC groups. (* $p < 0.05$; ** $p < 0.01$). **(c)** Mid-ventricular heart slices 4 weeks after cell transplantation. Thinning of the left ventricular free wall and dilation of the left ventricle were noted in the medium control and O-hMSC groups. **(d)** Scar size was significantly reduced in the Y-hMSC, O-VEGF, and O-TIMP3 groups compared with medium control and O-hMSC groups (** $p < 0.01$).

TIMP3 groups compared to the medium control and O-hMSC groups (Fig. 5c,d). Capillary density did not differ between the medium control and O-hMSC groups.

Collagen content and infarct size decreased with transplantation of genetically modified O-hMSCs

Masson's Trichrome staining showed that the collagen content in the infarcted region 4 weeks after cell transplantation was significantly lower in the Y-hMSC, O-VEGF, and O-TIMP3 groups than in the medium control and O-hMSC groups (Fig. 6a,b).

Four weeks following cell transplantation, computerized morphometric analysis demonstrated a significantly smaller infarct in the Y-hMSC, O-VEGF, and O-TIMP3 groups versus the medium control and O-hMSC groups (Fig. 6c,d). However, there were no significant differences within the Y-hMSC, O-VEGF, and O-TIMP3 groups.

Post-MI TNF- α level was suppressed with transplantation of genetically modified O-hMSCs

Myocardial TNF- α levels increased significantly in all groups after MI, especially in the medium control group (Fig. 7). The TNF- α level was highest in the medium control and O-hMSC groups compared to the Y-hMSC, O-VEGF, and O-TIMP3 groups at 3, 7, and 14 days after MI and cell transplantation. There were no differences in the myocardial TNF- α level within the Y-hMSC, O-VEGF, and O-TIMP3 groups.

Discussion

The beneficial role of cell-based therapy in cardiac repair has been confirmed in various animal models of MI, in contrast to the inconsistent and modest benefits of cell transplantation in clinical trials.^{29,30} Reviewing both pre-clinical animal experiments and clinical trials, we found that the age of both the recipients and donors significantly influenced the outcome.³¹ Previously, we have shown that hMSCs from old patients exhibited significantly less plasticity than hMSCs from young patients and conferred weaker functional benefits after implantation into the infarcted heart.¹¹ A number of studies have verified that transplantation of genetically modified MSCs into the infarcted heart maximized the cardiac functional benefits by increasing cytokine production.^{14–17,32} To this end, in our study, we genetically modified old hMSCs with VEGF- or TIMP3-expressing vectors to enhance the benefits of cell therapy with old hMSCs in a murine MI model. Our *in vitro* studies showed that overexpression of VEGF or TIMP3 did not affect the multi-differentiation potential of the old hMSCs, which retained their ability to differentiate into adipogenic, osteogenic, and myogenic lineages. We demonstrated that, following cell transplantation, overexpression of VEGF or TIMP3 in the infarcted myocardium increased survival of the transplanted old hMSCs, reduced infarct size, and restored cardiac function.

We found that injection of young hMSCs or old hMSCs overexpressing VEGF or TIMP3 significantly preserved ventricular volumes and systolic function following MI. We believe these functional benefits of TIMP3 and VEGF are mainly due to modification of matrix modulation and in-

creased angiogenesis, respectively. Infarct size was also reduced in the hearts injected with young hMSCs or genetically modified old hMSCs.

In the current study, it was observed that hMSC transplantation substantially suppressed TNF- α expression, especially in the young hMSCs and genetically modified old hMSCs. Several studies have demonstrated that inhibition of TNF- α restored cardiac function and reduced infarct size after MI due to matrix remodeling,^{33,34} and TNF- α can increase MMP gene expression in the infarcted heart.³⁵ In addition, an imbalance between MMPs and TIMPs has been shown to contribute to progressive ventricular dysfunction in heart failure.³⁶ In our study, we demonstrated that MMP2 and MMP9 expression and activity levels were lower in the hearts of rats that received young hMSCs or old hMSCs overexpressing VEGF or TIMP3. This shift in the MMP/TIMP ratio may be an explanation for the reduced infarct size and decreased cardiac fibrosis in these rats after MI.

Our research also showed that, compared to old hMSCs, more young hMSCs and genetically modified old hMSCs survived at 4 weeks after implantation. These data revealed that genetic modification of hMSCs with VEGF or TIMP3 effectively increases their survival after implantation. It has been demonstrated that the efficacy of MSC transplantation after MI was closely related to the hostile environment of hypoxia, inflammation, and scarring.¹⁷ Therefore, factors related to the survival of young hMSCs and genetically modified old hMSCs, including cytokines, chemokines, integrin, and adhesion molecules, need to be addressed further.

There are several mechanisms that contribute to the benefits of cell therapy for ischemic hearts, including paracrine effects, generation of new cardiomyocytes or smooth muscle cells, and induction of neovascularization. Considering that human cardiomyocyte-specific markers were not detected at 4 weeks (data not shown) and the reported high frequency of stem cell death within 4 days^{37,38} of injection, transdifferentiation of hMSCs in the ischemic myocardium seems unlikely. Similarly, in other studies, differentiation of MSCs into cardiomyocytes was not observed after implantation into rat hearts.^{39,40} In the current study, we found that the number of capillaries was greatest in the hearts injected with young hMSCs or old hMSCs overexpressing VEGF. RT-PCR demonstrated that VEGF was upregulated in the infarcted myocardium of animals injected with young hMSCs, and the cytokine level was similar in animals that received old hMSCs genetically modified to overexpress VEGF, which supports the mechanism of paracrine effects. Therefore, cell-based gene therapy may increase angiogenesis and prevent adverse matrix remodeling in the infarcted heart due to the constitutive secretion of cytokines.

The malignant potential of MSC-based gene therapy has been reported.⁴¹ However, the old hMSCs in our study were genetically modified with non-viral gene vectors, which have several advantages such as low toxicity, efficient transfection, and sufficient gene delivery. More importantly, the transient overexpression of VEGF and TIMP (1 week) may minimize the potential risk of tumorigenesis while maximizing myocardial regeneration. In our study, the expression of VEGF and TIMP3 peaked at 3 days, remained significantly higher at 7 days, and then dropped off by 14 days. After MI, ventricular modulation is active within the first 1–2 weeks

FIG. 7. Post-myocardial infarction (MI) tumor necrosis factor- α (TNF- α) level increased less with transplantation of genetically modified old human mesenchymal stem cells (O-hMSCs). TNF- α level was similar among groups before MI (day 0) but increased in all groups after MI. After MI and cell transplantation, TNF- α was significantly lower in the young (Y)-hMSC and O-hMSCs transfected with vascular endothelial growth factor (O-VEGF) and tissue inhibitor of matrix metalloproteinase-3 (O-TIMP3) groups. O-VEGF, and O-TIMP3 groups vs. the medium control and O-hMSC groups (* $p < 0.05$).

because of cardiomyocyte necrosis, lymphocyte infiltration, and myofibroblast proliferation. Overexpression of VEGF and TIMP3 genes within 14 days of an MI can improve the microenvironment of the infarcted heart by inducing new vessel formation and inhibiting MMP activity, respectively, to prevent adverse ventricular remodeling and improve regeneration. Therefore, it appears that the increased VEGF and TIMP3 expression we achieved falls within the key 1- to 2-week post-MI period for regeneration of the heart.

In conclusion, hMSC-based gene therapy ameliorated the paracrine microenvironment of the recipient myocardium, promoted local angiogenesis to augment the blood supply to the infarct border zone, reduced infarct size, modulated matrix remodeling, and restored cardiac function after MI. Genetic modification of hMSCs from aged patients rejuvenated the cells for cardiac repair. In the future, genetic modification of hMSCs may provide a novel and effective clinical approach to treating ischemic heart disease in the elderly.

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Author Disclosure Statement

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