A promising strategy for the treatment of ischemic heart disease: Mesenchymal stem cell-mediated vascular endothelial growth factor gene transfer in rats

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BACKGROUND: Treatment of ischemic heart disease (IHD) remains a worldwide problem. Gene therapy, and recently, cell transplantation, have made desirable progress. A combination of appropriate stem cells and angiogenic genes appears promising in treating IHD.

OBJECTIVE: To study the results of angiogenesis and myogenesis induced by transplantation of the adenovirus carrying human vascular endothelial growth factor 165 (Ad-hVEGF₁₆₅)-transfected mesenchymal stem cells (MSCs) in IHD compared with direct MSC transplantation or Ad-hVEGF₁₆₅ delivery.

METHODS: Cultured MSCs were transfected by Ad-hVEGF₁₆₅, and secreted VEGF was measured by ELISA *in vitro*. Ad-hVEGF₁₆₅-transfected MSCs (MSC/VEGF group), MSCs (MSC group), Ad-hVEGF₁₆₅ (VEGF group) or a serum-free medium (control group) was injected into syngeneic Wistar rats immediately after left coronary artery occlusion. All cells were marked with CM-Dil (Molecular Probes, USA) before transplantation. One week after treatment, messenger RNA expression of hVEGF₁₆₅ in the MSC/VEGF group was found to be significantly higher than in other groups by reverse transcriptase-polymerase chain reaction analysis. One month after cell transplantation, left ventricular (LV) ejection fraction, capillary density of the infarcted region, infarct size and hemodynamic parameters (including LV end-diastolic pressure, LV+dP/dt and LV-dP/dt) were measured and immunohistochemical analysis was performed.

RESULTS: A high level of VEGF was expressed by Ad-hVEGF₁₆₅-transfected MSCs. LV ejection fraction, mean capillary density of the infarcted region and hemodynamic parameters were significantly improved in the MSC/VEGF group compared with the MSC group, the VEGF group and the control group ($P < 0.001$ for all). Partly transplanted MSCs showed the cardiomyocyte phenotype, expressed desmin and cardiac troponin T, and resulted in angiogenesis in the ischemic myocardium. However, a few transplanted MSCs incorporated into the vascular structure and most of the new vascular components were host-derived.

CONCLUSIONS: The combined strategy of MSC transplantation and VEGF gene therapy can produce effective myogenesis and host-derived angiogenesis, resulting in the prevention of progressive heart dysfunction after myocardial infarction.

Key Words: Angiogenesis; Cell transplantation; Gene therapy; Ischemic heart disease; Mesenchymal stem cells; Vascular endothelial growth factor

Une stratégie prometteuse pour le traitement de la cardiopathie ischémique : Le transfert génique du facteur de croissance de l'endothélium vasculaire à médiation par les cellules souches mésenchymateuses chez les rats

HISTORIQUE : Le traitement de la cardiopathie ischémique (CPI) demeure un problème mondial. La thérapie génique et, récemment, la transplantation de cellules, ont fait des progrès souhaitables. Une association de cellules souches et de gène angiogénique semble prometteuse pour le traitement de la CPI.

OBJECTIF : Étudier les résultats de l'angiogenèse et de la myogenèse induite par la transplantation des cellules souches mésenchymateuses (CSM) transfectées par l'adénovirus porteur du facteur de croissance de l'endothélium vasculaire humain 165 (Ad-FCEVh₁₆₅) en cas de CPI par rapport à la transplantation de CSM ou à la livraison d'Ad-FCEVh₁₆₅.

MÉTHODOLOGIE : On a transfecté des cultures de CSM par Ad-FCEVh₁₆₅, et le FCEV sécrété a été mesuré par ELISA *in vitro*. On a injecté les CSM transfectées par Ad-FCEVh₁₆₅ (groupe CSM/FCEV), les CSM (groupe CSM), l'Ad-FCEVh₁₆₅ (groupe FCEV) ou un médium sans sérum (groupe témoin) à des rats Wistar syngéniques immédiatement après une occlusion de l'artère coronaire gauche. Toutes les cellules ont été marquées par CM-Dil (Molecular Probes, États-Unis) avant la transplantation. Une semaine après le traitement, l'expression mARN du FCEVh₁₆₅ par méthode PCR-CDNA au sein du groupe CSM/FCEV était significativement plus élevée qu'au sein des autres groupes. Un mois après la transplantation des cellules, on a mesuré la fraction d'éjection ventriculaire gauche (VG), la densité capillaire du foyer infarcté, la dimension de l'infarctus et les paramètres hémodynamiques (y compris la tension VG en fin de diastole, +dP/dt VG et -dP/dt VG) et procédé à une analyse immunohistochimique.

RÉSULTATS : Les CSM transfectées par FCEVh₁₆₅ exprimaient un FCEV élevé. La fraction d'éjection VG, la densité capillaire moyenne du foyer infarcté et les paramètres hémodynamiques s'amélioraient considérablement au sein du groupe CSM/FCEV par rapport au groupe CSM, du groupe FCEV et du groupe témoin ($P < 0,001$ pour tous). Des CSM partiellement transplantées révélaient le phénotype cardiomyocyte, la desmine exprimée et la troponine T cardiaque et entraînaient une angiogenèse dans le myocarde ischémique. Cependant, quelques CSM transplantées s'intégraient à la structure cardiovasculaire, et la plupart des nouveaux éléments vasculaires étaient dérivés de l'hôte.

CONCLUSIONS : L'association de transplantation de CSM et de thérapie génique par FCEV peut produire une myogenèse efficace et une angiogenèse dérivée de l'hôte, ce qui prévient l'évolution du dysfonctionnement cardiaque après un infarctus du myocarde.

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Despite advances in revascularization techniques, the treatment of ischemic heart disease (IHD) remains a challenge. Two main mechanisms are known for the generation of new blood vessels: angiogenesis (sprouting existing primitive vasculature from differentiated cells) and vasculogenesis (developing new blood vessels in situ by differentiation from precursors) (1). Angiogenesis is an integral part of many physiological and pathological processes, and is controlled by a number of factors that induce or inhibit blood vessel formation. Commonly adopted approaches for therapeutic angiogenesis include protein therapy, using direct introduction of recombinant cytokines, and gene therapy, based on the delivery of genes encoding for one or more angiogenic factors using injections of naked DNA, nonviral vectors and/or viral vectors into the myocardium (2,3). Among these angiogenic factors, the better documented are the vascular endothelial growth factor (VEGF) isoforms due to their pivotal role in angiogenesis (4). In addition to its angiogenic effect, VEGF may provide myocardial protection against ischemic injury (5).

More recent progress along these two basic strategies has been to achieve angiogenesis through the transplantation of cells (6). Stem cells derived from marrow stroma are pluripotent and have the ability to differentiate into several cell types, including osteoblasts, chondroblasts, fibroblasts, adipocytes, skeletal myoblasts, cardiomyocytes and endothelial cells (7-10). Moreover, mesenchymal stem cells (MSCs) are abundant in bone marrow and are easy to harvest, culture and expand in vitro, making them a desirable cell type for autologous regenerative cell therapy. Myoblast-mediated angiogenic gene transfer may concurrently achieve myogenesis and angiogenesis in infarcted myocardium and skeletal muscle, and it is therefore being studied as a potential alternative strategy for the treatment of IHD (11).

To pave the way for clinical applications of this new strategy for enhanced therapeutic effects, the present study tested whether cell transplantation using the adenovirus carrying human VEGF₁₆₅ (Ad-hVEGF₁₆₅)-transfected MSCs can enhance angiogenesis and myogenesis, resulting in the prevention of progressive heart dysfunction after myocardial infarction.

METHODS

Animals

All procedures were approved by the Animal Care Committee of the University Health Network and conformed with the National Institutes of Health guidelines. Both donor and recipient rats were syngeneic, inbred, male Wistar rats weighing 250 g to 300 g (SLAC Laboratory Animal Co Ltd, Shanghai, China).

Isolation, culture and characterization of MSCs

Isolation and culture of MSCs were performed as follows. Rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and bone marrow was extruded from tibias and femurs. Bone marrow MSCs were isolated by 1.073 g/mL Percoll (Pharmacia, USA), using density gradient centrifugation. Isolated cells were then cultured in a low-glucose Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, and incubated with 95% air and 5% carbon dioxide at 37°C. Nonadherent cells were removed by changing the medium after 48 h and every three days thereafter. The MSCs were kept subconfluent and expanded by a 1:2 split for three or four passages before transfection and transplantation.

Fluorescence-activated cell sorting analysis (Beckman Coulter, USA) was used to evaluate the lineage and surface marker phenotype of the cultured MSCs. The MSCs were trypsinized and subsequently stained with the following monoclonal antibodies:

anti-CD34 fluorescein isothiocyanate (FITC), anti-CD44-FITC, anti-CD45-FITC and anti-CD90-FITC.

Transfection of MSCs with adenoviruses

The recombinant replication-incompetent Ad-hVEGF₁₆₅ (provided by Dr Gang Li, Shanghai Stem Cell Institute, China) was propagated in cells of the human embryonic kidney line. At a density of approximately 70% to 80% confluence, the cells were infected with Ad-hVEGF₁₆₅ or a null adenovirus (Null-Ad) in 10% fetal bovine serum-supplemented Dulbecco's Modified Eagle Medium. Following full cytopathic effect development, cells were harvested, repeatedly frozen and thawed, and purified by cesium chloride gradient ultracentrifugation following standard procedures. A viral titre was estimated by an end point assay (Quantum Dot Corp, USA), and the quantity of virus particles was calculated based on one optical density (equivalent to 1.05×10^{12} particles per millilitre). MSCs were exposed to adenoviral particles at a multiplicity of infection of 50 for 12 h for gene transfer.

ELISA for VEGF expression in vitro

The Ad-hVEGF₁₆₅-transfected MSCs were grown in six-well tissue culture plates at a cell density of 2×10^5 cells per well, using MSCs transfected with Null-Ad and nontransfected MSCs as controls. The supernatant from each well was collected at two-day intervals from day 1 to day 14 and kept frozen at -20°C until used for the assay. hVEGF₁₆₅ secreted from transfected MSCs was measured using an hVEGF₁₆₅ Sandwich ELISA kit (Chemicon International Inc, USA) following the manufacturer's instructions.

Myocardial infarction model

Male Wistar rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and mechanically ventilated. Through a left thoracotomy in the fourth intercostal space, myocardial infarction was induced by ligating the left anterior descending coronary artery 2 mm from the tip of the left auricle with 6-0 polypropylene (Ethicon, USA). Successful performance of a coronary occlusion was verified by observation of a rapid occurrence of akinesia and/or discolouration in the distal myocardium after ligation. In total, 156 rats were randomly assigned into the MSC/VEGF group, the MSC group, the VEGF group or the control group.

MSC labelling and assay for cell viability

Cultured MSCs were labelled with fluorescent lipophilic dye CM-DiI (Molecular Probes, USA) for cell tracking according to the manufacturer's protocol. In brief, the CM-DiI cell-labelling solution (2 mg/mL) was directly added into Dulbecco's Phosphate-Buffered Saline at 2 µL/mL. MSCs were incubated in this working solution for 5 min at 37°C, and then for an additional 15 min at 4°C. After labelling, cells were washed with phosphate-buffered saline five times before transplantation; cell viability was determined by trypan blue exclusion assay. Labelled MSCs were stained with trypan blue; viable MSCs, with an intact membrane, excluded trypan blue and were therefore not stained.

MSC transplantation and Ad-hVEGF₁₆₅ delivery

CM-DiI-labelled MSCs were detached from the culture dish with 0.05% trypsin in D-Hanks solution. After centrifugation at 500 g for 5 min, cells were resuspended in a serum-free culture medium and injected into the border zone surrounding the infarcted area (total 8.0×10^6 cells in 50 µL) in rats with coronary occlusion with a tuberculin syringe. Ad-hVEGF₁₆₅ (total of 5×10^8 plaque-forming units in 50 µL) and a serum-free culture medium (total 50 µL) were

injected in the VEGF and control groups, respectively, following the same procedure.

Reverse transcriptase-polymerase chain reaction analysis for hVEGF₁₆₅ gene expression in treated hearts

One week after transplantation, the hearts from every group (n=5 from each) were used for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. The RT-PCR primers of hVEGF₁₆₅ 5'→3' (5'ATGAACCTTCTGCTGTCTTGGGTG3') and 3'→5' (5'TCACCCGCTCGGCTTGTCACA3'), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5'→3' (5'GTGGAGGAGTGGGTGTCGCTG3') and 3'→5' (5'CGGATTTGGTCGTATTGGGCG3') were used for amplification. The melting and annealing temperatures for hVEGF₁₆₅ were 68°C to 70°C and 64°C, respectively, and for GAPDH were 66°C to 70°C and 64°C, respectively. Total RNA was isolated using the Trizol Kit (Gibco, USA) according to the manufacturer's protocol. The purified RNA was kept at -80°C until use.

The RT-PCR analysis was performed using the OneStep RT-PCR Kit (Qiagen, Germany). In brief, 10 master mixtures were prepared from 10 samples (five with VEGF₁₆₅ primers, five with GAPDH primers). Each master mixture received 500 ng of total RNA as a template from each sample. Reverse transcription was carried out at 50°C for 30 min followed by the initial PCR activation step for 15 min at 95°C. The denaturation and annealing temperatures were 94°C and 64°C, respectively. The reaction was carried out for a total of 30 cycles, followed by a final extension at 72°C for 10 min.

Echocardiography and hemodynamics

One day and one month after transplantation, echocardiographic tests were performed using a high-frequency linear array transducer (Sonos 5500; Hewlett Packard, USA) in anesthetized animals. The anterior chest area was shaved and two-dimensional images and M-mode tracings were recorded from the parasternal short axis view at the level of papillary muscles. The end-diastolic left ventricular (LV) volume (EDV) and the end-systolic LV volume were obtained by the biplane area length method, and the percentage of LV ejection fraction (EF) was calculated as follows: $(EDV - \text{end-systolic LV volume})/EDV \times 100$. One month after transplantation, rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and the right carotid artery was cannulated with a microtip pressure transducer catheter (model SPR-671; Millar Instruments, USA) for measuring the LV end-diastolic pressure, and LV+dp/dt and LV-dp/dt in the closed-chest preparation. All measurements were averaged on three consecutive cardiac cycles and were analyzed blindly by two investigators.

Measurement of vascular density and histochemical staining analysis

One month after transplantation, the LVs of all experimental animals were fixed in 10% formaldehyde, embedded in paraffin and cut into 10 to 15 transverse sections from apex to base; some were processed for routine histology (hematoxylin and eosin) and Masson's trichrome staining for evaluating the collagen content. Sections from all slices were projected onto a screen for computer-assisted planimetry. The ratios of the infarcted area to the LV circumferences of the endocardium and epicardium were presented as percentages to define infarct size. To quantify the capillary density, the origin of capillaries and the destiny of transplanted MSCs in vivo, a polyclonal rabbit anti-von Willebrand factor antibody (Sigma, USA), monoclonal mouse antidesmin (Dako, Denmark) and antidiabetic troponin T (cTnT) (Santa Cruz Biotechnology

Inc, USA) were applied, followed by FITC-conjugated goat antirabbit and antimouse antibody (Santa Cruz Biotechnology Inc, USA), respectively. Immunoreaction was observed by fluorescence microscopy. The number of blood vessels was counted in 10 random fields (magnification $\times 200$). The average of the 10 high-power fields (hpf) was calculated, and mean vascular density (MVD) was defined as the number of blood vessels per hpf. The data were acquired and analyzed blindly by two investigators.

Statistical analysis

All data are presented as mean $\pm$ SD. Statistical analysis was carried out by *t* test using SPSS, version 10.0 (SPSS Inc, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Characterization and VEGF gene transfection of MSCs in vitro

Culture-expanded adherent MSCs were uniformly fibroblast-like in appearance in vitro. Analysis by flow cytometry performed at passage 10 revealed that MSCs expressed high levels of CD44 and CD90, but were virtually negative for CD34 and CD45, which is in agreement with previously published data on MSC surface markers (Figure 1A) (12). Ad-hVEGF₁₆₅-transfected MSCs showed the same pattern of surface molecule expression after transfection. Nearly 100% of MSCs can be effectively labelled with CM-DiI, and the viability of labelled MSCs was greater than 98% as indicated by dye exclusion using trypan blue staining after transfection (Figure 1B). The optimum level of transfection efficiency was achieved at a multiplicity of infection of 50. Transfected MSCs continued secreting hVEGF₁₆₅ for up to 14 days after transfection, with peak VEGF₁₆₅ in the cell culture supernatant on the seventh day (Figure 1C). Untransfected MSCs and Null-Ad-transfected MSCs also secreted hVEGF₁₆₅ at very low levels. RT-PCR analysis showed that the hVEGF₁₆₅ messenger RNA of Ad-VEGF₁₆₅-transduced MSCs was much stronger than the other groups one week after treatment (Figure 1D).

Angiogenesis and histochemical staining analysis

One month after MSC transplantation and Ad-hVEGF₁₆₅ delivery, MVD was quantified by immunohistochemical staining for the von Willebrand factor. The MVD in the MSC/VEGF group was 41 ± 5 capillaries/hpf compared with 31 ± 4 capillaries/hpf, 27 ± 2 capillaries/hpf and 16 ± 3 capillaries/hpf in the MSC, VEGF and control groups, respectively ($P < 0.001$). Angiogenesis was significantly increased in both the MSC and VEGF groups compared with the control group ($P < 0.01$ for both), and a significant difference between the MSC and VEGF groups ($P < 0.05$) (Figure 2A) was also found. There were many new capillaries in the border zone around the scar, but fewer were in the centre. One month after cell transplantation, the hearts of all rats exhibited large anterolateral wall infarctions. As demonstrated in Figure 3, the size of infarct area was significantly larger in the control group than in the other groups ($P < 0.001$ for both) (Figure 2B). Hematoxylin and eosin, Masson's trichrome and von Willebrand factor staining are shown in Figure 3.

Destiny of transplanted MSCs

Desmin and cTnT-positive cells derived from CM-DiI-labelled MSCs were found around the scar, expanding into viable host myocytes (Figures 4A and B). Of interest, a few transplanted MSCs were incorporated into new vascular components, and most of the new vascular components were host-derived (Figure 4C).

Figure 1 **A** Fluorescence-activated cell sorting analysis (Beckman Coulter, USA) of cultured mesenchymal stem cells (MSCs). MSCs were virtually negative for CD34 ($5 \pm 0.2\%$) and CD45 ($2 \pm 0.3\%$), but expressed high levels of CD44 ($92 \pm 0.5\%$) and CD90 ($88 \pm 0.2\%$). **B** Cultured MSCs, nearly 100% labelled by CM-DiI (Molecular Probes, USA), showed typical fibroblastic morphology. **C** Expression of human vascular endothelial growth factor 165 (hVEGF₁₆₅) in the adenovirus carrying hVEGF₁₆₅ (Ad-hVEGF₁₆₅)-transfected MSCs in vitro. The concentration of secreted VEGF was significantly higher than that of untransfected MSCs and null adenovirus (Null-Ad) MSCs (approximately 360-fold), and reached a peak on the seventh day after transfection. **D** Reverse transcriptase-polymerase chain reaction analysis for hVEGF₁₆₅ expression in treated hearts. The MSC/VEGF group was much stronger than the other groups. Sample 1 is the DNA ladder. Samples 2, 4, 6 and 8 are the MSC/VEGF, MSC, VEGF and control groups, respectively. Samples 3, 5, 7 and 9 are glyceraldehyde-3-phosphate dehydrogenase (GAPDH). bp base pair; MI Myocardial infarction

Figure 2 **A** Angiogenesis and **B** infarct size after mesenchymal stem cell (MSC) transplantation and delivery of the adenovirus carrying human vascular endothelial growth factor (VEGF) 165 after one month. The MSC/VEGF group showed significantly increased mean vascular density and significantly reduced infarct size compared with the MSC group, VEGF group and control group ($P < 0.001$ for each). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. hpf High-power field

Cardiac function after MSC transplantation and VEGF gene delivery

Measurements of LVEF during the progression of infarction and hemodynamics at the time of death showed that repair improved

ventricular performance. LVEF values were not significantly different among the four groups one day after treatment. One month later, significant differences on measured values, including higher LVEF, $LV \pm dP/dt$ and lower LV end-diastolic pressure, were seen in

Figure 3) Histochemical staining for hematoxylin and eosin, Masson's trichrome and von Willebrand factor after mesenchymal stem cell (MSC) transplantation and delivery of the adenovirus carrying human vascular endothelial growth factor (VEGF) 165 after one month. **A to D** Hematoxylin and eosin staining (original magnification $\times 20$); **E to H** Masson's trichrome staining (original magnification $\times 100$); **I to L** von Willebrand factor staining (original magnification $\times 200$)

Figure 4) Immunofluorescence staining after mesenchymal stem cell (MSC) transplantation and delivery of the adenovirus carrying human vascular endothelial growth factor 165 at one month. CM-DiI-labelled MSCs show a cardiomyocyte phenotype and express desmin and cardiac troponin C (cTnT). **A** Fluorescein isothiocyanate (FITC)-labelled cTnT (green)+ CM-DiI (red) merge (yellow, white arrows); **B** FITC-labelled desmin (green)+CM-DiI (red) merge (yellow, white arrows) (original magnification $\times 400$); a few transplanted MSCs were incorporated into the vascular structure, and most of the new vascular components were host-derived; **C** FITC-labelled von Willebrand factor (green)+CM-DiI (red) merge (yellow, white arrow) (original magnification $\times 200$)

the MSC/VEGF group compared with the MSC, VEGF and control groups ($P < 0.001$ for each) (Figure 5).

DISCUSSION

The present study demonstrated that the transplantation of Ad-hVEGF₁₆₅-transfected MSCs induces myocardial angiogenesis (new blood vessel formation) and cardiomyocyte regeneration,

and prevents progressive scar formation and heart dysfunction in a rodent model of myocardial ischemia. The study investigated the therapeutic angiogenic effects of the transplantation of Ad-hVEGF₁₆₅-transfected MSCs, MSCs and Ad-hVEGF₁₆₅ delivery only. The results prove that Ad-hVEGF₁₆₅-transfected MSC transplantation is a promising way to effectively increase therapeutic angiogenesis in myocardial infarction.

Figure 5) Cardiac function after mesenchymal stem cell (MSC) transplantation and adenovirus carrying human vascular endothelial growth factor (VEGF) 165 delivery. One month after treatment, left ventricular (LV) values and hemodynamic parameters (including LV end-diastolic pressure [LVEDP] and $LV\pm dP/dt$) were measured (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). The MSC/VEGF group showed significantly improved cardiac function compared with the MSC group, VEGF group and control group (all $P<0.001$)

VEGF is a major angiogenic factor among those involved in physiological and pathological angiogenesis. The imbalance between supply and demand for oxygen in the hypoperfused myocardium upregulates the expression of proangiogenic factors and their receptors (13). Under normal circumstances, however, the contribution of neoangiogenesis to the infarct-capillary bed network is insufficient to keep pace with the tissue growth required for contractile compensation and is unable to support the greater demands of the hypertrophied but viable myocardium. Previous studies have documented the MSC release of a wide array of cytokines (including VEGF) that support hematopoietic stem and progenitor cell development, as well as the secretion of other cytokines that are relevant to augmenting blood flow to ischemic tissue (14). Ad-hVEGF₁₆₅-transfected MSCs can release a higher level of VEGF, and the level of VEGF expression may be important to achieving successful angiogenesis. It has been documented (15) that a period of one to two weeks of VEGF overexpression, mediated by direct intramyocardial gene transfer, may be sufficient to induce collateral vessels in ischemic myocardium without tumorigenesis. The present study showed that the expression of VEGF significantly increased, reached a peak after one week and maintained a high level for at least two weeks in the MSC/VEGF group (Figure 1C). Remarkable new blood vessel density was observed one month after cell transplantation in the infarcted regions in the MSC/VEGF group. On the other hand, VEGF

therapy associated with untoward effects such as progression of atherosclerosis and inappropriate growth of blood vessels, leading to angioma formation, were not observed.

We found that the injection of MSCs into ischemic myocardium significantly enhanced perfusion of ischemic tissue and collateral remodelling, reduced tissue damage and improved cardiac function. Interestingly, these actions occurred with only a few observable MSCs incorporated into new blood vessels. Although the incorporation of freshly isolated bone marrow mononuclear cells and endothelial progenitor cells into the vessel wall has been documented (16), the incorporation of MSCs is less well characterized. MSCs isolated in the present study may represent a population that is less able to differentiate and incorporate through an endothelial lineage. We showed that the transplantation of Ad-hVEGF₁₆₅-transfected MSCs subcutaneously elicits a robust angiogenic response after one month, leading to a significant increase in vessel density (Figures 2A and 3I). We also observed that most of the neovascular response was host-derived (Figure 4C), reflecting the extraordinarily potent recruitment capability that MSCs possess, in addition to their direct contribution to new vessel formation via transdifferentiation. In this process, autocrine and/or paracrine effects of locally produced VEGF may lead to an MSC vasculogenic response and simultaneously initiate a potent host-derived angiogenic response. Local delivery of MSCs may also instigate circulating stem and progenitor

cells homing to the region of injury and contribute to healing. MSCs play an important hematopoietic supportive role and have an intimate relationship with stem and progenitor cells in the marrow cavity. Previous studies have demonstrated that MSCs release other stem and progenitor cell chemokines, including hepatocyte growth factor, stem cell-derived factor, VEGF and monocyte chemoattractant protein-1 (17). At the same time, the injured heart itself can release some cellular factors, such as myocardial chemokine stromal cell-derived factor-1, which, increasing only in the early phase following myocardial infarction, can recruit MSCs to the injured heart to enhance angiogenesis and improve cardiac function (18). Therefore, it is highly likely that the collateral enhancing effects are mediated through multiple pathways, including autocrine and paracrine effects on local vascular cells and chemoattractant effects leading to the homing of circulating stem and/or progenitor cells.

In the present study, we observed that partly transplanted MSCs differentiated into desmin and cTnT-positive cells in the peri-infarct area, and that these cells showed a cardiomyocyte phenotype (Figures 4A and B). This result indicated that partly transplanted MSCs can differentiate and express a cardiomyocyte phenotype in a specific microenvironment in the infarcted area and prevent myocardial remodelling by cardiomyocyte regeneration. We found more desmin and cTnT-positive cells in the MSC/VEGF group than in the MSC group, which might have been due to more angiogenesis and an improved local microenvironment favourable to transplanted MSCs in the MSC/VEGF group. Moreover, multiple sublineages of bone marrow cells include Lin-c-kit⁺, CD34⁺ and CD34⁻ sublineages (19,20). Besides effectively stimulating angiogenesis, CD 34⁺ and CD34⁻ bone marrow-derived MSCs also release VEGF to play an integral role in the angiogenic effect (21). The hematopoietic CD34⁻ 'side population' has the potential to differentiate into endothelial cells and cardiomyocytes, and there is histological evidence for increased neovascularization and cardiomyocytes (22).

The destiny of transplanted MSCs in vivo is still unknown. Besides cardiomyocyte transdifferentiation, Alvarez-Dolado et al (23) showed that transplanted MSCs can fuse with viable myocytes and exhibit a cardiomyocyte phenotype, while Balsam et al (24) demonstrated that Lin-c-kit⁺ bone marrow cells and c-kit⁺Thy1.1(lo) Lin-Sca-1⁺ long-term reconstituting hematopoietic stem cells adopt only traditional hematopoietic fates. These inconsistent findings may result from the different experimental protocols for isolation. We used 1.073 g/mL Percoll to obtain cells for transplantation that included multiple sublineages rather than a single sublineage. It would be interesting to investigate whether the interactions of sublineages are important in determining their destiny in vivo.

Timing and graft cell number appear to be important for optimal therapeutic results, although firm conclusions await more thorough investigation. We demonstrated that the injection of eight million MSCs immediately after coronary artery occlusion results in reduced infarct size and improved function. In addition, transplantation of MSCs transduced with Akt, a serine-threonine kinase in cardiomyocytes shown to protect against apoptosis after ischemia and reperfusion injury, can increase the number of viable MSCs and further enhance the repair of injured myocardium (25). VEGF induces nitric oxide production and cyclic GMP accumulation in cultured endothelial cells through the activation of endothelial nitric oxide synthase (26). The important functions of these two mediators include vasodilation, inhibition of smooth muscle proliferation, antiplatelet accumulation and leukocyte adhesion inhibition, leading to vascular protection. Therefore,

transplanting MSCs/VEGF immediately after coronary artery occlusion sufficiently brought an instantaneous high level of VEGF expression into full play, and thus more MSCs were viable and more stunned, and hibernating myocardium was salvaged in the infarcted area.

The present study used CM-DiI for effectively tracking transplanted MSCs in vivo. CM-DiI possesses strong and photostable fluorescence. With excellent cellular retention and minimal cytotoxicity among long-chain carbocyanine membrane probes, it is particularly suitable for long-term labelling and tracking of cells, especially in neurons. CM-DiI can be retained in cells throughout fixation, permeabilization and paraffin embedding procedures. In our study, CM-DiI was well retained in transplanted MSCs in the myocardium for at least six months. The advantages of using this method also include clearer tissue structure, more convenient experimental procedure and longer tissue conservation, all superior to optimum cutting temperature compound embedding and freezing.

CONCLUSIONS

The transplantation of Ad-hVEGF₁₆₅-transfected MSCs induced myocardial angiogenesis and cardiomyocyte regeneration, preventing progressive heart dysfunction in ischemic hearts in rats. A distinct advantage of combining VEGF and MSCs is the creation of a reservoir of myocardial cells that can form part of the myocardium. The transplantation of Ad-hVEGF₁₆₅-transfected MSCs provided a transient source of VEGF, which can help to maintain a therapeutic level of VEGF for angiogenesis in combination with myocardial repair. MSC-mediated VEGF gene transfer may therefore be a promising strategy for effectively increasing the effects of treatment for IHD.

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