Vascular Endothelial Growth Factor–Expressing Mesenchymal Stem Cell Transplantation for the Treatment of Acute Myocardial Infarction

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Objective—Vascular endothelial growth factor (VEGF) plays an important role in inducing angiogenesis. Mesenchymal stem cells (MSCs) may have potential for differentiation to several types of cells, including myocytes. We hypothesized that transplantation of VEGF-expressing MSCs could effectively treat acute myocardial infarction (MI) by providing enhanced cardioprotection, followed by angiogenic effects in salvaging ischemic myocardium.

Methods and Results—The human VEGF165 gene was transfected to cultured MSCs of Lewis rats using an adenoviral vector. Six million VEGF-transfected and LacZ-transfected MSCs (VEGF group), LacZ-transfected MSCs (control group), or serum-free medium only (medium group) were injected into syngeneic rat hearts 1 hour after left coronary artery occlusion. At 1 week after MI, MSCs were detected by X-gal staining in infarcted region. High expression of VEGF was immunostained in the VEGF group. At 28 days after MI, infarct size, left ventricular dimensions, ejection fraction, E wave/A wave ratio and capillary density of the infarcted region were most improved in the VEGF group, compared with the medium group. Immunohistochemically, α-smooth muscle actin–positive cells were most increased in the VEGF group.

Conclusions—This combined strategy of cell transplantation with gene therapy could be a useful therapy for the treatment of acute MI. (Arterioscler Thromb Vasc Biol. 2005;25:1168-1173.)

Key Words: angiogenesis • gene therapy • myocardial infarction • stem cell • transplantation

Cell transplantation has become a promising novel therapy for ischemic heart disease and heart failure. Recent studies have revealed that various types of cells are effective in cell transplantation after myocardial infarction (MI), such as skeletal myoblasts,1,2 smooth muscle cells,3 and bone marrow mononuclear cells.4 Bone marrow mononuclear cells are especially useful because they contain, among various lineage cells, hematopoietic cells and endothelial progenitor cells; therefore they have the ability to induce angiogenesis in ischemic tissue. A reported clinical trial of cell transplantation with skeletal myoblasts and mononuclear bone marrow cells showed that such therapies can have cardioprotective and angiogenic effects after MI.5,6 However, selection of the most appropriate cell types for transplantation is controversial.

Mesenchymal stem cells (MSCs) are isolated from bone marrow mononuclear cells and can be expanded ex vivo. Under appropriate culture conditions, MSCs have the potential to terminally differentiate into osteocytes, chondrocytes, adipocytes, tenocytes, myotubes, astrocytes, hematopoietic supporting stroma, and endothelial cells.7 MSCs have also been used in a model of cell transplantation,8,9 showing that these cells could differentiate into myogenic cells. Therefore, MSCs have many characteristics that make them useful for cellular therapy.

Vascular endothelial growth factor (VEGF) is a strong therapeutic reagent for treating ischemia by inducing angiogenesis.10 It has been reported that direct intramyocardial gene transfer results in localized enhancement of VEGF levels and successful angiogenesis in animal models of MI.11 Furthermore, recent human trials of angiogenesis gene therapy using naked plasmid DNA or an adenoviral vector coding for VEGF have shown favorable results.12,13 Cell-mediated gene transfer may also be useful for sustained local protein delivery.14 In addition to its angiogenic effect, VEGF may provide myocardial protection against ischemic injury.15,16

Considering these findings, we hypothesized that cell transplantation using VEGF-expressing MSCs could enhance
the cardioprotective effects of MSCs, followed by angiogenesis effects in salvaging host myocardium. The results of this study indicate a key role for transplantation of VEGF-expressing MSCs as a strategy for cellular cardiomyoplasty after MI.

Methods

Cell Isolation and Culture

Both donors and recipients were inbred male Lewis rats (SLC, Shizuoka, Japan) weighing 250 to 300 grams. Bone marrow was extruded from tibias and femurs. Bone marrow mononuclear cells were isolated using density gradient centrifugation (NycoPrep 1.077 Animal; AXIS-SHIELD PoC, AS, Oslo, Norway). Then, the mononuclear cells were cultured in low-glucose Dulbecco’s modified eagle medium containing 10% fetal bovine serum for MSC outgrowth. The nonadherent cells were removed by a medium change at 48 hours and every 4 days thereafter.

Adenoviruses and Gene Transfer

A cDNA fragment containing the full-length coding regions of human VEGF165 was obtained from mRNA of human umbilical vascular endothelial cells by a RT-PCR method, using a reverse-transcription PCR kit (Toyobo Co, Ltd, Osaka, Japan). The recombinant adenovirus, expressing either β-galactosidase (LacZ) or human VEGF165, was generated using cosmid cassettes and the adenovirus DNA-terminal protein complex method (COS/TPC method), with an Adenovirus Expression Vector Kit (Takara, Osaka, Japan). For adenovirus-mediated gene transfer, MSCs were exposed to adenoviral vectors at a multiplicity of infection (MOI) of ~10 to ~20 for 12 hours.

An In Vitro Characterization of MSCs

For immunofluorescence studies, 15-day cultured MSCs were washed once and fixed with 4% (v/v) paraformaldehyde in phosphate-buffered saline. Primary antibodies were anti-α smooth muscle actin, anti-vimentin (Sigma), and secondary antibodies were anti-gout polyvalent-fluorescein isothiocyanate (FITC) conjugate, anti-gout polyvalent-Cy3 conjugate, (Sigma), which were incubated for 30 minutes each at room temperature.

Two days after LacZ-expressing adenovirus transfection, the cells were fixed with 0.2% gluteraldehyde for 10 minutes, and X-gal staining was initiated. To evaluate the lineage and surface marker phenotype of the cultured MSCs, cells were detached and incubated in phosphate-buffered saline containing 1% bovine serum albumin with the following fluorescent antibodies: anti–CD45-FITC as a panleukocyte marker; anti-CD11b (Mac-1)-FITC as monocyte/macrophage maker; anti–HLA-DR-FITC as activated T lymphocytes or natural killer cells marker; anti-CD90 (Thy-1)-FITC as pan T cells or early progenitor cells maker; and anti–CD31 (PECAM)-FITC as endothelial marker (Beckman Coulter). Cells were analyzed on a fluorescence-activated cell sorter Calibur Instrument (Becton Dickinson) with 10,000 events stored.

Human VEGF Expression

The VEGF-transfected MSCs were metabolically labeled with 35S methionine and 38S cysteine. After 48 hours, both culture medium and cell lysates were prepared for immunoprecipitation with anti-human VEGF antibody (Santa Cruz Biotechnology). After SDS-gel electrophoresis using 15% polyacrylamide gels, the labeled proteins were analyzed using a phosphomager (FUJIX BAS 2000; Fuji). To confirm the level of secreted VEGFs, the culture medium was collected from MSCs (n=6 in each group) at 1, 3, 5, 7, 10 days after gene transfection. VEGF levels in the medium were quantified using a human VEGF immunoassay kit (R&D Systems Inc).

MI and Cell Transplantation

Male Lewis rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and mechanically ventilated. After the heart was exposed through a lateral thoracotomy, myocardial infarction was produced by transient ligation of the left coronary artery for 1 hour. Ischemia and reperfusion were confirmed by visible discoloration. Although this procedure was being performed, gene-transferred MSCs were harvested using trypsin and resuspended in serum-free Dulbecco’s modified eagle medium just before grafting to the heart. After left coronary artery reperfusion, the VEGF-transfected and LacZ-transfected MSCs (VEGF group), or LacZ-transfected MSCs (control group), or serum-free Dulbecco’s modified eagle medium only (medium group) were injected into the anterior and lateral border zone surrounding the infarct area (total 6.0×10^6 cells in 0.1 mL) with a 32-gauge needle. Because high expression of LacZ and/or VEGF were preliminarily observed at ~7 days after MI, and because it was better to evaluate the cardiac remodeling at least 4 weeks after MI, we euthanized these rats at 7 and 28 days after MI. A total of 174 rats were used in the present experiments. The level of circulating human VEGFs were quantified using a human VEGF immunoassay kit (R&D Systems Inc) at 3, 7, and 14 days after MI. mRNA levels of platelet-derived growth factor-B, angiopoietin-1, and transforming growth factor-β in infarct area were quantified by reverse-transcriptase PCR using Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences) and TaqMan quantitative PCR analysis with the ABI PRISM 7700 Detection System.

Doppler Echocardiographic Studies

At 1 and 28 days after cell transplantation, transthoracic echocardiographic studies were performed using an echocardiographic system equipped with a 12.0-MHz phased-array transducer (SONOS 5500; Philips Medical System, Best, the Netherlands) as previously described. The axial resolution provided by a 12.0-MHz transducer is ~0.18 mm.

Light and Electron Microscopic Study

For the light microscopic study, the specimens were fixed in 10% formaldehyde, embedded in paraffin, and cut into 4-μm-thick sections. The tissue sections were stained with Elastica van Gieson and Mallory-azan. Sections from all slices were projected onto a screen for computer-assisted planimetry. The ratio of infarct area to left ventricular circumferences of the endocardium and epicardium was expressed as a percentage to define infarct size.

For the transmission electron microscopic study, the specimens were fixed in 4% paraformaldehyde containing 0.25% glutaraldehyde and 4.5% sacrose, and ultrathin sections obtained from the embedded blocks were examined with a Hitachi H-7000 electron microscope.

Immunohistochemical Analysis

For immunohistochemical examination of VEGF, CD31, and α-SM actin, the heart embedded in OCT compound (Tissue Tek, Miles, Inc) was frozen and cut into 5-μm sections. Antibodies for human VEGF (Santa Cruz Biotechnology), CD31 (DAKO, Kyoto, Japan), and α-smooth muscle actin (Sigma) were used as the primary antibody, and the secondary antibody was peroxidase conjugate (Nichirei, Japan). Peroxidase activity was visualized using 3,3-diaminobenzidine as chromogen, and nuclei were counterstained with methyl green. The VEGF-positive area was calculated by computer-assisted planimetry. The number of capillaries and α-smooth muscle actin–positive cells were counted in randomly selected 5 high-power fields of each section and averaged. To identify LacZ expression, the sections (5-μm-thick) were stained with X-gal. For immunofluorescent staining, the sections were incubated with a polyclonal rabbit FITC-conjugated anti–human VEGF antibody, and the secondary antibody was peroxidase conjugate (Abcam). Then, anti-α-smooth muscle actin (Sigma) or anti-cardiac troponin T (TrT) (Santa Cruz Biotechnology) followed by anti-goat rhodamine-conjugated antibody (Santa Cruz Biotechnology) were applied. The number of LacZ-positive...
cells and LacZ-positive cells stained by α-smooth muscle actin or TnT were counted in randomly selected 5 high-power fields of each section and averaged.

Statistics
Results were expressed as mean±SEM. Statistical significance was determined using ANOVA and the Student-Newman-Keuls test. Differences were considered statistically significant at \( P<0.05 \).

Results
In Vitro Characterization of MSCs
MSCs appeared morphologically to be a homogenous population of fibroblast-shaped cells, and cells maintained a similar morphology with subsequent passages. As shown in Figure 1Aa, vimentin was immunohistochemically stained in all cultured cells, thereby showing their mesenchymal origin. Moreover, these cells expressed α-smooth muscle actin (Figure 1Ab), which is specific to smooth muscle cells. To characterize the phenotype of cultured MSCs, we analyzed the expression of surface molecules. Almost all MSCs expressed the bone marrow progenitor cell make CD90 (Thy-1). However, they did not express CD45, HLA-DR, CD11b (Mac-1), and CD31 (PECAM) (Figure 1B). VEGF and/or LacZ gene-transfected MSCs showed the same pattern of the surface molecules expression at least 28 days after adenovirus transfection. Figure 1Ac shows the X-gal staining of recombinant adenovirus carrying LacZ-transfected MSCs. Because MSCs were damaged at higher MOI (especially at MOI >50), we used adenovirus vectors for gene transfection at 10 to 20 MOI. Approximately 10% of MSCs were stained blue by X-gal staining.

In Vitro VEGF Gene Transfection of MSCs
Figure 1C shows the expression of human VEGF165 in adenovirus carrying VEGF-transfected MSCs. Metabolically labeled VEGF was observed mainly in the culture medium (upper panel). MSCs were transfected with VEGF165-adenoviral (Ad/VEGF) or LacZ-adenoviral vectors. VEGF levels in the culture medium were markedly higher in VEGF–transfected MSCs than LacZ-transfected MSCs. Five day after transfection, the VEGF level was increased ∼700-fold (lower graph). * \( P<0.01 \) compared with the VEGF levels in LacZ-transfected MSCs in each time course.

Cell Transplantation With Gene-Transfected MSCs in MI Heart
At 1 week after cell transplantation, rats were euthanized for histological assessment of the grafting MSCs and VEGF. As shown in Figure 1A (available online at http://atvb.ahajournals.org), the LacZ-labeled cells, injected in the ischemic myocardium, were blue after X-gal staining. Most of the X-gal–stained cells were observed in the infarct area and border zone. Immunohistochemically stained VEGFs in the infarcted region were increased in the VEGF group compared with the control and the medium groups (Figure 1B). Moreover, LacZ and α-smooth muscle actin–positive cells were observed in the infarct area (Figure 2, a, b, and c; also, please see http://atvb.ahajournals.org) at 7 days after cell transplantation of control and VEGF groups. The ratio of both LacZ and α-smooth muscle actin–positive cells in LacZ-positive cells of VEGF group was higher than that of control group.
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Infarct Size and Cardiac Function After Cell Transplantation

At 4 weeks after cell transplantation, the hearts of all rats had large anterolateral wall infarction. As shown in Figure 3, the size of the infarct area was significantly reduced in the VEGF group compared with the medium group. Figure II (available online at http://atvb.ahajournals.org) illustrates the results of echocardiography assessment. Left ventricular end-diastolic dimension was significantly lower in the VEGF group, compared with the Medium group. Left ventricular ejection fraction was significantly higher in the VEGF and the control groups. Diastolic function, as defined by an increased ratio of E wave to A wave (E/A ratio), was significantly improved in the VEGF group compared with the medium group. Systolic and diastolic functions were best preserved in the VEGF group after MI. These data indicated that VEGF-transfected MSCs had more cardioprotective effects than MSCs in control group and that the transplantation of these cells suppressed cardiac remodeling after MI.

Histological Analysis

As shown in Figure 4, an increased number of capillaries were observed in the sections of immunohistochemical staining for CD31 in the infarct area of the VEGF group and of the control group at day 28 after implantation, but obvious angiogenesis was not found in the medium group. Furthermore, immunohistochemically stained α-smooth muscle actin-positive cells in the peri-infarct area were increased in the control group compared with the medium group. In the VEGF group, the number of α-smooth muscle actin-positive cells was larger than in the control group. No evidence for the differentiation of MSCs to adipose, bone, cartilage, or tendon was found in any animal. Similarly, no significant inflammatory infiltrates, angiomia, and interstitial edema were identified at the site of implantation of VEGF-expressing MSCs.

Under electron microscopy, small cells containing myofilaments and many interstitial cells were observed adjacent to the infarct area in both the VEGF group and in the control group (Figure IIIa and IIIb, available online at http://atvb.ahajournals.org). The small cells containing myofilaments may be smooth muscle cells. Furthermore, spindle-shaped cells with protruding cytoplasm were often observed to have point-to-point contact with one another and were arranged helically around a lumen in the VEGF group (Figure IIIc and IIIId).
Discussion

The main advantage of using MSCs in treating MI is that they can be isolated from adult bone marrow by aspiration and expanded ex vivo before implantation. Bone marrow contains a small number of mesenchymal lineage cells, the MSCs, which grow as adherent myofibroblastic cells in culture. These cells can be expanded by cell culture from many species, including rats. Under specialized culture conditions, MSCs have the capacity to differentiate into cells such as bone, cartilage, adipocytes, myocytes, and even cardiomyocytes, when treated with 5-azacytidine. Recent studies have shown that MSCs transplanted into the myocardium environment could express myogenic-specific proteins, such as sarcomeric myosin heavy chain, desmin, TnT, and phospholamban. Furthermore, Toma et al have demonstrated that purified MSCs from adult bone marrow appear to be smooth muscle cell, in the peri-infarct area were increased after being engrafted in the myocardium. These results suggest that MSCs may be good candidates for cell transplantation. However, an additional technical approach to treat MI by cell transplantation has not been well discussed. In the present study, we demonstrated that the grafting of VEGF-expressing MSCs provided advanced benefits on treatment for MI, compared with MSCs transplantation alone.

The present study showed that myofilament-containing cells and interstitial cells were increased in the infarct area after MSCs transplantation. Immunohistochemical staining revealed α-smooth muscle actin–positive cells, which might be smooth muscle cell, in the peri-infarct area were increased in the VEGF groups. Because cardiac ventricles are exposed to hemodynamic stress, increased smooth muscle cells may be needed as tissue reinforcement. Furthermore, some spindle-shaped cells protruded a part of cytoplasm, which were arranged circular in the VEGF group. Because the transplantation of MSCs increased vascular density in the area, we believe that the implantation of purified MSCs into the ischemic myocardium enhances angiogenesis. In addition, we could observe engrafted TnT-positive cells, thereby suggesting that MSCs have the potential of transdifferentiation to cardiomyocyte in the damaged myocardium. Although VEGF is suggested to play important roles in embryonic ventricular development, the number of TnT-positive cells is not so much and VEGF did not change the number of the cells. These effects of cellular transplantation may contribute to the prevention of infarct expansion and progressive ventricular remodeling.

We have also shown that expression of VEGF was significantly increased in the infarct regions treated with VEGF gene-transfected MSCs at 1 week after cell transplantation. Therefore, we confirmed that grafted cells could supply a transient high level of VEGF to the ischemic areas in the acute phase. The expression of VEGF might therefore provide cardioprotective effects in ischemic myocardium. Therefore, the angiogenic effects of VEGF might contribute to the salvaging of ischemic myocardium and decreasing the infarct area that would result from an improved blood supply. In addition, it has been reported that VEGF is associated with smooth muscle cell differentiation and proliferation. In the present study, we showed that smooth muscle cells were observed most in the VEGF group, thereby suggesting that VEGF might be useful in improving graft survival and in differentiation from MSCs to smooth muscle cells in the tissue. Therefore, when combined with cell transplantation, VEGF could play an additional role in preventing cardiac remodeling and improving systolic and diastolic function after MI.

The level of VEGF expression may be important to achieve successful angiogenesis. It was recently reported that skeletal myoblasts, expressing VEGF by retroviral transduction, cause the formation of vascular tumors in nonischemic myocardium of immunodeficient mice because of persistent overexpression of VEGF. However, it has been documented that a period of 1 to 2 weeks of VEGF overexpression, mediated by direct intramyocardial gene transfer, might be sufficient to induce collateral vessels in ischemic myocardium without tumorigenesis. In the present study, although the direct transfection of adenovirus vector-expressing VEGF to ischemic myocardium might induce effective neovascularization, implantation of MSCs produced by transient transfection of the VEGF gene could provide adequate magnitude and duration of VEGF expression in the ischemic myocardium to induce functional collateral vessels, without vascular tumor formation.

The optimal graft cell number is that required to achieve the maximum attenuation of adverse remodeling or actual improvement in cardiac function after cell transplantation. In this study, we showed that the injection of 6 million MSCs results in reduced infarct size and improved function after MI. Shake et al demonstrated that a direct intramuscular injection of 60 million MSCs improved the function of swine MI model. Davani showed that 1 million MSCs improve cardiac function in Lewis rat after MI. However, the overall functional result may be affected by various factors, such as grafting cell survival capacity and the condition of host myocardium.

The timing and site of introduction of the grafting cells is also an important consideration. We injected the cells 1 hour after left coronary artery occlusion. Early grafting of VEGF-expressing cells could be useful in salvaging more myocardium, because of the cardioprotective effects of secreted VEGF. However, it is likely that grafted cells would experience greater adverse conditions if transplanted at this time point, compared with delayed injection after the myocardium has been stabilized. In the present study, we transplanted MSCs to the border area of infarcts for the purpose of salvaging the stunned or hibernating myocardium in this area. Investigating the effects of varying injection times or injection sites would provide new and interesting data on cell transplantation to the heart.

In conclusion, we have demonstrated that the transplantation of VEGF-expressing MSCs, produced by gene transfection, results in transient, high-level VEGF expression within rat myocardium with ischemic damage. This VEGF-expressing MSC transplantation may lead to reduction in the MI size, to successful angiogenesis, and to distinct increase of α-smooth muscle–positive cells, which may be associated with the improvement in cardiac function. MSCs can be easily isolated from adult bone marrow, expanded in vitro,
and re-administered to a patient without immunosuppressive therapy. For these reasons, this combined strategy of cell transplantation with gene therapy could be a useful therapy for the treatment of acute MI.

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Figure I.

A. X-gal staining of heart engrafted LacZ-transfected MSCs. Cells injected in the ischemic myocardium were observed as a blue area 1 week after cell transplantation.  B. Immunohistochemical staining for VEGF in the infarcted region 1 week after cell transplantation.  VEGF expression was significantly increased in the VEGF group (c) compared to the Medium group (a) and Control group (b).

Figure II.

Doppler echocardiographic assessment of left ventricular geometry and function.  LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular systolic dimension; LVEF, left ventricular ejection fraction; E/A ratio, E wave velocity/A wave velocity; *p<0.05 and ** p<0.01 between indicated groups, ## P<0.01 vs. parameter at day 1 in each group. Values are mean ± S.E.M.

Figure III.

Electron microscopy assessments. Small cells containing myofilaments (a) and many interstitial cells with protruding cytoplasm (b) were
observed adjacent to the site of cell transplantation. Furthermore, spindle-shaped cells with protruding cytoplasm were often found to make point-to-point contact with one another, and were arranged helically around a lumen in the VEGF group (c and d).