Enhanced Myocardial Angiogenesis by Gene Transfer With Transplanted Cells

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Background—The combination of myocardial cell transplantation and angiogenic gene transfer may improve postinfarction left ventricular (LV) perfusion. We evaluated the angiogenic effect of heart cells transfected with vascular endothelial growth factor (VEGF) and transplanted into a myocardial scar.

Methods and Results—Donor rat heart cells were transfected with plasmids encoding VEGF165 and green fluorescence protein. Syngeneic adult rats underwent LV cryoinjury to create a transmural scar. Three weeks later, 4×10^6 transfected heart cells (n=14), untransfected heart cells (n=13), or culture medium (n=16) were transplanted into the center of the scar. After 5 weeks, LV function, quantitative histology, and regional blood flow were evaluated. Plates of heart cells transfected with VEGF165 produced 6.1 times more intracellular VEGF than nontransfected cells. Capillary density (mean±SEM) per high-power field in the center of the myocardial scar was 1.1±0.02 in control rats, 3.9±0.11 in untransfected rats, and 6.3±0.11 in transfected rats (P=0.0002). Capillary density in the border zone around the scar was 1.9±0.03 in control rats, 6.4±0.10 in untransfected rats, and 8.7±0.16 in transfected rats (P=0.004). Regional blood flow within the scar was 8.8±0.8% of normalized flow in control hearts, 10.4±0.7% in hearts transplanted with untransfected cells, but 17.6±1.2% in hearts transplanted with transfected cells (P=0.03 versus control, P=0.07 versus nontransfected). There was no difference in LV function attributable to transplantation with transfected cells at the time point studied.

Conclusions—Transplantation of heart cells transfected with VEGF induced greater angiogenesis than transplantation of unmodified cells. Combined gene transfer and cell transplantation strategies may improve postinfarction LV perfusion and function. (Circulation. 2001;104[suppl I]:I-218-I-222.)

Key Words: gene therapy ■ cells ■ transplantation ■ angiogenesis

Cell transplantation has undergone intense investigation within the last several years as a potential novel therapy for postinfarction left ventricular (LV) dysfunction. A number of investigators have demonstrated that transplantation of skeletal myoblasts,1–4 bone marrow cells,5 stem cells,6 smooth muscle cells,7,8 and heart cells9–11 into scarred myocardium results in engraftment and can improve LV function. The first clinical case of skeletal myoblast transplantation as an adjunct to coronary artery bypass surgery has recently been reported by Menasché and colleagues (American Heart Association, New Orleans, November 2000), spurring further interest in this field.

We have noted a consistent but limited angiogenic effect of cell transplantation into myocardial scar, suggesting that cell transplantation may be developed into a novel revascularization strategy in patients with postinfarction ventricular dysfunction. We therefore evaluated the angiogenic effect of different cell types. Untransfected rat cardiomyocytes, smooth muscle cells, endothelial cells, and fibroblasts induced varying degrees of angiogenesis after transplantation into myocardial scar. Capillary density in the scar after cell transplantation had a linear relation to vascular endothelial growth factor (VEGF) levels in each cell type before transplantation (unpublished data, 2000). However, only cardiomyocyte and fibroblast transplantation increased arteriolar as well as capillary density. We therefore chose to investigate the angiogenic potential of transplanted cardiomyocytes further because of their ability to both induce angiogenesis and improve ventricular function. We reasoned that expression of an angiogenic transgene in these cardiomyocytes might further increase their angiogenic potential.

Vascular endothelial growth factor, basic fibroblast growth factor, angiopoietin, and hypoxia-inducible factor are promising candidate genes for therapeutic myocardial angiogenesis. Current strategies for myocardial gene transfer have used direct injection of naked DNA or use of viral vectors, most commonly adenoviruses.12,13 We reasoned that cells transplanted into the myocardium may serve as useful vehicles for the delivery of transgenes that may favorably affect the transplanted or the native cells.
In our current study, we transfected donor cardiomyocytes with VEGF and transplanted these cells into the scarred hearts of syngeneic rats to determine whether expression of an angiogenic transgene would enhance the angiogenic effect of cell transplantation alone. If this combination of therapeutic gene transfer and myocardial cell transplantation has additional benefits, it may become a novel therapeutic strategy for patients with ischemic LV dysfunction and perhaps for other pathologies as well.

**Methods**

**Animals**

All procedures were approved by the Animal Care Committee of the University Health Network and conformed to the guidelines in the “Guide to the Care and Use of Experimental Animals,” published by the National Institutes of Health (NIH publication 85-23, revised 1985). Both donor and recipient rats were syngeneic male Lewis inbred rats weighing 250 to 300 g (Charles River Canada Inc, Quebec, Canada).

**LV Cryoinjury**

A cryoinjury technique was used to generate a large transmural scar in the LV free wall of rat hearts, as we have previously described. Rats were anesthetized with ketamine hydrochloride (20 mg/kg IM) and sodium pentobarbital (30 mg/kg IP), intubated, and ventilated with room air supplemented with oxygen and isoflurane (0.2% to 1.0%) by a Harvard ventilator. The ECG was monitored continuously during operation.

The heart was exposed through a 2- to 3-cm left lateral thoracotomy. Cryoinjury of the LV free wall (LVFW) was performed with the use of an elliptical metal probe 8 × 10 mm in diameter cooled to −190°C by immersion in liquid nitrogen. It was applied to the LVFW for 1 minute, and this procedure was repeated 10 times to the same area of the LVFW to ensure a transmural injury. Penlog XL (benzathine penicillin G 150 000 U/mL and procaine penicillin G 150 000 U/mL; 1 mL/kg) and buprenorphine hydrochloride (0.01 mg/kg) were given intramuscularly after operation. The cryoinjured rats were randomly divided into each of the 3 experimental groups (control, n = 16; untransfected cell transplantation, n = 13; VEGF-transfected cell transplantation, n = 14).

**Cell Isolation**

Isolation and culture of adult rat heart cells was performed as previously described. We used an enzymatic digestion and preplating technique to deplete the culture of fibroblasts, resulting in 94±3.5% purity by immunofluorescence staining with monoclonal antibodies for cardiac myosin heavy chain (n = 8). These cells were maintained in Iscove’s modified Dulbecco’s medium containing 10% fetal bovine serum for 4 to 7 days before transfection and transplantation. Because isointransplantation in syngeneic rats was used in these experiments, there was an essentially unlimited source of donor cells, and the isolated heart cells were maintained in culture for only several days without further passaging or expansion.

**Transfection**

Cells were transfected by a lipid-based technique, in vitro, either with a plasmid encoding VEGF (VEGF<sub>165</sub>, pCEP4-VEG<sub>165</sub>) or with both pCEP4-VEG<sub>165</sub> and a plasmid encoding green fluorescence protein (GFP, pEGFP-N1) to monitor transfection efficiencies. Two micrograms of pCEP4-VEG<sub>165</sub> DNA or both 1.42 μg pCEP4-VEG<sub>165</sub> DNA and 0.61 μg pEGFP-N1 DNA (a 1:1 molar ratio) were diluted with DNA-condensation buffer, to which 16 μL enhancer reagent was added. This was incubated at 21°C for 3 minutes; 30 μL of Effectene transfection reagent was added, and this was incubated for 5 minutes before dilution with cell growth medium. This DNA mixture was added dropwise to each plate of heart cells (10<sup>6</sup> cells at 60% to 75% confluence) and incubated at 37°C overnight. Transfected cells were used for cell transplantation 24 hours after transfection.

**Analysis of Growth Factor Expression in Cell Culture**

VEGF protein levels were assayed by chemiluminescent slot blot analysis (MiniFold II, Schleicher & Schuell Inc). The supernatant culture medium was assayed for secreted VEGF, and heart cells were centrifuged and collected for lysis and quantification of intracellular VEGF. Briefly, 100 μL of culture supernatant or total soluble protein lysates was loaded into each slot (6 mm<sup>2</sup>), vacuum filtered, and protein immobilized on Immobilon-P membrane (Millipore). The membrane was probed with monoclonal primary antibody (VEGF<sub>165</sub> antibody, Sigma Diagnostics), washed, probed with goat anti-mouse IgG conjugated to horseradish peroxidase (BioRad), and washed free of unbound antibodies before reacting with luminol substrate (BM Chemiluminescence Blotting substrate, Boehringer Mannheim). Oxidation of luminol and light emission on radiographic film was used to densitometrically quantify secreted or intracellular VEGF.

**BrdU Prelabeling**

One day before transplantation, 25 μL of 0.4% BrdU solution was added to 10 mL of culture medium in 1 of every 6 culture dishes and incubated for 24 hours. Approximately 60% of the cultured heart cells in the labeled plates were labeled with BrdU at the time of transplantation, and approximately 10% of the total number of cells transplanted were therefore prelabeled in this manner. In cells scheduled to undergo transfection before transplantation, BrdU labeling was instead carried out starting 2 days before transplantation, again for a period of 24 hours. At the conclusion of the experiment, hearts were excised, fixed, and sectioned, and monoclonal antibodies against BrdU were used to localize the transplanted cells in the scar.

**Cell Transplantation**

Three weeks after myocardial cryoinjury, cell transplantation was performed. The cultured heart cells were detached from the culture dishes with 0.05% trypsin in PBS. After centrifugation at 580 g for 5 minutes, the cell pellets were resuspended in culture medium. Under general anesthesia, the rat hearts were exposed through a midline sternotomy. The suspension of heart cells (4 × 10<sup>6</sup> cells) was injected into the center of the scar tissue in the two transplantation groups with the use of a tuberculin syringe, and the same volume of culture medium was injected into the scar in control rats. Because syngeneic rats were used, immunosuppression with cyclosporine A was not used.

**Evaluation of LV Function**

Rat hearts were excised for assessment of function in a blood-perfused Langendorff apparatus as we<sup>15,18</sup> and Fremes et al<sup>19</sup> have previously described. Coronary blood flow was measured in triplicate by timed collection in the empty beating state. Systolic and diastolic function was evaluated by measuring systolic, diastolic, and developed pressures with a Millar micromanometer (Millar Instruments Inc) over a range of intraventricular balloon volumes by stepwise addition of saline (0.02 mL increments from 0.04 to 0.6 mL). After evaluation of LV function, hearts were subjected to sectioning for quantitative histology or evaluation of regional blood flow.

**Quantitative Histology**

In each experimental group, half of the rat hearts were subjected to quantitative histological analysis (control, n = 8; untransfected cell transplantation, n = 6; VEGF-transfected cell transplantation, n = 7). Heart sections were fixed in 5% glacial acetic acid in methanol, embedded in paraffin, and sectioned into 10-μm slices. Sections were stained with hematoxylin and eosin as described in the manufacturer specifications (Sigma Diagnostics) or immunohistochemical staining with antibodies against factor VIII<sub>20</sub> to facilitate quantification of vascular density in both the center of the scar.
(which was transmural) and the border zone (where the scar was only partial thickness). Identification of transplanted heart cells was confirmed by visualization of the cells prelabeled with BrdU in the myocardial scar. The number of vessels per high-power field (0.2 mm²) was counted by 2 blinded observers in 5 fields per slide; the mean number of vessels per field was used for analysis.

**Regional Blood Flow**
In each experimental group, the remaining half of the rat hearts underwent evaluation of regional blood flow (control, n=8; untransfected cell transplantation, n=7; VEGF-transfected cell transplantation, n=7). Hearts were arrested with potassium chloride in the Langendorff apparatus. Neutron-labeled microspheres were resuspended in 2 mL of 0.9% saline containing 5% sucrose and 0.05% Tween80. After vigorous vortexing for 2 minutes, the microspheres were injected into the aortic root over 5 seconds. The LV scar was excised, the tissue was weighed, and specimens were submitted for neutron activation and quantification of activity in the scar and in the normal myocardium. Counts per milligram of scar were compared with counts per milligram of normal myocardium in each rat.

**Results**

**VEGF Expression In Vitro**
Plates of rat heart cells were transfected with plasmids encoding VEGF165 and a marker green fluorescence protein at efficiencies of ~25% to 30%. In vitro, intracellular VEGF expression, evaluated by chemiluminescent slot blot analysis of cell lysates, was 6.1-fold greater in plates of transfected cells than in untransfected cells. Concentrations of VEGF secreted into the supernatant were 3.8-fold greater in plates of transfected cells than untransfected cells. The relative overexpression of VEGF in the 25% to 30% of cells per plate actually transfected with the plasmid would therefore have been proportionately greater, ~20- to 25-fold.

**Histology**
The LV scars of control hearts were relatively avascular, with only occasional capillary spaces (Figure 1a). BrdU-labeled cells were observed in the zone of transplantation of hearts transplanted with untransfected cells, which also demonstrated qualitatively greater vascular densities than in control hearts (Figure 1b). Hearts transplanted with the VEGF-transfected cells demonstrated both BrdU-labeled cells and the highest vascular density (Figure 1c).

**Quantitative Vascular Density**
Quantitative vascular densities in both the center of the scar (P=0.0002) and in the border zone around the scar (P=0.004) were lowest in control rats injected with culture medium alone, intermediate in rats transplanted with untransfected heart cells, and greatest after transplantation with cells transfected with VEGF (Figure 2).

**Regional Blood Flow**
Regional myocardial blood flow within the LV scar was lowest in the control rats, increased but not statistically significantly so in hearts transplanted with untransfected cells, and greatest in hearts transplanted with VEGF-transfected cells (Figure 3).

**LV Function**
Parameters of LV function, including LV developed pressure over a range of balloon volumes (Figure 4), did not differ between groups at 5 weeks. Control rats tended to have lower developed pressures than rats transplanted with either untransfected or transfected cells, but with the two cell-transplanted groups having essentially identical function curves, the group effect did not reach statistical significance.

**Discussion**
Several previous studies have used transfer of marker genes such as β-galactosidase to facilitate identification of cells transplanted into the myocardium. The combination of therapeutic gene transfer strategies with myocardial cell
transplantation may represent a promising strategy for patients with ischemic LV dysfunction but has received little attention to date. Koh and coworkers23 have reported that skeletal myoblasts transfected with a construct encoding transforming growth factor-β before transplantation into murine hearts expressed this transgene for up to 3 months. Our current experiments demonstrate that overexpression of an angiogenic VEGF transgene in cardiomyocytes transplanted into a myocardial scar induces greater angiogenesis than transplantation of unmodified cardiomyocytes alone and that the increased vascular densities persist 5 weeks after cell transplantation. Vascular densities in the border zone around the scar were greater than in the center of the scar. One possible explanation for this finding is that although the angiogenic stimulus of VEGF expression may be greatest at the center of the scar, the angiogenic response proceeds inward from the surrounding native myocardium and is therefore greatest in the border of the scar. The enhanced regional blood flow observed in this study may help to support the transplanted cells, although further studies to determine whether new vessel ingrowth occurs rapidly enough to influence the survival of the transplanted cells are required. Greater regional perfusion may also favorably affect remodeling and recruit function from surviving but hibernating native cardiomyocytes. These effects are likely to be demonstrated in models of coronary occlusion rather than of cryoinjury-induced scar formation.

We did not note any improvement in global LV function in rat hearts transplanted with VEGF-transfected cells compared with those transfected with untransfected cells. This finding probably is related to the nature of the scar induced by our cryoinjury model. Cryoinjury creates a largely fibrous, avascular scar with very few remaining native cardiomyocytes that might benefit from the improved perfusion afforded by VEGF-transfected cells. A model of coronary ligation, which would result in a less homogeneous infarct zone with islands of surviving cardiomyocytes, would be more likely to demonstrate an improvement in ventricular function related to improved regional blood flow. We have previously used a porcine model of left anterior descending coronary artery coil occlusion to demonstrate that transplantation of genetically unmodified cardiomyocytes improved LV end-systolic elastance, preload-recruitable stroke work, and regional perfusion.11 Further studies with this porcine model may demonstrate whether further improvement in these parameters of both function and perfusion can be achieved by transplantation of VEGF-transfected cardiomyocytes.

We noted greater LV developed pressures in both of the cell-transplanted groups compared with control rats injected with culture medium alone. The magnitude of these differences were not, however, statistically significant at the 5-week time point at which these rats were evaluated. In other experiments with syngeneic Lewis rats, we have demonstrated improvement in parameters of LV function 8 weeks after transplantation of unmodified cardiomyocytes, but these differences were not evident after only 4 weeks (unpublished data, 2000). It appears that the process of ventricular remodeling after cryoinjury-induced scar generation in these Lewis rats occurs over at least an 8-week period, and differences between cell-transplanted and control rats did not achieve significance until this time. These findings contrast with our previous studies in Sprague-Dawley rats, in which differences in LV function were noted only 4 weeks after transplantation.9,10 We would anticipate, therefore, that the differences in parameters of LV function between the cell-transplanted rats and the control rats in our current experiments would have been greater if they were evaluated at 8 weeks rather than at 5 weeks.

We were able to achieve transfection efficiencies of ≈25% with the lipid-based transfection technique used in these experiments, with an ≈6-fold overproduction of VEGF by plates of transfected cells compared with untransfected cardiomyocytes. Relative overexpression of VEGF in the 25% of cells actually transfected would therefore have been proportionately higher, ≈20- to 25-fold greater than untransfected cells. Greater transfection efficiencies may be achieved with viral vectors, including replication-deficient adenoviruses12,13 and the hemagglutinating virus of Japan. However, the immune response that occurs after in vivo transfection with adenoviral vectors, particularly earlier-generation vectors, limits the duration of transgene expression24 and is particularly undesirable when the persistence of the transfected cells is of paramount importance. We have therefore used our current lipid-based technique of transfer of naked plasmid DNA to minimize the likelihood or magnitude of any immune response to the transfected transplanted cells. Despite the
overexpression of the VEGF transgene and the absence of any pharmacological immunosuppression by cyclosporine or other agents, we did not note any overt histological evidence of rejection within the myocardial scar in these syngeneic Lewis rats. Other studies to determine whether expression of potentially antigenic proteins reduces the survival of transplanted cells, or, conversely, whether increased angiogenesis enhances their survival, will be required, but quantification of cell survival in transplanted hearts over time has so far proved difficult.

Further experiments will be required to determine the time course of transgene expression in these transplanted cells as well as its late effects over several months. Comparative studies to evaluate the relative effects of in vivo transfection of native cardiomyocytes with viral vectors or naked DNA or in vitro transfection of donor cells by either of these methods before transplantation are also necessary. However, our experiments demonstrate that the expression of an angiogenic transgene further enhances the angiogenic effect of cardiomyocyte transplantation. These findings support the premise that the combination of therapeutic gene transfer strategies with the emerging technique of cell transplantation may represent a promising new avenue of investigation in the treatment of patients with ischemic LV dysfunction.

References

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