Vascular endothelial growth factor transgene expression in cell-transplanted hearts

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Objective: We evaluated the effect of transplanted cell type, time, and region of the heart on transgene expression to determine the potential of combined gene and cell delivery for myocardial repair.

Methods: Lewis rats underwent myocardial cryoinjury 3 weeks before transplantation with heart cells (a mixed culture of cardiomyocytes, smooth muscle cells, endothelial cells and fibroblasts, n = 13), vascular endothelial growth factor–transfected heart cells (n = 13), skeletal myoblasts (n = 13), vascular endothelial growth factor–transfected skeletal myoblasts (n = 13), or medium (control, n = 12). Vascular endothelial growth factor expression in the scar, border zone, and normal myocardium was evaluated at 3 days and at 1, 2, and 4 weeks by means of quantitative polymerase chain reaction. Transplanted cells and vascular endothelial growth factor protein were identified immunohistologically on myocardial sections.

Results: Vascular endothelial growth factor levels were very low in control scars but increased transiently after medium injection. Transplantation with heart cells and skeletal myoblasts significantly increased vascular endothelial growth factor expression in the scar and border zone. Transplantation of vascular endothelial growth factor–transfected heart cells and vascular endothelial growth factor–transfected skeletal myoblasts further augmented vascular endothelial growth factor expression, resulting in 4- to 5-fold greater expression of vascular endothelial growth factor in the scar at 1 week. Peak vascular endothelial growth factor expression was greater and earlier in vascular endothelial growth factor–transfected heart cells than in vascular endothelial growth factor–transfected skeletal myoblasts. Vascular endothelial growth factor was primarily expressed by the transplanted cells. Some of the transplanted heart cells and vascular endothelial growth factor–transfected heart cells were identified in the endothelial layer of blood vessels in the scar.

Conclusions: Transplantation of heart cells and skeletal myoblasts induces vascular endothelial growth factor expression in myocardial scars and is greatly augmented by prior transfection with a vascular endothelial growth factor transgene. Vascular endothelial growth factor expression is limited to the scar and border zone for 4 weeks. Both heart cells and skeletal myoblasts may be excellent delivery vehicles for cell-based myocardial gene therapy.

Cell transplantation may be a novel therapeutic option for myocardial repair in hearts with postinfarction congestive heart failure, unreconstructable coronary atherosclerosis, or cardiomyopathy. We have previously studied the angiogenic effect of transplantation of endothelial cells or of a mixed culture of heart cells (predominantly cardiomyocytes, with smaller proportions of endothelial cells, smooth muscle cells, and fibroblasts). When these cells were transfected with
vascular endothelial growth factor (VEGF) 165 ex vivo and then transplanted into scarred rat hearts, regional blood flow and vascular densities were significantly greater than those observed after transplantation of unmodified cells or of culture medium alone. Ex vivo modification of cells before transplantation may therefore have the potential to enhance survival of the transplanted cells and modify their effect on angiogenesis, matrix remodeling, or restoration of function. However, the duration of transgene expression after transplantation is unknown and is likely to differ from that observed after gene transfer alone by means of intramyocardial injection of adenoviral vectors. Long-term or uncontrolled expression of angiogenic transgenes has been associated with angioma formation. Optimal transgene expression in transplanted cells would therefore be transient but of sufficient duration to ensure maximal therapeutic effect. It would also be limited spatially to the zone to which the transplanted cells were delivered to prevent undesirable angiogenesis in normal myocardium.

In this series of experiments, we hypothesized that a VEGF transgene would be expressed only transiently in cells transplanted into scarred rat hearts and that this VEGF expression would be limited to the zone of transplantation. We also evaluated the effect of 2 different cell types to determine whether there were cell-specific differences in transgene expression that might influence the suitability of varying cell types for future therapies.

**Methods**

**Animals and Experimental Model**

Animals were syngeneic adult Lewis rats (body weight of 225-250 g for female rats and 250-300 g for male rats; Charles River Canada Inc, Quebec, Canada). 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 Laboratory Animals” prepared by the National Research Council and published by the National Academy Press.

A large transmural scar was created in the left ventricular (LV) free wall of rat hearts by means of a cryoinjury technique, as previously described. Briefly, through a left lateral thoracotomy, the LV free wall was exposed, and cryoinjury was performed by means of 12 one-minute applications of an 8 × 10–mm elliptical metal probe cooled to −196°C by means of immersion in liquid nitrogen. After recovery, the cryoinjured rats were randomly divided into 5 experimental groups: control, injected with culture medium alone (control, n = 12), transplantation with a mixed culture of unmodified heart cells (HC group; n = 13), transplantation with VEGF-transfected heart cells (HC+ group; n = 13), transplantation with unmodified skeletal myoblasts (Sk group; n = 13), or transplantation with VEGF-transfected skeletal myoblasts (Sk+ group; n = 13).

**Cell Isolation and Culture**

A mixed primary culture of cardiomyocytes, smooth muscle cells, endothelial cells, and fibroblasts was isolated from the left ventri-
volume of culture medium without cells was injected into the scars of control rats.

Rats were put to death, and the hearts were excised 3 days \( (n = 15) \), 1 week \( (n = 15) \), 2 weeks \( (n = 15) \), and 4 weeks \( (n = 19) \) after cell transplantation. The atria and the right ventricular free wall were excised, leaving the left ventricle, which was divided into the scar zone (consisting of the transmural scar), the border zone (partial-thickness scar containing both fibrous tissue and surviving muscle), and the normal area. A portion of each zone was fixed in formalin for histologic evaluation, and the rest of the tissue was frozen in liquid nitrogen for analysis of gene expression.

**RNA Isolation and Reverse Transcription**

Myocardial specimens were snap-frozen in liquid nitrogen and powdered. A portion of each specimen was used immediately for total RNA isolation, whereas the remainder was stored at \(-80^\circ\text{C}\) for subsequent analysis of protein levels. Total RNA was isolated with TRIzol RNA extraction reagents (Invitrogen Corp, Carlsbad, Calif), according to the manufacturer’s specifications. Messenger RNA in this specimen was reverse transcribed to single-strand cDNA with SuperScript II reverse transcriptase (Invitrogen Corp). Briefly, 10 \( \mu \text{g} \) of total RNA was mixed with 1 \( \mu \text{g} \) of oligo (dT) 20-mer at 65\({\text{°C}}\) and incubated for 5 minutes. The sample was placed on ice, and 4 \( \mu \text{L} \) of 5X first-strand buffer, 2 \( \mu \text{L} \) of 10 mmol/L dithiothreitol, 40 units of RNase inhibitor, and 400 units of SuperScript II reverse transcriptase were added to a 20-\( \mu \text{L} \) final reaction volume. The mixture was incubated at 42\({\text{°C}}\) for 60 minutes, followed by enzyme inactivation at 70\({\text{°C}}\) for 15 minutes. The reverse transcribed cDNA was stored at \(-20^\circ\text{C}\) for later use.

**Quantitation of VEGF mRNA by Means of Real-Time Polymerase Chain Reaction**

Quantitation of VEGF mRNA expression was carried out by means of real-time polymerase chain reaction (PCR) on the 9700 HT System (Applied Biosystems Inc, Foster City, Calif). Two pairs of specific PCR primers were designed on the basis of VEGF sequences from the GeneBank (National Center for Biotechnology Information) to accurately evaluate the molecular weight of the PCR products. One primer (antisense, 5'-TGAAAGTTCCGAGTCACGAG-GTCCACCAC-3') generated a 107-bp sequence, and the other (antisense, 5'-TCATGGTTGTCTATCAGCGCAG-3'; sense, 5'-GTCCACCAC-3') generated a 277-bp sequence, and the other primer (antisense, 5'-TGAAGGTCGGAGTCCGCCG-3' and sense, 5'-CAACGGATTTGGT-3') generated a 107-bp product. Oligo(dT)20-mer at 65\({\text{°C}}\) was added to a 20-\( \mu \text{L} \) final reaction volume. The mixture was incubated at 42\({\text{°C}}\) for 60 minutes, followed by enzyme inactivation at 70\({\text{°C}}\) for 15 minutes. The reverse transcribed cDNA was stored at \(-20^\circ\text{C}\) for later use.

**Quantification of VEGF Protein**

VEGF protein in the scar, border zone, and normal myocardium was quantitated by means of chemiluminescent slot blot analysis (MiniFold II; Schleicher & Schuell Inc, Keene, NH), as previously described.8

**Histologic and Immunohistochemical Assays**

Myocardial specimens were fixed in formalin, embedded in paraffin, and sectioned into 6-\( \mu \text{m} \)-thick slices. One slide of each sample was stained with hematoxylin and eosin for morphologic evaluation of the scar and transplanted cells. Immunohistochemical staining with monoclonal antibodies directed against BrdU, Factor VIII, or VEGF was performed to localize the transplanted cells, VEGF protein, and vascular spaces. Slides were deparaffinized in xylene and rehydrated in graded ethanol, immersed in 3% \( \text{H}_2\text{O}_2 \) in 70% methanol to inhibit endogenous peroxidase, and incubated in 0.2% of Triton X-100. Nonspecific protein binding was blocked with 2% normal goat serum and 150 \( \mu \text{L} \) of primary antibodies against BrdU (1:2000), Factor VIII (1:1000), VEGF (1:3000), or phosphate-buffered saline (PBS; as a negative control) was added before incubation at 4\({\text{°C}}\) overnight. Slides were washed to remove unbound primary antibodies, and a biotin-labeled secondary antibody (1:2500; BioRad Laboratories, Richmond, Calif) was added. After washing, sections were incubated with an avidin-biotin complex conjugated with peroxidase. Visualization was performed with a diaminobenzidine solution. Cellular nuclei were counterstained with hematoxylin for 1 minute, and the samples were then covered with crystal mounts and photographed.

VEGF protein and the transplanted cells were also identified and localized by means of immunohistochemical double staining with a laser confocal microscopy system (Bio-Rad, Richmond, Calif), according to the manufacturer’s protocols (Molecular Probe Inc, Eugene, Ore). Briefly, the slides were treated with 3% \( \text{H}_2\text{O}_2 \) in 70% methanol, incubated in 0.2% Triton X-100, and blocked with 2% normal goat serum after rehydration. Slides were incubated with fluorescein isothiocyanate–labeled anti-BrdU antibodies (1:100), phycoerythrin-labeled anti-VEGF antibodies (1:100), or both overnight at 4\({\text{°C}}\). Unbound antibodies were washed away with PBS. Slides were treated with 10 mg/mL RNase (Qiagen, Mississauga, Ontario, Canada) for 30 minutes at room temperature, washed with PBS, and incubated for 5 minutes in 4,6-diamino-2-phenylindole (DAPI) diluted 1:300 in PBS to stain both host and donor cell nuclei. The fluorescein isothiocyanate, phyco-
were greater than those in the control group \((P \leq .0001)\), whereas the effect of cell type was not \((P = .8)\). All interactive effects except transfection \(\times\) cell type \((P = .13)\) were significant \((P < .05)\).

### VEGF Expression: Effect of Cell Transplantation Alone

**VEGF** mRNA and protein levels in the scars of control rat hearts injected only with medium were extremely low (Figure 1). **VEGF** mRNA expression in control rats was slightly greater at 3 days than at 1, 2, or 4 weeks. Levels in control hearts at 1, 2, and 4 weeks were similar. In hearts transplanted with unmodified heart cells or skeletal myoblasts, **VEGF** mRNA expression was greater than that in control rats at 3 days and 1 and 2 weeks (Figure 1). Peak **VEGF** mRNA expression in hearts transplanted with unmodified cells (heart cells or skeletal myoblasts) occurred at 3 days in both groups. **VEGF** mRNA expression was greater in the scar than in the border zone at 3 days and 1 and 2 weeks in the **HC** and **Sk** groups, whereas expression levels in normal myocardium in the **HC** and **Sk** groups were similar to those in the control group at all time points studied. By 4 weeks, **VEGF** mRNA expression had decreased in the scar and border zones of the hearts of the **HC** and **Sk** groups to levels that were similar to those observed in the control group.

### VEGF Expression: Effect of Ex Vivo Transfection with VEGF

Prior ex vivo transfection with a **VEGF** transgene in the **HC**+ and **Sk**+ groups was associated with dramatically increased levels of **VEGF** mRNA and protein expression in the scars and border zones of these hearts compared with **HC**, **Sk**, or control hearts at 3 days and 1 and 2 weeks (Figure 1). The overall effect of **VEGF** transfection on **VEGF** mRNA and protein levels in the cell-transplanted groups was highly significant \((P < .0001)\). **VEGF** mRNA and protein expression in the **HC**+ and **Sk**+ groups was substantially greater in the scar than in the border zone at 3 days and 1 and 2 weeks. By 4 weeks, **VEGF** mRNA and protein levels had decreased and were similar to those in the control group. **VEGF** mRNA levels in the normal myocardium were similar in all groups at all times.

### VEGF Expression: Effect of Cell Type

Peak **VEGF** mRNA expression in the scar areas of the **HC**+ hearts was observed at 3 days compared with 7 days for the **Sk**+ hearts (Figure 1, A and B). Maximum **VEGF** mRNA levels appeared to be greater in the scar areas of the **HC**+ hearts (at 3 days) than in the **Sk**+ hearts (at 1 week). In contrast, **VEGF** mRNA expression in the border zone appeared to be greater in the **Sk**+ hearts at 1 week than in the...
HC+ hearts (Figure 1, B). Although the overall effect of cell type on VEGF mRNA expression in cell-transplanted hearts was not significant ($P = .8$), significant interactive effects of cell type × time ($P = .02$) and cell type × zone ($P = .01$) were noted.

**Colocalization of VEGF Expression with Transplanted Cells**

Transplanted cells were localized within the myocardial scar in all cell-transplanted hearts by means of immunohistochemical staining with monoclonal antibodies directed against BrdU (Figure 2, A), as well as by means of confocal microscopy of sections stained with both green fluorescence-tagged antibodies against BrdU and blue fluorescence-tagged DAPI labeling of all cell nuclei (Figure 2, B). Photomicrographs of hearts stained with green fluorescence-tagged antibodies against BrdU and red fluorescence-tagged antibodies against VEGF demonstrated that VEGF was being expressed in the transplanted cells (Figure 2, C). The degree of staining for VEGF was qualitatively more intense in the HC+ and Sk+ groups at 3 days and 1 week, which is consistent with the real-time PCR observations.

**Angiogenesis**

Vascular spaces lined with Factor VIII–positive cells were observed in the scars and border zones of all rat hearts but were qualitatively fewer in the control hearts, intermediate in the HC and Sk hearts, and greatest in the HC+ and Sk+ hearts. BrdU-positive endothelial cells were identified in blood vessels within the scars of HC+ hearts and, to a lesser extent, within the scars of HC hearts (Figure 3) but were not apparent in the other groups.

**Discussion**

In this study our quantitative PCR results indicated that VEGF expression in the hearts transplanted with VEGF-transfected heart cells and VEGF-transfected skeletal myoblasts was dramatically greater than in hearts transplanted with unmodified heart cells and skeletal myoblasts. In all
cell-transplanted hearts, however, VEGF expression was temporally limited to a 4-week period after transplantation. We deliberately used a technique of gene transfer in which transient rather than long-term expression would be anticipated. Because persistent or uncontrolled VEGF expression might result in excessive angiogenesis or angioma formation, a limited duration of transgene expression may provide greater safety while still achieving the desired angiogenesis. Histologic examination did not reveal any evidence of angioma formation as a result of this gene delivery strategy at the time points studied.

The 4-week duration of transgene expression observed with these ex vivo transfected cells compares with the approximately 1- to 4-week duration of expression reported after adenoviral transfection techniques of direct gene transfer without cell transplantation. Host immune responses to the adenoviral vectors are thought to limit the duration of expression in such delivery strategies. In these experi-

Figure 2. Laser confocal micrographs of serial sections of the scar of a rat heart transplanted with VEGF-transfected heart cells (HC+) after DAPI (blue) staining of all cell nuclei (A), BrdU (green) staining of transplanted cell nuclei (B), DAPI (blue) staining of all cell nuclei and VEGF (red) staining (C), or BrdU (yellow) staining of transplanted cell nuclei and VEGF (red) staining (D). (Original magnification 1000×.) DAPI-positive and BrdU-positive nuclei (white arrows) represent transplanted cells. DAPI-positive and BrdU-negative nuclei (yellow arrows) might represent either BrdU-unlabeled transplanted cells or host cells. Cytoplasmic VEGF protein (red) surrounds both the BrdU-positive nuclei and the smaller number of BrdU-negative nuclei.

Figure 3. Conventional light photomicrograph of the scar of a rat heart transplanted with VEGF-transfected heart cells (HC+) after immunostaining for BrdU. BrdU-positive transplanted cells (arrows) are observed in the endothelial cell layer of this capillary. (Original magnification 600×.)
ments we attempted to minimize the immune response to the transplanted cells by using syngeneic donor cells. No immunosuppressive therapy was administered to these rats, and we did not observe any histologic evidence of an immune response, such as a lymphocytic infiltrate. Such an immune response could, however, occur because of the transplanted cells, the technique of DNA transfer, or an immunogenic transgene product. We used a lipid-based, rather than viral, technique of DNA transduction to minimize potential host responses to viral proteins. Obviously, if this technique of ex vivo gene transfer before cell transplantation resulted in increased immunogenicity and therefore led to the eventual rejection of the transplanted cells, it would be of very limited utility. Care will therefore have to be taken in future studies to select gene products that are not significantly antigenic.

Suzuki and colleagues\(^\text{14}\) have previously reported that skeletal myoblasts transduced with VEGF through hemagglutinating virus of Japan liposome and transplanted into the border zone of a circumflex artery infarct resulted in increased VEGF expression in whole LV specimens. They did not, however, examine the spatial distribution of this VEGF expression to determine whether inappropriate expression occurred in normal myocardium. In our current study enhanced VEGF expression was spatially limited to the scars and border zones of cell-transplanted hearts, regardless of the presence or absence of prior transfection with a VEGF transgene. In all cell-transplanted groups and at all times, VEGF levels in the scars were higher than levels in the border zones. VEGF mRNA levels in the normal myocardium were similar to control levels in all groups at all time points. At thoracotomy, donor cells were injected into multiple sites in what appeared visually to be the center of the scar and its immediate surroundings. At the time of histologic evaluation, transplanted cells were observed throughout the scar and, to a lesser degree, in the border zone. The local prevalence of the transplanted cells appeared to correlate with the level of VEGF expression, and confocal microscopy confirmed that VEGF expression was localized to the transplanted cells. It is likely, however, that a low level of VEGF expression will take place in host cells that have absorbed VEGF-coding plasmids from donor cells that die and undergo lysis after transplantation.

Interestingly, we also observed a low but distinct increase in VEGF expression at 3 days in control hearts in which only serum-free culture medium, without cells, was injected. This likely represents a response to the local trauma of injection and may be analogous to the angiogenic response to mechanical or laser perforation of the myocardium. Under the conditions used in this study, however, this response was dwarfed by the effects of cell transplantation and of VEGF gene transfer.

In this study peak VEGF expression occurred earlier in HC+ hearts than in Sk+ hearts (3 days vs 7 days), and peak VEGF levels were greatest in HC+ hearts. In contrast, VEGF levels in the border zone at 1 week appeared to be greater with Sk+ than HC+ hearts. Although the degree of angiogenesis induced by means of cell transplantation may represent an important determinant of transplanted cell survival and ultimate efficacy, it is unclear as to whether the differences in peak VEGF expression observed between heart cells and skeletal myoblasts in this study will have a significant effect on cell survival. Heart cells and skeletal myoblasts may also have differential susceptibilities to the ischemic conditions in the scar or differential survival rates after transplantation for other reasons, and the importance of this finding therefore remains unknown.

Histologic evaluation revealed BrdU-positive cells in the endothelial layer of capillaries and arterioles in the scars and border zones of HC and HC+ hearts, but not in the other groups. These cells are probably derived from the endothelial cell population in the mixed cultures of heart cells and VEGF-transfected heart cells used in this study. We did not expect, and did not observe, BrdU-positive endothelial cells in the Sk and Sk+ groups.

In summary, our study demonstrated that ex vivo transfection of either a mixed culture of heart cells or of skeletal myoblasts with a VEGF-encoding plasmid was associated with dramatically increased levels of VEGF mRNA expression. This VEGF expression occurred primarily in transplanted cells and was spatially limited to the scars and border zones of the rat hearts and temporally limited to a 4-week duration. These patterns of transgene expression suggest that both heart cells and skeletal myoblasts may be appropriate delivery vehicles for therapeutic transgenes. We are currently evaluating the effect of this VEGF transgene on survival of the transplanted cells, the durability of the angiogenic response, and its safety profile over a longer follow-up period. Transplantation of cells transfected with multiple genes to promote angiogenesis, to enhance integrin expression, and its safety profile over a longer follow-up period. Transplantation of cells transfected with multiple genes to promote angiogenesis, to enhance integration with host cells,\(^\text{15}\) or to minimize apoptosis\(^\text{16}\) may ultimately prove to be the most promising therapeutic application of cell transplantation.

References