Vascular Endothelial Growth Factor Receptor Upregulation in Response to Cell-Based Angiogenic Gene Therapy

Terrence M. Yau, MD, MS, Guangming Li, MD, Yaoguang Zhang, MD, Richard D. Weisel, MD, Donald A. G. Mickle, MD, and Ren-Ke Li, MD, PhD

Division of Cardiovascular Surgery, Toronto General Hospital, University Health Network, Department of Surgery, University of Toronto, Heart & Stroke Foundation, Richard Lewar Centre of Excellence, Toronto, Ontario, Canada

Background. **We have previously reported that transplantation of vascular endothelial growth factor transfected cells into myocardial scar enhances angiogenesis. We evaluated the effect of transplanted cell type, time, and region of the heart on expression of the vascular endothelial growth factor receptors fms-like tyrosine kinase-1 (flt-1) and fetal liver kinase-1 (flk-1).**

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), vascular endothelial growth factor transfected heart cells, skeletal myoblasts, vascular endothelial growth factor transfected skeletal myoblasts, or medium (controls) (N = 13 each). Flt-1 and flk-1 expression in the scar, border zone, and normal myocardium were evaluated at 3 days and 1, 2, and 4 weeks by quantitative polymerase chain reaction. Transplanted cells, vascular endothelial growth factor, flt-1, and flk-1 were identified by immunohistology.**

Results. **Flt-1 and flk-1 levels were low in all areas of control hearts. Upregulation of flt-1 and flk-1 after cell transplantation occurred primarily in host cells in the border zone rather than the scar (zone, $p < 0.0001$). Flt-1 and flk-1 expression was doubled by heart cells and skeletal myoblasts and increased eightfold by vascular endothelial growth factor transfected heart cells and skeletal myoblasts (group, $p < 0.0001$). Flk-1 expression peaked at 1 week, whereas flt-1 peaked at 2 weeks (time, $p < 0.0001$).**

Conclusions. **Flk-1 and flt-1 upregulation may mediate the angiogenic effect of cell transplantation and are augmented by vascular endothelial growth factor transgene expression, perhaps through a paracrine effect. Optimizing the angiogenic response to cell transplantation may maximize the benefit of cell transplantation strategies.**

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Myocardial cell transplantation has undergone intensive investigation as a potential therapy for postinfarction congestive heart failure, unreconstructable coronary arteriosclerosis, or cardiomyopathy [1–6]. We have reported that transplantation of endothelial cells induces angiogenesis, but does not alter ventricular function [7]. Even greater angiogenesis was observed in response to the transplantation of a mixed culture of heart cells (HC) (predominantly cardiomyocytes, with smaller proportions of endothelial cells, smooth muscle cells, and fibroblasts) after ex vivo transfection with vascular endothelial growth factor (VEGF) [8]. We have observed that VEGF transgene expression in these transplanted cells is limited to the scar and border zone, and lasts approximately 4 weeks [9]. Therefore, ex vivo modification of cells before transplantation may have the potential to enhance survival of the transplanted cells and modify their effect on myogenesis, angiogenesis, or matrix remodeling. However, the mechanisms by which the VEGF transgene acts on the transplanted and donor cells has not been evaluated. Because of interactions between cell transplantation and gene delivery strategies, these mechanisms of action may differ from those described for gene therapeutic approaches alone.

In this series of experiments, we hypothesized that a VEGF transgene, expressed transiently in cells transplanted into scarred rat hearts, would exert its angiogenic effects at least in part through the transient upregulation of the VEGF receptors fetal liver kinase-1 (flk-1) and fms-like tyrosine kinase-1 (flt-1). We also hypothesized that this receptor upregulation would occur primarily in the host cells, and would be spatially limited to the scar and border zone. Finally, we evaluated the effect of two different cell types to determine whether there were cell type-specific differences in the mechanisms by which angiogenesis was induced.

Material and Methods

Animals and Experimental Model

Animals were syngeneic adult Lewis rats (body weight, 225 to 250 g for female rats, 250 to 300 g for males) (Charles River Canada Inc, Quebec, Canada). All procedures were approved by the Animal Care Committee of

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Address reprint requests to Dr Yau, Division of Cardiovascular Surgery, Toronto General Hospital, University Health Network, 4N-470, 200 Elizabeth St, Toronto, Ontario M5G 2C4, Canada; e-mail: terry.yau@utoronto.ca.

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Abbreviations and Acronyms

Ctrl = controls
Flk-1 = fetal liver kinase-1
Flt-1 = fms-like tyrosine kinase-1
HC = heart cells
HC+ = vascular endothelial growth factor transfected heart cells
RNA = ribonucleic acid
PCR = polymerase chain reaction
Sk = skeletal myoblasts
Sk+ = vascular endothelial growth factor transfected skeletal myoblasts
VEGF = vascular endothelial growth factor

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 free wall of rat hearts by a cryoinjury technique as previously described [8]. After recovery, rats were randomly divided into 5 groups: controls (Ctrl), injected with culture medium, or transplantation with a mixed culture of unmodified HC, VEGF transfected heart cells (HC+), unmodified skeletal myoblasts (Sk), or VEGF transfected skeletal myoblasts (Sk+) (N = 13 each).

Cell Isolation and Culture

A mixed primary culture of cardiomyocytes, smooth muscle cells, endothelial cells, and fibroblasts was isolated from the left ventricles of donor rats as previously described [8, 9]. The cultured cells were depleted of fibroblasts by a pre-plating technique and were then maintained in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum for 5 to 7 days before transfection and transplantation. In a subset of plates, 71% of cells stained positively for myosin heavy chain were assumed to be cardiomyocytes, 13% of cells stained positively for α-smooth muscle actin were assumed to be smooth muscle cells, and 13% of cells stained positively for factor VIII were assumed to be endothelial cells. The remaining cells were assumed to be fibroblasts. Primary skeletal myoblasts were isolated and cultured by a modified single-muscle fiber culture technique [10].

Cell Transfection

Skeletal myoblasts and heart cells were transfected in 100 mm dishes at 60% to 70% confluence. Cells were transfected ex vivo for 24 hours by a lipid-based technique with a plasmid encoding $VEGF_{165}$ (pCEP4-VEGF) as previously described [8, 9].

Bromodeoxyuridine Pre-Labeling

One of every four plates of skeletal myoblasts and heart cells was pre-labeled with bromodeoxyuridine (BrdU) 2 days before transplantation and 1 day before transfection [8, 9]. A monoclonal antibody against BrdU was used to identify the transplanted cells within recipient hearts.

Cell Transplantation

Rats underwent cell transplantation 3 weeks after left ventricular cryoinjury. VEGF transfected or untransfected HC or Sk were detached from culture dishes with trypsin, centrifuged and re-suspended in serum-free medium. Three million cells in 0.05 mL of serum-free medium, or medium without cells, were injected at multiple points into the center of the cryoinjury-induced scar with a tuberculin syringe.

Rat hearts were excised 3 days, 1 week, 2 weeks, and 4 weeks (N = 15 each time point, 3 rats $\times$ 5 groups) after cell transplantation. The atria and the right ventricular free wall were excised, leaving the left ventricle, which was divided into the scar (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. Total RNA was isolated with TRIzol RNA extraction reagents (Invitrogen Corp, Carlsbad, CA) 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, Carlsbad, CA) [9].

Quantitation of Flk-1 and Flt-1 Messenger RNA by Real-Time PCR

Quantitation of flk-1 and flt-1 messenger RNA expression was carried out by real-time PCR on the 9700 HT System (Applied Biosystems Inc, Foster City, CA). Two pairs of specific PCR primers were designed based on flk-1 and flt-1 sequences from the GeneBank (NCBI) (flk-1 sense 5′ — GCTCCTGCAGTGCATAACCTGG — 3′, antisense 5′ — CTTAGATAGCCCGGAACGCTAC — 3′; flt-1 sense 5′ — TGGCTCACTGTAGTAGGCAGAG — 3′, antisense 5′ — GGTGTCTGCTTCTCACAGGATA — 3′). Standard PCR was first performed with these primers utilizing the single strand cDNA from the sample as a template. Gel electrophoresis confirmed that the PCR products comprised single bands of the correct size. These bands were excised, the PCR products were purified from the gel, sequenced to confirm their identities, and quantitated spectrophotometrically for use as standards.

Real time PCR was performed using the Master Mix SYBR Green I Kit (Applied Biosystems Inc, Foster City, CA) utilizing serial dilutions to generate standards ranging from 1,500 pg to 0.23 pg cDNA samples from the rats, which were diluted 200-fold, and 5 μL of each standard or sample were transferred to a 96 well PCR plate. Assays were performed in duplicate, and 5 μL of dd-water were assayed as a no-template control. Five μL of a 5 pmol flk-1 or flt-1 sense and antisense primer mixture and 10 μL of Master SYBR Green I Mix were added to each well. The reaction sequence included stabilization for 2 min-