Increasing Transplanted Cell Survival With Cell-Based Angiogenic Gene Therapy

Terrence M. Yau, MD, MS, Christopher Kim, BS, Dawn Ng, BS, Guangming Li, MD, Yaoguang Zhang, MD, Richard D. Weisel, MD, and Ren-Ke Li, MD, PhD

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

Background. **The majority of cells transplanted into infarcted myocardium do not survive. Maximizing cell survival should maximize the efficacy of cell transplantation for myocardial repair. We evaluated the role of apoptosis in cell loss after transplantation and the effect of angiogenesis on apoptosis and overall cell survival.**

Methods. **Female Lewis rats underwent myocardial cryoinjury 3 weeks before transplantation with male 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 (n = 6 each), or medium (control, n = 5). One week later, transplanted cell survival and apoptosis were quantitated by real-time polymerase chain reaction for Y chromosomal deoxyribonucleic acid, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling assay and deoxyribonucleic acid fragmentation.**

Results. **Approximately one third of heart cells and skeletal myoblasts survived 1 week after transplantation, and one half of vascular endothelial growth factor–transfected heart cells and skeletal myoblasts survived to this time ($p < 0.05$). Apoptosis was greatest in heart cell and skeletal myoblast-transplanted hearts ($p < 0.05$), reduced in the vascular endothelial growth factor–transfected groups ($p < 0.05$) and lowest in controls.**

Conclusions. **Ischemia and apoptosis both contribute to cell loss after transplantation. Transfection with vascular endothelial growth factor induced angiogenesis, which reduced both ischemic and apoptotic cell death. Our findings suggest that further strategies to reduce apoptosis may enhance the efficacy of cell transplantation in myocardial repair.**

(Ann Thorac Surg 2005;80:1779–86)

In the last decade, myocardial cell transplantation has undergone intensive investigation as a potential therapy for postinfarction congestive heart failure, unreconstructable coronary atherosclerosis, or cardiomyopathy [1–6]. We have previously 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 (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]. 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.

Greater survival of the transplanted cells may increase their efficacy for myocardial repair [10], although it may also increase potential arrhythmogenesis [11]. In addition, because of the generally limited clinical availability of autologous cells and the time required for their expansion in culture before transplantation, greater cell survival after transplantation may broaden the number of patients who may derive benefit from this approach.

In this series of experiments, we hypothesized that the angiogenic effect of a VEGF transgene, expressed transiently in cells transplanted into scarred rat hearts, would increase transplanted cell survival by reducing apoptosis. We also evaluated the influence of cell type on survival after cell transplantation, and the interactive effect of cell type and angiogenesis on this outcome.

Material and Methods

Animals and Experimental Model

Animals were syngeneic adult Lewis rats (body weight 225 g to 250 g for female rats, 250 g to 300 g for males [Charles River Canada, 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.

Accepted for publication April 21, 2005.

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

Published by Elsevier Inc

0003-4975/05/$30.00
doi:10.1016/j.athoracsur.2005.04.079

A large transmural scar was created in the left ventricular free wall of female recipient rat hearts by a cryoinjury technique as previously described [8]. Briefly, through a left lateral thoracotomy, the left ventricular free wall was exposed and cryoinjury performed by 12 1-minute applications of an 8 × 10 mm elliptical metal probe cooled to –196°C by immersion in liquid nitrogen. After recovery, the cryoinjured female recipient rats were randomly divided into 5 experimental groups: control, injected with culture medium alone (control, n = 5), transplantation with a mixed culture of unmodified heart cells (HC, n = 6), transplantation with VEGF-transfected heart cells (HC+, n = 6), transplantation with unmodified skeletal myoblasts (Sk, n = 6), or transplantation with VEGF-transfected skeletal myoblasts (Sk+, n = 6).

Cell Isolation and Culture

A mixed primary culture of cardiomyocytes, smooth muscle cells, endothelial cells and fibroblasts was isolated from the left ventricles of male donor rats as previously described [8]. The cultured cells were depleted of fibroblasts by a preplating technique and 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, 69% ± 14% of cells stained positively for myosin heavy chain and were assumed to be cardiomyocytes, 14% ± 12% of cells stained positively for alpha-smooth muscle actin and were assumed to be smooth muscle cells, and 11% ± 7% of cells stained positively for Factor VIII and were assumed to be endothelial cells. The remainder of the cells were assumed to be fibroblasts.

Primary skeletal myoblasts were isolated and cultured by a modified single-muscle fiber culture technique [12]. Briefly, 3 g muscle from the quadriceps femoris muscle of adult male donor Lewis rats underwent enzymatic digestion with protease and type I collagenase (Sigma, St. Louis, Missouri) before isolation and resuspension of single, intact muscle fibers in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum. After preplating, single skeletal muscle fibers were plated onto laminin-coated plates (Becton Dickinson Labware, Bedford, Massachusetts). Skeletal myoblasts dissociated from the muscle fibers, attached to the plate, and were allowed to proliferate for 48 to 72 hours as the original muscle fibers underwent cell death and lysis. The skeletal myoblasts were maintained in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum for a further 5 to 7 days and then transfected before fusion and myotube formation.

Cell Transfection

Skeletal myoblasts and heart cells were transfected in 100 mm dishes at 60% to 70% confluence. Cells were transfected ex vivo, by a lipid-based technique, with a plasmid encoding $VEGF_{165}$ (pCEP4-VEGF) as previously described [8]. Transfection efficiencies were monitored in a subset of plates by cotransfection with pEGFP-N2 (BD Biosciences Clontech, Palo Alto, California), a plasmid expressing green fluorescence protein. Cells were incubated with the transfection reagents for 24 hours before transplantation.

Cell Transplantation

Female recipient rats underwent cell transplantation 3 weeks after cryoinjury of the left ventricle free wall. The VEGF-transfected or untransfected male donor heart cells or skeletal myoblasts were detached from culture dishes with trypsin, centrifuged, and resuspended in serum-free medium. Under general anesthesia, recipient rat hearts were exposed through a midline sternotomy. Three million cells in 0.05 mL serum-free medium were injected at five points into the center of the cryoinjury-induced left ventricle scar with a tuberculin syringe. The same volume of culture medium without cells was injected into the scar of control rats.

Rats were sacrificed and the hearts were excised 1 week 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. In 3 rats per group, half of the scar, border zone, and normal myocardium were utilized for histologic evaluation and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining. The other half of the scar specimen, and all of the scar in the remaining rats, were snap-frozen in liquid nitrogen, powdered, and used for DNA isolation for polymerase chain reaction (PCR) and analysis of fragmentation.

Isolation of DNA

The DNA was isolated from snap-frozen and powdered myocardial specimens as well as heart cells from male Lewis rats (for use as standards) with the Qiagen Genomic-tip 20/G DNA isolation kit (Qiagen, Mississauga, Ontario). For the standards, male heart cells were detached from a culture plate with 0.25% trypsin, which was then inactivated with Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, and the cells washed and resuspended in phosphate-buffered saline. Cell numbers were counted in a Coulter counter (Beckman Coulter, Fullerton, California), and to each tube of 5×10^6 male cells, 0.5 mL buffer C1 containing 0.5 μg ribonuclease A and then 1.5 mL ice-cold distilled H_2O were added before incubation on ice for 10 minutes. Cell nuclei from the lysed cells were pelleted by centrifugation (1,300*g*, 15 minutes), the supernatant discarded, and nuclei resuspended in 0.25 mL C1 buffer and 0.75 mL ice-cold distilled water by vortexing. The nuclei were centrifuged again (1300*g*, 15 minutes), the supernatant discarded, and the pellet vortexed for 30 seconds into 1 mL buffer G2. Nuclear proteins were then digested with 25 μL QIAGEN Protease solution (50°C, 45 minutes). A Qiagen Genomic-tip 20G was equilibrated in 2 mL buffer QBT, and the DNA solution applied to the Genomic-tip after vortexing (10 seconds). The DNA was washed (1 mL buffer QC × 3), eluted (1 mL buffer QF × 2), and precipitated (0.7 vol isopropanol). The DNA solution was then centrifuged

Table 1. Summary of Statistical Analysis of Y Chromosomal Quantitative Polymerase Chain Reaction Assay

Group	Mean	Standard Deviation
HC	9.0×10^5	2.7×10^5
HC+	1.6×10^6	2.7×10^5
SK	8.4×10^5	1.3×10^5
SK+	1.4×10^6	1.7×10^5
Control	4.9×10^3	2.6×10^3
Summary of ANOVA		
Effect: group		
F Value: 60.51		
Pr > F: <0.0001		

HC = heart cells; HC+ = vascular endothelial growth factor–transfected heart cells; SK = unmodified skeletal myoblasts; SK+ = vascular endothelial growth factor–transfected skeletal myoblasts.

(12,000*g*, 15 minutes), washed in 70% EtOH, centrifuged again, and dissolved in 100 μL TE buffer (10 mM Tris-Cl, 2 mM EDTA, pH 8.0) overnight on a shaker before real-time PCR. The DNA from myocardial specimens was prepared in the same fashion except that 20 mg of snap-frozen tissue was homogenized in buffer G2 with ribonuclease (instead of cells being lysed in buffer C1), and digestion with protease was prolonged (120 minutes instead of 45 minutes).

Quantitation of Y Chromosomal DNA by Real-Time PCR

To accurately quantitate the number of Y chromosome–bearing male donor cells within the female recipient hearts, a pair of specific primers was designed based on the GeneBank sequence (NCBI, Washington, DC) of the sex-determining region of the Y chromosome (sry) [13] of the rat (sense 5′—GAGGCACAAGTTGGCTCAACA—3′, antisense 5′—CTCCTGCAAAAAGGGCCTTT—3′). Quantitative PCR was performed on the 9700 HT System (Applied Biosystems, Foster City, California) using the Master Mix SYBR Green Kit (Applied Biosystems) utilizing threefold serial dilutions of the sry2 DNA standards in dd-water to generate standards ranging from the sry2 DNA of 10,000 cells to the sry2 DNA of 41 cells. Briefly, 5 μL of each standard or sample (diluted fivefold) were transferred to a 96-well PCR plate. Each assay was performed in duplicate, and 5 μL of dd-water were assayed as a no-template control. Five μL of a 5 pmol sry2 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 10 minutes at 50°C before 45 cycles of denaturation for 15 seconds at 95°C, annealing and primer extension for 1 minute at 60°C, and dissociation for 15 seconds at 95°C, 15 seconds at 60°C, and 15 seconds at 95°C. The PCR products were labeled with SYBR Green and detected by a laser detector on the 9700 HT system. Real-time PCR data were analyzed with SDS 2.1 software (Applied Biosystems).

TUNEL Assay

In situ DNA nick-end fluorescence labeling [14] and propidium iodide nuclear counterstaining were performed with the APO-TUNEL assay kit (Molecular Probe, Eugene, Oregon) according to the manufacturer's instructions. Briefly, tissue sections were rehydrated in phosphate-buffered saline for 30 minutes. On each slide, double strand DNA breaks in apoptotic cells were labeled in the reaction with 50 μL DNA-labeling solution (10 μL reaction buffer, 0.75 μL terminal deoxynucleotidyl transferase [TdT], 8 μL Alexa Fluor 488-conjugated deoxyuridine triphosphate, and 31.25 μL dH_2O) incubated

Table 2. Summary of Statistical Analysis of Apoptosis

	Mean			Standard Deviation		
Group	Normal	Border	Scar	Normal	Border	Scar
HC	6.62	9.11	16.55	5.30	5.30	4.85
HC+	6.49	5.52	8.33	5.38	2.18	2.25
SK	6.02	8.58	17.80	4.09	3.45	4.84
SK+	6.21	5.35	7.37	4.76	2.65	3.30
Control	3.44	5.31	2.00	2.50	4.06	2.33
Summary of ANOVA						
Effect: group						
F Value						
Normal, 1.27						
Border, 3.96						
Scar, 48.42						
Pr > F						
Normal, <0.0001						
Border, <0.0001						
Scar, <0.0001						

HC = heart cells; HC+ = vascular endothelial growth factor–transfected heart cells; SK = unmodified skeletal myoblasts; SK+ = vascular endothelial growth factor–transfected skeletal myoblasts.

Table 3. Summary of Statistical Analysis of DNA Fragmentation

Group	Mean	Standard Deviation
HC	26.15	1.14
HC+	14.77	5.12
SK	30.22	2.17
SK+	17.16	0.46
Control	13.04	4.64
Summary of ANOVA		
Effect: group		
F Value: 15.681		
Pr > F: <0.0001		

HC = heart cells; HC+ = vascular endothelial growth factor–transfected heart cells; SK = unmodified skeletal myoblasts; SK+ = vascular endothelial growth factor–transfected skeletal myoblasts.

at 37°C for 60 minutes in a dark incubation chamber. Samples were rinsed in rinse buffer (3 × 5 minutes), incubated in propidium iodide/ribonuclease A staining buffer (100 μL, 30 minutes, 23°C) and washed in phosphate-buffered saline. Slides were covered with a cover slip mounted with 50% glyceryl. All procedures were performed in a dark box to protect samples from light during incubation and washing. After TUNEL and propidium iodide counter staining, DNA strand breaks in apoptotic cells were identified by their yellow color (green plus red fluorescence), and all other propidium iodide-labeled nuclei were identified as red, detected at 488 nm and 536 nm under laser confocal microscopy (Bio-Rad Laboratories, Hercules, California). Total cell nuclei and apoptotic nuclei were counted in five fields (×400) per slide, and the apoptotic index was calculated as the ratio of apoptotic cells to total cells, expressed as a percentage.

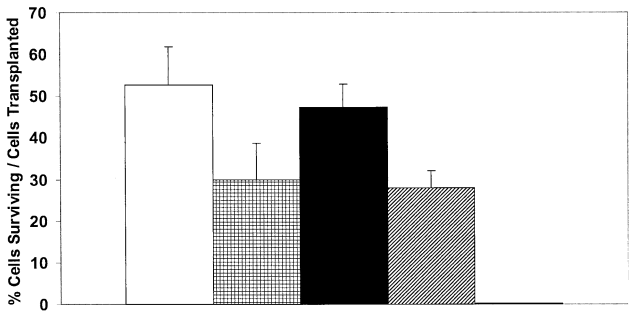

Fig 1. Percentage of cells surviving 1 week after transplantation, in rat hearts transplanted with heart cells (HC; checked bar*), vascular endothelial growth factor (VEGF)–transfected HC (HC+;* open bar*), skeletal myoblasts (Sk;* diagonal slash bar*), VEGF-transfected Sk (Sk+;* solid bar*), or medium alone (control;* vertical stripe bar*), evaluated by quantitative polymerase chain reaction for sry2. Approximately one third of the transplanted HC and Sk survived 1 week, compared with approximately one half of the transplanted HC+ and Sk+ (p < 0.05). There was a significant effect of transfection with VEGF (p < 0.0001), but the effect of cell type was nonsignificant (p = 0.5).*

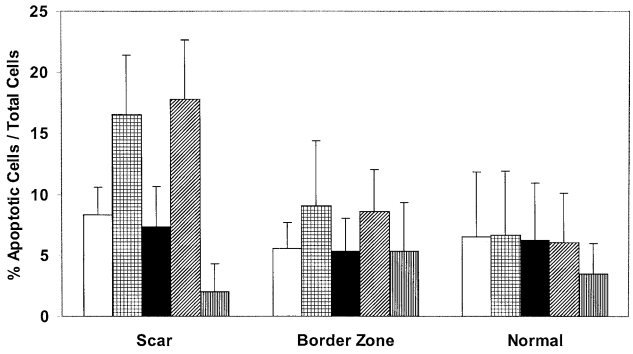

Fig 2. Percentage of apoptotic cells 1 week after transplantation in the scar, border zone, or normal myocardium, in rat hearts transplanted with heart cells (HC; checked bars*), vascular endothelial growth factor (VEGF)–transfected HC (HC+;* open bars*), skeletal myoblasts (Sk;* diagonal slash bars*), VEGF-transfected Sk (Sk+;* solid bars*) or medium alone (control;* vertical stripe bars*), evaluated by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling staining. Apoptotic cells were rare in the scar of control hearts. The highest apoptotic indices were observed in the scar of HC and Sk rats (p < 0.05 versus control). The HC+ and Sk+ hearts had significantly reduced apoptosis (p < 0.05 versus HC, Sk). Apoptotic cells were also more common in the border zone of the HC and Sk groups than in HC+, Sk+, or control rats (p < 0.05). There was a significant effect of transfection with VEGF (p < 0.0001), but the effect of cell type was nonsignificant (p = 0.9).*

DNA Fragmentation Analysis

Nuclease-mediated degradation of nuclear DNA in apoptotic cells was analyzed by electrophoretic methods [15, 16]. Forty milligrams of each powdered tissue sample was lysed in a lysis buffer (50 mM Tris, 10 mM EDTA, 2% SDS, 0.5 mg/mL proteinase K; Qiagen, Mississauga, Ontario) at 60°C for 3 hours. After treatment with deoxyribonuclease-free ribonuclease (50°C, 1 hour), DNA was extracted using a phenol-chloroform technique, precipitated with sodium acetate and dissolved in TE (10 mM Tris, pH 8.0, 1 mM EDTA). The concentration of DNA was quantified spectrophotometrically (Buckman International MI-SS, Corona, California). Five micrograms of DNA from each sample was subjected to electrophoresis on a 1.5% agarose gel, visualized by ethidium bromide staining, and analyzed with the Alpha Innotech Gel Document system (Alpha Innotech Corporation, San Leandro, California). Staining intensities of DNA fragments less than 5 kb versus total DNA staining intensity were measured and analyzed with AlphaImager 2200 software (Alpha Innotech Corporation).

Statistical Analysis

Data are presented as mean and standard deviation. All analyses were performed with SAS statistical software (SAS Institute, Cary, North Carolina). We analyzed data on sry2 DNA levels, percentage of apoptotic cells, and degree of DNA fragmentation by analysis of variance (ANOVA) for group effect. When the F-value for the ANOVA was statistically significant ($p < 0.05$), differ-

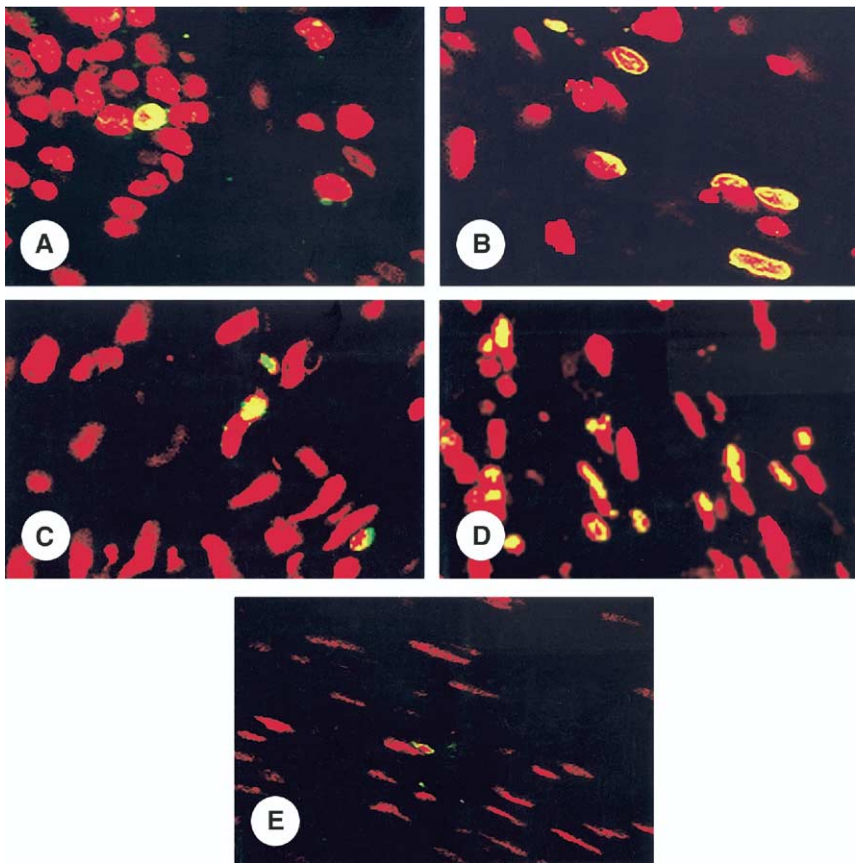

Fig 3. Laser confocal micrographs of the scar of rat hearts transplanted with (A) vascular endothelial growth factor (VEGF)-transfected heart cells (HC+); (B) untransfected heart cells (HC); (C) VEGF-transfected skeletal myoblasts (Sk+); (D) untransfected skeletal myoblasts (Sk); or (E) medium alone (control). (Original magnifications, ×600). Cell nuclei are stained red *by propidium iodide, while the nuclei of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling–positive apoptotic cells appear* yellow. *Apoptosis was more common in the HC and Sk hearts compared with HC+, Sk+, and control groups. Greater cellularity (density of nuclei) is noted in the cell-transplanted groups than in the control hearts.*

ences between groups were specified by Duncan's multiple range test.

To statistically evaluate the effect of VEGF transfection and cell type on apoptosis rather than group assignment in general, a second analysis was performed on the four cell-transplanted groups alone, evaluating the effects of VEGF transfection (yes/no), cell type (HC/Sk) and location by ANOVA. Again, main effects and all interactive effects (VEGF × cell type, VEGF × location, cell type × location, and VEGF × cell type × location) were modeled. Nonsignificant interactive effects were discarded, and the final model included only the statistically significant main and interactive effects. We presented the data graphically to visually depict obvious differences or lack of differences, in the context of the overall analysis of main and interactive effects, the details of which are presented in Tables 1, 2, and 3. Overall effects of a specific factor were subjected to Duncan's multiple range test.

Results

Cell Survival

Overall survival of the transplanted cells in the myocardial scar, quantitated by real-time PCR for sry DNA, was approximately 30% 1 week after transplantation of either unmodified HC or unmodified Sk (Fig 1). Cell survival was significantly greater in the HC+ and Sk+ hearts at 1 week, approximately 50% ($p < 0.05$ versus HC, Sk, or control). There was no difference between HC+ and Sk+, nor was there a difference between unmodified HC and unmodified Sk (p = not significant). Cell survival was predicted by VEGF transfection ($p < 0.0001$), but not by cell type ($p = 0.5$). There was no interactive effect of transfection with cell type ($p = 0.9$).

Apoptosis: TUNEL Staining

The percentage of TUNEL-positive, apoptotic cells was approximately 2% in the relatively acellular scar of control rats in which culture medium alone was injected, without cells (Fig 2, Fig 3E). Approximately 16% to 17% of nuclei in the HC and Sk-transplanted scars were TUNEL-positive ($p < 0.05$ versus control; Fig 2, Fig 3B and 3D). There was no difference between HC and Sk (p = not significant). In contrast, only 7% to 8% of nuclei in the HC+ and Sk+ hearts were TUNEL-positive ($p < 0.05$ versus HC, Sk, control; Fig 2, Fig 3A and 3C). Again, there was no difference between HC+ and Sk+ (p = not significant).

Nuclei in the scar of control hearts were primarily the elongated nuclei of fibroblasts in the fibrous scar (Fig 3E). Nuclei in the scars of the cell-transplanted groups were primarily the nuclei of the transplanted heart cells or skeletal myoblasts (Figs 3A to 3D).

Approximately 5% of the nuclei in the border zone of the HC+, Sk+, and control hearts were TUNEL-positive, while approximately 9% of the nuclei in the HC and Sk

Ann Thorac Surg
2005;80:1779–86

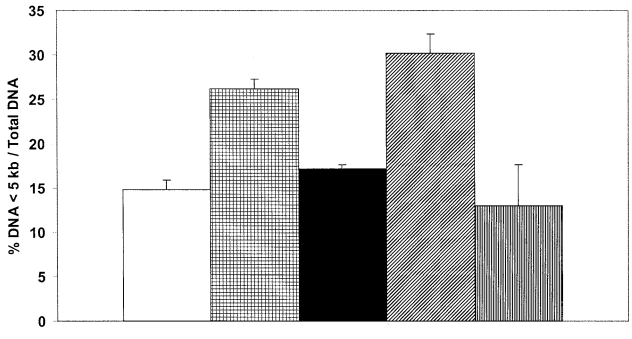

Fig 4. Fragmentation of DNA in the myocardial scar 1 week after cell transplantation, in rat hearts transplanted with heart cells (HC; checked bar*), vascular endothelial growth factor (VEGF)–transfected HC (HC+;* open bar*), skeletal myoblasts (Sk;* diagonal slash bar*), VEGF-transfected Sk (Sk+;* solid bar*), or medium alone (control;* vertical stripe bar*). The DNA fragmentation was significantly greater in the scar of HC and Sk rats compared with HC+, Sk+, or control hearts (*$p < 0.05$*), indicating greater apoptosis in HC and Sk hearts.*

groups were apoptotic ($p < 0.05$ versus HC+, Sk+, control; Fig 2).

There was no difference between any of the cell-transplanted groups in the percentage of apoptotic cells in the normal myocardium (Fig 2). Apoptosis was somewhat less frequent in the normal zone of the control hearts, but this difference was not statistically significant ($p = 0.3$).

Overall, the percentage of apoptotic cells was predicted by group, zone and the interaction between group x zone (all $p < 0.0001$). When only the four cell-transplanted groups were analyzed, VEGF transfection, zone, and the interaction between VEGF and zone were highly predictive of apoptosis (all $p < 0.0001$), while cell type ($p = 0.9$) and the other interactive effects were not predictive.

Apoptosis: DNA Fragmentation

Fragmentation of DNA in the scar of the control hearts at 1 week was low (Fig 4). The DNA fragmentation was significantly greater ($p < 0.05$ versus control) in the HC and Sk groups. In contrast, the HC+ and Sk+ hearts demonstrated a level of DNA fragmentation that was similar to that observed in control rats ($p =$ not significant versus control), and significantly lower than fragmentation levels in the HC and Sk rats ($p < 0.05$ versus HC, Sk). Fragmentation of DNA was predicted by VEGF transfection ($p < 0.0001$), but not by cell type ($p = 0.09$) or by the interaction between VEGF and cell type ($p = 0.6$).

Comment

Cell transplantation is a promising new therapy for hearts with ischemic ventricular dysfunction [1–4, 6]. Both the efficacy and the potential side effects of this novel therapy appear to be strongly related to dose, with both increasing as the number of cells implanted increases [10, 11]. Although it has so far been difficult to accurately quantify cell survival, it seems likely that the majority of cells transplanted into scarred myocardium do not survive the first several days. In addition, autologous cells, which avoid the problems of allogeneic cell transplantation, will generally be in limited supply in a clinical setting. For example, while patients may undergo harvesting of skeletal myoblasts for expansion in culture before reimplantation at the time of surgery, this process currently requires approximately 3 weeks. Patients requiring more urgent surgery are unfortunately ineligible for this therapy because of this time constraint. Strategies to increase the survival of transplanted cells may therefore make this therapy more widely applicable as well as increase its efficacy for both myogenesis and angiogenesis.

The degree of engraftment and survival of cells transplanted into a myocardial infarct or scar may be mediated by multiple factors. These may include properties of the transplanted cells such as resistance or susceptibility to ischemia, the ability to couple mechanically or electrically with surrounding cells, or the ability to induce changes in the local environment such as matrix reorganization [17] or angiogenesis. The local milieu will also influence transplanted cell survival. Any target environment for cell transplantation is likely to be a hostile one, with ischemia, abnormal mechanical properties, and a native cell population that may differ significantly from normal myocardium. Global environmental influences, such as immune responses or rejection, or perhaps even the population of stem cells recruitable from remote locations, may also influence transplanted cell survival.

The cryoinjury model that we employed results in an extremely hostile environment for transplanted cells, with predominantly fibrous tissue and collagen composition and very few remaining native cells, those being primarily fibroblasts [3]. The unmodified scar thins and dilates, with markedly abnormal regional mechanics. Cryoinjury therefore represents an extreme model for testing transplanted cell survival, and facilitates evaluation of interventions to increase survival. It may have limitations, however, for evaluation of interactions between the transplanted cells and surviving host cells. Such interactions are likely to be limited to the border zone, where the scar is not transmural and peninsulae of host cells extend into the scar. Follow-up studies employing a coronary ligation model will be required to determine whether the mechanism of myocardial injury influences the prosurvival effect of cell-based VEGF gene therapy.

In this study, we employed real-time PCR for the Y-chromosome–specific sry gene to quantitate the survival of male donor cells after transplantation into female recipient rats. The number of cells measured by this technique may reflect a combination of survival of the originally transplanted cells and proliferation of those cells after implantation. Using a similar methodology, Muller-Ehmsen and colleagues [18] reported 57% ± 9% survival of male neonatal cardiomyocytes 1 hour after transplantation into female recipient hearts, decreasing to 28% ± 11% survival at 1 week. These figures parallel

the approximately 30% survival at 1 week of unmodified HC and Sk observed in our current study. While this technique of PCR for sry could detect DNA from dead as well as live cells, Muller-Ehmsen and coworkers [18] noted that only 2.2% of DNA from a known quantity of dead male cells was detected 24 hours after injection. Therefore, the contribution of nonviable cells to the number that we measured at 7 days should be extremely low. Persistence of both neonatal [19] and fetal [20] male cardiomyocytes 6 months after transplantation into female recipient rats has also been reported using this methodology. In addition, Laflamme and coworkers [21] have used this technique to demonstrate repopulation of female donor hearts, in response to rejection, by extracardiac progenitors in male heart transplant recipients. This technique allows quantitation of donor cell Y chromosomal DNA within a background of female recipient cells in a manner that is not dependent on geometry. Previous techniques employed for quantitation of transplanted cells have relied on serial histologic sections and counting of donor cells that have been labelled by various techniques, including 4′,6-diamidino-2-phenylindole and green fluorescence protein, or the expression of green fluorescence protein or beta-galactosidase transgenes. However, it is impossible to determine with such techniques that every donor cell has been visualized and counted in one of the multiple sections, and just as importantly, that it has been counted only once despite traversing multiple sections. While our current methodology does not permit spatial localization of the transplanted cells within the volume of sample, it does yield a more reliable quantitative estimate of donor cell survival, and permits comparison between groups.

Our results suggest that both apoptotic and ischemic cell death contribute to early loss of the transplanted cells. Zhang and coworkers [22] have also evaluated apoptosis by TUNEL staining after transplantation of neonatal rat cardiomyocytes after acute cryoinjury. Cells transplanted acutely into the cryoinjured myocardium had peak TUNEL indices of 32% ± 4% at 1 day, falling to only 1.0% ± 0.2% at 1 week. The much more rapid rise and fall of apoptosis noted in their study likely relates to the effect of cell transplantation into an acute cryoinjury zone, in contrast to the more mature scar employed in this study. Interestingly, Zhang and colleagues [22] noted that both prior heat shock and overexpression of the cytoprotective kinase Akt tended to reduce TUNEL indices, although not to a statistically significant extent. They concluded, as we do, that early cell death was related both to ischemia and to apoptosis. While TUNEL staining has been widely employed to quantitate apoptosis, there is some controversy as to whether it is completely specific for apoptosis [23]. Therefore, while TUNEL positivity predominantly indicates apoptosis, a smaller component of necrotic cell death may also be incorporated into this outcome.

We also observed that ex vivo transfection of a mixed culture of heart cells or skeletal myoblasts with VEGF had a strongly protective effect on the survival of these cells 1 week after transplantation into a cryoinjured scar. Vascular endothelial growth factor may exert its protective effect through a variety of mechanisms. The most obvious mechanism is through induction of angiogenesis and vasculogenesis, which may support the transplanted cells in an ischemic environment and thereby increase survival. We have previously reported that heart cells transfected with VEGF and transplanted into a myocardial scar result in significantly greater regional blood flow and vascular densities than untransfected cells [8]. Expression of the VEGF transgene by the transplanted cells occurs early, with VEGF levels being significantly elevated at 3 days and peaking at 1 week in transfected heart cells, and 2 weeks in transfected skeletal myoblasts [9]. However, this angiogenic response requires time, and there are as yet no data on the time at which angiogenesis first results in significantly enhanced perfusion with this approach. An alternative multimodal approach which may be beneficial is to pretreat the infarct zone with angiogenic gene [24] or protein therapy before cell implantation. Prior induction of angiogenesis may result in greater regional perfusion, which is in place to support the transplanted cells immediately after implantation [25, 26]. Pretreatment of the target area in this fashion, followed by the implantation of cells expressing other mediators of host cell migration, integration, or response that must emanate from the transplanted cells specifically, rather than the zone of injury in general, may further increase cell survival and efficacy. Because these interventions must be separated in time, however, only a minimally invasive approach to this pretreatment is likely to be clinically useful [27].

Another mechanism by which VEGF may exert an effect more rapidly is through its vasodilatory effect [28, 29]. Vasodilation of arterioles and capillaries in the border zone of the scar may improve local perfusion and support the transplanted cells. Because these animals did not have coronary artery disease, inflow to these dilated arterioles and capillaries should be adequate to improve distal perfusion, and this mechanism may be important in the early period after cell transplantation before VEGF-mediated angiogenesis has fully developed.

Thirdly, VEGF may have as-yet-unrecognized direct effects on apoptosis and may thus have improved cell survival not through modulating regional perfusion but by directly inhibiting cell death. However, this possibility requires further investigation. Vascular endothelial growth factor may also have other effects on the host cells as well. Rafii and colleagues [30] have described a population of bone marrow-derived CD34+ stem cells expressing the VEGF receptor flt-1. These cells were able to repopulate the bone marrow after induction through flt-1-mediated signaling. If VEGF does in fact modulate signals for pluripotent cells in the bone marrow to proliferate or home to areas of injury, it is possible that an enhanced VEGF signal may increase the magnitude of this response. Further studies will be required, however, to examine the role of these potential mechanisms in the effects observed in our current series of experiments.

In conclusion, our study demonstrated that ex vivo transfection of heart cells and skeletal myoblasts with

VEGF before transplantation into scarred rat hearts significantly increases survival of the transplanted cells. This effect is achieved at least in part by decreased apoptosis in the transplanted cells, suggesting that other antiapoptotic strategies may further enhance cell survival and maximize the efficacy of cell transplantation for myocardial repair. We did not, however, evaluate cell survival at other timepoints to evaluate the durability of this response. It is possible, therefore, that VEGF transfection simply delays apoptotic or ischemic cell death, rather than preventing it. We are conducting further studies with longer followup in order to more fully characterize the effects of this cell-based gene therapy. It seems likely, however, that combined approaches utilizing both cell transplantation and gene therapy will increase the potential benefits of these strategies for myocardial repair.

This work was supported by grants from the Heart and Stroke Foundation of Ontario (NA4873 and T5305) to Terrence M. Yau.

References

1. Zibaitis A, Greentree D, Ma F, et al. Myocardial regeneration with satellite cell implantation. Transplant Proc 1994;26:3294.
2. Van Meter CH Jr, Claycomb WC, Delcarpio JB, et al. Myoblast transplantation in the porcine model: a potential technique for myocardial repair. J Thorac Cardiovasc Surg 1995; 110:1442–8.
3. Li RK, Jia ZQ, Weisel RD, Mickle DA, Zhang J, Mohabeer MK. Cardiomyocyte transplantation improves heart function. Ann Thorac Surg 1996;62:654–60.
4. Li RK, Weisel RD, Mickle DA, et al. Autologous porcine heart cell transplantation improved heart function after a myocardial infarction. J Thorac Cardiovasc Surg 2000;119: 62–8.
5. Yoo KJ, Li RK, Weisel RD, et al. Heart cell transplantation improves heart function in dilated cardiomyopathic hamsters. Circulation 2000;102(Suppl 3):204–9.
6. Yau TM, Tomita S, Weisel RD, et al. Beneficial effect of autologous cell transplantation on infarcted heart function: comparison between bone marrow stromal cells and heart cells. Ann Thorac Surg 2003;75:169–76.
7. Kim EJ, Li RK, Weisel RD, et al. Angiogenesis by endothelial cell transplantation. J Thorac Cardiovasc Surg 2001;122:963–71.
8. Yau TM, Fung K, Weisel RD, et al. Enhanced myocardial angiogenesis by gene transfer with transplanted cells. Circulation 2001;104(Suppl 1):218–22.
9. Yau TM, Li G, Weisel RD, et al. Vascular endothelial growth factor transgene expression in cell-transplanted hearts. J Thorac Cardiovasc Surg 2004;127:1180–7.
10. Pouzet B, Vilquin JT, Hagege AA, et al. Factors affecting functional outcome after autologous skeletal myoblast transplantation. Ann Thorac Surg 2001;71:844–50.
11. Menasche P, Hagege AA, Vilquin JT, et al. Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. J Am Coll Cardiol 2003;41: 1084–6.
12. Rosenblatt JD, Lunt AI, Parry DJ, Partridge TA. Culturing satellite cells from living single muscle fiber explants. In Vitro Cell Dev Biol Anim 1995;31:773–9.
13. Kakinoki R, Bishop AT, Tu YK, Matsui N. Detection of the proliferated donor cells in bone grafts in rats, using a PCR for a Y-chromosome-specific gene. J Orthop Sci 2002;7:252–7.
14. Li X, Melamed MR, Darzynkiewicz Z. Detection of apoptosis and DNA replication by differential labeling of DNA strand breaks with fluorochromes of different color. Exp Cell Res 1996;10;222:28–37.
15. Arends MJ, Morris RG, Wyllie AH. Apoptosis. The role of the endonuclease. Am J Pathol 1990;136:593–608.
16. Pedersen HG, Watson ED, Telfer EE. Analysis of atresia in equine follicles using histology, fresh granulosa cell morphology and detection of DNA fragmentation. Reproduction 2003;125:417–23.
17. Fedak PW, Altamentova SM, Weisel RD, et al. Matrix remodeling in experimental and human heart failure: a possible regulatory role for TIMP-3. Am J Physiol Heart Circ Physiol 2003;284:H626–34.
18. Muller-Ehmsen J, Whittaker P, Kloner RA, et al. Survival and development of neonatal rat cardiomyocytes transplanted into adult myocardium. J Mol Cell Cardiol 2002;34: 107–16.
19. Muller-Ehmsen J, Peterson KL, Kedes L, et al. Rebuilding a damaged heart: long-term survival of transplanted neonatal rat cardiomyocytes after myocardial infarction and effect on cardiac function. Circulation 2002;105:1720–6.
20. Yao M, Dieterle T, Hale SL, et al. Long-term outcome of fetal cell transplantation on postinfarction ventricular remodeling and function. J Mol Cell Cardiol 2003;35:661–70.
21. Laflamme MA, Myerson D, Saffitz JE, Murry CE. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. Circ Res 2002;90:634–40.
22. Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE. Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. J Mol Cell Cardiol 2001;33:907–21.
23. Hughes SE. Detection of apoptosis using in situ markers for DNA strand breaks in the failing human heart. Fact of epiphenomenon? J Pathol 2003;201:181–6.
24. Lee LY, Patel SR, Hackett NR, et al. Focal angiogen therapy using intramyocardial delivery of an adenovirus vector coding for vascular endothelial growth factor 121. Ann Thorac Surg 2000;69:14–23.
25. Sakakibara Y, Nishimura K, Tambara K, et al. Prevascularization with gelatin microspheres containing basic fibroblast growth factor enhances the benefits of cardiomyocyte transplantation. J Thorac Cardiovasc Surg 2002;124:50–6.
26. Yamamoto M, Sakakibara Y, Nishimura K, Komeda M, Tabata Y. Improved therapeutic efficacy in cardiomyocyte transplantation for myocardial infarction with release system of basic fibroblast growth factor. Artif Organs 2003;27: 181–4.
27. Sanborn TA, Hackett NR, Lee LY, et al. Percutaneous endocardial transfer and expression of genes to the myocardium using fluoroscopic guidance. Catheter Cardiovasc Interv 2001;52:260–6.
28. Sellke FW, Wang SY, Stamler A, et al. Enhanced microvascular relaxations to VEGF and bFGF in chronically ischemic porcine myocardium. Am J Physiol 1996;271:H713–20.
29. Laham RJ, Li J, Tofukuji M, Post M, Simons M, Sellke FW. Spatial heterogeneity in VEGF-induced vasodilation: VEGF dilates microvessels but not epicardial and systemic arteries and veins. Ann Vasc Surg 2003;17:245–52.
30. Rafii S, Avecilla S, Shmelkov S, et al. Angiogenic factors reconstitute hematopoiesis by recruiting stem cells from bone marrow microenvironment. Ann NY Acad Sci 2003;996: 49–60.