Enhanced Angiogenesis With Multimodal Cell-Based Gene Therapy

Terrence M. Yau, MD, MS, Christopher Kim, BS, Guangming Li, MD, Yaoguang Zhang, MD, Shafie Fazel, MD, PhD, Dan Spiegelstein, MD, Richard D. Weisel, MD, and Ren-Ke Li, MD, PhD

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

Background. **We evaluated the synergism of transient vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) overexpression on angiogenesis and left ventricular (LV) function after bone marrow cell (BMC) transplantation, to determine the potential of multimodal cell-based gene therapy for myocardial repair.**

Methods. **Female Lewis rats underwent coronary ligation 3 weeks before transplantation with male donor BMC, BMC transfected with VEGF (BMC+VEGF), bFGF (BMC+bFGF), VEGF and bFGF (BMC+VEGF+bFGF), or medium (control) (n = 3 each group at 3 days, 1 week and 2 weeks; n = 6 each group at 4 weeks; n = 75 total). Three days, 1 week, 2 weeks, and 4 weeks after transplantation, transgene expression was quantitated by real-time polymerase chain reaction, angiogenesis by quantitative histology, and LV function by echocardiography. At 4 weeks, regional perfusion was quantitated with microspheres.**

Results. **The VEGF and bFGF were expressed transiently over 4 weeks. At 1 week, VEGF expression was greatest in BMC+VEGF and BMC+VEGF+bFGF hearts ($p < 0.05$), while bFGF expression was greatest in BMC+bFGF and BMC+VEGF+bFGF rats ($p < 0.05$). Regional perfusion and vascular densities in the scar were lowest in control, intermediate in BMC, BMC+VEGF, BMC+bFGF, and greatest in BMC+VEGF+bFGF ($p < 0.05$). Four weeks after transplantation, LV ejection fraction was lowest in control, intermediate in BMC, BMC+VEGF and BMC+bFGF, and greatest in BMC+VEGF+bFGF ($p < 0.05$).**

Conclusions. **The VEGF and bFGF transgenes were expressed transiently and exerted a powerful synergism on the angiogenic effect of cell transplantation, but did not normalize perfusion or function. The future of cell transplantation may lie in multimodal cell-based gene therapy, in combination with other novel therapies.**

(Ann Thorac Surg 2007;83:1110–20)

Strategies to enhance the angiogenic and myogenic effect of cell transplantation may increase the efficacy of this approach for the repair of infarcted hearts. We have previously reported that angiogenesis after cell transplantation can be enhanced by transplantation of cells transiently overexpressing vascular endothelial growth factor (VEGF) [1]. This transgene is expressed by the transplanted cells over a 4-week period within the scar and border zone [2], and induces upregulation of the VEGF receptors fetal liver kinase-1 and fms-like tyrosine kinase-1 through a paracrine effect on surviving host myocardium [3]. Transplantation of smooth muscle cells expressing insulin-like growth factor-1 also induced VEGF expression, increased angiogenesis, and reduced apoptosis [4]. Ex vivo modification of cells may therefore have the potential to enhance transplanted cell survival, myogenesis, angiogenesis, or matrix remodeling. However, the optimal combination of therapeutic gene delivery with cell transplantation is unknown.

In these experiments, our objective was to determine whether the known synergism of VEGF and basic fibroblast growth factor (bFGF) on angiogenesis [5–8] could be utilized to further enhance the angiogenic effect of cell-based gene therapy. Another objective was to determine whether the combination of cell transplantation with two angiogenic transgenes, delivered 3 weeks after myocardial infarction, could normalize left ventricular (LV) function and perfusion.

Material and Methods

Animals and Experimental Model

Donor and recipient rats were syngeneic adult Lewis rats (Charles River Canada, Quebec, Canada [body weight 225 to 250 g for females, 250 to 300 g for males]). 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" published by the National Acad-

Accepted for publication Oct 20, 2006.

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@uhn.on.ca.

Published by Elsevier Inc

0003-4975/07/$32.00
doi:10.1016/j.athoracsur.2006.10.050

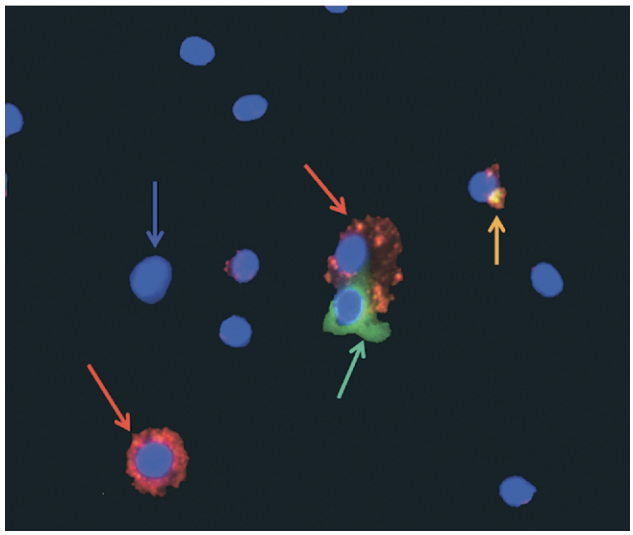

Fig 1. Bone marrow cells immunostained for Sca-1, CD45, and CD34 (original magnification, ×1,000). Mesenchymal stem cells (red arrow; approximately 10% of cultured cells) were Sca-1 (red) positive, CD45 (green) negative, and CD34 negative. The Sca-1 and CD45 positive cells appear brown (brown arrow). Nuclei were stained with DAPI (blue).

emy Press. Myocardial infarctions were induced in female Lewis rats by ligation of the left anterior descending coronary artery.

Bone Marrow Cell Isolation

Donor bone marrow cells (BMC) were isolated from the femurs of male rats and cultured in 100-mm dishes in Iscove's modified Dulbecco's medium with 10% fetal bovine serum for 7 to 10 days before harvesting (at 60% to 70% confluence) for transfection and transplantation. The cultures were partially depleted of erythroid progenitor cells by removal of nonadherent cells with each change of medium, every 3 days. No further fractionation of BMC was performed.

Cell Transfection

Cells were transfected ex vivo, by a lipid-based technique, with a plasmid encoding $VEGF_{165}$ (pCEP4-VEGF) or bFGF (pcDNA3.1-bFGF), or both, 24 hours before transplantation [1–4]. Transfection efficiencies were monitored in a subset of plates by cotransfection with pEGFP-N2 (BD Biosciences Clontech, Palo Alto, California), encoding green fluorescence protein.

Cell Transplantation

Three weeks after infarct creation, rats were randomly divided into five experimental groups and injected with culture medium alone (control), or transplanted with 3×10^6 untransfected BMC, BMC transfected with VEGF (BMC+VEGF), BMC transfected with bFGF (BMC+bFGF), or BMC transfected with VEGF and bFGF (BMC+VEGF+ bFGF) into multiple points in the center of the infarct (n = 3 each group at 3 days, 1 week, and 2 weeks; n = 6 each group at 4 weeks; n = 75 total) [1–4]. Rats were sacrificed, and the hearts were excised 3 days, 1 week, 2 weeks, or 4 weeks after cell transplantation [1–3]. The atria and the right ventricular free wall were excised, and the LV was divided into the scar zone (transmural scar), the border zone (partial-thickness scar containing both fibrous tissue and surviving muscle) and the normal myocardium.

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°C for subsequent analysis of protein levels. Total RNA was isolated with TRIzol RNA extraction reagents (Invitrogen Corp, Carlsbad, California). Messenger RNA in this specimen was reverse transcribed to single-strand complementary DNA (cDNA) with SuperScript II reverse transcriptase (Invitrogen Corp) [2, 3].

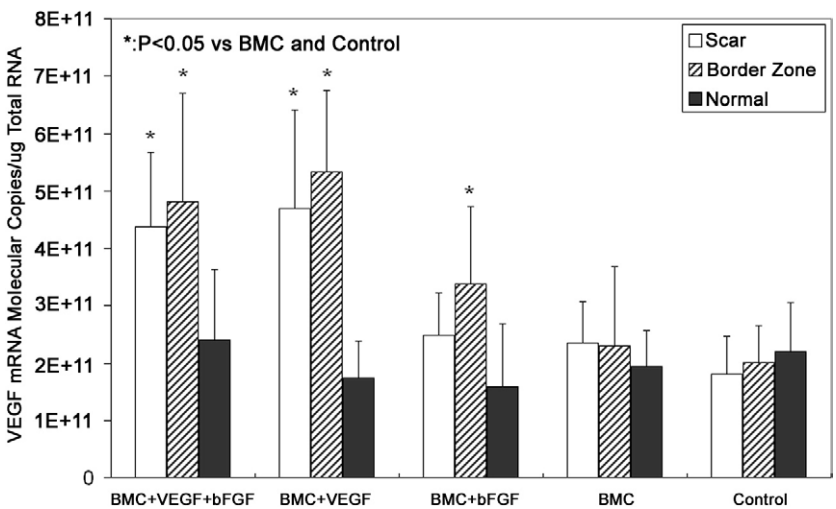

Fig 2. Vascular endothelial growth factor (VEGF) transgene expression in the scar (open bars), border zone (hatched bars), and normal myocardium (solid bars) after bone marrow cells (BMC) transplantation. One week after transplantation, VEGF expression in the scar and border zone was greatest in BMC+VEGF+bFGF and BMC+VEGF hearts ($p < 0.05$). (bFGF = basic fibroblast growth factor.)

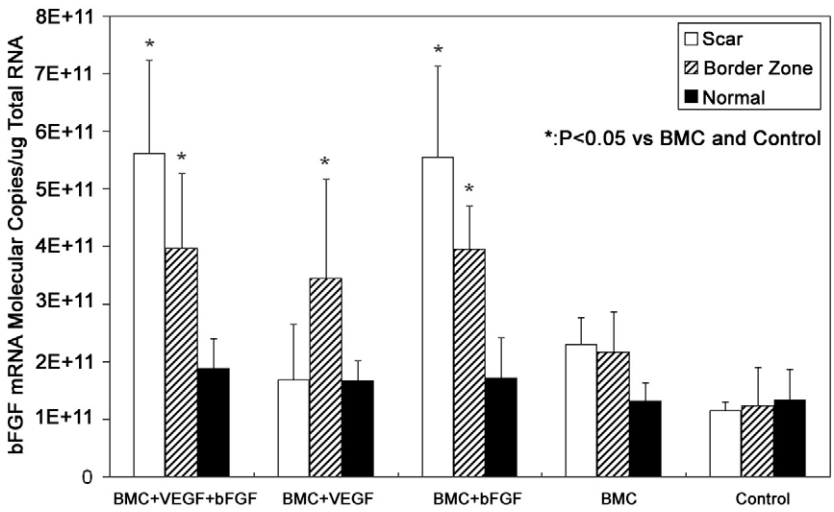

Fig 3. Basic fibroblast growth factor (bFGF) transgene expression in the scar (open bars), border zone (hatched bars), and normal myocardium (solid bars) after bone marrow cells (BMC) transplantation. One week after transplantation, bFGF expression in the scar and border zone was greatest in BMC+VEGF+bFGF hearts and BMC+bFGF hearts ($p < 0.05$). (VEGF = vascular endothelial growth factor.)

Quantitation of VEGF and bFGF mRNA by Real-Time Polymerase Chain Reaction

Quantitation of VEGF and bFGF messenger RNA expression was carried out by means of real-time polymerase chain reaction (PCR) [2, 3]. Specific PCR primers were designed from VEGF and bFGF GeneBank sequences. VEGF primers (antisense, 5′-TCATGGTTGTCTATCAGCGCAG-3′; sense, 5′-GCACACAGGATGGCTTGAAGAT-3′) generated a 107-base pair fragment. bFGF primers (antisense, 5′-CCCGTTTTGGATCCGAGTTTA-3′; sense, 5′-GAACGCCTGGAGTCCAATAACTAC-3′) generated a 107-base pair fragment. Standard PCR was first performed with these primers using single-strand cDNA from the sample as a template. Gel electrophoresis confirmed that each PCR product comprised a single band of the correct size, which was excised and sequenced to confirm its identity. The purified products were quantitated spectrophotometrically for use as standards for subsequent real-time PCR assays, which were performed with these primers [2, 3].

Immunohistochemical Assays

The Sca-1, CD45, and CD34 were localized in cultured BMC by immunohistochemical double staining with a laser confocal microscopy system (Bio-Rad, Richmond, California), according to the manufacturer's protocols (Molecular Probes, Eugene, Oregon) [2, 3]. Slides were incubated with phycoerythrin-labeled anti-Sca-1 antibodies (1:100), Alexa Fluor 488-labeled anti-CD45 antibodies (1:100), and Cy5-labeled anti-CD34 antibodies (1:100; all Molecular Probes) overnight at 4°C.

Quantitative Histology

Myocardial specimens were fixed in formalin, embedded in paraffin, and sectioned into 6-μm-thick slices. Samples were stained with hematoxylin and eosin for morpho-

Table 1. Summary of Statistical Analysis of VEGF

	Mean			Standard Deviation		
Group	Normal	Border	Scar	Normal	Border	Scar
BMC+VEGF+bFGF	2.40E + 11	4.80E + 11	4.37E + 11	1.23E + 11	1.89E + 11	1.30E + 11
BMC+VEGF	1.73E + 11	5.34E + 11	4.70E + 11	6.47E + 10	1.41E + 11	1.72E + 11
BMC+bFGF	1.59E + 11	3.37E + 11	2.48E + 11	1.10E + 11	1.36E + 11	7.50E + 10
BMC	1.94E + 11	2.29E + 11	2.35E + 11	6.19E + 10	1.39E + 11	7.13E + 10
Control	2.20E + 11	2.00E + 11	1.80E + 11	8.46E + 10	6.50E + 10	6.66E + 10

Summary of ANOVA	*F* Value	*p* Value
Group	5.77	0.0014
Zone	7.55	0.0022
Interaction	1.59	0.1707

ANOVA = analysis of variance; BMC = bone marrow cells; BMC+bFGF = bone marrow cells transfected with basic fibroblast growth factor; BMC+VEGF = bone marrow cells transfected with vascular endothelial growth factor; BMC+VEGF+bFGF = bone marrow cells transfected with vascular endothelial growth factor and basic fibroblast growth factor.

Table 2. Summary of Statistical Analysis of bFGF

	Mean			Standard Deviation		
Group	Normal	Border	Scar	Normal	Border	Scar
BMC+VEGF+bFGF	1.89E + 11	3.97E + 11	5.62E + 11	5.19E + 10	1.63E + 11	1.62E + 11
BMC+VEGF	1.66E + 11	3.44E + 11	1.68E + 11	3.52E + 10	4.29E + 10	9.60E + 10
BMC+bFGF	1.72E + 11	3.95E + 11	5.55E + 11	6.98E + 10	1.30E + 11	1.59E + 11
BMC	1.31E + 11	2.16E + 11	2.31E + 11	3.21E + 10	2.20E + 10	4.53E + 10
Control	1.34E + 11	1.24E + 11	1.15E + 11	5.26E + 10	1.89E + 10	1.42E + 10
Summary of ANOVA						
	F Value	*p* Value				
Group	14.45	< 0.0001				
Zone	14.73	< 0.0001				
Interaction	4.21	0.0018				

ANOVA = analysis of variance; BMC = bone marrow cells; BMC+bFGF = bone marrow cells transfected with basic fibroblast growth factor; BMC+VEGF = bone marrow cells transfected with vascular endothelial growth factor; BMC+VEGF+bFGF = bone marrow cells transfected with vascular endothelial growth factor and basic fibroblast growth factor.

logic evaluation of the scar and transplanted cells (Sigma Diagnostics, St. Louis, Missouri) or immunohistochemical staining with antibodies against factor VIII, to facilitate quantitation of vascular density. The number of vessels per medium power field (×400) in the scar, border zone, or normal myocardium was counted by two

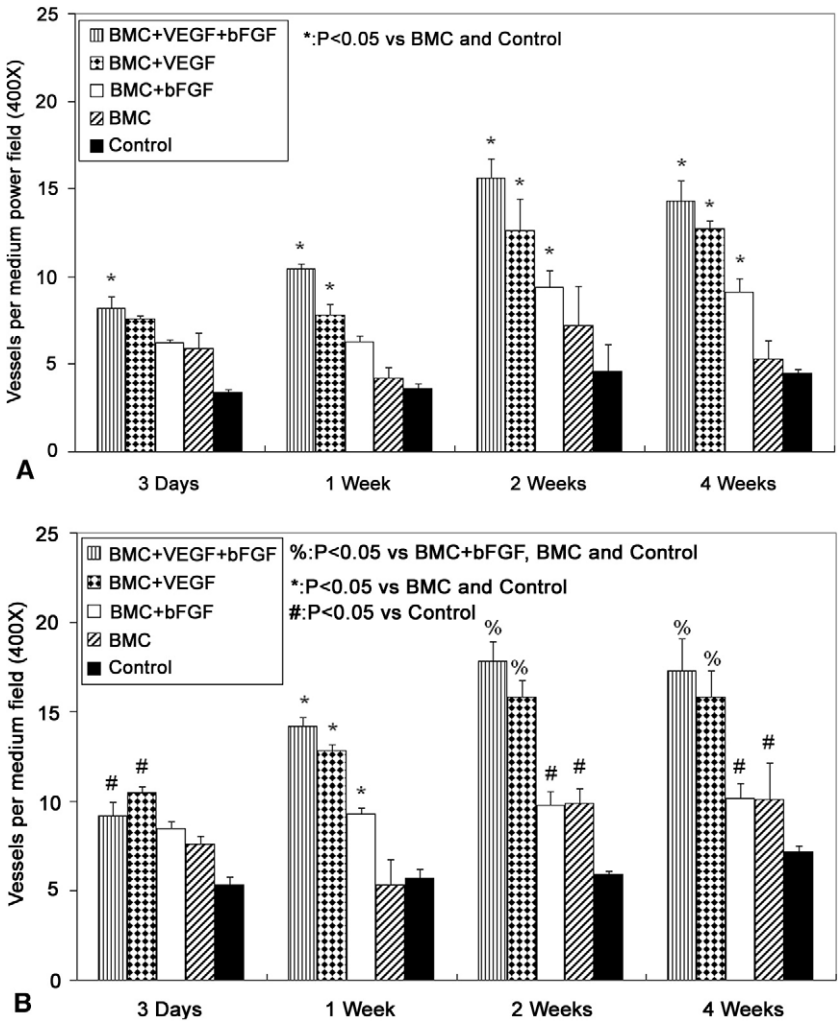

Fig 4. Vascular densities in the border zone and scar after bone marrow cells (BMC) transplantation. At all timepoints, vascular densities in the scar and border zone were lowest in control rats. (A) In the scar, vascular densities were slightly but nonsignificantly greater in rats transplanted with unmodified BMC (hatched bars), progressively greater in BMC+bFGF hearts (open bars), BMC+VEGF hearts (diamond bars), and greatest in BMC+VEGF+bFGF hearts (striped bars; $p < 0.05$). (B) In the border zone, unmodified BMC induced greater vascular densities than control rats at 2 and 4 weeks ($p < 0.05$), and BMC+bFGF had greater vascular densities than controls from 1 to 4 weeks ($p < 0.05$). The greatest vascular densities in the border zone were observed in the BMC+VEGF and BMC+VEGF+bFGF groups, from 1 to 4 weeks after cell implantation ($p < 0.05$). (bFGF = basic fibroblast growth factor; VEGF = vascular endothelial growth factor.)

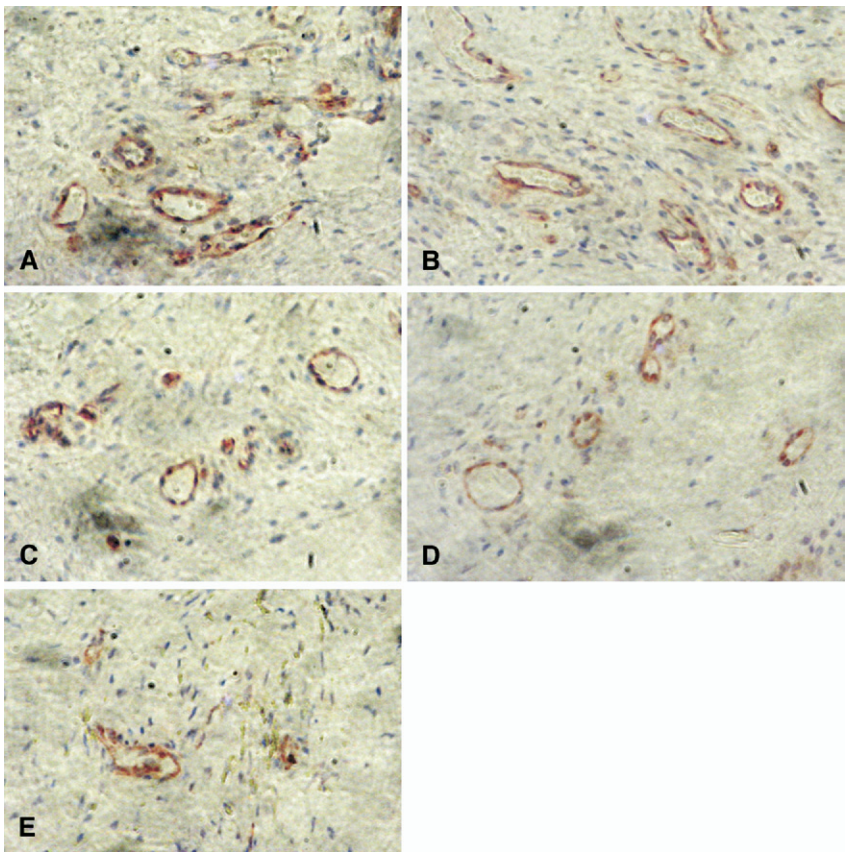

Fig 5. (A–E) Vascular density photomicrographs of the scar region in rat hearts 2 weeks after transplantation with (A) BMC+VEGF+bFGF, (B) BMC+VEGF, (C) BMC+bFGF, (D) BMC, or (E) control, after immunohistochemical staining for factor VIII. (Original magnification, ×400.) (bFGF = basic fibroblast growth factor; BMC = bone marrow cells; VEGF = vascular endothelial growth factor.)

masked observers in five fields per slide, and the mean number of vessels per field subjected to analysis.

Microsphere Analysis of Regional Blood Flow

Regional myocardial perfusion was evaluated by FluoSpheres polystyrene microspheres in 3 rats per group, 4 weeks after cell transplantation (n = 3 per group × 5 groups × 1 timepoint = 15). Under general anesthesia, the heart and great vessels were exposed through a median sternotomy. The ascending aorta was ligated and the right atrium was incised. One milliliter of a suspension containing 3.6×10^6 10 μm yellow-green fluorescent microspheres (Molecular Probes) were injected into the ascending aorta over 60 seconds. The heart was then

Table 3. Summary of Statistical Analysis of Vascular Density

	Border Mean ± SD				Scar Mean ± SD			
Group	3 Days	1 Week	2 Weeks	4 Weeks	3 Days	1 Week	2 Weeks	4 Weeks
BMC+VEGF+bFGF	9.2 ± 0.8	14.2 ± 0.5	17.8 ± 1.1	17.3 ± 1.8	8.2 ± 0.6	10.5 ± 0.2	15.6 ± 1.0	14.3 ± 1.1
BMC+VEGF	10.5 ± 0.3	12.8 ± 0.4	15.8 ± 1.0	15.8 ± 1.5	7.6 ± 0.2	7.8 ± 0.6	12.6 ± 1.8	12.7 ± 0.5
BMC+bFGF	8.5 ± 0.4	9.3 ± 0.3	9.8 ± 0.8	10.2 ± 0.8	6.2 ± 0.2	6.3 ± 0.3	9.4 ± 0.9	9.1 ± 0.8
BMC	7.6 ± 0.5	5.3 ± 1.4	9.9 ± 0.8	10.1 ± 2.0	5.9 ± 0.9	4.2 ± 0.6	7.2 ± 2.3	5.3 ± 1.0
Control	5.3 ± 0.5	5.7 ± 0.5	5.9 ± 0.2	7.2 ± 0.3	3.4 ± 0.2	3.6 ± 0.3	4.6 ± 1.5	4.5 ± 0.2

Summary of ANOVA

	Border Zone			Scar	
	F Value	*p* Value		*F* Value	*p* Value
Group	180.40	< 0.0001	Group	79.87	< 0.0001
Interaction	9.04	< 0.0001	Interaction	5.88	0.0002
Time	59.76	< 0.0001	Time	62.53	< 0.0001

ANOVA = analysis of variance; BMC = bone marrow cells; BMC+bFGF = bone marrow cells transfected with basic fibroblast growth factor; BMC+VEGF = bone marrow cells transfected with vascular endothelial growth factor; BMC+VEGF+bFGF = bone marrow cells transfected with vascular endothelial growth factor and basic fibroblast growth factor.

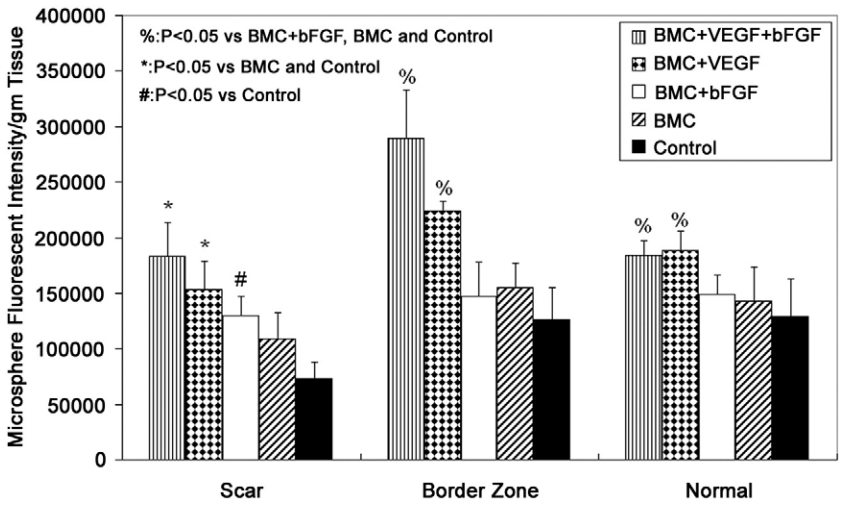

Fig 6. Regional perfusion 4 weeks after cell implantation, in the scar, border zone, and normal myocardium. Regional perfusion in the scar increased progressively from control rats (solid bars) to bone marrow cells (BMC) rats (hatched bars) to BMC+bFGF (open bars) to BMC+VEGF (diamond bars) to BMC+VEGF+bFGF (striped bars; $p < 0.05$). Regional perfusion in the border zone was generally higher than that in the scar, but this difference was most marked in the BMC+VEGF and BMC+VEGF+bFGF rats. Perfusion of the normal myocardium was slightly increased in the BMC+VEGF and BMC+VEGF+bFGF groups ($p < 0.05$). (bFGF = basic fibroblast growth factor; VEGF = vascular endothelial growth factor.)

excised, the atria and right ventricle removed, and the left ventricle was divided into scar, border zone, and normal regions. The weight of each specimen was recorded before digestion with 4 M potassium hydroxide (300 μL per 100 mg tissue) at room temperature for 24 hours. After complete digestion of the myocardial specimens, the microspheres were extracted and fluorescent intensities measured in a CytoFluor Multi-Well Plate Reader Series 4000 (PerSeptive Biosystems, Stafford, Texas).

Left Ventricular Function

Left ventricular function was evaluated in vivo in rats by transthoracic echocardiography (Siemens ACUSON Sequoia C256, Malvern, PA), as described by Sahn and colleagues [9] and Schiller and associates [10].

Statistical Analysis

Data are presented as mean ± SD. Categorical data were subjected to χ^2 analyses, and continuous data to analysis of variance (ANOVA). When the ANOVA F value was significant, Duncan's multiple range test was employed to specify differences.

Results

Bone Marrow Cell Characterization

We have characterized a subpopulation of BMC, approximately 10%, by immunohistochemistry and laser confocal microscopy to express Sca-1, but not CD45 or CD34. This cell population was characterized as mesenchymal cells. An additional subgroup of approximately 20% of the BMC expressed both Sca-1 and CD45, while the majority of BMC did not express Sca-1, CD34, or CD45 (Fig 1) [11]. No further characterization of the BMC was performed.

VEGF and bFGF Transgene Expression

Both VEGF and bFGF were expressed transiently by BMC+VEGF, BMC+bFGF, and BMC+VEGF+bFGF, within the scar and border zone, with levels peaking at 1

Table 4. Summary of Statistical Analysis of Regional Perfusion

	Mean			Standard Deviation		
Group	Normal	Border	Scar	Normal	Border	Scar
BMC+VEGF+bFGF	184302.2	289027.6	182873.9	12974.3	43558.1	30896.2
BMC+VEGF	187996.3	224213.7	153465.4	17551.5	8042.7	25547.3
BMC+bFGF	149166.4	147020.2	129962.4	17610.9	30917.9	16982.6
BMC	142539.7	154845.8	108543.7	31296.9	22413.9	23870.5
Control	128730.1	126351.9	72968.4	34426.3	28765.4	14697.6
Summary of ANOVA						
	F Value	*p* Value				
Group	26.49	< 0.0001				
Zone	19.64	< 0.0001				
Interaction	2.65	0.0251				

ANOVA = analysis of variance; BMC = bone marrow cells; BMC+bFGF = bone marrow cells transfected with basic fibroblast growth factor; BMC+VEGF = bone marrow cells transfected with vascular endothelial growth factor; BMC+VEGF+bFGF = bone marrow cells transfected with vascular endothelial growth factor and basic fibroblast growth factor.

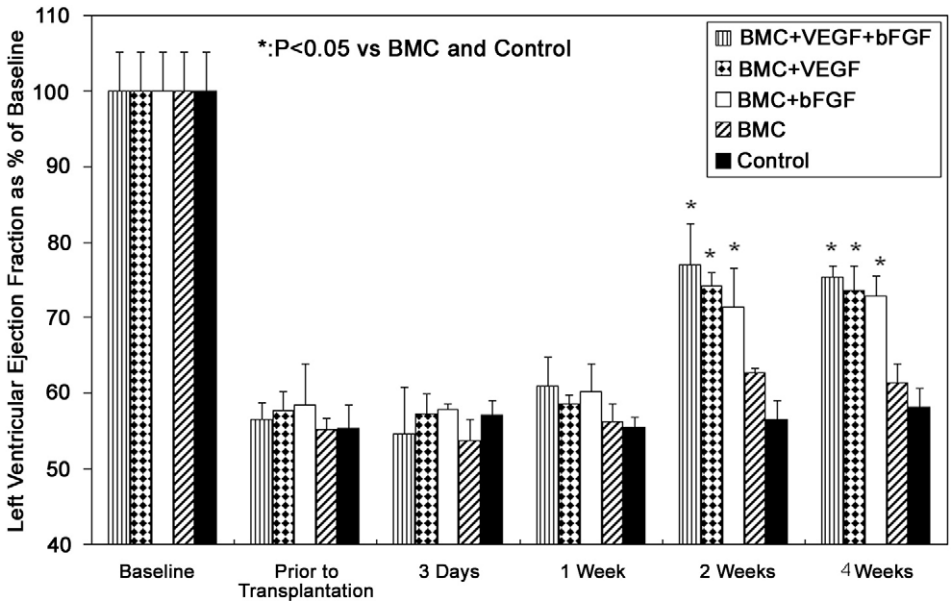

Fig 7. Left ventricular ejection fraction (LVEF) before infarction, before transplantation, and 3 days and 1, 2 and 4 weeks after transplantation. Two and 4 weeks after transplantation, LVEF was greater in BMC+VEGF+bFGF hearts (striped bars), BMC+bFGF hearts (open bars), and BMC+VEGF hearts (diamond bars) than in BMC rats (hatched bars) or control rats (solid bars). (bFGF = basic fibroblast growth factor; BMC = bone marrow cells; VEGF = vascular endothelial growth factor.)

week and declining to baseline by 4 weeks after cell transplantation. One week after transplantation, VEGF mRNA levels in the scar and border zone of the infarcted hearts were greatest in the BMC+VEGF and BMC+VEGF+bFGF groups ($p < 0.05$ versus BMC and control; Fig 2). One week after transplantation, bFGF mRNA levels in the scar were greatest in the BMC+bFGF and BMC+VEGF+bFGF groups ($p < 0.05$ versus BMC and control; Fig 3). Levels of VEGF and bFGF mRNA did not differ between the unmodified BMC and control groups. Levels of VEGF and bFGF mRNA in the normal myocardium were also similar in all groups (Tables 1 and 2).

Quantitative Vascular Densities

Vascular densities in both the scar (Fig 4A) and border zone (Fig 4B) were lowest in control rat hearts injected with culture medium alone, intermediate in BMC and BMC+bFGF rats, and greatest in BMC+VEGF and BMC+VEGF+bFGF ($p < 0.05$; Figs 5A to 5E). Vascular densities increased from 3 days to 2 weeks, and then plateaued. Densities at 4 weeks did not differ from those at 2 weeks (Table 3).

Regional Myocardial Perfusion

Four weeks after cell transplantation, regional perfusion in the scar was lowest in controls and increased from BMC to BMC+bFGF to BMC+VEGF, with the greatest perfusion in BMC+VEGF+bFGF (Fig 6). Perfusion in the border zone was similar between the control, BMC, and BMC+bFGF groups, but was greater in the BMC+VEGF group and greatest in the BMC+VEGF+bFGF rats ($p < 0.05$). In the normal myocardium, there was a slight but significant increase in perfusion in the BMC+VEGF and

Table 5. Summary of Statistical Analysis of Left Ventricular Ejection Fraction

	Mean ± SD					
Group	Baseline	Before Transplantation	3 Days	1 Week	2 Weeks	4 Weeks
BMC+VEGF+bFGF	100.0 ± 5.2	56.4 ± 2.3	54.6 ± 6.1	60.9 ± 3.8	77.0 ± 5.5	75.4 ± 1.5
BMC+VEGF	100.0 ± 5.2	57.7 ± 2.4	57.2 ± 2.6	58.6 ± 1.2	74.2 ± 1.8	73.6 ± 3.2
BMC+bFGF	100.0 ± 5.2	58.4 ± 5.5	57.8 ± 0.8	60.1 ± 3.7	71.4 ± 5.2	72.9 ± 2.6
BMC	100.0 ± 5.2	55.2 ± 1.4	53.7 ± 2.7	56.2 ± 2.3	62.7 ± 0.5	61.4 ± 2.4
Control	100.0 ± 5.2	55.4 ± 3.0	57.1 ± 1.9	55.5 ± 1.3	56.5 ± 2.6	58.1 ± 2.4
Summary of ANOVA						
	F Value	*p* Value				
Group	31.74	< 0.0001				
Interaction	7.59	< 0.0001				
Time	662.50	< 0.0001				

ANOVA = analysis of variance; BMC = bone marrow cells; BMC+bFGF = bone marrow cells transfected with basic fibroblast growth factor; BMC+VEGF = bone marrow cells transfected with vascular endothelial growth factor; BMC+VEGF+bFGF = bone marrow cells transfected with vascular endothelial growth factor and basic fibroblast growth factor.

BMC+VEGF+bFGF hearts compared with unmodified BMC (Table 4).

Left Ventricular Function

Myocardial infarction by left anterior descending artery ligation resulted in significant depression of left ventricular ejection fraction (LVEF), to approximately 55% to 60% of baseline (Fig 7). The LVEF did not decline further, nor recover, in any of the groups, 3 days or 1 week after transplantation. At 2 and 4 weeks, LVEF was greater in hearts transplanted with unmodified BMC than in controls, but this difference did not reach statistical significance. Rats transplanted with BMC+VEGF, BMC+bFGF, or BMC+VEGF+bFGF all had significantly greater LVEF, both 2 and 4 weeks after implantation, than did the BMC or control groups ($p < 0.05$). At both 2 and 4 weeks, LVEF was greatest in the BMC+VEGF+bFGF rats, although the differences relative to BMC+VEGF and BMC+bFGF did not reach statistical significance (Table 5). Other echocardiographic variables, including fractional area shortening and fractional shortening, showed similar changes over time and differences between groups (data not shown).

Comment

The ultimate goal of cell transplantation strategies is to normalize myocardial function, perfusion, and geometry after infarction. Although the myogenic and angiogenic effects of transplantation of a host of varying cell types have been widely reported, and result from cell engraftment and perhaps the secretion of as-yet-unidentified adaptive cytokines in response to the local milieu, normalization of ventricular perfusion or function has not been achieved. We initially hypothesized that the angiogenic effect of cell transplantation could be enhanced by transient expression of a proangiogenic VEGF transgene. We have observed that VEGF-overexpressing cells induced greater vascular densities and regional perfusion than unmodified cells [1]. However, because long-term expression of VEGF may result in angioma formation [12, 13], only transient expression is desired. We noted that expression of the VEGF transgene after transplantation of a mixed culture of heart cells (predominantly cardiomyocytes, with smaller proportions of smooth muscle cells, endothelial cells, and fibroblasts), or skeletal myoblasts, was limited spatially to the scar and the border zone surrounding the scar [2]. Expression was also limited temporally to a 4-week period [2]. While the complete sequence of mechanisms by which this enhanced angiogenesis is effected is unknown, an initial response appears to be the sequential upregulation of the VEGF receptors fetal liver kinase-1 and fms-like tyrosine kinase-1 in surrounding host cells through a paracrine effect [3].

The enhanced angiogenesis induced by transplantation of VEGF-transfected cells was not, however, sufficient to normalize either perfusion or function [1]. Because angiogenesis is a complex process involving multiple cytokines, we hypothesized that augmentation of the angiogenic stimulus, through the well-described synergism of VEGF and bFGF [5–8], could further increase both regional perfusion and function. We utilized a model of myocardial infarction in syngeneic Lewis rats to eliminate the issue of rejection of the transplanted cells [2, 3]. Because previous experiments have demonstrated that transplanted BMC can improve postinfarction LV function [14], and that BMC may have greater plasticity in myocardial repair than heart cells [15], we utilized BMC as our transgene delivery vehicles.

As we had previously observed with a VEGF transgene alone, the expression of the VEGF and bFGF transgenes after transplantation of the BMC was limited spatially to the scar and border zone, and temporally to a 4-week period. Expression of VEGF in the BMC+VEGF group induced a moderate increase in bFGF expression in the border zone of those hearts, and similarly, a moderate increase in VEGF expression was noted in the border zone of the BMC+bFGF hearts. The VEGF and bFGF mRNA levels did not differ between any of the groups in the normal myocardium. We have previously noted that transplantation of unmodified heart cells or skeletal myoblasts results in a moderate but appreciable increase in VEGF expression compared with controls receiving only an injection of culture medium [2]. In contrast, transplantation of unmodified BMC in this study did not result in increased VEGF expression. There was a small and nonsignificant increase in bFGF expression in the scar and border zone of BMC rats.

Transgene expression of VEGF and bFGF peaked at 1 week, before the peak in vascular densities, which was observed 2 weeks after transplantation. Vascular densities were stable from 2 weeks to 4 weeks, the last timepoint in our study, when VEGF and bFGF expression had declined to baseline levels. In other studies, we have noted that angiogenesis in response to transiently VEGF-overexpressing heart cells and skeletal myoblasts persists for at least 6 months, and does not result in angioma formation [16]. However, our current data do not allow us to comment on whether BMC expressing VEGF or bFGF, or both, elicit a similarly durable angiogenic response.

The synergistic effect of VEGF and bFGF on vascular densities was most pronounced in the scar region, where a progressive increase from control to unmodified BMC to bFGF, VEGF, and VEGF and bFGF-expressing BMC was noted. The number of copies of bFGF mRNA in the groups transfected with this plasmid was roughly equivalent to the number of copies of VEGF mRNA in VEGF-transduced groups, but similar levels of expression of bFGF appeared to induce less of an increase in vascular densities than that of VEGF.

In the control rats, regional perfusion was lower in the scar than in the border zone and normal myocardium, as would be anticipated. A similar pattern was noted in the BMC group. In contrast, myocardial perfusion was greater in the border zone of the BMC+VEGF and BMC+VEGF+bFGF hearts than in either the scar or the normal myocardium. Vascular densities followed a similar pattern. That may reflect the localization of new vessel formation, developing from the border zone and

extending toward the scar, where the angiogenic stimulus was centered.

Two and 4 weeks after cell transplantation, rats transplanted with unmodified BMC had greater LVEF than controls, consistent with the findings of Tomita and coworkers [14] at 8 weeks. However, because of the small number of rats in each group, these differences did not reach statistical significance. The addition of a VEGF or bFGF transgene, or both, resulted in significantly greater LVEF at both 2 and 4 weeks than the BMC or control groups. The LVEF was stable from 2 weeks to 4 weeks, but our study did not include later timepoints to determine whether the functional benefit of cell-based VEGF and bFGF gene transfer was durable beyond this point.

We did not evaluate the effect of the VEGF and bFGF transgenes on the survival of the transplanted BMC in this study. However, in previous studies, we have noted that expression of a VEGF transgene reduced apoptosis and increased the survival of transplanted heart cells and skeletal myoblasts at 1 week from approximately 30% to 50% [17]. We also noted that the simultaneous expression of both VEGF and insulin-like growth factor-1, or VEGF or insulin-like growth factor-1 alone in transplanted BMC had a synergistic effect on myogenesis, enhancing transplanted cell survival and reducing apoptosis [11]. We would anticipate, therefore, that BMC survival was increased in our current study in at least the BMC+VEGF+bFGF and BMC+VEGF groups, but cannot comment on the effect of bFGF alone.

While these types of multimodal cell-based gene therapeutic strategies may significantly enhance the benefit of cell transplantation in hearts with ischemic cardiomyopathy, the addition of complementary transgenes, cell types, and other reparative strategies may still be required to achieve complete myocardial repair after infarction. One such potential intervention is angiogenic pretreatment of the scar before cell implantation. Yamamoto and colleagues [18] have reported that prevascularization of a rat myocardial infarction by slow-release bFGF protein resulted in greater maximal elastance than either bFGF or cell transplantation alone. Sakakibara and associates [19] also noted greater cell survival with prevascularization than without. Sato and colleagues [20, 21] reported that intracoronary bFGF delivery results in significant improvement in collateralization and regional and global function in infarcted porcine hearts and also that intracoronary $VEGF_{165}$ infusion improved collateralization and increased myocardial blood flow in the same model. Retuerto and associates [22] injected infarcted rat hearts with an adenoviral vector carrying $VEGF_{121}$ before fetal cardiomyocyte transplantation, and noted greater exercise capacity and an increased cell survival index compared with pretreatment with empty vectors or saline. Scar pretreatment may thus extend the efficacy of cell-based gene therapeutic approaches to myocardial repair.

The results of our study must be tempered by the lack of statistical significance of some trends that we observed. Despite using a total of 109 rats in this experiment (75 recipients, 25 donors, and 9 replacements for rats that died after infarct creation), the division of these rats into five groups and four timepoints for evaluation resulted in only a small number of rats in each combination of group and timepoint. Thus, while vascular densities and regional perfusion in the border zone were significantly greater in the BMC+VEGF+bFGF rats than in the BMC+bFGF group, differences between the BMC+VEGF+bFGF and BMC+VEGF groups did not reach statistical significance, nor did those in the scar itself. However, our data were internally consistent, with trends favoring the BMC+VEGF+bFGF group over both the BMC+VEGF and BMC+bFGF rats at all timepoints for all variables studied. Although a greater number of rats would have been desirable, it seems reasonable to conclude, in light of the known biology of VEGF and bFGF, that these two transgenes acted synergistically in the current study.

In conclusion, our data demonstrate that VEGF and bFGF transgenes are transiently expressed by transplanted BMC, resulting in significantly increased vascular densities and regional perfusion in the scar and border zone of an infarcted heart, compared with unmodified BMC or controls. There was a powerful synergistic effect of VEGF and bFGF on this angiogenic response. In addition, LVEF was significantly increased by expression of VEGF or bFGF, compared with unmodified BMC or controls. Although the synergism between VEGF and bFGF significantly increased the angiogenic effect of BMC transplantation, it was still insufficient to normalize myocardial perfusion or function.

This work was supported by a grant from the Heart and Stroke Foundation of Ontario (T5305) to Terrence M. Yau. Doctor Yau is also supported by the Angelo and Lorenza DeGasperis Chair in Cardiovascular Surgery Research.

References

1. Yau TM, Fung K, Weisel RD, et al. Enhanced myocardial angiogenesis by gene transfer with transplanted cells. Circulation 2001;104(Suppl 1):218–22.
2. 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.
3. Yau TM, Li G, Zhang Y, Weisel RD, Mickle DA, Li RK. Vascular endothelial growth factor receptor upregulation in response to cell-based angiogenic gene therapy. Ann Thorac Surg 2005;79:2056–63.
4. Liu TB, Fedak PW, Weisel RD, et al. Enhanced IGF-1 expression improves smooth muscle cell engraftment after cell transplantation. Am J Physiol Heart Circ Physiol 2004; 287:H2840–9.
5. Starvri GT, Zachary IC, Baskerville PA, Martin JF, Erusalimsky JD. Basic fibroblast growth factor upregulates the expression of vascular endothelial growth factor in vascular smooth muscle cells. Synergistic interaction with hypoxia. Circulation 1995;1:11–4.
6. Mandriota S, Pepper M. Vascular endothelial growth factor-induced in vitro angiogenesis and plasminogen activator

expression are dependent on endogenous basic fibroblast growth factor. J Cell Sci 1997;18:2293–302.
7. Goto F, Goto K, Weindel K, Folkman J. Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels. Lab Invest 1993;5:508–17.
8. Couper LL, Bryant SR, Eldrup-Jorgensen J, Bredenberg CE, Lindner V. Vascular endothelial growth factor increases the mitogenic response to fibroblast growth factor-2 in vascular smooth muscle cells in vivo via expression of fms-like tyrosine kinase-1. Circ Res 1997;6:932–9.
9. Sahn DJ, DeMaria A, Kisslo J, Weyman A. Recommendations regarding quantitation in M-mode echocardiography: results of a survey of echocardiographic measurements. Circulation 1978;58:1072–83.
10. Schiller NB, Shah PM, Crawford M, et al. Recommendations for quantitation of the left ventricle by two-dimensional echocardiography. J Am Soc Echocardiogr 1989;2:358–67.
11. Yau TM, Kim C, Li G, Zhang Y, Weisel RD, Li RK. Maximizing ventricular function with multimodal cell-based gene therapy. Circulation 2005;112(Suppl 1):123–8.
12. Lee RJ, Springer ML, Blanco-Bose WE, et al. VEGF gene delivery to myocardium: deleterious effects of unregulated expression. Circulation 2000;22:898–901.
13. Arbiser JL, Larsson H, Claesson-Welsh L, et al. Overexpression of VEGF 121 in immortalized endothelial cells causes conversion to slowly growing angiosarcoma and high level expression of the VEGF receptors VEGFR-1 and VEGFR-2 in vivo. Am J Pathol 2000;156:1469–76.
14. Tomita S, Li RK, Weisel RD, et al. Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 1999;100(Suppl 19):II247–56.
15. 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.
16. Kim C, Li RK, Li G, Zhang Y, Weisel RD, Yau TM. Effects of cell-based angiogenic gene therapy at 6 months: persistent angiogenesis and absence of oncogenicity. Ann Thorac Surg 2007;83:640–6.
17. Yau TM, Kim C, Ng D, et al. Increasing transplanted cell survival with cell-based angiogenic gene therapy. Ann Thorac Surg 2005;80:1779–86.
18. 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.
19. 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.
20. Laham RJ, Laham K, Pearlman JD, et al. Efficacy of intracoronary versus intravenous FGF-2 in a pig model of chronic myocardial ischemia. Ann Thorac Surg 2000;70:2113–8.
21. Sato K, Wu T, Laham RJ, et al. Efficacy of intracoronary or intravenous VEGF165 in a pig model of chronic myocardial ischemia. J Am Coll Cardiol 2001;37:616–23.
22. Retuerto MA, Schalch P, Patejunas G, et al. Angiogenic pretreatment improves the efficacy of cellular cardiomyoplasty performed with fetal cardiomyocyte implantation. J Thorac Cardiovasc Surg 2004;127:1041–9.

INVITED COMMENTARY

Yau and colleagues [1] report that the combination of bone marrow cell (BMC) transplantation with vascular endothelial growth factor/basic fibroblast growth factor (VEGF/bFGF) transgenes yields a synergistic effect on angiogeneisis in a rodent model of myocardial infarction. The authors found that mRNA of VEGF/bFGF was transiently expressed in BMC transfected with VEGF/bFGF transgenes, which leads to enhanced vascular density and improved regional perfusion and left ventricular contractile function. These novel findings suggest that multimodal cell-based gene therapy may be more useful in myocardial repair than unmodified cell-based therapy.

This is a well-designed and executed experimental study from a group that has previously published a number of elegant works in this exciting field of cell-based and gene-based cardiac repair. The results of the present study appear quite promising; however these results must be interpreted with caution because of the very small sample size. The authors performed a careful statistical analysis of these relatively small study groups. At 4 weeks, 6 rats were used and this number was sufficient to confirm their major findings. This is one of the first studies to investigate potentially synergistic effects of the combination of therapeutic VEGF/bFGF gene delivery with cell transplantation on angiogensis in an experimental model of the infarcted rat heart.

There is a growing body of evidence supporting the hypothesis that the release of paracrine growth factors and other potentially unknown cytokines from stem cells can promote neoangiogenesis [2]. The VEGF and bFGF are well-known angiogenic growth factors, and certainly the combination of the two may help to maximize the proliferation and differentiation of transplanted BMC and endogenous stem cells [3]. The combination of the two growth factors may synergistically protect the ischemic myocardium through similar or different molecular pathways. Although cell survival and VEGF/bFGF-protein expression were not evaluated, based on their previous findings, one speculates that the combination of the two factors may have an additive effect on improving cell survival and VEGF/bFGF-protein expression.

If gene or cell therapy is still in its infancy, multimodel cell-based gene therapy is just a newborn. Future studies should focus, in addition to other things, on developing less immunogenic and toxic vectors; controlling the level of transgene expression within transfected cells; optimizing the differentiation and proliferation of the stem cells; and regulating stem cell delivery, survival, and tissue integration. Another challenge will be to choose the appropriate applications that will allow a single stage, simple, and effective therapeutic approach with minimal potential for side effects [4, 5]. Thus more experimental and pre-clinical

0003-4975/07/$32.00
doi:10.1016/j.athoracsur.2006.11.053