

Maximizing Ventricular Function With Multimodal Cell-Based Gene Therapy

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Background—Angiogenesis is enhanced after transplantation of vascular endothelial growth factor (VEGF)-expressing cells into a myocardial scar. Insulin-like growth factor I (IGF-I) may induce hypertrophy and inhibit apoptosis. We evaluated the effect of cell-based IGF-I and VEGF multigene therapy on left ventricular (LV) function, cell survival, and apoptosis after bone marrow cell (BMC) transplantation.

Methods and Results—Female Lewis rats underwent left anterior descending ligation 3 weeks before transplantation with male donor BMC, BMC transfected with VEGF (BMC+VEGF), IGF-I (BMC+IGF-I), VEGF and IGF-I (BMC+VEGF+IGF-I), or medium without cells (control) (n=4 per group×5 groups×4 time points). Three days and 1, 2, and 4 weeks after transplantation, VEGF and IGF-I expression was quantitated by real-time polymerase chain reaction, cell survival by polymerase chain reaction for *sry2*, apoptosis by TUNEL staining, LV function by echocardiography and myosin heavy chain, and light chain and troponin I by Western blot. One week after transplantation, IGF-I expression in the scar and border zone was greatest in BMC+IGF-I and BMC+VEGF+IGF-I rats ($P<0.05$). VEGF expression in the scar and border zone was greatest in BMC+VEGF and BMC+VEGF+IGF-I hearts ($P<0.05$). Transplanted cell survival was lowest in BMC, intermediate in BMC+VEGF and BMC+IGF-I, and greatest in BMC+VEGF+IGF-I ($P<0.05$). Apoptotic indices were significantly reduced in BMC+VEGF+IGF-I, BMC+VEGF, and BMC+IGF-I ($P<0.05$). Two and 4 weeks after transplantation, LV ejection fraction was lowest in control, intermediate in BMC, BMC+VEGF, and BMC+IGF-I, and greatest in BMC+VEGF+IGF-I ($P<0.05$).

Conclusions—Transplantation of VEGF- and IGF-I-expressing BMC reduced apoptosis, maximized transplanted cell survival, and enhanced LV function. Multimodal cell-based gene therapy may maximize the benefits of cell transplantation. (*Circulation*. 2005;112[suppl I]:I-123–I-128.)

Key Words: cells ■ gene therapy ■ growth substances ■ angiogenesis

We have reported previously that the angiogenic effect of cell transplantation can be enhanced by transplantation of cells transfected *ex vivo* with vascular endothelial growth factor (VEGF)₁₆₅.¹ Expression of VEGF in these transplanted cells is limited to the scar and border zone, and lasts ≈4 weeks,² while upregulating the VEGF receptors *flk-1* and *flt-1* through a paracrine effect on donor cells.³ Transplantation of smooth muscle cells expressing insulin-like growth factor I (IGF-I) 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, and angiogenesis. However, the optimal combination of transgenes and cells is unknown.

In this study, our objective was to define potential synergisms of myogenic and angiogenic enhancements to cell transplantation by cell-based gene therapy. Our specific hypothesis was that the combination of VEGF and IGF-I transgenes, expressed in transplanted bone marrow cells

(BMC), would combine VEGF-induced angiogenesis with IGF-I-induced myogenesis, increase transplanted cell survival, and optimally preserve left ventricular (LV) function.

Methods

Animals and Experimental Model

Donor and recipient rats were syngeneic adult Lewis rats (body weight 225 to 250 g for females and 250 to 300 g for males) (Charles River Canada Inc.). All of the 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 Academy Press. Myocardial infarctions were induced in female Lewis rats by ligation of the left anterior descending coronary artery.

BMC Isolation

Male donor BMCs were isolated from rat femurs and cultured in Iscove's modified Dulbecco's medium with 10% FBS for 7 to 10 days before harvesting (at 60% to 70% confluence). The cultures were partially depleted of erythroid progenitor cells by the removal

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DOI: 10.1161/CIRCULATIONAHA.104.525147

of nonadherent cells with each change of medium, every 3 days. No additional fractionation was performed.

Cell Transfection

Cells were transfected *ex vivo* with a plasmid encoding VEGF₁₆₅ (pCEP4-VEGF) and/or IGF-I (PUC18-IGF-I) 24 hours before transplantation.¹⁻⁴ Transfection efficiencies were monitored by cotransfection with pEGFP-N2 (BD Biosciences Clontech) encoding green fluorescence protein.

Cell Transplantation

Three weeks after the infarct creation, rats were randomly divided into 5 experimental groups and injected with culture medium alone (control) or transplanted with 3×10^6 untransfected BMCs, BMC transfected with VEGF (BMC+VEGF), BMC transfected with IGF-I (BMC+IGF-I), or BMC transfected with VEGF and IGF-I (BMC+VEGF+IGF-I)¹⁻⁴ ($n=4$ per group $\times 5$ groups $\times 4$ time points = 80). Rat hearts were excised 3 days or 1, 2, or 4 weeks after transplantation.¹⁻³ An additional 3 rats in each group were euthanized immediately after cell injection to quantitate the baseline cell retention. 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, powdered, and total RNA isolated with TRIzol RNA extraction reagents (Invitrogen Corp.). mRNA was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen Corp).^{2,3}

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

Specific polymerase chain reaction (PCR) primers were designed from *VEGF* and *IGF-I* GeneBank sequences for real-time PCR.^{2,3} VEGF primers (antisense, 5'-TCATGGTTGTCTATCAGCGCAG-3'; sense, 5'-GCACACAGGATGGCTTGAAGAT-3') generated a 107-bp fragment. IGF-I primers (antisense, 5'-CTCGGCATAGCTTTGCCAT-3'; sense, 5'-GGTGACCCTTGTCCAGTT-3') generated a 131-bp fragment. Standard PCR was 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 of VEGF and IGF-I mRNA.^{2,3}

Quantitation of Y Chromosomal DNA by Real-Time PCR

DNA was isolated from snap-frozen and powdered myocardial specimens, as well as heart cells from male Lewis rats (for use as standards) as described previously.⁴ To quantitate the number of male donor cells within the female recipient hearts, specific primers were designed based on the GeneBank sequences of the sex-determining region of the Y chromosome⁵ of the rat (sense 5'-GAGGCACAAGTTGGCTCAACA-3'; antisense 5'-CTCCTGCAAAAAGGGCCTT-3'). Quantitative PCR was performed and analyzed as described previously.⁴

TUNEL Assay

In situ TUNEL staining⁶ and propidium iodide nuclear counterstaining were performed with the APO-TUNEL assay kit (Molecular Probe Inc.). DNA strand breaks in apoptotic cells were identified by their yellow color (green plus red fluorescence), and all of the other propidium iodide-labeled nuclei were identified as red, detected at 488 and 536 nm by laser confocal microscopy (Bio-Rad Laboratories). Total cell nuclei and apoptotic nuclei were counted in 5 fields ($\times 400$) per slide by 2 blinded observers.

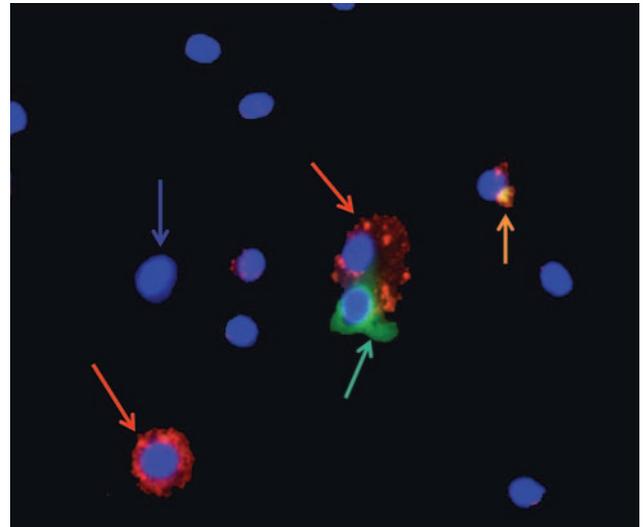


Figure 1. BMCs immunostained for Sca-1, CD45, and CD34 ($\times 1000$). Mesenchymal stem cells (red arrow, $\approx 10\%$ of cultured cells) were Sca-1 (red) positive, CD45 (green) negative, and CD34 negative. Sca-1- and CD45-positive cells appear brown (brown arrow). Nuclei were stained with 4',6-diamidino-2-phenylindole (blue).

Western Blot Analysis

Western blot analysis was performed⁷ with monoclonal primary antibodies against α -myosin heavy chain (MHC)(1:500), β -MHC (1:500), myosin light chain (1:1500), troponin I (1:1500; Biogenesis), and secondary goat antimouse IgG horseradish peroxidase conjugate (1:1000; Santa Cruz Biotech).

Immunohistochemical Assays

Myocardial specimens were fixed in formalin, embedded in paraffin, and sectioned into 6- μ m-thick slices. Sca-1, CD45, and CD34 were localized in cultured BMC by immunohistochemical double staining under laser confocal microscopy (Bio-Rad), according to the manufacturer's protocols (Molecular Probe Inc.).^{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).

LV Function

LV function was evaluated *in vivo* in rats by transthoracic echocardiography (Sequoia C256, Acuson), as described previously.^{8,9}

Statistical Analysis

Data are presented as mean \pm SD. Continuous data were analyzed by ANOVA. When the ANOVA F-value was significant, Duncan's multiple range test was used to specify the differences.

Results

BMC Characterization

Four to 7 days after harvesting, $\approx 10\%$ of the cultured BMCs were Sca-1+, CD45-, and CD34- (Figure 1), and were characterized as mesenchymal stem cells. Approximately 20% of BMCs expressed both Sca-1+ and CD45+ markers. The majority of the cells were Sca-1-, CD45-, and CD34-.

VEGF and IGF-I Transgene Expression

The expression of both VEGF and IGF-I peaked 1 week after cell implantation and declined to baseline by 4 weeks. One week after transplantation, VEGF mRNA levels in the scar and border zone were greatest in the BMC+VEGF and

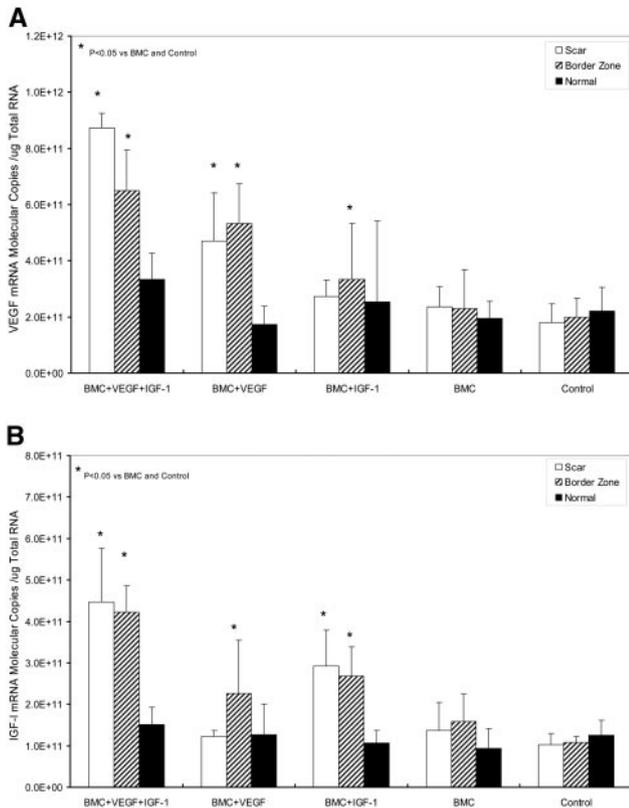


Figure 2. (a) At 1 week, VEGF expression in the scar and border zone was greatest in BMC+VEGF+IGF-I and BMC+VEGF hearts. (b) At 1 week, IGF-I expression in the scar and border zone was greatest in BMC+VEGF+IGF-I and BMC+IGF-I hearts.

BMC+VEGF+IGF-I hearts ($P < 0.05$ versus BMC and control), with the highest VEGF levels observed in the scar of the BMC+VEGF+IGF-I group (Figure 2a). One week after transplantation, the IGF-I mRNA levels in the scar and border zone were greatest in the BMC+VEGF+IGF-I and BMC+IGF-I hearts ($P < 0.05$ versus BMC and control) (Figure 2b). The levels of VEGF and IGF-I mRNA did not differ between the unmodified BMC and control groups.

Apoptosis

The apoptotic indices in control rats were low at all of the time points (Figure 3a and 3b). Unmodified BMC had high apoptotic indices in the scar and border zone 3 days and 1 week after implantation ($P < 0.05$ versus control). The apoptotic indices were significantly reduced in BMCs transfected with VEGF, IGF-I, or both VEGF and IGF-I ($P < 0.05$ versus BMC) at 3 days and 1 week. Two and 4 weeks after transplantation, the apoptotic indices were similar in all of the groups.

Cell Survival

The baseline cell retention after implantation ranged from 69% to 73% in the BMC-transplanted groups (Figure 4). Transplanted BMC survival declined progressively, but at 3 days and 1 and 2 weeks, BMCs expressing VEGF or IGF-I transgenes had greater survival than unmodified BMCs ($P < 0.05$). BMCs expressing both VEGF and IGF-I had an

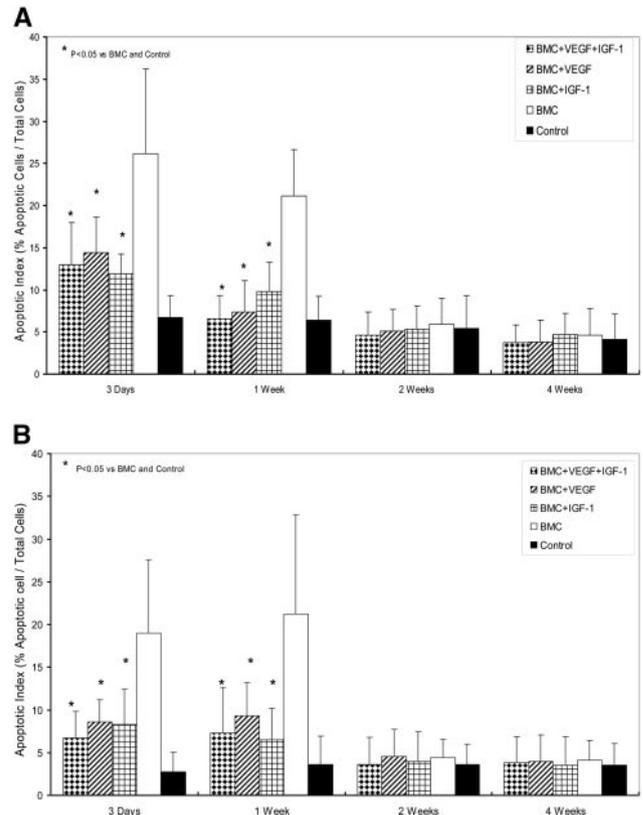


Figure 3. Apoptotic indices in the (a) scar and (b) border zone were low in the control but markedly higher in BMCs at 3 days and 1 week ($P < 0.05$). Apoptotic indices were significantly lower in BMC+VEGF+IGF-I, BMC+VEGF, and BMC+IGF-I hearts than in BMC rats ($P < 0.05$).

additional increase in cell survival at all of the time points, although this difference (relative to the BMC+VEGF and BMC+IGF-I groups) did not reach statistical significance. Four weeks after transplantation, only 16% to 23% of the original 3×10^6 cells, or 23% to 32% of the cells retained immediately after implantation, were detected in the BMC-transplanted hearts. Only BMC+VEGF+IGF-I had significantly greater survival than unmodified BMCs at 4 weeks.

LV Function

LV ejection fraction (EF) was reduced by left anterior descending ligation to 55% to 60% of baseline (Figure 5) and was stable at 3 days and 1 week. At 2 and 4 weeks, BMC had greater LVEF than controls, but this difference did not reach statistical significance, whereas rats transplanted with BMCs expressing VEGF, IGF-I, or both transgenes had significantly greater LVEF than the unmodified BMC or control groups ($P < 0.05$). At 4 weeks, LVEF was greater in BMC+VEGF+IGF-I rats than in BMC+IGF-I rats ($P < 0.05$). At 2 and 4 weeks, LVEF was greatest in the BMC+VEGF+IGF-I rats, although the differences relative to BMC+VEGF did not reach statistical significance. Other echocardiographic parameters, including fractional area shortening and fractional shortening, showed similar changes over time and differences between groups (data not shown).

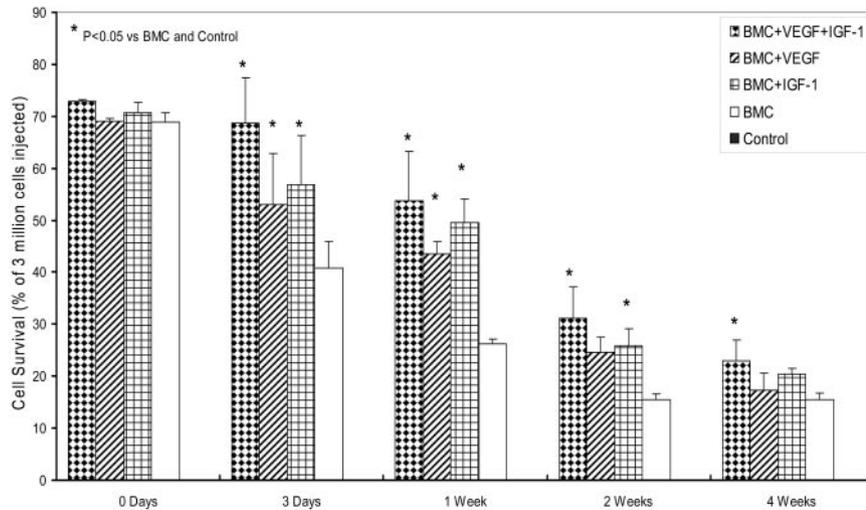


Figure 4. Transplanted donor cell survival was significantly greater in BMC+VEGF+IGF-I, BMC+VEGF, and BMC+IGF-I than unmodified BMCs ($P<0.05$). Survival was greatest in BMC+VEGF+IGF-I, although the differences relative to BMC+VEGF and BMC+IGF-I did not reach statistical significance.

α - and β -MHC, Myosin Light Chain, and Troponin I

At 2 weeks, α -MHC mRNA expression, evaluated by PCR, was lower in the scar than the border zone in all of the groups, but the expression in the scar was highest in the BMC+VEGF+IGF-I group ($P<0.05$). At 4 weeks, α -MHC content, evaluated by Western blotting, was greatest in the scar in the BMC+VEGF+IGF-I group ($P<0.05$ versus all of the other groups), and α -MHC in the border zone increased progressively from the control rats to BMC, BMC+IGF-I, and BMC+VEGF and was highest in BMC+VEGF+IGF-I ($P<0.05$ BMC and control versus other groups) (Figure 6). Myosin light chain, β -MHC, and troponin I showed similar changes (data not shown).

Discussion

Cell transplantation can induce myogenesis and angiogenesis and partially restore LV function. The extent of myogenesis and angiogenesis varies by the type of cell implanted, however, and normalization of LV function and morphology has not yet been achieved. The expression of selected transgenes in transplanted cells may augment myogenesis and angiogenesis and increase the efficacy of cell transplantation. Transplanted cells may express a host of adaptive cytokines

in response to their milieu, but overexpression of ≥ 1 transgenes may enhance their efficacy.

We have reported previously that the angiogenic response to cell transplantation can be augmented by implantation of VEGF-transfected cells.¹ Expression of VEGF, although transient and localized,² is sufficient to induce the sequential upregulation of the VEGF receptors flk-1 and flt-1 in host cells through a paracrine effect³ and leads to enhanced angiogenesis. In this series of experiments, we sought to augment the myogenic effect of cell transplantation by expression of IGF-I, which induces myocyte hypertrophy^{10,11} and inhibits apoptosis.^{12,13} We hypothesized that IGF-I would reduce apoptosis and enhance cell survival, induce hypertrophy of the transplanted cells, and lead to greater restoration of LV function. We also hypothesized that the effects of IGF-I might be synergistic with those of VEGF.

Because the transplantation of unfractionated BMCs results in improvement in ventricular function¹⁴ and BMCs may have greater plasticity after engraftment than myocytes,¹⁵ we used BMCs as carrier cells in these experiments. Although a subpopulation of BMC, for example, Sca-1+ cells, may have greater effects per cell on function or perfusion, this potential advantage may be offset by the smaller number of cells

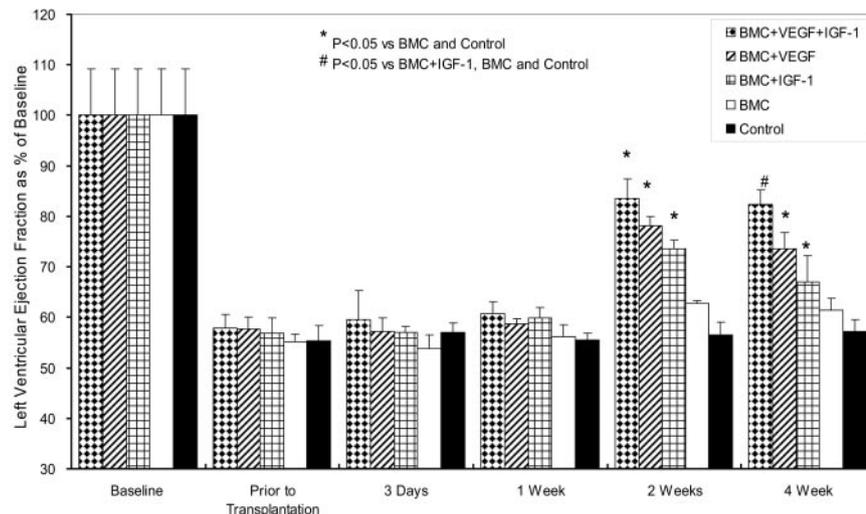


Figure 5. Two and 4 weeks after transplantation, LVEF was greater in BMC+VEGF+IGF-I, BMC+IGF-I, and BMC+VEGF hearts than in BMC or control rats ($P<0.05$). Four weeks after transplantation, LVEF was greater in BMC+VEGF+IGF-I hearts than in BMC+IGF-I rats ($P<0.05$).

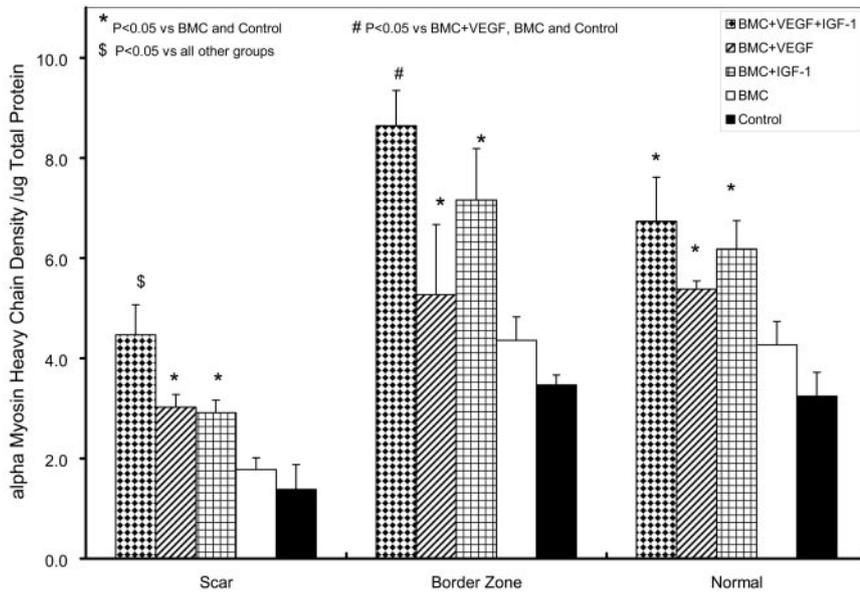


Figure 6. In both the scar and border zone, α -MHC content was greatest in BMC+VEGF+IGF-I, intermediate in BMC+VEGF and BMC+IGF-I, and lowest in BMC and control rats ($P < 0.05$).

available for transplantation, even after ex vivo expansion. We, therefore, elected only to deplete BMCs of hematopoietic precursors, without additional fractionation.

Expression of the VEGF and IGF-I transgenes was limited to the scar and border zone, over a period of 4 weeks, as we have observed previously with VEGF-expressing skeletal myoblasts or heart cells.² Although a greater duration of transgene expression might increase their effects on cell survival, apoptosis, and LV function, prolonged expression of a VEGF transgene has been reported previously to result in angioma formation,^{16,17} and, therefore, must be avoided. Similar precautions should apply to prolonged expression of IGF-I.

VEGF expression was greater in the BMC+VEGF+IGF-I group than the BMC+VEGF group, although this difference was not statistically significant. We have observed previously that transfection of smooth muscle cells with IGF-I also resulted in upregulation of VEGF expression.⁴ In this study, IGF-I appeared to have a synergistic effect on VEGF expression in the BMC+VEGF+IGF-I group. Our ongoing studies (unpublished data, 2004) have suggested that the expression of a VEGF transgene in skeletal myoblasts or a mixed culture of heart cells has an antiapoptotic effect. This synergism of IGF-I and VEGF transgenes on VEGF expression in the BMC+VEGF+IGF-I rats and the antiapoptotic effects of both transgenes may explain the greatly enhanced cell survival in this group.

Cell survival after implantation was quantitated by real-time PCR analysis of a Y-chromosomal gene, *sry*,⁵ from male donor cells in female recipient hearts. Muller-Ehmsen et al¹⁸ quantitated the survival of neonatal male rat cardiomyocytes into normal adult female rat hearts and noted cell survival of 57% at 0 to 1 hour, 23% to 28% from 24 hours to 4 weeks, and 13% at 12 weeks. We noted a loss of $\approx 30\%$ of the cells immediately after injection, presumably because of mechanical factors, followed by a progressive decrease over time. Whereas the expression of VEGF and IGF-I each improved early cell survival, and the combination of both

additionally increased survival (although not to a level that was statistically significant), VEGF and IGF-I did not completely prevent a gradual loss of most of the transplanted BMCs. Most studies have suggested that the majority of cell loss occurs early after implantation, implying that strategies to maximize early cell survival will ultimately result in greater late survival. In contrast, our study suggests that dramatically enhanced early survival may still result in only relatively modest increases in cell survival after several weeks. This progressive decline in cell numbers is unlikely to be related to rejection of the transplanted BMC, because syngeneic rats were used as donors and recipients. Our TUNEL data suggested that most of the cell loss because of apoptosis occurred early, at the 3-day and 1-week time points. At subsequent time points, apoptotic indices in the scar and border zone did not differ significantly between groups. Therefore, the pathophysiology underlying the continuing loss of transplanted BMC after 1 week remains unclear.

In contrast to cell survival, which diminished over time, we noted improvement in LVEF only at 2 and 4 weeks. The mechanical trauma of cell injection may have transiently depressed LV function in all of the groups, masking the beneficial effect of cell implantation on LVEF until after 1 week. Like cell survival, LVEF differed between groups, with greater LVEFs in hearts transplanted with BMCs expressing IGF-I, VEGF, or both transgenes. LVEFs were greatest in the BMC+VEGF+IGF-I group and greater than BMC+IGF-I at 4 weeks, although differences relative to BMC+VEGF did not reach statistical significance. These trends suggest, however, that the synergistic effect of VEGF-I and IGF-I on cell survival, and a potential effect of IGF-I on cellular hypertrophy, may enhance the restoration of LVEF.

We noted significantly greater mRNA and protein expression of contractile proteins in the scar and border zone of hearts transplanted with BMCs expressing VEGF, IGF-I, or both transgenes. Slight increases were noted in the normal myocardium of the transfected BMC-transplanted groups, but this may have been attributable to the incorporation of small

areas of border zone into the sample of normal myocardium during dissection of the heart. The greatest α -MHC levels were observed in the BMC+VEGF+IGF-I group. These patterns mirrored those of the cell survival results, although differences between groups were greater in the protein concentration data, suggesting that they may have been because of more than just the number of surviving cells. However, because hypertrophy of the individual cells after transplantation is extremely difficult to evaluate, it is not possible for us to determine whether IGF-I expression actually induced hypertrophy of the transplanted cells.

This study has some limitations. Although a total of 138 rats were used in this study (95 recipient rats plus 32 donor rats plus 11 recipient rats that did not survive infarct creation and which were replaced), the relatively small number of animals in each of 5 groups and at each of 4 time points hindered the identification of statistically significant differences between groups, particularly between the BMC+VEGF+IGF-I group and the BMC+VEGF and BMC+IGF-I groups. The trends within our data are generally consistent with an incremental benefit of expressing both VEGF and IGF-I, with plausible but statistically insignificant differences in cell survival, LVEF, and α - and β -MHC, myosin light chain, and troponin I content, always favoring the BMC+VEGF+IGF-I group over both the BMC+VEGF and BMC+IGF-I rats. If it had been feasible to double the number of rats used to 276, these differences would likely have attained statistical significance, but our conclusions must clearly be tempered by limited statistical power.

In summary, BMCs expressing IGF-I, VEGF, or both transgenes demonstrated reduced apoptosis early after transplantation and dramatically enhanced cell survival, greater contractile protein content, and greater LV systolic function compared with unmodified BMCs. VEGF and IGF-I appeared to have a synergistic effect on many of these outcomes. These data suggest that multimodal cell-based gene therapy may maximize the benefit of cell transplantation. Additional studies to identify other useful transgenes and the role of pretreatment before cell implantation¹⁹ may lead to refinements of this potential therapy for myocardial repair.

Acknowledgments

This work was supported by a grant from the Heart and Stroke Foundation of Ontario (T5305) to Dr Yau.

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