Angiogenesis by endothelial cell transplantation

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Purpose: Myocardial angiogenesis may improve regional perfusion and perhaps function after cardiac injury. We evaluated the effect of endothelial cell transplantation into a myocardial scar on angiogenesis and ventricular function, as an alternative to angiogenic gene or protein therapy.

Methods and Results: A transmural myocardial scar was created in the left ventricular free wall of rat hearts by cryoinjury. Allogeneic aortic endothelial cells were injected into the scar 2 weeks after cryoinjury. A cluster of transplanted cells was identified at the site of injection 1 day and 1 week after transplantation, but not after 2 weeks. The size of this cluster of transplanted cells decreased as vascular density in the transplanted scar tissue increased with time. Six weeks after transplantation, vascular density was significantly greater in transplanted hearts than in control hearts. Regional blood flow, by microsphere analysis, was greater in the transplanted rats. Systolic and diastolic ventricular function was similar between groups. In a second series of experiments, syngeneic aortic endothelial cells labeled with bromodeoxyuridine were transplanted 2 weeks after cryoinjury. Vascular density in the transplanted scar was greater than in controls. Labeled transplanted endothelial cells were identified forming part of the newly developed blood vessels. No difference in vascular density was found between allogeneic and syngeneic cell transplantation. Vascular endothelial growth factor was not expressed at greater levels in the transplanted cells or the myocardial scar.

Conclusion: Transplanted endothelial cells stimulated angiogenesis, were incorporated into the new vessels, and increased regional perfusion in myocardial scar tissue, but did not improve global function in this cryoinjury rat model.

An increasing number of patients are being referred for coronary artery bypass surgery despite extensive atherosclerosis that precludes complete revascularization.¹ A variety of novel angiogenic therapies²⁻⁷ are being evaluated as adjuncts to bypass grafting or as sole therapies for patients who are not candidates for coronary artery bypass. The goal of these interventions is to induce angiogenesis and increase perfusion to ischemic regions of the heart that cannot be adequately revascularized by conventional means. Transmyocardial laser revascularization improves anginal symptoms, probably by inducing angiogene-

sis.^{2,3} Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor have been given as protein therapy or as gene therapy by transfection by naked DNA or with adenoviral vectors.⁴⁻⁹ Growth factors have been given as a direct injection, in biodegradable capsules or by transcatheter delivery. The limitations of growth factor therapy include the risks of systemic effects inducing problematic angiogenesis in the retina or the potentiation of growth and metastasis of occult tumors. In addition, stability and adverse response to transfection vectors may limit the benefits of growth factor gene therapy.

Cell transplantation into an infarcted region was intended to restore elasticity to the injured region and prevent cardiac thinning and dilatation.¹⁰⁻¹³ Several types of cultured cells have been transplanted into the normal and infarcted myocardium of various species, and the survival of the cells has been confirmed with improvements in global and regional function.¹⁰⁻¹³ Menasché and colleagues^{13a} recently reported the first clinical case of skeletal myoblast transplantation into an infarct region with return of regional function (American Heart Association, New Orleans, Louisiana, November 14, 2000). The survival of the transplanted cells in animals and the first clinical trial were facilitated by a profuse angiogenesis induced in and around the transplanted cells.¹⁰⁻¹³ However, transplantation of cells to induce angiogenesis to an injured cardiac region has not been reported. We investigated endothelial cell transplantation because these cells may secrete a variety of vascular growth factors and because they may also participate in capillary formation. We hypothesized that transplanted endothelial cells would improve perfusion in a myocardial scar. We evaluated the magnitude of the angiogenesis induced by both allogeneic and syngeneic endothelial cell transplantation and the effects of transplantation on global left ventricular function.

Methods

Experimental Animals

All procedures performed on animals were approved by the Animal Care Committee of the Toronto General Hospital. Animal studies were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996. Sprague-Dawley rats (male, 300-350 g, Charles River Canada Inc, Quebec, Canada) were used as both recipients and donors for the allogeneic cell transplantation (n = 16) or media control (n = 15). Syngeneic Lewis rats were used for the syngeneic cell transplantation (n = 10) or media transplantation without cells (n = 8).

Cell Culture and Identification

Endothelial cells were isolated and cultured from rat aortas by the methods of Jaffe and associates.¹⁵ Cultured cells were passaged twice before transplantation. More than 95% of the cultured cells stained positively with antibodies against factor VIII-related antigen, confirming the purity of the cell cultures.¹⁶

Quantification of VEGF Protein Levels in Cultured Cells and Scar Tissue

Endothelial cells (purity 95%) were cultured in serum-free medium for 24 hours and the cells and the conditioned medium were separately harvested for quantification of VEGF protein at the same time that other culture dishes were being harvested for transplantation. VEGF protein concentrations in serum-free medium, conditioned medium (medium cultured with cells for 24 hours), and cultured cell lysates were quantitated by enzyme-linked immunosorbent assay, aliquots of VEGF protein at known concentrations being used as standards for comparison. One week after syngeneic cell transplantation (experiment 2), the scar tissue from the transplanted (n = 6) and media control (n = 6) groups was excised, homogenized, and sonicated. VEGF levels in the scar tissue were also quantitated. The data were expressed as nanograms of VEGF per milligram of total protein.

Myocardial Scar Generation

Rats were anesthetized with ketamine (20 mg/kg body weight, intramuscularly) and sodium pentobarbital (30 mg/kg body weight, intraperitoneally). Anesthetized rats were intubated and their lungs were ventilated with room air supplemented with oxygen and isoflurane (0.2%-1.0%) with a Harvard ventilator (Harvard Apparatus, Inc, Holliston, Mass).

Via a left thoracotomy, cryoinjury of the left ventricular free wall was performed with an elliptical metal probe 8 × 10 mm in diameter cooled to -190°C by immersion in liquid nitrogen.¹² The probe was applied to the left ventricular free wall for 1 minute and the procedure was repeated 10 times, to the same area of the left ventricular free wall, to ensure a transmural injury. Penlog XL (benzathine penicillin G 150,000 U/mL and procaine penicillin G 150,000 U/mL; 1 mL/kg) and buprenorphine hydrochloride (0.01 mg/kg) were given intramuscularly after surgery. Cryoinjured rats were randomly divided into transplantation and control groups.

Endothelial Cell Transplantation

Cultured endothelial cells were passaged twice and transplanted into rat hearts 2 weeks after cryoinjury. The cells were detached from the culture dish with trypsin, centrifuged, and resuspended in culture medium at a concentration of 1.0×10^6 cells/10 μ L. Via a sternotomy, the cell suspension (60 μ L, 6×10^6 cells) was injected into the center of the scar in the transplantation group with a tuberculin syringe and the same volume of culture medium was injected into the scar in control rats. Antibiotics and analgesics were given as described above. In the first study (experiment 1), in which allogeneic cells were transplanted, cyclosporine A (INN: ciclosporin; 15 mg · kg⁻¹ · d⁻¹) was administered subcutaneously to both the allotransplanted and control groups.

Identification of Transplanted Endothelial Cells

One, 7, and 14 days after the transplantation (experiment 1), 2 animals from each of the allogeneic endothelial cell transplanted and media control groups were put to death and their hearts were fixed and sectioned. After staining with hematoxylin and eosin, a cluster of transplanted cells could be easily identified in the center of the otherwise nearly acellular, transmural myocardial scar.

In addition, in experiments in which syngeneic endothelial cells were used (experiment 2), the cultured cells were incubated with

Figure 1. A, Phase contrast photomicrograph of cultured endothelial cells showing their typical “cobblestone” appearance (40 $\times$). B, Photomicrograph of cultured endothelial cells (arrows) stained positively (dark brown) for factor VIII–related antigen (400 $\times$).

bromodeoxyuridine (BrdU), to be incorporated into proliferating cells to facilitate subsequent identification after transplantation. Two days before transplantation, 25 μ L of 0.4% BrdU solution was added to 10 mL of culture medium in the 100-mm culture dishes and incubated until transplantation. Immediately before cell transplantation, a portion of the BrdU-labeled cells were fixed and stained with an antibody against BrdU. The total number of cells and the number of BrdU-positive cells per high-power field were counted, and the percentage of cells labeled with BrdU was calculated. Approximately 60% of the endothelial cells had incorporated BrdU after 48 hours, and the cultured cells were then harvested and transplanted into syngeneic adult rats as described previously (n = 10). These syngeneic rats did not receive cyclosporine to prevent immunorejection. Six weeks after transplantation, the animals were put to death and monoclonal antibodies against BrdU were used to localize the transplanted endothelial cells in the scar.¹⁷

Measurement of Myocardial Blood Flow

Six weeks after transplantation, blood flow to the normal and scar tissue was measured with radionuclide-labeled microspheres.¹⁸ A suspension of ⁵⁷Co-labeled microspheres (5 μ Ci; New England Nuclear, Boston, Mass) was infused into the aortic root of the isolated hearts. The ventricle was dissected into normal tissue, scar, and border zone. Each section was weighed and the radioactivity in each fraction was measured with a gamma counter using a window of 110 to 138 KeV. The ratio of counts per minute per milligram in the scar and in the border zone was calculated as a percentage of values in the normal myocardium.

Measurement of Vascular Density in Scar Tissue

Heart sections were processed as previously described.¹² The vascular density within the scar was counted by an observer blinded to the treatment group at 200 $\times$ magnification. Five fields of each section

Figure 2. Photomicrographs (100 $\times$) of hematoxylin-eosin-stained rat myocardial scar (*S*) in hearts excised 1 day (a, b), 1 week (c, d), and 2 weeks (e, f) after injection of culture medium (control, a, c, e,) or endothelial cells (transplanted, b, d, f). A cluster of endothelial cells (*TC*) was observed at the transplantation site on days 1 (b) and 7 (d), but not on day 14 (f). There was no difference in the number of capillaries (*arrows*) between the control and transplanted scar 1 day after transplantation (a, b), but capillary density was markedly increased by 7 (d) and 14 (f) days after endothelial cell transplantation compared with controls (c, e). Red blood cells are present in the capillaries.

Figure 3. Photomicrographs of control (A) and endothelial cell transplanted (B) myocardial scar 6 weeks after transplantation, stained for factor VIII-related antigen. Endothelial cells in the capillaries stained positively (arrows, 100 $\times$). A greater number of blood vessels was observed in the transplanted group than in controls.

were randomly selected and the vascular density was averaged and expressed as number of blood vessels per field (0.6 mm²).

Measurement of Left Ventricular Remodeling

The epicardial and endocardial surfaces of the normal and scar tissue in the left ventricular free wall were measured as described previously.¹²

Evaluation of Ventricular Function

Six weeks after transplantation, left ventricular function was measured in a Langendorff preparation as we have previously described.¹²

Statistical Analysis

Data were expressed as the mean $\pm$ standard deviation and analyzed with SAS software (SAS Institute, Inc, Cary, NC). Between-group comparisons were performed with the Student *t* test. Left ventricular function was evaluated by analysis of covariance.

Results

Experiment 1: Allotransplantation With Cyclosporine Administration

Cultured endothelial cells were distinguished from fibroblasts and vascular smooth muscle cells by morphologic criteria, growth characteristics, and immunohistochemistry. The endothelial cells were oval, in distinction to the spindle-shaped fibroblasts and vascular smooth muscle cells, and grew in a typical cobblestone appearance distinct from the “whirling” pattern of fibroblasts and the “hill and valley” pattern of vascular smooth muscle cells (Figure 1, A). The endothelial cells stained positively for factor VIII-related antigen (Figure 1, B). The purity of all endothelial cell cultures used for transplantation was greater than 95%.

Two rats in the allotransplanted group were each put to death 1, 7, and 14 days after transplantation to identify the

Figure 4. Vascular density 6 weeks after allogeneic and syngeneic endothelial cell transplantation. Capillary densities resulting from either allogeneic or syngeneic endothelial cell transplantation were significantly higher ($P = .002$ and $P = .04$, respectively) than those in control rats. Capillary density in the allogeneic transplantation group was not significantly different from that in the syngeneic transplantation group.

transplanted cells within the scar. One day after transplantation, a large cluster of endothelial cells was observed within the scar in the transplanted rats (Figure 2, *b*), but not in the control rats (Figure 2, *a*). At 1 day, there was no increase in vascular density in the scar around the cluster of transplanted endothelial cells compared with the controls. One week after transplantation, blood vessels, mainly capillaries and postcapillary venules, were noted in the scar near the cluster of transplanted endothelial cells (Figure 2, *d*), but no corresponding angiogenesis was noted in the control rats (Figure 2, *c*). Over the week after transplantation, the cluster of transplanted endothelial cells had diminished in size as the number of blood vessels surrounding it had increased. Two weeks after transplantation, the vascular density within the scar of transplanted rats was greater than that in controls (Figure 2, *e* and *f*) and the cluster of transplanted endothelial cells had completely disappeared.

Six weeks after transplantation, vascular structures in myocardial scar tissue were identified by staining the vascular endothelial cells with antibody against factor VIII (Figure 3). Vascular density in the transplanted scar (9.6 ± 5.5 vessels per 0.6 mm, $n = 10$) was greater ($P = .002$) than that in the control scar (2.7 ± 1.8 vessels per 0.6 mm, $n = 9$) (Figure 4). The blood vessels were more numerous in the periphery of the scar than in the center. Six weeks after transplantation, regional perfusion by microsphere measurements was similarly increased in the transplanted group ($6.85\% \pm 2.27\%$ of normal myocardium, $n = 9$) compared to the control group ($3.78 \pm 0.99\%$, $n = 8$) ($P = .008$).

Experiment 2: Syngeneic Transplantation Without Cyclosporine Administration

Although there was no mononuclear cell infiltration indicative of rejection in the allotransplanted animals treated with cyclosporine A, we also transplanted syngeneic animals with syngeneic endothelial cells to eliminate inflammation related to rejection as a possible cause of angiogenesis. BrdU-labeled syngeneic endothelial cells were transplanted into the cryoinjury-induced myocardial scar tissue of adult rats. Six weeks after transplantation, no leukocyte infiltration or other evidence of inflammation was observed in the myocardial scar. Many endothelial cells in the blood vessels in the scar stained positively for BrdU, indicating that the transplanted BrdU-labeled endothelial cells had been incorporated into the newly formed blood vessels (Figure 5). Vascular density in the scar tissue of the syngeneic transplanted group (7.5 ± 5.1 vessels per 0.6 mm², $n = 10$) was greater ($P = .03$) than that in the control group (2.9 ± 2.0 vessels per 0.6 mm², $n = 8$). No differences in vascular density was found between allogeneic cell transplantation with immunosuppression (9.6 ± 5.5 vessels per 0.6 mm²) and syngeneic cell transplantation (7.5 ± 5.1 vessels per 0.6 mm²).

One potential mechanism of angiogenesis was evaluated by measuring VEGF levels in cultured cells in vitro and myocardial scar tissue in vivo 1 week after transplantation. The concentration of VEGF in conditioned medium, in which cells had been incubated for 24 hours, was not different from that in unconditioned medium. Lysates of cultured cells contained 4.1 ± 1.4 ng of VEGF per milligram of total protein ($n = 7$). VEGF concentrations in the transplanted scar were 16.2 ± 5.1 ng of VEGF per milligram of total protein ($n = 6$) and were not different from controls (19.3 ± 7.4 ng of VEGF per milligram of total protein, $n = 6$).

In both the allotransplanted and syngeneically transplanted rats, there were no differences in the weights of the hearts, left ventricles, scar tissue, or body weights between the transplanted and control groups. Ventricular volume and scar size were also similar between the transplanted and control groups. There were also no differences in systolic, end-diastolic, or developed pressures between transplanted and control hearts (Figure 6). There were no significant differences in total coronary blood flow and heart rate between groups.

Discussion

The limitations of current strategies of inducing angiogenesis in the infarcted or ischemic myocardium that cannot be revascularized by standard methods led us to explore cell transplantation to stimulate neovascularization. In previous studies, we demonstrated that transplantation of cardiomyocytes¹² and smooth muscle cells¹⁹ into a myocardial scar can alter the postinjury remodeling process and improve global left ventricular function. Accompanying these changes in ventricular function, we noted a modest degree of angiogenesis.²⁰ Therefore, we undertook a series of experiments to

Figure 5. Photomicrograph (400 $\times$) of BrdU-labeled cells transplanted into the myocardial scar. The scar was stained 6 weeks after transplantation, using hematoxylin and antibodies against BrdU. Some endothelial cells in both the large (A) and small (B) blood vessels stained positively (*brown colored nuclei, arrows*), indicating that some of the transplanted endothelial cells were incorporated into the newly formed capillaries.

define the potential for cell transplantation to induce angiogenesis for patients with extensive coronary atherosclerosis who cannot be adequately revascularized. We selected endothelial cells to be transplanted, speculating that this cell type might participate in creating the capillary structure necessary for angiogenesis. These cells may also secrete a variety of growth factors that could stimulate a profuse vascular network. In addition, we compared the transplantation of allogeneic and syngeneic endothelial cells to determine the effects of immune rejection on angiogenesis.⁵

We used a rat model of cryoinjury,^{12,19} which provides an extreme challenge for any angiogenesis therapy. If cell transplantation induces angiogenesis in this extreme model, then treatment in an infarct model should be even more beneficial.

Cell transplantation was delayed until 2 weeks after myocardial cryoinjury to avoid loss of the transplanted cells in the post-necrosis inflammatory process. Preliminary studies suggested

that cells transplanted immediately or 7 days after cryoinjury were eliminated by an intense inflammatory reaction.

In this study, we used an ex vivo technique of microsphere injection to quantitate regional myocardial blood flow but avoided the contribution of noncoronary collaterals. Injection of microspheres into the left atrium or left ventricle in vivo may more closely approximate physiologic regional blood flows but may result in the majority of microspheres being delivered into the systemic, noncardiac capillaries, as well as permitting contamination of coronary blood flow by flow through the vascular pericardial adhesions resulting from the prior surgical procedures. Ex vivo microsphere injection in an isolated heart allowed us to maximize the delivery of microspheres into the myocardium, as well as to isolate the effect of intramyocardial collateral formation caused by cell transplantation from that of epicardial collaterals resulting from operative trauma. With this technique, we noted excellent mixing of

Figure 6. Developed pressures of the transplanted and control hearts measured in a Langendorff preparation over a range of intraventricular balloon volumes. There was no difference in developed pressures between the transplanted and control groups. The angiogenesis induced by endothelial cell transplantation did not improve the function of hearts with a transmural scar.

the microspheres in the aortic root during retrograde Langendorff perfusion and good reproducibility with low variability in our measurements of regional blood flow.

The microsphere data demonstrated increased regional perfusion in the scar area. The histologic studies also demonstrated increased vascular density in the scar region. However, no difference in perfusion of the myocardium outside the scar would be anticipated. The increase in perfusion within the scar, although statistically significant, would have been impossible to distinguish in the vastly greater volume of total coronary effluent measured in the Langendorff apparatus, which was not different between groups.

Endothelial cell transplantation resulted in significantly greater angiogenesis, identified by immunohistochemical staining for factor VIII, evaluated by vascular density and regional blood flow by microsphere injection, than control animals injected with culture medium alone. Vascular density was approximately 3-fold greater in the transplanted rats than in control animals, but regional blood flow was increased only marginally from 3.8% to 6.9% of normalized values. This improvement represents a doubling or tripling of the values noted in control rats, but our model of cryoinjury may still have limited the degree of angiogenesis achievable with endothelial cell transplantation. A coronary ligation model of myocardial infarction, in which a more extensive peri-infarct border zone could harbor viable but hypoperfused cardiomyocytes and endothelial cells, might result in greater angiogenesis. In addition, chronically ischemic cardiomyocytes in a peri-infarct penumbra may secrete signaling factors that potentiate the effect of the transplanted cells.

Potential mechanisms by which angiogenesis may be induced after endothelial cell transplantation include the following: (1) formation of blood vessels by transplanted endothelial cells; (2) stimulation of angiogenesis by growth factors expressed or stimulated by the transplanted endothelial cells; and (3) stimulation of angiogenesis by an inflammatory reaction caused by the transplanted endothelial cells.

The hypothesis that transplanted endothelial cells may play a structural role in induction of neovascularization is supported by the observation that many BrdU-labeled endothelial cells were noted in the newly formed blood vessels 6 weeks after transplantation. It is possible that a low level of unincorporated cytoplasmic BrdU may have remained in the endothelial cells and that this BrdU may have been available for uptake by native cells. However, because the BrdU incorporated into DNA must compete with non-BrdU labeled uridine in the cytoplasm, cells must be incubated with high levels of BrdU to be labeled effectively. The very low levels of BrdU that might have diffused from the transplanted endothelial cells into the interstitium of the myocardial scar would be unlikely to have been sufficient to label the native cells. After sectioning of the transplanted rat hearts, we noted a moderate number of cells that stained intensely positive, surrounded by unstained cells, consistent with BrdU being retained in the transplanted endothelial cells, rather than a larger number of weakly positive cells, a pattern that might be more consistent with leakage of BrdU from the transplanted cells and uptake by the surrounding native cells. Therefore, the BrdU-labeled capillary cells were likely to have resulted from cell transplantation rather than labeling of native cells after cell transplantation. These results are in agreement with data reported by other investigators, who have also demonstrated incorporation of endothelial cells into newly formed blood vessels in non-cardiac tissues.²¹⁻²⁴

We found no difference in VEGF protein levels between the transplanted and control hearts. This finding may be explained by the hypothesis that the angiogenesis induced by endothelial cell transplantation is independent of changes in VEGF expression. Alternatively, VEGF expression may have been upregulated, but to a degree that was not detectable by the relatively gross evaluation of total tissue levels by our enzyme-linked immunosorbent assay technique. We would speculate that even relatively low-level upregulation of VEGF expression in the transplanted hearts may exert significant effects on local angiogenesis, by autocrine or paracrine mechanisms. However, this hypothesis cannot be proved or disproved by our current study.

Inflammation may induce angiogenesis by release of cytokines and other mediators. However, no histologic evidence of inflammation was noted either in the hearts of rats transplanted with allogeneic endothelial cells (and given cyclosporine) or in those of rats transplanted with syngeneic endothelial cells (without cyclosporine). There was no sig-

nificant difference in vascular density after allogeneic versus syngeneic transplantation, suggesting that rejection did not affect angiogenesis in this series of experiments. A non-specific inflammatory response may result from the mechanical trauma of injection into the myocardial scar, but this should also occur in the control rats, in which culture medium without cells was injected. Our results suggest that the transplanted endothelial cells participated in the formation of new blood vessels.

The increased perfusion noted after endothelial cell transplantation did not affect ventricular remodeling or improve global left ventricular function in our experimental model. Previous studies with muscle cells (heart cells, smooth muscle cells, or skeletal muscle myoblasts) demonstrated improved global and regional function.^{11,13,19} The cells prevented cardiac thinning and dilatation, improved systolic function, and developed pressures at any ventricular volume. In contrast, previous studies with nonmuscle cells (fibroblasts) did not show an improvement in either global or regional function. The elastic elements of the muscle cells may be necessary to modify remodeling and prevent thinning and dilatation. Increasing blood flow by endothelial cell transplantation in this model did not improve contractility or prevent ventricular dilatation. These data suggest that angiogenesis alone, without restoration of cardiomyocyte numbers, will have less benefits in patients with extensive, transmural myocardial infarctions. We plan to perform autologous endothelial cell transplantation in our clinically relevant porcine model of occlusion of the left anterior descending coronary artery.¹³ We anticipate that angiogenesis may improve regional and global function.

Endothelial cell transplantation may become an alternative to gene and protein therapy to induce angiogenesis in patients who cannot be adequately revascularized by standard techniques. Short segments of peripheral vein can be taken from patients under local anesthesia, the endothelial cells isolated, expanded in culture, and then transplanted into a cardiac infarct region without the need for immunosuppression. Endothelial cells transplanted into a nontransmural myocardial infarction or into hibernating myocardium may improve myocardial function by improving regional perfusion. Cell transplantation may also be the ideal vehicle for therapeutic gene transfer.²⁶ The full potential of cell transplantation, however, remains to be clarified.

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