Impairment of human cell–based vasculogenesis in rats by hypercholesterolemia-induced endothelial dysfunction and rescue with L-arginine supplementation

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Objective: Clinical efficacy of cardiac cell therapy may be compromised by its target population, patients with endothelial dysfunction. In vivo inhibition by endothelial dysfunction has been demonstrated for protein angiogenesis but remains unclear for cell therapy. We examined whether hypercholesterolemia inhibits vasculogenic effects of transplanted human circulating progenitor cells in ischemic tissue and whether L-arginine, a nitric oxide donor, might prevent impairment.

Methods: Athymic rats were fed either normal (group A) or high-cholesterol diets, the latter without (group B) or with (group C) oral L-arginine supplementation. Two weeks later, these rats underwent left femoral artery ligation followed by injection of 2×10^6 human circulating progenitor cells into left hind-limb muscle. A fourth group (group D) received supplemented high-cholesterol diets but no cells.

Results: Group B had biochemical evidence of endothelial dysfunction and reduced tissue endothelial nitric oxide synthase expression, whereas group A levels were the same as in group C. By 21 postoperative days, left hind-limb perfusion had recovered fully in groups A and C, partially in D, and not at all in B (38% lower than group A, $P \leq .004$). Lower arteriolar densities were found in groups B and D than in groups A and C ($P \leq .02$). Engrafted human cell numbers were equivalent in all cell-transplanted groups after 3 weeks.

Conclusions: Endothelial dysfunction inhibited effects of cell therapy, specifically vasculogenesis, suggesting a role for substrate modification to overcome this inhibition. Involved mechanisms appear related to use of cells but not engraftment and require further investigation. (J Thorac Cardiovasc Surg 2010;139:209-216)

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Cardiac cell therapy encompasses all cell-based regenerative approaches for refractory heart failure or intractable myocardial ischemia. To date, this new modality has been investigated by in vitro, in vivo, and phase I and II clinical trials (Table E1).^{1,2} At this writing, however, cell therapy arguably has not resulted in long-term benefits or major recovery

of myocardial function in human beings, despite impressive results in animal models.^{3,4}

There are biologic issues that might explain the relative lack of efficacy of cardiac cell therapy in human beings. The recovery of injured or ischemic tissue may depend on the active recruitment of regenerative cells, on the survival of the cells, and on their phenotypes and functions within the target tissue. Cell recruitment and retention are mediated by the release of cytokines, such as stromal cell–derived factor 1 or vascular endothelial growth factor, which are upregulated by ischemia and depend on local nitric oxide (NO) levels.⁵⁻⁷

In this context, it is relevant to consider that patients with coronary artery disease, who constitute the majority of patients for whom cardiac cell therapy is intended, have abnormal coronary endothelial function and decreased NO bioavailability.^{7,8} Protein angiogenesis with fibroblast growth factor 2⁹ and vascular endothelial growth factor¹⁰ and also local endogenous angiogenesis^{7,11} depend on NO bioavailability. In addition, a decreased in vivo therapeutic effect of animal bone marrow cells (BMCs) on angiogenesis in the presence of hypercholesterolemia and hypertension has been reported, with an associated reduction in the NO pathway.^{12,13} Recently, it was shown that transplanted cells can quickly disappear from target tissue, and that it is the

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Abbreviations and Acronyms

bET-1	= big endothelin 1
BMC	= bone marrow cell
CPC	= circulating progenitor cell
eNOS	= endothelial nitric oxide synthase
ET-1	= endothelin 1
GM-CSF	= granulocyte macrophage–colony stimulating factor
IFN	= interferon
IL	= interleukin
NO	= nitric oxide
TNF	= tumor necrosis factor

induced humoral effects that play a crucial role in tissue recovery after cell therapy.¹⁴ The relationship between endothelial dysfunction and transplanted cell engraftment, an important consideration, has not previously been examined. Furthermore, no study had yet investigated the effects of hypercholesterolemia and endothelial dysfunction on transplanted human circulating progenitor cells (CPCs), which constitute a more clinically relevant, specific, and possibly potent vasculogenic cell population than BMCs of healthy or pathologic animal origin.^{15,16}

We report here for the first time that, in a rat model of acute hind-limb ischemia treated with human CPCs, hypercholesterolemia-induced endothelial dysfunction inhibited vasculogenesis in response to human cell therapy and that dietary supplementation with L-arginine, the substrate for NO production,⁹ reversed this inhibition. We also demonstrated that endothelial dysfunction had no effect on transplanted human cell engraftment after 3 weeks and that other mechanisms are likely responsible for the inhibition of vasculogenesis.

MATERIALS AND METHODS**Cell Isolation and Culture**

Procurement methods were approved by the Human Research Ethics Board of the University of Ottawa Heart Institute. After informed consent was obtained, total peripheral blood mononuclear cells were isolated from 30-mL samples of fresh blood from healthy young donors by means of Histopaque 1077 (Sigma, Oakville, Canada) density gradient centrifugation of buffy coats and cultured to obtain CPCs as described previously.¹⁷

Animal Model

Procedures were performed with the approval of the University of Ottawa Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care. All animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* (www.nap.edu/catalog/5140.html).

Athymic male Sprague–Dawley rats (250–300 g; Charles River Laboratories International, Inc, Wilmington, Mass) were divided into 3 dietary groups: (1) rats in the control group (group A, n = 12) were continuously maintained on a standard diet; (2) rats in the high-cholesterol group (group B, n = 12) were fed a 2% high-cholesterol diet; and (3) rats in the high-cho-

lesterol and L-arginine group (group C, n = 12) were also fed a 2% high-cholesterol diet. Two weeks after the initiation of this dietary modification, the left proximal femoral artery of each rat was ligated and segmentally excised to induce hind-limb ischemia, as described previously.¹⁸ Shortly thereafter, each rat received 2.0×10^6 4',6-diamidino-2-phenylindole-labeled human CPCs in 200 μ L phosphate-buffered saline solution, administered during the same surgical session by 10 equally distributed injections into the ischemic thigh adductor muscle with a 27-gauge needle. As an additional control, a second group of rats fed a high-cholesterol and L-arginine diet underwent the same surgical procedure as described but did not receive a cell injection (group D, n = 6).

Immediately after surgery, rats in groups C and D were maintained on the high-cholesterol diet and started on 2.25% L-arginine supplementation in the drinking water, both of which were continued for the remainder of the study. This L-arginine administration regimen was previously shown to increase serum levels of L-arginine and tissue contents of stable NO metabolites in rats.^{7,19} In all rats, the right hind limb was not operated on and served as a within-subject control. Rats were killed 21 days after the operation, and their serum and bilateral hind-limb skeletal muscles were collected. One animal died prematurely and was lost from group C (n = 11). Data for all procedures and analyses were collected from all experimental animals (n = 11–12 animals/group for all groups, except for group D, where n = 6).

Laser Doppler Blood Perfusion Analysis

The blood flow perfusion ratio of the ischemic (left) to the nonischemic (right) hind limb was determined before and after femoral artery ligation as well as on postoperative days 7, 14, and 21 by using a multifiber needle probe containing 8 separate collecting fibers and a laser Doppler blood flow monitor (Moor Instruments Ltd, Axminster, UK). To this end, rats were anesthetized with isoflurane, and the hind limbs were shaved and cleaned. The probe was calibrated with a physical standard according to the manufacturer protocol. The probe was then placed on the skin over the main rat hind-limb adductor muscle and, after the flux readings were allowed to stabilize for 2 to 3 minutes, a 30-second recording was taken with data-acquisition software (moorLAB V1.3; Moor Instruments). Readings were made for both limbs, and the average flux from the 8 separate probes during the 30-second recording was used to calculate flux ratio.

Serum Biochemical Analyses

Serum samples (approximately 230 μ L) were analyzed for circulating levels of endothelin 1 (ET-1) and big ET-1 (bET-1) according to the procedure reported by Kumarathasan and associates.²⁰ Briefly, serum samples stabilized with 3,4-dichloroisocoumarin (Sigma) were deproteinized with acidified acetone and clarified by molecular weight cutoff filtration (30 kDa). Samples were reconstituted with phosphate-buffered saline solution (Sigma) and endothelins were separated with a Gilson high-performance liquid chromatograph (Mandel Scientific Company Inc, Guelph, Ontario, Canada) on a LC-318 Zorbax column (Agilent Technologies, Inc, Santa Clara, Calif) by gradient elution with a water/acetonitrile mobile phase, followed by fluorescence detection at an excitation wavelength of 280 nm with an emission wavelength of 340 nm. Endothelin calibration standards ET-1 (Sigma) and bET-1 (Bachem California, Inc, Torrance, Calif) were used. Ratios of ET-1 to nitrite and ET-1 to bET-1 were calculated.

Nitrite analysis was also conducted according to a previously reported procedure.²⁰ Briefly, serum samples (50 μ L) treated with ethylenediamine-tetraacetic acid and diethylenetriaminepentaacetic acid (Sigma) were thermolyzed at 86°C, cooled, and filtered through 10-kDa molecular weight cutoff filters. The samples were treated with nitrate reductase (Sigma) at room temperature for 1 hour and were broken into derivatives with 2,3-diaminonaphthalene (Molecular Probes, Eugene, Ore). Fluorescence measurements were made at an excitation wavelength of 360 nm and an emission wavelength of 460 nm on a Cytofluor 2350 multiplate fluorescence detector (Millipore, Bedford, Mass).

Serum samples (150 μ L) treated with diethylenetriaminepentaacetic acid and butylated hydroxytoluene (to prevent autooxidation) were analyzed for 3-nitrotyrosine with a previously reported high-performance liquid chromatography CoulArray method.²¹ In summary, stabilized serum samples were deproteinized with acidified acetone and clarified by molecular weight cut-off filtration (30 kDa). Samples were reconstituted with acidified deionized water, and the 3-nitrotyrosine peak was separated with high-performance liquid chromatography (ESA, Chelmsford, Mass) on a LC-18 Zorbax column (Agilent Technologies) by isocratic elution with a citrate/acetate buffer as mobile phase, followed by coulometric array detection at 600 mV with the CoulArray detector (ESA). The calibration standard for 3-nitrotyrosine was obtained from Sigma.

The cytokines interleukin (IL) 1 α , IL-1 β , IL-2, IL-4, IL-6, interferon γ (IFN- γ), tumor necrosis factor α (TNF- α), IL-10, and granulocyte macrophage-colony stimulating factor (GM-CSF) in plasma were analyzed with a Bioplex liquid antibody array (Bio-Rad Laboratories, Hercules, Calif) according to manufacturer protocol. All treatment groups had the plasma level for each cytokine normalized to the mean level calculated for group A. An inflammatory index²² was calculated from the ratio of the average changes of proinflammatory cytokines IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IFN- γ , and TNF- α to the average changes of anti-inflammatory cytokines IL-10 and GM-CSF, as follows: Inflammatory index = $(\Delta\text{TNF-}\alpha + \Delta\text{IFN-}\gamma + \Delta\text{IL-1}\alpha + \Delta\text{IL-1}\beta + \Delta\text{IL-2} + \Delta\text{IL-4} + \Delta\text{IL-6}) / (\Delta\text{GM-CSF} + \Delta\text{IL-10})$, where Δ represents the average change in concentration.

Histologic Assessment

Animals were killed on postoperative day 21 with an overdose of isoflurane gas and an intracardiac injection of potassium chloride. Ischemic (left) and nonischemic (right) hind-limb main thigh adductor muscles were harvested, fixed with 4% paraformaldehyde, stored in 10% neutral buffered formalin, serially sectioned, and embedded in paraffin. Slides were prepared from 4- μ m serial sections at different levels. Sections were stained with hematoxylin–phloxine–safran and Masson trichrome staining.

Immunohistochemical localization of endothelial nitric oxide synthase (eNOS) was performed with the use of anti-eNOS antibody (1:100; Abcam plc, Cambridge, Mass), with subsequent development in the DAKO LSAB2 system followed by diaminobenzidine (DAKO Corporation, Carpinteria, Calif), as described previously.²³ The level of eNOS expression was calculated as the percentage of the area in sections staining positive for eNOS. As an endothelial cell marker, CD31 was localized in transplanted cells by immunohistochemical staining with anti-CD31 (1:50; Millipore Bioscience Research Reagents, Temecula, Calif) according to manufacturer's protocol.

The extent of vascularization was assessed by measuring arteriolar density in light microscopic sections stained with hematoxylin–phloxine–safran and was calculated as the number of arterioles per square millimeter. Arteriolar density was further confirmed by direct staining with an α -smooth muscle actin antibody (1:400; Millipore Bioscience Research Reagents) with visualization by fluorescence microscopy. An index of vascularity was also calculated, consisting of the ratio between arteriolar counts in the ischemic and nonischemic limbs. Capillary density was also assessed

by determining the ratio of the number of capillaries (stained CD31⁺) to that of muscle fibers. Arteriolar and capillary densities were determined from 6 random microscopic fields (at a minimum of 3 tissue levels) from each animal by an observer blinded to group.

Fluorescent in Situ Hybridization

Xenogeneic transplant allowed the use of the fluorescent in situ hybridization technique to detect specifically donor-derived cells by using a Cy3-labeled pancentromeric human chromosome probe (Cambio Ltd, Cambridge, UK) according to the manufacturer protocol. Briefly, sections were dewaxed, rehydrated, incubated in sodium thiocyanate, and then treated with pepsin. The reaction was quenched with 0.2% glycine, and sections were postfixed in 4% paraformaldehyde, dehydrated and air-dried. The pancentromeric chromosome paint was applied to the slide, sealed with a glass coverslip, heated to 80°C for 10 minutes, and incubated overnight at 37°C. The slides were then washed successively with formamide, standard saline citrate solution, and 10% polysorbate 20 in standard saline citrate solution, all provided by the manufacturer. Slides were then treated with mounting medium containing 4',6-diamidino-2-phenylindole. Slides were examined with fluorescence microscopy where the nuclei of the transplanted human cells in combination with the 4',6-diamidino-2-phenylindole stained purple. The number of positive cells was determined from 6 random microscopic high-power fields in a blinded fashion.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Statistical analyses were performed in Intercooled Stata 9.2 (Stata, College Station, Tex). Comparisons of continuous data between groups were performed with a 1-way analysis of variance or a 2-tailed Student *t* test, both with Bonferroni correction for repeated measures as appropriate. Doppler data were analyzed with a linear regression model that used the number of days after the procedure and the response group as the independent variables and the standardized Doppler signal as the dependent variable.

RESULTS

Tissue eNOS Expression

Expression of eNOS (as a percentage of total tissue area) in the ischemic hind limb was significantly reduced in group B (0.79% $\pm$ 0.09%) relative to groups A (1.91% $\pm$ 0.23%, *P* < .001) and C (1.62% $\pm$ 0.16%, *P* = .006; Figure 1). L-Arginine treatment restored hind-limb tissue eNOS in rats fed a hypercholesterolemic diet to a level equivalent to that observed in the control group (*P* = .73).

Circulating Endothelins and Nitrites

The ratio of ET-1 to nitrite in group A was 3.6% $\pm$ 0.4%, and the high-cholesterol diet significantly increased this

FIGURE 1. Immunohistochemical analysis of tissue endothelial nitric oxide synthase expression. Representative images of endothelial nitric oxide synthase levels in ischemic muscle of rats in groups A (A), B, (B), and C (C). Scale bar represents 150 μ m.

FIGURE 2. Biochemical analysis of endothelial function. Ratio of endothelin 1 (ET1) to nitrite (A), ratio of endothelin 1 (ET1) to big endothelin 1 (bET1) (B), and nitrotyrosine level (C) were all significantly increased in serum of group B (CHOL) relative to groups A (NORM) and C (CHOL-ARG). Asterisk indicates $P < .05$ versus other groups.

ratio to $8.1 \pm 1.4\%$ in group B ($P < .02$; Figure 2, A). L-Arginine supplementation in group C restored the ratio ($4.2 \pm 0.7\%$) to control level ($P > .999$). The ratio of ET-1 to bET-1 was also examined (Figure 2, B). There was a trend for the ratio of circulating ET-1 to bET-1 in groups A ($54\% \pm 10\%$) and C ($59\% \pm 11\%$) to be lower than that in group B ($88\% \pm 17\%$, $P = .08$ and $P = .11$ for B vs A and B vs C, respectively).

Serum Nitrotyrosine Levels

Serum nitrotyrosine levels were significantly higher in group B (950 ± 226 pmol/mL) than in groups A (371 ± 119 pmol/mL) and C (391 ± 86 pmol/mL, $P < .04$).

Inflammatory Index

The inflammatory index, calculated from the ratio between proinflammatory and anti-inflammatory cytokine levels, was elevated in group B (2.40 ± 0.70) relative to group A (1.25 ± 0.25 , $P = .15$). Treatment with L-arginine restored the index to a level equivalent to that in group A (group C 1.30 ± 0.32 , $P = .9$; Figure 3). Examination of individual cytokine levels revealed a trend toward decreased expression of several proinflammatory cytokines with L-arginine treatment (group C) relative to group B (Table E2).

Vascularization

Arteriolar density was reduced in group B relative to values in groups A and C (Figure 4, A–C). The number of arterioles in group B (6.8 ± 0.6 arterioles/mm²) was signif-

icantly reduced relative to the numbers in groups A (8.7 ± 0.8 arterioles/mm²) and C (8.8 ± 0.7 arterioles/mm², $P < .04$; Figure 4, D). When comparing groups according to the ratio of ischemic (left) to nonischemic (right) hind-limb arteriolar count, the same result was observed; that is, the arteriolar count ratio in group B (0.81 ± 0.10) was significantly less than that in groups A (1.23 ± 0.15) and C (1.31 ± 0.16 , $P < .01$; Figure 4, E). The number of arterioles and the arteriolar count ratio (ischemic/nonischemic hind limb) in group D (6.2 ± 0.5 arterioles/mm² and 0.74 ± 0.10 , respectively) were significantly reduced relative to groups A and C ($P < .05$).

FIGURE 3. Inflammatory index. Ratio of proinflammatory to anti-inflammatory cytokine expression was greater in group B (CHOL) than in groups A (NORM) and C (CHOL-ARG).

FIGURE 4. Arteriolar density. Representative images of α -smooth muscle actin–stained arterioles in ischemic muscle of rats in groups A (A, NORM), B (B, CHOL), and C (C, CHOL-ARG). Both arteriolar density (D) and ratio of ischemic to nonischemic arteriolar count (E) were significantly reduced in group B relative to groups A and C. Scale bar represents 250 μ m. Asterisk indicates $P < .04$ versus other groups; double asterisk indicates $P < .01$ versus other groups.

Capillary density, measured as the number of capillaries per muscle fiber, was also significantly reduced in group B (0.96 ± 0.06) relative to groups A and C (1.44 ± 0.10 and 1.59 ± 0.13 , respectively, $P < .02$; Figure 5).

Hind-Limb Perfusion

Laser Doppler analysis (Figure 6) revealed that the impaired blood flow (measured as ischemic/nonischemic flow ratio) after left femoral ligation was restored in group A after 3 weeks (0.97 ± 0.06) but did not recover in group B (0.59 ± 0.03). This represents a flow reduction of 38% in group B relative to group A ($P < .001$). Rats fed the high-cholesterol diet but receiving L-arginine (group C) had restoration of hind-limb blood flow that was significantly improved at 14 and 21 days (0.86 ± 0.03 and 0.98 ± 0.04 , respectively) relative to group B (0.64 ± 0.03 and 0.60 ± 0.03 at 14 and 21 days, respectively, $P < .001$). Hind-limb perfusion in group D with time (0.74 ± 0.1 and 0.86 ± 0.04 at 14 and 21 days, respectively) was significantly lower than in groups A and C ($P = .004$) and greater than in group B ($P = .02$).

Human Cell Engraftment

The number of transplanted human cells (as determined by in situ hybridization with the human-specific pancentromeric probe) within the ischemic hind limb at 3 weeks after injection was not different among treatment groups (Figure 7). Specifically, there were average numbers of 0.43 ± 0.12 , 0.38 ± 0.11 and 0.38 ± 0.11 positive transplanted human cells/field of view for groups A, B, and C, respectively ($P = .9$).

DISCUSSION

In this study, the effects of hypercholesterolemia-induced endothelial dysfunction on the efficacy of human cell therapy to improve revascularization of ischemic tissue in immunocompromised rats were examined. The main findings were as follows: (1) Hypercholesterolemia-induced endothelial dysfunction had an adverse effect on the vasculogenic response to human progenitor cell therapy. (2) Endothelial dysfunction did not affect the number of engrafted donor human cells in the subacute period; that is, at 3 weeks after injection. (3) The NO donor L-arginine rescued animals from the hypercholesterolemia-induced endothelial dysfunction and its associated oxidative stress and inflammatory state. (4) Finally, L-arginine supplementation prevented the hypercholesterolemia-induced impairment in human cell–based vasculogenesis. This study demonstrates that rescue from endothelial dysfunction restores the host response to human cell–based vasculogenic therapy in vivo, suggests a role for the cells themselves, and implies that mechanisms other than engraftment and differentiation of the transplanted cells are involved.

Previous work has established the in vivo inhibitory effects of endothelial dysfunction on the effectiveness of protein-based angiogenesis.^{9,10} A few studies have also suggested that cell-based vasculogenesis could be affected by endothelial dysfunction. For example, Aicher and colleagues⁵ demonstrated that eNOS deficiency resulted in defective mobilization of stem and progenitor cells, which contributed to impairment of endogenous ischemia-induced neovascularization. This finding indicates that NO, the availability of which is altered in endothelial dysfunction, has

FIGURE 5. Capillary density. Representative images of CD31-stained capillaries (arrowheads) in ischemic muscle of rats in groups A (A, NORM), B (B, CHOL), and C (C, CHOL-ARG). D, Capillary density was significantly reduced in group B relative to groups A and C. Scale bar represents 75 μ m. Asterisk indicates $P < .02$ versus other groups.

a role in endogenous progenitor cell recruitment and spontaneous collateral formation. Similarly, deNigris and associates^{12,13} transplanted BMCs into ischemic hind limbs of hypercholesterolemic and hypertensive mice and observed

FIGURE 6. Hind-limb perfusion. Laser Doppler analysis was used to measure perfusion before (*Pre-op*) and after (*Post-op*) operation and at 7, 14, and 21 postoperative days. Data are presented as average ratio of ischemic to nonischemic limb blood flow. Asterisk indicates $P < .001$ between groups B (CHOL) and C (CHOL-ARG). NORM, Group A.

an enhanced angiogenic response when cells were delivered in combination with metabolic intervention, with an associated increase in NO bioavailability. The BMCs used in these studies were isolated from the pathologically affected animals, in contrast to the clinically relevant human source used in our experiments.

In our study, we directly confirmed in an animal model with CPCs from healthy human donors that endothelial dysfunction as a substrate negatively influenced the in vivo response to cell therapy. Similarly, autologous stem or progenitor cells derived from patients with cardiovascular disease may have defects in their regenerative activities,^{24,25} and modification of the transplanted cell might be a prerequisite for efficacy in cell therapy. We showed that manipulating the host environment, which includes addressing the role of endothelial dysfunction as investigated by our group, will likely be required to improve the regenerative potential of cell therapy, independently of whether the transplanted cell has compromised function or is fully healthy. In this study, despite the use of cells with preserved functional activity,^{15,16} the mechanism did not relate to the number of engrafted transplanted cells at 3 weeks.

Recent studies have raised questions regarding engraftment and transdifferentiation of transplanted cells, and the

FIGURE 7. Cell engraftment at 3 weeks after cell transplant. A, Representative image of transplanted human circulating progenitor cell (arrowhead), stained with human-specific pancentromeric probe (purple nuclei), found engrafted in ischemic hind-limb muscle of rat 3 weeks after injection. All cell nuclei are stained with 4',6-diamidino-2-phenylindole (blue). Inset shows higher magnification of stained human cell. Scale bar represents 75 μ m. B, Number of transplanted human cells found engrafted in ischemic hind limb after 2 weeks was not different ($P = .9$) between groups A (NORM), B (CHOL), and C (CHOL-ARG).

premise that these are the primary mechanisms responsible for therapeutic effects is being challenged.^{14,26} Rather, it is believed that neovascularization of the dysfunctional myocardium from paracrine or humoral factors and secondary recruitment of host stem or progenitor cells are the likely mechanisms leading to functional improvement.^{14,16,26} Recently, Cho and colleagues¹⁴ demonstrated that transplanted cells can quickly disappear from the target tissue and that it is the induced humoral effects, which are sustained by the host tissues, that play a crucial role in tissue recovery after cell therapy. If humoral effects sustained by the host stand as the main mechanism for the therapeutic benefit of cell transplant, it is therefore reasonable to expect that endothelial dysfunction will minimize or abolish the effects of cell therapy. In this study, the level of human cell engraftment at 3 weeks was not affected by endothelial dysfunction, suggest-

ing that mechanisms other than engraftment and differentiation, such as host-sustained humoral effects, may have been responsible for the observed inhibitory effects. This notion is further corroborated by the observation that animals fed a high-cholesterol diet with L-arginine supplementation but no cell transplant (group D) demonstrated only partial restoration of perfusion. This suggests that L-arginine treatment improves an endogenous angiogenic response, as has been previously reported²⁷ and that cell transplant may augment this endogenous response, possibly through the interaction of host and transplanted cells and the associated humoral effects. The exact mechanisms by which transplanted cells confer their benefits in this context remain incompletely understood, and this is an area for future, more targeted, investigation.

One limitation of the study is that tissues were examined after 3 weeks, and therefore the possibility that early engraftment, allogenic immune response, and differentiation may have differed between the groups and resulted in the observed effects cannot be excluded. Nevertheless, minimal allogenic response is typically observed with the use of an athymic rat model, according to our data and previous studies,^{18,28} and this would be anticipated to be equivalent in the 3 treatment groups because all received equal numbers of transplanted human cells. Additionally, the persistences of the observed effects were not examined beyond 3 weeks, and the long-term benefits of substrate modification to reverse the inhibitory action of endothelial dysfunction on cell therapy constitutes a focus of future study. Notwithstanding these caveats, it remains to be elucidated whether an improvement in cell retention, from the use of delivery matrices for instance,¹⁸ might augment the host response to human cell therapy in the context of endothelial dysfunction.

Several parameters helped establish that the hypercholesterolemic animals used in this study had endothelial dysfunction. First, ET-1 is an important player in the development of vascular dysfunction.²⁹ The ratio of ET-1 to nitrite constitutes an index of vasoconstriction and indicates a mismatch between endothelin expression and the eNOS pathway; this ratio was increased in group B. Second, bET-1 is the precursor to formation of ET-1, and the high ratio of ET-1 to bET-1 observed in group B indicates either reduced de novo synthesis of bET-1 or increased conversion of bET-1 to the ET-1 peptide relative to the control group. Third, the level of the peroxynitrite marker nitrotyrosine, an indicator of oxidative stress and also of vascular function,³⁰ was elevated in group B relative to the normal control group. Finally, eNOS level in the ischemic hind-limb tissue was reduced in group B relative to the control group and was restored by L-arginine supplementation. Overall, the ratio of serum ET-1 to nitrite ratio, the ratio of serum ET-1 to bET-1, the nitrotyrosine level, and the eNOS level indicate the presence of endothelial dysfunction in the animals fed

a high-cholesterol diet, which was rescued by the NO donor L-arginine with respect to each parameter. In addition, examination of the ratio between proinflammatory and anti-inflammatory cytokines present in the serum suggests a proinflammatory state in the group B rats, which again was improved by supplementation with L-arginine.

This study indicates that endothelial dysfunction inhibits vasculogenic cell therapy in vivo with healthy functional human cells, which provide an effect but not through a subacute transplanted cell engraftment mechanism, and suggests a role for substrate modification to improve the results of therapy. Improving our understanding of the role of endothelial dysfunction in the host responses to ischemia and to cell treatment may expedite progress toward development of more effective cell-based vasculogenic and possibly myogenic therapies in human beings.

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TABLE E1. Completed trials that have used bone marrow mononuclear cells or EPCs after myocardial infarction (partial list)

Study	No.*	Cell type	Delivery	Time after MI (d)	Outcome
Nonrandomized (primarily safety)					
Strauer et al ^{E1}	10	BM-MNCs	IC	8 ± 2	Stroke volume ↑; infarct size ↓; wall motion ↑; perfusion ↑
TOPCARE-AMI ^{E2,E3}	59	BM-MNCs, PB-EPCs	IC	4.9 ± 1.5	LVEF ↑; remodeling ↓; infarct size ↓; perfusion ↑
Fernandez-Aviles et al ^{E4}	20	BM-MNCs	IC	13.5 ± 5.5	LVEF ↑; wall motion ↑; ESV ↓
Bartunek et al ⁵	19	CD133 ⁺ BM-MNCs	IC	11.6 ± 1.4	LVEF ↑; perfusion ↑; myocardial viability ↑
Randomized					
Ge et al ^{E6}	10	BM-MNCs	IC	<1	LVEF ↑; perfusion ↑
Li et al ^{E7}	35	PB-EPCs	IC	7 ± 5	LVEF ↑; wall motion ↑
BOOST ^{E8,9}	30	BM-MNCs	IC	4.8 ± 1.3	LVEF ↑ (6 mo only)
Janssens et al ^{E10}	33	BM-MNCs	IC	<1	No increase in LVEF; infarct size ↑; regional systolic function ↑
REPAIR-AMI ^{E11,E12}	102	BM-MNCs	IC	4.3 ± 1.3	LVEF ↑; greater benefit in <49% LVEF and >5 d after MI
ASTAMI ^{E13}	50	BM-MNCs	IC	6.0 ± 1.3	No benefit

MI, Myocardial infarction; BM-MNC, bone marrow mononuclear cell; IC, intracoronary; PB-EPC, peripheral blood-endothelial progenitor cells; LVEF, left ventricular ejection fraction; ESV, end-systolic volume. *Number of patients receiving cells.

TABLE E2. Levels of circulating proinflammatory and anti-inflammatory cytokines

	Control	HCD	HCD with arginine	P value*
Proinflammatory cytokines				
IL-1 α	1.00 ± 0.22	0.60 ± 0.18	0.39 ± 0.11	.06
IL-1 β	1.00 ± 0.15	0.97 ± 0.21	0.63 ± 0.09	.17
IL-2	1.00 ± 0.68	0.28 ± 0.20	0.68 ± 0.52	.6
IL-4	1.00 ± 0.58	1.70 ± 1.13	0.77 ± 0.48	.67
IL-6	1.00 ± 0.21	0.32 ± 0.15	0.07 ± 0.04	<.001
IFN- γ	1.00 ± 0.26	0.52 ± 0.23	0.37 ± 0.19	.16
TNF- α	1.00 ± 0.15	0.83 ± 0.15	0.85 ± 0.21	.78
Anti-inflammatory cytokines				
IL-10	1.00 ± 0.45	0.70 ± 0.20	0.61 ± 0.05	.54
GM-CSF	1.00 ± 0.29	0.40 ± 0.24	0.31 ± 0.11	.08
P/A ratio	1.25 ± 0.25	2.40 ± 0.70	1.30 ± 0.32	.16

Levels of individual cytokines are mean ± SEM, obtained from serum 3 weeks after cell transplant into ischemic hind-limb muscle and normalized to mean level of control (normal diet) group. HCD, High-cholesterol diet; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; P/A ratio, ratio of proinflammatory to anti-inflammatory cytokines. *P values calculated with 1-way analysis of variance.

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