Endothelial Progenitor Cells Are Decreased in Blood of Cardiac Allograft Patients With Vasculopathy and Endothelial Cells of Noncardiac Origin Are Enriched in Transplant Atherosclerosis

David Simper, MD*; Shaohua Wang, MD*; Arjun Deb, MD; David Holmes, MD; Christopher McGregor, MD; Robert Frantz, MD; Sudhir S. Kushwaha, MD; Noel M. Caplice, MD, PhD

Background—Recent studies in animals suggest that circulating recipient endothelial precursors may participate in the biology of transplant vasculopathy. It is currently unknown whether a similar interaction between recipient endothelial cells and the vessel wall occurs in human subjects undergoing allogeneic cardiac transplantation.

Methods and Results—Circulating endothelial cells and endothelial progenitor cells (EPCs) were quantified in 15 cardiac transplantation subjects with and without angiographic evidence of vasculopathy. In a separate series of experiments, the origin (donor or recipient) of transplant plaque endothelial cells was assessed in subjects who had undergone a gender-mismatched cardiac transplantation and had histological evidence of severe vasculopathy at the time of heart explantation. Circulating EPC outgrowth colonies in peripheral blood were significantly reduced in subjects with transplant vasculopathy compared with those without angiographic evidence of disease (EPC colony-forming units [CFUEPC]: 4.5 ± 1.9 versus 15.1 ± 3.7, \( P < 0.05 \)). There was no significant difference in circulating endothelial cell numbers as defined by day 4 culture acetylated LDL/lectin assay in either of these patient groups. In a separate group of 5 subjects who underwent gender-mismatched cardiac transplantation, there was a significant seeding of recipient endothelial cells (range: 1% to 24% of all luminal endothelial cells) in large-vessel lumen and adventitial microvessel lumen of arteriopathic vessels. No opposite-sex chimeric cells were observed in control gender-matched transplantation scenarios.

Conclusions—These data suggest that the human cardiac transplant arteriopathy is associated with reduction in circulating endothelial precursors and with seeding of recipient-derived endothelial cells at the site of plaque development. (Circulation. 2003;107:143-149.)

Key Words: blood cells ▪ endothelium ▪ transplantation

Cardiac transplant atherosclerosis has traditionally been regarded as a chronic form of vascular rejection consisting of concentric intimal hyperplasia with perivascular inflammation in both epicardial and smaller intramyocardial coronary arteries.1 Until recently, the origin of endothelial cells within transplant atherosclerotic plaque was presumed to be the local vessel wall.2 However, in light of several studies suggesting a recipient origin for plaque endothelial cells in animal models of transplant atherosclerosis,3–5 a question has arisen as to what role circulating endothelial cells (CECs) may play in graft atherosclerosis. For instance, Hillebrands and colleagues6 have demonstrated that recipient endothelial cells effectively replace all donor endothelial cells of plaque in rat models of aortic allograft rejection. This endothelial cell replacement was also shown in a renal allograft model to be a direct response to vascular injury.6 The precise origin of these recipient endothelial cells recruited to plaque currently remains unknown, but possibilities include circulating vessel wall–derived adult endothelial cells, angioblasts, or recipient bone marrow–derived cells.

Indeed, the exact nature of all CECs in vivo is currently unclear, but there seem to be at least 2 distinct endothelial phenotypes that can be derived from human blood.7 CECs can be cultured early (within 1 week) from buffy coat mononuclear cells (MNCs), but these cells have limited growth capacity in vitro, whereas endothelial progenitor cells (EPCs), which appear as colonies \( \approx 1 \) month after initial MNC culture, have a much greater (\( \approx 50- \) to 100-fold)
capacity for extended exponential growth. These latter cells have many of the characteristics of progenitors undergoing multiple population doublings and exhibit an angioblastic phenotype. In a series of elegant experiments, Lin and colleagues have also shown that CECs originate from the adult vessel wall, whereas EPCs most likely derive from circulating angioblasts.

In the present study, we hypothesized that if CECs are implicated in the biology of human atherosclerotic plaque formation after cardiac transplantation, then CECs and EPCs in the peripheral blood of these subjects may reflect this process. Thus, we determined the numbers of CECs or EPCs in cardiac transplantation subjects with established coronary arteriopathy compared with matched transplantation subjects without evidence of vascular disease. We also hypothesized that recipient-derived endothelial cells would be present in transplant atherosclerotic plaque of donor hearts. To test this, we determined the origin of atherosclerotic plaque endothelial cells (donor or recipient) in gender-mismatched cardiac transplantation subjects who had developed severe arteriopathy.

Methods

Study Subjects, Blood Sampling, Endomyocardial Biopsy, and Autopsy Tissue

We examined blood samples from 15 human cardiac transplantation subjects who had either angiographic evidence of transplant coronary arteriopathy (n=8) or no evidence of arteriopathy (n=7). "Angiographic evidence of transplant arteriopathy" was defined as >20% stenosis in a main, branch epicardial, or intramyocardial coronary artery. "No disease" was defined as normal-appearing coronary artery anatomy. Subjects were matched for the presence or absence of cardiac risk factors and for the presence of coexistent statin administration, a therapy known to alter endothelial progenitor number. Fresh blood was collected by venipuncture and anticoagulated in citrate phosphate dextrose solution (CPD) (Baxter). In addition, healthy human volunteer donors (n=6) not taking medications had blood taken for similar analysis.

In a separate series of experiments, coronary artery specimens from explanted hearts of 5 male subjects who had received a gender-mismatched cardiac transplant were studied. Two same-gender cardiac transplantation subjects were examined as controls. In addition, endomyocardial biopsy tissue from 2 gender-mismatched subjects included in the circulating progenitor cell study was also analyzed. The institutional review board approved all protocols, and all patients gave consent before transplantation for their tissue to be used in research studies.

Buffey Coat Preparation and Endothelial Cell Culture

Human MNCs were initially isolated from peripheral buffey coat blood in Histopaque-1077 followed by washing in MCDB 131 supplemented with hydrocortisone, antibiotics, and 10 ng/mL vascular endothelial growth factor (VEGF). MNCs were then suspended in endothelial cell growth medium-2, placed on a 6-well plate coated with collagen type I (Becton Dickinson), and grown in culture as previously described. CEC Culture and Quantitative Analysis

Initial cultures of CECs were established, and at 4 days the numbers of CECs were quantified using acetylated LDL (AcLDL) and isolectin B4 incorporation as previously described. Cells were classified as CECs if they showed dual positivity for both intracellular dyes, and the number of cells was determined by photographic analysis of 4 random high-powered fields in which positive fluorescent cells were counted on a predetermined grid. Each counting was performed at least 3 times for each subject. We and others have previously characterized these cells as having limited growth potential, becoming senescent after 2 to 3 weeks in culture.

EPC Culture and Quantitative Analysis

EPCs were established from endothelial outgrowth colonies, which began to appear ~3 to 4 weeks after initial MNC culture. An EPC colony was defined by the appearance of a colony rosette of >30 polygonal cells, which subsequently expanded to a confluent "cobblestone" endothelial monolayer. New colonies were counted for each subject between the time of first colony appearance to 6 weeks after initial MNC culture. The number of EPC colonies appearing over the 6-week culture period was expressed as CFUEPC (EPC colony-forming units).

Fluorescence-Activated Cell Sorter and Immunofluorescent Analysis

In Situ Hybridization

Formalin-fixed, paraffin-embedded blocks of gender-matched and gender-mismatched transplant atherosclerotic plaque were cut into 4-μm sections and placed on microscope slides. In additional experiments, formalin-fixed paraffin blocks of endomyocardial biopsy specimens from subjects in the CEC study who had reduced EPC colony formation and had a gender-mismatched cardiac allograft were also analyzed. All sections were deparaffinized using Citrisolv (Fisher Scientific) and rehydrated in an ethanol series. Immunohistochemical analysis was performed using a monoclonal antibody against CD31 (Dako). The secondary antibody used was an antimouse antibody conjugated to Cy-3 (Molecular Probes) (red stain). After immunostaining, fluorescent in situ hybridization (FISH) was immediately performed. The tissue was dehydrated twice in 100% ethanol for 1 to 2 minutes and then heated in a steamer in preheated 1 mmol/L EDTA (pH 8.0) for 20 minutes, followed by 0.05 μg/μL proteinase K (Sigma) or pepsin A (2100 U/mg) in buffer (0.05 mol/L Tris HCl, 2 mmol/L CaCl₂ [pH 7.8], 0.01 mol/L EDTA, 0.01 mol/L NaCl) at 37°C for 15 minutes. The tissue was then rinsed in an ethanol series. Subsequently, the hybridization probe cocktail (Vysis Inc, 30-804824) was applied to the sections. The DNA probe used was specific for the α satellite region of the Y chromosome and was fluorescently labeled. The Y chromosome probe (CEPY, Vysis Inc, B-6927) was labeled with fluorescein isothiocyanate (FITC-green). For combined immunostaining and FISH, CD31, CD34, VEGFR2, or Tie-2 R-Cy3 staining with Y chromosome FITC labeling was used.

Coverslips were affixed and sealed with rubber cement, and the slides were incubated at 80°C for 3 minutes to denature all DNA and then were incubated at 37°C overnight. After hybridization, the slides were washed in 2× SSC/0.1% NP 40 solution at room temperature, counterstained with 0.03 μg/mL 4′,6-diamidino-2-phenylindole (DAPI), and mounted with Vectashield (Vector Laboratories, H-1200). FISH signals were enumerated by using a Zeiss Axiosplan microscope equipped with a triple-pass filter (Vysis).

Data and Statistical Analysis

All data are presented as the mean±SEM. Comparison between groups was made using 1-way ANOVA. A P value <0.05 was considered statistically significant. The χ² test was used for comparison of categorical variables.
Experienced independent observers blinded to the sex of the donor and recipient reviewed each section. Sections were reviewed on the same microscope to ensure the image was consistent and reproducible. Only sections that contained clear morphology with adequate immunohistochemical and FISH staining were considered for analysis. Cells were considered endothelial in origin if they were positioned on the luminal side of plaque, thin and elongated in shape, and surrounded by CD31 staining. A nucleus was considered of male origin if there was a clear green signal.

The number of recipient-derived cells that were CD31 positive was expressed as a percentage of all plaque luminal and adventitial microvessel endothelial cells counted in the arteriopathic vessel. The summation of counts from each subject was averaged and expressed as percentages ($\pm$SD) of the total cell counts within these vascular regions. A total of 35,300 nuclei were analyzed, approximating 6000 nuclei per sex-mismatched subject ($n=5$) and 2000 nuclei per sex-matched subject ($n=2$). Finally, an additional 1300 nuclei were analyzed to determine the level of endothelial cell chimerism in nondiseased coronary arteries of allografts also containing vessels with atherosclerosis.

Results

Clinical Characteristics of Study Subjects

The clinical characteristics of all CEC study subjects are summarized in Table 1. Briefly, all study subjects were nonsmokers, were treated with statins at equivalent dosages, and had similar blood pressure levels. Subjects were well matched for all other baseline risk factors, including age and circulating C-reactive protein levels, and there was no statistically significant difference between the diseased and nondiseased groups in any of the clinical or laboratory parameters studied. There was also no significant difference between groups in the time interval between initial transplantation and blood MNC isolation. In the case of coronary artery tissue analysis, the baseline characteristics of all study subjects are shown in Table 2.

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<th>TABLE 1. Baseline Clinical and Laboratory Characteristics of Endothelial Progenitor Study Patients</th>
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<td>Male sex, No. (%) of patients</td>
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<td>Time from transplantation to MNC isolation, mo</td>
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<th>TABLE 2. Baseline Clinical Characteristics of Gender-Mismatched Cardiac Transplantation Subjects</th>
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CMP indicates cardiomyopathy; OKT3, Muromonab-CD3; TLI, total lymphoid irradiation; cyclo, cyclosporine; and AZA, azathioprine.

No chimeric endothelial cells were detected in same-gender control cardiac transplantation subjects.
EPC Colony Analysis

EPC colonies were observed in diseased and nondiseased groups; they began to appear at \( \sim 2 \) to 3 weeks after initial culture and were observed over the next 3 weeks. There was a significant reduction in the number of endothelial outgrowth colony-forming units (CFUEPC) in subjects with vasculopathy (filled bar) compared with subjects without disease (open bar). FACS showing strongly positive CD31 labeling of EPCs (open histogram) compared with immunoglobulin G control (filled histogram). C and E, Positive immunofluorescence labeling (green) of EPCs using antibodies to VE-cadherin (C) and von Willebrand factor (E) with lack of labeling using isotype-matched immunoglobulin G control antibodies (D and F). The nuclei in panels C through F are indicated by Hoechst blue stain.

CEC Analysis

CECs were assessed at day 4 after initial culture by digital microphotography of attached endothelial cells on collagen type-1, positive AcLDL uptake, and positive fluorescence for isolectin B4 fluorochromes. There was no significant difference in the number of attached CECs (measured by numerous criteria, including AcLDL, lectin, or combined staining) between diseased and nondiseased subjects (Figure 2, A through F). There also was no significant difference between the CEC count in a healthy volunteer group \((n=6)\) without medications \((6.3 \pm 4.6)\) and the diseased transplantation subjects.

Detection of Recipient Endothelial Cells in Diseased and Undiseased Vessels of Gender-Mismatched Cardiac Transplantation Subjects

Five male subjects who had undergone gender-mismatched cardiac transplantation were studied using combined endothelial marker immunohistochemistry and FISH. Two subjects who had received same-gender cardiac transplantation served as controls. The clinical characteristics of all subjects studied are shown in Table 2. Recipient endothelial cells that were CD31 and Y chromosome positive were detected in eNOS, VEGF R1, VEGFR, and Tie-2 receptor. The CFU_{EPC} count in a healthy volunteer group \((n=6)\) without medications was \(6.3 \pm 4.6\).
diseased vessels of gender-mismatched female cardiac transplantation subjects (Figure 3). These chimeric cells lined the lumen of medium-sized coronary arteries and microvessels within the adventitia of the vessel wall. The mean percentages of recipient-derived luminal and adventitial microvascular endothelial cells in diseased segments of allografts with atherosclerotic coronary arteries (1100 nuclei analyzed) was 0.2 ± 0.02%. One subject (patient 3) died 4 days after transplantation but still showed evidence of recipient endothelial cell chimerism in the coronary vessel wall. No correlation was seen between the level of recipient endothelial cell chimerism and the time from transplantation when the donor heart was explanted. No evidence of opposite-sex chimerism was detected in same-gender cardiac transplantation scenarios (data not shown).

The efficiency of combined FISH/immunostaining in detecting male CD31-positive endothelial cells in same-gender male transplants (4200 nuclei analyzed) was 41.2 ± 5.6%. To further characterize the potential angioblastic lineage of recipient endothelial cells in donor coronary vessels, combined FISH for Y chromosome and CD34, VEGFR-2, and Tie-2R staining was performed. Recipient male endothelial cells within female donor epicardial coronary arteries and microvessels were positive for each of these markers (Figure 5).

Finally, to correlate recipient endothelial cell chimerism seen in the FISH study (Table 2) with reductions in CFU_EPC seen in the CEC study (Table 1), additional FISH analysis of graft biopsies from any female cardiac allografts transplanted in male recipients that were included in the circulating endothelial study were performed. Two subjects met these gender-mismatch criteria, and both had angiographic evidence of arteriopathy, reduced CFU_EPC count, and FISH/immunostaining evidence of recipient CD31-positive endothelial cells within microvessels in the biopsy tissue (Figure 6).

**Discussion**

Recent studies in animals show that atherosclerotic plaque endothelium in animal models of transplant vasculopathy may derive from the recipient circulation. In the present study, we
present evidence that human circulating EPCs, but not CECs, are significantly decreased in subjects with transplant atherosclerosis compared with matched transplantation subjects without evidence of disease. We also show that recipient endothelial cells are significantly recruited to the lumen of epicardial vessels and adventitial microvessels of coronary artery segments after cardiac transplantation. These are, to our knowledge, the first human data showing differential circulating EPC numbers in subjects who have developed transplant atherosclerosis and seeding of recipient endothelial cells to donor coronary arteries and areas of transplant atherosclerosis.

There are several potential mechanisms to explain the different levels of circulating EPCs seen in our 2 study groups. It is possible that EPCs or their putative progenitors may have been recruited into the plaque during development of transplant atherosclerosis. Previous animal data\(^3,4\) and our chimeric analysis would support such endothelial recruitment from the recipient circulation. Previous studies have suggested qualitative differences in EPCs of subjects with coronary artery disease compared with subjects without disease.\(^1\) It is currently unknown why EPCs might be reduced in primary atherosclerosis or transplant atherosclerosis, but possibilities include ongoing recruitment of EPCs to sites of endothelial injury or dysfunction in the transplanted heart. Our data provide circumstantial evidence for but not proof of depletion of a circulating pool of EPCs (decreased colonies in transplant arteriopathy patients) with seeding of (most likely) circulating recipient-derived endothelial cells to areas of vascular injury (recipient CD31-positive cells in areas of transplant vasculopathy). Although our study design cannot prove a circulating origin for the CD31/Y chromosome-positive cells seen in arteriopathic vessel segments, numerous animal studies already support this concept.\(^3,5\) Moreover, higher levels of endothelial cell chimerism seen in diseased (1% to 25%) compared with nondiseased segments (0.2%) support preferential seeding of these cells to sites of atherosclerotic plaque development.

An alternative explanation for diminished EPCs may involve molecules released (in the context of transplant arteriopathy or chronic low-grade rejection) that alter mobilization, migration, cell survival, or intravascular seeding functions of EPCs after transplantation. Recent in vitro data, for instance, suggest that C-reactive protein can downregulate endothelial cell survival, leading to premature senescence and predisposition to apoptosis.\(^12\) It is conceivable that proinflammatory molecules might also alter circulating EPC number and function. The exact biological role of circulating angioblasts is still not completely clear, but on the basis of a range of animal and human studies, it seems that these cells play an active part in vascular homeostasis. Indeed, while this article was in preparation, another group reported that in healthy men, circulating EPC number may offer a surrogate marker of vascular function. Hill and coworkers\(^13\) showed an inverse correlation between the number of EPC colonies in peripheral blood and endothelial dysfunction (measured by brachial artery ultrasound) or cardiovascular risk (measured by Framingham risk score). Our data showing diminished EPC number in arteriopathic subjects are consistent with this finding and extend to transplant atherosclerosis a paradigm of abnormal EPC regulation in the presence of vascular disease.

The present study would also suggest that the biological contribution of CECs may differ from EPCs during the development of transplant arteriopathy. The similar CEC numbers seen in both groups of patients in our study is not surprising if we consider that these cells expand poorly in vitro and become senescent or undergo apoptosis at 2 to 3

**Figure 5.** Recipient endothelial cells stain positive for angioblastic markers in mildly and moderately diseased atherosclerotic vessels and microvessels of subjects with gender-mismatched cardiac allografts. A, Recipient male endothelial cells (arrows) stain positive for Tie-2 receptor (Cy3-red). Asterisk denotes a microvessel within the plaque (65× objective). B, Recipient male endothelial cell staining positive for VEGFR2 (red). Closed arrowhead indicates the internal elastic lamina of the vessel wall; open arrowhead, male recipient cells staining negative for VEGFR2 (65× objective). C, Recipient male endothelial cells (arrows) lining an atherosclerotic plaque and staining positive for CD34 (red). Bloodborne recipient cells (open arrowhead) within the lumen also stain positive for CD34. I indicates intimal plaque (65× objective). D, Recipient endothelial cells (arrows) line a microvessel and stain positive for CD34 (red). Bloodborne cells (open arrowheads) within the lumen of the microvessel also stain positive for CD34 (100× objective). In panels A through D, the lumen is indicated by L.

**Figure 6.** Recipient male cells within endomyocardial biopsy tissue of 2 male endothelial progenitor study subjects (Table 1) who had female cardiac allografts and had evidence of reduced CFU<sub>EC</sub> count. A and B, Male recipient cells staining positive for CD31 (arrows) within microvascular structures (*) in endomyocardial biopsy tissue. Open arrowhead in each panel indicates a male recipient cell staining negative for CD31 (65× objective).
weeks after initiation of culture. This limited replication capacity suggests that these cells are similar to the adult vessel wall–derived endothelial cells previously described by Lin and colleagues. We speculate that these CECs may represent the remnants of injured endothelium removed from the vessel lumen after allotransplantation, becoming freely detectable in the circulation.

The origin of recipient endothelial cells in experimental models of transplant atherosclerosis is still unclear, but numerous possibilities exist, including circulating or myocardial progenitors, or adult endothelial cells from recipient vascular or rump atrial tissue. Regardless of the precise origin of these cells, there seems to be progressive recruitment of recipient vascular cells after donor cell loss early after transplantation. In the present study, seeding of these recipient endothelial cells seems to have occurred as early as day 4 after transplantation. Although later time points after transplantation seem in general to have more vessel wall accumulation of these cells, a conclusive temporal seeding relationship for recipient endothelial cells cannot be inferred from this small number of subjects. It is conceivable, however, that precursor cells are recruited early (as in patient 3, Table 2) and late either during or after development of transplant arteriopathy. It is also tempting to speculate that precursor cells may be continuously recruited to areas of donor endothelial dysfunction in a cycle of continuous repair and in the context of ongoing donor-recipient alloimmune interactions.

In conclusion, we have shown that EPC colonies are significantly decreased in the circulation and recipient endothelial cells are enriched in the donor coronary arteries of subjects who develop transplant vasculopathy. These data support a role for EPCs in the transplant arteriopathic process, but further studies will be required to elucidate the nature and mechanism of circulating EPC participation in the cardiac transplant vascular biology.

Acknowledgments

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References

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