Human Umbilical Cord Blood–Derived Endothelial Cells
Reendothelialize Vein Grafts and Prevent Thrombosis


Objective—To accelerate vein graft reendothelialization and reduce vein graft thrombosis by infusing human umbilical cord blood–derived endothelial cells (hCB-ECs) because loss of endothelium contributes to vein graft thrombosis and neointimal hyperplasia.

Methods and Results—Under steady flow conditions in vitro, hCB-ECs adhered to smooth muscle cells 2.5 to 13 times more than ECs derived from peripheral blood or human aorta (P<0.05). Compared with peripheral blood and human aorta ECs, hCB-ECs had 1.4-fold more cell surface α5β1 integrin heterodimers per cell (P<0.05) and proliferated on fibronectin 4- to 10-fold more rapidly (P<0.05). Therefore, we used hCB-ECs to enhance reendothelialization of carotid interposition vein grafts implanted in NOD.CB17-Prkdcscid/J mice. Two weeks postoperatively, vein grafts from hCB-EC–treated mice demonstrated approximately 55% reendothelialization and no luminal thrombosis. In contrast, vein grafts from sham-treated mice demonstrated luminal thrombosis in 75% of specimens (P<0.05) and only approximately 14% reendothelialization. In vein grafts from hCB-EC–treated mice, 33±10% of the endothelium was of human origin, as judged by human major histocompatibility class I expression.

Conclusion—The hCB-ECs adhere to smooth muscle cells under flow conditions in vitro, accelerate vein graft reendothelialization in vivo, and prevent vein graft thrombosis. Thus, hCB-ECs offer novel therapeutic possibilities for vein graft disease. (Arterioscler Thromb Vasc Biol. 2010;30:2150-2155.)

Key Words: endothelial function ■ thrombosis ■ vascular biology ■ vascular surgery ■ integrin

Saphenous vein graft failure from thrombosis occurs with remarkable frequency: approximately 3% to 12% within the first postoperative month1 and 28% within the first postoperative year.2 Vein graft thrombosis is generally attributed to alterations in the vessel wall and flow dynamics, vein graft endothelial damage associated with both vein harvest and vein graft distension by arterial pressure, and consequent compromise of anticoagulant vessel properties.1 Among these factors, the most practical target for therapy appears to be maintenance or restoration of the vein graft endothelium.

Reendothelialization of vein grafts is a relatively slow process that involves proliferation and migration of both graft-intrinsic endothelial cells (ECs) and graft-extrinsic ECs,3 which may derive from adjacent arterial endothelium or circulating endothelial progenitor cells (EPCs).4,5 Consequently, accelerating EC growth or increasing EC number with exogenous ECs may accelerate vein graft reendothelialization. Exogenous ECs derived from peripheral and umbilical cord blood EPCs are relatively easy to obtain and possess substantial replicative capacity.6

Most studies that have examined adhesion of ECs to damaged vasculature have used ECs obtained from early-outgrowth cultures of EPCs, which are of a myeloid lineage and do not exhibit the antithrombotic properties of ECs.7–9 Few vascular repair experiments have examined ECs derived from late-outgrowth cultures of EPCs isolated from adult or umbilical cord blood. These late-outgrowth EPCs, or endothelial colony-forming cells, exhibit characteristics only of ECs and not of monocytes.10,11 ECs obtained from late-outgrowth cultures of peripheral blood EPCs (PB-ECs) from healthy donors and patients with coronary artery disease (CAD) are similar to aortic ECs with regard to proliferation, viability, adhesion, and antithrombotic function.12 However, the clinical use of autologous PB-ECs is limited to elective procedures because of the time required to isolate and expand them in culture. The suitability of PB-ECs for vein graft repair may be further limited because PB-ECs cannot be isolated from a substantial minority of patients with CAD.12 This limitation may be circumvented by using ECs derived from late-outgrowth cultures of human umbilical cord blood EPCs (hCB-ECs), which have demonstrated a variety of EC properties13 and can be cultured in advance so that sufficient numbers are available for emergency procedures. To facilitate
nonautologous transplantation, hCB-ECs can be matched to donors with compatible major histocompatibility class (MHC) I proteins.\textsuperscript{14,15} Compared with hCB lineage-negative mononuclear cells previously used to reduce vein graft neoimal hyperplasia,\textsuperscript{16} hCB-ECs would be expected to exhibit more uniform EC function and to lack monocytc function.

To assess the suitability of hCB-ECs for therapeutic reendothelialization of vein grafts, we first compared the adhesion, integrin expression, and growth of hCB-ECs with ECs derived from late-outgrowth cultures of adult human PB-ECs and with human aortic ECs in vitro. Subsequently, we used hCB-ECs in immunocompromised mice subjected to vein grafting.

**Methods**

Expanded methods are provided in the supplemental material (available online at http://atvb.ahajournals.org).

**Cell Culture and Adhesion Experiments**

We isolated and cultured hCB-ECs, PB-ECs, and human aorta ECs (HAECs), as previously described, and characterized them regarding the expression of CD31, Krüppel-like factor (KLF)-2, endothelial NO synthase, and cyclooxygenase-2 and the lack of CD14 expression.\textsuperscript{2,13} Smooth muscle cells (SMCs) were prepared, as previously described,\textsuperscript{17} and were plated at 80 000 cells per centimeter squared, with SMC growth media on fibronectin-coated polystyrene. After seeding, SMCs were maintained in serum-free medium for 7 days. One day before the experiment, the medium was replaced with serum-free medium containing extracellular matrix proteins (fibronectin, collagen I, and collagen III). To test adhesion, hCB-ECs, PB-ECs, or HAECs were superfused over SMCs in a parallel plate flow chamber for 4 days. The number of adherent cells per centimeter squared was determined.

In integrin-blocking studies, ECs were incubated with anti–integrin α1 integrins, present on the plasma membrane of hCB-ECs, PB-ECs, and HAECs, respectively, was determined by cell surface immunofluorescence and flow cytometry, as previously described.\textsuperscript{13,19} ECs were seeded at subconfluent density (25 000 cells/cm\(^2\)); after 15 minutes of static incubation, the ECs were placed inside a parallel plate flow chamber\textsuperscript{18} at a shear stress of 0.5 dyne/cm\(^2\). After 10 minutes, the number of adherent cells per centimeter squared was determined. In integrin-blocking studies, ECs were incubated with anti–integrin IgGs for 30 minutes and then superfused over SMCs for 5 minutes. The net force imposed on the ECs during the dynamic adhesion experiments was determined by assuming that the ECs were spherical when initially adhered, as described in the supplemental methods.

**Flow Cytometry and EC Proliferation**

The expression of α\(_{\beta_1}\), α\(_{\beta_2}\), and α\(_{\beta_3}\) integrins, present on the plasma membrane of hCB-ECs, PB-ECs, and HAECs, respectively, was determined by cell surface immunofluorescence and flow cytometry, as previously described.\textsuperscript{13,19} ECs were seeded at subconfluent density (25 000 cells/cm\(^2\)); after 15 minutes of static incubation, the ECs were placed inside a parallel plate flow chamber for 4 days. The number of cells present on days 0, 2, and 4 for static conditions and on days 0 and 4 for flow conditions was used to find the approximate doubling time of the cells.

**Vein Graft Surgery and hCB-EC Injections**

All animal experiments conformed to protocols approved by the Duke Institutional Animal Care and Use Committee. Interposition vein grafting of the common carotid artery was performed as previously described, except that NOD.CB17-Prkdc\textsuperscript{-/-}J (“SCID”) mice were used, to avoid immune-mediated rejection of the hCB-EC xenotransplantation. Before infusion into mice, hCB-ECs were trypsinized and rinsed with trypsin-neutralizing solutions. The hCB-ECs were then resuspended in Iscove-modified Dulbecco medium (Invitrogen, Carlsbad, Calif) at 6.7 × 10\(^6\) cells per milliliter; 1 × 10\(^6\) hCB-ECs (or a corresponding volume of cell-free medium) were injected at surgery (33% directly into the vein graft lumen for 15 minutes before establishing vein graft blood flow and 67% intravenously). Four days postoperatively, 1 × 10\(^6\) hCB-ECs were again injected intravenously. Vein grafts were harvested 2 weeks postoperatively, placed in optimal cutting temperature medium, and frozen at −80°C before sectioning.

**Histology**

Serial vein graft sections were stained for CD31, which stains all ECs, and human MHC I, using hematoxylin-eosin and routine methods.\textsuperscript{2,22} To quantitate vein graft endothelialization and hCB-EC–derived endothelialization, we used computer software (ImageJ) from the National Institutes of Health to measure the vein graft luminal perimeter and the percentage of this perimeter that stained for CD31 alone or CD31 and (on serial sections) human MHC I, respectively (n=8 for both hCB-EC– and control-treated mice).

**Data Analysis**

Data are presented as mean±SD in the text and mean±SE in the figures. Proportions were analyzed by the Fisher exact test. Multiple group means were compared by 1-way ANOVA with the Tukey post hoc test for multiple comparisons.

**Results**

**hCB-ECs Demonstrate Superior Dynamic Adhesion and Proliferation**

Previously, we found that hCB-ECs express characteristic EC proteins (ie, von Willebrand factor, CD31, and VE-cadherin) but not the monocyte marker CD14.\textsuperscript{15} Furthermore, we found that hCB-ECs responded to flow in a manner characteristic of vascular endothelium: by increasing NO release and upregulating mRNA for KLF-2, endothelial NO synthase, cyclooxygenase-2, and thrombomodulin.\textsuperscript{13} For the current study, we first compared hCB-ECs, PB-ECs, and HAECs with regard to CD31 and VE-cadherin expression and found all EC types to be homogenously equivalent (Figure 1A and data not shown).

Because the goal of this study was to mitigate vein graft disease with exogenous ECs, we sought to test EC adhesion by using a model of the vein graft’s subendothelial surface exposed after the vein is overdistended by arterial pressure, when ECs and their basement membrane no longer cover the entire luminal surface of the vein graft.\textsuperscript{23} This subendothelial surface of the vein comprises interstitial collagens I and III and fibronectin,\textsuperscript{24,25} in addition to basement membrane constituents, such as laminin and collagen IV.\textsuperscript{24} We used cultured quiescent SMCs to model this subendothelial surface and found, by immunofluorescence, that the endogenous SMC matrix includes fibronectin, laminin, and collagens I, III, and IV (supplemental Figure I and data not shown). To augment EC adhesion to SMC monolayers, we adsorbed exogenous fibronectin, collagen I, and collagen III to the SMCs (supplemental Figure II). Next, we compared dynamic adhesion among hCB-ECs, PB-ECs from healthy subjects, PB-ECs from subjects with CAD, and HAECs, at a shear stress of 0.5 dyne/cm\(^2\). After dynamic adhesion for 10 minutes, hCB-ECs adhered approximately 3 times greater than HAECs or PB-ECs from healthy adults (PB-ECs[healthy]) (Figure 1B).

One possible explanation for the superior adhesion of hCB-ECs derives from their size: the cross-sectional area of hCB-ECs was significantly smaller than that of other ECs: 239±10 μm\(^2\) for hCB-ECs and 464±46, 692±40, and 428±22 μm\(^2\) for PB-ECs(CAD), PB-ECs[healthy], and HAECs, respectively (P<0.05). Consequently, the force exerted by flowing medium on hCB-ECs was only half that
exerted on other ECs (Equations 2a–2c in the supplemental materials). Indeed, an inverse relationship between adherence and net force per cell was observed (Figure 1C).

Another potentially important factor in determining EC adherence under shear stress is EC integrin activity. By using IgG to block integrins αβ1, αβ2, or αβ3, we found that dynamic adhesion of hCB-ECs to an SMC/matrix substrate was strongly dependent on the αβ1 and αβ3 integrins (Figure 2A). By using SMC monolayers in the absence of exogenous matrix proteins, concordant findings were previously obtained: αβ1 and αβ3 integrins mediated static adhesion of hCB-ECs. To determine whether EC type-specific expression levels of αβ1 or αβ3 integrins could explain why hCB-ECs adhere to the SMC/matrix substrate more avidly than other ECs (Figure 1B), we quantitated cell surface integrin expression on all of our EC types by flow cytometry. The cell surface expression of the αβ1 integrin was approximately 45% higher on hCB-ECs than on PB-ECs or HAECs (Figure 2B), and the cell surface expression of αβ1 was similar on all EC types (Figure 2B). Thus, because all EC types expressed similar αβ1 integrin levels, the superior adhesion of hCB-ECs seems unlikely to derive from their αβ1 integrin expression. Rather, the superior adhesion of hCB-ECs may derive in part from higher expression levels of cell surface αβ3 integrins. These experiments do not exclude the possibility that superior hCB-EC dynamic adhesion also derives from additional integrins or hCB-EC-specific differences in integrin-promoted signaling.

The adhesion of ECs to denuded vascular surfaces would optimally be followed by EC proliferation to facilitate reendothelialization. Accordingly, we tested the ability of hCB-ECs, PB-ECs, and HAECs to proliferate under static and flow conditions on fibronectin-coated plastic. Although EC proliferation declined with shear stress, hCB-ECs demonstrated doubling times that were 4 to 13.7 times shorter than those of PB-ECs and HAECs under flow conditions (Table). Because hCB-ECs demonstrated adhesion and proliferation that was superior to PB-ECs and HAECs, we tested the ability

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Table. EC Proliferation as a Function of Shear Stress

<table>
<thead>
<tr>
<th>Shear Stress, dyne/cm²</th>
<th>hCB-EC</th>
<th>PB-EC(CAD)</th>
<th>HAEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (static)</td>
<td>1.7±0.2 †</td>
<td>3.0±0.7 †</td>
<td>3.0±0.6 †</td>
</tr>
<tr>
<td>5</td>
<td>3.4±1.6 ‡</td>
<td>15.6±7.8</td>
<td>31.8±5.5</td>
</tr>
<tr>
<td>15</td>
<td>6.0±1.5 ‡</td>
<td>24.0±9.5 ‡</td>
<td>82.4±26.0</td>
</tr>
</tbody>
</table>

*Data are given as mean±SE.
†P<0.05 vs ECs exposed to shear stress.
‡P<0.01 vs PB-EC(CAD) and/or HAEC.
of hCB-ECs to reendothelialize vein grafts. Thus, we performed interposition vein grafting to the common carotid artery with inferior vena cavae (IVCs) in SCID mice, which we treated with cell-free or hCB-EC (xenograft) infusions. We harvested the vein grafts 2 weeks postoperatively when vein graft reendothelialization is incomplete in C57BL/6 mice.20–22 Unlike C57BL/6 mice,16,20–22 NOD.CB17-Prkdcscid mice demonstrated a remarkable thrombotic diathesis: in control mice infused with just cell-free medium, 6 of 8 vein grafts thrombosed. In contrast, 0 of the 8 vein grafts implanted in hCB-EC–treated mice demonstrated thrombosis (P < 0.007). Thus, hCB-EC infusion prevented vein graft thrombosis. Representative vein graft explants demonstrate vein graft thrombosis, whereas cross-sectional photomicrographs demonstrate analogous findings (Figure 3).

To test the hypothesis that hCB-ECs prevented vein graft thrombosis by accelerating vein graft reendothelialization, we immunostained vein grafts for the EC marker CD31, using an IgG that recognizes both human and mouse proteins. Judging by the percentage of vein graft luminal surface that stained with CD31, we found endothelialization of vein grafts from hCB-EC–injected mice to be approximately 4-fold greater than that in control-injected mice with patent vein grafts (Figure 4): 54 ± 6% versus 14 ± 1% of the luminal surface was covered with CD31-staining cells in vein grafts from hCB-EC– and sham-treated mice, respectively.

To determine the contribution of injected hCB-ECs to vein graft reendothelialization, we calculated the percentage of CD31-positive cells that stained for human MHC I antigen on serial sections of the vein grafts. Vein grafts from mice that were not injected with hCB-ECs demonstrated no staining for human MHC I (data not shown). In contrast, the endothelium in vein grafts from mice that were injected with hCB-ECs did stain for human MHC I (ie, 33 ± 6% of the endothelium present in the 2-week-old vein grafts) (Figure 4). Thus, injected hCB-ECs engrafted on the vein graft subendothelial surface and contributed approximately 33% of the ECs during their reendothelialization.

Discussion

To our knowledge, these studies demonstrate for the first time that hCB-ECs adhere to extracellular matrix or SMCs and proliferate under flow conditions not only in vitro but also in vein grafts in vivo. The adhesive properties of the EPC-derived hCB-ECs in vitro proved superior to those of several
lines of adult EPC-derived ECs and aortic ECs. Furthermore, the adhesion of hCB-ECs to vein grafts in vivo prevented vein graft thrombosis.

The greater adhesion of hCB-ECs compared with adult primary ECs is likely influenced by the higher expression of α5β1 integrins and the smaller relative size of hCB-ECs. The inverse relation between the force and number of adherent ECs per centimeter squared (Figure 1C) suggests that the force acting on the cell strongly influences adhesion. EC size affects adhesion in 2 potentially competing ways. First, larger cells experience a greater fluid force and the net fluid force on the cell increases with the square of the cell radius (Equations 2a–2c in the supplemental methods). Second, the contact area between the EC and surface increases with EC radius and equals 2πR, where R is the distance over which adhesion bonds can form and R is the cell radius. The contact area and receptor number are related to the force exerted by the bonds (f b), which is approximately N b A c f b = N b 2πR f b, where N b represents the number of receptors per unit cell surface area and f b is the force per bond. The bond force increases linearly with cell radius, whereas the fluid force increases with R 2. After accounting for the effect of force and contact area on adhesion, increasing cell size should lead to reduced adhesion, as long as receptor expression levels are similar. The relative change in force per cell for 2 cell types of different sizes is N b R c /N b R a. By using Figure 2B to obtain the α5β1 integrin levels and measurements of the cell radius, this ratio between hCB-ECs and the other EC types is close to 1 for all cell types, except for PB-ECs from healthy individuals (0.6 ± 0.2). Thus, the increased contact area for larger cells is offset by the higher receptor expression on hCB-ECs, and the net fluid force acting on the cells is the dominant factor influencing adhesion of the different cell types.

Cell size also affects the likely retention of ECs in the microcirculation. The larger PB-ECs and HAECS are more likely to be limited in their ability to pass through capillaries than the smaller hCB-ECs. However, the hCB-ECs (radius, 8.4 μm) are larger than monocytes (radius, 6.1 μm) and neutrophils (radius, 4.1 μm). Thus, ECs injected into the circulation may be trapped predominantly in the microcirculation. Indeed, autologous EPC-derived ECs infused intravenously have been sequestered in the spleen. Therefore, the most critical steps in promoting EC attachment to the vein graft may occur during static incubation with the vein graft and initial adhesion after injection.

By demonstrating the antithrombotic therapeutic efficacy of hCB-EC infusion, our vein graft studies exploited an unusual tendency to thrombosis in NOD.CB17-Prkd c−/−J mice. Although thrombosis occurred in approximately 75% of our IVC carotid interposition isografts in NOD.CB17-Prkd c−/−J mice, thrombosis occurs in only approximately 2% of our IVC carotid interposition isografts in C57BL/6 mice, with or without the SCID mutation. The mechanisms by which hCB-EC infusion achieves antithrombotic efficacy in NOD.CB17-Prkd c−/−J mice remain to be determined. However, based on our current data, it seems likely that these mechanisms include a reduction in the area of the subendothelial surface exposed to blood and the host of antithrombotic properties inherent in hCB-ECs. Previous studies have shown that thrombosis is inhibited in vitro by EPC-derived ECs. Furthermore, thrombosis of decellularized iliac arteries is inhibited in vivo when these arteries are preseeded in vitro with autologous EPC-derived ECs in a manner that achieves full reendothelialization. Our results are consistent with these studies and demonstrate that infused hCB-ECs adhere to the vein graft luminal surface and inhibit thrombosis, even without complete coverage of the luminal surface. The inhibition of thrombosis in the context of incomplete reendothelialization may rely in part on prostaclin secreted by the hCB-ECs. Of course, the optimal hCB-EC dose and route of administration for preventing vein graft thrombosis remain to be established.

The adhesion of hCB-ECs to the vein grafts is likely facilitated by the reduction in shear stress attendant to the relatively large diameter of the vein grafts used. Assuming unilateral common carotid blood flow of 0.28 mL/min and a common carotid artery radius of 0.15 mm, carotid shear stress would be approximately 52 dyne/cm 2, which is slightly higher than another report of 35 dyne/cm 2. However, if we assume that overall carotid flow and pressure are not altered by the vein graft, we can infer that shear stress in the vein graft is only approximately 1.7 dyne/cm 2 because the radius of the IVC carotid interposition graft is approximately 0.47 mm postoperatively.

Because it was performed in a highly thrombogenic mouse model, our study may overestimate the true benefit of hCB-EC therapy for vein graft thrombosis in humans. Moreover, from a therapeutic perspective, using hCB-ECs to prevent human vein graft thrombosis would require matching donor hCB-ECs and the recipient for MHC antigens. When stimulated with interferon γ or tumor necrosis factor, even late-outgrowth hCB-ECs have expressed significant levels of MHC I, MHC II, and adhesion molecules; and have stimulated proliferation of allogeneic CD4+ T lymphocytes. Moreover, EC tubes formed in SCID mice by hCB-ECs are destroyed when these mice are injected with PB mononuclear cells that are allogeneic to the hCB-ECs. The high proliferative capacity of hCB-ECs may facilitate matching these ECs for MHC antigens to subjects undergoing vein grafting.

In a physiological model of vein grafting, our hBC-EC reendothelialization and antithrombotic data accord well with studies that demonstrate enhanced reendothelialization of injured arteries with administration of PB-ECs. However, it seems likely that there would be a therapeutic advantage associated with the superior adhesive and proliferative properties of hCB-ECs. It remains to be determined whether in vivo comparisons of hCB-ECs with other EPC-derived ECs will support this hypothesis.

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SUPPLEMENTAL MATERIAL

Expanded Methods

Cell Culture
Human cord blood derived endothelial cells (hCB-ECs) and peripheral blood derived endothelial cells (PB-ECs) were isolated as previously described by Ingram et al.\textsuperscript{1,2} Umbilical cord blood was obtained from the Carolina Cord Blood Bank at Duke University per protocols approved by the Duke University Institutional Review Board (IRB). Prior to receipt, all patient identifiers were removed. Peripheral blood samples were obtained from two groups of subjects: (1) young healthy, non-smoking volunteers taking no medicines (n = 4); (2) patients undergoing left heart catheterization that gave consent at Duke University Medical Center (n = 4). The Duke University Institutional Review Board approved the protocol for collection and use of human blood employed in this study. The patients’ medical histories are described in Stroncek et al.\textsuperscript{3}

After collection, blood was diluted 1:1 with Hanks Balanced Salt Solution (HBSS, Invitrogen), placed onto Histopaque 1077 (Sigma), and centrifuged at 740×\textit{g} for 30 minutes. Buffy coat mononuclear cells were collected and washed three times with “complete EC growth medium,” comprising 8% (vol/vol) fetal bovine serum (FBS) added to “EC growth medium”: Endothelial Basal Media-2 (Cambrex) supplemented with Endothelial Growth Media-2 SingleQuots (containing 2% FBS [final] plus growth factors, Cambrex), and 1× antibiotic/antimycotic solution (Invitrogen). Mononuclear cells were plated on plastic coated with collagen I (rat tail, BD Biosciences) in complete EC growth medium. Medium was exchanged every 24 hours for the first week in culture, to remove non-adherent cells. Colonies of EPC-derived ECs appeared 7-10 days after the initial isolation. The ECs grew to confluence and were serially passaged onto collagen I-coated plates. The EPC-derived PB-ECs and hCB-ECs were cultivated in complete EC growth medium, and used at passages 4-9 for all experiments.

Human aortic endothelial cells (HAECs) (Cambrex) were also cultivated in complete EC growth medium. HAECs were used at passage 7-10 for all experiments.

Human aortic smooth muscle cells (SMCs) (Cambrex) were expanded with SMC growth media containing Smooth Muscle Basal Media (Cambrex) supplemented with Smooth Muscle Growth Media-2 SingleQuots (Cambrex) and 1× antibiotic/antimycotic solution. The serum-free “quiescent media” was composed of DMEM/F12 (Invitrogen) supplemented with 1× insulin/transferrin/selenium (Invitrogen) and 1× antibiotic/antimycotic solution. SMCs were used at passages 8-11 for all experiments.

Substrates for Adhesion
SMCs to be used for dynamic adhesion were prepared as previously described.\textsuperscript{4} Briefly, SMCs were plated in SMC growth medium at 80,000 cells/cm\textsuperscript{2} on polystyrene slideflasks (NUNC) that were pre-incubated with 3.3 μg/ml human fibronectin (Sigma) for one hour. One day after seeding, the SMCs were changed into serum-free medium. After 6 days in serum-free medium (and 1 day prior to dynamic adhesion experiments), SMC monolayers were exposed overnight to serum-free medium lacking (control) or containing extracellular matrix proteins. Extracellular matrix proteins were used at the following concentrations: 3.3 μg/ml fibronectin (Sigma); 5 μg/ml collagen I (BD Bioscience); 5 μg/ml collagen III (BD Bioscience); 5 μg/ml collagen IV (BD Bioscience); and 5 μg/ml laminin ("ultrapure," extracted from Engelbreth-Holm-Swarm mouse tumors; BD Bioscience).
Dynamic Adhesion

HCB-ECs, PB-ECs, or HAECs were stained with Cell Tracker Green, trypsinized and resuspended in Dulbecco’s PBS (DPBS) at 500,000 cells/ml. In DPBS, ECs flowed over the SMC/matrix substrate in a parallel plate flow channel, which was heated to 37 °C and connected to a syringe pump. The flow channel was mounted on an inverted microscope (Zeiss Axiovert). The shear stress was computed by equation 1.

\[
\tau = \frac{6Q\mu}{wh^2} \tag{1}
\]

where \(\tau\) is the shear stress (dyn/cm²), \(Q\) is the flow rate (cm³/s), \(\mu\) is the fluid viscosity (g/cm/s), \(w\) is the channel width and \(h\) is the channel height. Flow lasted for 10 minutes, after which images of adherent cells were recorded at a magnification of 10× at 4 positions within a distance of 10 cm from the inlet of cells. The number of adherent cells per field was converted to adherent cells/cm² using a conversion of 0.54 pixels/µm. Experiments were performed quadruplicate.

Blocking studies were performed by using blocking murine IgG₁s specific for integrins \(\alpha_5\beta_1\) (clone JBS5, Millipore, at 10 µg/ml), \(\alpha_v\beta_3\) (clone LM609, Millipore, at 20 µg/ml), and \(\alpha_2\beta_1\) (clone BHA2.1, Millipore, at 10 µg/ml). After the cells were trypsinized as described above they were incubated with identical concentrations of an anti-integrin IgG₁ or cognate non-immune IgG₁ isotype control (Caltag) for 30 minutes with gentle rotation, and then flowed over SMCs for 5 minutes and analyzed as described above.

Cell Area

To determine the area of the cells in suspension, hCB-ECs, PB-ECs, and HAECs were stained with Cell Tracker Orange (Invitrogen, 2 µM, 15 minutes, 37 °C), detached and immediately imaged to determine the area of suspended cells. Ten random fluorescent images were captured at 20× and the projected cell area was measured using ImageJ (version 1.36, NIH) software. For each experiment, we averaged data from 75 cells of each type. Three independent experiments were performed and the means from each experiment were averaged. Cell area was used to determine the cell radius, assuming that the cells were spherical.

Net Hydrodynamic Force Acting on Cells

In order to compare the adhesion of cells of different sizes, the net fluid force imposed on the cells during the dynamic adhesion experiments was determined (Supplemental Figure 3). Adhesion of hCB-ECs occurs when the bond force \(F_b\) exceeds the net fluid force. The bond force is assumed to be localized in a microvillus of length \(L\). For dynamic adhesion experiments, the cells were modeled as spheres of radius \(R\) when initially adhered. The net fluid force acting on the cells is dependent upon the \(x\) component of the force, determined in equation 2a, and the torque, determined in equation 2b and equal to the net force \(F_b\) acting on the bonds holding the microvillus of the cell to the surface.

\[
F_x = F_b \cos \theta = 32.054R^2 \tau \tag{2a}
\]

\[
T = F_b \sin \theta = 43.916R^3 \tau \tag{2b}
\]

\[
\theta = \tan^{-1} \left( \frac{R}{L} \right) + \cos^{-1} \left( \frac{L^2 + L^2}{2L\sqrt{L^2 + R^2}} \right) \tag{2c}
\]
In these equations, $\tau$ is the wall shear stress, $l$ is the lever arm between the center of the cell and the microvillus, $R$ is the cell radius, and $\theta$ is calculated using equation 2c, in which $L$ is the length of the microvillus, which we assumed to be 0.3 $\mu$m.

**Flow Cytometry**

The surface expression of $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_2\beta_1$ integrins present on hCB-ECs, PB-ECs, and HAECs was determined by cell surface immunofluorescence and flow cytometry as previously described. Cells were trypsinized, incubated with either 10 $\mu$g/ml mouse-anti-$\alpha_5\beta_1$, 20 $\mu$g/ml mouse-anti-$\alpha_v\beta_3$, or 10 $\mu$g/ml mouse-anti-$\alpha_2\beta_1$ antibodies (Millipore) for 1 hour, washed, incubated with AlexaFluor™-488 goat-anti-mouse secondary antibody (1:500, Invitrogen), washed, and fixed in 3.7% formaldehyde. Preliminary experiments determined that 10 $\mu$g/ml of these IgG’s was more than sufficient to saturate the integrin binding sites.

Fluorescence intensity per cell produced by the bound antibodies was measured using a FACSCalibur flow cytometer (BD Bioscience). Typically 10,000 cells were measured for each experiment. In addition, isotype controls (Mouse IgG, Caltag) were performed for each sample condition, and the mean fluorescence intensity per cell found for the isotype control IgG was subtracted from the mean fluorescence intensity per cell obtained with anti-integrin IgG’s.

**Proliferation Under Laminar Flow**

HCB-ECs, PB-ECs, and HAECs stained with Cell Tracker Orange™ were seeded onto plastic slide flasks that were previously incubated with 3.3 $\mu$g/ml of fibronectin for 1 hour. All three cell types were seeded at sub-confluent densities of 25,000 cells/cm² (~25% of the confluent cell density) and allowed to adhere for 15 minutes. The slides were then placed inside a parallel plate flow chamber, imaged at 10 random locations using epi-fluorescence, and exposed to steady flow at 5 or 15 dyn/cm² for 4 days using Dulbecco’s Modified Eagle Medium (DMEM), 10% FBS, and antibiotic/antimycotic. Initial experiments were stopped at 1 day to ensure that cells did not detach from the flow chamber, which suggests that we are truly measuring proliferation of the cells. At the end of the experiment, cells were imaged to determine the number of cells present. Static samples were also run for all cell types. However, since the ECs under static conditions grew at a faster rate than those exposed to flow, images were taken every day until confluent. The number of ECs present at days 0, 2, and 4 for static conditions and 0 and 4 for flow conditions were used to find the approximate doubling time of the ECs. The number of ECs at the various time points examined were fit to an exponential function $N = N_0e^{t/c}$, where $t_c$ represents the characteristic time for the number of ECs to increase by $e^1$. The doubling time $t_d$ was found as $t_d = t_c\ln(2)$.

**Mouse Vein Graft Surgery and hCB-EC Injections**

Both female and male NOD.CB17-Prkdc<sup>scid</sup>/J mice (Jackson Laboratory) ages 10-15 weeks were used for all experiments. Mice were paired according to age and gender for all experiments. All animal experiments were performed according to protocols approved by the Duke Institutional Animal Care and Use Committee.

Carotid interposition vein isograft surgery was performed as we previously described, except that one cohort of mice received hCB-EC infusions intra- and post-operatively while the control cohort received infusions of only cell culture medium. All operative procedures were performed aseptically; anesthesia consisted of pentobarbital sodium (50 mg/kg, i.p.). A 1-cm segment of inferior vena cava (IVC) was harvested from a donor NOD.CB17-Prkdc<sup>scid</sup> mouse, and placed as an interposition graft in the right common carotid artery of a recipient NOD.CB17-Prkdc<sup>scid</sup> mouse. HCB-ECs used to treat the vein-grafted mice were detached from cell culture dishes by incubation with 0.025% trypsin (Clonetics) for 5 minutes, rinsed with trypsin neutralizing solutions (Clonetics) and centrifuged. Cells were resuspended in Iscove’s Modified Dulbecco Medium (Invitrogen) at 1x10<sup>6</sup> hCB-ECs per 150 $\mu$L.
The IVC to be grafted was stored after harvest in heparinized lactated Ringer’s solution at room temperature until it was transplanted (15 ± 5 min). In the recipient mouse, the right common carotid artery was exposed by ventral midline incision from the lower mandible to the sternum. A 15-mm segment of the carotid was isolated from the brachiocephalic to its bifurcation. A 5-0 silk suture was used to gently occlude the proximal and distal ends of this segment of common carotid artery. A bent 28-gauge needle and a Vannas angled scissors (Roboz Surgical, Inc.) were used to create two arteriotomies (about 2 mm long) proximally and distally, 0.8 to 1 cm apart. The carotid artery was then flushed with heparinized (50 U/mL) saline through arteriotomies and end-to-side anastomosis between the IVC and carotid was performed with 11-0 nylon (Ethicon, Inc.), using two fixed sutures at the proximal and distal corners of each arteriotomy, and two running sutures, each 180° around the circumference (with 4-6 bites/180°). Once the proximal end was sutured in place and before the distal end was secure, approximately 50 μL of Iscove’s solution containing approximately 3.33×10⁵ hCB-ECs (or Iscove’s solution with no hCB-ECs as the control) were injected into the graft lumen with a 27 gauge needle. After vein graft distal anastomosis was complete, the common carotid segment intervening between vein graft anastomoses was ligated and resected; blood flow through the graft was established by releasing proximal and distal ligatures on the common carotid. On average ~10-15 min elapsed between hCB-EC injection into the IVC graft and the onset of vein graft blood flow. Once both ends of the IVC were Anastomosed with the carotid, another injection of Iscove’s solution with or without hCB-ECs (~100 μL containing ~6.6×10⁵ hCB-ECs) was made into the jugular vein. The incision was closed in two layers with running 5-0 polypropylene sutures. The total time for interposition vein grafting averaged 90 minutes; total ischemic time for the vein graft was less than 60 minutes.

At four days postoperatively, mice were injected via tail vein with 150 μL of Iscove’s medium lacking (control) or containing 1×10⁶ hCB-ECs.

**Vein Graft Harvest**

At two weeks postoperatively, mice were anesthetized as above. Vein grafts were exposed through the previous surgical incision and the thoracic cavity was opened. The right atrium was incised, and the graft was perfused via the left ventricle with PBS until right atrial flow was no longer sanguinous. The aortic root and arch were excised along with the right carotid artery up to the bifurcation (including the vein graft), embedded in OCT, and then frozen and stored at -80 ºC. Sections of 5 μm thickness were obtained roughly 0.5 mm from the distal anastomosis and subjected to staining.

**Immunofluorescence and Microscopy**

Serial sections were stained for mouse and/or human CD31 (rabbit IgG (ab27510, Abcam) and human MHC I (rabbit IgG sc-30204, Santa Cruz Biotechnology). Briefly, sections were thawed at room temperature and fixed for 5 minutes in an acetone/methanol mixture (1:1) at room temperature. The OCT was then washed away from the sections by gently rinsing the slides in deionized water for 20 minutes. Sections were then incubated with either rabbit anti-CD31, rabbit anti-human MHC I (1:20), or equivalent concentrations of non-immune rabbit IgG (negative control) in 0.2% gelatin for 1 hour at room temperature. Sections were then rinsed and incubated with goat anti-rabbit Alexa Fluor®-488 and Hoescht 33342 (10 μg/ml) for 1 hour at room temperature. After washing, sections were mounted with Fluorsave (Calbiochem) and coverslipped. Mouse aorta was used as a positive control for CD31 and human lung sections were used as a positive control for human MHC I staining. Sections were also stained with hematoxylin and eosin by routine methods. To quantitate vein graft endothelialization and hCB-EC-derived endothelialization, we used NIH ImageJ to measure the vein graft luminal perimeter and the percentage of this perimeter that stained for CD31 alone or CD31 and (on serial sections) human MHC I, respectively (n = 8 for both hCB-EC- and control-treated mice).

**Aortic SMCs**—SMCs were grown as specified above for adhesion experiments. For immunostaining, SMCs were fixed and incubated with IgGs at 22 ºC. After fixation with 4% formaldehyde in PBS for 10 min, SMCs were stained for laminin or collagen IV with 3 serial 1-hr incubations: (a) 5% goat serum in
PBS ("blocking solution"), (b) mouse anti-laminin β-1 (A-1, Santa Cruz Biotechnology, Inc.) diluted 1:50 in blocking solution or mouse anti-human collagen IV (Millipore) diluted 1:100 in blocking solution, and (c) goat anti-mouse IgG conjugated to Alexa-Fluor-546 (Invitrogen) diluted 1:250 in blocking solution. Washing in blocking solution and PBS followed the second and third incubations. Slides were mounted in VECTASHIELD with DAPI mounting media (Vector Labs, Burlingame, CA). For collagen IV staining, fixed SMCs were permeabilized in 0.1% Triton-X 100 in PBS for 5 min before blocking. On nonspecific control slides, SMCs were incubated with blocking buffer in lieu of primary IgG, but otherwise were treated just as laminin- and collagen IV-stained SMCs. All specimens were imaged at identical exposure times on a CCD camera.

**Efficiency of hCB-EC Engraftment in Vein Grafts**

To estimate the efficiency of hCB-EC engraftment into the vein grafts, we estimated the total number of vein graft hCB-ECs present in 2-wk-old vein grafts, and divided this number by the total number of hCB-ECs injected into each mouse. First, we calculated the total number of ECs lining H&E-stained cross sections of mature (6-wk-old) mouse vein grafts (from C57BL/6 mice, n = 6). Next, we estimated the total number of ECs required to surface the entire (1-cm-long) vein graft by assuming that, on average, each cross section represents 50% of the length of each EC. We assumed an EC length of 44 ± 4 μm. (To arrive at this figure, we performed planimetry on CD-31-immunostained ECs grown under flow conditions, measured the area of each EC, and calculated dimensions by assuming an elliptical shape for each EC, with the long axis in the direction of blood flow.) To estimate the total number of hCB-ECs that engrafted, we multiplied the total number of ECs in a fully endothelialized 1-cm vein graft (1.2×10⁵ ECs) by the percentage of total vein graft circumference occupied by hCB-ECs in the 2-wk-old vein grafts (0.54 × 0.33 = 18%), to get 2.2×10⁴ hCB-ECs engrafted. Dividing the number of engrafted hCB-ECs by the number of hCB-ECs injected (2×10⁶) gives us an engraftment rate of only 1.1%.

We also estimated the total number of ECs in each vein graft by dividing our measured vein graft perimeter (2.8±0.3 mm) by the calculated width of the average EC. EC width was calculated from the area of ECs grown under flow conditions, by assuming ellipsoid geometry as specified above, and assuming the short axis of the ellipse represents EC width in the vein graft cross section: 19 ± 2 μm. With this method, we estimate that only 0.6% of hCB-ECs engrafted into the vein grafts. It is important to note that these estimates assume that, once engrafted, the hCB-ECs neither proliferate nor die.

**Vessel Shear Stress Calculations**

The time-averaged shear stress acting on the wall of the carotid artery and vein graft was determined with Equation 3 below,9 by using measured inner circumferences of the mouse carotid artery and vein graft, and reported flow rates within the carotid artery.13 In using Equation 3, we assume that the flow rate and pressure are not significantly different in the carotid artery compared to the vein graft:

\[
\tau = \frac{4\mu Q}{\pi R^4} \quad (3)
\]

where \(\mu\) is the fluid viscosity, \(R\) is the radius of the vessel, and \(Q\) is the volumetric flow rate.

The Reynolds number was calculated to assess whether flow is laminar or turbulent. The Reynolds number (Re) represents the ratio of viscous to inertial forces and, for a circular tube, equals \(2Q/\pi R\nu\) where \(Q\) is the flow rate in the carotid or vein graft (0.00467 mL/s),13 \(R\) is the vessel radius (0.015 cm for the carotid and 0.047 cm for the graft) and \(\nu\) is the kinematic viscosity of blood (0.033 cm²/s). The Reynolds numbers are 6.0 and 1.9 for the carotid artery and the vein graft, respectively. Since these values are much less than the value of 2100 at which flow begins the transition to turbulence,9 the flow is laminar. A limitation of Equation (3) is that it applies for straight vessels and away from branches, so the estimate of the shear stress is approximate.
Supplemental References


Supplemental Figure 1. Cultured human SMCs express basement membrane proteins. SMCs were immunostained with the indicated IgG, and subsequently with anti-mouse IgG/Alexa-546 and DAPI. Immunofluorescence images are shown on the left, and corresponding differential interference contrast (DIC) images are shown on the right. Scale bar = 50 μm (original magnification ×200).
Supplemental Figure II. Dynamic adhesion of hCB-ECs to SMC monolayers is altered by exogenous ECM proteins. Human SMC monolayers were rendered quiescent for 6 days, and then incubated without (control) or with the indicated ECM proteins in serum-free medium as described in Methods. One day later, SMC monolayers were placed in a parallel plate flow chamber and used to test hCB-EC adhesion in the presence of 0.5 dyn/cm² shear stress. The number of adherent ECs are plotted as the mean ± SE of 2-8 determinations. FN, fibronectin; Col, collagen; LN, laminin. Compared with adhesion to control SMCs: *, p < 0.05. Compared with adhesion to SMCs treated with FN/Col I/Col III: #, p < 0.05.
Supplemental Figure III. Schematic to explain the calculation of net forces on ECs. A model cell is depicted as if attached to a substrate while subjected to hydrodynamic force (under flow conditions). For an explanation of terms, please see the text subsumed under “Net Hydrodynamic Force Acting on Cells.”