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Late-outgrowth endothelial progenitors from patients with coronary artery disease: Endothelialization of confluent stromal cell layers

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Abstract

Patients with coronary artery disease (CAD) are the primary candidates to receive small-diameter tissue-engineered blood vessels (TEBVs). Peripheral blood derived endothelial progenitor cells (EPCs) from CAD patients (CAD EPCs) represent a minimally invasive source of autologous cells for TEBV endothelialization. We have previously shown that human CAD EPCs are highly proliferative and express many of the hallmarks of mature and healthy endothelial cells; however, their behavior on stromal cells that comprise the media of TEBVs has not yet been evaluated. Primary CAD EPCs or control human aortic endothelial cells (HAECs) were seeded over confluent, quiescent layers of human smooth muscle cells (SMCs) using a direct co-culture model. The percent coverage, adhesion strength, alignment under flow and generation of flow-induced nitric oxide of the seeded CAD EPCs were compared to that of HAECs. The integrin-binding profile of CAD EPCs was also evaluated over a layer of confluent, quiescent SMCs. Direct comparison of our CAD EPC results to analogous co-culture studies with cord blood EPCs show that both types of blood-derived EPCs are viable options for endothelialization of TEBVs.

Keywords

Endothelial progenitor cells; Smooth muscle cells; Tissue-engineered blood vessel; Co-culture; Coronary artery disease

1. Introduction

Growing an intact layer of autologous endothelium in the lumen of small-diameter blood vessel substitutes has been a long-sought goal in cardiovascular medicine [1–3]. The adhesion and proliferation of endothelial cells (ECs) onto the lumen of synthetic vascular grafts has been well characterized [4–8]; however, forming a layer of intact endothelium on the luminal surface of tissue-engineered blood vessels (TEBVs) has been met with varying degrees of success and inconsistent results have been reported [9–13].

Autologous ECs are most commonly obtained from patients through invasive methods such as trypsin or collagenase digestion from a patient's excised jugular, radial or saphenous vein, or by obtaining microvascular ECs from liposuctioned adipose tissue [14]. In contrast, cultured endothelial progenitor cells (EPCs) obtained from patient peripheral blood offer a noninvasive source of autologous ECs.

EPCs were first reported by Asahara et al. [15]. Depending on the method of culture, EPCs may be defined as colony-forming unit ECs (CFU-ECs) or blood outgrowth endothelial cells (BOECs) [16]. CFU-ECs appear earlier in the ex vivo expansion process, and differentiate into monocytic cells that can exhibit phagocytosis, and can function as antigen-presenting cells [17]. BOECs appear at least 2 weeks later during ex vivo expansion [18] and appear phenotypically as endothelial cells that exhibit robust proliferation capacity. Because of their early and late appearance during ex vivo expansion, CFU-ECs and BOECs, respectively, are more simply referred to as "early-outgrowth" and "late-outgrowth" EPCs [19].

Our group has characterized the phenotypic properties of late-outgrowth EPCs derived from the peripheral blood of patients with significant coronary artery disease (CAD) [20]. These "CAD EPCs" were shown to have similar proliferative capacity and surface antigen expression to late-outgrowth EPCs derived from healthy patients. Further, they function similarly to EPCs derived from healthy individuals and human aortic endothelial cells. We have also shown that CAD EPCs may be successfully grafted onto the lumen of small-diameter ePTFE vascular grafts, and that the CAD EPC-treated grafts dramatically improved the short- and long-term patency compared to bare grafts in a rodent femoral artery model [21].

Late-outgrowth EPCs have been studied as a cell source for TEBV endothelialization because of their ease of isolation from peripheral blood. The majority of work on the attachment and growth of late-outgrowth EPCs on the stromal cells that make up the TEBV media has involved cord blood derived EPCs (CB EPCs) [22–24]. These studies, mainly out of the Truskey group, have shown that CB EPCs have the capacity to be cultured to confluence over human aortic smooth muscle cells (SMCs) [22]. Late-outgrowth EPCs from healthy volunteers have also been seeded over medial surfaces made of dermal fibroblasts entrapped within tubular fibrin gels [25].

Individuals with CAD are the primary candidates for receiving an endothelialized small-diameter TEBV, and therefore the target source of autologous cells. While much of the work on EPCs involves cells isolated from healthy individuals, understanding the behavior of peripheral blood EPCs isolated from CAD patients is critical toward the implementation of autologous endothelium for TEBVs. In the current study, we examined if CAD EPCs also have suitable capacity to be cultured to confluence using the same direct co-culture system employed for CB EPCs and HAECs [22,26,27]. CAD EPCs were evaluated for their potential to create and maintain a confluent endothelium, and their ability to align and produce nitric oxide after exposure to long-term laminar shear stress. The strength of cell adhesion was evaluated by assessing cell retention after exposure to a burst of high shear stress. Additionally, the adhesive properties of CAD EPCs over confluent, quiescent SMCs

were evaluated by blocking with various integrin antibodies. Parallel studies were also performed using HAECs.

2. Materials and methods

2.1. Cell isolation and culture

The Duke University Institutional Review Board approved a protocol for collection of human blood from consenting patients undergoing left heart catheterization at Duke University Medical Center. All patients were males over the age of 55 with advanced CAD documented by angiography. CAD EPCs were isolated and grown as previously stated [20,28]. Approximately 50 ml of blood was diluted 1:1 with Hanks' balanced salt solution (HBSS; Gibco). Equal volumes of blood-HBSS mixture were slowly layered over an equal volume of Histopaque (Sigma) and centrifuged at 740 g for 30 min. Afterward, the platelet-rich plasma layer was aspirated, and the buffy coat cells collected and washed with complete EC media. Mononuclear cells were then plated onto 6-well TCPS plates (BD Biosciences) precoated with 50 $\mu\text{g ml}^{-1}$ rat tail collagen type I (BD Biosciences) in 0.02 N acetic acid. For the first 7 days after isolation, media was changed daily to remove non-adherent cells. Colonies of CAD EPCs appeared 2 weeks post-isolation. Colonies were passaged onto TCPS T-25 flasks (BD Biosciences) and allowed to grow to confluence. CAD EPCs were proliferative and exhibited healthy morphology through P11.

Human aortic endothelial cells (HAECs) were purchased from Lonza (Walkersville, MD). Cells were passaged every 4 days. Both CAD EPCs and HAECs were maintained in EC media as described below. SMCs were purchased from Lonza and maintained in SMC growth media. A low trypsin concentration was used as previously indicated to increase EC adhesion to substrates [29]. All cells were passaged with 0.025% trypsin/EDTA (Lonza) and trypsin neutralizing solution (Lonza). SMCs were plated on T-75 flasks (BD Biosciences) that had been coated with 10 $\mu\text{g ml}^{-1}$ human serum fibronectin (Millipore) for 1 h. Media was changed every other day. CAD EPCs, HAECs and SMCs were used from passage 6 to 10. CAD EPCs from four different donors were used.

2.2. Cell media

CAD EPCs and HAECs were maintained in EC media comprised of Endothelial Basal Medium-2 (EBM-2) with EGM-2 Single Quots kit (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) (Gibco) and 1% Pen/Strep (Lonza). SMCs were maintained in Smooth Muscle Basal Medium (SmBM) supplemented with SmGM-2 Single-Quots kit (Lonza). The quiescent phenotype of SMCs was induced using a serum-free media made of DMEM/F-12 (Lonza) with 1 $\times$ insulin-transferrin-selenium supplement and 1% Pen/Strep. Co-cultures were maintained after 24 h of EC seeding in co-culture media made from EBM-2 supplemented with 3.3% HI-FBS and 1% Pen/Strep. Flow studies were conducted with flow media made from low-glucose DMEM supplemented with 3.3% HI-FBS, 1 $\times$ insulin-transferrin-selenium supplement, and 1% Pen/Strep.

2.3. CAD EPC/HAEC co-culture with SMCs

CAD EPCs and HAECs were directly cultured over SMCs as previously described [26]. Briefly, SMCs were seeded at a density of at least 70,000 cells cm⁻² and allowed to become confluent. Quiescent phenotype was induced after 24 h in culture by changing media to serum-free quiescent media. Cells were maintained in quiescent media for 1–2 days, and then ECs were seeded at various densities in EC media. For co-cultures lasting longer than 24 h, media was changed to co-culture media after 24 h and changed every other day. Cells were maintained in an incubator at 37 °C and 5% carbon dioxide/95% air.

2.4. CAD EPC/HAEC confluency measurements

CAD EPCs were evaluated for their potential to maintain a confluent endothelium over a confluent, quiescent SMC layer compared to HAECs. HAECs and CAD EPCs were stained with 2 µM Cell Tracker Green (CMFDA) and seeded over confluent, quiescent SMCs at 30,000, 50,000, 70,000, 90,000 and 110,000 cells cm⁻². Co-cultures were imaged at day 1, 3, 5 and 7 with fluorescence microscopy. ECs were stained with Ac-LDL-DiI at day 5 after the CMFDA had metabolized. Five random images per sample were taken using fluorescence microscopy at 10× magnification (Nikon TE2000U, Tokyo, Japan) and a digital camera (DS-Qi1Mc, Nikon) at days 1, 3, 5 and 7. On day 7 cells were washed with DPBS and fixed in methanol for 10 min at –20 °C. Cells were rinsed with DPBS and incubated with 10% goat serum (Gibco) for 30 min at room temperature to block non-specific binding. ECs were incubated with mouse anti-human primary antibody (Platelet-EC adhesion molecule, 1:200, BD Pharminogen) in 10% goat serum for 1 h at room temperature. Cells were rinsed several times with DPBS and incubated with a goat anti-mouse Alexa Fluor 488 secondary antibody (1:500, Invitrogen) in 10% goat serum for 1 h at room temperature. Samples were rinsed with DPBS and maintained in VectaShield (Vector Labs) containing DAPI. Each experiment was performed three times. Confluence of the cultures was analyzed using Image J software (version 10.2, National Institutes of Health, Bethesda, MD) and normalized to 100% confluence over SMCs.

2.5. Flow chamber

A previously described parallel-plate flow chamber connected to a circular flow loop was used for both the long-term flow studies as well as the short-term cell adhesion studies [27]. A steady, laminar fluid flow provided the desired wall shear stress, τ_w , defined as follows:

$$\tau_w = \frac{6\mu Q}{wh^2}, \quad (1)$$

where Q is the volumetric flow rate, μ is the fluid viscosity (0.86 Cp), w is the channel width (1.9 cm) and h is the local channel height (212 µm).

2.6. Strength of adhesion study

The strength of adhesion of CAD EPCs and HAECs over confluent, quiescent SMCs were compared in the parallel-plate flow chamber. Slide flasks (Nunc) were seeded with SMCs as described above. CAD EPCs or HAECs were stained with Calcein-AM and seeded subconfluently over confluent, quiescent SMC surfaces at a density of 20,000 cells cm⁻² to

easily visualize individual adhered cells and the co-culture was sealed into the flow chamber. Cells were allowed to adhere for either 15 min or 24 h and then exposed to a steady laminar shear stress of 100 dynes cm⁻² for 10 min. This supraphysiological level of shear stress was used to demonstrate the strength of adhesion of the ECs. Ten random images per sample were taken on a fluorescence microscope at 4×. Adhered cells before and after exposure to shear stress were counted with ImageJ software. The percentage of cells retained was determined by dividing the average number of remaining cells post-flow by the average number of cells initially adhered.

2.7. Cell alignment under flow

The alignment of CAD EPCs and HAECs over confluent, quiescent SMCs was measured in the parallel-plate flow chamber. SMCs were seeded on slide flasks and allowed to become confluent and quiescent as described above. CAD EPCs or HAECs were seeded over confluent, quiescent SMCs at 110,000 cells cm⁻² in EC media. After 24 h, the media was changed to co-culture media. Co-cultures were maintained another 24 h, sealed into the flow chamber, then exposed to a physiological level of steady laminar shear stress of 15 dynes cm⁻² for 48 h. A static control was maintained in flow media for 48 h.

Samples were fixed in methanol for 10 min at -20 °C. Confluence and alignment of ECs was evaluated by examining the presence of CD31, or platelet-EC adhesion molecule (PECAM) within the cell junctions. Cells were rinsed with DPBS and incubated with 10% goat serum (Gibco) for 30 min at room temperature to block non-specific binding. ECs were incubated with mouse anti-human primary antibody (PECAM, 1:200, BD Pharminogen) in 10% goat serum for 1 h at room temperature. Cells were rinsed several times with DPBS and incubated with a goat anti-mouse Alexa Fluor 488 secondary antibody (1:500, Invitrogen) in 10% goat serum for 1 h at room temperature. Samples were rinsed with DPBS and maintained in VectaShield (Vector Labs) containing DAPI and covered with a cover glass. Each sample was imaged using a Zeiss LSM 510 inverted confocal microscope at 20×. Four random images per sample were taken and 15 random cells per field-of-view were analyzed for cell roundness and cell orientation angle with ImageJ as previously described [22]. Cell roundness was calculated using the following equation:

$$\text{Roundness} = \frac{4A}{\pi L^2}, \quad (2)$$

where A is the cell area measured and L is the maximum chord length. A cell roundness of 1 refers to a circle, and a cell roundness of 0 refers to a straight line, where roundness values closer to 0 indicate more elongated cells. The cell orientation angle was computed in reference to 0° being the flow direction, where an angle of 0° indicated complete alignment and an angle of 45° indicates no alignment.

2.8. Nitric oxide quantification

Production of nitric oxide (NO) by CAD EPCs seeded over confluent, quiescent SMCs was evaluated through direct measurement of nitrite (NO_2^-), a stable byproduct of NO oxidation in the presence of oxygen. At the 48 h time point 100 µl media samples were collected from the CAD EPC cell alignment studies for both cells exposed to flow and static controls.

Media samples were frozen down to -80°C immediately following collection. Samples were later brought to room temperature and an Ionics/Sievers Nitric Oxide Analyzer (NOA 280, Sievers Instruments, Boulder, CO) was used to measure the nitrite concentration using chemiluminescence as previously described [30]. Nitrite concentrations released per million CAD EPCs seeded were evaluated as a representation of NO production under flow as previously described [31]. Three samples per condition were analyzed.

2.9. Integrin blocking assays

The integrin binding of CAD EPCs and HAECs was evaluated by blocking with anti-integrin antibodies as previously demonstrated [32]. CAD EPCs or HAECs were stained with Cell Tracker Green CMFDA, trypsinized and resuspended in DPBS (Gibco). ECs were then incubated with $10\text{ }\mu\text{g ml}^{-1}$ mouse-anti- $\alpha_5\beta_1$, $20\text{ }\mu\text{g ml}^{-1}$ mouse-anti- $\alpha_v\beta_3$ antibodies (Abcam), alone or in combination, for 30 min at 37°C with gentle rotation. Controls were ECs incubated with DPBS.

Cells were seeded onto confluent, quiescent SMCs in 48-well TCPS culture plates (BD Biosciences) at a density of $20,000\text{ cells cm}^{-2}$ and allowed to adhere in a humidified incubator at 37°C for 15 min, then rinsed with DPBS and fixed with 3.7% paraformaldehyde at room temperature for 10 min. Cells were imaged on a fluorescent microscope at $10\times$. Ten images per well were captured and analyzed for the number of cells adhered using ImageJ.

3. Statistical analysis

Results are expressed as mean $\pm$ SEM. Statistical analyses were performed using MATLAB (MathWorks, version R2012a). A two-way analysis of variance (ANOVA) followed by Tukey–Kramer post hoc tests or Student's *t*-test with a Bonferroni correction was used to determine the significance between groups. All measurements were performed in triplicate.

4. Results

4.1. CAD EPC confluence over SMCs in co-culture

CAD EPCs or HAECs were seeded over confluent, quiescent SMCs and evaluated at 1 day (24 h) and 7 days for the formation and maintenance of a confluent endothelium (Fig. 1). Networks characteristic of EC vessel formation were most prevalent at low seeding densities for CAD EPCs and HAECs, both of which transitioned to confluence with increased seeding density. The average percent cell coverage of both CAD EPCs and HAECs on SMCs increases steadily with seeding density. Two-way ANOVA tests showed these trends to be significantly different between CAD EPCs and HAECs for both 1 and 7 days ($P < 0.05$). On average CAD EPCs also exhibited greater percent coverage over SMCs for all but one seeding density at both 1 and 7 days; however, this difference within a given seeding density was only statistically significant at $110,000\text{ cells cm}^{-2}$ for both 1 and 7 days ($P < 0.05$, Student's *t*-test with Bonferroni correction), with the largest difference occurring at 7 days ($87.65 \pm 1.67\%$ confluence CAD EPCs vs. $63.03 \pm 0.89\%$ confluence for HAECs).

4.2. Percent retention

CAD EPCs and HAECs seeded subconfluently over SMCs were allowed to adhere for 15 min or 24 h and then exposed to $100 \text{ dynes cm}^{-2}$ shear stress for 10 min. 70–80% of both CAD EPCs and HAECs remained attached after 15 min and > 90% remained attached after 24 h (Fig. 2). The difference in percent retention between 15 min and 24 h was significant for both CAD EPCs and HAECs ($P < 0.05$).

4.3. Cell alignment under flow and in static conditions

The alignment (cell orientation angle) and elongation (cell roundness) of CAD EPCs and HAECs over confluent, quiescent SMCs were measured after 48 h exposure to 15 dynes cm^{-2} shear stress and under static conditions (Figs. 3 and 4). CAD EPCs exposed to flow exhibited significantly greater alignment and elongation compared to HAECs exposed to flow and to CAD EPCs cultured under static controls ($P < 0.05$). HAECs showed significantly greater alignment ($P < 0.05$) but not cell elongation compared to HAEC static controls. There were no significant differences in average cell area between CAD EPCs and HAECs measured following exposure to flow or under static conditions (Fig. 5). CAD EPCs from three different donors exhibited significantly greater production of nitrite under flow than under static conditions ($P < 0.05$) (Fig. 6).

4.4. Integrin-blocking studies

CAD EPCs and HAECs incubated with various anti-integrin antibody solutions exhibited a similar binding profile over confluent, quiescent SMCs (Fig. 7). All antibody solutions had significant blocking effects compared to controls ($P < 0.05$). For all antibody solutions, the blocking behavior of HAECs and CAD EPCs was not significantly different. Blocking with anti- $\alpha_5\beta_1$ significantly decreased adhesion compared to blocking with anti- $\alpha_v\beta_3$ for both CAD EPCs and HAECs ($P < 0.05$). The combination of both antibodies significantly decreased adhesion compared to anti- $\alpha_5\beta_1$ alone for both CAD EPCs and SMCs ($P < 0.05$).

5. Discussion

In previous studies we demonstrated that a homogeneous population of late-outgrowth CAD EPCs can be isolated from patient peripheral blood and cultured to display mature and fully differentiated EC characteristics [20]. CAD EPCs are highly proliferative and exhibit the characteristics of healthy ECs. CAD EPCs are positive for EC markers CD31 and CD105 and negative for CD133 and hematopoietic markers CD45 and CD14. We also showed that a layer of CAD EPCs dramatically improved the patency of small-diameter ePTFE vascular grafts in a rat femoral artery model [21].

One of the primary concerns voiced about the use of adult late-outgrowth EPCs as an endothelial source is their rare occurrence in peripheral blood [16]. CB-EPCs have been favored by some because they are relatively abundant and highly proliferative, likely due to increased telomerase activity [18]. However, we have shown that CAD EPCs are also highly proliferative and comparable to late-outgrowth EPCs from young, healthy patients [20]. Peripheral blood EPC isolations exhibit a success rate of approximately 50%. However, all successfully isolated CAD EPC cultures expanded to $>10^7$ cells over 3–6 passages, while

late-outgrowth EPCs from healthy donors exhibited limited proliferation in about half of the successfully isolated colonies. This was consistent with the observation that peripheral blood EPC counts are increased in patients with angiographically documented CAD [33].

The current study employed a direct co-culture model to examine whether late-outgrowth CAD EPCs were also suitable candidates for endothelializing small-diameter TEBVs. This direct co-culture model has been used previously with HAECs [26,27] and CB EPCs [22,23]. To our knowledge this study is the first examination of the interaction between adult peripheral blood EPCs and SMCs in general, and between CAD EPCs and SMCs in particular. These studies are a necessary first step towards employing CAD EPCs as an autologous source for TEBV endothelialization.

Previously, the direct co-culture model employed in the current study was used to characterize the attachment and growth of CB EPCs to layers of quiescent SMCs [22,23]. Our studies followed the co-culture protocol described in these studies. Table 1 directly compares results from our recent publication on CAD EPCs and the current study with recently reported data on CB EPCs mostly from the Truskey group. All CAD EPC and CB EPC studies used HAECs as control cells. This comparison indicates that CAD EPCs performed similarly to CB EPCs.

CAD EPCs maintained stable attachment and growth on confluent layers of SMCs over 1 and 7 days (Fig. 1). The extent of cell coverage increased with seeding density for CAD EPCs and HAECs, with CAD EPCs exhibiting significantly greater coverage over SMCs than did HAECs at 1 and 7 days when complete data sets were taken into consideration ($P < 0.05$). For any given cell seeding density, however, there were no significant differences in CAD EPCs or HAECs on average, except for SMCs seeded with 110,000 cells CAD EPCs HAECs for 1 or 7 days ($P < 0.05$). Because a seeding density of 110,000 cells cm^{-2} CAD EPCs resulted in ~90% coverage on SMCs at both 1 and 7 days, all subsequent co-cultures used this seeding density.

Greater capillary-like network formation was noted after 7 days in co-cultures with HAECs and CAD EPCs seeded at 30,000 cells cm^{-2} . Others have noted greater network formation with blood derived EPCs on PLLA scaffolds or Matrigel in the presence of perivascular cells such as SMCs [34,35] or mesenchymal stem cells [36]. Our observations reinforce the observation that ECs seeded at low densities result in greater network formation [24], and that EC-SMC interactions are critical for vascular development [37–39]. Furthermore, late-outgrowth EPCs from either adult peripheral blood or umbilical cord blood form networks more readily than ECs derived from arterial or venous endothelium [36], indicating that less differentiated or less mature endothelial cells are more favorable for tissue engineering applications.

Both CAD EPCs and HAECs exhibited >90% retention when cultured over SMCs for 24 h and exposed to a short burst of supraphysiological shear stress of 100 dynes cm^{-2} (Fig. 2). CB EPCs also exhibited >90% retention over confluent, quiescent SMCs upon exposures to bursts of supraphysiological shear stress [22]. This indicates that EPCs seeded onto SMCs remain strongly adhered after initial attachment.

Alignment in the direction of flow is a key characteristic of endothelium. We have previously showed that CB EPCs and CAD EPCs aligned in the direction of flow when seeded over fibronectin-coated Teflon-AF, which was used to mimic the surface properties of an ePTFE synthetic vascular graft surface [20,40]. However, ECs are known to exhibit different adhesion characteristics over substrates of a softer modulus, such as a SMC layer or other TEBV substrates [41]. Furthermore, biological substrates such as a monolayer of other cells have been shown to behave differently to passive substrates of similar modulus such as polyacrylamide gels [23]. Therefore, it was important to evaluate the behavior of CAD EPCs over a monolayer of SMCs in order to evaluate their potential as an endothelial source for TEBVs.

ECs seeded over SMCs in static culture follow the alignment of the SMC layer [41], but reorient toward the direction of flow after long-term exposure to physiological levels of steady, laminar, shear stress. Both CAD EPCs and HAECs exposed to physiological shear stress for 48 h aligned in the direction of flow, with CAD EPCs exhibiting greater elongation and alignment than HAECs. Neither CAD EPCs nor HAECs exhibited a change in area before or after exposure to long-term shear stress when cultured over SMCs. This was consistent with results reported for CB EPCs cultured over SMCs [23]. We have shown previously that CAD EPCs exhibit greater mRNA expression of Krüppel-like factor 2 (KLF-2) under flow than under static conditions when seeded over fibronectin-coated Teflon-AF [20]. The increased alignment with flow exhibited by CAD EPCs may be due in part to the expression of KLF-2.

Vascular endothelial cells secrete NO in response to fluid shear stress, which serves to promote flow-mediated vasodilation[42,43]. We have previously confirmed that CAD EPCs seeded on fibronectin-coated Teflon-AF released significantly greater NO after 24 h of flow at physiological shear stress than under static conditions [20]. In the current study, nitrite production was directly measured as a representation of NO production due to the short half-life of NO in aqueous solutions. After 48 h of flow at physiological shear stresses, average nitrite production of CAD EPCs increased from an average of 0.14 nmol to 46 nmol per million cells seeded (Fig. 6). A recently published study of nitrite production of CB EPCs seeded on fibronectin-coated glass showed that CB EPCs produced approximately 20 nmol nitrite per million ECs seeded after 48 h [31].

CAD EPC adhesion over confluent, quiescent SMCs was significantly blocked by antibody solutions against $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins alone, and by combinations of antibodies against $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins ($P < 0.05$). This behavior is similar to that exhibited by HAECs. CB EPCs and HAECs have also exhibited binding to fibronectin-coated surfaces that was dependent only on the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins [22,27]. These results further demonstrate that EPCs isolated from CAD patients exhibit healthy EC behavior.

Finally, CB EPCs may be attractive due to their potential for allogeneic transplantation [44,45], though it is important to note that CB EPCs are not immunocompetent. Late-outgrowth EPCs from rat peripheral blood elicited substantially less alloimmune reaction than rat aortic ECs [46]. However, a comparison of CB EPCs and human umbilical vein endothelial cells (HUVECs) from the same patient found that CB EPCs expressed the same

pattern of class I and II MHC molecules as HUVECs, indicating that they would likely be rejected in the case of an allogeneic implant without matching MHC types [47]. For this reason alone, use of CAD EPCs as an autologous endothelial source may be preferred to avoid potential immune rejection.

6. Conclusions

CAD EPCs exhibit confluence over SMCs for 7 days and maintained a confluent layer more effectively compared to HAECs, attached to SMCs within 15 min and remain strongly adhered, and exhibited greater alignment with flow compared to HAECs. These results were essentially equivalent to that observed for CB EPC adhesion on SMCs. CAD EPCs exhibited binding dependence on the $\alpha_5\beta_1$ integrin on confluent, quiescent SMCs as did CB EPCs. These results demonstrate that peripheral blood EPCs isolated from CAD patients may be a viable source of autologous endothelium for use in TEBVs.

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Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 1 and 3, are difficult to interpret in black and white. The full color images can be found in the on-line version, at <http://dx.doi.org/10.1016/j.actbio.2013.10.004>.

References

- [1]. Kakisis JD, Liapis CD, Breuer C, Sumpio BE. Artificial blood vessel: the Holy Grail of peripheral vascular surgery. *J Vasc Surg.* 2005; 41:349–54. [PubMed: 15768021]
- [2]. Heyligers JM, Arts CH, Verhagen HJ, de Groot PG, Moll FL. Improving small-diameter vascular grafts: from the application of an endothelial cell lining to the construction of a tissue-engineered blood vessel. *Ann Vasc Surg.* 2005; 19:448–56. [PubMed: 15864472]
- [3]. Melchiorri AJ, Hibino N, Fisher JP. Strategies and techniques to enhance the in situ endothelialization of small-diameter biodegradable polymeric vascular grafts. *Tissue Eng Part B Rev.* 2013; 19:292–307. [PubMed: 23252992]
- [4]. Dudash LA, Kligman F, Sarett SM, Kottke-Marchant K, Marchant RE. Endothelial cell attachment and shear response on biomimetic polymer-coated vascular grafts. *J Biomed Mater Res A.* 2012; 100:2204–10. [PubMed: 22623267]
- [5]. Walluscheck KP, Steinhoff G, Kelm S, Haverich A. Improved endothelial cell attachment on ePTFE vascular grafts pretreated with synthetic RGD-containing peptides. *Eur J Vasc Endovasc Surg.* 1996; 12:321–30. [PubMed: 8896475]
- [6]. Williams SK, Rose DG, Jarrell BE. Microvascular endothelial cell sodding of ePTFE vascular grafts: improved patency and stability of the cellular lining. *J Biomed Mater Res.* 1994; 28:203–12. [PubMed: 8207032]
- [7]. Meinhardt JG, Schense JC, Schima H, Gorlitzer M, Hubbell JA, Deutsch M, et al. Enhanced endothelial cell retention on shear-stressed synthetic vascular grafts precoated with RGD-cross-linked fibrin. *Tissue Eng.* 2005; 11:887–95. [PubMed: 15998228]

- [8]. Deutsch M, Meinhart J, Zilla P, Howanietz N, Gorlitzer M, Froeschl A, et al. Long-term experience in autologous in vitro endothelialization of infrainguinal ePTFE grafts. *J Vasc Surg.* 2009; 49:352–62. discussion 62. [PubMed: 19110397]
- [9]. Dahl SL, Kypson AP, Lawson JH, Blum JL, Strader JT, Li Y, et al. Readily available tissue-engineered vascular grafts. *Sci Transl Med.* 2011; 3:68ra9.
- [10]. Teebken OE, Bader A, Steinhoff G, Haverich A. Tissue engineering of vascular grafts: human cell seeding of decellularised porcine matrix. *Eur J Vasc Endovasc Surg.* 2000; 19:381–6. [PubMed: 10801371]
- [11]. Kaushal S, Amiel GE, Guleserian KJ, Shapira OM, Perry T, Sutherland FW, et al. Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. *Nat Med.* 2001; 7:1035–40. [PubMed: 11533707]
- [12]. Kerdjoudj H, Boura C, Moby V, Montagne K, Schaaf P, Voegel JC, et al. Re-endothelialization of human umbilical arteries treated with polyelectrolyte multilayers: a tool for damaged vessel replacement. *Adv Funct Mater.* 2007; 17:2667–73.
- [13]. L'Heureux N, Dusserre N, Konig G, Victor B, Keire P, Wight TN, et al. Human tissue-engineered blood vessels for adult arterial revascularization. *Nat Med.* 2006; 12:361–5. [PubMed: 16491087]
- [14]. Tiwari A, Salacinski HJ, Hamilton G, Seifalian AM. Tissue engineering of vascular bypass grafts: role of endothelial cell extraction. *Eur J Vasc Endovasc Surg.* 2001; 21:193–201. [PubMed: 11352676]
- [15]. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science.* 1997; 275:964–7. [PubMed: 9020076]
- [16]. Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood.* 2007; 109:1801–9. [PubMed: 17053059]
- [17]. Asakage M, Tsuno NH, Kitayama J, Kawai K, Okaji Y, Yazawa K, et al. Early-outgrowth of endothelial progenitor cells can function as antigen-presenting cells. *Cancer Immunol Immunother.* 2006; 55:708–16. [PubMed: 16133110]
- [18]. Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood.* 2004; 104:2752–60. [PubMed: 15226175]
- [19]. Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, et al. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. *Arterioscler Thromb Vasc Biol.* 2004; 24:288–93. [PubMed: 14699017]
- [20]. Stroncek JD, Grant BS, Brown MA, Povsic TJ, Truskey GA, Reichert WM. Comparison of endothelial cell phenotypic markers of late-outgrowth endothelial progenitor cells isolated from patients with coronary artery disease and healthy volunteers. *Tissue Eng Part A.* 2009; 15:3473–86. [PubMed: 19435420]
- [21]. Stroncek JD, Ren LC, Klitzman B, Reichert WM. Patient-derived endothelial progenitor cells improve vascular graft patency in a rodent model. *Acta Biomater.* 2012; 8:201–8. [PubMed: 21945828]
- [22]. Brown MA, Wallace CS, Angelos M, Truskey GA. Characterization of umbilical cord blood-derived late outgrowth endothelial progenitor cells exposed to laminar shear stress. *Tissue Eng Part A.* 2009; 15:3575–87. [PubMed: 19480571]
- [23]. Cao L, Wu A, Truskey GA. Biomechanical effects of flow and coculture on human aortic and cord blood-derived endothelial cells. *J Biomech.* 2011; 44:2150–7. [PubMed: 21683362]
- [24]. Peters EB, Christoforou N, Leong KW, Truskey GA. Comparison of mixed and lamellar coculture spatial arrangements for tissue engineering capillary networks in vitro. *Tissue Eng Part A.* 2013; 19:697–706. [PubMed: 23171167]
- [25]. Ahmann KA, Johnson SL, Hebbel RP, Tranquillo RT. Shear stress responses of adult blood outgrowth endothelial cells seeded on bioartificial tissue. *Tissue Eng Part A.* 2011; 17:2511–21. [PubMed: 21599543]

- [26]. Lavender MD, Pang Z, Wallace CS, Niklason LE, Truskey GA. A system for the direct co-culture of endothelium on smooth muscle cells. *Biomaterials*. 2005; 26:4642–53. [PubMed: 15722134]
- [27]. Wallace CS, Champion JC, Truskey GA. Adhesion and function of human endothelial cells co-cultured on smooth muscle cells. *Ann Biomed Eng*. 2007; 35:375–86. [PubMed: 17191127]
- [28]. Hirschi KK, Ingram DA, Yoder MC. Assessing identity, phenotype, and fate of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol*. 2008; 28:1584–95. [PubMed: 18669889]
- [29]. Brown MA, Wallace CS, Anamelechi CC, Clermont E, Reichert WM, Truskey GA. The use of mild trypsinization conditions in the detachment of endothelial cells to promote subsequent endothelialization on synthetic surfaces. *Biomaterials*. 2007; 28:3928–35. [PubMed: 17570483]
- [30]. Allen JD, Miller EM, Schwark E, Robbins JL, Duscha BD, Annex BH. Plasma nitrite response and arterial reactivity differentiate vascular health and performance. *Nitric Oxide*. 2009; 20:231–7. [PubMed: 19371597]
- [31]. Kang SD, Carlon TA, Jantzen AE, Lin FH, Ley MM, Allen JD, et al. Isolation of functional human endothelial cells from small volumes of umbilical cord blood. *Ann Biomed Eng*. 2013; 41:2181–92. [PubMed: 23604849]
- [32]. Brown MA, Wallace CS, Angelos M, Truskey GA. Characterization of umbilical cord blood derived late outgrowth endothelial progenitor cells exposed to laminar shear stress. *Tissue Eng Part A*. 2009; 15:3575–87. [PubMed: 19480571]
- [33]. Guven H, Shepherd RM, Bach RG, Capoccia BJ, Link DC. The number of endothelial progenitor cell colonies in the blood is increased in patients with angiographically significant coronary artery disease. *J Am Coll Cardiol*. 2006; 48:1579–87. [PubMed: 17045891]
- [34]. Wu X, Rabkin-Aikawa E, Guleserian KJ, Perry TE, Masuda Y, Sutherland FW, et al. Tissue-engineered microvessels on three-dimensional biodegradable scaffolds using human endothelial progenitor cells. *Am J Physiol Heart Circ Physiol*. 2004; 287:H480–487. [PubMed: 15277191]
- [35]. Melero-Martin JM, Khan ZA, Picard A, Wu X, Paruchuri S, Bischoff J. In vivo vasculogenic potential of human blood-derived endothelial progenitor cells. *Blood*. 2007; 109:4761–8. [PubMed: 17327403]
- [36]. Melero-Martin JM, De Obaldia ME, Kang SY, Khan ZA, Yuan L, Oettgen P, et al. Engineering robust and functional vascular networks in vivo with human adult and cord blood-derived progenitor cells. *Circ Res*. 2008; 103:194–202. [PubMed: 18556575]
- [37]. Brown DJ, Rzucidlo EM, Merenick BL, Wagner RJ, Martin KA, Powell RJ. Endothelial cell activation of the smooth muscle cell phosphoinositide 3-kinase/Akt pathway promotes differentiation. *J Vasc Surg*. 2005; 41:509–16. [PubMed: 15838487]
- [38]. Xia Y, Bhattacharyya A, Roszell EE, Sandig M, Mequanint K. The role of endothelial cell-bound Jagged1 in Notch3-induced human coronary artery smooth muscle cell differentiation. *Biomaterials*. 2012; 33:2462–72. [PubMed: 22204979]
- [39]. Rzucidlo EM, Martin KA, Powell RJ. Regulation of vascular smooth muscle cell differentiation. *J Vasc Surg*. 2007; 45(Suppl A):A25–32. [PubMed: 17544021]
- [40]. Anamelechi CC, Truskey GA, Reichert WM. Mylar and Teflon-AF as cell culture substrates for studying endothelial cell adhesion. *Biomaterials*. 2005; 26:6887–96. [PubMed: 15990164]
- [41]. Wallace CS, Strike SA, Truskey GA. Smooth muscle cell rigidity and extracellular matrix organization influence endothelial cell spreading and adhesion formation in coculture. *Am J Physiol Heart Circ Physiol*. 2007; 293:H1978–1986. [PubMed: 17644568]
- [42]. Buga GM, Gold ME, Fukuto JM, Ignarro LJ. Shear stress-induced release of nitric oxide from endothelial cells grown on beads. *Hypertension*. 1991; 17:187–93. [PubMed: 1991651]
- [43]. Andrews AM, Jaron D, Buerk DG, Kirby PL, Barbee KA. Direct, real-time measurement of shear stress-induced nitric oxide produced from endothelial cells in vitro. *Nitric Oxide*. 2010; 23:335–42. [PubMed: 20719252]
- [44]. Yang C, Zhang ZH, Li ZJ, Yang RC, Qian GQ, Han ZC. Enhancement of neovascularization with cord blood CD133+ cell-derived endothelial progenitor cell transplantation. *Thromb Haemost*. 2004; 91:1202–12. [PubMed: 15175808]

- [45]. Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, Eguchi H, et al. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. *J Clin Invest.* 2000; 105:1527–36. [PubMed: 10841511]
- [46]. Ladhoff J, Fleischer B, Hara Y, Volk HD, Seifert M. Immune privilege of endothelial cells differentiated from endothelial progenitor cells. *Cardiovasc Res.* 2010; 88:121–9. [PubMed: 20388638]
- [47]. Suarez Y, Shepherd BR, Rao DA, Pober JS. Alloimmunity to human endothelial cells derived from cord blood progenitors. *J Immunol.* 2007; 179:7488–96. [PubMed: 18025193]
- [48]. Sabatino M, Ren J, David-Ocampo V, England L, McGann M, Tran M, et al. The establishment of a bank of stored clinical bone marrow stromal cell products. *J Transl Med.* 2012; 10:23. [PubMed: 22309358]

Fig. 1. Co-culture of CAD EPCs or HAECs with SMCs for 1 week. (A–D) Representative images of co-cultures after 7 days. ECs are stained with CD31 antibody. EC and SMC nuclei are stained with DAPI. Scale bars represent 100 μm. Bottom: percent EC coverage after 1 day (E) and 7 days (F), normalized against 100% coverage. A two-way ANOVA indicated significant effects due to both cell density and cell type ($P < 0.05$, $n = 3$).

Fig. 2.

Short-term supraphysiological shear stress studies. CAD EPCs or HAECs cultured over SMCs for either 15 min or 24 h were exposed to 10 min of 100 dynes cm^{-2} steady, laminar shear stress. Both CAD EPCs and HAECs experienced greater adhesion to SMCs after 24 h ($*P < 0.05$, $n = 3$).

Fig. 3.

Fluorescence micrographs of CAD EPCs and HAECs after 48 h of static culture or exposure to physiological shear stress. CAD EPCs or HAECs were seeded over confluent, quiescent SMCs. Cell junctions are indicated with a CD31 antibody. Arrow indicates direction of flow. Scale bars represent 100 μm .

Fig. 4.

Orientation (A) and elongation (B) of CAD EPCs and HAECs on SMCs exposed to 48 h of 15 dynes cm^{-2} shear stress or maintained in static culture. CAD EPCs experience greater alignment toward the direction of flow (** $P < 0.05$) and greater elongation (* $P < 0.05$) than HAECs ($n = 3$). 0 refers to perfect alignment with the direction of flow, and 0 refers to a perfect line, while 1 refers to a perfect circle. Results reported as mean $\pm$ SEM, $n = 3$.

Fig. 5.
Cell area of both CAD EPCs and HAECs in co-culture did not decrease with flow. Data reported as mean $\pm$ SEM ($n = 3$).

Fig. 6.

Nitrite release of CAD EPCs under static vs. flow conditions. The amount of nitrite released was calculated taking into account the volume of media in the static and flow samples and the number of CAD EPCs seeded per sample. Data reported as mean $\pm$ standard deviation (* $P < 0.05$, $n = 3$).

Fig. 7.

Integrin blocking indicates functional CAD EPC binding mechanisms. A reduction in the number of cells bound to a confluent, quiescent SMC layer after blocking the functional integrins for fibronectin indicates the functional presence of these integrin subsets on the surface of the CAD EPCs (* $P < 0.05$, $n = 3$).

Table 1

Characteristic properties of CAD EPCs vs. CB EPCs.

	CAD EPCs	CB EPCs
Proliferation potential	High [19]	High [18]
Capillary-like network formation in Matrigel	Yes [20]	Yes [31,48]
Average cell area over SMCs after 4 days (μm^2)	1878 ± 244	1700 ± 100 [23]
Percent retention on SMCs after high shear stress	>90%	>90% [22]
Alignment with flow over fibronectin-coated Teflon-AF	Yes [20]	Yes [22]
Alignment with flow over confluent, quiescent SMC layer	Yes	Yes [22,23]