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Tissue-engineered microvessels on three-dimensional biodegradable scaffolds using human endothelial progenitor cells

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Tissue-engineered microvessels using human endothelial progenitor cells. Am J Physiol Heart Circ Physiol 287: H480–H487, 2004; 10.1152/ajpheart.01232.2003.—Tissue engineering may offer patients new options when replacement or repair of an organ is needed. However, most tissues will require a microvascular network to supply oxygen and nutrients. One strategy for creating a microvascular network would be promotion of vasculogenesis in situ by seeding vascular progenitor cells within the biopolymeric construct. To pursue this strategy, we isolated CD34+/CD133+ endothelial progenitor cells (EPC) from human umbilical cord blood and expanded the cells ex vivo as EPC-derived endothelial cells (EC). The EPC lost expression of the stem cell marker CD133 but continued to express the endothelial markers KDR/VEGF-R2, VE-cadherin, CD31, von Willebrand factor, and E-selectin. The cells were also shown to mediate calcium-dependent adhesion of HL-60 cells, a human promyelocytic leukemia cell line, providing evidence for a proinflammatory endothelial phenotype. The EPC-derived EC maintained this endothelial phenotype when expanded in roller bottles and subsequently seeded on polyglycolic acid-poly-L-lactic acid (PGA-PLLA) scaffolds, but microvesSEL formation was not observed. In contrast, EPC-derived EC seeded with human smooth muscle cells formed capillary-like structures throughout the scaffold (76.5 ± 35 microvessels/mm²). These results indicate that 1) EPC-derived EC can be expanded in vitro and seeded on biodegradable scaffolds with preservation of endothelial phenotype and 2) EPC-derived EC seeded with human smooth muscle cells form microvessels on porous PGA-PLLA scaffolds. These properties indicate that EPC may be well suited for creating microvascular networks within tissue-engineered constructs.

Blood vessels; tissue engineering; polyglycolic acid-poly-L-lactic acid scaffolds; tissue engineering; polyglycolic acid-poly-L-lactic acid (PGA-PLLA) scaffolds, but microvessel formation was not observed. In contrast, EPC-derived EC seeded with human smooth muscle cells formed capillary-like structures throughout the scaffold (76.5 ± 35 microvessels/mm²). These results indicate that 1) EPC-derived EC can be expanded in vitro and seeded on biodegradable scaffolds with preservation of endothelial phenotype and 2) EPC-derived EC seeded with human smooth muscle cells form microvessels on porous PGA-PLLA scaffolds. These properties indicate that EPC may be well suited for creating microvascular networks within tissue-engineered constructs.

Our goal was to advance feasibility studies by evaluating 1) rejection, TE blood vessels and cardiovascular structures have been made from autologous vascular cells (12, 14, 35). In studies involving small-diameter vascular grafts, an endothelialized lumen has been sufficient for viability, but larger vessels and, especially, whole organs will require a microvascular network to deliver oxygen and nutrients to the tissue. Strategies such as embedding angiogenic factors into the scaffold material to promote ingrowth of microvessels and fabrication technologies to create polymers containing vascular-like networks have been proposed (19, 24, 26). For example, VEGF incorporated into polyglycolic acid-poly-L-lactic acid (PGA-PLLA) matrices was found to increase the ingrowth of microvessels from the host vasculature (26). In the same study, functional human EC-derived microvessels were formed when VEGF-containing matrices were seeded with human dermal microvascular endothelial cells (HDMEC). More recently, investigators explored the possibility of using endothelial progenitor cells (EPC) from peripheral blood or bone marrow to create TE small-diameter blood vessels (14).

Human EPC are best defined as a CD133+/KDR+ subpopulation of CD34+ cells that can be isolated from peripheral blood, bone marrow, or fetal liver (28). CD133, also known as AC133 and human prominin-1 (10), is a stem/progenitor cell marker of unknown function. When cultured in vitro, the EPC rapidly differentiate into mature endothelial cells (EC) and express virtually all the well-known markers of the endothelial lineage (4, 6, 9). The number of EPC in adult peripheral blood is extremely low, but, when isolated and expanded in culture, EPC can undergo >1,000 population doublings (20). This doubling ability is in contrast to that of mature EC, which are also present in peripheral blood. These cells, which appear to be shed from the vessel wall in response to injury, grow in culture but senesce after ~30 population doublings (20). We speculate that human EPC isolated from peripheral blood and expanded in vitro as mature EC provide a safe and robust source of autologous cells for promoting vascularization of TE organs and tissues with dimensions that exceed limits of oxygen diffusion. Indeed, umbilical cord blood represents an excellent source of autologous EPC for infants diagnosed in utero with defects that may be corrected by TE.

Our goal was to advance feasibility studies by evaluating 1) the ease with which highly purified and phenotypically defined

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human EPC can be obtained from peripheral blood, 2) the ability to expand these cells as phenotypically stable mature EC, and 3) the ability of EPC-derived mature EC to form microvessels in vitro in a three-dimensional TE construct. Our results strongly support the therapeutic potential of using human EPC to form, in situ, a vascular network within TE organs and tissues.

MATERIALS AND METHODS

Purification of mononuclear cells from human umbilical cord blood. Human umbilical cord blood (HUCB) from umbilical and placental tissue was obtained from the Brigham and Women’s Hospital in accordance with an Institutional Review Board-approved protocol. Mononuclear cells (MNC) were isolated by Ficoll-Hyphaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient sedimentation.

Flow cytometry. To determine the percentages of CD34+/CD133+, CD34+/CD133+ cells in HUCB, the MNC fraction was incubated with 10 μl of phycoerythrin-labeled anti-CD133 MAAb and 10 μl of FITC-labeled anti-CD34 MAAb for 10 min. MNC were also incubated with control phycoerythrin-labeled mouse IgG1 and FITC-labeled mouse IgG2a. All fluorescent antibody conjugates were purchased from Miltenyi Biotec. Two-color flow cytometric analyses were performed by FACSscan and Cell Quest software (Becton-Dickinson). Each analysis included ≥10,000 events.

Positive selection of CD34+/CD133+ cells using MAAb-coated magnetic beads. MNC were plated onto 1% gelatin-coated (Difco) 100-mm tissue culture plates at 3.3 × 106 cells per 100-mm plate in EC basal growth medium-2 (EBM-2; Clonetics) supplemented with EC growth medium-2, SingleQuots (except hydrocortisone), 1× glucose-penicillin-streptomycin (GIBCO), 20% heat-inactivated FBS (Hyclone), and 15% human plasma. This nutrient- and growth-factor rich culture medium will be referred to as EBM-2/20%/15%. After they were cultured for 2 days, CD34+ cells were purified from the nonadherent cell population with the use of anti-CD34-conjugated superparamagnetic microbeads (CD34 isolation kit, Miltenyi Biotec). Endothelial outgrowth from CD34+ cells has been shown to be more homogenous than that from EC derived from unselected MNC (9). The CD34+ population was further purified using anti-CD133/1-conjugated magnetic microbeads. The yield of CD34+/CD133+ cells was typically 1–2 × 106 cells per 60 ml of HUCB.

Cell culture. The CD34+/CD133+ cells were grown on 1% gelatin-coated 96-well plates at a density of 1.0 × 104 cells/well in EBM-2/20%/15%. On day 14, the cells reached confluence and were passaged for 24-well gelatin-coated dishes; after 21 days, the cells were cultured in larger dishes in EBM-2/20%/15%. On day 21, the cells reached confluence and were passaged for 24-well gelatin-coated dishes; after 21 days, the cells were cultured in larger dishes in EBM-2/20%/15% human plasma.

For phenotypic analyses, HEDMEC were used as a positive control for mature EC (17). The retinoblastoma cell line WERI-RB-1 (American Type Culture Collection) was used as a positive control for detection of CD133 (37). Human saphenous vein smooth muscle cells (SMC) (2, 30) were grown in DMEM, 10% FBS, 1× glucose-penicillin-streptomycin, and 250 μg/ml amphoterin B (Fungizone, Invitrogen).

Indirect immunofluorescence. EPC and EPC-derived EC were plated onto 1% gelatin-coated glass coverslips for ≥24 h, fixed with –20°C methanol, and incubated with primary antibody diluted 1:1,000 followed by FITC-conjugated or Texas red-conjugated secondary antibody at 5 μg/ml of Willebrand factor (vWF) (Dako). CD31 was detected with goat anti-human CD31 (Santa Cruz). VE-cadherin was detected using a mouse MAAb against human VE-cadherin (clone TEA131, Immunotech). To induce E-selectin, cells were treated with LPS (1 μg/ml) for 3–6 h before they were stained with mouse anti-human E-selectin (18).

RT-PCR. Total RNA was isolated from CD34+/CD133+ cells using the RNeasy kit and RNase-free DNase (Qiagen). RT-PCR was performed with Superscript II RNase H– RT (Invitrogen). Oligonucleotide probes were synthesized for CD133 mRNA: 5'-CCAATT-TCTACCTCATGTGTTGG-3' (5' site) and 5'-ACCAACAGG-GAGATTGCAAAGC-3' (3' site). PCR amplification was performed with Taq DNA polymerase (Invitrogen); denaturation for 30 s at 94°C, annealing for 45 s at 60°C, and elongation for 40 s at 72°C for 35 cycles. For kinase insert domain-containing receptor (KDR) mRNA, oligonucleotide probes were synthesized: 5'-ACCAACGTCATG-CATCACC-3' (5' site) and 5'-TTACACACCTGTGTCGTA-3' (3' site). PCR amplification was carried out as follows: 30 cycles of 92°C for 45 s, 55°C for 45 s, and 72°C for 1 min. RNA levels were normalized by amplification of GAPDH.

Adhesion assay and quantitation of cell surface adhesion molecules. EPC-derived EC and HDMEC plated on 35-mm dishes were assayed at 80% confluency as described elsewhere (8). Briefly, cells were fed fresh medium, with or without TNF-α (10 ng/ml), for 5 h. Medium was removed, and cell monolayers were washed once with or without 2.5 mM EGTA. In a volume of 0.6 ml, 2 × 106 HL-60 cells, with or without 2.5 mM EGTA, were added to the monolayers and incubated at 4°C on a rocking platform for 45 min, washed five times with or without 2.5 mM EGTA, and fixed with 2.5% glutaraldehyde for 10 min to analyze cell surface leukocyte adhesion molecules in TNF-α-stimulated cells. EPC-derived EC were removed from the cell culture plate by brief trypsinization, resuspended in PBS-0.1% BSA-1 mM EDTA, incubated with MAAb directed against human E-selectin, human VCAM-1, or human ICAM-1, and then analyzed by flow cytometry as described elsewhere (8). Flow cytometric analyses were performed using a Beckon-Dickinson FACScan flow cytometer.

PGA-PLLA biodegradable scaffold. Mult-extruded fibers of PGA and PLLA were purchased from Purac and were assembled into nonwoven mesh textile at 65 mg/ml (Albany International, Mansfield, MA). The rationale for this composite is that PLLA stabilizes the mesh and prolongs the normally rapid biodegradation time of PGA (1, 15, 22). PGA-PLLA conduits were cold gas sterilized by exposure to ethylene oxide.

Cell expansion and seeding on PGA-PLLA. After 21 days, the cells expanded on dishes and then in roller bottles at 37°C. Each roller bottle was filled with 200 ml of EBM-2/20%. Fresh medium and CO2 were supplied every 5–6 days. CO2 was added when the medium was changed. Cells were seeded on PGA-PLLA constructs (0.5 cm diameter × 5 cm long × 1 mm thick) at 1 × 106–1 × 107 EC/cm2 under rotating dynamic conditions (12 g) for 48 h in 25 ml of EBM-2/20% and then placed in roller bottles so that continuous rotation could be maintained. At the end of 6 wk, cells were recovered from the conduit for phenotypic analysis or lysed for Western blot analysis, or the conduits were sectioned for histological examination.

Seeding of EPC-derived EC and SMC on PGA-PLLA. EPC-derived EC, alone or in combination with human SMC (106 cells/cm2 of each cell type for a total of 2 × 106 cells/cm2), were seeded onto PGA-PLLA constructs (0.16 cm diameter × 1 cm long × 1 mm thick) in a sequential process that involved 30 h of rotating dynamic seeding (12 g) and 6 days of roller bottle culture in EBM-2/20%. Subsequently, EPC-derived EC (106 cells/cm2) were seeded a second time under dynamic rotating conditions, and the cultures were continued for another 6 days to determine whether an endothelial monolayer would form on the surface of the scaffold.

Western blot. Cells that had been seeded and cultured on PGA-PLLA scaffolds for 6 wk were lysed by addition of 0.5 ml of lysis buffer per gram of TE scaffold. Lysates were subjected to 4–12% NuPAGE Bis-Tris gel (Invitrogen) electrophoresis (10 μg protein/lane) and transferred to Immobilon-P membranes (Millipore). Membranes were incubated with goat anti-human CD31 in PBS, 5% dry milk, and 0.1% Tween 20 and then with peroxidase-conjugated anti-goat IgG. Antigen-antibody complexes were visualized using SuperSignal West Pico (Pierce). The proteins were visualized by chemiluminescence according to the manufacturer's instructions. The following antibodies were used: anti-CD31 (Novocea) for EC, anti-CD34 (Serotec) for common leukocytes, anti-CD133 (BD Biosciences) for EPC, and anti-CD99 (Novocea) for EC and EPC.
Lumiglo (Kirkegaard and Perry Laboratory) and chemiluminescent-sensitive Hyperfilm ECL (Amersham Pharmacia Biotech). Cell lysates from HDMEC were used as a positive control.

Histology and immunohistochemistry. The midsections of TE constructs were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm. Sections were stained with hematoxylin and eosin for overall morphology and with Movat pentachrome stain for evaluation of the extracellular matrix (ECM). Immunohistochemistry was performed by avidin-peroxidase complex (Vectastain ABC kit, Vector Laboratories) using goat anti-human CD31 diluted at 1:5 (Dako) and mouse anti-human α-smooth muscle actin (α-SMA) at 1:200 (Dako). The reaction was visualized with 3-amino-9-ethylcarbazole substrate and sensitive Hyperlumiglo (Kirkegaard and Perry Laboratory) and chemiluminescent-substrate. The entire cross section of the TE constructs (n = 4) was counted and normalized to the total area.

Microvessel density analysis. Microvessels were quantitated by counting lumens containing a cell nucleus and CD31 immunostain. The entire cross section of the TE constructs (n = 4) was counted and normalized to the total area.

RESULTS

CD34+/CD133+ cells in HUCB. The fraction of cells in HUCB that expresses CD34 and CD133 was determined by flow cytometry. A representative analysis is shown in Fig. 1 (n = 3). Incubation of cells with control fluorescent antibody conjugates showed a very low level of binding to the cells (Fig. 1A). Double-label antibody staining showed that 3.0% of the cells expressed CD34 alone, 0.14% of the cells expressed CD133 alone, and 0.53% of the cells expressed CD34 and CD133 (Fig. 1B). This CD34+/CD133+ population of cells (Fig. 1B, top right) contains EPC and, possibly, a small number of hematopoietic stem cells, which also express CD34 and CD133 (16, 36). The percentage of CD34+/CD133+ cells was 0.16% and 0.32% in two other isolations. Analysis of the forward and side scatter indicated that the cell population was homogenous in size (data not shown).

Characterization of CD34+/CD133+ cells. CD34 is the only marker known that distinguishes EPC from mature EC (28). Therefore, it is essential to show directly that cells that bound to the anti-CD133-coated magnetic beads do indeed express CD133. To do this, CD34+/CD133+ cells obtained from the two-step immunoselection procedure (Fig. 1B, top right) were analyzed by indirect immunofluorescence using a second anti-CD133 MAb. CD133 was detected on the cell surface of every individual cell in the sparse population (Fig. 2A). To further confirm CD133 expression in the CD34+/CD133+ cells, the cells were analyzed by RT-PCR (Fig. 2B). The WERI-RB-1 retinoblastoma cell line was used as a positive control for CD133 (21), whereas HDMEC, mature human EC, were used as a negative control. CD133 mRNA was readily detected in EPC after 2 days in culture. As expected from previous studies showing loss of CD133 as EPC are cultured in vitro, CD133 was no longer detected by RT-PCR...
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factor, epidermal growth factor, and insulin-like growth factor
contains growth factors such as VEGF, basic fibroblast growth factor, epidermal growth factor, and insulin-like growth factor I, which are known to influence EC differentiation. The cells exhibited a cobblestone morphology, which is characteristic of mature EC (Fig. 3A).

Expression of endothelium-specific proteins was shown by indirect immunofluorescence: the cells uniformly expressed VE-cadherin (Fig. 3C) and CD31 (Fig. 3D). The localization of these proteins at the cell-cell borders is consistent with their functions as EC adhesion molecules. vWF was detected in rod-shaped granules that appear to be Weibel-Palade bodies (Fig. 3E). E-selectin was induced in cells treated with LPS for 4 h (Fig. 3F). Background staining observed with a control IgG is shown in Fig. 3B. To further confirm the endothelial phenotype, total RNA was extracted from the EPC-derived EC and assayed for KDR/VEGF-R2 mRNA (Fig. 3G). Robust expression of KDR was detected in these EPC-derived EC. In this analysis, WERI-RB-1 RNA served as a negative control, and HDMEC served as a positive control. In summary, the cells no longer expressed CD133 but continued to express KDR, VE-cadherin, CD31, vWF, and E-selectin. This combination of markers provides a definitive demonstration that the cells isolated from HUCB are endothelial.

EPC-derived EC exhibit a proinflammatory phenotype. To determine whether EPC-derived EC were capable of expressing a functional endothelial phenotype, we used a static cell adhesion assay to test whether EPC-derived EC monolayers, stimulated with the cytokine TNF-α, would mediate adhesion of the HL-60 cells. HL-60 cells are a human promyelocytic cell line used to model neutrophil interactions with endothelium. EPC-derived EC monolayers (Fig. 4, A–C) and, for comparison, HDMEC (Fig. 4, D–F) were treated without or with TNF-α (10 ng/ml) for 5 h. HL-60 cells in the absence or presence of 2.5 mM EGTA were added to the cell monolayers. As expected, HL-60 cells did not adhere to the nonstimulated cells (Fig. 4, A and D). This is expected on the basis of the low-to-undetectable levels of E-selectin, VCAM-1, and ICAM-1 in EPC-derived EC and HDMEC (Fig. 4G). Robust binding of HL-60 cells was seen in TNF-α-treated cells (Fig. 4, B and E), consistent with the upregulation of leukocyte adhesion molecules (Fig. 4G). HL-60 cell adhesion to TNF-α-treated cells was abolished when EGTA was included in the adhesion assay (Fig. 4, C and F), indicating the calcium/magnesium-dependent binding. In summary, EPC-derived EC upregulate leukocyte adhesion molecules in response to an inflammatory cytokine and mediate calcium-dependent adhesion of a neutrophil-like cell line in a manner very similar to that of normal human microvascular EC. This suggests that microvessels formed from EPC-derived EC will express appropriate proinflammatory properties.

Phenotypic analysis of EPC-derived EC after seeding on PGA-PLLA scaffolds. The generation of TE cardiovascular structures has typically required 3–6 wk of in vitro development. To determine whether EPC-derived EC could maintain an endothelial phenotype during prolonged in vitro culture, we analyzed the EPC-derived EC after 6 wk of in vitro development. PGA-PLLA scaffolds were prepared (Fig. 4), and the scaffolds were seeded with EPCs. The scaffolds were cultured for 6 wk, and the cells were then analyzed for the expression of endothelial markers. VCAM-1, ICAM-1, and E-selectin were detected in the EPC-derived EC monolayers (Fig. 5). However, little or no ECM was detected with Movat pentachrome stain, even when up to 1 × 10^7 cells/cm^2 were seeded on the scaffolds.
To reexamine cell phenotype after the extended in vitro culture period, the cells were removed from the PGA-PLLA scaffolds with trypsin and returned to two-dimensional cell culture. The cells formed a cobblestone morphology (Fig. 5B) and expressed VE-cadherin (Fig. 5D), vWF (Fig. 5E), and inducible E-selectin (Fig. 5F). Cells incubated with a control IgG showed only background staining (Fig. 5C).

To further confirm the endothelial phenotype, the cells were shown to express CD31 by lysis of the cells on the scaffold with the urea-based buffer and analysis by Western blot (Fig. 5G). The ability of EPC-derived EC to maintain a stable endothelial phenotype during long-term culture on PGA-PLLA indicates that these cells are highly suitable for the rigors of many TE applications.

**Microvessel formation in PGA-PLLA scaffolds.** To mimic more closely the cellular interactions in the blood vessel wall, EPC-derived EC were seeded with human SMC onto PGA-PLLA under the same dynamic seeding and culture conditions used for EPC-derived EC alone. At the end of the 6-day period, the constructs were seeded again with EPC-derived EC to promote formation of an endothelial monolayer on the surface of the construct. This cell seeding protocol resulted in layered tissue formation (Fig. 6A and C), accumulation of ECM (Fig. 6B), and formation of microvessels within the tissue (Fig. 6D, arrows). In Fig. 6A, hematoxylin-eosin staining revealed viable cells throughout the scaffold as well as along the surface of the scaffold (PGA-PLLA fibers were visible as uniformly sized holes). Cell viability was confirmed by immunostaining for the cytoskeletal protein vimentin (data not shown) (27). In Fig. 6B, Movat pentachrome staining revealed proteoglycans and extracellular collagen. An example of a PLLA fiber that stained (data not shown). To reexamine cell phenotype after the extended in vitro culture period, the cells were removed from the PGA-PLLA scaffolds with trypsin and returned to two-dimensional cell culture. The cells formed a cobblestone morphology (Fig. 5B) and expressed VE-cadherin (Fig. 5D), vWF (Fig. 5E), and inducible E-selectin (Fig. 5F). Cells incubated with a control IgG showed only background staining (Fig. 5C).

**Fig. 4.** Proinflammatory properties of EPC-derived EC. HL-60 cell adhesion to EPC-derived EC (A–C) and HDMEC (D–F) is shown. EC were treated without (A and D) or with TNF-α for 5 h (B, C, E, and F). In C and F, 2.5 mM EGTA was included during HL-60 adhesion and washes. G: control and TNF-α-stimulated cells analyzed for expression of leukocyte adhesion molecules E-selectin, VCAM-1, and ICAM-1. Percentage of EPC-derived EC and HDMEC expressing each antigen was determined by flow cytometry.

**Fig. 5.** EPC maintain EC phenotype on polyglycolic acid-poly-L-lactic acid (PGA-PLLA). A: paraffin cross section of EPC-seeded PGA-PLLA stained with hematoxylin and eosin. When recovered from the PGA-PLLA construct and returned to culture, cells displayed an endothelium-like cobblestone morphology (B) and continued to express EC markers: cells immunostained with mouse IgG1 (C), VE-cadherin (D), von Willebrand factor (E), and E-selectin (F). G: cell lysates from EPC-seeded PGA-PLLA analyzed by Western blot for CD31.
yellow in this procedure is shown in Fig. 6B. In Fig. 6C, α-SMA+ cells were found throughout the scaffold. In Fig. 6D, most CD31+ cells formed microvessels and were present throughout the construct. In some cases, the CD31+ cells appeared to be wrapped around a residual polymer fiber (Fig. 6D, asterisk), as observed in Fig. 5A. The number of microvessels detected in whole cross sections of EPC-derived EC/SMC constructs was 76.6 ± 35/mm² (n = 4). No microvessels or CD31+ cells were detected when human SMC were seeded alone (n = 2; data not shown). Thus EPC-derived EC were able to spontaneously form microvessel-like structures when seeded with human SMC on PGA-PLLA scaffolds.

**DISCUSSION**

We have described methods for expanding highly purified EPC from HUCB. Importantly, we have rigorously demonstrated that the cells isolated from blood and placed in culture express the stem/progenitor cell marker CD133 as well as the endothelial markers CD34 and KDR. The EPC differentiate to mature EC on the basis of the loss of CD133 expression but remain viable and phenotypically stable after seeding on a biodegradable scaffold, i.e., PGA-PLLA, which is currently being tested for construction of TE heart valves. We show that EPC-derived EC have an inherent ability to assemble into a microvascular network when seeded on PGA-PLLA with human SMC. This vasculogenic potential suggests that EPC-derived EC can be used to introduce a microvascular network in TE organs and tissues. The rationale is that the engineered microvessels might readily form anastomoses with existing vessels in the host, thereby accelerating vascularization and improving the construct viability. Support for this rationale has been suggested by previous investigations: preformed human microvascular networks in collagen/fibronectin gels were shown to form complex vascular structures perfused by the host circulation within 31 days of implantation into immunodeficient mice (31). Kim and Mooney (15) and Nor et al. (23) have reported similar results with human microvascular EC seeded on biodegradable polymer matrices: functional microvessels were evident 7–10 days after implantation into mice. Ideally, tissue perfusion would occur within hours after implantation.

We speculate that microvessels could be preformed in many types of TE constructs with use of EPC-derived EC. One application would be to prefabricate a vasa vasorum in the conduit wall of TE heart valves. The development of TE heart valves is an active area of investigation (12), yet little attention has been devoted to how to establish a vasa vasorum in the thick-walled arterial conduit needed for TE heart valves.

Our results are the first to demonstrate the vasculogenic potential of EPC in vitro in a TE setting. In the absence of SMC, EPC-derived EC adhered to the PGA-PLLA fibers and remained viable for several weeks. However, very little tissue formed when EPC-derived EC were seeded alone. Seeding with SMC dramatically enhanced the assembly of CD31+ cells into microvessel-like structures. This finding is consistent with the extensive literature on endothelium-SMC interactions in vascular development (7).

The purity of the EPC population used in this study was likely to have been a critical factor in the vasculogenic potential displayed by the cells. Previous studies have shown that the CD133+ subpopulation in CD34+ cells has an increased capacity for endothelial differentiation and decreased apoptosis (11, 13). In addition, selection of CD34+ cells from MNC has been shown to yield cells with an endothelial phenotype, whereas nonsellected MNC resulted in cultures with endothelial and monocytic phenotypes (9). Indeed, Rehman et al. (29) showed that the majority of adherent cells cultured from MNC, often characterized as “endothelial” on the basis of uptake of acetylated-LDL and binding of the lectin Ulex europeus I, express markers specific for the monocye/macrophage lineage and do not grow ex vivo in the presence of endothelial mitogens. Interestingly, these cells secrete angiogenic factors. Therefore, the phenotypic complexity of the adherent cell population...
obtained from MNC is further illustrated by experiments suggesting that these cells can transdifferentiate into functional active cardiomyocytes (3). For TE applications, it will be critical to isolate defined populations of cells so that growth and differentiation can be controlled and regulated during ex vivo tissue development. In our study, we obtained $5 \times 10^5$ EPC-derived EC cells, after 21 days in culture, from $1 \times 10^4$ CD34+/CD133+ cells; $5 \times 10^5$ cells would be sufficient to form microvessels in a PGA-PLLA rectangular patch with the dimensions of 1 cm x 0.5 cm x 0.1 mm. Furthermore, our data demonstrate that these cells maintain their endothelial phenotype, both the expression of endothelial markers and a proinflammatory endothelial phenotype, through prolonged periods in culture and can be expanded to yield $\approx 3 \times 10^6$ cells.

An important advantage of using CD34+/CD133+ cells to isolate EPC-derived EC for these applications is that the cells can be obtained from peripheral blood, i.e., from a newborn’s own cord blood, perhaps banked for later use, or from juvenile or adult peripheral blood. The use of cells from peripheral blood eliminates the need to sacrifice a blood vessel or tissue to obtain EC. Although the number of EPC in adult blood is much lower (25), this should not be an impediment, inasmuch as improvements in the cell purification and culture methods have been rapid in recent years.

Previous studies have shown that human peripheral blood-derived EPC can endothelialize a gelatin-coated polyurethane microporous vascular graft: the cells elongated and aligned with the direction of flow and produced a nonthrombogenic surface (32). Shirota and colleagues (33) induced EPC migration from a cell-seeded stent to adjacent hybrid vascular tissue in vitro, suggesting that delivery of EPC on stents may lead to novel treatments for arterial occlusion. Our previous work also demonstrated use of autologous EPC from peripheral blood for endothelialization of small-diameter vascular grafts (14). In the present study, the porous architecture of the PGA-PLLA scaffold appeared to provide a favorable environment for formation of microvessels. This is in contrast to previous studies in which EC adhesion to PGA was thought to impair the three-dimensional organization of microvessels (34). This suggests that EC interactions with various biopolymers may result in different microvasculature structures.

Formation of a microvasculature within a TE organ or tissue will depend on many factors: EC type, the microarchitecture presented by the scaffold material, the biochemical environment, and mechanical signals (5, 26). Our results suggest that EC source, EC phenotypic stability, and EC and SMC interactions, as well as EC-biopolymer interactions, play critical roles in the formation of TE microvasculature. Further effort is required to implement strategies for controlled vasculogenesis in TE constructs so that oxygen and nutrients can properly nourish the tissue as quickly as possible after implantation in vivo.

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REFERENCES


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