Shear Stress Responses of Adult Blood Outgrowth Endothelial Cells Seeded on Bioartificial Tissue

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Human blood outgrowth endothelial cells (HBOECs) are expanded from circulating endothelial progenitor cells in peripheral blood and thus could provide a source of autologous endothelial cells for tissue-engineered vascular grafts. To examine the suitability of adult HBOECs for use in vascular tissue engineering, the shear stress responsiveness of these cells was examined on bioartificial tissue formed from dermal fibroblasts entrapped in tubular fibrin gels. HBOECs adhered to this surface, deposited collagen IV and laminin, and remained adherent when exposed to 15 dyn/cm² shear stress for 24 h. The shear stress responses of HBOECs were compared to human umbilical vein endothelial cells (HUVECs). As with HUVECs, HBOECs upregulated vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 when exposed to tumor necrosis factor (TNF)- α and shear stress decreased the expression of these adhesion molecules on TNF- α -activated monolayers. Nitric oxide production was elevated by shear stress, but did not vary between cell types. Both cell types decreased platelet adhesion to the bioartificial tissue, whereas pre-exposing the cells to flow decreased platelet adhesion further. These results illustrate the potential utility for HBOECs in vascular tissue engineering, as not only do the cells adhere to bioartificial tissue and remain adherent under physiological shear stress, they are also responsive to shear stress signaling.

Introduction

HUMAN BLOOD OUTGROWTH endothelial cells (HBOECs) are a promising cell source for vascular tissue engineering. These cells can be isolated by outgrowth of circulating endothelial progenitor cells (EPCs) from a patient blood sample^{1–3} and thus could provide a convenient source of autologous endothelial cells for seeding on tissue-engineered vessels before implantation. HBOECs, also called endothelial colony forming cells and late outgrowth EPCs, have been shown to uniformly express endothelial cell markers, form tubular structures *de novo*, and have typical endothelial cell morphology and a robust proliferative capacity, expanding from 20 cells to 10¹⁹ cells in 9 weeks.¹ HBOECs are negative for hematopoietic cell markers CD45, CD14, and CD115 and do not phagocytize bacteria.^{1,3–5} This is in contrast to other putative EPCs, termed early outgrowth endothelial cells or colony forming unit endothelial cells (CFU-ECs), which do not form vessels *in vivo*, will ingest bacteria, and express hematopoietic, as well as endothelial, cell markers.⁴ These CFU-ECs are likely of hematopoietic origin,^{4,6–9} but may express endothelial cell markers due to the uptake of platelet microparticles by mononuclear cells.¹⁰

This distinction between various cells termed EPCs in the literature is an important one, as unlike CFU-ECs, HBOECs have the potential to provide a highly proliferative, autologous endothelial cell source for vascular grafts.

Rigorous assessment of HBOECs is required to ensure successful use of these cells for vascular grafts. The endothelial layer needs to be confluent and remain adherent under normal physiological shear stresses, acting as a barrier between the blood and the procoagulant, sub-endothelial surface. Further, these cells need to be in a nonactivated, quiescent state, producing protective molecules that suppress platelet adhesion and activation, such as prostacyclin (PGI₂) and nitric oxide (NO), anticoagulation factors, such as thrombomodulin (TM), and fibrinolytic factors. In conjunction with this phenotype, HBOECs should show limited expression of proinflammatory molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), P-Selectin, and E-Selectin.^{11,12}

In vitro and *in vivo* studies have examined EPCs derived by a variety of methods for seeding on synthetic grafts,^{13–16} decellularized grafts,¹⁷ and tissue-engineered blood vessels.^{18,19} Fewer studies have examined the specific HBOEC population for this same purpose.^{20,21} However, these initial

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reports are promising as good HBOEC retention is reported on synthetic materials. Eighty percent of ovine BOECs were retained on cholesterol-modified polyurethane after 2 h of flow exposure at 75 dyn/cm².²¹ BOECs derived from cord blood have been shown to adhere on fibronectin or smooth muscle cell (SMC) monolayers at supraphysiological shear stresses (up to 300 dyn/cm²) for short durations and align with flow after 48 h of shear stress exposure at a physiological shear stress (15 dyn/cm²).²² Importantly, HBOECs have been successfully isolated from patients with coronary artery disease,²³ indicating that isolation of adult HBOECs may be a viable option for seeding of vascular grafts for bypass surgery.

To examine the suitability of adult HBOECs for use in vascular tissue engineering, the shear stress responsiveness of these cells was examined on bioartificial tissue formed from neonatal human dermal fibroblasts (HDFs) entrapped in tubular fibrin gels. During 4 weeks in culture, the HDFs degrade the initial fibrin scaffold and deposit new extracellular matrix (ECM), producing bioartificial tissue. The tissue strengthens and stiffens over time, producing a matrix that more closely mimics native vascular tissue.^{24–26} Immunohistochemistry was used to identify predominate ECM on the surface of the bioartificial tissue, as well as ECM deposited by the HBOECs postseeding. Cell density, elongation, alignment, NO production, and expression of the proinflammatory molecules ICAM-1 and VCAM-1 by HBOECs under shear stress were compared to human umbilical vein endothelial cells (HUVECs) to determine if HBOEC flow responses were similar to mature endothelial cells. A whole blood assay was used to examine platelet adhesion to HBOEC- and HUVEC-seeded constructs pre-exposed to shear stress.

Materials and Methods

Cell source

HBOECs were isolated and expanded from the peripheral blood of healthy, human volunteers, as previously described.¹ The protocol for blood collection was approved by the University of Minnesota Institutional Review Board. The cells were maintained in endothelial growth medium (EGM; EBM-2 medium with EGM Singlequot supplement kit; Clonetics) supplemented with 8% additional fetal bovine serum (FBS; Hyclone), and 1% penicillin/streptomycin (Invitrogen). HBOECs were plated onto rat tail collagen I (BD Biosciences) and subcultured at 80%–90% confluence using 0.05% trypsin EDTA (Gibco Invitrogen Co.). Flow cytometry of HBOEC isolations at passage 2 ensured positive staining for endothelial cell markers, including VE-Cadherin and P1H12, and negative staining for CD14 and CD45, as described previously.¹ These cells also displayed the characteristic cobblestone morphology of ECs, formed tubules in Matrigel, and took up acetylated low-density lipoprotein (Biomedical Technologies, Inc.). HBOECs were used from passage 8–12 for all experiments. For comparison, HUVECs (Clonetics) were maintained in the same culture medium and used from passage 4–6.

Neonatal HDFs (Clonetics) were maintained in 50:50 Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Invitrogen) supplemented with 10% FBS (Hyclone), 100 U/mL penicillin (Invitrogen), 100 µg/mL streptomycin (Invitrogen), and 2.5 µg/mL amphotericin-β (Invitrogen). HDFs were pla-

ted at 8500 cells/cm², passaged at 100% confluence, and used at passage 9.

Fibrin-based vascular graft fabrication

Tubular fibrin constructs were made as previously reported.²⁷ Briefly, HDFs suspended in DMEM were mixed into a solution of bovine fibrinogen (Sigma) in 20 mM HEPES-buffered saline. A mixture of bovine thrombin (Sigma) and calcium chloride in DMEM was then added to the suspension, mixed well, and injected into tubular glass molds measuring 8 cm long and 9 mm in inner diameter and containing 2 mm glass rods with stoppers on either end. The glass rods had been coated in 5% Pluronic F127 (Sigma) for 3 hours to reduce adhesion between the fibrin gel and the mandrel. Final gel component concentrations were 3.3 mg/mL fibrin, 500,000 HDF/mL, 0.2 U/mL thrombin, and 1.2 mM CaCl₂. The solution was allowed to gel at 37°C for 30 min, at which time the outer mold was removed and the constructs placed in DMEM supplemented with 10% FBS, 10 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin-β. About 2 µg/mL insulin and 50 µg/mL ascorbic acid were added to the medium to enhance fibrin gel remodeling into tissue. Construct medium was changed 3 times per week until use at 4 weeks. Vascular constructs at 4 weeks were 3–4 cm long and 2 mm in diameter, with a wall thickness of ~200 µm.

HBOEC and HUVEC seeding on bioartificial tissue

After 4 weeks, remodeled fibrin tissue constructs were slit axially and placed luminal surface down on a polycarbonate sheet using a method adapted from Kladakis and Nerem.²⁸ A polycarbonate cap with a 3 mm high groove was placed around the piece of tissue and filled with a solution of 4% low gel temperature agarose (A0701; Sigma). The agarose was allowed to gel and then the polycarbonate sheet was removed, exposing the luminal surface of the construct. A Teflon ring was placed around the cap to form a well for cell culture medium. HBOECs or HUVECs were labeled with 2.5 µM Cell-Tracker Green (Invitrogen) and then were seeded on the luminal surface of the tissue constructs. The cells were seeded slowly, drop-wise onto the tissue at a concentration of 2 × 10⁶ cells/mL so as to achieve a seeding of 100,000 cells/cm². One hour after seeding the tissue was rinsed with Hank's balanced salt solution (HBSS) to remove nonadherent cells and 4 mL of EGM medium was added to each well. For tumor necrosis factor (TNF)-α activation or shear stress experiments, cells were allowed to adhere for 24 h before start of the experiment.

Cell staining

HBOEC and HUVEC expression of TM, tissue factor (TF), endothelial nitric oxide synthase (eNOS), VCAM-1, ICAM-1, and VE-Cadherin was examined by immunocytochemistry. Cells seeded for 48 h in EGM medium on tissue culture plastic (TCP) coated with type I rat tail collagen or on tissue constructs were rinsed with HBSS, fixed in 4% paraformaldehyde for 10 min, and blocked with 5% normal donkey serum for 1 h at room temperature. Samples were incubated with mouse anti-human TM (5 µg/mL; American Diagnostica), goat anti-human TF (10 µg/mL; American Diagnostica), rabbit anti-human eNOS (4 µg/mL; Santa Cruz),

mouse anti-human VCAM-1 (5 µg/mL; Chemicon), mouse anti-human ICAM-1 (5 µg/mL; Chemicon), goat anti-human VE-Cadherin (10 µg/mL; Santa Cruz), and isotype-matched controls for 1 h. After rinsing, Dylight 549 conjugated, host-matched secondary antibody (1:400; Jackson Immuno) was applied for 1 h at room temperature. Hoechst 33342 (1 µg/mL; Invitrogen) was used for staining of nuclei. Images were captured using epi-fluorescent microscopy (Olympus IX700) and confocal microscopy (Olympus Fluoview 1000). Measurement of the integrated fluorescent density per image for VCAM-1 and ICAM-1 was obtained with ImageJ software (NIH), as were cell counts per image. The integrated density per cell was calculated for each image and background values of the matched species IgG controls were subtracted.

Vascular graft ECM characterization

Constructs for histology were fixed in 4% paraformaldehyde for 3 h at 4°C, followed by overnight infiltration with 30% sucrose and 5% dimethyl sulfoxide in phosphate-buffered saline (PBS). Samples were frozen in OCT (Tissue-Tek), sectioned into 9-µm-thick cross sections, and then stained with Lillie's trichrome.²⁹

Construct sections were also stained for the presence of various ECM proteins by immunohistochemistry. Sections were incubated with rabbit anti-human collagen I (2 µg/mL; Novus Biologicals), collagen IV (6 µg/mL; Abcam), laminin (10 µg/mL; Abcam), and fibronectin (1 µg/mL; American Diagnostica), goat anti-human fibrinogen (2 µg/mL; American Diagnostica), and isotype-matched controls in 5% normal donkey serum in PBS (Jackson Immuno) overnight. After rinsing, sections were incubated with Dylight 549 donkey anti-rabbit or anti-goat (1:400 dilution; Jackson Immuno) in PBS for 45 min and counterstained for 10 min with Hoechst 33342 (1 µg/mL; Invitrogen).

Shear stress experiments

The polycarbonate caps with the embedded tissue seeded with HBOECs or HUVECs were placed in parallel plate flow chambers (PPFCs) modified from the design of Sakariassen *et al.*³⁰ The PPFC had a slot height of 0.5 mm, with a tapered inlet and outlet, reaching a final width of 1 cm. The chamber was assembled in a closed loop consisting of a medium reservoir allowing for gas exchange, a peristaltic pump (Cole-Parmer), and a pulse dampener (Cole Parmer). The system was primed with the EGM medium before placing the tissue within the system and then the flow rate was increased 0.5 dyn/cm² every minute, starting at 3 dyn/cm² and increasing to 15 dyn/cm². Steady laminar shear stress was applied for 24 h and then the constructs were harvested for analysis. In the case of TNF-α exposure, 10 U/mL TNF-α was added to static and flow samples at the time flow was started.

For short-duration shear stress experiments, the shear stress was ramped as described; however, at 0, 1, 5, 10, 15, and 25 dyn/cm² the shear stress was held for 20 min and then the construct surface was imaged. HBOECs were seeded on bioartificial tissue and type I collagen-coated TCP for 24 h before application of shear stress. Twelve images per sample were obtained of the CellTracker Green-labeled HBOECs using an Olympus IX70 inverted microscope. Images were thresholded at a fixed level and analyzed with

ImageJ software (NIH) for percent area coverage. Unseeded TCP and bioartificial tissue were used as negative controls.

Cell retention, orientation and alignment

HBOECs and HUVECs were stained and imaged for VE-Cadherin and Hoechst as described above. Confocal images were taken at 20× and 40× to eliminate imaging of Hoechst staining of the HDF nuclei. The numbers of nuclei per image were counted using ImageJ software and normalized to the area of the image to give a cell density. To quantitate cell orientation and alignment, ImageJ software was used to outline each cell, based on the VE-Cadherin staining, and calculate the area, perimeter, major axis, minor axis, and angle of the major axis (cell alignment) with the flow direction. The shape index was calculated using:

$$\text{Shape Index} = \frac{4\pi \text{Area}}{\text{Perimeter}^2}$$

The shape index is a measure of the elongation of a cell and is equal to one for a circle and zero for a line. Decreasing values thus indicate that cells are becoming more elongated.

NO production

Medium samples were collected from HUVEC and HBOEC seeding bioartificial tissue after 24 h of static culture of exposure to 15 dyn/cm² shear stress. Samples were frozen at -80°C, lyophilized, and reconstituted at 2.5× the initial concentration. A commercially available kit was used to convert nitrate to nitrite, followed by the spectrophotometric quantitation of nitrite levels using Griess Reagent (Calbiochem).

Whole blood assay

A platelet adhesion assay was performed with 1 U/mL heparinized whole blood in the PPFCs described above. Whole blood was collected from healthy, consenting donors under University of Minnesota Institutional Review Board approval and used at 37°C within 2 h of the blood draw. The system was primed with HBSS at 37°C before loading with whole blood. The initial 5–10 mL of blood was discarded to remove blood that had mixed with the HBSS and then the system was run as a closed loop at a shear rate of 400 s⁻¹. After 20 min of whole blood exposure, HBSS was run through the system for 2 min to remove unbound platelets and leukocytes. The samples were removed from the flow chamber and fixed with 4% PFA for 10 min and then rinsed with HBSS. The tissue was simultaneously stained for EC junctions with goat anti-human VE-Cadherin and platelets with mouse anti-human GP-1b (1 µg/mL; Abcam) for 1 h at room temperature. After rinsing with PBS, Dylight 549 donkey anti-mouse and Dylight 649 donkey anti-goat secondary antibody (1:400; Jackson Immuno) were applied for 45 min, followed by staining with Hoechst 33342 for 10 min. Samples were imaged on an Olympus Fluoview 1000 confocal microscope and platelet and leukocyte coverage quantified using ImageJ software. Images were converted to binary by use of a fixed threshold. These images were used to calculate the area coverage of platelets. Values were normalized to the HBOEC static group.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software for Windows (GraphPad Software, Inc.). One-way and multivariate analysis of variance (ANOVA) with Bonferroni *post hoc* analysis was conducted to evaluate significant differences between groups. A significance level of $\alpha=0.05$ was used for all tests. All graphs indicate mean $\pm$ standard error of the mean.

Results

HBOECs formed a confluent monolayer on bioartificial tissue

HBOECs adhered to the bioartificial tissue and formed a confluent monolayer (Fig. 1A–C). The cells had typical cobblestone morphology, stained for VE-Cadherin, and maintained cell-to-cell contact (Fig. 1B, C). The HBOECs also expressed TM (Fig. 1D), an important endothelial anticoagulant molecule also expressed by HUVECs (Fig. 1E), and displayed limited TF expression (Fig. 1F) until exposed to the agonist TNF- α (Fig. 1G). Staining throughout the thickness of the tissue showed that TNF- α stimulated TF expression by the HBOECs on the luminal surface as well as by the underlying fibroblasts.

Nonendothelialized and HBOEC-seeded bioartificial tissue was stained for ECM molecules to assess the protein composition at the luminal surface. The tissue was embedded in agarose with the luminal surface exposed and cultured with or without HBOECs for 7 days. Immunostaining for fibrin, fibronectin, collagen I, collagen IV, and laminin

highlighted the presence of all of these ECM proteins throughout the unseeded (Fig. 2A–E) and HBOEC-seeded tissue (Fig. 2F–J). Staining for IgG controls was negative, whereas staining for ECM components showed positive, diffuse staining through the tissue. Residual fibrin, fibronectin, and collagen I were present on the luminal surface (Fig. 2A–C); however, collagen IV and laminin staining was limited on the luminal surface of the unseeded tissue (Fig. 2D, E). This is in contrast to the HBOEC-seeded tissue, where distinct bands of collagen IV and laminin were present on the luminal surface (Fig. 2I, J). This alteration on the luminal surface of the HBOEC-seeded tissue is indicative of new ECM deposition by the HBOECs.

HBOECs remained adherent on bioartificial tissue under physiological shear stress and elongated in the flow direction

HBOECs were seeded on collagen-coated TCP or bioartificial tissue and allowed to adhere for 24 h. Surfaces were then exposed to increasing shear stress (1, 5, 10, 15, and 25 dyn/cm²) and cell retention quantified after 20 min at each shear stress level. Surface coverage was improved by seeding on bioartificial tissue, with a surface coverage after exposure to 25 dyn/cm² of $94.4\% \pm 0.6\%$ as compared with $82.6\% \pm 1.2\%$ on collagen-coated TCP (Fig. 3). The surface coverage was statistically lower for HBOEC on TCP beginning at 15 dyn/cm², but was not lower for HBOEC on bioartificial tissue until 25 dyn/cm².

HBOECs and HUVECs were seeded on the bioartificial tissue and allowed to adhere for 24 h. After 24 h, seeded tissue was either maintained in static culture for another 24 h

FIG. 1. HBOECs seeded on bioartificial tissue. **(A)** Cross-sectional and **(B, C)** *En face* images of CellTracker Green-labeled HBOECs seeding on bioartificial tissue show that the HBOECs form a confluent monolayer on the tissue surface. Nuclei of HBOEC and human dermal fibroblast in the tissue are stained blue, and VE-Cadherin staining is pseudo-colored red. **(D)** HBOECs and **(E)** HUVECs on the bioartificial tissue express thrombomodulin. **(F)** Tissue factor expression by HBOEC on unstimulated tissue and **(G)** after exposure to TNF- α . **(B–E)** Scale bar = 50 μ m. **(F, G)** Scale bar = 25 μ m. HBOEC, human blood outgrowth endothelial cell; HUVEC, human umbilical vein endothelial cell; TNF, tumor necrosis factor. Color images available online at www.liebertonline.com/tea

FIG. 2. ECM staining of bioartificial tissue. (A–E) Bioartificial tissue stained for ECM components. (F–J) Bioartificial tissue seeded with HBOECs for 7 days before staining. (A, F) Fibrin staining. (B, G) Fibronectin staining. (C, H) Collagen I staining. (D, I) Collagen IV staining. (E, J) Laminin staining. The luminal surface is on the left side of each image, nuclei counterstained with Hoechst 33342. Scale bar = 100 μ m. ECM, extracellular matrix. Color images available online at www.liebertonline.com/tea

(Fig. 4A, C) or placed in a PPFC and laminar, steady shear stress applied at 15 dyn/cm² (Fig. 4B, D). After 24 h under flow, HBOECs and HUVECs had begun to elongate in the flow direction, as illustrated with VE-Cadherin staining. VE-Cadherin staining of the cell junctions was visible in all

FIG. 3. HBOEC retention under physiological shear stress for short durations. HBOECs were seeded on type I collagen-coated TCP or bioartificial tissue and incrementally exposed to 1, 5, 10, 15, or 25 dyn/cm² for 20 min at which time HBOEC surface coverage was calculated based on the Cell-Tracker Green staining. Plot shows mean $\pm$ SE for $n=3$ independent experiments. *Significant difference compared to 1 dyn/cm² samples on the same substrate ($p<0.05$). #Significant difference between HBOEC on TCP and bioartificial at the same shear stress ($p<0.05$). TCP, tissue culture plastic; SE, standard error of the mean.

samples and confirmed that HBOEC and HUVEC under flow still maintained a confluent monolayer. Although the HBOECs and HUVECs exposed to flow showed a marked change in cell morphology, the cell density was not significantly altered by application of shear stress (Fig. 4E). HBOEC and HUVEC elongation and alignment were quantified for comparison by calculation of the cell shape index and alignment angle. These measurements confirmed that by 24 h, HBOECs and HUVECs were elongating and aligning in the flow direction (Fig. 4F, G). There was no significant difference between HBOECs and HUVECs in shape index or the strength of alignment with flow by 24 h.

Expression of proinflammatory molecules VCAM-1 and ICAM-1 was modulated with shear stress and TNF- α stimulation

Application of 15 dyn/cm² shear stress for 24 h did not significantly alter VCAM-1 or ICAM-1 expression by HBOECs or HUVECs compared to static culture (Fig. 5). Background due to nonspecific binding of the VCAM-1 and ICAM-1 antibodies was subtracted from adhesion molecule expression during quantification by subtraction of mouse IgG control values; thus, the values near zero indicate minimal expression of either VCAM-1 or ICAM-1, under static or shear stress culture, as these groups could not be distinguished statistically from the IgG controls.

To examine the responsiveness of the HBOECs to an inflammatory stimulus, 10 U/mL TNF- α was added to the cultures for 24 h, during static culture or shear stress exposure. Addition of TNF- α increased VCAM-1 and ICAM-1 expression for both cell types, whereas shear stress was able to modulate this effect; shear stress conditioning combined

FIG. 4. Retention, elongation, and alignment of HBOECs and HUVECs seeded on bioartificial tissue and exposed to shear stress. VE-Cadherin staining of (A, B) HBOECs and (C, D) HUVECs. (A, C) Statically cultured cells. (B, D) Cells cultured under 15 dyn/cm² for 24 h. (E) Cell density, (F) elongation, and (G) alignment of HBOECs and HUVECs after exposure to 15 dyn/cm² shear stress or static culture for 24 h. The elongation is given as a shape index where 0 is a line and 1 is a circle. Cell alignment is given on a scale from perfectly aligned (0°) to no alignment (45°). **p* < 0.05 compared to static conditions. All plots show mean ± SE for *n* = 3 independent experiments. Scale bar = 50 μm and applies to all images. Color images available online at www.liebertonline.com/tea

with TNF- α stimulation decreased VCAM-1 and ICAM-1 expression compared to the static, TNF- α -treated cells. VCAM-1 and ICAM-1 expression in the flow-conditioned, TNF- α -stimulated cells remained higher than the static or flow-cultured, nonstimulated HBOECs and HUVECs. This indicates that HBOECs, like HUVECs, are responsive to

biochemical inflammatory stimuli and modulate this response under steady laminar shear stress of physiological value.

Based on multivariate ANOVA, the major source of variation in VCAM-1 and ICAM-1 expression was accounted for by the application of shear stress or TNF- α ; however, cell

FIG. 5. VCAM-1 and ICAM-1 expression by HBOECs and HUVECs seeded on bioartificial tissue and cultured under static or flow conditions for 24 h, in the presence or absence of TNF- α . (A) VCAM-1 expression. (B) ICAM-1 expression. Plots show mean $\pm$ SE for $n=3$ tissue samples. *Significant increase over static and flow groups of the same cell type ($p < 0.05$). **Significant increase over all other groups of the same cell type ($p < 0.05$). ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

type was a significant source of variability for VCAM-1 expression as well ($p < 0.05$). VCAM-1 expression by HBOECs was elevated compared to HUVECs when TNF- α was applied to statically cultured cells. Cell type was not a significant source of variability for ICAM expression.

eNOS expression and NO_x production were elevated by exposure to shear stress

After 24 h of static culture or shear stress exposure at 15 dyn/cm², HBOECs seeded on the bioartificial tissue were stained for eNOS (Fig. 6A, B). eNOS was localized to the HBOEC layer on the luminal surface of the tissue. While eNOS staining of the statically cultured HBOECs was sporadic throughout the HBOEC layer (Fig. 6A), flow conditioning increased the eNOS intensity and created a continuous band of eNOS throughout the HBOEC monolayer (Fig. 6B).

FIG. 6. eNOS expression and NO_x production by HBOECs and HUVECs seeded on bioartificial tissue and cultured under static and flow conditions for 24 h. eNOS staining of HBOEC cross sections after (A) static culture or (B) flow exposure. (C) NO production was quantified by measurement of total nitrate and nitrite in the HBOEC and HUVEC culture medium. Values are normalized to the statically cultured HBOEC value. Plot shows mean $\pm$ SE for $n=3$ independent experiments. *NO production by HBOECs and HUVECs cultured under flow conditions increased compared to HBOEC static culture ($p < 0.01$). Scale bar = 100 μ m. eNOS, endothelial nitric oxide synthase. Color images available online at www.liebertonline.com/tea

To assess the effect of higher eNOS expression by HBOEC or HUVEC, NO production was measured for these same samples. NO production was measured via the quantitation of NO byproducts, nitrate and nitrite, in the cell culture medium after 24 h of static culture or flow conditioning at 15 dyn/cm² (Fig. 6C). Similar basal levels of NO were seen in HUVEC and HBOEC static culture. NO production was elevated ~19-fold in HBOEC and HUVEC cultures when shear stress was applied for 24 h.

Platelet adhesion was lower after exposure to shear stress

As an *in vitro* method for examining the functional effect of HBOEC and HUVEC seeding on the bioartificial tis-

sue, endothelialized and nonseeded tissue was exposed to 1 U/mL heparinized whole blood in PPFCs for 20 min and platelet adhesion to the exposed surfaces was then quantified (Fig. 7A, B). Activation of HBOECs with TNF- α for 4 h before application of the whole blood increased platelet adhesion to the surface (Fig. 7A). Platelet surface coverage was higher on nonseeded tissue ($11.0\% \pm 2.8\%$) than on HBOEC-seeded tissue ($0.5\% \pm 0.1\%$) or HUVEC-seeded tissue ($0.5\% \pm 0.1\%$). Thus, HBOECs, like HUVECs, show decreased platelet adhesion compared to unseeded tissue.

A subset of the HBOEC- and HUVEC-seeded tissue was exposed to 15 dyn/cm^2 shear stress for 24 h before whole blood exposure. Preconditioning of the HBOECs and HUVECs with shear stress decreased platelet adhesion compared to the statically cultured cells. Higher platelet adhesion on the static samples could be due to application of shear stress for the first time during the whole blood study, which causes an initial loss of ECs that had not been pre-exposed to shear stress and allows platelet adhesion to the exposed ECM. To examine the possibility that monolayer integrity was lost during whole blood exposure to statically cultured HBOECs or HUVECs, tissue samples were stained *en face* or after sectioning for VE-Cadherin and the platelet marker, GP-1b (Fig. 7C–E). Images indicated HBOEC and HUVEC monolayer coverage after whole blood exposure for samples that were statically cultured or shear stress-conditioned before the whole blood assay. Platelet binding most often appeared co-localized with the VE-Cadherin stain (Fig. 7C). Platelets adhered near the junctions of cells, primarily as individual platelets (Fig. 7C, D) and occasionally as small aggregates (Fig. 7E).

An assessment of leukocyte binding to the HBOECs using a CD45 antibody, performed in parallel with these platelet binding studies under whole blood flow, revealed no leukocyte binding unless the HBOECs were TNF- α stimulated (data not shown).

Discussion

The fabrication of a small-diameter vascular graft will require the incorporation of a nonthrombogenic luminal surface, most likely through the formation of a functional neoendothelium, to obviate the need for anti-coagulation therapy. HBOECs are a promising cell source for this application, as they can be easily and noninvasively isolated, are highly proliferative, and do not suffer from SMCs contamination issues. The successful use of HBOECs for vascular tissue engineering will require (1) formation of a confluent monolayer on the tissue surface, (2) retention of the HBOECs under physiological shear stress, and (3) presentation of a nonthrombogenic and anti-inflammatory phenotype. As with native endothelium, responsiveness of the HBOECs to physiological shear stress will likely be necessary for the maintenance of a thromboresistant phenotype.

To address these requirements, bioartificial tissue was fabricated for HBOEC seeding. Tubular fibrin gels with entrapped HDF were allowed to remodel for 4 weeks as the HDFs degraded the fibrin and deposited new ECM, including fibronectin, laminin, and collagen I and IV, as shown in this study. The luminal surface of the tissue stained positively for fibrin, fibronectin, and collagen I; however, collagen IV and laminin were primarily localized on the abluminal surface. Fibronectin,^{31–34} laminin,^{31,35} collagen I,^{31,32} and fibrin^{36,37} are all adhesion proteins that have been utilized to improve retention of ECs on vascular grafts, suggesting that a cell-produced matrix rich with these components would be conducive to EC adhesion and retention. HBOECs were seeded on the luminal surface of the HDF-remodeled tissue, where the HBOECs deposited new ECM, which appeared as localized bands of the basement membrane proteins collagen IV and laminin. These bands were not present in the nonendothelialized tissue. The deposition of laminin and collagen IV was shown after 7 days of

FIG. 7. Platelet adhesion on HBOEC- and HUVEC-seeded bioartificial tissue preconditioned with 15 dyn/cm^2 shear stress for 24 h. (A) Normalized platelet coverage. HBOEC-seeded tissue was compared to HBOEC-seeded tissue that was pre-exposed to TNF- α for 4 h or nonseeded tissue. All groups were normalized to the statically cultured HBOEC group. * $p < 0.001$ compared to unstimulated HBOEC. # $p < 0.001$ compared to stimulated and nonstimulated HBOEC-seeded tissue. (B) HBOEC- and HUVEC-seeded tissue was statically cultured or exposed to 15 dyn/cm^2 shear stress for 24 h before whole blood flow exposure. * $p < 0.05$ compared to the statically cultured cells of the same EC type. (C) *En face* image of platelet localization. Scale bar = $25 \mu\text{m}$. Cross-sectional views of (D) platelet localization and (E) platelet aggregation on the surface of statically cultured HBOECs. Scale bar = $50 \mu\text{m}$. Plots show mean $\pm$ SE for one representative experiment with $n = 3$ tissue samples. Color images available online at www.liebertonline.com/tea

HBOEC culture since increased fluorescent intensity at the luminal surface was not obvious at the 2-day time point. While all of the flow studies were started after only 24 h of seeding, these staining results do show that HBOECs were depositing collagen IV and laminin on bioartificial tissue over the course of the first week. The delayed expression could be a confounding issue with immunohistochemistry. We can visually detect an increase in ECM deposition by 7 days; however, ECM was likely being deposited earlier. A high concentration of ECM deposition may be required before an obvious difference in the immunostained tissue is apparent. Alternatively, the cells may need to adhere and form a complete monolayer with tight junctions before increasing their ECM deposition.

HBOEC adhesion and morphology was assessed on the bioartificial tissue. By 24 h postseeding, HBOECs formed a confluent monolayer of cells, with cell-to-cell junction formation, as assessed by VE-Cadherin staining. In short-duration shear stress experiments, the HBOEC monolayer was better retained on the bioartificial tissue than on collagen-coated TCP. To assess HBOEC retention and alignment under flow conditions, a shear stress of 15 dyn/cm² was applied to mimic physiological arterial flow. When exposed to 15 dyn/cm² shear stress for 24 h, HBOECs remained adherent, maintained a similar cell density and VE-Cadherin staining, and elongated and aligned with flow. These results compared well with the HUVEC control cells used in these experiments, as well as with cell retention reported for cord blood-derived HBOECs²² and short-duration, high-shear-stress experiments with HBOECs.^{20,21,23}

While HBOECs grown as monolayers on collagen-coated TCP show limited expression of the proinflammatory markers ICAM-1 and VCAM-1,¹ expression of these markers may be altered depending on the underlying ECM as well as the presence of secondary cell types. SMC-EC co-culture has been shown to increase gene and protein expression of activation markers such as VCAM-1 and ICAM-1, whereas shear stress decreases this expression.³⁸ Shear stress also alters activation marker expression in TNF- α -stimulated and unstimulated EC monolayers.^{39–44} Like mature ECs, HBOECs upregulated VCAM-1 and ICAM-1 when exposed to TNF- α , a proinflammatory cytokine.⁴⁵ Similar to results found with HBOECs seeded on type I collagen,¹ HBOECs and HUVECs seeded on bioartificial tissue expressed low levels of VCAM-1 and ICAM-1 until exposed to TNF- α . TNF- α -stimulated ICAM-1 and VCAM-1 expression was diminished by shear stress when TNF- α was administered during steady laminar shear flow. These studies demonstrate that shear stress conditioning may be useful in decreasing surface expression of various procoagulation molecules on HBOECs, thereby promoting an anti-inflammatory phenotype, as reported for mature ECs.

Shear stress can also be used to increase eNOS expression and NO production in mature ECs.^{46–48} NO promotes blood vessel vasodilation, inhibits platelet adhesion and aggregation, and is constitutively expressed in native ECs, primarily through eNOS activity.⁴⁹ Thus, production of NO is a necessary phenotype for functional HBOECs seeded on bioartificial tissue. Similar basal levels of NO were produced by HUVECs and HBOECs cultured statically. HBOECs increased total NO production when cultured with steady laminar shear stress for 24 h, as did the HUVEC control cells.

This correlated with increased staining for eNOS in tissue cross sections.

Although assessment of HBOEC retention and function *in vivo* will be necessary for a full consideration of HBOEC function, *in vitro* studies allow us to compare HUVECs and HBOECs for a number of quantifiable parameters. As a functional test, we developed a whole blood study to examine platelet adhesion under flow. Bioartificial tissue, either seeded with HBOECs or HUVECs or left unseeded, was exposed to whole blood at a shear rate of 400 s⁻¹ to mimic blood flow in human arteries. This equated to a shear stress of ~15 dyn/cm². Platelet coverage was dramatically reduced on HBOEC-seeded bioartificial tissue as compared to non-seeded tissue. HBOECs and HUVECs had similar levels of platelet coverage, both when statically cultured and when preconditioned with shear stress for 24 h before whole blood exposure.

Preconditioning of the HBOECs or HUVECs with 15 dyn/cm² shear stress for 24 h in EGM medium reduced platelet coverage compared to statically cultured samples. Preconditioning may have improved monolayer integrity, whereas blood flow over statically cultured HBOECs and HUVECs may have disrupted the endothelium, exposing the subendothelial matrix and increasing platelet adhesion on these substrates. Although a limited loss of statically cultured HBOECs or HUVECs during exposure to whole blood flow could not be fully ruled out, VE-Cadherin staining of cross sections indicated that monolayer coverage by the HBOECs and HUVECs was preserved. Increased thromboresistance of ECs pre-exposed to shear stress may also be responsible for decreased platelet adhesion. We have shown that HBOECs and HUVECs exposed to shear stress express higher levels of NO, whereas other groups have reported increased EC expression of PGI₂, NO, TM, and tissue plasminogen activator, and decreased expression of plasminogen activator inhibitor type-1 with shear stress.⁵⁰ Lund *et al.* showed that HBOECs have lower TF and higher eNOS gene expression when exposed to shear stress.⁵¹ This switch to a more thromboresistant phenotype with shear stress exposure could account for the decreased platelet adhesion on the shear stress preconditioned surfaces. Either of these possibilities, improved monolayer integrity or increased EC thromboresistance, leads to the same conclusion: preconditioning with shear stress has the potential to improve the functional outcome with an HBOEC or HUVEC neoendothelium.

Methods to improve HBOEC function before implantation will be important in developing a functional tissue-engineered vascular graft. Our results with HBOECs seeded on bioartificial tissue indicate that (1) HBOECs are shear stress responsive and (2) HBOECs can be preconditioned with shear stress *in vitro* to increase NO production and decrease platelet adhesion. These results illustrate the potential utility for HBOECs in vascular tissue engineering, as not only do the cells adhere to the bioartificial tissue and remain adherent under physiological shear stress, but they also exhibit low expression of activation markers and reduced platelet binding compared to unseeded tissue.

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Disclosure Statement

No competing financial interests exist.

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