SUPPLEMENTAL MATERIALS

Supplemental Methods

Isolation of hCB-ECs

hCB-ECs were derived from human umbilical cord blood, obtained from the Duke University Cord Blood Bank, according to our approved Institutional Review Board protocol. Blood was collected with Cord Blood Collection Units (Pall Corporation, NY), which contained 30-45% of citrate phosphate dextrose (CPD). For each ml of blood and CPD solution, 20 USP of heparin (APP Pharmaceuticals, IL) was added. Mononuclear cells (MNCs) were collected via density centrifugation,[1] cultured in EBM-2 base medium plus EGM-2 SingleQuots (Lonza, Basel Switzerland) supplemented with 5% fetal bovine serum on human collagen I (Sigma-Aldrich, MO) pre-coated 6-well plates (Celltreat, MA), and incubated at 37°C, 5% CO_2 (Heraeus, Germany). To remove non-adherent cells, the medium was changed daily for the first 4 days and then every other day on day 6 onwards. Colonies of hCB-ECs appeared after 7 to 10 day culture of the initially isolated MNCs.

Characterization of hCB-ECs by Flow Cytometry

Flow cytometry was used to confirm EC phenotype. The hCB-ECs were suspended at a concentration of $1x10^6$ cells/ml in 1% bovine serum albumin in phosphate buffered saline (Gibco, CA) and incubated at room temperature (RT) for 30 min with FITC-conjugated or AlexaFluor488-conjugated antibodies for EC markers CD31 (AbD Serotec, UK), CD105 (AbD Serotec, UK) and CD146 (BD Biosciences, CA), as well as monocyte markers CD14 (AbD Serotec, UK) and CD45 (BioLegend, CA). Respective isotype controls IgG_1 (AbD Serotec, UK) and IgG_2 (BioLegend, CA) were used as appropriate. Cells were subsequently rinsed, pelleted and fixed in 3.7% paraformaldehyde. To measure the fluorescent intensity of each antibody,

approximately 10,000 cells were acquired on a FACSCalibur flow cytometry (BD Biosciences, US) and analyzed with CellQuest software (BD Biosciences, US).

Characterization of hCB-ECs by Immunocytochemistry

Isolated hCB-ECs were tested for DiI-Acetylated LDL (Biomedical Technologies, MA) uptake by incubating the cell culture with 5 μg/mL solution (4 hr at 37°C, 5% CO_2). In addition, the cells were also characterized by immunolabeling for EC specific markers. Specifically, the cells were fixed in 3.7% paraformaldehyde (10 min, RT), permeabilized with 0.2% Triton-X (5 min, RT) and blocked with 10% goat serum (30 min, RT). Following, the cells were incubated with CD31 (AbD Serotec, UK) at 1:100 (1 hr, RT) or von Willebrand Factor (vWF, Santa Cruz, CA) at 1:1000 (1 hr, RT). After rinsing, the labeled cells were incubated with Alexa Fluor 488 (Invitrogen, NY) at 1:500 (45 min at 37^{o}C). Images were taken by a fluorescent microscope (Leica DMRB, Germany).

Cell Quantification with Cell Counting Kit – 8 Assay

To quantify the number of hCB-ECs retained on the Ti tubes after flow exposure, hCB-ECs were analyzed with Cell Counting Kit – 8 assay (CCK-8 Sigma–Aldrich) diluted at 1:15 in L-15 Leibovitz medium (Life Technologies, NY). The samples were incubated for 3 hours (37°C, 5% CO_2) and analyzed for absorbance readings at 450nm with a microplate reader (Thermo Scientific, Waltham, MA). Several points of absorbance values for known concentrations of adherent hCB-ECs were initially measured to create a standard curve, which was subsequently used to interpolate the absorbance values of the samples.

Visualization of hCB-ECs on Sintered Ti with a Confocal Microscope

hCB-ECs on sintered Ti were stained with 1 mM Cell Tracker Green (Life Technologies), fixed in 3.7% formaldehyde (Ricca Chemical) and permeabilized with

0.1% Triton X (Sigma-Aldrich). The ECs were blocked in 10% goat serum, incubated with primary antibody mouse anti-CD31 (Invitrogen, 1:100 dilution) and then secondary antibody goat anti-mouse IgG AlexaFluor 488 (Invitrogen, 1:500 dilution). Nuclei were counterstained with Hoechst 34580 (Invitrogen, 1:1000 dilution). Subsequently, the stained samples were imaged on a confocal microscope (Zeiss 780 upright, Oberkochen, Germany).

Visualization of hCB-ECs on Sintered Ti with a Scanning Electron Microscope

hCB-ECs on sintered Ti were fixed in 3.7% formaldehyde (Ricca Chemical, TX), dehydrated in an ethanol series, dried in a Pelci CPD2 critical point dryer (Ted Pella, CA) and sputter coated with Au/Pd (60/40%) for 30 sec in a Hummer 6.2 sputter coater (Anatech, NY) prior to imaging. Following, the samples were imaged with a scanning electron microscope (Philips XL30 Environmental SEM) at 3-5 kV.

Sintered Ti Materials

Sintered Ti tubes were provided by Thoratec Corporation (CA) and used in this study. Each tube had the following dimensions: length of 1.90 cm, outer radius of 0.91 cm, inner radius of 0.63 cm and unadjusted surface area of 7.46 cm^2. In order to allow luminal view of the sintered Ti surface, Ti tubes were precut longitudinally into 3 sections and reassembled into a Ti tube by heat-shrinking PVC tubing (McMaster-Carr, GA) over a mandrel. After rapid-seeding, the 3 sections composing Ti tube were dismantled for imaging purposes.[2]

Preparation of Clean and Sterile Sintered Ti

To remove any organic compounds, Ti sections were cleaned with aqua regia (a 1:3 solution of concentrated nitric and hydrochloric acid) for 5 min, rinsed in deionized (DI) water for 5 min, sonicated in DI water for 5 min and then sonicated in an Alconox solution.[3] Following, the Ti sections were air dried inside a laminar flow hood and

reassembled into a Ti tube by heat shrinking PVC tubing (McMaster-Carr) over a mandrel. The reassembled Ti tube was sterilized with ethylene oxide gas sterilization (18 hr, 55°C) prior to use.

Flow Experiments

A modified cardiopulmonary bypass circuit was assembled with COBE 3/8" x 3/32" SMARxT cardiopulmonary bypass tubing, a 500 mL reservoir bottle (Nalgene), a modified cardiopulmonary bypass pump (Stöckert, Germany), pulse dampener (Cole-Parmer, IL), and custom-made aluminum inlet and outlet adaptors with an inside diameter of 1.26 cm and outside diameter of 1.82 cm. A seeded Ti tube was placed in between the aluminum inlet and outlet adaptors. Two sections of silastic tubing (1/2" diameter, 3cm long) were used to connect the Ti tube between the aluminum connectors.

To develop near laminar flow at the point where the perfusate entered the sintered Ti tube, our flow circuit utilized an inlet of equal geometry. Under this assumption, the shear stress acting tangentially on hCB-ECs adhering to the luminal Ti surface was calculated as 4.36 dynes/cm^2 according to equation (1):

$$(1) \quad \tau = \frac{4 \times \mu \times Q}{\pi \times r^3}$$

where τ denotes the wall shear stress (dynes/cm^2), μ the medium viscosity at 37°C (0.02 g/cm·s), Q the volumetric flow rate (cm^3/s) and r the inner radius of the Ti tube (0.625 cm).

As perfusion medium, 450 mL Leibovitz's L-15 medium (Life Technologies, NY) containing 3% Dextran (Thermo Fisher Scientific, NC), 5% FBS (Thermo Fisher

Scientific, NC) and 10 mmol of HEPES (Life Technologies, NY) was used. Flow was maintained at 2.5 L/min. To maintain the temperature at 37°C, the reservoir was placed in a water bath (37°C) and pliable transparent thermal insulator sheets were used to insulate the tubing. A small hole was drilled into the top of the reservoir lid for gas exchange during flow and then covered with gas-permeable membrane to avoid contamination of the perfusate.

Nitric Oxide Quantification

To quantify nitric oxide (NO) production by hCB-ECs, the primary oxidation product (NO_2^-) was directly measured in flow circuit medium samples by chemiluminescence with an Ionics/Sievers Nitric Oxide Analyzer (NOA 280, Sievers Instruments, Boulder, CO) as previously described.[4] Medium samples were frozen at -80°C until analysis. The total amount of nitrite was calculated as the product of concentration to the total circuit volume. Note that nitrite is a commonly used surrogate marker of NO availability and has been shown to reflect changes in NO bioavailability following physical stimuli more effectively than nitrate (NO_3^-) or the combined quantity of nitrite and nitrate (NO_x).[4,5] Our nitrite analysis utilized 0.05M potassium iodide in 14.5M acetic acid as reductant, which has the potential to convert nitrite to NO but is insufficient to reduce any higher oxides of nitrogen such as nitrate and thus is relatively specific for nitrite.[4]

Platelet Adhesion Assay

Platelets were obtained from the Duke University Medical Center Blood Bank following Institutional Review Board approval, and the number of platelets was determined with a Complete Blood Count Analyzer (Abbott Cell Dyn 3700 Hematology System, Abbott Diagnostics). As previously described,[4] platelets were washed twice at 2,500g for 5 min (22°C) with a buffered saline glucose citrate (BSGC) solution containing 8.6mM Na_2HPO_4, 1.6mM KH_2PO_4, 0.12M NaCl, 0.9mM

EDTA, 13.6mM Na_3Citrate, 11.1mM Glucose, titrated to a pH of 7.1 in deionized water and subsequently sterilized via vacuum filtration. The platelets were labeled with 1mM Cell Tracker Orange CMRA (Life Technologies, C34551) for 45 min (dark, 22°C). Following, the platelets were centrifuged at 2,500g for 5 min (22°C), resuspended in fresh BSGC to the starting volume and incubated for additional 30 min (dark, 22°C) to allow any excess unconjugated dye to diffuse out of the platelets, as recommended by the manufacturer. To maximize the diffusion of excess unconjugated dye, we repeated this step once and then resuspended the platelets in 1:1 solution of DMEM/Tyrodes buffer (20mM Hepes, 137mM NaCl, 2.7mM KCl, 1mM $CaCl_2$, 11.9mM $NaHCO_3$, 5.5mM Glucose) to activate the platelets. A solution of $5x10^8$ platelets/ml was incubated with either sintered Ti surfaces without hCB-ECs. or hCB-EC-seeded Ti for 30 min (static, 37°C, 5% CO_2). Samples were then rinsed 3 times with D-PBS (1X) containing calcium and magnesium (Life Technologies) prior to imaging. A total of 3 images (20× magnification) along the Ti surface were obtained by Zeiss 780 upright confocal microscope (Zeiss, Oberkochen, Germany). The number of platelets was determined by a researcher blinded to experimental conditions, specifically by counting (Cell Counter) with ImageJ software.

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