Neovascularization Potential of Blood Outgrowth Endothelial Cells From Patients With Stable Ischemic Heart Failure Is Preserved

Dieter Dauwe, MD, PhD; Beatriz Pelacho, PhD; Arief Wibowo, MD; Ann-Sophie Walravens; Kristoff Verdonck, MD; Hilde Gillijns; Ellen Caluwe; Peter Pokreisz, PhD; Nick van Gastel, PhD; Geert Carmeliet, MD, PhD; Maarten Depypere, PhD; Frederik Maes, PhD; Nina Vanden Driessche; Walter Droogne, MD; Johan Van Cleemput, MD, PhD; Johan Vanhaecke, MD, PhD; Felipe Prosper, MD, PhD; Catherine Verfaillie, MD, PhD; Aernout Luttun, PhD; Stefan Janssens, MD, PhD

Background—Blood outgrowth endothelial cells (BOECs) mediate therapeutic neovascularization in experimental models, but outgrowth characteristics and functionality of BOECs from patients with ischemic cardiomyopathy (ICMP) are unknown. We compared outgrowth efficiency and in vitro and in vivo functionality of BOECs derived from ICMP with BOECs from age-matched (ACON) and healthy young (CON) controls.

Methods and Results—We isolated 3.6±0.6 BOEC colonies/100×10⁶ mononuclear cells (MNCs) from 60-mL blood samples of ICMP patients (n=45; age: 66±1 years; LVEF: 31±2%) versus 3.5±0.9 colonies/100×10⁶ MNCs in ACON (n=32; age: 60±1 years) and 2.6±0.4 colonies/100×10⁶ MNCs in CON (n=55; age: 34±1 years), P=0.29. Endothelial lineage (VEGFR2+/CD31+/CD146+) and progenitor (CD34+/CD133+) marker expression was comparable in ICMP and CON. Growth kinetics were similar between groups (P=0.38) and not affected by left ventricular systolic dysfunction, maladaptive remodeling, or presence of cardiovascular risk factors in ICMP patients. In vitro neovascularization potential, assessed by network remodeling on Matrigel and three-dimensional spheroid sprouting, did not differ in ICMP from (A)CON. Secretome analysis showed a marked proangiogenic profile, with highest release of angiopoietin-2 (1.4±0.3×10⁵ pg/10⁶ ICMP-BOECs) and placental growth factor (5.8±1.5×10³ pg/10⁶ ICMP BOECs), independent of age or ischemic disease. Senescence-associated β-galactosidase staining showed comparable senescence in BOECs from ICMP (5.8±2.1%; n=17), ACON (3.9±1.1%; n=7), and CON (9.0±2.8%; n=13), P=0.19. High-resolution microcomputed tomography analysis in the ischemic hindlimb of nude mice confirmed increased arteriogenesis in the thigh region after intramuscular injections of BOECs from ICMP (P=0.025; n=8) and CON (P=0.048; n=5) over vehicle control (n=8), both to a similar extent (P=0.831).

Conclusions—BOECs can be successfully culture-expanded from patients with ICMP. In contrast to impaired functionality of ICMP-derived bone marrow MNCs, BOECs retain a robust proangiogenic profile, both in vitro and in vivo, with therapeutic potential for targeting ischemic disease. (J Am Heart Assoc. 2016;5:e002288 doi: 10.1161/JAHA.115.002288)

Key Words: arteriogenesis • blood outgrowth endothelial cells • cell transplantation • ischemic heart disease • therapeutic neovascularization

Blood outgrowth endothelial cells (BOECs) represent an easy accessible, culture-expandable cell source with endothelial lineage specification. BOECs belong to the family of endothelial progenitor cells (EPCs), which harbors a heterogeneous selection of different cell types, participating in and modulating neovascularization. Distinct EPC subtypes are defined using different culture techniques and/or combinations of cell surface markers.¹–³ BOECs, also referred to as circulating endothelial colony-forming cells (ECFCs) or late outgrowth EPCs, are the only cell type featuring the

From the Department of Cardiovascular Sciences, Clinical Cardiology (D.D., A.W., A.-S.W., H.G., E.C., P.P., N.V.D., W.D., J.V.C., J.V., S.J.); Center for Molecular and Vascular Biology (K.V., A.L.); Department of Clinical and Experimental Endocrinology (N.v.G., G.C.); Department of Electrical Engineering, Center for the Processing of Speech and Images (M.D., F.M.); and Department of Development and Regeneration, Stem Cell Biology and Embryology (C.V.), KU Leuven, Leuven, Belgium; Cell Therapy Department, Center for Applied Medicine Research (B.P., F.P.) and Hematology Department (F.P.), Clinica Universidad de Navarra, University of Navarra, Pamplona, Spain.

Correspondence to: Dieter Dauwe, MD, PhD, or Stefan Janssens, MD, PhD, Department of Cardiovascular Diseases, University Hospitals Leuven, KU Leuven, Herestraat 49, 3000 Leuven, Belgium. E-mails: dieter.dauwe@med.kuleuven.be; stefan.janssens@med.kuleuven.be

Received July 5, 2015; accepted February 11, 2016.

© 2016 The Authors. Published on behalf of the American Heart Association, Inc., by Wiley Blackwell. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
characteristics of a “true” EPC, given that these cells are self-renewing, clonogenic, and able to form capillary-like structures and integrate into functional blood vessels in vitro and in vivo, respectively.4

Refractory angina is an increasingly prevalent clinical syndrome with an estimated incidence between 5% and 10% of patients undergoing cardiac catheterization.5 In patients with ischemic heart disease, refractory to optimal medical treatment and percutaneous or surgical revascularization, progenitor cell transfer may constitute a promising biological alternative (“biological bypass”).6 Autologous BOECs are able to induce neovascularization not only by incorporation into functional vessels, but also through paracrine modulation.7,8

One of the major concerns when envisioning autologous (progenitor/stem) cell therapy in cardiovascular medicine, is the presumed dysfunctional progenitor cell phenotype in older and diseased patients burdened with risk factors, as evidenced for early outgrowth EPCs of monocytic origin (also known as circulating angiogenic cells),9–12 CD133+/VEGFR2+ circulating EPCs,10,13 colony-forming-unit EPCs (CFU-Hill),13,14 bone-marrow–derived mononuclear cells (MNCs)15 and hematopoietic stem cells.11

Because no data are currently available on BOEC outgrowth and functionality in patients with the greatest unmet clinical need, we compared outgrowth efficiency and in vitro and in vivo functional characteristics of BOECs from patients with severe, but stable, ischemic cardiomyopathy (ICMP) and BOECs from both healthy age-matched (ACON) and young (CON) control subjects.

Methods

Study Design

BOECs were isolated from peripheral blood of patients with stable ischemic cardiomyopathy (left ventricular ejection fraction [LVEF] ≤45%; 18–80 years) (ICMP: n=45; Table 1) in follow-up at the outpatient heart failure clinic of the Department of Cardiovascular Diseases at University Hospitals (Leuven, Belgium). The study protocol was approved by the Medical Ethics Committee (S51940), and informed consent was obtained from all patients. The control group consisted of healthy individuals (18–80 years; Table 1) with no evidence/history of coronary artery disease, divided in an age-matched (ACON: n=32) and young (CON: n=55) control populations.

BOEC Isolation and Expansion

BOECs were isolated and expanded as previously described, with minor modifications.4,8 In short, the mononuclear fraction from 40- to 60-mL venous blood samples was isolated using Ficoll Paque Plus (GE Healthcare, Little Chalfont, UK) density centrifugation. Cells were resuspended in EBM2 supplemented with a bullet kit (Lonza, Basel, Switzerland), excluding the provided gentamicin and amphotericin singlequots. In addition, the medium was supplemented with an extra 13% of FBS, MEM-NEAA (Gibco, Grand Island, NY), 1% penicillin/streptomycin (Gibco), and 1:550 β-Mercaptoethanol (Gibco). Cells were cultured on collagen type I precoated dishes (Cellcoat; Greiner Bio-One GmbH, Kremsmünster, Austria) in a 21% O2, 5% CO2 incubator.

Table 1. Clinical Characteristics of Study Subjects

<table>
<thead>
<tr>
<th></th>
<th>ICMP (n=45)</th>
<th>CON (n=55)</th>
<th>ACON (n=32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>66±1</td>
<td>34±1</td>
<td>60±1</td>
</tr>
<tr>
<td>M/F (%)</td>
<td>91/9</td>
<td>59/41</td>
<td>25/75</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>31±2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>56±1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cardiovascular risk factors</td>
<td>ND</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Hypertension</td>
<td>29/45</td>
<td>7/32</td>
<td>—</td>
</tr>
<tr>
<td>Diabetes</td>
<td>17/45</td>
<td>0/32</td>
<td>—</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>38/45</td>
<td>7/32</td>
<td>—</td>
</tr>
<tr>
<td>Obesity</td>
<td>14/45</td>
<td>6/32</td>
<td>—</td>
</tr>
<tr>
<td>Smoking</td>
<td>38/45</td>
<td>10/32</td>
<td>—</td>
</tr>
<tr>
<td>Familial cardiovascular risk</td>
<td>23/45</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Extent of coronary artery disease</td>
<td>NA</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>One-vessel disease</td>
<td>8/45</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Two-vessel disease</td>
<td>12/45</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Three-vessel disease</td>
<td>25/45</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Medical treatment</td>
<td>0/55</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Aspirin</td>
<td>26/45</td>
<td>0/32</td>
<td>—</td>
</tr>
<tr>
<td>Thienopyridin</td>
<td>11/45</td>
<td>0/32</td>
<td>—</td>
</tr>
<tr>
<td>Statin</td>
<td>42/45</td>
<td>2/32</td>
<td>—</td>
</tr>
<tr>
<td>Beta-blocker</td>
<td>42/45</td>
<td>0/32</td>
<td>—</td>
</tr>
<tr>
<td>ACE inhibitor/ARB</td>
<td>38/45</td>
<td>1/32</td>
<td>—</td>
</tr>
<tr>
<td>Spirolactone</td>
<td>23/45</td>
<td>0/32</td>
<td>—</td>
</tr>
<tr>
<td>Device treatment</td>
<td>0/55</td>
<td>0/32</td>
<td>—</td>
</tr>
<tr>
<td>CRT-P</td>
<td>3/45</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CRT-D</td>
<td>8/45</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ICD</td>
<td>7/45</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Blood outgrowth endothelial cell (BOEC) isolations were performed in 45 patients with ischemic cardiomyopathy (ICMP), 55 young healthy controls (CON), and 32 age-matched controls (ACON). Left ventricular ejection fraction (LVEF) and left ventricular end-diastolic diameter (LVEDD) were measured by transthoracic echocardiography. ACE inhibitor indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CRT-D, cardiac resynchronization therapy incorporating an implantable cardioverter defibrillator; CRT-P, cardiac resynchronization therapy without implantable cardioverter defibrillator; ICD, implantable cardioverter defibrillator; NA, not applicable; ND, not determined.

isolated using Ficoll Paque Plus (GE Healthcare, Little Chalfont, UK) density centrifugation. Cells were resuspended in EBM2 supplemented with a bullet kit (Lonza, Basel, Switzerland), excluding the provided gentamicin and amphotericin singlequots. In addition, the medium was supplemented with an extra 13% of FBS, MEM-NEAA (Gibco, Grand Island, NY), 1% penicillin/streptomycin (Gibco), and 1:550 β-Mercaptoethanol (Gibco). Cells were cultured on collagen type I precoated dishes (Cellcoat; Greiner Bio-One GmbH, Kremsmünster, Austria) in a 21% O2, 5% CO2 incubator.
Individual BOEC colonies were picked at days 21 to 28 and pooled for further polyclonal expansion up to passage 7 or growth arrest. Phenotyping and functional evaluation was performed at passage 3 to 5 (Figure 1). Mycoplasma contamination was excluded, using the MycoAlert Mycoplasma detection kit (Lonza), following the manufacturer’s instructions.

**Confirmation Endothelial Phenotype**

Cells were stained with *Ulex europeus* agglutinin (UEA)-lectin (Sigma-Aldrich), Dil-AcLDL uptake (Invitrogen, Carlsbad, CA) and cell surface markers were quantified using flow cytometry (BD FACS Canto II; BD Biosciences, San Jose, CA). CD309 (vascular endothelial growth factor receptor 2; VEGFR-2), CD146 (melanoma cell adhesion molecule), and CD31 (platelet endothelial cell adhesion molecule 1) were selected as endothelial markers, CD34 and CD133 as progenitor markers, and CD45 as a panleukocyte exclusion marker. Antibodies and isotype controls were from Miltenyi Biotec (Cambridge, MA). Gene expression was studied using real-time quantitative PCR (RT-qPCR; StepOnePlus; Applied Biosystems, Foster City, CA) and TaqMan Gene Expression Assays (Life Technologies, Carlsbad, CA), focusing on angiogenic growth factors and endothelial markers (Table 2, top). Results are expressed as a $2^{-\Delta CT}$-value, relative to HPRT as the housekeeping gene. In addition, the angiogenic secretome was studied in 24-hour conditioned medium (EBM$_2$/2%FBS) by ELISA (PLGF, angiopoietin-2 [ANG-2], fibroblast growth factor [FGF] 2, platelet-derived growth factor-BB [PDGF-BB], VEGF165, insulin-like growth factor 1 [IGF-1], and HGF, hepatocyte growth factor [HGF]; Quantikine; all from R&D Systems, Minneapolis, MN; Table 2, bottom).

**Proliferation Potential**

Cell numbers were calculated at every passage in order to derive expansion curves and calculate population doubling times throughout the entire expansion process. In addition to growth kinetics, senescence was quantified by senescence-associated β-galactosidase staining (Senescence Detection Kit; BioVision, Inc., Milpitas, CA). The number of blue cells was manually counted and reported as a percentage of the entire cell population.

**In Vitro Oxidative Stress**

Production of reactive oxygen species (ROS) was quantified using CM-H$_2$DCFDA (Molecular Probes, Eugene, OR) as a fluorescent ROS indicator (Ex/Em: 492-495/517-527). Cells were loaded with a final dye concentration of 10 μmol/L for 30 minutes at 37°C. Intensity of the fluorescence was...
ICMP BOECs Retain Neovascularization Potential  Dauwe et al

Table 2. Transcriptional Analysis and Secretome of BOECs

<table>
<thead>
<tr>
<th>RT-qPCR (2−ΔCT vs HPRT)</th>
<th>ICMP (n=15)</th>
<th>CON (n=9)</th>
<th>ACON (n=10)</th>
<th>1-Way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vWF</td>
<td>7.120±2.620</td>
<td>7.330±4.370</td>
<td>3.390±0.655</td>
<td>P=0.69</td>
</tr>
<tr>
<td>NOS-3</td>
<td>0.101±0.029</td>
<td>0.072±0.044</td>
<td>0.184±0.045</td>
<td>P=0.05</td>
</tr>
<tr>
<td>Angiogenic/arteriogenic growth factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLGF</td>
<td>1.010±0.319</td>
<td>0.639±0.230</td>
<td>0.755±0.216</td>
<td>P=0.97</td>
</tr>
<tr>
<td>ANG-2</td>
<td>1.140±0.592</td>
<td>0.642±0.216</td>
<td>1.475±0.381</td>
<td>P=0.06</td>
</tr>
<tr>
<td>FGF-2</td>
<td>0.337±0.110</td>
<td>0.344±0.082</td>
<td>0.243±0.040</td>
<td>P=0.46</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>0.918±0.362</td>
<td>0.648±0.131</td>
<td>0.809±0.309</td>
<td>P=0.65</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.063±0.017</td>
<td>0.087±0.037</td>
<td>0.083±0.015</td>
<td>P=0.20</td>
</tr>
<tr>
<td>IGF-1</td>
<td>0.015±0.008</td>
<td>0.006±0.002</td>
<td>0.016±0.007</td>
<td>P=0.81</td>
</tr>
<tr>
<td>HGF</td>
<td>0.007±0.004</td>
<td>0.007±0.003</td>
<td>0.031±0.008</td>
<td>P=0.05</td>
</tr>
</tbody>
</table>

In Vitro Neovascularization Potential

In a matrigel “network remodeling” (Tube Formation) assay (BD Matrigel Basement Membrane Matrix, 354234; BD Biosciences), a total of 75 000 BOECs were seeded per 24-well, coated with 300 μL of Matrigel. Phase contrast mosaic images were acquired of the complete well after 6 hours of incubation. The cellular network was manually delineated in order to semiautomatically quantify total network length and the number of intersections using ImageJ software (National Institutes of Health, Bethesda, MD). Additionally, sprouting angiogenesis was studied using a three-dimensional (3D) spheroid sprouting assay in collagen gel, as previously described with minor modifications.16 In short, BOEC spheroids containing 1000 cells were generated by suspension culture in EBM2 culture medium containing 0.25% (w/v) carboxymethylcellulose, seeded in nonadherent round-bottomed 96-well plates. Under these conditions, all suspended cells contribute to the formation of a single BOEC spheroid per well. These spheroids were harvested within 24 hours and embedded in collagen gels (Rat Tail Collagen Type I; Corning, Corning, NY), as previously described.10 The length of the 3 longest capillary-like sprouts that had grown out of each spheroid was measured after 24 hours. A total of 20 ± 2 spheroids per sample were analyzed.

In Vivo Neovascularization Potential

All animal procedures were approved by the Ethics Committee on Animal Use of KU Leuven (Leuven, Belgium) and conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. Female 8- to 10-week-old athymic NMRI nude mice (Janvier Labs, Le Genest-Saint-Isle, France) were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (15 mg/kg). The common, superficial, and deep femoral artery of the right leg were ligated and transected in between. Laser Doppler measurements (PIM-II; Lisca Development AB, Linköping, Sweden) were performed under 2% isoflurane gas anesthesia and temperature-controlled conditions (37°C) 5 days after hindlimb ischemia surgery to assess degree of flow reduction. Only animals with ≥55% flow reduction in the ischemic versus nonligated control limb were included in the study.

DOI: 10.1161/JAHA.115.002288
Figure 2. BOEC outgrowth characteristics. BOECs can be isolated to the same extent from patients and controls (A), irrespective of age (B), severity of the disease process (C, D, and E), and cardiovascular risk factors (F and G). The linear regression line is surrounded by 95% confidence bands (C). 1/2/3-VD indicates 1/2/3-vessel disease; ACON, age-matched controls; CON, healthy young controls; CVRF, cardiovascular risk factors; DM, diabetes mellitus; ICMP, ischemic cardiomyopathy patients; LVEDD, left ventricular end diastolic diameter; LVEF, left ventricular ejection fraction; y, years.
Intramuscular injections of 250,000 BOECs from patients (ICMP) or young controls (CON) versus vehicle control (PBS) were performed in the ischemic hindlimb, 5 days after the ligation procedure. Cells were resuspended in 100 µL of PBS and subsequently injected into the thigh region (5×10 µL) and gastrocnemius muscle (5×10 µL) using a 1-mL 29G syringe. Intraperitoneal anti-asialo-GM1 injections (Wako Chemicals, Inc., Dallas, TX) were performed once a week to suppress residual natural killer cell activity.

Mice were euthanized 21 days after femoral artery ligation. At sacrifice, mice were sedated with an intraperitoneal injection of Nembutal 70 mg/kg. A midline sternotomy was performed to expose the heart, followed by insertion of a winged 25G needle (Mycroflex; Vygon, Ecouen, France) into the LV apex and removal of the right auriculum. Vessels were perfused by the LV cavity with 20 mL of saline, containing 100 IU/mL of unfractionated heparin, in order to remove the blood and prevent clotting. Tissue was subsequently fixed by a 15-mL perfusion with neutral buffered formalin 10%, followed by 20 mL of saline to wash out the fixative. A final perfusion with a preheated mixture of 30% barium sulphate (Micropaque; Guerbet, France) and 2% gelatin was performed in order to visualize the vessel tree on post-mortem angio

Figure 3. BOEC phenotype. BOECs display the typical cobblestone morphology (A) (scale bar, 200 µm), stain positive for UEA-lectin and the endothelial marker, CD31 (B) (scale bar, 20 µm), and take up acetylated LDL (C) (scale bar, 20 µm). Representative flow cytometry analysis (D) for endothelial markers (CD31+/CD309+/CD146+), progenitor markers (CD34+/CD133+), and a panleukocytic marker (CD45+) on ICMP-BOECs. Flow cytometry data (red line in histogram) are expressed in relation to respective isotype controls (black line in histogram) and a quantitative comparison with CON-BOECs is shown in the table. BOEC indicates blood outgrowth endothelial cell; CON, healthy young controls; DAPI, 4',6-diamidino-2-phenylindole; ICMP, ischemic cardiomyopathy patients; LDL, low-density lipoprotein; UAE, Ulex europeus agglutinin.

<table>
<thead>
<tr>
<th>Flow Cytometry</th>
<th>ICMP (n=17) (% Positive Cells)</th>
<th>CON (n=13) (% Positive Cells)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>/</td>
</tr>
<tr>
<td>CD146</td>
<td>98.4±0.3</td>
<td>96.1±1.4</td>
<td>0.38</td>
</tr>
<tr>
<td>CD31</td>
<td>98.5±0.3</td>
<td>97.9±0.7</td>
<td>0.26</td>
</tr>
<tr>
<td>CD309</td>
<td>81.7±2.2</td>
<td>70.5±6.4</td>
<td>0.08</td>
</tr>
<tr>
<td>CD34</td>
<td>57.2±7.3</td>
<td>71.6±9.3</td>
<td>0.17</td>
</tr>
<tr>
<td>CD133</td>
<td>0.1±0.0</td>
<td>0.0±0.0</td>
<td>0.72</td>
</tr>
</tbody>
</table>
ICMP BOECs Retain Neovascularization Potential  Dauwe et al

microCT (computed tomography). Mice were stored on ice overnight to solidify the gelatin and fix the contrast agent in the vessels.

The hind limbs were scanned with a high-resolution microCT (50 kV, 200 μA, 10 W; SkyScan-1172; SkyScan, Virginia Beach, VA) at a pixel size of 8 μm, providing highly detailed, quantitative 3D structural data of the perfused “vessel tree” of the complete limb. The individual two-dimensional (2D) images were reconstructed into a 3D data set with isotropic voxel size of 8 μm using the manufacturer’s dedicated software (NRecon; SkyScan). Analysis was performed using in-house developed custom-made software (MeVisLab based; MeVis Medical Solutions AG, Bremen, Germany) after an additional downsampling step by a factor of 4, for reasons of computational feasibility. The 3D vessel tree was automatically segmented for semiautomatic quantification. Data in the ligated leg were analyzed versus the nonligated control leg.

Histology: *Arthrobacter luteus* Repeats and Combined hCD31/BS-I Lectin Staining

Adductor and gastrocnemius muscles, as well as lung, spleen, liver, kidney and heart on a separate set of animals not perfused with gelatin/barium-sulfate were collected and paraffin-embedded to study BOEC incorporation and biodistribution. BOEC-incorporation into the vasculature of adductor and gastrocnemius muscles, was studied by combined chromogenic in situ hybridization for *Arthrobacter luteus* (ALU)-repeats (ALU Positive Control Probe-II and ISHiVIEW Blue Plus Detection Kit; Ventana Medical Systems, Inc., Oro Valley, AZ), as the primate-specific sequence, and dual immunofluorescence staining for human (h) CD31 (anti-hCD31 IgG1, m0823; Dako, Carpinteria, CA) and BS-I Lectin (L3759; Sigma-Aldrich) on adjacent sections. An initial acute retention and biodistribution study was performed, 24 hours after intramuscular injections (6 days after ligation) of

Figure 4. BOEC expansion potential. Growth kinetics, studied by population doubling times (A3) and the slope of the log10(expansion curves) (A1,2), showed similar proliferation rates in BOECs from ICMP patients and controls. Growth kinetics were studied from the start of the polyclonal phase, defined as day 0, the time point at which individual BOEC colonies were picked and pooled for culture expansion. The number of senescent cells, quantified as the percentage of blue cells in the senescence-associated β-galactosidase staining (B1) (scale bar, 200 μm), did not differ between patients and controls (B2). ACON indicates age-matched controls; BOEC, blood outgrowth endothelial cell; CON, healthy young controls; ICMP, ischemic cardiomyopathy patients.
250 000 BOECs labeled by lentiviral overexpression of Cherry fluorescent protein. Moreover, chronic engraftment was quantified at 21 days.

Statistical Analysis

All data are presented as mean±SEM. Intergroup differences were analyzed using 2-tailed unpaired t tests or 1-way ANOVA followed by a Bonferroni post-hoc test for normally distributed data. Non-normally distributed data were compared using a nonparametric Mann–Whitney test or nonparametric Kruskal–Wallis test followed by Dunn’s post-hoc test. Pearson correlations were performed to analyze linear covariation between normally distributed data sets, whereas Spearman nonparametric correlation was performed for nonparametric datasets. A probability value of P<0.05 was considered statistically significant. All analyses were performed using Prism 5.0a software (version 5.0a; GraphPad Software Inc., La Jolla, CA).

Results

Patient Characteristics

BOEC isolations were performed in 45 patients (ICMP, 66±1 years) and 32 healthy age-matched (ACON, 60±1 years) and 55 healthy young (CON, 34±1 years) controls (Table 1). Global systolic LV function was impaired (LVEF 31±2%) without concomitant adverse LV remodeling (left ventricular end diastolic diameter [LVEDD], 56±1 mm) in the majority of patients. Adverse LV remodeling, defined as an LVEDD ≥60 mm on a transthoracic acquired echocardiography parasternal long axis view, was present in 12 of 45 (26.7%) patients. Four patients were excluded from the study because blood was taken less than 1 month after device implantation.

BOEC Outgrowth Efficiency

Starting from 40 to 60 mL of whole blood, a mean of 3.6±0.6 BOEC colonies/100×10^6 MNCs could be obtained from patients versus 3.5±0.9 from age-matched controls and 2.6±0.4 from healthy young controls (P=0.29; Figure 2A). Colonies could be obtained in 84% of ICMP, 72% of ACON and 82% of CON. The number of BOEC colonies obtained in our ICMP population increased with age (r=0.42; P<0.01; Figure 2B), but was not affected by severity of the disease process, assessed by LVEF (r=0.12; Figure 2C) or adverse LV remodeling (Figure 2D). BOEC outgrowth was independent of the extent of coronary artery disease, number of cardiovascular risk factors, and presence of diabetes mellitus (Figure 2E through 2G).

BOEC Phenotype

BOECs uniformly expressed CD31, bound lectin, and took up Dil-AcLDL (representative examples are shown in Figure 3A through 3C). Flow cytometry evaluation of endothelial (CD31+, CD309+, and CD146+) and progenitor (CD34+, CD133+) cell surface markers did not differ between groups (Figure 3D). The panleukocyte marker, CD45, was absent in all groups. RT-qPCR neither showed differences in expression for additional endothelial markers, such as VWF and NOS3 (Table 2, top).
BOEC Progenitor Potential

We aimed to expand our cells to a target dose of \( \approx 30 \times 10^6 \) BOECs from 60 mL of whole blood, a cell number with proven functional benefit in our porcine myocardial ischemia/reperfusion model.\(^7\) Progenitor potential, studied by growth kinetics and senescence-associated \( \beta \)-galactosidase staining, did not show differences between BOECs from ICMP and (A) CON. Population doubling times (\( P=0.16 \)) and slopes of the \( \log_{10} \)-(expansion curves) (\( P=0.38 \)) showed comparable growth kinetics (Figure 4A). The number of senescent cells was 5.8±2.1\% in ICMP (\( n=17 \)) versus 3.9±1.1\% and 9.0±2.8\% of total BOECs in ACON (\( n=7 \)) and CON (\( n=13 \)), respectively (\( P=0.19; \) Figure 4B). Starting from a single 60-mL blood sample in our patient population, expansion up to \( 30 \times 10^6 \) BOECs was achieved in 52\% of isolations, if initial BOEC colonies were obtained. The number of initial BOEC colonies (\( P<0.0001 \)) and the time of appearance of the first clone (\( P=0.005 \)) were major predictors of expansion success. If expansion up to \( 30 \times 10^6 \) cells could be achieved, the average number of BOEC colonies to start from was 9.9±2.3 isolation with an appearance at day 12±1. In contrast, if expansion up to \( 30 \times 10^6 \) cells failed, on average only 2.5±0.5 colonies/isolation were initially obtained with an appearance at day 16±1. Clinical, biochemical, or hematological predictors of expansion potential could not be identified.

BOEC Response to Oxidative Stress

No differences were noted in baseline reactive oxygen species (ROS) production in BOECs isolated from ICMP (\( n=5 \)) and CON (\( n=6; \) \( P=0.37; \) Figure 5A). Moreover, induction by 500 \( \mu \)mol/L of \( H_2O_2 \) showed comparable increase in ROS production in ICMP and CON, 10 and 45 minutes after exposure (\( P=0.60; \)
ICMP BOECs Retain Neovascularization Potential  Dauwe et al

Lo fH2O2 did not show significant differences in in vitro network formation potential. On the other hand, increased arteriogenesis was observed after transplantation of BOECs from healthy young controls ([A]CON) (P=0.66 and 0.13 for ANG-2 and PLGF, respectively).

**BOEC In Vivo Neovascularization Potential**

Initial acute retention and biodistribution studies, using Cherry-labeled BOECs, documented multiple clusters and singlets of transplanted cells in the femoral and tibiofibular region 24 hours after intramuscular cell delivery (average of 312 BOECs/4.5 mm²), mainly localized in perimuscular connective tissue (Figure 8). No detectable distribution was observed in lungs, spleen, liver, kidneys, and heart.

Doppler flow index at inclusion (5 days after hindlimb ischemia surgery) was similar in all groups (vehicle control: 35.9±1.5; CON-BOECs: 35.5±1.8; ICMP-BOECs: 35.9±2.0; P=0.98). High-resolution angio microCT of the upper leg confirmed enhanced BOEC-mediated arteriogenesis in comparison to vehicle control, 21 days after surgery (Figure 9). Although total vessel length and the number of vessel branch points tended to be increased after BOEC delivery from both CON and ICMP (Figure 9B), therapeutic effects were confined to smaller arterioles with a diameter ranging from 32 to 64 μm (Figure 9D), corresponding to collateral arteries, as documented by Scholz and Schaper.17,18 Importantly, increased arteriogenesis was observed after transplantation of BOECs from healthy young controls (P=0.048) and ICMP patients (P=0.025) over vehicle control, both to a similar extent (P=0.831). Clinically, BOECs were able to prevent additional amputations after cell delivery (5 days after hindlimb ischemia surgery). A total of 3 of 14 animals suffered from additional amputations after injections of vehicle control versus none in the BOEC-injected groups.

Immunostaining for human CD31 and in situ hybridization for primate specific ALU-repeat sequences could detect incorporation of rare human BOECs into murine vessels in the ischemic hindlimb 21 days after surgery (Figure 10). Engraftment, studied in 3 versus 3 mice, showed 4±2

Figure 5A). Additionally, 24 hours of exposure to 1000 μmol/L of H₂O₂ did not show significant differences in BOEC viability between ICMP (n=6) and CON (n=6; P=0.11; Figure 5B).

**BOEC In Vitro Neovascularization Potential**

We subsequently focused on functional neovascularization potential of the obtained cell populations. In vitro vasculogenic potential, explored using the Matrigel network remodeling assay (Figure 6A), did not show differences in the number of network intersections between cells from ICMP and (A)CON (P=0.32; Figure 6B). Total network length did not differ between cells from ICMP and (A)CON. An increase in network length was, however, noted in aged versus young controls. Severity of the disease process, assessed by LVEF, did not impair in vitro network formation potential. On the contrary, LVEF inversely correlated with total network length (r=−0.57; P=0.01; n=16) and the number of intersections between ICMP and (A)CON (r=−0.58; P=0.02; n=16). No other major clinical, biochemical, or hematological predictors could be identified. Additionally, in vitro angiogenesis potential, explored using a 3D spheroid sprouting assay in collagen gel, did not show differences in sprouting angiogenesis between BOECs from ICMP and CON (P=0.70; Figure 7).

RTqPCR and ELISA documented a marked proangiogenic profile with high expression of angiogenic growth factors (ANG-2, PLGF, PDGF-BB, FGF-2, and VEGF) both in BOECs from patients and controls (Table 2). ANG-2 and PLGF were the most abundant angiogenic growth factors in 24-hour conditioned medium, with similar amounts secreted by BOECs from ICMP and healthy subjects ([A]CON) (P=0.66 and 0.13 for ANG-2 and PLGF, respectively).
incorporated ICMP-BOECs/4.5 mm² versus 3/C6 incorpo-
rated CON-BOECs/4.5 mm². This limited incorporation
after injection of 250 000 BOECs/mouse suggests a
concomitant paracrine contribution of the transplanted
cell population to their documented neovascularization
potential.7,8

Discussion
We report that BOECs can be successfully isolated and
culture-expanded from patients with severe, but stable,
ischemic cardiomyopathy, irrespective of age, severity of
myocardial systolic dysfunction, or degree of maladaptive LV
remodeling. These cells show a robust and consistent
endothelial progenitor phenotype with comparable prolifera-
tion kinetics and without evidence of increased senescence
when compared to cells isolated from young or age-matched
healthy subjects. We did not document differences in in vitro
neovascularization potential of BOECs from patients or
controls, because they form comparable vascular networks
in Matrigel, demonstrate similar sprouting potential in a 3D
spheroid-sprouting angiogenesis assay, and secrete high

Figure 8. Acute BOEC retention. Confocal microscopy documents multiple clusters (A1) and singlets (A2) of Cherry-labeled BOECs (red),
24 hours after intramuscular injection into the ischemic hindlimb. These findings were confirmed by immunofluorescence microscopy for human
CD31 (red; labeling only transplanted cells) and BS-I-Lectin (green; labeling host endothelium) (B1) and in situ hybridization for primate-specific
ALU-repeat sequences (dark blue) on the respective adjacent slides (B2) (scale bar, 100 μm). Clusters and singlets of human BOECs are
indicated by white arrows. ALU indicates Arthrobacter luteus; BOEC(s), blood outgrowth endothelial cell(s); CON indicates healthy young
controls; ICMP, ischemic cardiomyopathy patients.
levels of potent angiogenic and arteriogenic factors. After in vivo cell transfer, BOECs from patients and healthy donors increase collateral vascular remodeling in the thigh region of nude mice, with significant induction of arterioles 32 to 64 μm in diameter and prevention of imminent amputations after cell delivery.

In contrast to various other EPC subtypes studied as biomarkers of cardiovascular risk and disease,14,19 we did not observe inverse correlations between the number of BOEC outgrowth colonies and severity of the ischemic atherosclerotic disease process. We did not observe a disease-related increase in the number of BOEC colonies obtained, as was previously shown during active inflammation, tissue damage, and intense vascular remodeling (acute myocardial infarction,20 acute ischemic stroke,21,22 rheumatoid arthritis,23 neovascular age-related macular degeneration,24 or extensive burns25). In our patient population with stable ischemic heart failure, optimally treated according to current guidelines, a similar number of BOEC colonies were obtained in patients and healthy controls. These findings are consistent with earlier data in statin-treated patients with premature coronary artery disease.26 Within our patient cohort, we did, however, note a mild positive correlation between age and BOEC outgrowth, absent in our control population.

Because of limited cell retention after intracoronary or intramyocardial delivery, a sufficient cell mass is needed to obtain therapeutic effects in the target region as previously shown in domestic swine.7 Starting from a single 60-mL blood sample, we were able to achieve our expansion goal of $30 \times 10^6$ cells in 52% of patients, with similar growth kinetics in controls. Given that we showed expansion success to be highly dependent on the number of initial BOEC colonies.

---

**Figure 9.** In vivo neovascularization potential assessed by angio microCT: Representative 3D reconstructions (left panel) and quantitative analysis (right panel). Representative 3D angio microCT reconstructions illustrate an enhanced neovascularization response in the thigh region after intramuscular injections of BOECs from both patients (ICMP) and CON (A). Twenty-one days after hindlimb ischemia surgery, angio microCT quantifications documented a trend toward increased vessel length (B1) and number of vessel branch points (B2) in the thigh region (A and C) after intramuscular BOEC injections of patients (ICMP) and CON in comparison to vehicle control (PBS). BOEC-mediated effects were confined to arterioles 32 to 64 μm in diameter (2-tailed unpaired t tests) (D). All results from the ischemic hindlimb (HLI) are expressed in relation to the contralateral control leg (CONT). 3D indicates three-dimensional; BOEC, blood outgrowth endothelial cell; CON indicates healthy young controls; CT, computed tomography; ICMP, ischemic cardiomyopathy patients; ROI, region of interest.
obtained, increasing the number of MNCs by increasing the volume of the blood sample or using leukapheresis could further enhance expansion success.

In contrast to various other cell types, we observed preserved neovascularization potential of BOECs derived from an older patient population with severe ICMP. Ingram et al. reported that BOECs contain a complete hierarchy of low- and high-proliferation potential cells, analogous to the hematopoietic cell system. Moreover, long-term culture conditions may erase the functional “fingerprint” of various microenvironmental factors, present in the donor milieu. Cell types with a previously documented dysfunctional phenotype in an older patient population with ischemic heart disease were only exposed to short-term in vitro cell culture (e.g., circulating angiogenic cells and colony-forming-unit EPCs) or no in vitro environment at all (e.g., CD133+/VEGFR2+ circulating EPCs and bone-marrow–derived MNCs). Hence, “weaning off” donor microenvironmental influences and positive selection based on proliferation potential were only minimally or not present. Specific reports on BOEC functionality and proliferation potential in various other disease states are, however, not uniform. Functional impairment has been reported, related to age, type 2 diabetes, obesity, pulmonary arterial hypertension, and smoking/chronic obstructive pulmonary disease. On the other hand, previous reports in treated coronary artery disease and arterial hypertension documented similar BOEC functionality in comparison to healthy controls. Moreover, improved BOEC functionality has been demonstrated in neovascular age-related macular degeneration, ischemic stroke, and extensive burns. In comparison to healthy controls, our findings did not demonstrate differences in BOEC functionality.

Figure 10. Human BOECs incorporate into murine blood vessels. Immunofluorescence costaining for human CD31 (A1, B1), BS-I-Lectin (A2, B2), and DAPI nuclear counterstain (A3, B3) demonstrates human BOEC incorporation (red) into murine blood vessels (green) in the adductor (A) and gastrocnemius (B) region, 21 days after hindlimb ischemia surgery (scale bar, 20 µm). These findings were confirmed by in situ hybridization for primate-specific ALU-repeat sequences on adjacent slides, showing incorporated human BOECs in dark blue (A5, B5) (A5 scale bar, 100 µm; B5 scale bar, 50 µm). Field of view of the immunofluorescence images is indicated in red. Engrafted human BOECs are indicated by white arrows. ALU indicates Arthrobacter luteus; BOEC(s), blood outgrowth endothelial cell(s); DAPI, 4’,6-diamidino-2-phenylindole.
and proliferation potential in a patient population with severe, but stable, ischemic heart failure. We noted, however, mild positive correlations between Matrigel network formation potential and both degree of systolic LV dysfunction and BMI within our patient cohort. No differences were noted in response to oxidative stress, studied by ROS induction and cell viability.

We studied in vivo cell-mediated neovascularization potential using high-resolution angio microCT because of its ability to quantitatively assess the complete "vessel tree" in 3D. Histology is mainly restricted to evaluating vascular density in a limited 2D area of view, whereas laser Doppler flowmetry is only reporting superficial blood flow (200–300 μm penetration depth), classically in the foot region.36 Collateral vascular remodeling (arteriogenesis) has been postulated as the most important mechanism to compensate for bulk perfusion loss. Efficient collateral formation is a critical determinant of tissue vulnerability and preservation during ischemia. Occlusion of the femoral artery in a hindlimb ischemia model results in development of collateral vessels in the upper leg, relatively close to the site of occlusion. In contrast, ischemia and angiogenesis occur in the lower leg and foot.37 We consequently focused on the thigh region as the readout for collateral vascular remodeling, and observed that cells from both patients and controls support the arteriogenesis process to a similar extent, especially at the level of arterioles (32–64 μm in diameter). Extensive (ultra)structural histological analysis by Scholz and Schaper in murine hindlimbs previously identified collateral arteries in the 40- to 70-μm diameter range.17,18 We documented a significant BOEC-mediated increase in vascular length within this specific size range. A trend toward a BOEC-mediated increase of vessel length and number of branch points of the complete vessel tree in the thigh region of the ligated leg was noted. These results did, however, not reach statistical significance, given that the therapeutic effects were confined to one specific size range (arterioles 32–64 μm in diameter). Arteriogenesis relies on a complex interplay of many growth factors, of which ANG-2, PLGF, FGF, and PDGF-BB were all demonstrated to be secreted by BOECs.37–40

Acute retention studies documented BOECs, mainly clustered within the perimuscular compartment 24 hours after intramuscular injection. At 21 days, we noted disappearance of the BOEC clusters in the perimuscular compartment and appearance of isolated BOECs in the vascular compartment. We observed only limited incorporation/engraftment of BOECs into functional blood vessels after intramuscular delivery of 250 000 cells per mouse, suggesting a concomitant paracrine contribution of the transplanted cell population to their neovascularization potential. These findings indicate migration toward and engraftment within the vascular compartment to perform their biological effects, “sustained” release of a proangiogenic/-arteriogenic growth factor “cocktail” with or without incorporation into functional vessels. ANG-2 and PLGF were found to be the most abundantly secreted angiogenic growth factors, independent of underlying ischemic disease. Both ANG-2 and PLGF play an important role in the complex cytokine cocktail, mediating the overall neovascularization process in ischemic disease.37–47

In conclusion, we report that BOECs can be isolated and culture-expanded from patients with severe, but stable, ICMP and offer an easily accessible, clonally expandable cell source with profound neovascularization potential. In contrast to impaired functionality of other progenitor cell types derived from patients with advanced cardiovascular disease, BOECs retain a robust proangiogenic profile in vitro and in vivo and hold promise for autologous cell therapy in severe or refractory ischemic heart disease.

Sources of Funding
This work was supported by the Research Foundation Flanders (FWO), a KULeuven Research Grant (PF/10/014) and funds from the ISCI (P13/02144, CP09/00332). Nick van Gastel is funded by BOF-KU Leuven GOA project 3M120209. Aer�nout Luttun is supported by an Interuniversity Attraction Poles (IUAP) grant (IUAP/P7/07).

Disclosures
None.

References
ICMP BOECs Retain Neovascularization Potential

Dauwe et al


20. Massa M, Campanelli R, Bonetti E, Ferrario M, Marinoni B, Rosti V. Rapid and


Neovascularization Potential of Blood Outgrowth Endothelial Cells From Patients With Stable Ischemic Heart Failure Is Preserved


*J Am Heart Assoc.* 2016;5:e002288; originally published April 18, 2016; doi: 10.1161/JAHA.115.002288

The *Journal of the American Heart Association* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Online ISSN: 2047-9980

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://jaha.ahajournals.org/content/5/4/e002288

Subscriptions, Permissions, and Reprints: The *Journal of the American Heart Association* is an online only Open Access publication. Visit the Journal at http://jaha.ahajournals.org for more information.