Supplement Material.

Methods

Characterization of blood-derived CACs

Blood was drawn from the cubital vein into vacuum tubes pre-filled with a liquid density gradient medium and MNCs were isolated based on the Ficoll method (Vacutainer CPT, Becton Dickinson, Franklin Lakes, NJ). In order to remove mature endothelial cells from the harvested cell population, the cells were preplated on fibronectin-coated culture plates for 1 day in EBM-2 MV (supplemented with Singlequots, 20% fetal bovine serum, HyClone, Logan, UT). The initially firmly adherent cells were discarded and the non-adherent cells (>95%) were moved to a new dish and cultured for another 6 days, during which time many cells (10% on average) became newly adherent.

To confirm that the *in vitro* adhesion selection of initially weakly or non-adherent MNCs gives rise to pro-angiogenic CACs, we characterized newly adherent cells and non-adherent cells at day 7 by their ability to take up acLDL and bind to UEA lectin, specific surface markers, VEGF, and eNOS expression.[1,2] Adherent CACs on fibronectin-coated glass slides (Nalge NUNC, Naperville, IL) were incubated for 1 h with 2 μg/mL DiI-acLDL (Invitrogen, Carlsbad, CA) in EBM-2 MV, washed twice with PBS and fixed in 2% formaldehyde/PBS. After blocking with 2% goat serum/PBS for 1 h, cells were washed and incubated with 23 μg/mL FITC-conjugated *Ulex europeus* agglutinin-1 (UEA-1, Sigma, St. Louis, MO). The nuclei were stained with 125 ng/mL Hoechst 33258 (Invitrogen). The slides were observed using a Nikon E800 fluorescence microscope and Openlab software (Improvision, Lexington, MA). To further characterize the cells, FACS analysis was performed with the CACs (large mainly spindle shaped, firmly adherent)

and compared to the small weakly or non-adherent cells (non-CACs) in the same cultures. After harvesting weakly or non-adherent cells and rinsing the dish with PBS, adherent cells were detached by repetitive flushing with cold 1 mM EDTA/PBS. CACs and non-adherent cells were pelleted, adjusted to 10^6 cells/mL, and incubated for 20 min with normal human IgG (1 mg/mL, Zymed, San Francisco, CA) to block F_C receptor. Staining was performed for 20 min with 100 μL cell suspension and the following fluorescently labeled antibodies: CD45-PerCP, CD34-PE, CD133-PE (Miltenyi Biotech, Auburn, CA), KDR-APC, CD31-PC5, CXCR4-APC, CD14-PerCP, and CD11b-APC (Pharmingen, San Diego, CA). After washing with FACS buffer, cells were fixed with 1% formaldehyde/PBS and stored at 4°C until flow-cytometry analysis. 10,000 events were counted (FACSCalibur, BD, San Diego, CA). Further characterization was performed by measuring eNOS protein levels in cell lysates before and after 2, 4, 7, and 14 days culture using a commercially available ELISA kit (Quantikine, R&D) following the recommended protocol. Lysates were produced by addition of supplied lysis buffer to frozen cell pellets.

Cell proliferation and apopotosis assays

BrdU incorporation assays were performed following the manufacturer's protocol (Cell Proliferation BrdU Assay, Roche). Cells were detached, resuspended in EBM-2 supplemented with 1% BSA, and plated at 10^4 /well in 96-well cell culture plates (Corning). The cells were preincubated with test mitogens for 48 h. BrdU was added and cells were incubated for another 24 h. BrdU incorporation was determined in an ELISA plate reader by light absorption at 450 nm after incubation with anti-BrdU antibodies conjugated with horseradish peroxidase. Apoptosis assays were performed with FACS

essentially as described in the manufacturer's (Guava, Hayward, CA) protocol. Day 7 CACs were washed 2x with PBSE (phosphate buffered saline, 1 mM EDTA), detached, and resuspended in EBM-2 (without supplements other than 1% BSA). 100 uL of cell suspension containing 40,000 CACs were incubated with the apoptosis inductor camptothecin (0.01-100 μmol/l), SNAP (0.01-100 μmol/l), or VEGF (50 ng/mL) at 37˚C. After 3 h, cells were washed in assay buffer on ice and resuspended in 40 uL assay buffer. 5 uL of AnnexinV-PE/7-amino actinomycin D (7-AAD) staining solution was added to cell suspension and incubated 20 min on ice. After addition of 450 uL assay buffer, cells were run on a flow cytometer (Guava). Analyses were performed automatically (Nexin, Guava). Apoptotic cells were positive for AnnexinV binding ($AnnV^+$). Additionally, $AnnV^+$ cells that excluded 7-AAD ($AnnV^+$ $7\text{-}AAD^-$), indicating an intact cell membrane, were defined as early apoptotic. 7-AAD positivity ($AnnV^+/7\text{-}AAD^+$) indicated disrupted cell membrane integrity and late apoptosis.

Additional information for chemotaxis

In preparation for the migration experiments, bottom chambers were blocked with 10% BSA/PBS for 10 min and rinsed with PBS 3 times, as our preliminary experiments have shown that VEGF binds to the plastic and the concentration in the solution drops precipitously if the wells are not blocked. Preliminary recovery experiments were performed by measuring VEGF in the upper and lower chamber after adding VEGF to the lower chamber. These experiments confirmed that there was significantly higher VEGF concentration in the lower chamber for up to 12 h.

Both CACs and HUVECs were detached non-enzymatically by flushing with cold EDTA-containing dissociation buffers (Invitrogen) to avoid digestion of receptors by

trypsin. After detachment, cells were resuspended in EBM-2 (without supplements, 1% BSA) and $2x10^4$ plated in the upper of two chambers divided by a membrane with 8 μm pores (Corning Transwell). The bottom of the membrane was coated with vitronectin, fibronectin, and gelatin (Sigma). Chemoattractants specific to the experiment were added to the lower chamber only. The following were added to both the upper and lower chamber: NOS substrate L-arginine (100 μmol/l), NOS inhibitor L-NNA (100 μmol/l), NO scavenger PTIO (2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, 100 μmol/l), guanylyl cyclase inhibitor ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one, 100 μmol/l), and the PI3 Kinase inhibitor Wortmannin (100 nmol/l). We tested the chemotactic properties of vascular endothelial growth factor (VEGF, Sigma) and stromal cell-derived factor (SDF-1α; Sigma), and pleiotrophin (PTN, Sigma) at 10-500 ng/mL, monocyte chemoattractant protein-1 (MCP-1, Sigma), sphingosine-1-phosphate (S1P, Sigma), and interleukin-6 (IL6, Sigma) at 10-100 ng/ml, and S-nitroso-N-acetylpenicillamine (SNAP, Sigma) at 1 nmol/l-10 μmol/l. To test the chemokinetic properties of SNAP inducing random cell movement, SNAP was added to the upper and lower chambers. Each experimental condition was performed in triplicate and the number of migrated cells was determined on 5 random 100x optical fields (0.998 mm^2) per membrane.

Flow-mediated dilation (FMD)

Endothelium-dependent dilation of the brachial artery (BA) was measured by ultrasound (Sonosite Micromax, Bothell, WA) in combination with an automated analysis system (Brachial Analyzer, Medical Imaging Applications, Iowa City, IA). Baseline data for diameter and blood-flow velocity of the BA were quantified after 10 min of supine rest in

a 21°C room. A forearm blood-pressure cuff was placed distal to the antecubital fossa and inflated to 250 mmHg for 5 min. Diameter was measured immediately after cuff deflation, at 20, 40, 60, and 80 sec. FMD was expressed as: ($diameter_{max}$-$diameter_{baseline}$)/$diameter_{baseline}$.

Plasma nitrite level

The plasma nitrite levels, representing a sensitive read-out of NOS activity, were measured by chemiluminecence. In brief, venous blood supplemented with heparin (10 IU/mL), and EDTA (2 mmol/L) was centrifuged for 10 minutes at 800 *g* and 4°C immediately after sample drawing. The separated plasma samples were stored on ice. Nitrite was measured using a mixture of iodine/iodide in glacial acetic acid and subsequent detection of the liberated NO by its gas-phase chemiluminescence reaction with ozone. Concentrations of nitrite were determined by the difference in peak areas of untreated aliquots and those subjected to preincubation with 0.5% sulfanilamide/HCl.

RNA expression analyses

RNA was isolated by using an RNAeasy mini kit (Qiagen) following the manufacturer's instructions. The RNA (0.5 or 1 μg) was then reverse transcribed in a MyCycler personal thermal cycler (Bio-Rad Laboratories GmbH, Munich, Germany) using a QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. DNA (5 ng) or control RNA was used as a template for real-time PCR performed in triplicate using TaqMan universal PCR master mix in a ABI PRISM 7900 system (Applied Biosystems, Foster City, CA). The following primers and probes were purchased from Applied Biosystems: nitric oxide synthase 3, endothelilal (Hs00167166_m1), nitric oxide-synthase 2, inducible (Hs01075527_m1), and 18s rRNA, which was chosen as a

housekeeping gene. As a positive control HUVECs were activated by incubation with TNFα 500 U/mL, INFγ 500 U/mL, and IL-1β 500 U/mL over 24 h.

Results

CAC characterization. To confirm that the *in vitro* adhesion selection of initially weakly or non-adherent MNCs gave rise to pro-angiogenic CACs, we characterized large, firmly adherent, mainly spindle shaped or round flat cells as well as small non-adherent cells by their ability to take up acLDL and bind to UEA lectin, and by expression of specific surface markers, the angiogenic growth factor VEGF, and eNOS.[1] During culture of MNCs from healthy subjects, one population of cells firmly adhered to fibronectin and progressively increased eNOS protein content up to 19.3±6.1 ng/10^6 cells plateauing after 7 days. Expression of eNOS by the non-adherent cells was very low, below the detection limit of the ELISA assays (Figure 1a). The eNOS protein content in the CACs was significantly lower than that measured in HUVECS (33.5 ng/10^6 cells, p=0.03). We have previously shown by experiments with the NO-sensitive fluorescent dye DAF-2DA that CACs isolated under our conditions express a functional NOS.[1] Furthermore, adherent cells progressively secreted increasing amounts of VEGF (Figure 1b). The majority of firmly adherent cells also expressed both endothelial (KDR, CD31) and monocyte markers (CD11b, CD14) (Figure 1c). The majority of non-adherent cells expressed CD3, consistent with an identity of lymphocytes. Both populations expressed the hematopoietic marker CD45 and CXCR4. 92±8% of the firmly adherent cells took up acLDL and stained positive with UEA lectin, whereas none of the non-adherent cells took up acLDL (data not shown). Neither one of the populations differentiated into endothelial cells as defined by formation of tube-like structures or contact-inhibited monolayers (data not

shown). Taken together, the adherent cell population that was studied in the present paper and is herein referred to as CACs is consistent with the early pro-angiogenic hematopoietic EPC type, whereas non-adherent cells which were not further studied herein were mainly consistent with lymphocytes.[3-5]

SNAP also dose-dependently inhibited camptothecin-induced apoptosis of CACs (Figure 2). Early apoptosis was identified by annexinV-binding ($AnnV^+$) along with 7-AADexclusion ($7\text{-}AAD^-$) showing that the cell membrane was intact. AnnexinV binding along with 7-AAD uptake (i.e. disrupted membrane) identified late apoptosis. Vital cells were identified as being negative for annexin V-binding and 7-AAD. Camptothecin dose-dependently induced apoptosis in CACs. Maximal apoptosis of CACs was achieved with >10 μmol/l camptothecin at 3 h (40% early apoptosis $AnnV^+/7\text{-}AAD$ 20% late apoptosis $AnnV^+/7\text{-}AAD^+$). Coincubation of SNAP at 0.1-100 μmol/l led to dose-dependent inhibition of camptothecin-induced apoptosis at 50 μmol/l with significantly higher numbers of vital cells ($AnnV^-/7\text{-}AAD^-$). Maximal effects were observed at 1 μmol/l SNAP. The degree of apoptosis inhibition was similar to that induced by VEGF (50 ng/ml), which is known to inhibit apoptosis. Similar results were obtained when apoptosis was induced by staurosporin (data not shown).

Supplement Table I Characteristics of study population

	Healthy	CAD	*p*
N (m/f)	10 (7/3)	10 (7/3)	
Age (yr)	30±1	56±3	*<0.001*
BMI (kg/m^2)	24.3±1.1	27.8±1.8	*0.098*
Diabetes mellitus (%)	0	40	
Hypertension (%)	0	80	
Hyperlipidemia (%)	0	100	
Prior smoking (%)	0	50	
ACE inhibitor/angiotensin receptor blocker (%)	0	90	
Aspirin (%)	0	100	
Beta blocker (%)	0	80	
Statin (%)	0	100	
Heart rate (/min)	63±3	59±2	*0.206*
Systolic blood pressure (mmHg)	100±3	128±5	*0.002*
Diastolic blood pressure (mmHg)	58±2	80±3	*<0.001*
Total cholesterol (mg/dL)	179±7	145±7	*0.005*
LDL cholesterol (mg/dL)	121±9	75±5	*<0.001*
HDL cholesterol (mg/dL)	51±5	50±4	*0.821*
Triglycerides (mg/dL)	77±9	102±15	*0.188*
Fasting glucose (mg/dL)	73±3	94±4	*0.001*
Flow-mediated dilation (%)	6.8±0.3	4.7±0.7	*0.007*
Plasma nitrite (nmol/L)	60±3	38±3	*<0.001*

Data given as mean±SEM

References

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