Nitric Oxide Synthase Expression and Functional Response to Nitric Oxide Are Both Important Modulators of Circulating Angiogenic Cell Response to Angiogenic Stimuli

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***Objective*—**Circulating angiogenic cells (CACs), also termed endothelial progenitor cells, play an integral role in vascular repair and are functionally impaired in coronary artery disease (CAD). The role of nitric oxide (NO) in CAC function is poorly understood. We hypothesized that CAC migration toward angiogenic signals is modulated by both NO synthase (NOS) expression and functional response to NO.

***Methods and Results*—**Similar to endothelial cells, CAC chemotaxis to vascular endothelial growth factor (VEGF) was blocked by inhibition of NOS, phosphatidylinositol 3-kinase, or guanylyl cyclase or by treatment with an NO scavenger. Addition of an NO donor (*S*-nitroso-*N*-acetylpenicillamine) and the NOS substrate L-arginine increased random cell migration (chemokinesis) and enhanced VEGF-dependent chemotaxis. Healthy CACs expressed endothelial NOS, but endothelial NOS was not detected in CAD patient CACs. Both chemokinesis and chemotaxis to VEGF of patient CACs were decreased compared with healthy CACs but were restored to healthy values by *S*-nitroso-*N*-acetylpenicillamine. In parallel, CAD patients exhibited lower flow-mediated vasodilation and plasma NO source nitrite than young, healthy subjects, indicating endothelial dysfunction with reduced NO bioavailability.

***Conclusion*—**NOS activity is required for CAC chemotaxis. In CAD patients, impairment of NOS expression and NO bioavailability, rather than response to NO, may contribute to dysfunction of CACs and limit their regenerative capacity. **(*Arterioscler Thromb Vasc Biol*. 2010;30:2212-2218.)**

Key Words: cell physiology ■ coronary artery disease ■ endothelium ■ nitric oxide ■ nitric oxide synthase ■ circulating angiogenic cells

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NO is an important signaling molecule in vascular biology.[1] Physiologically, many integral functions of the vascular endothelium are modulated by endothelial nitric oxide synthase (eNOS)-derived NO, including the inhibition of platelet and leukocyte adhesion, smooth muscle relaxation, and proliferation. Newer literature shows that NO not only acts in paracrine manner but may also exert systemic effects via reversible formation of more stable storage forms, including nitrite and nitoso-adducts. The disruption of this pathway in endothelial cells is associated with chronic vascular disease.[2] Risk factors appear to selectively damage the vascular endothelium, leading to a dysfunctional, maladaptive endothelial phenotype.[3,4] Studies suggest that eNOS activity and expression as well as circulating NO storage forms in blood are progressively decreased with cardiovascular risk factors including aging, hypertension, hypercholesterolemia, diabetes, and smoking and cigarette smoke exposure.[2,5–7] Over time, chronic endothelial dysfunction leads to intimal hyperplasia and enhanced plaque formation in predisposed areas of the vascular tree. Notably, the functional capacity of the vascular endothelium not only depends on the degree of damage but also on the presence and status of repair systems, including circulating angiogenic cells (CACs).[8]

Vascular repair involves not only local migration and proliferation of mature endothelial cells but also angiogenic cells that circulate in blood and the recruitment of the latter cells to sites of injury. Literature from the last 10 years suggests that circulating proangiogenic blood cells can enhance angiogenesis and the replacement of vascular endothelium.[8–10] These cells were initially termed endothelial progenitor cells because of their phenotypic similarities to mature endothelial cells, including kinase insert domain

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receptor, eNOS, and platelet endothelial cell adhesion molecule (CD31), but also to stem cells (CD34 and CD133) and myeloid cells (CD14 and CD45). Newer literature suggests that these early outgrowth angiogenic cells temporarily aid endothelial repair, rather than developing into mature endothelial cells, in contrast to late outgrowth endothelial colony-forming cells, which can form endothelial tubes and monolayers.[11,12] Therefore, these cells are herein referred to as CACs rather than early endothelial progenitor cells. Clinical and experimental studies show that the reparative and therapeutic potency of CACs is determined by their functional status, which, in turn, are characterized by migratory capacity toward chemotactic signals, such as vascular endothelial growth factor (VEGF).[13,14]

Several studies suggest that cardiovascular disease not only may be caused by endothelial damage but also may cause or be caused by CAC dysfunction. The number or function of these cells is reduced with aging,[6] hypertension, diabetes,[15] smoking,[16] and environmental smoke exposure.[17] CAC dysfunction was shown to limit the therapeutic potency of these cells when transplanted.[13] It is conceivable that the functional capacities of CACs in patients may also be affected by the pathomechanisms that impair endothelial cell dysfunction, including decreased NO production and bioavailability, further facilitating vascular disease progression.[18] The role of NO activity in fundamental functional CAC capacities is not well studied. We hypothesized that CAC migration is modulated by NO and that CAC dysfunction in coronary artery disease (CAD) patients is a result of reduced NO bioavailability in blood or decreased NOS expression.

We first characterized the chemotactic response of CACs and compared the results with human umbilical vein endothelial cells (HUVECs), serving as a standard endothelial cell system. We then studied the effect of the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) on migration of CACs and HUVECs. Finally, we measured eNOS expression and migratory responses in ex vivo–differentiated CACs isolated from CAD patients, who were shown to experience endothelial dysfunction with impaired NO bioavailablity, and compared them with results from young, healthy volunteers.

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Methods

Study Subjects

CACs were isolated from 10 young, healthy subjects without cardiovascular risk factors (hypertension, diabetes mellitus, smoking, and hypercholesterolemia, which are associated with impaired number and function of CACs), and with normal endothelial function (as measured by flow-mediated dilation [FMD] of the brachial artery of greater than 6%).[2,6,19] (See Supplemental Table I, available online at http://atvb.ahajournals.org, for characteristics.) We also isolated CACs from 10 patients with angiographically documented CAD as defined by >70% stenosis of at least 1 coronary artery on optimal medical therapy according to current secondary prevention guidelines[20] and endothelial dysfunction with FMD <5%. The characterization of CACs, including mechanistic experiments, was performed in CACs isolated from the healthy subjects. The protocol was approved by the University of California, San Francisco, Committee on Human Research, and volunteers gave written informed consent.

Cell Culture and Characterization of Blood-Derived CACs

CACs were differentiated ex vivo from peripheral blood mononuclear cells as previously described (see supplemental material for more detailed characterization protocols).[11,21] CACs were isolated from mononuclear cells as adherent cells on fibronectin-coated dishes after 7 days. Culture was preceded by 1 day of preplating to remove platelets and shed endothelial cells. eNOS protein was quantitated in cell lysates of CACs at day 7 and VEGF in cell medium of adherent and nonadherent cells using commercially available ELISA kits following the manufacturer's protocol (Quantikine, R&D Systems). Marker expression (CD45, CXCR4, CD31, kinase insert domain receptor, CD11b, CD14, CD3, CD34, CD133) of day 7 cells was determined by flow cytometry.

Pooled HUVECs were purchased from Cambrex (Walkersville, Md), cultured in EBM-2 (supplemented with Singlequots 5% FBS) and used no later than passage 3.

Chemotaxis and Chemokinesis Assay

Cell migration was quantified by a transwell chemotaxis assay using a modified Boyden chamber.[13,22,23] Migration of both CACs and HUVECs was measured as follows: cells (2×10^4) were plated in EBM-2 medium (0.5% BSA, without other supplements, containing 63 mg/L L-arginine) in the upper of 2 chambers divided by a membrane with 8-μm pores (Corning Transwell). We tested the chemotactic properties of the following chemoattractants in only the lower chamber: vascular endothelial growth factor (VEGF, Sigma), stromal cell–derived factor (SDF-1α; Sigma), and pleiotrophin (PTN; Sigma) at 10 to 500 ng/mL; and monocyte chemoattractant protein 1 (Sigma), sphingosine-1-phosphate (Sigma), and interleukin 6 (Sigma) at 10 to 100 ng/mL. The following were added to both the upper and lower chamber: the NOS substrate L-arginine (100 μmol/L), NOS inhibitor N^G-nitro-L-arginine (100 μmol/L), NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO; 100 μmol/L), guanylyl cyclase inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ; 100 μmol/L), PI3K inhibitor wortmannin (WM; 100 nmol/L), and NO donor SNAP (Sigma) at 1 nmol/L to 10 μmol/L. The number of migrated cells was determined on 5 random $\times100$ optical fields per membrane. To distinguish chemokinetic from chemotactic properties of VEGF and SNAP, both substances were added to upper and lower chambers in a checkerboard fashion.

Cell Proliferation and Apoptosis Assays

5-Bromodeoxyuridine incorporation assays were performed in 96-well dishes following the manufacturer's protocol (Cell Proliferation BrdU Assay, Roche). Apoptosis assays were performed with fluorescence-activated cell sorting essentially as described in the manufacturer's protocol (Guava, Hayward, Calif). cGMP levels were measured in 10^5 cells under baseline unstimulated conditions and after incubation with SNAP at 1 μmol/L for 30 minutes using an ELISA kit following the manufacturer's protocol (GE Healthcare).

FMD

Endothelium-dependent dilation of the brachial artery was measured by ultrasound (Sonosite Micromax, Bothell, Wash) in combination with an automated analysis system (Brachial Analyzer, Medical Imaging Applications, Iowa City, Iowa) as described (see supplemental material for details).[17]

Plasma Nitrite Level

The plasma nitrite levels, representing a sensitive readout of NOS activity, were measured as recently described using gas-phase chemiluminescence (see supplemental material for details).[24]

Statistical Analyses

Data are presented as mean±standard error of the mean. Group differences were calculated with repeated measurements ANOVA and consecutive post hoc test. Probability values of less than 0.05 were regarded as significant. Correlations were by the Pearson *r*. All experiments were performed in triplicate.

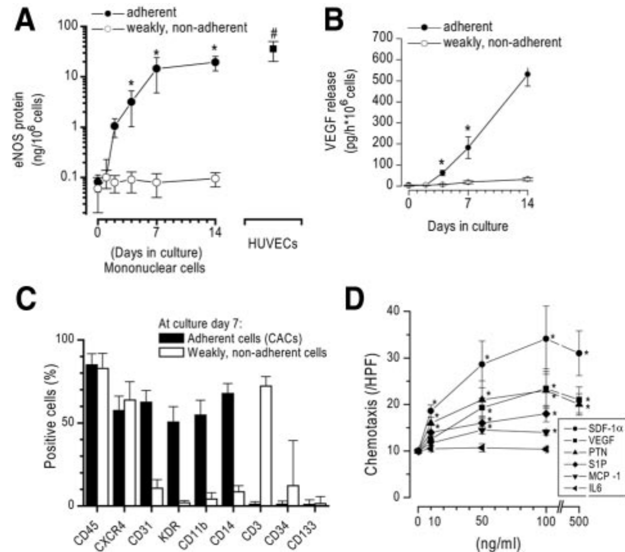

Figure 1. Characterization of CACs. Adherence-selected mononuclear cells progressively expressed eNOS (A); released VEGF (B); and expressed hematopoietic, monocytic, and endothelial markers (C). D, Day 7 CACs exhibited chemotaxis toward VEGF, PTN, SDF-1α-, monocyte chemoattractant protein 1 (MCP-1), and sphingosine-1-phosphate (S1P) but not toward interleukin 6. *$P<0.05$ versus control.

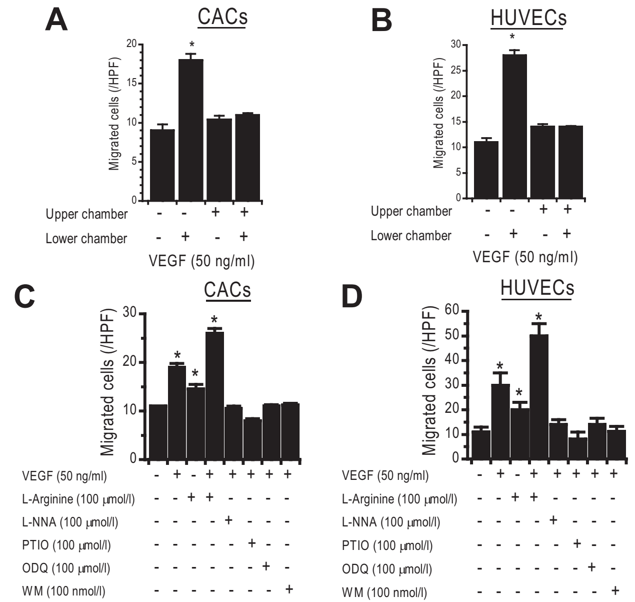

Figure 2. Mechanisms of CAC chemotaxis are similar to those of HUVECs. VEGF is a chemotactic stimulus for CACs (A) and HUVECs (B). Chemotaxis to VEGF was inhibited by L-NNA (NOS inhibitor), PTIO (NO scavenger), ODQ (guanylyl cyclase inhibitor), and WM (PI3K inhibitor) and was increased by L-arginine (NOS substrate). *$P<0.05$ versus control. (C and D, VEGF was added only to the lower chamber; L-NNA, ODQ, PTIO, WM, and L-arginine were added to the upper and lower chambers.) HPF indicates high-power field.

Results

CAC Characterization

During culture, mononuclear cells gave rise to adherent cells progressively expressing eNOS and releasing VEGF (Figure 1). At day 7, the majority of adherent cells expressed markers consistent with the early proangiogenic hematopoietic endothelial progenitor cell type as described in the literature, whereas nonadherent cells, which were not further studied herein, were mainly consistent with lymphocytes.[11,25,26] CACs migrated dose-dependently to a number of chemokines including VEGF, SDF-1α, PTN,[23] sphingosine-1-phosphate, and monocyte chemoattractant protein 1 but not to interleukin 6 at the concentrations tested (Figure 1D). Of the investigated chemokines, SDF-1α exerted the strongest migratory response (SDF-1α>VEGF=PTN>monocyte chemoattractant protein 1=sphingosine-1-phosphate).

Mechanisms of CAC Chemotaxis: Similarity With Endothelial Cells (HUVECs)

Further experiments showed that random cell movement in the presence of VEGF (50 ng/mL) in the upper and lower chambers (that is, no gradient) did not lead to significantly more cells on the lower side of the membrane compared with the negative control lacking VEGF (Figure 2A and 2B). This confirms that VEGF does not merely induce a significant chemokinetic response but stimulates specific chemotactic responses in CACs and HUVECs.[23]

To gain mechanistic insight into chemotaxis of CACs, we performed inhibitor studies to attempt to block the VEGF-induced chemotaxis (Figure 2C and 2D). Chemotaxis toward VEGF at 50 ng/mL was inhibited in the presence of a NOS inhibitor (L-NNA), an NO scavenger (PTIO), a phosphatidylinositol 3-kinase inhibitor (WM), and a guanylyl cyclase inhibitor (ODQ). Nondirectional cell movement, without addition of chemokines, remained unaffected by these inhibitors, suggesting that the observed lack of chemotaxis is due to specific inhibition of the pathways in question and cannot be explained by unspecific cell toxicity or globally disabled cell motility. Furthermore, our experiments show that the mechanisms involved in CAC chemotaxis are similar in HUVECs. Interestingly, L-arginine in the upper and lower chambers (no gradient) not only increased chemotaxis toward the VEGF gradient but enhanced chemokinesis.

NO Donor Induces Chemokinesis and Enhances Chemotaxis

To test how NO itself affects CAC motility and whether CACs follow a gradient of NO, we performed migration assays with the NO donor SNAP (Figure 3). As opposed to VEGF, SNAP induced a strong increase in random cell movement (chemokinesis). However, the number of migrated cells when SNAP (1 μmol/L; measured NO concentration in medium, 2.7 and 0.8 nmol/L at 0 and 3 hours, respectively) was present in both the upper and lower chambers was greater than when SNAP was present only in the lower chamber, suggesting that NO is a stronger inductor of chemokinesis than chemotaxis. Dose-dependent chemokinesis at 0.01 to 50 μmol/L showed a maximum at 1 μmol/L (28±3 cells/high-power field). Directional cell movement toward a VEGF gradient was also present at these SNAP concentrations. The highest absolute number of migrated cells was observed with a VEGF gradient (50 ng/mL) in the presence of SNAP at 1 μmol/L (34±4 cells/high-power field). These findings illustrate that SNAP-mediated chemokinesis acts synergisti-

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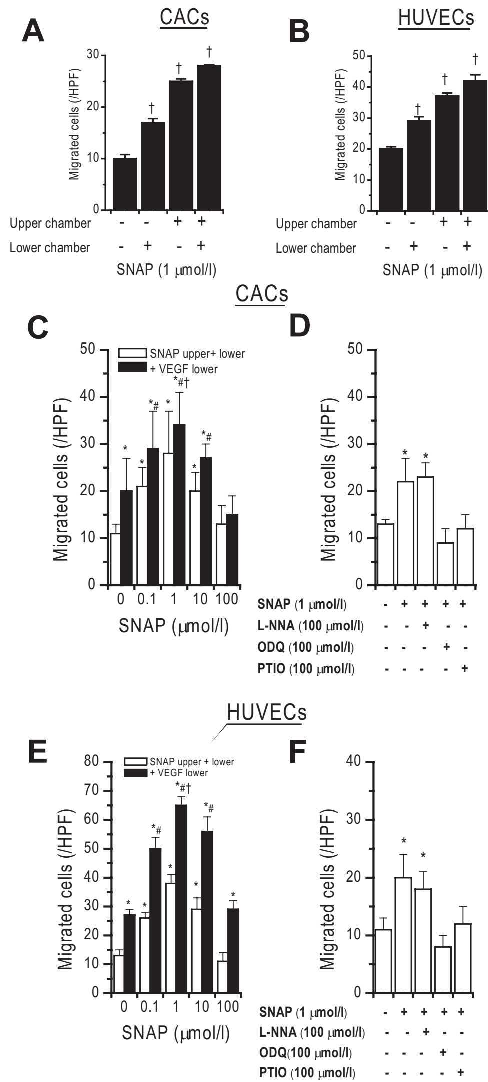

Figure 3. NO donor SNAP caused dose-dependent chemokinesis and enhanced VEGF-mediated chemotaxis. A and B, SNAP stimulated random cell movement (chemokinesis). C and E, Dose-dependent stimulation of chemokinesis and chemotaxis toward VEGF. D and F, Inhibition by ODQ (guanylyl cyclase inhibitor) and PTIO (NO scavenger) but not by NOS inhibitor L-NNA. †$P<0.05$ versus the respective column to the left; *$P<0.05$ versus control; #$P<0.05$ versus the respective white column; †$P<0.05$ versus VEGF alone. HPF indicates high-power field.

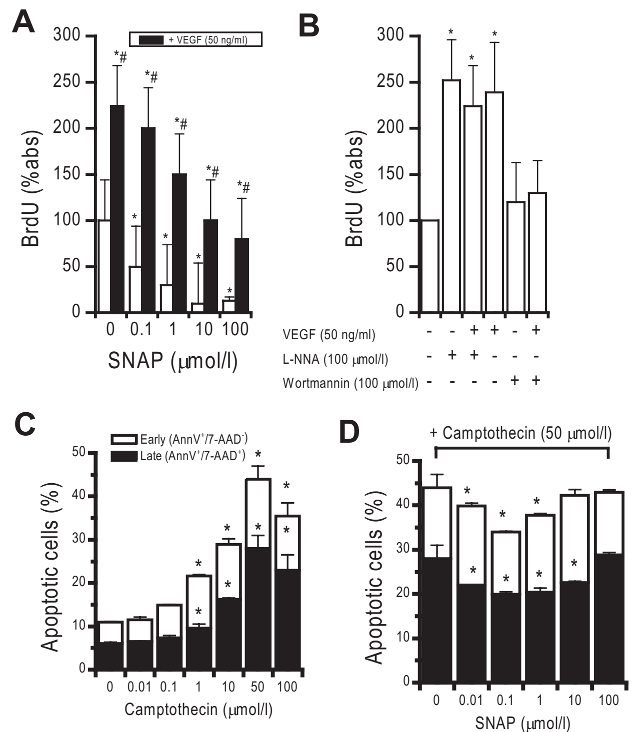

Figure 4. Effect of SNAP on CAC proliferation and apoptosis. A, Proliferation of CACs. B, VEGF-induced proliferation was increased by addition of L-NNA (NOS inhibitor), and inhibited by WM (PI3K inhibitor). C, Dose finding for camptothecin-induced apoptosis (early apoptotic cells: AnnV$^+$/7-AAD$^-$ [white columns]; late apoptotic cells: AnnV$^+$/7-AAD$^+$ [black columns]). D, SNAP dose-dependently inhibited apoptosis. %abs, percent absorption.

cally with VEGF-induced chemotaxis to enhance the number of net migrated cells, suggesting that exogenous NO facilitated directional cell movement to a chemokine stimulus. Addition of ODQ and PTIO but not L-NNA inhibited the chemokinetic SNAP (1 μmol/L) response, suggesting guanylate cyclase dependence, NO specificity, and NOS independence. This shows that both CACs and HUVECs similarly distribute faster in the presence of NO but are still responsive toward chemotactic stimuli.

In addition to its effects on cell migration, SNAP dose-dependently inhibited both VEGF-induced and spontaneous CAC proliferation (Figure 4). Conversely, inhibition of NOS by L-NNA stimulated proliferation to a degree similar to VEGF, suggesting opposite effects of VEGF and NO with respect to proliferation, as previously shown.[23] VEGF-induced proliferation was inhibited by WM, suggesting dependence of this effect on phosphatidylinositol 3-kinase but not NOS.

SNAP also dose-dependently inhibited camptothecin-induced apoptosis of CACs (Figure 4). Early apoptosis was identified by annexin V binding (AnnV$^+$) along with 7-AAD exclusion (7-AAD$^-$), showing that the cell membrane was intact. AnnV$^+$, along with 7-AAD uptake (ie, disrupted membrane), identified late apoptosis. Vital cells were identified as being negative for AnnV$^+$ and 7-AAD. Camptothecin dose-dependently induced apoptosis in CACs. Maximal apoptosis of CACs was achieved with >10 μmol/L camptothecin at 3 hours (40% early apoptosis AnnV$^+$/7-AAD; 20% late apoptosis AnnV$^+$/7-AAD$^+$). Coincubation of SNAP at 0.1 to 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-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).

Impaired CAC Migration and NOS Expression in CAD Patients

To show the clinical relevance of these findings to human cardiovascular disease, we measured CAC migration as a

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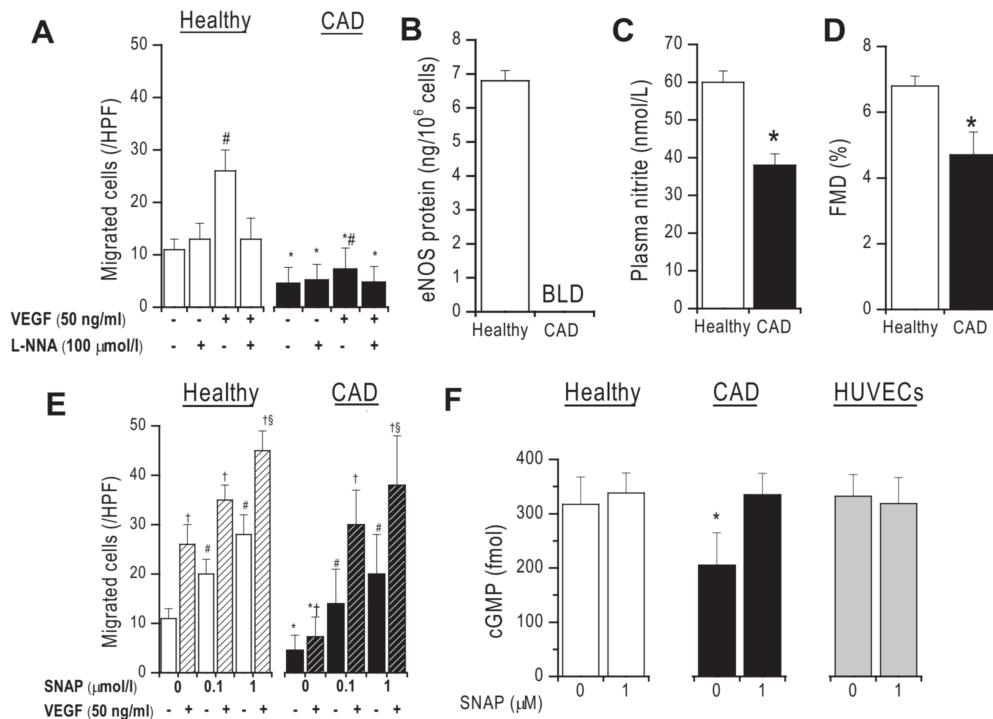

Figure 5. Impaired chemotaxis and eNOS expression in CAD patients. A, L-NNA-inhibitable CAC migration was significantly reduced in CAD patients as compared with healthy controls. B, eNOS protein expression in CAD CACs was below the limit of detection (BLD). C and D, Plasma nitrite (C) and flow-mediated vasodilation (FMD) (D) were impaired in CAD, reflecting endothelial dysfunction. E, CAC random cell movement and chemotaxis (toward 50 ng/mL VEGF; hatched columns) dose-dependently increased in CACs from both groups. F, The respective cGMP levels. *$P<0.05$ versus the respective condition in the healthy group; #$P<-0.05$ versus random cell movement without additives in the same group; †$P<0.05$ versus the respective no-VEGF condition; §$P<0.05$ versus VEGF alone in the same group. HPF indicates high-power field.

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readout of therapeutic potency and quantitated eNOS protein in CACs from older CAD patients compared with those from younger, healthy subjects (age, 56±3 versus 30±2 years; $P<0.001$). Supplemental Table I summarizes the clinical baseline characteristics. Flow-mediated vasodilation (FMD; 4.7±0.7 versus 6.8±0.3%, $P<0.001$, Figure 5) and plasma nitrite (38±3 versus 60±3 nmol/L, $P<0.001$) were significantly lower in the patients, demonstrating endothelial dysfunction and impaired systemic NO bioavailability in these patients despite optimal medical therapy including statins.

In vitro assays showed that in CAD both unstimulated random CAC movement and chemotaxis toward VEGF (5±3 and 7±4 cells/high-power field, respectively) were significantly impaired compared with healthy subjects (11±2 and 26±4 cells/high-power field, each $P<0.001$; Figure 5). Importantly, eNOS levels were also significantly reduced (below the detection limit of the assay) in older CAD patients compared with healthy volunteers, offering a feasible explanation for the decreased functional capacity of these patients' CACs. SNAP added to both chambers at 0 to 1 μmol/L led to a similar dose-dependent chemokinetic migratory response in both groups and enhanced chemotaxis to VEGF (healthy n=10, CAD n=5, because of insufficient number of cells from 5 of the patients). In the presence of SNAP at >0.1 μmol/L, the migratory response was not significantly different between healthy and CAD patients. Whereas baseline intracellular cGMP levels were significantly lower in CAD, there was no significant difference between healthy and CAD after incubation with SNAP (1 μmol/L). No significant differences were seen in CD45 (98±2%, 97±1%) and CD31 (26±4%, 34±9%) expression by fluorescence-activated cell sorting, and inducible NOS mRNA was not detected in either kind of cell (data not shown). This suggests that response to exogenous NO was preserved in CAD patients despite a reduction in NOS-dependent response to endogenous NO.

Discussion

Our data show that both endogenous NOS activity and exogenous NO modulate CAC motility. NOS activity is required for chemotactic migration of CACs to angiogenic chemokines, whereas exogenous NO induces chemokinesis, enhancing directional chemotaxis toward VEGF, without directly acting as a chemoattractant itself. Notably, the effects of the NO donor SNAP and NOS on the CACs were qualitatively similar to their effects on HUVECs, despite the presumption that these early proangiogenic CACs do not function as direct endothelial precursors. We show clinical relevance in that CAC migration in CAD patients is limited by decreased endogenous NOS activity because of impaired expression rather than impaired response to exogenous NO.

NOS plays an important regulatory role in vascular biology, and defective endothelial NO synthesis may limit angiogenesis in patients with endothelial dysfunction.[27] An impairment of the endogenous NO signaling in endothelium is coupled with the inability to produce an angiogenic

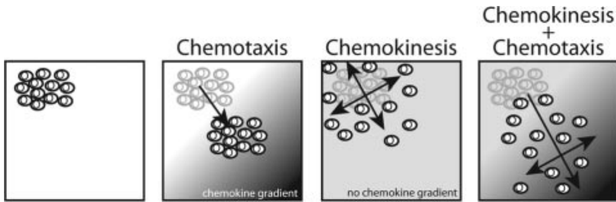

Figure 6. Schematic to illustrate the proposed additive effects of directional (chemotaxis) and nondirectional (chemokinesis) random cell movement.

response to VEGF.[28,29] The effects of NO on CACs, which are important cells for endothelial repair, are not well understood. We describe here how the presence or absence of NO affects CAC motility. Corroborating previous studies,[17,23,30] we demonstrate that CACs NOS-dependently migrate to a number of chemokines. This supports the notion that NOS represents an integral pathway for cell migration. We have recently shown that CACs migrate to a gradient of PTN in a manner that is dependent on NOS, cGMP, NO, and PI3K.[23] Similarly, it was previously shown by others that SDF-1α induces CAC migration in an eNOS-, Akt-, and PI3K -dependent manner.[30] We show here that the migration to VEGF involves the same pathways as migration to PTN and SDF-1α. This is important because a number of risk factors promoting arteriosclerosis and poor tissue regeneration, including smoking, aging, diabetes, hypertension, and hypercholesterolemia, have been shown to also inhibit NO production.[2,17] These factors may mediate part of their vascular pathology by affecting vascular maintenance exerted by lowered NOS activity potentially via oxidative stress not only in endothelial cells but also in CACs, leading to dysfunction of these cells. This is supported by several studies in animal models and human clinical studies. In a recent clinical report, we have shown that passive smoke may decrease CAC migration by blocking NO production.[17] Animal hindlimb ischemia experiments have revealed that angiogenesis is impaired in eNOS$^{-/-}$ mice and that the eNOS substrate L-arginine can enhance angiogenesis in rabbits.[27] Another study suggests that diabetes may impair reendothelialization by impaired CAC function due to decreased eNOS expression.[31] More recently, it was shown in diabetic rats and patients that diabetes may impair CAC functions by uncoupling eNOS.[15] Taken together, our data suggest that dysfunctional CAC migration in CAD patients may be due to lower eNOS expression rather than impaired response to exogenous NO in CACs.

To our knowledge, this is the first report to show the effect of exogenous NO on CAC migratory function. We show that an NO donor induces chemokinesis. It is important to note that this does not impair the CACs' capacity to sense chemoattractant gradients and follow them, but actually significantly also increases the net number of migrated cells at the site of higher chemokine concentration (Figure 6). This is in agreement with previously published results by others showing that HUVECs cGMP-dependently migrate toward a gradient of NO using different NO donors, 1,1-diethyl-2-hydroxy-2-nitroso-hydrazine and 2,2′-(Hydroxynitrosohydrazono)bis-ethanimine, which have a longer half-life than SNAP.[32] To methodically exclude the possibility that SNAP merely increases cell proliferation at the lower side of the membrane, we performed proliferation assays showing that NO in fact decreases proliferation.[33] Corroborating results by others, we show that SNAP also decreased apoptosis.[34] In the context of the present study, we cannot exclude the possibility that NO increases survival of CACs and may thereby explain part of the migration results, potentially contributing to more cells recovered at the lower side of the membrane. Mechanistically, both CACs and HUVECs release NO, and chemotaxis of both cell types is enhanced by NO-related chemokinesis. This suggests that NO may serve as a signal coordinating and potentially stimulating endothelial and proangiogenic cell interactions. NO and chemokines released by proangiogenic CACs that have homed to sites of injury may further attract new cells in a positive feedback loop by facilitating chemotaxis and chemokinesis. Once cells reach each other, higher NO levels may enhance adhesion (Heiss et al, unpublished results, 2007) and inhibit proliferation and apoptosis while facilitating even dispersal via chemokinesis of CACs and endothelial cells. As the observed effects were dose dependent, the effect may likely differ between sites with different NO levels, such as inflammation with expression of high-output inducible NOS (micromolar range) or vascular endothelium (low nanomolar range). Furthermore, these results may have clinical importance in disease states with lowered NO bioavailability, eg, decreased levels of plasma *S*-nitrosothiols or nitrite, which represent physiological NO donors with cardiovascular risk factors.[2,24,35,36]

Our data further support the concept that the NOS/NO pathway is a strong modulator of CAC functions, as it is in endothelial cells. CAC functions are likely to be affected both by factors that impair this pathway of endothelial cells in patients with cardiovascular disease in vivo and by reduced NO bioavailability.[2]

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Disclosures

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Nitric Oxide Synthase Expression and Functional Response to Nitric Oxide Are Both Important Modulators of Circulating Angiogenic Cell Response to Angiogenic Stimuli

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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-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

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