Published in final edited form as:

Nat Clin Pract Cardiovasc Med. 2008 August ; 5(8): 489–496.

Circulating CD34+ Cell Subsets in Patients with Coronary Endothelial Dysfunction

Barry A. Boilson, MB, BCh, MRCPI [Research Fellow], Thomas J. Kiernan, MB, BCh, MRCPI [Research Fellow], Adriana Harbuzariu, MD [Research Fellow], Rebecca E. Nelson, RN [Study Coordinator], Amir Lerman, MD [Professor of Medicine], and Robert D. Simari, MD [Professor of Medicine]

Division of Cardiovascular Diseases, Mayo Clinic, Rochester, Minnesota, 55905

Abstract

Introduction—Circulating cells that express CD34 including hematopoietic progenitors and endothelial progenitor cells may have a role in the development and progression of atherosclerosis. Endothelial dysfunction is an early manifestation of atherosclerotic disease. The aim of this study was to evaluate the association between coronary endothelial dysfunction (CED) and circulating CD34+ subsets.

Methods—The presence of CED was determined by the response to intracoronary acetylcholine in 57 consecutive patients having undergone diagnostic coronary angiography and without significant obstructive lesions. Mononuclear cells were extracted from blood, analyzed by flow cytometry, and cultured for functional analysis (CFUs). CD34 subsets were compared between patients with and without CED.

Results—Patients determined to have CED demonstrated a significant decrease in the number of circulating CD34+ CD45_{dim} VEGFR2[−] and CD34+ CD45_{dim} CD133+ VEGFR2[−] cells and in the total CFU count, but not in CD34+ CD45[−] VEGFR2⁺, compared to patients with normal coronary endothelial function.

Conclusions—This is the first study to demonstrate a differential regulation of CD34 subsets with CED. These data suggest that changes in specific subsets of circulating progenitor cells may be an early manifestation of atherosclerosis.

Keywords

circulating progenitor cells; coronary disease; endothelial function

Introduction

There exists a dynamic interface between circulating blood elements and the vascular wall. The importance of this interface may be accentuated in the presence of chronically diseased vessels or following acute vascular injury. The presence of circulating endothelial cells¹ as well as cells capable of assuming an endothelial phenotype has been supported by modern studies of human and animal chimeras.^{2, 3} These studies and many others support the concept

Address for correspondence: Robert D. Simari, MD, Professor of Medicine, Mayo Clinic College of Medicine, Division of Cardiovascular Diseases, 200 First Street SW, Rochester, Minnesota, 55905, USA, Fax: 507-538-6418, Phone: 507-284-3727, E-mail: simari.robert@mayo.edu.

Disclosures:

None.

of the circulating “endothelial progenitor cell” (EPC) and its therapeutic and diagnostic potential in the setting of vascular disease. It has been hypothesized that the EPC and the hematopoietic progenitor cell (HPC) have a common precursor, the hemangioblast,^{4–6} and both may be subsets of bone marrow-derived progenitor cells expressing CD34.

Endothelial dysfunction is an early manifestation of vascular disease and is a marker of atherosclerotic risk burden.⁷ Moreover, coronary endothelial dysfunction (CED) in the absence of obstructive coronary disease has been shown to be associated with increased cardiac events^{8–10} and may be a manifestation of systemic vascular injury or impaired vascular repair. Previous studies have shown decreased numbers of EPCs both in established coronary disease¹¹ and in patients with increased burden of cardiovascular risk.^{12–14} Furthermore decreased levels of EPCs are associated with increased cardiovascular risk.^{13, 14} Decreases have also been shown in patients with peripheral endothelial dysfunction and more recently in patients with coronary endothelial dysfunction in the setting of established coronary artery disease.^{15, 16} However, the concept of reduced circulating EPCs in both early and overt vascular disease has been also challenged by controversy regarding the definition of EPCs^{17, 18} and recent work which has demonstrated increased EPC numbers in advanced coronary disease¹⁹. Recently conflicting evidence has also been presented in the setting of cardiac syndrome X as defined by the presence of chest pain, an abnormal functional test and normal coronary angiography, where reduced numbers of EPCs as colony forming units were observed in despite increased numbers of circulating EPCs detected by flow cytometry.^{20, 21} These conflicting data further highlight the controversy which exists regarding how circulating EPCs are defined and enumerated.²²

Internationally standardized techniques have rigorously defined circulating HPCs utilizing their expression of CD34. The ISHAGE criteria define a flow cytometric technique which uses a precise definition of circulating HPCs as CD34 positive cells which express the common leukocyte antigen CD45 at a reduced intensity compared to mature leukocytes and which backscatter to the lymphocyte region.²³ A subset of these cells also expresses CD133. Unfortunately, the field of vascular biology lacks a standardized set of criteria for the enumeration of circulating EPCs as there has been no uniformly accepted definition to date. Furthermore, short-term culture of peripheral blood mononuclear cells generates spindle shaped cells originally referred to as EPCs. More recently, these cells have been demonstrated to represent cells of myelomonocytic origin and should be considered as progeny of HPCs.^{24, 25}

Recent evidence has defined that circulating EPCs (circulating cells capable of generating outgrowth endothelial cells in culture) differ from HPCs in CD45 expression – it is absent on EPCs, whereas dim CD45 expression is detectable on HPCs.²⁶ Additionally, EPCs express VEGFR2 which is not expressed by HPCs.^{26, 27} Thus, the current study was designed to test the hypothesis that CED without significant coronary obstruction in humans is associated with an alteration in circulating CD34+ cell subsets, which may include cells involved directly or indirectly in vascular repair.

Methods

Patients

This study was approved by the Mayo Clinic Institutional Review Board and informed written consent was obtained from all patients in advance of cardiac catheterization. Consecutive patients undergoing diagnostic coronary angiography for evaluation of coronary artery disease were eligible for inclusion in the study if they had no significant epicardial coronary artery stenoses (stenosis <30%) and had been willing to sign informed consent. Patients with hematological malignancy, left ventricular ejection fraction <50%, significant renal, hepatic,

and endocrine disease and those with ongoing infection were excluded. Nitrates and calcium channel blockers were withheld for 48 hours prior to the study.

Five ml of whole blood was obtained from the first cohort of 38 patients at the time of cardiac catheterization and analyzed by flow cytometry for expression of CD14, CD34, CD133, CD45, and VEGFR2. In order to refine the detection of CD133 positive cells and to enumerate colony forming units in vitro, a further group of 19 patients had 50 ml of whole blood drawn at the time of cardiac catheterization, and buffy coat was extracted using density gradient centrifugation (Ficoll). This was subjected to flow cytometric analysis and plated on fibronectin coated wells. Analysis of whole blood samples was not performed on the latter group. Colony forming units were counted at 7 days.

Intracoronary acetylcholine challenge testing

Intracoronary acetylcholine challenge testing was performed as described previously and outlined briefly below.^{8,9} Coronary artery diameter was measured off-line by an independent investigator in three segments, proximal, mid, and distal, using a quantitative coronary angiography program (Medis Imaging Systems, Leiden, the Netherlands) as was previously described.²⁸ CBF was calculated from the Doppler-derived time velocity integral and vessel diameter as: $\pi \times (\text{coronary artery diameter}/2)^2 \times (\text{APV}/2)$.⁸ According to previous studies at our center, we defined coronary epicardial endothelial dysfunction as a decrease in diameter >20% in response to the maximal dose of acetylcholine. Microvascular endothelial dysfunction was defined as $\geq 50\%$ increase in CBF in response to the maximal dose of acetylcholine compared to baseline CBF.^{8,9,29} As in the prior studies, the syndrome of coronary endothelial dysfunction (CED) was defined as the presence of microvascular and/or epicardial coronary endothelial dysfunction.

Flow Cytometry

Whole Blood—Five ml of whole blood was obtained from each patient at the time of cardiac catheterization and analysed by flow cytometry for expression of CD14, CD34, CD133, CD45, and VEGFR2. Eight 100 μL aliquots of whole blood were incubated with the following fluorochrome conjugated antibodies: CD34-FITC (BD Biosciences), mouse anti-human antibody CD133 (Miltenyi-Biotec) conjugated to rat anti-mouse IgG1-PerCP (BD Biosciences), CD45-APC (BD Biosciences), VEGFR2-PE (R&D Biosystems), and CD14-PECy7 (BD Biosciences). Murine IgG₁ (R&D Systems) conjugated to Alexa 488, PE (Molecular Probes), and Rat anti-mouse PerCP (BD Biosciences) was used as isotype controls as well as IgG₁-APC and IgG₁-PECy7 from BD Biosciences. Red cells were lysed by addition of 1X H-Lyse solution (R&D Biosystems) and cells were washed twice with phosphate buffered saline.

Buffy Coat—An additional group of patients had 50 ml of whole blood drawn at the time of cardiac catheterization, and buffy coat was extracted using density gradient centrifugation (Ficoll). Of 50 ml of whole blood drawn at the time of cardiac catheterization, 25 ml was decanted over an equal volume of Ficoll-PaqueTM (Amersham Biosciences, Uppsala, Sweden) and centrifuged for 25 minutes at 1600 RPM without brake as previously described.²⁴ Buffy coat was extracted and washed twice. Cells were counted and re-suspended in seven 100 μL aliquots of PBS for FACS analysis, each containing approximately 10^6 cells. After addition of Fc receptor blocking antibody (Miltenyi Biotec) to each tube, cells were incubated with fluorochrome-conjugated antibodies to CD34-FITC, CD45-APC, CD133-PE (Miltenyi Biotec), and a biotinylated goat anti-human VEGFR2 antibody (R&D Systems), subsequently labeled with Streptavidin-PerCP (BD Biosciences). Murine IgG₁ (R&D Systems) conjugated to Alexa 488, PE (Molecular Probes), and Rat anti-mouse PerCP (BD Biosciences) was used as isotype controls as well as IgG₁-APC from BD Biosciences.

CD34⁺ cells were analyzed using a sequential gating strategies to enumerate CD45_{dim} (ISHAGE criteria – figure 1) and CD45[–] cell subsets (figure 2) and 60,000 events were analyzed from each tube. The equipment used was a FACS Canto benchtop analyzer (BD, Franklin Lakes, NJ, USA) and then the files were analyzed using WinMDI version 2.8 software (The Scripps Research Institute, La Jolla, CA, USA). Standardized cell counts are presented as a percentage of total leukocytes, which were identified as the total number of all CD45⁺ cells (CD45_{dim} and CD45^{bright}) in the sample.

The ISHAGE criteria: The ISHAGE criteria are a single platform sequential gating analysis technique for flow cytometric evaluation of hematopoietic progenitor cells (HPCs) devised by Sutherland et al in 1996.²³ This technique was subsequently adopted as the standard HPC enumeration protocol by the International Society for Hematotherapy and Graft Engineering (ISHAGE) in 1999.

In essence, hematopoietic progenitor cells must fulfill specific criteria i.e. positive staining for a hematopoietic stem cell marker (CD34 $\pm$ CD133), CD45_{dim} positive staining and specific light scatter characteristics (low side scatter and intermediate to high forward scatter). These criteria are equally applicable to CD34 and CD133 positive cells. The application of ISHAGE criteria is described in detail in figure 1.

Cell culture and enumeration of colony forming units (CFUs)

As described above, buffy coat was extracted from 25 ml whole blood and washed twice. Cells were counted and plated on 6 fibronectin coated wells in a 12 well plate at a density of 2 to 3×10^6 cells per well and incubated at 37°C in EGM2 growth medium (Cambrex, Walkersville, MD, USA). After 48 hours, unattached cells were re-plated onto 6 new fibronectin coated wells.¹³ Media was changed every 2 days and colonies counted at day 7.

Statistical methods

All data were analyzed using JMP version 6.0 (SAS Institute, Inc., Cary, NC, USA). Demographic data were compared between groups using the Student t-test for continuous variables and the Fisher's exact test for categorical variables. Cell counts were not normally distributed (skewed to the left) and therefore they were compared between groups using the Wilcoxon test. Data are expressed as median {25%, 75%} for continuous variables. Using a Bonferroni correction for multiple comparisons, differences in cell counts were only considered statistically significant at $p < 0.01$ and $p < 0.05$ for other analyses.

Results

Thirty-seven patients of the total population of 57 patients were found to have CED as assessed by intracoronary acetylcholine administration. The fifty seven patients were studied in two cohorts. In the initial cohort of 38 patients, a significant decrease in the CD34⁺CD45_{dim} counts standardized by % leukocytes in whole blood was noted among patients with abnormal response to acetylcholine (CED) (median 0.05%, {25% percentile 0.03%, 75% percentile 0.07%} vs 0.02% {0.01%, 0.03%} respectively in whole blood, $p = 0.003$). (Figure 3) However, standardized CD34⁺CD45[–] cell counts did not differ between the groups (0.009% {0.005%, 0.02%} vs. 0.003% {0.002%, 0.02%} in whole blood in normal and CED patients respectively, $p = 0.41$). In addition, no differences were seen in CD14⁺ cells between groups. These data suggest that specific CD34 subsets may be decreased in patients with CED.

Utilization of ISHAGE criteria did not allow for enumeration of CD133 cells in whole blood due to excessive non-specific binding of antibody to granulocytes, lysed red cells, platelets and debris. In order to increase the sensitivity of flow cytometric analysis and to provide cells

for *in vitro* CFU analysis, additional studies were performed using buffy coat as a substrate. When buffy coat was used, a subset ($61.1 \pm 9.1\%$) of CD34+ CD45_{dim} cells were identified which were also positive for CD133, which is in keeping with the findings of previous investigators.^{30,31} A significant decrease in the standardized CD34+ CD45_{dim} counts ($0.14\% \{0.13\%, 0.21\% \}$ vs $0.08\% \{0.07\%, 0.1\% \}$, $p=0.005$) and CD34+ CD45_{dim} CD133+ counts ($0.11\% \{0.07\%, 0.13\% \}$ vs $0.04\% \{0.03\%, 0.07\% \}$, $p=0.006$) was noted among patients with an abnormal response to acetylcholine (CED). (Figure 4). VEGFR2 expression was not detected on CD34+ CD45_{dim} or CD34+ CD133+ CD45_{dim} cells enumerated using ISHAGE criteria but could be detected on CD34+ CD45_{dim} cells. In distinction to differences found in standardized CD34+ CD45_{dim} cell numbers, no differences were detected in standardized CD34+ CD45_{dim} cell counts ($0.0008\% \{0\%, 0.002\% \}$ vs $0.001\% \{0.00009\%, 0.002\% \}$, $p=0.90$) between the two groups.

The demographic data of the study population are outlined in Table 1. Notably, there was no significant difference detected in differential leukocyte count between patients with and without CED. No significant differences were observed between patient groups in terms of age, medications, or cardiovascular risk factors with the exception of gender ($p=0.02$) and the serum C-reactive protein (CRP) level ($p=0.02$). Neither gender nor CRP level correlated with either CD34+CD45_{dim} or CD34+CD45_{dim} counts.

In vitro studies of colony formation have been shown to be a manifestation of the hematopoietic nature of these cells.^{24–26} The total number of colony forming units (CFUs) counted at 7 days from cells derived from normal patients showed a significant decrease compared to those with CED ($11 \{5, 23\}$ vs $4 \{2, 6\}$ CFUs per well, $p=0.02$). (Figure 5). Taken together, these data suggest quantitative differences exist among CD34+ CD45_{dim} VEGFR2_{dim} cells and their progeny (which may include CFU forming cells) but not among CD34+ CD45_{dim} VEGFR2_{dim} cells in patients with CED compared to normals.

Discussion

The current study demonstrates for the first time that specific subsets of circulating CD34+ cells are decreased in patients with coronary endothelial dysfunction and early coronary atherosclerosis. The data support an association between CD34+ CD45_{dim} VEGFR2_{dim} cells and CED in a population without significant obstructive disease. Furthermore, a decrease in CFUs (derived from HPCs) was also seen. However, no relationship was seen with CD34+, CD45_{dim} VEGFR2_{dim} cells when they were rigidly defined and quantified. Taken together, these data support the concept that even in early atherosclerosis there are decreases in specific circulating subsets of CD34 cells.

In spite of the current findings, the relevance of these CD34+ subsets remains somewhat controversial. Endothelial progenitor cells (EPCs) have previously been defined using two distinct methods for their characterization - flow cytometry and cell culture, but the validity of both definitions has recently been questioned. The flow cytometric definition has centered on the expression of CD34 on circulating cells since the landmark publication by Asahara in 1997 which characterized blood-derived cells which form endothelial cells in culture as being derived from cells expressing this antigen.³² In studies which have used flow cytometry to enumerate these cells, most have quantified cells that express CD34 and VEGFR2 or KDR, and a minority have also included expression of CD133.^{5, 11–15, 20, 33, 34}

An alternative definition of EPCs described colonies of spindle shaped cells appearing after culture of blood derived mononuclear cells for 7 days.^{12, 13, 19} These cells have been termed EPCs owing to the appearance of some endothelial characteristics in culture. However, evidence has accumulated that these spindle-shaped cell appearing early in culture are in fact

of hematopoietic lineage and do not represent true endothelial progenitor cells.^{24, 25, 35} These cells have now been referred to as circulating angiogenic cells (CACs) or culture modified mononuclear cells (CMMCs) by these groups.

Most studies to date which have used flow cytometry to characterize and enumerate circulating CD34⁺ progenitors have not described expression of the common leukocyte marker CD45 on these cells. Recent data has shown that this information is critical, as it likely defines two distinct circulating subpopulations that may be detected – those CD34⁺ cells of hematopoietic lineage and differentiation potential, and on the other hand, those whose differentiation potential is distinctly endothelial.^{26, 36} These groups demonstrated that CD34⁺ cells from bone marrow, umbilical cord blood, and mobilized peripheral blood have different differentiation potential depending on the presence or absence of CD45 expression on these cells. CD34⁺ cells which express CD45 do not form true endothelial cells in culture, but do form colonies of spindle-shaped cells at 7 days which express a mixture of hematopoietic and endothelial surface markers. These early spindle-shaped cells are in keeping with the more current definition in use which describes these cells as CACs or CMMCs rather than EPCs, as these cells are essentially hematopoietic in origin and differentiation potential. On the other hand, CD34⁺ cells which do not express CD45 do not form early spindle-shaped cells in culture (CACs/CMMCs) but after more prolonged culture give rise to sheets of cells of cobblestone morphology which are distinctly endothelial (outgrowth endothelial cells, OECs). Therefore, CD34⁺ cells which do not express CD45 by flow cytometry may include cells capable of developing *bona fide* endothelial cells.

Our work has extended these observations further by studying patients with chest pain and coronary endothelial dysfunction. CD34 expressing cells in peripheral blood were analyzed based on their CD45 expression. Those CD34⁺ cells which expressed CD45 were in the majority and the expression of CD45 on these cells was in a very narrow dim fluorescence range (i.e. CD34⁺ CD34_{dim}), in keeping with previous standardized definitions of circulating hematopoietic progenitor cells.²³ A minority of circulating CD34⁺ cells did not express CD45 but did express VEGFR2. Only those circulating CD34⁺ cells which co-expressed CD45 (but not VEGFR2) were found to be significantly reduced in number in patients with coronary endothelial dysfunction. This could not be shown for CD34⁺ cells which did not express CD45. When early colony forming units were studied in this patient cohort, a similar correlation was found with presence of coronary endothelial dysfunction as with CD34⁺ CD45_{dim} cells. This likely reflects the hematopoietic origin of these cells as recently described by others.^{24–26, 35}

The findings of this study support the hypothesis that endothelial dysfunction and reduced circulating cell subsets are integrally related. Unlike the recent work of Werner and colleagues which demonstrated an association between CED and CD34⁺/KDR⁺ cells in later stages of coronary artery disease, these data support this association is present at a much earlier stage¹⁶. The reduced numbers of circulating cells, which has been shown in various settings of vascular disease and elevated risk, and now extended to patients with coronary endothelial dysfunction may reflect homing and seeding of these cells in sites of vascular disease and repair. The presence of increased progenitor cells in atherosclerotic plaques has been demonstrated by Torsney in human vascular tissue obtained at the time of coronary bypass surgery.³⁷ The role of bone marrow derived cells in sites of endothelial disease has yet to be elucidated, but there is growing evidence that it may include paracrine effects.^{38, 39} Indeed, there is increasing evidence to suggest that vascular progenitors may be resident within the vessel wall itself⁴⁰ which may be quiescent unless triggered by a cytokine or growth factor signal perhaps from a circulating cell of bone marrow origin. Alternatively, the reduced number of cells found in this study may reflect impaired production from bone marrow as a systemic

manifestation of endothelial dysfunction. Additional studies will be required to determine the basis for this reduction.

Limitations

Any study of rare peripheral blood cells risks nonspecificity and the inability to discriminate between signal and noise. Quantification of rare CD34⁺ CD45[−] cells represents such a challenge. The low frequency of these rare cells renders their differentiation from cells which have bound antibodies non-specifically and from background autofluorescence difficult. Alternative methods to detect such rare cells will be required to overcome these inherent potential limits of flow cytometry. Additionally, the size of the population studied prevents further detailed analysis of covariates that might generate hypotheses concerning the mechanism of this associated decrease in CD34⁺ subsets.

Conclusions

A reduction in circulating CD34⁺CD45^{dim} VEGFR2[−] and CD34⁺CD133⁺CD45^{dim} VEGFR2[−] subsets was found in patients with coronary endothelial dysfunction and early atherosclerosis. Similar decreases were not shown for the CD34⁺ CD45[−] VEGFR2⁺ subset. Demonstration of this differential regulation required utilization of rigorous international standards of analysis. These results support the hypothesis that decreases in specific circulating CD34⁺ subsets are an early manifestation of atherosclerosis and is associated with coronary endothelial dysfunction. Additionally, this study highlights the need for rigid definitions of circulating subsets if the use of cellular biomarkers is to be advanced in cardiovascular disease.

Acknowledgements

Special thanks to James E. Tarara and staff at the Mayo Clinic Flow Cytometry Core Facility personnel for their assistance with processing the samples, to Tanya Hoskin and Darrell Schroeder for biostatistical advice, and to Megan E. Crouch for her secretarial support.

Funding Sources:

This study was funded by the National Institutes of Health (HL76611, 75566) and the Minnesota Partnership for Biotechnology and Medical Genomics.

References

1. Stump MM, et al. Endothelium grown from circulating blood on isolated intravascular dacron hub. *Am J Pathol* 1963;43:361–363. [PubMed: 14057632]
2. Gunsilius E, et al. Evidence from a leukaemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. *Lancet* 2000;355:1688–1691. [PubMed: 10905245]
3. Werner N, et al. Bone marrow-derived progenitor cells modulate vascular reendothelialization and neointimal formation: Effect of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibition. *Arterioscler Thromb Vasc Biol* 2002;22:1567–1572. [PubMed: 12377731]
4. Sabin FR. Preliminary note on the differentiation of angioblasts and the method by which they produce blood-vessels, blood-plasma and red blood-cells as seen in the living chick. 1917. *J Hematother Stem Cell Res* 2002;11:5–7. [PubMed: 11846999]
5. Peichev M, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood* 2000;95:952–958. [PubMed: 10648408]
6. Zambidis ET, et al. Emergence of human angiohematopoietic cells in normal development and from cultured embryonic stem cells. *Ann N Y Acad Sci* 2007;1106:223–232. [PubMed: 17360801]
7. Bonetti PO, et al. Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol* 2003;23:168–75. [PubMed: 12588755]
8. Suwaidi JA, et al. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. *Circulation* 2000;101:948–954. [PubMed: 10704159]

9. Hasdai D, et al. Coronary endothelial dysfunction in humans is associated with myocardial perfusion defects. *Circulation* 1997;96:3390–3395. [PubMed: 9396432]
10. Kunz GA, et al. Circulating endothelial progenitor cells predict coronary artery disease severity. *Am Heart J* 2006;152:190–195. [PubMed: 16824855]
11. Werner N, et al. Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med* 2005;353:999–1007. [PubMed: 16148285]
12. Vasa M, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001;89:E1–7. [PubMed: 11440984]
13. Hill JM, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003;348:593–600. [PubMed: 12584367]
14. Schmidt-Lucke C, et al. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. *Circulation* 2005;111:2981–2987. [PubMed: 15927972]
15. Murphy C, et al. Vascular dysfunction and reduced circulating endothelial progenitor cells in young healthy UK South Asian men. *Arterioscler Thromb Vasc Biol* 2007;27:936–942. [PubMed: 17255539]
16. Werner N, et al. Endothelial progenitor cells correlate with endothelial function in patients with coronary artery disease. *Basic Res Cardiol* 2007;102:565–571. [PubMed: 17932708]
17. Ingram DA, et al. Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells. *Blood* 2005;106:1525–1531. [PubMed: 15905185]
18. Doyle B, et al. Endothelial progenitor cells. *Endothelium* 2006;13:403–410. [PubMed: 17169772]
19. Guven H, et al. The number of endothelial progenitor cell colonies in the blood is increased in patients with angiographically significant coronary artery disease. *J Am Coll Cardiol* 2006;48:1579–1587. [PubMed: 17045891]
20. Shmilovich H, et al. Circulating endothelial progenitor cells in patients with cardiac syndrome X. *Heart* 2007;93:1071–1076. [PubMed: 17194713]
21. Huang P, et al. Vascular endothelial function and circulating endothelial progenitor cells in patients with cardiac syndrome. *Heart* 2007;93:1064–1070. [PubMed: 17488770]
22. Leor J, Marber M. Endothelial progenitors: a new Tower of Babel? *J Am Coll Cardiol* 2006;48:1588–1590. [PubMed: 17045892]
23. Sutherland DR, et al. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. *J Hematother* 1996;5:213–226. [PubMed: 8817388]
24. Gulati R, et al. Diverse origin and function of cells with endothelial phenotype obtained from adult human blood. *Circ Res* 2003;93:1023–1025. [PubMed: 14605020]
25. Yoder MC, et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood* 2007;109:1801–1809. [PubMed: 17053059]
26. Timmermans F, et al. Endothelial outgrowth cells are not derived from CD133+ cells or CD45+ hematopoietic precursors. *Arterioscler Thromb Vasc Biol* 2007;27:1572–1579. [PubMed: 17495235]
27. Duda DG, et al. A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. *Nat Protoc* 2007;2:805–810. [PubMed: 17446880]
28. Al Suwaidi J, et al. Measuring maximal percent area stenosis poststent placement with intracoronary Doppler and the continuity equation and correlation with intracoronary ultrasound and angiography. *Am J Cardiol* 1999;84:650–654.
29. Yang EH, et al. Lipoprotein-associated phospholipase A2 is an independent marker for coronary endothelial dysfunction in humans. *Arterioscler Thromb Vasc Biol* 2006;26:106–111. [PubMed: 16239595]
30. Yin AH, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* 1997;90:5002–5012. [PubMed: 9389720]
31. de Wynter EA, et al. CD34+AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell progenitors. *Stem Cells* 1998;16:387–396. [PubMed: 9831864]

32. Asahara T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;275:964–967. [PubMed: 9020076]
33. Heiss C, et al. Impaired progenitor cell activity in age-related endothelial dysfunction. *J Am Coll Cardiol* 2005;45:1441–1448. [PubMed: 15862416]
34. Friedrich EB, et al. CD34-/CD133+/VEGFR-2+ endothelial progenitor cell subpopulation with potent vasoregenerative capacities. *Circ Res* 2006;98:e20–25. [PubMed: 16439688]
35. Lin Y, et al. Origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest* 2000;105:71–77. [PubMed: 10619863]
36. Case J, et al. Human CD34+AC133+VEGFR-2+ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors. *Exp Hematol* 2007;35:1109–1118. [PubMed: 17588480]
37. Torsney E, et al. Characterisation of progenitor cells in human atherosclerotic vessels. *Atherosclerosis* 2007;191:259–264. [PubMed: 16787646]
38. Urbich C, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. *J Mol Cell Cardiol* 2005;39:733–742. [PubMed: 16199052]
39. Sharpe EE 3rd, et al. The origin and in vivo significance of murine and human culture-expanded endothelial progenitor cells. *Am J Pathol* 2006;168:1710–1721. [PubMed: 16651636]
40. Zengin E, et al. Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. *Development* 2006;133:1543–1551. [PubMed: 16524930]

Figure 1. PC counts (ISHAGE criteria)

CD45+ cells are gated in R1 (**A**) and CD34+ in R2 (**B**). CD45_{dim} cells from R1×R2 are gated R3 (**C**). R4 defines the lymphocyte population (**D**), displayed by scatter (**E**) and used to define phenotypic limits for HPCs in R5. R1×R2×R3 are also displayed by scatter (**F**) and cells falling within R5 are counted as CD34+ CD45_{dim} HPCs.

Figure 2. EPC counts – enumeration strategy

Quadrants are set using isotype controls (A). CD34⁺, CD45⁻ events are gated R1 (B). CD34⁺ + VEGFR2⁺ cells from R1 are gated in R2 (C). In D, cells R1 × R2 are displayed by scatter demonstrating low side and forward scatter characteristics of these cells.

Figure 3.

Numbers of CD34+, CD45_{dim} VEGFR2- counted by flow cytometry from whole blood using the ISHAGE criteria and CD34+, CD45-, VEGFR2+ cells across the normal and CED groups. The cell counts are displayed as a percentage of total CD45+ cells (leukocytes) in the sample, as described in the methods. (n=38)

Figure 4.

Numbers of CD34+, VEGFR2-, CD45_{dim} and CD34, CD133+, CD45_{dim} VEGFR2- cells counted by flow cytometry from buffy coat using the ISHAGE criteria and CD34+, VEGFR2+, CD45- cells across the normal and CED groups. The cell counts are displayed as a percentage of total CD45+ cells (leukocytes) in the sample. (n=19)

Figure 5.

A. Typical colony forming unit (CFU) at day 7 in culture and 100× magnification showing a nidus of round cells surrounded by spindle shaped cells. **B.** Total numbers of CFUs counted at 7 days (n=19) across the normal and CED groups.

Table 1

Patient Characteristics	All subjects (n=57)	Normal Coronary Endothelial Function (n=20)	Coronary Endothelial Dysfunction (n=37)	p
Age (years)	45.8±1.5	45.9±2.1	45.8±2.0	0.75
Gender - no (%)				* 0.02
Female	39 (68)	18 (90)	21 (57)	
Male	18 (32)	2 (10)	16 (43)	
BMI (kg/m ²)	28.4±0.8	26.7±1.4	29.4±1.0	0.10
Diabetes - no (%)	4 (7)	1 (5)	3 (8)	0.83
Hypertension - no (%)	19 (33)	7 (35)	12 (32)	0.54
Mean arterial pressure (mmHg)	95.7±1.6	95.6±3.0	95.8±2.0	0.60
Smoking status - no (%)				
Current	6 (11)	2 (10)	4 (11)	0.70
Never	31 (54)	13 (65)	18 (49)	0.28
Medications – no (%)				
Aspirin	33 (58)	11 (55)	22 (59)	0.78
Beta blockers	21 (37)	9 (45)	12 (32)	0.40
ACE inhibitors	11 (19)	4 (20)	7 (19)	0.92
Statins	22 (39)	6 (30)	16 (43)	0.40
Nitrates	27 (47)	8 (40)	19 (51)	0.58
Calcium channel blockers	24 (42)	7 (35)	17 (46)	0.58
Oral anticoagulants	7 (12)	1 (5)	6 (16)	0.40
L-Arginine	2 (4)	1 (5)	1 (3)	0.66
Vitamin E	1 (2)	0 (0)	1 (3)	0.46
Laboratory values				
Total Cholesterol (mg/dL)	185.2±6.6	178.3±9.4	189.1±8.9	0.41
LDL (mg/dL)	104.0±5.7	97.4±7.9	107.8±7.6	0.48
HDL (mg/dL)	55.5±2.1	58.5±4.0	53.8±2.3	0.37
Triglycerides (mg/dL)	131.1±10.3	111.9±14.1	141.7±13.8	0.16
Lipoprotein a (mg/dL)	18.9±3.7	18.1±5.7	19.4±4.9	0.2
Serum CRP (mg/L)	1.9±0.3	1.2±0.4	2.3±0.4	* 0.02
Serum BNP (ng/ml)	24.1±2.6	24.7±4.9	23.7±3.1	0.90
Serum homocysteine (mmol/l)	8.4±1.2	7.1±0.4	9.0±1.7	0.66
Serum Insulin level µIU/ml)	5.0±0.4	4.4±0.8	5.3±0.5	0.21
L-Arginine level (mmol/l)	61.0±2.1	58.5±3.1	62.3±2.8	0.47
Left ventricular ejection fraction (%)	63.2±0.7	63.1±1.2	63.2±1.0	0.97
Serum creatinine (mg/dL)	1.00±0.02	0.95±0.03	1.02±0.03	0.21
Leukocyte count (cells*10 ³ /µL)	6.6±0.3	6.6±0.5	6.7±0.3	0.46
Neutrophils	3.8±0.2	3.8±0.3	3.8±0.3	0.96
Basophils	0.04±0.02	0.03±0.004	0.04±0.002	0.62
Eosinophils	0.17±0.02	0.18±0.03	0.16±0.02	0.93
Monocytes	0.47±0.02	0.48±0.02	0.46±0.02	0.51
Lymphocytes	2.4±0.4	1.9±0.2	2.6±0.7	0.39
Platelets (*10 ³ /µL)	262±6.7	262.7±15.0	261.6±6.6	0.65
Hematocrit (fl)	40.5±0.5	39.6±0.8	41.0±0.6	0.13