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Common Endothelial Progenitor Cell Assays Identify Discrete EPC Populations

Thomas J. Povsic, M.D., Ph.D.[#], Katherine L. Zavodni, B.A., Enrikas Vainorius, M.D.[#], Jennifer F. Kherani, B.S.[#], Pascal J. Goldschmidt-Clermont, M.D.[†], and Eric D. Peterson, M.D., M.P.H.^{*}

[#] Division of Cardiology, Duke University Medical Center, Durham, NC 27710

[†] Miller School of Medicine, University Of Miami, Miami, FL 33136

^{*} Duke Clinical Research Institute, Duke University Medical Center, Durham, NC 27710

Abstract

Background—Multiple measures of endothelial progenitor cells (EPCs) have been described, but there has been limited study of the comparability of these assays. We sought to determine the reproducibility of and correlation between alternative EPC assay methodologies.

Methods—We simultaneously assessed EPC numbers in 140 patients undergoing cardiac catheterization using the two most commonly used culture techniques: endothelial cell outgrowth and colony forming unit outgrowth (CFUs). In the final 77 patients, EPCs were also identified on the basis of cell surface marker expression (CD133, CD34, and VEGFR-2) and aldehyde dehydrogenase (ALDH) activity.

Results—EPC enumeration based on flow cytometry (FACS) was more precise than culture assays. There was limited correlation between EPC numbers determined using the two common culture based assays; however, endothelial CFUs correlated with VEGFR-2 and CD34/VEGFR-2 expressing cells. EPCs defined by expression of CD133, CD34, CD133/CD34, and ALDH activity correlated with each other, but not with VEGFR-2⁺ cells.

Conclusions—EPCs can be broadly classified into two classes: VEGFR-2 expressing cells which give rise to endothelial CFUs, and CD133/CD34 or ALDH^{br} cells. These observations underscore the need for better assay standardization and a more precise definition of EPCs in cell therapy research.

Introduction

The discovery of circulating cells capable of differentiating into endothelium and homing to sites of ischemia¹ has focused interest on cellular repair processes which might be harnessed to promote vascular healing. Considerable work has focused on the role the loss of vascular repair capability plays in the development and progression of atherosclerosis.

Corresponding Author: Thomas J. Povsic M.D. Ph.D., Department of Cardiology, Box 3126, Duke University Medical Center, Durham, NC 27710, Ph: 919-681-5821, Fax: 919-681-9607, Povsi001@mc.duke.edu.

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Central to this paradigm is the hypothesis that progenitor cell depletion is antecedent to clinically evident disease. Multiple groups have demonstrated an association between circulating EPC numbers and the presence of clinical risk factors in patients without evident coronary artery disease.² These studies were extended to patients with coronary disease,^{3, 4} and as predictors of future vascular events.^{5, 6}

Despite the immense interest in this field, there still is no standard definition for EPCs nor accepted methodology for their enumeration. Methods for enumeration include (a) outgrowth of colony forming units (CFUs) on fibronectin,² (b) growth of individual endothelial cells,⁴ and (c) FACS based enumeration of cells expressing either single or combinations of cell surface markers.^{5–8} The degree of discrepancy is highlighted by the fact that the two most widely published culture based protocols differ on the necessity for a pre-plating step, medium used (M-199 vs. EBM), time of culture (4 days vs. 9 days), and types of cells counted (CFUs vs. stained endothelial cells).^{2, 4} Assays based on cell surface marker expression vary in the markers used, with most studies employing a combination of CD133 (expressed on progenitor cells, but not mature cells), VEGFR2 (mature and immature endothelial cells), and CD34 (hematopoietic stem cells, immature and mature endothelial cells).⁹

We have recently proposed using a novel methodology for identifying EPCs based on aldehyde dehydrogenase (ALDH) activity,¹⁰ a property common to multiple progenitor cell types¹¹ which may be responsible for maintaining progenitor cell characteristics.¹²

To date, we are unaware of any systematic assessment of the reliability, temporal stability, or correlation of EPCs as defined by each of these assays. We addressed these issues by measuring EPCs in (a) simultaneously obtained duplicate blood samples and in (b) samples obtained 24 hours apart in healthy volunteers. To determine the extent to which these assays identify similar or differing EPC populations, we simultaneously identified EPCs using each assay in a large cohort of cardiac patients and compared EPC types with each other.

Methods

Patient enrollment

After consent and insertion of an arterial sheath, 30 cc of blood was collected in EDTA containing tubes and processed within 4 hours. Normal volunteers were recruited by advertisement and represented healthy patients without known medical conditions. This investigation conforms with the principles outlined in the Declaration of Helsinki.

Mononuclear Cell Isolation

Mononuclear cells (MNCs) were recovered by density centrifugation over Ficoll-Paque (Amersham). MNCs were isolated by centrifugation at 200g for 20 min., washed extensively and dispersed for EPC assays.

Culture Based Assays

EPCs were enumerated using the two most commonly reported culture based assays.^{2, 4} For the CFU assay, 5×10^6 MNCs were layered onto fibronectin coated 6-well plates and cultured in M199 medium supplemented with 20% fetal calf serum (FCS) for 48 hours, after which the supernatant was removed, and 1×10^6 cells replated into 24-well plates. EPC colonies were counted after an additional 7 days of culture.² To minimize the effect of alternate pre-plating strategies, we assessed EPC CFUs arising before and after re-plating.

To quantitate endothelial cell outgrowth, MNCs (4×10^6) were plated onto a fibronectin coated 4-chamber slide plate in EBM supplemented with SingleQuotes and 20% FCS (Cambrex

Walkersville, MD). After four days, cells were stained with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine-labeled acetylated LDL (DiLDL,) and FITC-labeled *Ulex europaeus* agglutinin I (lectin, Sigma), and double stained cells were counted.

Analysis of EPCs based on cell surface marker expression

Mononuclear fractions (4×10^6 cells) were incubated with FcR blocking reagent (Miltenyi Biotec) for 10 min, then incubated with CD133-APC (Miltenyi Biotec), CD34-FITC (Miltenyi Biotec) and VEGFR-2-PE for 60 min. at 4°. Dead and dying cells were excluded with 7-AAD (1 µg/ 10^6 cells, Molecular Probes). Isotype control antibodies were used to set baseline fluorescence levels.

Analysis of EPCs based on ALDH activity

MNCs were aliquoted into tubes and suspended in Aldefluor assay buffer. Freshly prepared and aliquoted BODIPY-aminoacetaldehyde (B-AAD, Aldagen Inc., Durham, NC) was added to the reaction tube (1 µM), and the cells incubated at 37° C for 30 min., after which cells were maintained on ice. Baseline activity levels were established based on control samples incubated with diethylaminobenzaldehyde (DEAB), a potent inhibitor of ALDH.

FACS Analysis

All flow cytometry was performed by trained technicians blinded to other EPC analyses. Compensation was performed daily. Analysis was performed by first gating lymphocytes and monocytes on the basis of light scattering properties, then enumerating the numbers of ALDH^{br} cells or cells expressing each marker in the appropriate channel.

Statistical analysis

Patient characteristics are reported as percentages unless otherwise stated. The median and interquartile ranges of EPCs are reported.

To assess assay precision, EPC numbers determined in independent experiments from duplicate samples were graphically plotted, and Pearson correlation coefficient determined. A similar analysis was performed for the temporal stability assays.

A D'Agostino and Pearson omnibus normality test was employed to determine normality of each EPC distribution. Correlation amongst EPC assays was determined using a Spearman correlation.

Results

Between October of 2004 and April of 2005, consent was obtained from 140 patients undergoing elective cardiac catheterization, with clinical characteristics delineated in Table 1.

EPC CFUs and endothelial cell outgrowth were successfully determined in each patient in the cohort. We additionally enumerated EPC numbers based on expression of cell surface markers CD133, CD34, and VEGFR-2 and on the basis of ALDH activity in the final 77 patients enrolled. Importantly, there were no differences between the patients in the complete cohort and the final 77 patients who had EPCs defined by all techniques (Table 1).

To ensure that CFUs observed reflected endothelial differentiation, colonies were stained with acetylated LDL (DiLDL,) and FITC-labeled lectin. All colonies which were examined displayed staining with both markers.

ALDH Assay and Characterization of ALDH^{br} Cells

In addition to conventional EPC identification, we enumerated EPC numbers based on ALDH activity using Aldefluor®. A typical staining profile is shown in Fig. 1. Identification of ALDH^{br} cells resulted in an identifiable population of cells characterized by high levels of ALDH activity and low light side scatter. This population is not observed in the presence of DEAB, a potent ALDH inhibitor (left panel).

Test Characteristics of EPC Assays

To assess the precision of each assay, we compared EPC numbers determined in duplicate samples drawn at the same time point. There is a strong statistical correlation between EPCs determined in duplicate samples using each assay, although the correlation is higher for FACS based analysis. To compare the precision of the assay, a comparison of correlation coefficients was performed, demonstrating a greater correlation in the FACS based assays than in the culture based assays ($p < 0.02$ for comparison of CFU vs. CD133/CD34 or ALDH^{br} assay, $p < 0.001$ for comparison of endothelial cell outgrowth vs. CD133/CD34 or ALDH^{br} assay (Fig. 2)).

We assessed the temporal stability of EPCs as enumerated on the basis of a culture based assay (endothelial CFUs) and a FACS based assay (CD133⁺CD34⁺ cells). To minimize biological variability, we enrolled healthy volunteers who were clinically stable and assessed samples drawn in a fasting state 24 hours apart. The temporal variability of culture based assays exceeds that of FACS based EPC assessments ($p < 0.05$ for comparison of correlation coefficients, Fig. 3).

Distribution of EPC Numbers Using Each Assay

To assess the degree to which each EPC assay identified similar or variant EPC populations, we sought to perform the first large cohort study to simultaneously determine EPC numbers using each of the previously described assays.

EPCs enumerated using each assay failed all tests of normality by wide margins ($p < 0.0001$ for all analyses), with a predominance of patients displaying low levels of EPCs (Fig. 4). EPCs identified on the basis of culture based assays showed the greatest deviation from normality, with the highest proportion of patients in the first interval.

Correlation of EPCs amongst the Assays

We next sought to determine the extent to which each assay identified similar or varying EPC populations by assessing the association between EPC types (Table 2).

Culture Based Assays

EPCs were enumerated using two commonly used culture based assays: endothelial cell outgrowth⁴ and endothelial cell CFU assay.² We find a statistically significant, but weak correlation between EPCs enumerated based on these strategies (Fig. 5a), suggesting that culture based assays identify similar cells.

We correlated the numbers of EPCs identified using the CFU assay, since this is the most commonly used assay to assess EPCs in culture. The endothelial CFU assay correlated with both the numbers of VEGFR2⁺ (Fig. 5b) and CD34⁺-VEGFR2⁺ (Fig. 5c) cells, but not with the numbers of CD133⁺, CD34⁺, or CD133⁺CD34⁺ positive cells (Figures 5d, 5e and 5f), suggesting that endothelial CFUs arise from circulating VEGFR-2 expressing cells, while CD133⁺ or CD133⁺ and CD34⁺ cells represent a distinct progenitor populations.

EPC Analysis based on Cell Surface Marker Expression

We next assessed the correlation of EPCs as identified on the basis of the most commonly used cell surface markers. We observe an association between the numbers of CD133⁺ and CD34⁺ cells⁺, consistent with the known expression of these markers on early circulating progenitor cells (Fig. 6A); however, there is no correlation between either of these cell populations with the numbers of VEGFR-2⁺ cells ($p=0.35$ and 0.45 respectively, Figure 6C). VEGFR-2⁺ cells fail to correlate with the numbers of CD133⁺CD34⁺ cells ($p=0.70$), although, as expected given the expression of a common marker, there is a strong association with CD34⁺VEGFR-2⁺ cells ($p<0.0001$, $r = 0.55$). EPCs as identified by the most commonly used double marker combinations, CD34/CD133 and CD34/VEGFR-2, failed to correlate with one another, despite the fact that CD34 expression is common to these cell types (Fig 6B). This finding, in context with the previously described correlation of CD34⁺VEGFR-2⁺, but not CD34⁺CD133⁺, cells with endothelial CFU outgrowth (Fig. 5c and 5f), suggests that these analysis techniques identify different EPCs.

EPCs Identified on the Basis of ALDH activity

We also determined the correlation of ALDH^{br} cells with EPCs enumerated using conventional methods. ALDH^{br} cells correlated with endothelial CFUs (Fig 7a), CD133⁺ cells (Fig 7b), CD34⁺ cells (Fig 7c), and CD133⁺-CD34⁺ cells (Fig 7d), suggesting that ALDH^{br} cells (as assessed using Aldefluor) may include several progenitor cell populations. Interestingly, we did not find a correlation with VEGFR-2⁺ cells.

Discussion

Since the initial description of EPCs capable of vascular repair, multiple reports have reported an association between EPC numbers and an array of clinical risk factors.^{2, 3, 5, 6, 8} More recently, the use of EPC levels to predict cardiac risk has been explored.^{5, 6} Nonetheless, the methodologies for EPC enumeration continue to vary from study to study, and little is known about the performance characteristics of each assay or the extent to which these assays identify similar populations.

We are unaware of any systematic comparison of EPCs as identified by all EPC assays, but several investigators have commented that there may be discrepancies between EPCs as identified by different techniques.^{13–17} Powell et al. noted that there was no correlation between EPCs identified on the basis of CD133-VEGFR2 expression and EPC colony-forming units.¹⁵ Heiss reported no correlation between CD34⁺/VEGFR2⁺ cells, CD133⁺/VEGFR2⁺ cells, and endothelial cells viable after 4 days in culture,¹⁴ and Vasa reported that statins increase CD34⁺VEGFR-2⁺, but not CD133⁺CD34⁺ cells.¹⁶ In a small study, George et al. compared the numbers of endothelial CFUs with the numbers of CD34⁺/VEGFR2⁺ cells in limited group of healthy patients, and reported no correlation.¹³ In addition, VEGF levels correlated weakly only with CD34⁺/VEGFR2⁺ cells, while no correlation with CD34⁺/CD133⁺ cells or endothelial CFUs was noted.¹³

This work represents the largest systematic comparison of EPCs enumerated by both commonly used culture based methodologies as well as common EPC cell surface markers. We further assess the correlation ALDH^{br} cells, which enhanced capability for endothelial differentiation,^{10, 11} with EPCs identified by conventional methodologies.

We find that culture based assays are less precise and display more daily variability than do assays based on cell surface markers or ALDH activity. In addition, there is weak correlation between EPCs identified using the two most commonly used culture based assays.^{2, 4} Endothelial CFUs correlate with the numbers of VEGFR-2 and CD34/VEGFR-2 expressing

cells, suggesting that “early EPCs” which arise in culture after short periods^{18, 19} may arise from VEGFR-2 expressing cells in the circulation. These findings are consistent with previous reports that CFUs fail to exhibit potential for long term expansion,^{20, 21} and may derive from monocytic cells.¹⁸ The observation that these cells have limited capacity for expansion in culture suggests that cells which express VEGFR-2 and act as endothelial CFU precursors may represent EPCs with limited proliferative potential and represent a more differentiated endothelial precursor.

Both CD34 and CD133 have been used to delineate cells with progenitor cell potential in bone marrow, cord blood and mobilized peripheral blood sources, and both are markers expressed on immature cells. The strong correlation observed between the numbers of CD34⁺ and CD133⁺ cells in peripheral blood suggests that these markers identify either overlapping cell populations or distinct precursors whose numbers are closely correlated to one another. EPCs defined by these markers do not correlate with any EPCs identified using culture based assay. We conclude that CD133⁺CD34⁺ EPCs are not precursors to early outgrowth EPC.^{2, 16}

The numbers of VEGFR-2⁺ and CD34⁺/VEGFR-2⁺ cells do not correlate with the numbers of CD34⁺, CD133⁺, or CD133⁺/CD34⁺ cells. As these are two commonly used sets of markers for EPC identification, it is important to note that they likely identify distinct classes of EPCs.

Several authors have noted a lack of correlation amongst EPCs enumerated on the basis of cell surface marker expression and culture based assays. One interpretation for this discrepancy is that FACS analyses enumerate EPCs, while culture based assays reflect EPC proliferative capacity. For instance, Herbrig et al. found higher numbers of EPC CFUs but lower numbers of CD133/CD34 cells in hemodialysis patients, concluding that hemodialysis may favor differentiation of early progenitor cell precursors.¹⁷ Heiss et al. noted lower numbers of endothelial cell CFUs, but no difference in the numbers of CD34/VEGFR-2 and CD133/VEGFR-2 cells in older subjects, concluding that absolute numbers of EPCs in the elderly are conserved, but that aging leads to impairments in EPC functional capacity.¹⁴

Recent work has suggested that “long term EPCs” are more frequent in the circulation of patients who have coronary disease²² and may have a fundamentally different relationship with cardiovascular disease than the more frequently measured EPC CFUs, which are almost uniformly inversely correlated with angiographically documented coronary disease.^{3, 23}

Our findings suggest that the lack of correlation amongst EPC types may reflect the enumeration of distinct EPC populations, pointing to the importance for more precise nomenclature in this field.

Identification of ALDH^{br} Cells

The demonstration that ALDH^{br} cells correlate with EPCs as defined on the basis of multiple other assays suggests that this technique may identify a variety of progenitor cell phenotypes, consistent with the ability of ALDH^{br} cells to give rise to multiple types of progeny¹¹ and the known importance of ALDH activity in progenitor cell biology.¹² The strong correlation of ALDH^{br} cells with CD133⁺CD34⁺ cells implies that these EPC identification strategies either identify cells representing populations with considerable overlap or which are biologically correlated to one another.

We used Aldefluor™ a research reagent used to enumerate ALDH^{br} cells in this study. This identified a large portion of MNCs as high ALDH expressors. In our study the mean number of ALDH^{br} cells was 2.37% of MNCs, as compared with CD133⁺/CD34⁺ cells (0.042% of MNCs) and CD34⁺/VEGFR-2⁺ cells (0.013% of MNCs), which were found at rates more consistent with previously published frequencies. A new reagent (Aldecou) specifically

formulated for the enumeration ALDH^{br} cells is now available. Aldecoum identifies a more distinct population present with a mean frequency of 0.05%.¹⁰ The results presented here corroborate our previous results correlating cells identified using AldecoumTM reagent with CD133⁺CD34⁺ cells. Interestingly, we find no correlation between ALDH^{br} cells and cells expressing VEGFR-2. This is consistent with our previous observations that circulating ALDH^{br} cells lack VEGFR-2 expression,¹⁰ again suggesting that CD133⁺CD34⁺ and ALDH^{br} cells identify one population of EPCs, distinct from cells expressing VEGFR-2.

Limitations

This work represents our single center experience in the measurement of EPCs by a variety of techniques. To maximize reproducibility, all work was performed by a single technician in identical fashion, and culture based assays were performed with single lots of culture medium and serum. It is possible that the lack of correlation observed amongst the assays may represent errors introduced by inconsistencies in sample analysis and preparation. This in of itself would represent an important contribution to the field, pointing to the lack of easily conducted and readily reproducible EPC assays.

The extent to which any of these cell populations represent true endothelial progenitors as opposed to mixed populations with variable differentiation potential dependent on growth conditions remains of some debate. While cells expressing VEGFR-2 in combination with CD34, CD133, or both have previously exhibited capacity for endothelial differentiation,^{9, 24, 25} a direct comparison of the endothelial and hematopoietic potential of these cells showed limited endothelial outgrowth.²⁶

The endothelial potential of EPCs is best assessed via determination of the differentiation potential of purified progenitor cell populations. Due to the paucity of these cells in unstimulated peripheral blood, we and others have relied on progenitor enriched sources such as bone marrow, cord blood, or mobilized peripheral blood to define EPCs based on capacity for endothelial outgrowth. These studies have suggested that ALDH^{br} cells,^{11, 27} CD34⁺ cells,^{1, 9, 28, 29} CD133⁺ cells,^{29, 30} as well as VEGFR-2⁺ cells^{9, 29, 31} all display potential for endothelial differentiation. Progenitor cell populations derived from such enriched sources may not reflect similar populations identified in non-mobilized circulating blood. We were, however, unable to directly compare the endothelial potential of sorted populations of cells from peripheral blood. Nonetheless, our observation that there is limited correlation between EPC numbers in peripheral blood as defined by the various assays is consistent with other findings that the term EPC is loosely applied to a variety of very different cell populations and likely identifies disparate populations.

Conclusions

This represents the first large scale study to correlate EPCs enumerated using all commonly described EPC assays. We demonstrate that EPCs fall into two broad classes: cells expressing VEGFR-2, which correlated with EPC CFUs, and cells identified on the basis of CD133, CD34 or CD133/CD34 expression, which also correlate with ALDH^{br} cells. These results will serve as a basis of a greater understanding of EPC sub-types which are identified using various assays. Our findings imply that the field would benefit from more precise terminology to define EPCs, as well as further studies to clearly define the role of each EPC type in vascular repair.

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References

1. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of Putative Progenitor Endothelial Cells for Angiogenesis. *Science* 1997;275:964–967. [PubMed: 9020076]
2. Hill JM, Zalos G, Halcox JPJ, Schenke WH, Waclawiw MA, Quyyumi AA, et al. Circulating Endothelial Progenitor Cells, Vascular Function, and Cardiovascular Risk. *N Eng J Med* 2003;348:593–600.
3. Kunz G, Liang G, Cuculi G, Gregg D, Vata K, Shaw L, et al. Circulating endothelial progenitor cells predict coronary artery disease severity. *American Heart Journal* 2006;152(1):190–95. [PubMed: 16824855]
4. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, et al. Number and Migratory Activity of Circulating Endothelial Progenitor Cells Inversely Correlate with Risk Factors for Coronary Artery Disease. *Circ Res* 2001b;89:e1–e7. [PubMed: 11440984]
5. Schmidt-Lucke C, Rossig L, Fichtlscherer S, Vasa M, Britten M, Kamper U, 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(22):2981–2987. [PubMed: 15927972]
6. Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, et al. Circulating Endothelial Progenitor Cells and Cardiovascular Outcomes. *N Engl J Med* 2005;353(10):999–1007. [PubMed: 16148285]
7. Fadini GP, Miorin M, Facco M, Bonamico S, Baesso I, Grego F, et al. Circulating Endothelial Progenitor Cells Are Reduced in Peripheral Vascular Complications of Type 2 Diabetes Mellitus. *Journal of the American College of Cardiology* 2005;45(9):1449–1457. [PubMed: 15862417]
8. Schattteman G. Are Circulating CD133+ Cells Biomarkers of Vascular Disease? *Arterioscler Thromb Vasc Biol* 2005;25(2):270–271. [PubMed: 15681306]
9. Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, et al. Expression of VEGFR-2 and AC133 by circulating CD34+ cells identifies a population of functional endothelial precursors. *Hemostasis, Thrombosis, and Vascular Biology* 2000;95:952–58.
10. Povsic T, Zavodni K, Kelly F, Zhu S, Goldschmidt-Clermont P, Dong C, et al. Circulating Endogenous Progenitor Cells Can Be Reliably Identified on the Basis of Aldehyde Dehydrogenase Activity. *J Am Coll Card* 2007;53(20):2243–48.
11. Gentry T, Foster S, Winstead L, Deibert E, Fiordalisi M, Balber A. Simultaneous isolation of human BM hematopoietic, endothelial and mesenchymal progenitor cells by flow sorting based on aldehyde dehydrogenase activity: implications for cell therapy. *Cytotherapy* 2007;9(3):259–274. [PubMed: 17464758]
12. Chute JP, Muramoto GG, Whitesides J, Colvin M, Safi R, Chao NJ, et al. Inhibition of aldehyde dehydrogenase and retinoid signaling induces the expansion of human hematopoietic stem cells. *PNAS* 2006;103(31):11707–11712. [PubMed: 16857736]
13. George J, Shmilovich H, Deutsch V, Miller H, Keren G, Roth A. Comparative Analysis of Methods for Assessment of Circulating Endothelial Progenitor Cells. *Tissue Engineering* 2006;12(2):331–335. [PubMed: 16548691]
14. Heiss C, Keymel S, Niesler U, Ziemann J, Kelm M, Kalka C. Impaired Progenitor Cell Activity in Age-Related Endothelial Dysfunction. *Journal of the American College of Cardiology* 2005;45(9):1441–1448. [PubMed: 15862416]
15. Powell TM, Paul JD, Hill JM, Thompson M, Benjamin M, Rodrigo M, et al. Granulocyte Colony-Stimulating Factor Mobilizes Functional Endothelial Progenitor Cells in Patients With Coronary Artery Disease. *Arterioscler Thromb Vasc Biol* 2005;25(2):296–301. [PubMed: 15569821]
16. Vasa M, Fichtlscherer S, Adler K, Aicher A, Martin H, Zeiher AM, et al. Increase in Circulating Endothelial Progenitor Cells by Statin Therapy in Patients With Stable Coronary Artery Disease. *Circulation* 2001;103:2885–2890. [PubMed: 11413075]
17. Herbrig K, Pistrosch F, Oelschlaegel U, Wichmann G, Wagner A, Foerster S, et al. Increased Total Number but Impaired Migratory Activity and Adhesion of Endothelial Progenitor Cells in patients on Long-Term Hemodialysis. *Am J Kid Dis* 2004;44(5):840–9. [PubMed: 15492950]

18. Rehman J, Li J, Orschell CM, March KL. Peripheral Blood “Endothelial Progenitor Cells” Are Derived From Monocyte/Macrophages and Secrete Angiogenic Growth Factors. *Circulation* 2003;107(8):1164–1169. [PubMed: 12615796]
19. Romagnani P, Annunziato F, Liotta F, Lazzeri E, Mazzinghi B, Frosali F, et al. CD14+CD34low Cells With Stem Cell Phenotypic and Functional Features Are the Major Source of Circulating Endothelial Progenitors. *Circ Res* 2005;97(4):314–322. [PubMed: 16020753]
20. Hur J, Yoon C–H, Kim H–S, Choi J–H, Kang H–J, Hwang K–K, et al. Characterization of Two Types of Endothelial Progenitor Cells and Their Different Contributions to Neovasclogenesis. *Arterioscler Thromb Vasc Biol* 2004;24:288–293. [PubMed: 14699017]
21. Gulati R, Jevremovic D, Peterson TE, Chatterjee S, Shah V, Vile RG, et al. Diverse Origin and function of Cells With Endothelial Phenotype Obtained From Adult Human Blood. *Circ Res* 2003;93:1023–25. [PubMed: 14605020]
22. Guven H, Shepherd RM, Bach RG, Capoccia BJ, Link DC. The Number of Endothelial Progenitor Cell Colonies in the Blood Is Increased in Patients With Angiographically Significant Coronary Artery Disease. *Journal of the American College of Cardiology* 2006;48(8):1579–1587. [PubMed: 17045891]
23. Eizawa T, Ikeda U, Murakami Y, Matsui K, Yoshioka T, Takahashi M, et al. Decrease in circulating endothelial progenitor cells in patients with stable coronary artery disease. *Heart* 2004;90:685–86. [PubMed: 15145881]
24. Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, et al. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. *Blood* 2000;95:3106–12. [PubMed: 10807776]
25. Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelial progenitors in human postnatal bone marrow. *J Clin Invest* 2002;109(3):337–346. [PubMed: 11827993]
26. Case J, Mead LE, Bessler WK, Prater D, White HA, Saadatzadeh MR, et al. Human CD34+AC133+VEGFR-2+ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors. *Experimental Hematology* 2007;35(7):1109–1118. [PubMed: 17588480]
27. Nagano M, Yamashita T, Hamada H, Ohneda K, Kimura K-i, Nakagawa T, et al. Identification of functional endothelial progenitor cells suitable for the treatment of ischemic tissue using human umbilical cord blood. *Blood* 2007;110(1):151–160. [PubMed: 17379743]
28. Redondo S, Hristov M, Gordillo-Moscoso AA, Ruiz E, Weber C, Tejerina T. High-reproducible flow cytometric endothelial progenitor cell determination in human peripheral blood as CD34+/CD144+/CD3– lymphocyte sub-population. *Journal of Immunological Methods* 2008;335(1–2):21–27. [PubMed: 18402976]
29. Friedrich EB, Walenta K, Scharlau J, Nickenig G, Werner N. CD34–/CD133+/VEGFR-2+ Endothelial Progenitor Cell Subpopulation With Potent Vasoregenerative Capacities. *Circ Res* 2006;98(3):e20–25. [PubMed: 16439688]
30. Quirci N, Soligo D, Caneva L, Servida F, Bossolasco P, Delilieri GL. Differentiation and expansion of endothelial cells from human bone marrow CD133+ cells. *Br J Haematology* 2001;115:186–94.
31. Nowak G, Karrar A, Holmen C, Nava S, Uzunel M, Hultenby K, et al. Expression of Vascular Endothelial Growth Factor Receptor-2 or Tie-2 on Peripheral Blood Cells Defines Functionally Competent Cell Populations Capable of Reendothelialization. *Circulation* 2004;110:3699–3707. [PubMed: 15381639]

Fig. 1. EPC Identification Based on ALDH Activity Using Aldefluor

ALDH activity is shown on the X-axis, and side scatter on the Y-axis in the absence and presence (left panel) of DEAB.

Fig. 2. Correlation between Independent EPC Enumeration Assays

Analysis of the precision of EPC enumeration on the basis of endothelial cell outgrowth⁴ [top left panel], endothelial CFUs² [top right panel], CD133 and CD34 expression [bottom left panel], and ALDH activity [bottom right panel]. Duplicate samples were obtained at the same time point from patients and analyzed in duplicate. Pearson correlation coefficients and p values are shown.

Fig. 3. Temporal stability of EPCs measured by ALDH or FACS

Blood samples obtained from volunteers 24 hours apart were analyzed for EPC content on the basis of a culture based assay (endothelial CFUs) and FACS based assay (CD133/CD34 expression), n = 11. A Pearson correlation value and p-value for the correlation are shown.

Fig. 4. Distribution of EPCs Identified by each technique

Distribution of EPCs as identified on the basis of endothelial cell outgrowth ⁴ [A], endothelial CFUs ² [B], CD133 and CD34 expression [C], CD34 and VEGFR-2 expression [D], and ALDH activity [E]. EPC numbers are not normally distributed based on D'Agostino & Pearson omnibus normality test ($p < 0.0001$ for each).

Fig. 5. Correlation of Endothelial CFU Numbers with other EPC assays

Correlation of EPCs identified on the basis of the endothelial CFUs (x-axis) vs. EPC numbers as determined using endothelial outgrowth assay (Panel A), EPCs identified on the basis of VEGFR-2 expression (Panel B), CD34 and VEGFR-2 expression (panel C), CD133 expression (panel D), CD34 expression (Panel E), or CD133 and CD34 expression (panel F). A Spearman correlation coefficient is shown for those correlations which reached statistical significance.

Fig. 6. Correlation of EPCs Identified Based on Cell Surface Protein Expression

Correlation of EPCs identified on the basis of CD34 expression with those expressing CD133 (panel A), and correlation of CD133⁺CD34⁺ cells with CD34⁺ VEGFR-2⁺ cells (panel B). Correlation of cells expressing VEGFR-2 with cells expressing CD133 and CD34 (panels C and D). A Spearman correlation coefficient is shown for those correlations which reached statistical significance.

Fig. 7. Correlation of ALDH^{br} EPCs with other EPC types

Correlation of EPCs identified on the basis of ALDH activity (x-axis) vs. EPC numbers as determined on the basis of endothelial CFUs (Panel A), CD133 expression (Panel B), CD34 expression (Panel C) and CD133⁺CD34⁺ expression (Panel D). A Spearman correlation coefficient is shown for those correlations which reached statistical significance.

Table 1

Table 1A. Patient Demographics and Clinical Characteristics.

	All Patient (n=140)	Patients Analyzed via FACS Analysis (n=77)
Age	61.1	62.3
Race (Caucasian)	81%	81%
Gender (Male)	66.9%	68%
Hyperlipidemia	76.6%	82.3%
Total Cholesterol	182.4	179.3
LDL	102.4	97.4
HTN	73.7%	72%
Diabetes	36.2%	36%
Current Tobacco	19.7%	21.3%
Family History of CAD	41.7%	39.8%
Prior CABG	22.5%	27%
# of Risk Factors	2.727	2.89%

Table 2
Summary of Correlations between Progenitor Populations.

	Endothelial CFUs	CD133 ⁺ Cells	CD34 ⁺ Cells	VEGFR2 ⁺ Cells	CD133 ⁺ -34 ⁺ Cells	CD34 ⁺ - VEGFR2 ⁺ Cells	ALDH ^{br} Cells
Endothelial Cell Outgrowth	p<0.02 r=0.22	p=.21	p=.23	p=.77	p=.78	p=.35	p=.27
Endothelial CFUs		p=.87	p=.75	p<0.0001 r=0.50	p=0.08	p<0.005 r=0.31	p<0.05 r=0.23
CD133 ⁺ Cells			p<0.05 r=0.27	p=0.35	p=.16	p=0.09	p<0.006 r=0.32
CD34 ⁺ Cells				p=.45	p<0.0001 r=0.51	p<0.0001 r=0.55	p<0.005 r=0.34
VEGFR2 ⁺ Cells					p=.70	p<0.0001 r=0.60	p=0.17
CD133 ⁺ -34 ⁺ Cells						p=.78	p<0.05 r=0.26