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Circulating Angiogenic Cell Populations, Vascular Function, and Arterial Stiffness

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Abstract

Objective—Several bone marrow-derived cell populations have been identified that may possess angiogenic activity and contribute to vascular homeostasis in experimental studies. We examined the extent to which lower quantities of these circulating angiogenic cell phenotypes may be related to impaired vascular function and greater arterial stiffness.

Methods—We studied 1,948 Framingham Heart Study participants (mean age, 66±9 years; 54% women) who were phenotyped for circulating angiogenic cells: CD34+, CD34+/KDR+, and early outgrowth colony forming units (CFU). Participants underwent non-invasive assessments of vascular function including peripheral arterial tone (PAT), arterial tonometry, and brachial reactivity testing.

Results—In unadjusted analyses, higher CD34+ and CD34+/KDR+ concentrations were modestly associated with lower PAT ratio (β=−0.052±0.011, $P<0.001$ and β=−0.030±0.011, $P=0.008$, respectively) and with higher carotid-brachial pulse wave velocity (β=0.144±0.043, $P=0.001$ and β=0.112±0.043, $P=0.009$), but not with flow-mediated dilation; higher CD34+ was also associated with lower carotid-femoral pulse wave velocity (β=−0.229±0.094, P=0.015)

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However, only the association of lower CD34+ concentration with higher PAT ratio persisted in multivariable analyses that adjusted for standard cardiovascular risk factors. In all analyses, CFU was not associated with measures of vascular function or arterial stiffness.

Conclusions—In our large, community-based sample of men and women, circulating angiogenic cell phenotypes largely were not associated with measures of vascular function or arterial stiffness in analyses adjusting for traditional risk factors.

Keywords

angiogenesis; vascular function; risk factors; endothelium; epidemiology

INTRODUCTION

Accumulating evidence suggests that select circulating cell populations have endothelial reparative and angiogenic properties.[1] Derived from the bone marrow, these cell phenotypes have been identified using cell surface markers and culture-based assays.[1] In models of arterial ischemia and myocardial infarction, CD34+ cells are capable of promoting both neovascularization and angiogenesis.[2,3] Similarly, selectively cultured peripheral blood mononuclear cells appear to facilitate re-endothelialization and minimize neointimal formation in arterial injury models.[4–6] In humans, lesser quantity of circulating angiogenic cells has been associated with both greater burden of cardiovascular risk factors[7–9] and a higher incidence of cardiovascular events.[9–12] Thus, these cell-based phenotypes have been hypothesized to represent angiogenic potential, depletion of which predisposes to impaired vascular function and the development of vascular disease.

To elucidate the role of circulating angiogenic cell phenotypes in the development of vascular disease, several clinical studies have examined the association of these cell phenotypes with measures of vascular function. However, these prior studies have been limited to referral samples of fewer than 100 subjects each and have reported conflicting results.[8,13–17] Whereas some studies have observed a relation of reduced quantities of angiogenic cells with impaired endothelial function[8,13,14] and higher arterial stiffness,[15] other studies have observed no association.[16] Therefore, we used several methods to assess circulating angiogenic cells and investigated their association with a comprehensive set of non-invasive measures of vascular function and arterial stiffness in a large, community-based sample of men and women.

METHODS

Study Sample

In 1971, the Framingham Offspring Study enrolled 5,124 individuals who were either offspring of the Original cohort or spouses of offspring.[18] Offspring study participants receive routine examinations approximately every 4 years, and a total of 3,021 participants attended the eighth examination cycle (2005 through 2008). Of this sample, 1,948 had phenotyping of circulating angiogenic cells and a complete assessment of cardiovascular risk factors, at the eighth examination and also had available vascular function measures (brachial reactivity at the seventh examination [1998–2001], and arterial tonometry and peripheral arterial tonometry [PAT] at the eighth examination). Systolic and diastolic blood pressures were the average of two physician-measured readings. Body mass index was calculated as weight divided by height squared (kg/m^2). Blood was drawn for glucose, total and high-density lipoprotein (HDL) cholesterol, and triglycerides after overnight fast. Use of medications and cigarette smoking (regular smoker within the past year) were self-reported. Diabetes was defined as having a fasting glucose ≥126 mg/dL or taking medications to treat

diabetes. Prevalent cardiovascular disease (CVD) was adjudicated by a panel of 3 investigators and defined as prior history of myocardial infarction, heart failure, or stroke.

Assessment of Cell Phenotypes

Blood specimens were collected from fasting participants in the morning between 8 and 9 A.M. to assay the following angiogenic cell phenotypes: CD34+ cells, CD34+/KDR+ cells, and colony forming unit (CFU). Each blood specimen was initially centrifuged and the resulting buffy coat was further processed for cell phenotyping within 4 hours of blood collection as previously described,[8,19] with modifications. Specifically, buffy coats were diluted to 10.5 mLs using PBS (Invitrogen) and then layered over 5 mLs of Ficoll (Amersham Pharmacia Biotech). Each specimen was then centrifuged at 2200 rpm for 15 minutes at 10°C. Using Ficoll density-gradient centrifugation, peripheral blood mononuclear cells were isolated from the buffy coat and then processed for flow cytometry and CFU assay.[8]

Flow Cytometry—Peripheral blood mononuclear cells were incubated on ice for 15 minutes with FcR blocking agent (Miltenyi Biotec), and then for an additional 25 minutes with anti-KDR PE (R&D Systems) and anti-CD34 FITC (BD Biosciences) anti-human antibodies. Specimens were washed and then fixed in 2% paraformaldehyde. Surface marker expression was evaluated by fluorescence-activated cell sorter (FACS) analysis, and positive cells were quantified using a Becton-Dickinson FacsCalibur flow cytometer using fluorochrome-matched IgG isotype controls. The frequency of CD34+ cells was identified within the nucleated cell gate using population gating; KDR+ events within the CD34 population were also analyzed via population gating. FlowJo analysis software (Treestar, Inc.) was used to quantify CD34+ and CD34+/KDR+ cells,[19] where quantities of each cell type were reported as percent of the total number of gated nucleated cells. To ensure consistency, all flow analysis plots were reviewed by an investigator blinded to identity and risk factor status of the participant (KSC).

Colony Forming Unit Assay—Peripheral blood mononuclear cells were washed with PBS, and remaining red blood cells were then lysed with ACK lysis buffer (Fisher Scientific). In each well of a 6 well fibronectin coated tissue culture plate (BD Biosciences), viable mononuclear cells (5 million per specimen) were plated in M199/20% FBS and cultured at 37°C/5% CO_2. After 2 days, non-adherent cells were collected and 2 million viable cells in M199/20% FBS were re-plated in wells of a 24-well fibronectin coated tissue culture plate. After an additional 5 days of culture, the number of colonies in each well was counted by a single, blinded technician. Colony number was reported as the average number of colonies per well across up to 12 wells; in wells where the number of colonies was too numerous to count (mean of 4.7 wells from 63 individuals), the number of colonies per well was censored at 300. Following initial cell plating and colony counting, one of two technicians performed replating of all cells; to minimize the effects of operator variation, colony counts were standardized by identity of the replating technician.

Assessment of Vascular Function

Flow-Mediated Dilation—Fasting participants who attended the seventh examination cycle (1998 to 2001) underwent assessment of brachial artery flow-mediated dilation (FMD) as previously described and reported as the percent change in brachial artery diameter from its baseline value to that at 60 seconds after cuff deflation.[20] A subset of participants had flow assessment with Doppler to assess the hyperemic flow velocity after cuff deflation, a measure of microvessel dilation (the protocol was implemented part of the way through the examination, accounting for the smaller sample size).[20,21]

Peripheral Arterial Tonometry—PAT was assessed in fasting participants attending their eighth examination cycle (2005 to 2008) by measuring digital pulse amplitude using a PAT device placed on the tip of each index finger, as previously described.[22] The index finger pulse amplitude before and after hyperemia was measured in standard fashion. The ratio of post- to pre-ischemia pulse amplitudes was calculated for the hyperemic and control (contralateral, non-hyperemic) index fingers. The PAT ratio was defined as the natural log-transformed ratio of the hyperemic to the control finger in the 90–120 second period after cuff deflation.[23]

Arterial Stiffness—Non-invasive hemodynamics and measures of arterial stiffness were assessed by using arterial tonometry in fasting participants who attended their eighth examination cycle (2005 to 2008) as previously described.[24] Calibrated carotid pressure was considered a surrogate for central pressure. Carotid-femoral and carotid-brachial pulse wave velocity (m/s) were calculated from tonometry waveforms and body surface measurements according to a standardized protocol.[21]

Statistical Analyses

All analyses were performed using SAS statistical software, version 9.2. For the primary analyses, a two-tailed $P<0.05$ was considered statistically significant. Due to right-skewed distributions, natural logarithmically transformed values were used for triglycerides, PAT, CD34+/KDR+, and CD34+. Square-root transformed values were used for CFU after standardization by the replating technician identification.

Associations of cell phenotypes (predictor variables: CD34+, CD34+/KDR+, and CFU) with vascular measures (outcome variables) were characterized using unadjusted linear regression analyses. Multivariable linear regression models were adjusted for clinical covariates previously associated with vascular function:[20,22,24] age, sex, systolic blood pressure, diastolic blood pressure, body mass index, total/HDL cholesterol ratio, log triglycerides, fasting glucose, diabetes mellitus, smoking, hypertension medication, lipid-lowering treatment, hormonereplacement therapy, and prevalent CVD.

In secondary analyses, we evaluated for effect modification by age (<65 versus ≥65 years), sex, diabetes, and prevalent CVD status with a two-sided P value threshold of 0.01 used to assess significance given multiple testing.

We also estimated power to detect significant associations between vascular and angiogenic cell phenotypes in our sample. The PROC POWER function in SAS was used to determine the minimum difference in R-squared (R^2) value for 1 predictor change that could be detected with 80% power and alpha threshold of 0.05, given the observed R^2 value, the number of predictors included, and the sample size from the fully-adjusted multivariable linear regression models.

All analyses were performed using SAS v9.2 (Cary, NC).

RESULTS

Clinical characteristics of the study sample are shown in Table 1. The mean age was 66±9 years, and 52% were women. The mean and median values of the circulating cell phenotypes are shown in Table 2. The unadjusted Pearson correlation coefficient between CD34+ and CD34+/KDR+ was r=0.31 ($P<0.0001$). The correlation coefficient between CFU and CD34+ was r=0.06 ($P=0.03$), and between CFU and CD34+/KDR+ was r=0.02 ($P=0.54$).

In unadjusted linear regression analyses (Table 3), higher levels of CD34+ and CD34+/KDR + cells were associated with lower PAT ratio, indicating lower small vessel vasodilator response. Higher CD34+ was also associated with lower central arterial stiffness, as represented by lower carotid-femoral pulse wave velocity. Higher quantities of circulating CD34+ and CD34+/KDR+ cells were also associated with higher muscular artery stiffness, as represented by higher carotid-brachial pulse wave velocity. In multivariable regression analyses that further adjusted for standard risk factors, only the relationship between CD34+ and PAT ratio persisted (Table 4).

In uadjusted analyses, CFU number was not associated with measures of vasodilator function or arterial stiffness. These results did not change in multivariable analyses that adjusted for age, sex, and standard cardiovascular risk factors.

We performed a secondary analysis to evaluate the clinical covariates that attenuated the associations of CD34+ and CD34+/KDR+ with vascular measures. Stepwise regression analyses demonstrated that the association of CD34+ with carotid-brachial pulse wave velocity was attenuated by the addition of diastolic blood pressure to a model adjusting for age, sex, heart rate, systolic blood pressure, and smoking. The relation of CD34+ with carotid-femoral pulse wave velocity was attenuated by the addition of age and sex. The relation of CD34+/KDR+ with carotid-brachial pulse wave velocity was attenuated by inclusion of diastolic blood pressure to a model adjusting for age and sex. The association of CD34+/KDR+ with PAT ratio was attenuated with the addition of body mass index to a model adjusting for age and sex.

In multivariable models, the pre-specified interaction tests between age group, sex, and prevalent CVD status and each of the cell phenotype measures for each of the vascular measures were all not statistically significant (all $P>0.01$, Supplemental Tables Ia and b). Prior studies have suggested an association of angiogenic cell phenotypes with vascular measures in participants with certain clinical characteristics (e.g. men only, or patients with high CVD risk). Thus, we conducted a secondary analysis stratifying by sex, presence of diabetes, or presence of clinical CVD. The results in stratified analyses were largely similar to the primary pooled analyses (Supplemental Tables IIa and b).

Other investigators have observed associations between circulating angiogenic cell phenotypes and measures of vascular dysfunction[8,13,14] or arterial stiffness.[15] Given the results of our analyses, we were interested in estimating the statistical power to detect associations in our study sample. At a significance level of $\alpha=0.05$, our sample had >80% power to detect a change in the model R^2 of 0.005 or smaller for the angiogenic cell variables in the multivariable linear regression models, corresponding to a partial correlation of 0.072. Thus, we had power to detect associations of a small magnitude.

DISCUSSION

In a large, community-based sample of middle-aged to older men and women, we investigated the relations of circulating angiogenic cell phenotypes with a comprehensive set of vascular function measures. We observed modest associations of higher CD34+ and CD34+/KDR+ concentrations with lower digital microvessel dilation but with higher peripheral arterial stiffness in unadjusted models. In addition, higher CD34+ concentrations were associated with lower central arterial stiffness in unadjusted models. Only the modest relation of higher CD34+ and lower microvessel dilation measured by PAT persisted in models adjusting for standard cardiovascular risk factors. In all models, we did not observe associations of CFU with any of the vascular measures. Overall, our findings suggest that these circulating angiogenic cell phenotypes are largely not associated with conventional

measures of vasodilator function or arterial stiffness after accounting for standard cardiovascular risk factors.

Our findings stand in contrast to prior work indicating that depletion of progenitor cell phenotypes relates to vascular dysfunction. Prior human studies that related angiogenic cell phenotypes to vasodilator function and arterial stiffness have been restricted to small and selected samples, and the results from these studies have not been consistent. Lower CFU has been associated with impaired brachial artery flow mediated dilation in men and with lower arterial elasticity in older individuals.[8,15] In healthy young men[17] and patients with Type 1 diabetes mellitus,[13] higher levels of CD34+/KDR+ cells have been associated with higher flow-mediated dilation. In patients with coronary artery disease, one study found a relation of CD34+/KDR+ cells with coronary endothelial dysfunction[25] whereas another did not.[26]

There are several potential explanations for our findings. The present study included a large, unselected sample with comprehensive vascular testing and risk factor evaluation. Thus, the observed lack of association may reflect lower vascular disease burden. In addition, the assessment of associated cardiovascular risk factors in a large sample facilitated adjustment in multivariable models to reduce the potential for confounding. Therefore, it remains possible that variation in circulating quantities of angiogenic cells are associated with vascular dysfunction through pathways that also involve the development of certain traditional risk factors, such as diastolic blood pressure and body mass index. In addition, available techniques for identifying progenitor cell-related phenotypes in humans remain non-specific,[1] even though many of these same cell-based phenotypes have been demonstrated to exhibit angiogenic capacity in other settings, most notably in the setting of acute or chronic ischemia.[27–29] Furthermore, the angiogenic cell phenotypes, as measured, may also vary in individuals in response to exogenous or endogenous stressors. Thus, current methods for detecting variation in angiogenic cell capacity may lack adequate specificity, particularly in an ambulatory cohort.

Relatively little is known about the behavior of circulating angiogenic cell phenotypes in relation to the progression of chronic vascular disease. Although we did not observe effect modification by CVD status, it remains possible that variability in both measured and unmeasured cell-based phenotypes are important but not as easily detectable in individuals who are relatively healthy or have subclinical disease, compared to individuals with clinically manifest acute or chronic illness.[27,28] In addition, mild abnormalities in vascular function occurring in association with depletion of angiogenic cells may, in turn, promote their increased production and/or mobilization into the circulation in the effort to maintain relative vascular homeostasis.[30] This could account for the unexpected inverse association observed between CD34+ cells and PAT ratio.

The possibility that production and mobilization might compensate for depletion of any particular cell type to a greater extent in milder rather than more severe vascular disease states could explain the more consistent associations observed in studies relating CFU and/or CD34+ cells with measures of atherosclerosis.[31–33] Indeed, we observed in this same Framingham cohort that variation in CFU was significantly associated with measures of coronary artery and abdominal aortic calcification.[33] Compared to the measures of vascular function in the present study, measures of arterial calcification are likely to remain more stable in each individual. In addition, large artery calcification represents more advanced vascular disease reflecting cumulative alterations in the arterial wall that appear to relate more directly to angiogenic cell populations. On the other hand, subclinical alterations in vascular function, existing in the absence of marked anatomic disease, constitute early and potentially reversible vascular disease.[34,35] Interpreting the present findings in this context,

we speculate that measurable variation in circulating angiogenic cells may associate more closely with advanced structural rather than early and dynamic functional vascular abnormalities. Vascular dysfunction that precedes the development of anatomic atherosclerosis may yet be associated with alterations in angiogenic cell turnover; however, current techniques for accurately quantifying supply versus mobilization of these cellular phenotypes remain limited.[36]

Limitations

Since our analyses were cross-sectional, our findings do not exclude the possibility that variation in angiogenic cell quantity is related to later development of vascular dysfunction in a time-dependent manner. This limitation applies particularly to analyses of flow-mediated dilation measurements, which were measured at the prior examination cycle to the angiogenic cell populations. There is limited data regarding the stability of flow-mediated dilation (or angiogenic cell phenotypes) over a similar period of time in community-based cohorts. In contrast to flow-mediated dilation, arterial tonometry and digital vascular function measures were performed concurrently with the cell assays. With respect to cell phenotyping, the assays used in the present analysis differed somewhat from those used in prior studies. For CFU phenotyping, our analyses were performed on buffy coat as opposed to whole blood specimens;[8] however, this difference is not expected to alter variation in colony formation significantly. For the CD34+ and CD34+/KDR+ assays, we isolated non-adherent rather than adherent peripheral blood mononuclear cells;[7,10] however, prior studies have demonstrated consistent findings when using these two approaches.[1] The lack of significant associations observed in our analyses could relate, in part, to overfitting of models that included multiple cardiovascular risk factors as covariates. Our sample included middle-aged to older adults of predominantly European ancestry; thus, the generalizability of our findings to other age groups and racial/ethnic populations remains untested.[17]

Conclusions

The main results of our analysis in a large community-based sample indicate lack of an association between angiogenic cell phenotypes, assessed using established assays, and vascular functional phenotypes after adjustment for traditional cardiovascular risk factors. These findings suggest that our understanding of the relationship between circulating angiogenic cells and vascular function remains limited.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Sample Characteristics

	Total Sample N=1,948
Clinical Characteristics	
Age, years	66±9
Women, %	54
Systolic blood pressure, mm Hg	128±17
Diastolic blood pressure, mm Hg	74±10
Body mass index, kg/m^2	28.1±5.1
Total cholesterol/HDL, ratio	3.5±1.0
Fasting glucose, mg/dL	106±23
Diabetes, %	16
Smoking, %	9
Hormone replacement therapy, %†	10
Hypertension medication, %	47
Lipid-lowering medication, %	43
Prevalent cardiovascular disease, %	14
Vascular Measures	
Flow mediated dilation, %	3.0±2.9
Hyperemic mean flow velocity, cm/s	51.6±20.8
Peripheral arterial tonometry ratio	0.69±0.43
Carotid-femoral pulse wave velocity, m/s	10.5±3.8
Carotid-brachial pulse wave velocity, m/s	9.0±1.7

All values are presented as means ± standard deviations or percentages, unless otherwise noted.

*Values presented as median (25th, 75th percentile) for non-normally distributed variables.

†Percent of women.

Table 2

Circulating Angiogenic Cell Populations in the Study Sample

	Mean ± SD	Median (IQR)	Range
CD34+, %	0.087 ± 0.050	0.075 (0.053 – 0.110)	0.479
CD34+/KDR+, %	0.004 ± 0.004	0.003 (0.002 – 0.005)	0.047
CFU, no.	55.5 ± 59.8	38.8 (20.5 – 65.2)	300

Table 3

Unadjusted Relation of CD34+ and CFU Phenotypes with Vascular Measures

	CD34+		CD34+/KDR+		CFU	
	β (SE)	*P*	β (SE)	*P*	β (SE)	*P*
Flow-mediated dilation, %	−0.050 (0.075)	0.51	−0.053 (0.075)	0.48	−0.012 (0.073)	0.87
Hyperemic flow velocity, cm/s	0.129 (0.598)	0.83	−0.509 (0.597)	0.39	0.145 (0.605)	0.81
PAT ratio, unitless	−0.052 (0.011)	<0.001	−0.030 (0.011)	0.008	−0.001 (0.011)	0.94
Carotid-femoral pulse wave velocity, m/s	−0.229 (0.094)	0.015	−0.116 (0.094)	0.22	0.015 (0.093)	0.87
Carotid-brachial pulse wave velocity, m/s	0.144 (0.043)	0.001	0.112 (0.043)	0.009	0.082 (0.042)	0.05

Sample size range is 1175 to 1639, maximized for each analysis. Regression coefficients represent difference in the vascular measure per 1-SD difference in log CD34+, log CD34+/KDR+, and square-root CFU.

Table 4

Multivariable-Adjusted Relation of CD34+ and CFU Phenotypes with Vascular Measures

	CD34+		CD34+/KDR+		CFU	
	β (SE)	*P*	β (SE)	*P*	β (SE)	*P*
Flow-mediated dilation, %	−0.057 (0.073)	0.44	−0.034 (0.070)	0.63	−0.020 (0.069)	0.77
Hyperemic flow velocity, cm/s	−0.332 (0.535)	0.53	−0.573 (0.521)	0.27	−0.021 (0.531)	0.97
PAT ratio, unitless	−0.023 (0.010)	0.03	−0.017 (0.010)	0.09	0.006 (0.010)	0.58
Carotid-femoral pulse wave velocity, m/s	−0.003 (0.072)	0.97	−0.059 (0.071)	0.41	0.128 (0.072)	0.07
Carotid-brachial pulse wave velocity, m/s	0.075 (0.040)	0.06	0.067 (0.039)	0.08	0.072 (0.038)	0.06

Regression coefficients represent difference in the vascular measure per 1-SD difference in log CD34+, log CD34+/KDR+, and square-root CFU. All analyses are adjusted for age, sex, systolic blood pressure, diastolic blood pressure, body mass index, total/HDL cholesterol ratio, triglycerides, glucose, diabetes mellitus, smoking, hypertension medication, lipid-lowering treatment, hormone-replacement therapy, prevalent CVD.