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Differentiation Capacity of Endothelial Progenitor Cells Correlates With Endothelial Function in Healthy Young Men

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Background: Endothelial progenitor cells (EPCs) have been assumed to maintain vascular endothelial integrity, so the present study investigated whether the functional capacity of EPCs correlates with endothelial function in healthy young subjects, as has been confirmed in aged subjects with atherosclerotic disease.

Methods and Results: EPCs in 41 healthy, young male nonsmokers (age 33.1±3.9 years, mean±SD) were characterized. The correlation between flow-mediated vasodilation (FMD) and the number of EPCs or the plasma concentrations of growth factors, such as vascular endothelial growth factor, did not reach statistical significance. However, FMD was significantly correlated with the EPC differentiation index, defined as the ratio of the number of EPCs to the total number of adherent cells (r=0.391, P=0.011) and the abundance of endothelial nitric oxide synthase mRNA (r=0.340, P=0.030).

Conclusions: In healthy young men, despite a lack of correlation of the number or colony counts of EPCs, the ability of circulating progenitor cells to differentiate into an endothelial lineage is closely correlated with endothelial function. This cell function assay may serve as a novel biomarker for vascular function in healthy subjects in the pre-atherosclerotic stage. (*Circ J* 2009; **73:** 1324 – 1329)

Key Words: Angiogenesis; Atherosclerosis; Endothelial function; Endothelial progenitor cells

Circulating progenitor cells (PCs) are potent in differentiating into many different cellular lineages, including all of the cells comprising the vascular wall (ie, endothelial cells, vascular smooth muscle cells, and fibroblasts)[1,2] and are involved in tissue repair and organ viability[3–6] Recently, endothelial PCs (EPCs) were characterized in detail[3–7] We have also found that, in patients with primary acute myocardial infarction, the ability of EPCs to differentiate positively correlated with functional improvement and infarct size reduction[8] Although it is traditionally assumed that replacement of the damaged endothelium results only from outgrowth of preexisting endothelial cells, recent studies have found that PCs mobilized from bone marrow and other putative tissue niches appear to contribute to vascular homeostasis and repair[9] with the number and colony formation of circulating EPCs inversely correlating with the risk of cardiovascular diseases and positively correlating with endothelial function[10] The ability of circulating EPCs to form colonies progressively decreases as coronary risk factors increase![11]

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However, little is known about the degree to which these circulating EPCs are involved in the maintenance of vascular function in healthy young subjects. Furthermore, it is unclear whether the functional characteristics of EPCs correlate with endothelial function in healthy young subjects without obvious atherosclerosis. We consider that circulating EPCs may be more involved in vascular reparative processes than expected, with rapid turnover at endothelial lesions, and that the function of EPCs would directly influence arterial endothelial function. We thus hypothesized that functional EPC characteristics reflect endothelial function, and thereby could serve as an initial biomarker of arteriosclerosis. In the present study, we measured flow-mediated endothelium-dependent vasodilation (FMD) as a marker of endothelial function[12–14] and assessed the association between various EPC characteristics and endothelial function in vivo.

Methods

Study Subjects

We studied 41 healthy young men (age 33.1±3.9 years, range, 25–35), who were all volunteers and free from hypertension, diabetes mellitus, and dyslipidemia. None of them had ever smoked[15,16] The protocol was reviewed and approved by the Ethics Committee of Nagoya University and the study was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent and filled out a questionnaire on their physical

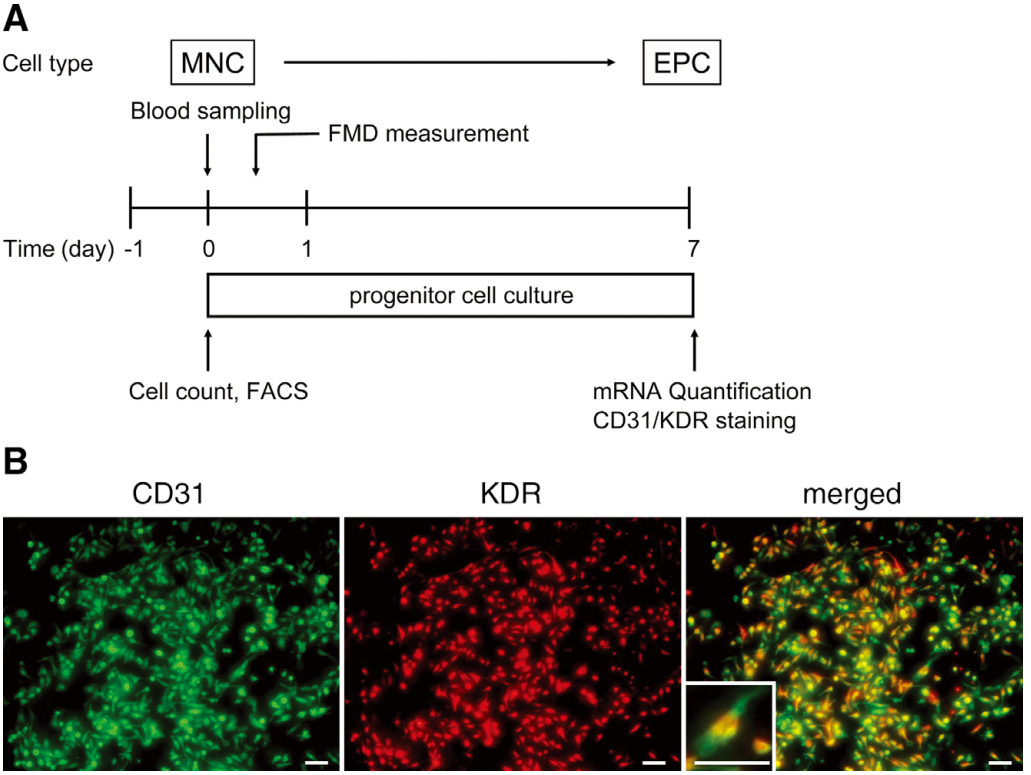

Figure 1. Protocol and immunocytochemistry. (**A**) Summary of the protocol. Blood samples were drawn while in the fasting state, followed by measurement of flow-mediated dilation (FMD) of the brachial artery. eNOS, endothelial nitric oxide synthase; FACS, fluorescence-activated cell sorter. (**B**) Mononuclear cells (MNCs) were cultured for 7 days and endothelial progenitor cells (EPCs) were confirmed as endothelial lineage by immunostaining for CD31 and KDR. Inset shows magnified views of double-positive staining of CD31 and KDR. Bars = 50 μm.

activity. The score for total physical activity was calculated according to the Compendium of Physical Activities, which defines the amount of energy expenditure on each physical activity[17] and the average energy expenditure was expressed by metabolic equivalent (MET = 4.184 kJ·kg^{-1}·h^{-1})-h per week.[18]

EPC Preparation

Peripheral blood samples were collected while the subjects were in a fasting condition between 7.00 and 9.00 h (**Figure 1A**). Mononuclear cells (MNCs) were isolated from 50 ml of peripheral blood by a density gradient centrifugation method[3,7] Cell-surface markers, such as CD34, CD45, and CD133, and vascular endothelial growth factor receptor type 2 (VEGFR2/KDR) were analyzed by flow cytometry (FACS: FACScan, Beckton-Dickinson, Franklin Lakes, NJ, USA) as previously described[8,16] The numbers of CD45^{low}CD34^{+}CD133^{+}cells/ml and CD45^{low}CD34^{+}CD133^{+}VEGFR2^{+}cells/ml were counted.[8,16,19,20] Plasma levels of vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and erythropoietin were measured by ELISA kits (R&D Systems, Minneapolis, MN, USA).

Cell Culture and Characterization of PCs

MNCs were cultured on 2% gelatin/fibronectin-coated 6-well dishes (BioCoat, Becton-Dickson) at 10^6 cells/cm^2, as described previously[3,8,16] Cells were cultured in M199 supplemented with 20% fetal bovine serum (GIBCO-Invitrogen, Carlsbad, CA, USA), 2 mmol/L glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and bovine pituitary extract (GIBCO-Invitrogen). To visualize the upregulation of endothelial markers, cells were fixed at 7 days of culture, incubated with the anti-human antibodies listed below, and stained with biotinylated anti-mouse or rabbit Ig antibodies, and FITC and Texas red-avidin with DAPI counterstaining, then observed using an epifluorescence microscope (Olympus, Tokyo, Japan). The marker proteins were VEGFR2/KDR (LabVision, Fremont, CA, USA), and platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) (Invitrogen, Carlsbad, CA, USA)[8,19,20] A colony forming unit (CFU) was defined as a cell cluster of EPCs consisting of multiple thin, flat cells emanating from a central cluster of rounded cells. A central cluster alone without associated emerging cells was not counted as a colony. Colonies were counted manually in a minimum of 5 wells by observers who were unaware of the subjects' clinical profiles.[11]

Measurement of mRNA Expression

Total RNA was extracted from cultured EPCs using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. EPCs were lysed with lysis buffer containing 1% β-mercaptoethanol, and the first cDNA strand was synthesized using the SuperScript™ First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed using the LightCycler™ System (Roche Diagnostics, Mannheim, Germany) and QuantiTect™ SYBR Green PCR kit (Qiagen). The reaction was carried out under the following conditions for human endothelial nitric oxide synthase (eNOS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 1 cycle of 95°C for 15 min, followed

Table 1. Clinical Characteristics of the Study Subjects

	Young subjects (n=41)
Age, years	33.1±3.9
Physiological values	
Systolic BP, mmHg	120±14
Diastolic BP, mmHg	69±8
Heart rate, beats/min	62.1±1.0
Body mass index, kg/m^2	22.8±2.5
Physical activity, MET-h/week	33.5±3.5
Blood cell counts and fraction	
White blood cells, /μl	5,490±1,600
Red blood cells, $\times10^4$/μl	510±35
MNCs, /μl	801±654
Metabolic markers	
Lipid profile	
Total cholesterol, mmol/L	5.10±0.68
LDL-cholesterol, mmol/L	3.07±0.60
HDL-cholesterol, mmol/L	1.56±0.37
Triglycerides, mmol/L	1.17±0.77
MDA-LDL, IU/L	91.6±30.2
Apolipoprotein A-I, mg/dl	139±24
Apolipoprotein A-II, mg/dl	30.8±4.9
Apolipoprotein B, mg/dl	87.7±17.9
Glucose profile	
Fasting glucose, mmol/L	5.41±0.42
Hemoglobin A_{1c}, %	4.60±0.26
Fasting insulin, pmol/L	55±29
HOMA-IR	2.25±1.32
HOMA-β	96.8±46.8
C-reactive protein, mg/dl	0.18±0.09
Folic acid, nmol/L	16.4±10.4
Homocysteine, μmol/L	11.4±4.9
Growth factors	
pVEGF, pg/ml	240±35
pPlGF, pg/ml	615±222
Erythropoietin, mmol/L	21.3±9.2
Angiographic values	
Brachial artery diameter, mm	4.28±2.4
FMD (Δ%)	4.19±2.11
GTN (Δ%)	15.7±5.2

Values expressed as mean±SD.
BP, blood pressure; MET, metabolic equivalent; MNC, mononuclear cell; LDL, low-density lipoprotein; HDL, high-density lipoprotein; MDA, malondialdehyde-modified; HOMA, homeostasis model assessment; VEGF, vascular endothelial growth factor; PlGF, placental growth factor; FMD, flow-mediated dilation; GTN, glyceryl-trinitrate-induced dilation.

by 50 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s. Analyses were carried out with the LightCycler™ 3.5 software (Roche Diagnostics Japan, Tokyo, Japan). The level of expression of each mRNA was normalized to the GAPDH expression level. The nucleotide sequences for primers are as follows: eNOS, forward: 5'-TTC CGG AAG GCT TTT GAT CC-3', reverse: 5'-TGC AAA GCT CTC TCC ATT CTC C-3'; GAPDH, forward: 5'-ATC ACC ATC TTC CAG GAG CGA-3', reverse: 5'-GAG GGG GCA GAG ATG ATG AC-3'.

Evaluation of Arterial Endothelial Function

Brachial artery endothelial function was assessed according to a previously described noninvasive technique![14] Using high-resolution ultrasound sonography (SONOS 5500, Agilent Technology, Palo Alto, CA, USA) with a 7- to 11-MHz linear-array transducer, the end-diastolic diameter of the right brachial artery and the blood flow were measured by pulsed-wave Doppler ultrasound. The diameter of the right brachial artery was measured from the anterior to the posterior interface between the media and adventitia just before the QRS wave on the ECG, using the mean of three measurements.

FMD was measured at baseline, 1 min after forearm hyperemia (produced by releasing a forearm cuff inflated to 200 mmHg for 5 min), and at rest after the subject had been lying quietly for 10 min. After the diameter had recovered to baseline, glyceryl-trinitrate-induced dilation (GTN), which is endothelium-independent, was assessed 5 min after sublingual application of 300 μg of GTN. Vasodilation was then calculated as the percentage change in diameter from baseline. All of the FMD and GTN measurements were performed by a single investigator (A.I.) who was unaware of the subjects' characteristics: the mean intraobserver variabilities were 6.8% for FMD and 5.6% for GTN. Participants were asked to avoid sidestream smoke from cigarettes or cigars within 24 h of the measurements![16]

Statistical Analysis

Data are reported as means±SD. Data were analyzed using SPSS version 13 (SPSS Inc, Chicago, IL, USA). Continuous variables were tested for normal distribution by the Kolmogorov-Smirnov test, and all variables passed it ($P<0.05$). Pearson correlation analysis was used to evaluate the bivariate relationship between individual variables with FMD. Multivariate analysis was not undertaken because of the small sample size. A level of $P<0.05$ was accepted as statistically significant.

Results

The baseline characteristics of the volunteers are listed in **Table 1**. All subjects were nonsmokers and had no signs of hypertension, diabetes mellitus or dyslipidemia. The average physical activity was 33.5±3.5 MET-h/week, indicating a mild to moderate level of physical activity![17,18]

Circulating Cell Count and Vascular Function

To find other novel biomarkers of endothelial function, we first counted the number of blood cells in peripheral blood and compared this with vascular function (**Table 2**). Pearson correlation analysis revealed that the numbers of white blood cells, red blood cells, and MNCs had no correlation with FMD. Regarding cell cluster formation, unlike several other studies in old and atherosclerotic subjects![10–13] in the present study the correlation with FMD did not reach statistical significance ($P=0.23$).

Glucose and Lipid Metabolic Levels and Vascular Function

It is proven that several metabolic disorders and, especially, the overlap of these disorders (metabolic syndrome), cause endothelial dysfunction. We assessed the relationship between representative metabolic values and endothelial function (**Table 2**). There were no significant relationships between the level of the following substances and vascular function: total cholesterol ($P=0.73$), high-density lipoprotein-cholesterol ($P=0.05$), low-density lipoprotein-cholesterol ($P=0.66$), triglycerides ($P=0.72$), fasting blood glucose ($P=0.71$), and hemoglobin A_{1c} ($P=0.32$).

In addition to the classical coronary risk factors, such as hyperglycemia, hypertriglyceridemia, and hypercholesterolemia, novel risk factors for endothelial dysfunction have emerged, such as C-reactive protein and homocysteine![11] We measured the following diverse circulating metabolic factors in peripheral blood to assess the potential relation-

Table 2. Correlations Between Characteristics and FMD in Young Healthy Men

	r	P value
Age	0.220	0.17
Physiological values		
Systolic BP	–0.097	0.55
Diastolic BP	–0.038	0.81
Heart rate	–0.100	0.53
Body mass index	–0.057	0.72
Physical activity	0.372	0.017
Blood cell counts and fraction		
White blood cells	–0.246	0.12
Red blood cells	–0.001	0.99
MNCs	–0.370	0.017
Metabolic markers		
Lipid profile		
Total cholesterol	0.055	0.73
LDL-cholesterol	0.071	0.66
HDL-cholesterol	–0.109	0.50
Triglycerides	0.057	0.72
MDA-LDL	0.109	0.50
Apolipoprotein A-I	–0.146	0.36
Apolipoprotein A-II	–0.198	0.22
Apolipoprotein B	0.141	0.38
Glucose profile		
Fasting glucose	0.059	0.71
Hemoglobin A_{1c}	0.160	0.32
Fasting insulin	–0.103	0.52
HOMA-IR	–0.095	0.56
HOMA-β	–0.182	0.26
C-reactive protein	0.007	0.97
Folic acid	0.062	0.70
Homocysteine	–0.016	0.92
Growth factors		
Plasma VEGF	0.102	0.53
Plasma PlGF	–0.317	0.043
Erythropoietin	–0.109	0.50
Angiographic values		
Brachial artery diameter	–0.381	0.014
Cell fraction analysis		
$CD45^{low}CD34^{+}CD133^{+}$	–0.157	0.33
$CD45^{low}CD34^{+}CD133^{+}KDR^{+}$	0.250	0.12
Functions after cell culture		
eNOS mRNA abundance	0.340	0.030
Cluster formation (colonies/HPF)	0.191	0.23
Adherent cell count (/HPF)	–0.172	0.28
Adherent cell count/MNC count	0.105	0.51
EPC count/adherent cell count	0.391	0.011

r, linear (Pearson) correlation coefficient; HPF, high-power field; EPC, endothelial progenitor cell. Other abbreviations see in Table 1.

ship between them and endothelial function (FMD), and found no significant relations: malondialdehyde-modified low-density lipoprotein (P=0.50), apolipoprotein A-I (P=0.36), apolipoprotein A-II (P=0.22), apolipoprotein B (P=0.38), insulin (P=0.52), C-reactive protein (P=0.97), folic acid (P=0.70), homocysteine (P=0.92), homeostasis model assessment (HOMA)-IR (P=0.56), and HOMA-β (P=0.26).

Cell-Surface Antigens, Growth Factors, and Vascular Function

Recent studies have shown that EPCs are mainly derived from $CD45^{low}CD34^{+}CD133^{+}VEGFR2^{+}$ PCs[8,16] so we used those fractions of MNCs for the measurement of circulating EPCs. There were no significant relationships between the number of cells expressing these markers and vascular function as assessed by FMD.

We next measured the plasma concentrations of several growth factors: VEGF, PlGF, and erythropoietin. Similar to cell-surface antigens, we did not find any relationship between FMD and these growth factors, except for PlGF (**Table 2**).

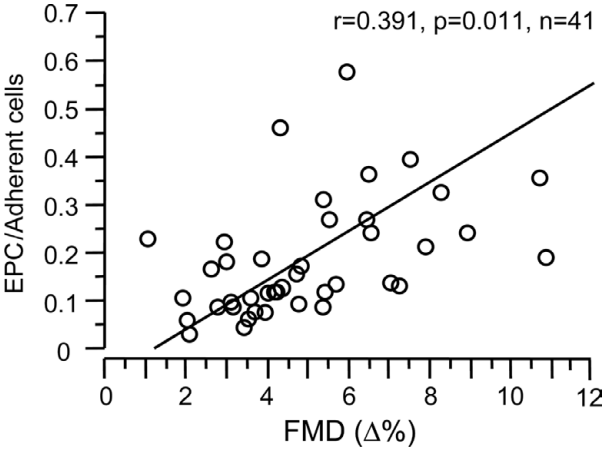

Figure 2. Relationship between flow-mediated dilation (FMD) and cell function. FMD does not correlate with the numbers of colonies formed and attached cells, but it is correlated with the ability to differentiate into an endothelial lineage (r=0.391, P=0.011). EPC, endothelial progenitor cell.

Differentiation Ability of Circulating PCs and Endothelial Function

To determine the angiogenic functions of PCs in culture, we cultured MNCs for 7 days and counted the number of colony formation units per well. The EPC characteristics were assessed by upregulation of CD31 and KDR[8,21] We compared these angiogenic functions with endothelial function in the brachial artery.

Some circulating MNCs attached to the culture dish and differentiated into EPCs, as confirmed by immunostaining for CD31 and KDR (**Figure 1B**). We observed that the degree of differentiation of attached cells into EPCs varied among the study samples. We then defined the ratio of EPCs counted to the number of adherent cells as the EPC differentiation index. This index correlated significantly with FMD (r=0.391, P=0.011) (**Figure 2**). Regarding the differentiation of EPCs into an endothelial cell lineage, eNOS mRNA abundance in the cultured cells also correlated with FMD (r=0.340, P=0.030).

No correlation was observed between GTN-induced dilation and individual variables measured in the present study (data not shown).

In order to assess the methods of evaluating endothelial function and circulating EPCs used in this study, we compared the results from the 41 healthy young nonsmoking men with those from 21 male patients with coronary heart disease (age 67.7±7.7 years). The patients showed a significantly lower FMD (2.43±2.00%, P=0.004) and a significantly reduced ratio of EPCs counted to the number of adherent cells (0.05±0.09 vs 0.19±0.17% in young men, P=0.001). In addition, the colony counts of EPCs per well were reduced in the patients with coronary heart disease compared with the healthy young men (18.1±29.4 vs 1.4±2.5, P=0.006). There was a significant relationship between FMD and the ratio of EPCs to the number of adherent cells even in patients with coronary heart disease (r=0.560, P=0.013).

Discussion

The present study has identified a positive correlation between the ability of circulating PCs to differentiate into an endothelial lineage and endothelial function, as measured by FMD, in healthy young men. This finding suggests that the engraftment and functioning of PCs are pivotal in the maintenance of the vascular endothelium, and that circulating PCs can be used as a novel biomarker of endothelial function. Using this novel marker may allow us to predict early-stage atherosclerosis and prevent its further development in young adults, as many studies have provided evidence that endothelial dysfunction leads to subsequent atherosclerosis.[22–27]

To elucidate the relationship between basal levels of cytokines and endothelial function, we measured the plasma concentrations of growth factors such as VEGF, PlGF, and erythropoietin, and found only a modest correlation between PlGF and FMD. Recently, Taguchi et al reported that angiogenic growth factors, such as VEGF, basic fibroblast growth factor, hemopoietic growth factor, and insulin-like growth factor, did not correlate with vascular function or the number of $CD34^+$ cells in patients with cerebrovascular disease.[28] Although these growth factors are believed to be involved in various steps of PC engraftment, such as mobilization, attachment, and migration, and to have many functions in bone marrow and blood vessels, their plasma levels seem to be minimally affected in the setting of pre-atheromatous arteries in young men.

Recent studies have demonstrated that physical activity also affects endothelial function via increased basal and maximal NO production in the vessels.[29] In our analysis of the correlation between average physical activity and FMD in young subjects, there was a significant correlation ($r=0.372$), which is consistent with previous reports.[12,13,18,28]

As total calorie intake and the proportion of fat in the daily diet increase, the risk and incidence of cardiovascular disease increase in parallel. Many investigators have studied metabolic disorders and the overlap of these disorders (metabolic syndrome) for the prevention of atherosclerosis. In the present study, to elucidate the effect of metabolic background on vascular function in young subjects, we calculated the glucose and lipid metabolic indices of HOMA-IR, as well as measuring the serum levels of glucose, cholesterol, triglyceride, homocysteine, and folic acid. As with the growth factors, none of these values or indices correlated with FMD. We assume that unless the glucose and lipid metabolism of the subjects are normal, these values and indices would be potential markers for endothelial function, even in healthy young men without cardiovascular diseases.

Regarding the EPC number and function, differing from previous reports of aged and/or atheromatous subjects,[9–13] we found no correlation between EPC number as $CD34^+$ $CD133^+KDR^+$ or cluster formation and FMD. In particular, Hill et al reported that FMD is significantly associated with the number of EPC colonies in healthy men at the mean age of 50 years.[11] The difference in results could be attributed to the age and race of the study subjects. Attachment of circulating cells to the extracellular matrix (ECM) depends on the interaction between leukocytes and the ECM.[22,30] Because the ECM concentration in culture plates is the same among samples, the dominant factors that regulate leukocyte adhesion are assumed to be the levels of integrins and some other cell-surface antigens that are relevant to leukocyte rolling and adhesion. In this study, all young subjects were healthy and were not smokers. The levels of humoral factors that may stimulate cell adhesion and the expression levels of cell-surface antigens such as ICAM-1 were relatively low, and so the distribution of those factors varied less. Therefore, we might not be able to obtain the statistically significant correlation of FMD with cell cluster formation or number of PCs as often seen in older or atherosclerotic subjects. Recently, George et al collected MNCs from 38 individuals (mean age 34 years) and compared them with cell-surface antigens such as CD34/CD133/KDR;[30] the CFU numbers did not correlate with CD34/KDR or CD34/CD133/KDR, and negatively correlated with CD34/CD133 numbers, and they concluded that CFU numbers are more likely to reflect their ability to proliferate, but not differentiate. Those findings are consistent with our results.

Finally and most importantly, we found a strong correlation between the ability of PCs to differentiate into an endothelial lineage and FMD in healthy young men. Peripheral blood was collected from subjects who were not taking drugs such as statins, which are known to promote the differentiation of PCs into an endothelial lineage[5,9] and improve endothelial function.

Study Limitations

We observed that most of the attached cells in cultures were EPCs rather than platelets or white blood cells, as confirmed by their spindle shape, and positive staining with CD31 and VEGFR2/KDR.[8] However, some EPCs may differentiate from other fractions.[32] Our findings are at best tentative because we have identified associations rather than proved a cause-and-effect relationship, and hence further investigation is required.

In conclusion, the ability of circulating PCs to differentiate into an endothelial lineage correlates with the degree of endothelial function in healthy young men, which suggests that measurement of the functional characteristics of PCs might be used as a novel biomarker of atherosclerotic diseases, and that circulating EPCs, as well as differentiated endothelial cells in the preexisting vasculature, play a pivotal role in the maintenance of vascular function.

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