Peripheral Blood CD34⁺KDR⁺ Endothelial Progenitor Cells Are Determinants of Subclinical Atherosclerosis in a Middle-Aged General Population

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Background and Purpose—Disruption of the endothelial layer is the first step in the atherogenic process. Experimental studies have shown that endothelial progenitor cells (EPCs) are involved in endothelial homeostasis and repair. Conversely, EPC depletion has been demonstrated in the setting of established atherosclerotic diseases. With this background, we evaluated whether variations in the number of EPCs are associated with subclinical atherosclerosis in healthy subjects.

Methods—Carotid intima-media thickness (IMT), high-sensitive C-reactive protein (hsCRP), levels of circulating EPCs, and cardiovascular risk were compared in 137 healthy subjects. Six subpopulations of progenitor cells were determined by flow cytometry on the basis of the surface expression of CD34, CD133, and KDR antigens: CD34⁺, CD133⁺, CD34⁺CD133⁺, CD34⁺KDR⁺, CD133⁺KDR⁺, and CD34⁺CD133⁺KDR⁺.

Results—Among different antigenic profiles of EPCs, only CD34⁺KDR⁺ cells were significantly reduced in subjects with increased IMT. Specifically, CD34⁺KDR⁺ cells were inversely correlated with IMT, even after adjustment for hsCRP and 10-year Framingham risk and independently of other cardiovascular parameters.

Conclusions—Depletion of CD34⁺KDR⁺ EPCs is an independent predictor of early subclinical atherosclerosis in healthy subjects and may provide additional information beyond classic risk factors and inflammatory markers. (Stroke. 2006; 37:2277-2282.)

Key Words: atherosclerosis ■ endotnhelium ■ progenitor cells

The discovery that a subset of circulating immature cells physically contributes to the homeostasis of the vascular tree has been a major achievement in cardiovascular research.¹ Endothelial progenitor cells (EPCs) are bone marrow–derived cells that share characteristics of both hematopoietic stem cells, including expression of CD34, and endothelial cells, such as expression of endothelial markers and the ability to differentiate into mature endothelium.² EPCs have been shown to be involved in physiological and pathological angiogenesis, as they are actively recruited at sites of new vessel growth.³ Moreover, EPCs have a prominent role in reendothelization phenomena at sites of endothelial damage.⁴,⁵ Therefore, EPCs constitute a circulating pool of cells able to form a “patch” that actively repairs the denuded or dysfunctional endothelium, whereas a reduced EPC pool may determine the inability to maintain adequate endothelial homeostasis. In this context, because endothelial injury represents the primus movens for the development of the atherosclerotic plaque, it has been hypothesized that EPC depression can be causally linked to the atherogenic process.⁶

Consistent with this hypothesis, cardiovascular risk factors, as well as coronary, cerebral, and peripheral atherosclerosis, have been associated with low EPC levels.⁷–¹⁰ Moreover, low EPC levels have been shown to represent an independent risk factor for future cardiovascular events.¹¹,¹²

Until now, very few studies have been performed in the healthy population to determine whether EPC depletion also characterizes the earliest phases of atherogenesis.¹³ This study was designed to assess the relationships between an established marker of subclinical atherosclerosis, ie, carotid in tima-media thickness (c-IMT),¹⁴ and levels of circulating EPCs, defined by the surface expression of CD34, CD133, and KDR antigens.

Subjects and Methods

Study Population
On the basis of a previous study,⁸ we calculated that a sample size of ~120 subjects would be needed to detect a significant correlation between progenitor cell count and c-IMT (α=0.05, β=0.05). One hundred thirty-seven healthy subjects were recruited from a local community of office employees on the basis of an agreement with
the University of Padova. The study was approved by the local ethics committee, informed consent was obtained from all subjects, and the procedures followed were in accordance with institutional guidelines.

The following data were recorded: age, sex, systolic and diastolic blood pressures, waist circumference, smoking habit (>1 cigarettes per day), and family history of cardiovascular disease (defined as a major cardiovascular event in 1 or more first-degree relatives). On the day of the study, after an overnight fast, blood samples were drawn for the determination of progenitor cell counts and plasma insulin, glucose, lipids, and high-sensitive C-reactive protein (hsCRP) concentrations. The Framingham risk was calculated as previously described.15

**Measurement of c-IMT**

The extent of subclinical atherosclerosis was measured by quantitative high-resolution B-mode ultrasound of the far wall of the right and left common carotid arteries. The measurements were carried out as previously described16 according to a validated procedure with an HDI-5000 SONO CT ultrasound machine (Philips Medical Systems/ATL Spa) equipped with a phased-array 4- to 7-MHz transducer. Readings were done independently by 2 trained operators. The flow divider between the internal and external carotid arteries was identified, and the common carotid arteries was explored starting 1 cm below the flow divider. Measurements were done by tracing the leading edge of the lumen-intima and the media-adventitia interfaces. Maximum right and left IMTs were averaged to obtain the c-IMT measure. Subjects with carotid plaques, defined as c-IMT >1.5 mm, were excluded from the study.

**Quantification of Circulating EPCs**

Peripheral blood cells were analyzed for the expression of surface antigens by direct flow cytometry as previously described.8 Before being stained with specific monoclonal antibodies, cells were treated with fetal calf serum for 10 minutes, and then the samples were washed with buffer containing phosphate-buffered saline and 0.5% bovine albumin. This approach is used to saturate sites for non-specific binding. Then, blood cells were stained with fluorescein isothiocyanate (FITC)–conjugated anti-human CD34 monoclonal antibody (mAb) (Becton Dickinson), phycoerythrin (PE)-conjugated anti-human KDR mAb (R&D Systems), and allophycocyanin (APC)–conjugated anti-human CD133 (Miltenyi Biotec). Control isotype IgG1 and IgG2a Abs were obtained from Becton Dickinson. The frequency of peripheral blood cells positive for the aforementioned reagents was determined by a 2-dimensional side-scatter fluorescence dot-plot analysis. A morphological gate was used to exclude granulocytes. Then, we gated CD34+ or CD133+ peripheral blood cells in the mononuclear cell fraction and examined the resulting population for the dual expression of KDR. At the intersection of the CD34 and CD133 gates, we identified CD34+CD133+ cells, whereas total KDR+ mononuclear cells were identified separately as cells with high KDR expression and low side scatter. Triple-positive cells were identified by the dual expression of KDR and CD133 in the CD34 gate. For fluorescence-activated cell sorting (FACS) analysis, 5 × 10^6 cells were acquired and scored with a FACSCalibur analyzer (Becton Dickinson). Data were processed with the aid of the Macintosh CELLQuest software program (Becton Dickinson). The instrument setup was optimized daily by analyzing the expression of peripheral blood lymphocytes labeled with an anti-CD4 FITC/CD8 PE/CD3 PECy5/CD45 APC 4-color combination.

**Statistical Analyses**

Data are expressed as mean±SEM. Results from flow cytometry are expressed as cells per 10^6 cytometric events. Comparison between 2 groups was performed with a 2-tailed Student’s t test. Linear-regression analysis was used to describe the correlation between 2 variables. To account for inflation of the experiment-wise type 1 error owing to multiple testing, we implemented the Bonferroni test when only 1 test was significant and the Hochberg procedure when >1 test was significant. To identify variables independently associated with c-IMT, a multiple linear-regression analysis was performed. Statistical significance was accepted at P<0.05.

**Results**

**Factors Associated With High c-IMT**

The study sample was representative of a healthy, middle-aged, general population (Table 1). Median c-IMT was 0.65 mm, and subjects were divided according to their c-IMT into low IMT (below the median) and high IMT (equal or above the median). As expected, subjects in the high–c-IMT group were older, had higher blood pressure and plasma glucose, a worse lipid profile, and a 2.7-fold higher predicted 10-year risk than did subjects in the low–c-IMT group.

As a continuous variable, c-IMT was significantly correlated with age; waist circumference; systolic and diastolic blood pressures; total, LDL, and HDL cholesterol; triglycerides; and Framingham risk, after adjusting for α inflation because of multiple testing (Table 2).

**Relations Between Progenitor Cell Counts and c-IMT**

With the use of 3 surface markers, we identified and enumerated 6 progenitor cell phenotypes (CD34+, CD1333, CD34+CD1333, CD34+KDR3, CD133+KDR+, and CD34+CD1333KDR+) and total KDR+ cells. Of these populations, only the CD34+KDR+ cell count was significantly different between the low– and high–c-IMT group: subjects in the high–c-IMT group showed a 32% reduction in the levels of circulating CD34+KDR+ cells (62.0±4.2 versus 90.7±6.6, P<0.001; Figure 1A). Also, when we used 0.8 mm as the cutoff value for c-IMT, CD34+KDR+ was the only cell population significantly different between groups (Figure 1B). Consistently, c-IMT was significantly negatively correlated only with the CD34+KDR+ phenotype (r = −0.28,

**TABLE 1. Characteristics of the Study Population**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>IMT&lt;Median (n=66)</th>
<th>IMT&gt;Median (n=71)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>42.1±0.8</td>
<td>47.7±0.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Male sex, %</td>
<td>26</td>
<td>63</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>83.2±1.3</td>
<td>94.1±1.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>119.2±1.3</td>
<td>128.1±1.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>81.2±0.9</td>
<td>87.2±1.3</td>
<td>0.0003</td>
</tr>
<tr>
<td>Smoking habit, %</td>
<td>14</td>
<td>21</td>
<td>0.208</td>
</tr>
<tr>
<td>Family history, %</td>
<td>52</td>
<td>44</td>
<td>0.361</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>77.7±4.3</td>
<td>101.2±5.9</td>
<td>0.002</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>56.7±1.7</td>
<td>50.4±1.5</td>
<td>0.018*</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>114.3±4.0</td>
<td>134.8±3.4</td>
<td>0.0001</td>
</tr>
<tr>
<td>Plasma glucose, mg/dL</td>
<td>86.2±1.5</td>
<td>91.4±1.6</td>
<td>0.019*</td>
</tr>
<tr>
<td>Plasma insulin, µg/L</td>
<td>6.84±0.78</td>
<td>9.21±1.2</td>
<td>0.111</td>
</tr>
<tr>
<td>hsCRP, g/L</td>
<td>0.96±0.14</td>
<td>1.27±0.20</td>
<td>0.218</td>
</tr>
<tr>
<td>Framingham risk, %</td>
<td>3.46±0.36</td>
<td>9.48±1.30</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Patients were divided into 2 groups according to their c-IMT (median value, 0.65 mm).

*Not statistically significant after adjustment for α inflation due to multiple testing.
In this study, we demonstrate that the level of circulating CD34⁺KDR⁺ EPCs is an independent determinant of the extent of early subclinical atherosclerosis, measured as c-IMT, in a sample representative of the healthy general population.

Measurement of c-IMT is accepted as the best way to detect early atherosclerosis in asymptomatic subjects; it is considered a strong biomarker of cardiovascular risk and has been extensively used as a surrogate end point in primary intervention studies of risk reduction. As expected, in our study sample, a high c-IMT (equal or above the median value) was associated with a worse cardiovascular profile. In fact, c-IMT was strongly correlated with the 10-year Framingham risk, which is, to date, 1 of the most reliable indicators of risk in asymptomatic individuals. However, it has been pointed out that some young subjects at low or intermediate Framingham risk may be at significantly increased risk in the long term. Because novel theories have redefined atherosclerosis as a chronic inflammatory disease of the arterial wall, inflammatory markers, namely hsCRP, have been suggested to add prognostic information beyond traditional risk factor assessment to predict cardiovascular events. However, this hypothesis has not been substantiated in all subsequent series, and also in our study, hsCRP was not significantly correlated with c-IMT and Framingham risk.

The main aim of our study was to determine whether EPCs might represent a novel prototype of cardiovascular risk biomarkers. The conceptual revolution in the field of progenitor cells implies that EPC depletion represents at the same time a pathogenic step of atherogenesis and a biomarker of cardiovascular risk. Such a comprehensive role in cardiovascular biology has been hardly reported before, even with respect to other established markers. Unfortunately, there is no clear consensus on which antigenic profile best identifies progenitor cells with the potential to repair the endothelium. With the consideration that EPCs should express at least 1 marker of immaturity and 1 additional marker reflecting endothelial commitment, 3 main antigenic profiles have been proposed. CD34 is an adhesion molecule expressed on hematopoietic stem cells and is typically considered a marker of immaturity; CD133 is a surface antigen of unknown function that identifies more immature progenitor cells than CD34 alone; and KDR represents type 2 vascular endothelial growth factor receptor and indicates early endothelial differentiation. Therefore, CD34⁺CD133⁺KDR⁺ and CD133⁺KDR⁺ cells may be considered more immature EPCs, such as those recently mobilized from bone marrow. Once in the peripheral blood, EPCs progressively lose CD133, and CD34⁺KDR⁺ cells thereafter become the main constituent of the circulating EPC pool. Consistently, in steady-state conditions (ie, without any stimulus for bone marrow mobilization), peripheral blood CD34⁺KDR⁺ cells are 3-fold and 10-fold more frequent than CD133⁺KDR⁺ and CD34⁺CD133⁺KDR⁺ cells, respectively. Progenitor cells lacking KDR expression (CD34⁺, CD133⁺, and CD34⁺CD133⁺ cells) should be considered undifferentiated and do not strictly correspond to EPCs. Those populations may undergo further differentiation toward the endothelial lineage as well as other phenotypes, such as smooth muscle cells and fibrocytes.

In the present study, we used flow cytometry to analyze the expression of the 3 most commonly used surface markers to obtain 6 subsets of progenitor cells and measured the levels of EPCs. As a control, we also enumerated total KDR⁺ cells, which cannot be considered EPCs because they lack the immature antigens. Even if progenitor cells are rare in the peripheral circulation, flow cytometry represents the “gold standard” for EPC enumeration, has been extensively used in the clinical setting, and has proved to be a sensitive, reproducible, and reliable technique. Among the 6 progenitor cell subtypes, only CD34⁺KDR⁺ cells were significantly reduced in the high- versus low-c-IMT group and were negatively correlated with c-IMT. This result is consistent with 2 recent observations indicating that the CD34⁺KDR⁺ cell level independently predicts cardiovascular events and atherosclerosis progression in patients with coronary artery disease. The lack of correlation between...
c-IMT and CD34⁺, CD133⁺, and CD34⁺CD133⁺ cells is consistent with the aforementioned theory and suggests that undifferentiated progenitors are distinct from EPCs and do not reflect endothelial homeostasis in healthy subjects. We have recently proposed that the percentage expression of KDR on CD34⁺ cells quantitatively reflects the extent of endothelial differentiation of generic progenitors.⁸,³⁴ In this study, the CD34⁺KDR⁺ /CD34⁺ percentage ratio was significantly negatively correlated with c-IMT, indicating that the endothelial commitment of circulating progenitors is important for vascular homeostasis. We speculate that differentiation toward phenotypes other than endothelial, such as smooth muscle or fibrocyte, may trigger the progression of atherogenesis, leading to plaque growth. The lack of correlation of c-IMT with CD133⁺KDR⁺ and CD34⁺CD133⁺KDR⁺ cells suggests that those immature cells are not very relevant in steady-state conditions, as they provide a limited contribution to the circulating EPC pool, which is made up mainly of CD34⁺KDR⁺ cells. Even if we have no data directly indicating that CD34⁺KDR⁺ cells functionally correspond to EPCs, our results suggest that this exact phenotype may be preferred in future studies in which EPC count is intended as a cardiovascular biomarker. Finally, the lack of correlation between c-IMT and KDR⁺ cells is not surprising, because those cells cannot be considered progenitors.

Remarkably, in our experience, the correlation between CD34⁺KDR⁺ cells and c-IMT remained significant even after adjustment for 10-year risk and hsCRP. This result suggests that EPC count provides additional information that goes beyond classic risk assessment and the use of inflammatory markers as novel risk factors. Therefore, what we report is not simply the correlation between 1 candidate biomarker and an established surrogate of cardiovascular risk. Rather, we show that EPC depletion is a determinant of the anatomic remodeling of the common carotid artery wall,³⁵ which is a strong indicator of generalized atherosclerosis, and we provide proof of principle for the role of EPCs in vascular homeostasis, confirming previous studies in animals.⁴,⁵

### TABLE 3. Linear Correlations Between c-IMT and Progenitor Cell Counts According to the Different Antigenic Phenotypes

<table>
<thead>
<tr>
<th>Antigenic Phenotype</th>
<th>Pearson’s r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34⁺</td>
<td>0.147</td>
<td>0.087</td>
</tr>
<tr>
<td>CD133⁺</td>
<td>0.065</td>
<td>0.480</td>
</tr>
<tr>
<td>CD34⁺CD133⁺</td>
<td>0.003</td>
<td>0.974</td>
</tr>
<tr>
<td>CD34⁺KDR⁺</td>
<td>−0.277</td>
<td>0.001*</td>
</tr>
<tr>
<td>CD133⁺KDR⁺</td>
<td>0.085</td>
<td>0.375</td>
</tr>
<tr>
<td>CD34⁺CD133⁺KDR⁺</td>
<td>0.007</td>
<td>0.944</td>
</tr>
<tr>
<td>KDR⁺</td>
<td>−0.043</td>
<td>0.652</td>
</tr>
<tr>
<td>CD34⁺KDR⁺ /CD34⁺ % ratio</td>
<td>−0.234</td>
<td>0.006*</td>
</tr>
</tbody>
</table>

*Significant after adjustment for α inflation.
In summary, we demonstrate that the level of circulating CD34+/KDR+ endothelial progenitors is a determinant of early atherosclerosis in the general population. The emerging concept is that EPC depletion reduces the ability to repair the endothelium, thus triggering subsequent steps in the development of the atherosclerotic plaque.

Disclosures

None.

References

14. Lorenz MW, von Kegler S, Markus HS, Sitzer M. Carotid intima-media thickening indicates a higher vascular risk across a wide age range.


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