Comparative Analysis of Methods for Assessment of Circulating Endothelial Progenitor Cells

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ABSTRACT

The number and properties of endothelial progenitor cells (EPC) in disease states is of considerable interest due to the importance attributed to this distinct cell population. However, there has been no study comparing each of the methods employed in the same sampled individuals. Herein, we performed an analysis of several methods used for circulating EPC assessment and correlated them with humoral factors known to influence their numbers. Thirty-eight individuals (mean age of 34 ± 9 years) were tested. Peripheral blood mononuclear cells were obtained and stained for FACS analysis with antibodies to CD34, CD45, CD133, and KDR and the remaining cells grown under endothelial cell conditions for assessment of colony-forming unit (CFU) numbers and adhesive properties. Levels of circulating vascular endothelial growth factor (VEGF), erythropoietin (EPO), and C-reactive protein (CRP) were determined and correlated with each of the EPC markers. CFU numbers did not correlate with CD34/KDR or CD34/CD133/KDR and negatively correlated with CD34/CD133 numbers. CD34/KDR numbers correlated with CD34/CD133/KDR, but not with CD34/CD133. Only CD34/KDR and CD34/CD133/KDR correlated with VEGF serum levels. The number of EPC adhering to fibronectin and endothelial cells correlated with CFU numbers and not with either of the EPC membrane markers. Current methods for quantitatively assessing numbers of circulating EPC are not correlated. VEGF serum levels are associated only with CD34/KDR and CD34/CD133/KDR, whereas CFU numbers correlate with EPC functional properties. These findings may suggest that CD34/KDR is more appropriate for the definition of circulating EPC, whereas CFU numbers are more likely to reflect their ability to proliferate.

INTRODUCTION

Endothelial progenitor cells (EPC) are a distinct population of cells that has also been found in the peripheral circulation.1,2 These findings have prompted a series of experimental studies that demonstrated that EPC contribute to angiogenesis and vasculogenesis, thereby leading to attenuation of myocardial remodeling, rescuing of limb ischemia, and promotion of corneal vessel formation.2

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thrombopoietin (EPO), C-reactive protein (CRP), medications (HMG CoA reductase inhibitors), physical training, and estrogen treatment were suggested to influence EPC numbers and may be partially responsible for their recruitment following vascular trauma, myocardi- cal infarction, and ischemia.

However, a major obstacle to the understanding and interpretation of this growing number of reports is the lack of fully corroborated and mutually compared methods for characterizing the putative endothelial progenitor cell. Whereas some investigators refer to colony-forming units as measures for estimating EPC numbers, others employ flow cytometry for assessment of circulating cells positive for either CD34/KDR, CD34/CD133, or CD34/CD133/KDR. Both KDR (VEGF receptor 2) and AC-133 positive cells were shown to differentiate into endothelial cells and were thus suggested as identifying AC-133 positive cells were shown to differentiate into endothelial cells and were thus suggested as identifying endothelial in nature.

For determination of fibronectin binding properties, EPC (day 7) were placed onto fibronectin-coated culture dishes and incubated for 30 min at 37°C. For assessment of binding to mature cultured endothelial cells, day-7 EPCs were labeled with DiI and incubated with human umbilical vein endothelial cell monolayer (Clonetics, East Rutherford, NJ) with or without pretreatment with tumor necrosis factor alpha (1 ng/mL) for 12 hours.

In 10 patients, repeated analysis of circulating EPC employing all markers was carried out 1 month after the primary analysis. The study was approved by the local institution ethics committee, and all patients gave informed consent to the study.

Serum VEGF levels were determined by ELISA according to the manufacturer’s instruction (BioSource, Camarillo, CA). EPO levels were determined by ELISA kit (R&D Systems).

The assay for high-sensitivity CRP was conducted according to the manufacturer’s instructions (Dade Behring, Deerfield, MA). Levels of VEGF, EPO, and CRP were evaluated on sera that were frozen immediately after blood drawing and defrozen immediately prior to assay performance.

Due to the non-Gaussian distribution of values, correlations were made using the Spearman test. The level of significance was set at 0.05.

RESULTS

Median CFU number was 20/well (CI 19–41), median number of total CD34 cells was 0.14% (0.13–26), median CD34/CD133 was 0.04% (0.05–0.14), median CD34/CD45 was 0.05% (0.06–0.16), and median CD34/CD133/KDR was 0.002% (0.001–0.02). Median number of CD34/KDR was 0.01% (0.001–0.03). Mean number of fibronectin adhering EPC was 9 ± 3 cell/× 40 magnification, whereas mean number of EPC adhering to culture endothelial cells was 22.3 ± 4.1 cell/× 40 magnification.

In 10 patients repeated evaluation of EPC markers was carried out 1 month after the primary analysis. No significant differences were evident in either of the EPC markers (CFU numbers, CD34/CD133, CD34/CD133/KDR, or CD34/KDR) between the two time points. Median VEGF level was 41 pg/mL (38–50), median EPO
level was 6.8 pg/mL (4.8–8.1), and median CRP level was 1.1 mg/dL (1.15–2.47).

The number of CD34/CD133 cells positively correlated with CD34/CD133/KDR and with total CD34/CD45 but not with CD34/KDR (Table 1). Interestingly, a negative correlation was found between CFU numbers and CD34/CD133 numbers. CD34/KDR numbers correlated with CD34/CD133/KDR but not with other markers of EPC, such as the CFU or CD34/CD133 numbers, nor was it associated with CD34 or CD34/CD45 numbers. Circulating CFU, a method employed for estimation of circulating EPC, did not correlate with CD34/KDR numbers but did negatively correlate with CD34, CD34/CD133, and CD34/CD45 numbers.

CFU numbers were significantly associated with fibronectin binding and TNF stimulated endothelial cell adherence (Table 1). A correlation between other markers of EPC and adhesive properties was not found.

Circulating VEGF concentrations correlated with numbers of CD34/KDR and CD34/CD133/KDR, but not with CD34, CD34/CD45, CD34/CD133, or CFU numbers (Fig. 1). EPO serum levels correlated with total CD34 cell numbers ($r = 0.41$, $p = 0.001$), but not with any of the EPC phenotypic markers or with CFU numbers. No correlation was found between circulating VEGF and each of the phenotypic markers of EPC (data not shown).

**DISCUSSION**

We performed a comparative assessment of the different methods employed for studying circulating EPC numbers. The growing interest in the field of EPCs led to the publication of many recent papers in which the characterization of EPCs was performed employing different methods, thereby precluding a valid comparison.

Several groups have employed modification of the CFU assay. However, other groups have used FACS markers, suggesting that the cells positive for CD34/CD133 are early EPCs and that loss of the CD133 is associated with commitment toward endothelial cell lineage. Other investigators favor the definition of EPC as circulating peripheral mononuclear cells positive for the CD34/VEGF receptor 2 (KDR), whereas still others suggest that the population of cells positive for the triple markers (CD34/CD133/KDR) can also be labeled as EPC. The main obstacle to establishing common background to the different definitions is the lack of a gold standard method. Since VEGF, erythropoietin, and CRP have been shown to associate with EPC numbers, we reasoned it would be of relevance to explore which of the different phenotypic subgroups most closely associates with VEGF, EPO, and CRP serum levels.

An interesting finding of this study is that no correlation was evident between CFU and CD34/KDR or CD34/CD133/KDR numbers. Additionally, there was no association between CD34/KDR and CD34/CD133 circulating cell numbers. Surprisingly, a negative association was found between CFU and CD34/CD133 numbers. These findings suggest that interpretation of the studies that define EPC employing each of these methods should be done with caution.

Several studies have shown that VEGF is a potent mobilizer of EPCs from the bone marrow to the peripheral circulation. We have found that circulating VEGF levels correlated only with the CD34/KDR and CD34/CD133/KDR cells and not with CFU numbers or CD34/CD133. Interestingly, EPO serum levels correlated with the total CD34 number and not with each of the cell populations currently regarded as EPCs, suggesting EPO drives a nonselective recruitment of progenitors from the bone marrow. It should be emphasized that for this study we have chosen apparent healthy subjects to allow assessment of "physiological" determinants of EPC numbers, and this finding may explain the lack of correlation between EPO and CRP levels and EPC numbers that have been suggested in previous studies.

We also found that CFU numbers, but not other phe-

**Table 1. Comparative Analysis Correlating EPC Phenotype Markers and Functional Properties in 40 Healthy Subjects**

<table>
<thead>
<tr>
<th>Phenotype Markers</th>
<th>CD34/CD133</th>
<th>CD34/KDR</th>
<th>CD34/CD133/KDR</th>
<th>CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34 total</td>
<td>0.86 (&lt;0.0001)</td>
<td>0.07 (0.60)</td>
<td>0.53 (0.0008)</td>
<td>−0.42 (0.009)</td>
</tr>
<tr>
<td>CD34/CD133</td>
<td>−0.19 (0.26)</td>
<td>0.19 (0.26)</td>
<td>0.55 (0.0002)</td>
<td>−0.54 (0.0005)</td>
</tr>
<tr>
<td>CD34/KDR</td>
<td>0.17 (0.26)</td>
<td>—</td>
<td>0.38 (0.02)</td>
<td>−0.06 (0.34)</td>
</tr>
<tr>
<td>CD34/CD133/KDR</td>
<td>0.51 (0.0002)</td>
<td>0.37 (0.02)</td>
<td>—</td>
<td>−0.22 (0.14)</td>
</tr>
<tr>
<td>CD34/CD45</td>
<td>0.86 (&lt;0.0001)</td>
<td>−0.01 (0.56)</td>
<td>0.31 (0.04)</td>
<td>−0.63 (&lt;0.0001)</td>
</tr>
<tr>
<td>CFU</td>
<td>−0.53 (0.0005)</td>
<td>−0.06 (0.34)</td>
<td>−0.24 (0.14)</td>
<td>—</td>
</tr>
<tr>
<td>AD-Fbn</td>
<td>−0.35 (0.06)</td>
<td>0.005 (0.92)</td>
<td>0.08 (0.66)</td>
<td>0.37 (0.01)</td>
</tr>
<tr>
<td>AD-EC</td>
<td>−0.19 (0.13)</td>
<td>0.30 (0.09)</td>
<td>−0.02 (0.90)</td>
<td>0.05 (0.42)</td>
</tr>
<tr>
<td>AD-TNF-EC</td>
<td>0.009 (0.99)</td>
<td>0.33 (0.09)</td>
<td>0.37 (0.14)</td>
<td>0.53 (0.01)</td>
</tr>
</tbody>
</table>

AD, adhesion; Fbn, fibronectin; CFU, colony-forming units; EC, endothelial cell; TNF, tumor necrosis factor.
notypic markers, correlated with EPC adhesive properties. This may be explained by the fact that CFU numbers represent a proliferative phenotype of cells rather than a marker for a distinct cell population.

Although EPCs have been extensively studied in the context of vascular dysfunction, they have been shown to play a role in other disorders such as cancer and also to provide a possible tool by which to deliver genes (as reviewed by Rafii and Lyden2). Moreover, it should be emphasized that several recent reports have also pointed to possible side effects associated with EPC use.18,19

Thus, the aim of accomplishing standardized methods by which to define EPC numbers is of further importance.

In conclusion, we found that in healthy subjects, there is no correlation between the different methods employed for estimating EPC numbers. Although this study does not address the reason for the apparent discrepancy, it is possible that the different methods sample EPC at different stages of differentiation and therefore cannot be reported as standard measures of EPC numbers. This is supported, at least in part, by the disappearance of CD133 as EPC differentiate into endothelial lineage.

FIG. 1. Correlation of circulating level of VEGF with each hemangioblastic and EPC markers. VEGF serum levels were determined by ELISA for each of the subjects, and EPC phenotype was determined by FACS or by CFU assay (as described in Materials and Methods).
CD34/KDR and CD34/CD133/KDR most closely associate with VEGF levels, whereas the CFU number correlates with adhesive properties, suggesting that the former are more consistent with the definition of circulating EPC and the latter more appropriately represents their proliferative properties.

REFERENCES


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