Endothelial Colony Forming Capacity is Related to C-Reactive Protein Levels in Healthy Subjects

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Abstract: The majority of clinical studies on endothelial progenitor cells (EPCs) focuses on the role of these cells in cardiovascular diseases and no systematic studies exist regarding their variations in healthy subjects. In order to define the burden of angiogenesis in physiological conditions we assessed the frequency of peripheral blood endothelial colonies (PB-ECs) and their relation with other factors possibly involved in their function such as high-sensitivity C-reactive protein (hs-CRP), endothelial cell-specific mitogen factor (VEGF) and tissue inhibitor of metalloproteinases-1 (TIMP-1) in a highly selected healthy population. A PB sample was obtained from 37/47 healthy subjects (age 40.2±15.0yrs; M/F 15/22) without known cardiovascular risk factors. The serum level of hs-CRP, VEGF, TIMP-1, the frequency of PB-ECs by clonogenic assay, and the number of early EPCs and late EPCs by flow cytometry analysis were evaluated. PB-ECs were formed by 40.5% of studied subjects with a mean of 0.40±0.82 colonies/10^6 cells. The differences in the frequency of colony formation between genders were not statistically significant. The subjects with PB-ECs were characterized by higher values of hs-CRP, when compared with those not forming colonies, 0.276±0.230 vs 0.095±0.077 mg/l (p=0.003) respectively, and of VEGF, 328.3±162.9 vs 202.6±118.5 pg/ml (p=0.02). No significant differences were found in TIMP-1 values. The EPC clonogenic potential seems to be related to hs-CRP and VEGF levels even in healthy population supporting the concept that these mediators are involved in physiological ECs function.

Key Words: Endothelial progenitor cells, vascular endothelial growth factor, high-sensitivity c-reactive protein, reparative medicine.

INTRODUCTION

The endothelium is a dynamic structure lining the circulatory system and composed of approximately 1 to 6 x 10^13 endothelial cells (EC). This area is maintained by a continuous self-renewal of the ECs that in basal conditions accounts for 0.1% replications per day (Cines et al. 1998). The basal replication rate of ECs is difficult to define since several physiological and pathological conditions, by eliciting an additional demand of ECs, could continuously interfere with their replication and activity. The number of vital and apoptotic circulating endothelial cells (CECs) detached from the endothelium is roughly related to the turnover of endothelial progenitor cells (EPCs) in physiological conditions (Hunting et al. 2005). However, several pathological conditions requiring tissue repair re-endothelialization of injured vessels and neoangiogenesis could overtake the self-renewal capacities of the endothelium. The identification in the peripheral blood (PB) of EPCs derived from bone marrow (BM) and the demonstration of their prompt mobilization, incorporation, and differentiation to the sites of injury has suggested that EPCs could serve as endothelial reparative reserve during stress injury of the vascular endothelium (Asahara et al. 1999; Iwami et al. 2004; Hristov et al. 2003a). In addition, it has been demonstrated in an experimental model of tissue injury that, even when injected peripherically, cells derived from BM are able to home to the site of damage (Ciulla et al. 2003) and contribute to neovessel formation (Crosby et al. 2000). Therefore, the frequency of PB-ECs has been proposed as diagnostic, therapeutic or prognostic marker of vascular injury and neovascularization (Shintani et al. 2001; Vasa et al. 2001; Gill et al. 2001; Hristov et al. 2003b). Unfortunately, EPCs are extremely rare in normal PB, representing between 0.01 and 0.0001% of peripheral mononuclear cells, and their accurate detection and enumeration is a technical challenge especially when high sensitive techniques are used, such as flow cytometry (Khan et al. 2005). Culture assays that allow a quantification of EPCs and are able to give rise to colonies are highly warranted to improve the reliability of flow cytometry, and to develop a functional evaluation of these cells (Hill et al. 2003). In order to define the burden of normal angiogenesis, we used a clonogenic assay to establish the frequency of PB-ECs and their relation with potential covariates in a highly selected healthy population at ascertained low cardiovascular risk. Since angiogenesis is a quite complex process involving several specific molecules, including inflammatory mediators, vascular
growth factors, and matrix metalloproteinases, we tested, in the same subjects, the relationship between PB-ECs, high-sensitivity C-reactive protein (hs-CRP), endothelial cell-specific mitogen factor (VEGF). Furthermore, the VEGF angiogenic effect requires the remodeling of the extracellular matrix (ECM) in order to allow endothelial cells to migrate and invade into the surrounding tissues (Lakka et al, 2005) and since this effect is known to be highly dependent on matrix metalloproteinases (MMP) activity of which effect is inhibited in vivo by both synthetic and natural MMP inhibitor (Seandel et al, 2001), we therefore measured tissue inhibitor of metalloproteinases-1 (TIMP-1) in our subjects as a rate limiting angiogenic regulator. Finally, since colony forming capacity is still poorly understood in physiological conditions (Khan et al. 2005), we categorized our population on the basis of colony forming capacity in order to characterize subjects who formed colonies.

MATERIALS AND METHODS

Subjects Selection

We enrolled 47 caucasian healthy non-smokers, non-obese, normo-cholesterol, normotensive, not currently under pharmacological treatment volunteers, 22 males and 25 females matched for age, between our hospital’s staff. All subjects gave informed consent and underwent a clinical examination including Body Mass Index (BMI) determination, a measurement of blood pressure at rest, Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP), with a standard sphygmomanometer, a 12 leads ECG at rest and a standard Color Doppler Echocardiogram examination. To exclude underlying cardiac disease, only the subjects with normal results were included in the study. The study was approved by the Ethical Committee of our hospital.

Carotid Artery Intima-Media Thickness

The mean carotid artery Intima-media thickness (IMT) was obtained in each subject from B-mode ultrasonographic measurements (GE Vivid 7, Wisconsin, USA) of the common carotid artery (CCA) IMT. The examination was performed with a 13 MHz linear array probe (focus depth set at 1.0 cm). IMT was measured on at least 2 segments and was expressed as mean value. Subjects with IMT > 1.3 mm were excluded from the study.

Blood Sampling

A 30 mL sample of PB was obtained from each subject. All the samples were taken between h 11:00 and 12:00 AM, a time interval when the maximum release of progenitor cells from BM is supposed (Smaaland et al. 2002). Physical exercise was not allowed in the week prior to measurements; females were not menstruated during the sampling procedure.

High-Sensitivity C-Reactive Protein Determination

Within 30 minutes of collection, 7 ml of PB were centrifuged at 2500 rpm for 10 minutes. Serum was divided into two aliquots and stored at –80°C until analysis. On each collected sample the hs-CRP was assessed to exclude subjects at increased cardiovascular risk. The assay for hs-CRP evaluation was conducted according to the manufacturer’s instructions (Cobas Integra, Roche Diagnostics, Germany). The lower detection limit of the assay was 0.071 mg/dl and the intra- and inter-assay coefficients of variation were 1.8% and 2.9% respectively. According to clinical criteria (Pearson et al. 2003), subjects with hs-CRP >1 mg/l were considered at increased risk and excluded from the study.

VEGF and TIMP-1 Determinations

Since the study was conducted in healthy subjects where no or little changes in angiogenic factors are expected, VEGF and TIMP-1 were tested in our subjects among a panel of 8 angiogenic factors to ascertain if their levels were within the detectability levels suggested by the manufacturer (data not shown). VEGF and TIMP-1 in the serum of each subject were determined by using the Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. All assays were performed in duplicate. The lower detection limit for VEGF was 9 pg/ml. The intra- and inter-assay coefficients of variation were 4.5% and 7.0% respectively. The lower detection limit for TIMP-1 was 0.08 ng/ml. Both the intra- and inter-assay coefficients of variation were 3.9 %.

Detection and Analysis of EPCs by Flow Cytometry

One hundred µl of PB samples were incubated for 20 minutes with 10 µl of a panel of fluorescein isothiocyanate (FITC)–, R-phycocerythrin (R-PE), or peridinin chlorophyll (PerCP) protein conjugated antibodies. Appropriate analysis gates were used to enumerate early and late EPCs. In a CD45 negative population, the total early EPCs were defined as KDR+/CD144– cells, while late EPCs as P1H12+/CD31+ cells. Five-parameter, 3-color flow cytometry was performed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The sensitivity of fluoroscope detectors was set and monitored using Calibrite Beads (Becton Dickinson) according to manufacturer’s recommendations. For sample analysis, 100,000 events per sample were acquired. Data were analyzed with CellQuest software (Becton Dickinson).

Isolation of Endothelial Progenitor Cells and PB-ECs Assay

Samples were processed after collection, and mononuclear cells were isolated by Lymphohyte-H (Cedarlane, Hornby, Ontario) density gradient centrifugation. Recovered cells were washed twice with phosphate-buffered saline and once in endothelial growth medium (Endomed, Biochrom, UK). Isolated cells were subsequently resuspended in endothelial growth medium and plated on dishes coated with human fibronectin (Biocat, Becton Dickinson Labware). To avoid the possibility of contaminating the assay with mature circulating endothelial cells, we performed an initial pre-plating step in a fibronectin-coated six-well plate using 5x10⁶ mononuclear cells per well. After 48 hours, the nonadherent cells were collected and 1x10⁶ cells were replated onto a fibronectin-coated 4-well chamber slide for a final assessment of the number of colonies. Endothelial growth medium was supplemented with FGF-2 (10ng/ml) and VEGF (100ng/ml) (Peprotech Inc., New Jersey, USA), and was changed every three days. The number of colonies
PB-ECs was counted manually by two operators in blind seven days after plating, in eight wells. Confirmation of endothelial-cell lineage was performed in all samples by immunofluorescence.

Immunofluorescence

After washing with phosphate buffered-saline (PBS; Gibco) only cells stained for von Willebrand factor (vWF) were permeabilized with 0.1% Triton X-100, for 10 minutes. All cells were blocked for 30 minutes with PBS + 2% of Bovine Serum Albumin (BSA; Sigma-Aldrich). After block, cells were stained with primary antibodies: mouse monoclonal anti-human Ve-Cadherin (Ve-Cad) (Chemicon; 1:25) at room temperature for 1 hour, mouse monoclonal anti-human vWF (Clone F8/86; Dako; 1:50) at 37°C for 1 hour and primary goat monoclonal anti human Ulex europaeus (Vector; 5 µg/ml) at room temperature for 1 hour. The primary antibody was removed, the chamber slides were washed with PBS and incubated for 1 hour with secondary antibodies: goat anti mouse FITC-conjugated IgG (Chemicon; 1:500) for Ve-Cad and vWF and rabbit anti goat Texas Red-conjugated IgG (Vector; 20µg/ml) for Ulex europaeus staining. The cells were counterstained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI; Roche Diagnostic; 1:10000) for 15 min at room temperature. Cells were viewed by fluorescence microscopy using a Nikon Eclipse 80i microscope.

Reproducibility of PB-ECs Culture Assay

Intra-assay and inter-assay-over-time variability of PB-ECs was tested with the method of Bland and Altman (Bland and Altman 1986) in which the difference between two repeated measurements is plotted against the mean of the same two measurements to calculate a mean bias with 95% confidence interval (95% CI). For the intra-assay, the first and the second PB-ECs measurements were obtained from the first and the second well of each subject respectively. The inter-assay-over-time of PB-ECs was assessed by performing a new analysis at 90 days in 8 randomly selected subjects.

Table 1.  Data from the Male and Female Subjects Evaluated in the Study

<table>
<thead>
<tr>
<th>Subject</th>
<th>All subjects n = 37</th>
<th>Males n = 15</th>
<th>Females n = 22</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>40.2 ± 15.0</td>
<td>42.2 ± 15.0</td>
<td>40.0 ± 15.0</td>
<td>0.52 ns</td>
</tr>
<tr>
<td>BMI (unit)</td>
<td>22.1 ± 2.6</td>
<td>23.9 ± 2.1</td>
<td>20.7 ± 2.0</td>
<td>&lt;0.0002 ***</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>115.0 ± 11.5</td>
<td>118.5 ± 12.1</td>
<td>113.2 ± 11.2</td>
<td>0.33 ns</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>75.4 ± 7.2</td>
<td>80.0 ± 7.6</td>
<td>73.2 ± 6.0</td>
<td>0.03 *</td>
</tr>
<tr>
<td>hs-CRP (mg/dl)</td>
<td>0.15 ± 0.16</td>
<td>0.18 ± 0.20</td>
<td>0.13 ± 0.13</td>
<td>0.35 ns</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>246.0 ± 145.7</td>
<td>213.5 ± 122.9</td>
<td>268.9 ± 159.5</td>
<td>0.32 ns</td>
</tr>
<tr>
<td>TIMP-1 (ng/ml)</td>
<td>156.2 ± 42.7</td>
<td>165.8 ± 34.1</td>
<td>149.9 ± 47.4</td>
<td>0.34 ns</td>
</tr>
<tr>
<td>PB-ECs/10⁶ cells</td>
<td>0.40 ± 0.82</td>
<td>0.56 ± 1.20</td>
<td>0.28 ± 0.42</td>
<td>0.32 ns</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD.

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, hs-CRP: high-sensitivity C-reactive protein, VEGF: endothelial cell-specific mitogen factor, TIMP-1: tissue inhibitor of metalloproteinases-1, PB-ECs: peripheral blood endothelial colonies.
Formation of PB-ECs Colonies

Typical PB-ECs colonies were formed by 40.5% of studied subjects (Yes/No = 15/22) with a mean of 0.40 ± 0.82 colonies/10^6 cells. Only colonies consisting of multiple thin, flat cells originating from a central cluster of rounded cells were counted. The colonies coexpressed Ve-Cad, vWF, Ulex, providing a confirmation of endothelial lineage (Fig. 1).

The frequency of formation of PB-EC was 46% in males and 36% in females, while males showed a higher absolute value of PB-ECs (p=0.32). The subjects forming PB-ECs (n=15) compared with those not forming PB-ECs (n=22)

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Table 2. Endothelial Circulating Progenitor Cells in Males and Females

<table>
<thead>
<tr>
<th></th>
<th>Males n = 15</th>
<th>Females n = 22</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Late EPCs [P1H12+/CD31+] (n°cells/µl)</td>
<td>16.93 ± 11.48</td>
<td>18.44 ± 13.35</td>
<td>0.73</td>
</tr>
<tr>
<td>Early EPCs [KDR+/144-] (n°cells/µl)</td>
<td>6.69 ± 5.97</td>
<td>6.71 ± 6.50</td>
<td>0.99</td>
</tr>
</tbody>
</table>

The number of cells/µl is obtained from >100,000 events

EPCs: endothelial progenitor cells

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Fig. (1). Morphological and immunofluorescence features of endothelial cells.
Formation of endothelial monolayer after 7 days of culture in subjects without colonies (a: brightness; a1: immunofluorescence for vWF (40x)). In vitro isolated endothelial colony (PB-EC) obtained after 7 days of culture (panel b and c) (40x). All colonies were positive for vWF (b1), Ve-cad and Ulex europaeus (c1). Panels (a2,b2,c2) 3D-rendering from panels (a1,b1,c1) based on colour level intensities.
showed a higher value of hs-CRP (0.276 ± 0.230 vs 0.095 ± 0.077 mg/l; p = 0.003). Similarly, higher values of VEGF were detected in colony formers as compared with non-formers (328.3 ± 162.9 vs 202.6 ± 118.5 pg/ml; p = 0.02). A direct correlation was found between hs-CRP values and the number of colonies in subjects with colonies (r = 0.36; p = 0.04). No significant differences in TIMP-1 values were found when comparing non-formers with formers (Table 3). Furthermore, there was no significant correlation of hs-CRP, VEGF and TIMP-1 with age, sex, BMI, SBP and DBP.

**Reproducibility of PB-ECs Assay**

When the Bland-Altman method was applied to define the intra-assay variability the mean bias between the first and the second measurements of colonies obtained from the first and the second well in each subject was -0.52 ± 4.25 (95% CI, from -1.6 to 0.6). For the inter-assay-over-time, the mean bias between the first and the second measurements obtained at 90 days from the first well in 8 randomly selected subjects was -1.27 ± 1.96 (95% CI, from -2.24 to 0.3).

**DISCUSSION**

Endothelial progenitor cells are responsible for the maintenance of the complex network of vessels as a component of the cardiovascular system in health and disease. Several factors are known to be involved in pathologic conditions in the regulation of endothelial compartment, while very limited information exists on its physiological homeostasis. Indeed, the role of bone marrow-derived cells in the regenerative mechanisms after vascular damage has been extensively evaluated in a number of in vivo studies (Asahara et al. 1999; Ciulla et al. 2003) and recently it has been also exploited in several phase I/II clinical trials (Stamm et al. 2003). In addition, many factors are known to be involved in the regulation of angiogenesis, in particular after ischemic damage. In this context, reliable and sensible methods aimed at the evaluation of EPCs are warranted. Flow cytometric analysis has been recently suggested as a quantitative method for the enumeration of EPCs, although the rarity of these cells and the lack of a clear definition of their immunophenotype still represent a technological challenge. In order to measure the burden of normal angiogenesis and to evaluate the physiological mechanisms involved in the regulation of the vascular system, we used a clonogenic assay to assess the number of endothelial colony forming units in the PB of healthy subjects. This method allows also a functional evaluation of the endothelial potential and improves the reliability of the information that could be obtained by flow cytometry.

We assessed the frequency of PB-ECs, together with the level of several factors involved in their regulation such as VEGF, TIMP-1 and hs-CRP, in a healthy population at very low cardiovascular risk in order to define the burden of normal angiogenesis and the influence of demographic variables such as age and gender.

**Circulating Endothelial Growth Factors and Precursors**

In the present study, the frequency of PB-ECs and the ECs cell-specific mitogen VEGF level seem to be unrelated to age and gender. This finding is an important difference between healthy and pathologic conditions, since aging has been recently correlated to an impairment of endothelial function in subjects affected by cardiovascular diseases (Weinsaft and Edelberg 2001). On the lack of sex related differences, no studies are available in normal subjects except experimental data on mice suggesting that estrogens could improve endothelial function and accelerate re-endothelialization after vascular injury (Strehlow et al. 2003). Our data support the concept that in healthy individuals, where a normal vascular homeostasis is supposed, PB-ECs and VEGF should be considered as individual variables, as also suggested by recent findings addressing the existence of common genetic pathways involved in the heritability of circulating growth factors (Pantsulaia et al. 2004). Accordingly, we found a relative constant colony forming capacity over time, as shown by the low mean bias between two measurements over time.

**Table 3. Multivariate Analysis Comparing Subjects with and Without Colonies**

<table>
<thead>
<tr>
<th></th>
<th>Without Colonies</th>
<th>With Colonies</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>37.4 ± 14.6</td>
<td>44.3 ± 15.0</td>
<td>0.17 ns</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>8/14</td>
<td>7/8</td>
<td>0.53 ns</td>
</tr>
<tr>
<td>BMI (unit)</td>
<td>21.7 ± 2.5</td>
<td>22.6 ± 2.7</td>
<td>0.33 ns</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>114.3 ± 11.9</td>
<td>116.2 ± 11.5</td>
<td>0.71 ns</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>74.6 ± 6.2</td>
<td>76.8 ± 8.8</td>
<td>0.50 ns</td>
</tr>
<tr>
<td>hs-CRP (mg/dl)</td>
<td>0.095 ± 0.077</td>
<td>0.276 ± 0.230</td>
<td>0.003 **</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>202.6 ± 118.5</td>
<td>328.3 ± 162.9</td>
<td>0.02 *</td>
</tr>
<tr>
<td>TIMP-1 (ng/ml)</td>
<td>155.2 ± 45.8</td>
<td>158.0 ± 38.9</td>
<td>0.87 ns</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD.
BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, hs-CRP: high-sensitivity C-reactive protein, VEGF: endothelial cell-specific mitogen factor, TIMP-1: tissue inhibitor of metalloproteinases-1.
When attempting to characterize our population in respect of colony forming capacity, associated with higher VEGF values we found, for the first time, higher hs-CRP values in colony formers supporting the concept that hs-CRP is a mediator involved in ECs function and not solely a biomarker of cardiovascular risk (Verma et al. 2005). In fact, the low levels of hs-CRP, formerly not associated with an increased cardiovascular risk, suggest that hs-CRP could contribute to normal EC homeostasis in physiological conditions causing minimal stress to the endothelium or following subclinical damages requiring minimal tissue repair.

Inflammation-Angiogenesis Cross-Talk

The physiological response to cell injury is characterized by a reaction of blood vessels initiated by regional vasodilatation, leading to the accumulation of fluid and leukocytes in extravascular tissues and followed by enhanced angiogenesis to facilitate the healing process (Frantz et al. 2005). These events are orchestrated by several cytokines activated in cascade and triggered by the inflammatory stimulus that, potentially, includes all causes of cell injury. The major cytokines that mediate inflammation are IL-1 and TNFα, β. They are produced by activated macrophages and T cells, and their most important actions are the effects on endothelium, leukocytes, and fibroblasts. The effects on endothelium, referred as to endothelial activation, are considered to give rise to the angiogenic response establishing the link between inflammation and angiogenesis, where EC is the main cellular player since it is involved in the secretion of angiogenic factors, mainly VEGF acting as a growth factor-related cytokine, and, at the same time, in response to them. The same activated T cells responsible for the production of TNFβ have been demonstrated, following experimental hypoxia, to produce directly VEGF and to express the specific VEGF receptor, making sense to the well-known redundancy of the cytokine system (Mor et al. 2004). Regarding the significance of VEGF elevation, it is noteworthy that specific isoforms of this cytokine are involved both in vasculogenesis and hematopoiesis; VEGF has been shown to be elevated both in physiological (e.g. altitude hypoxia, Tissot et al. 2005) and pathological ischemic conditions, such as myocardial infarction (Shintani et al. 2001), and peripheral arterial occlusive disease (Sandri et al. 2005).

Not only resident EC are involved in the angiogenetic response; the interaction between VEGF, chemokines and cellular pathways potentially involved in adult angiogenesis have been recently elucidated (Grunewald et al. 2006), suggesting that VEGF is involved also in EC recruitment, homing and retention in the sites of damage. In a murine model of soft tissue ischemia, BM-derived circulating EPCs have been locally recruited to ischemic tissue within 72 hours producing new blood vessels (Tepper et al. 2005), and pathologic tissue ischemia in experimental animal models has been demonstrated to increase the frequency of EPCs, thereby contributing to neovascularization (Takahashi et al. 1999). Furthermore, in vitro induced anoxia has been shown to enhance the differentiation of PB mononuclear cells from healthy subjects into EPCs (Akita et al. 2003). In this regard, the administration of a topical VEGF formulation has been shown to accelerate cutaneous wound healing of diabetic mice by increasing both circulating and wound-related levels of EPCs (Michaels et al. 2005). In physiological conditions, exercise is known to upregulate EPCs and to decrease the rate of EPC apoptosis (Laufs et al. 2004). Unfortunately, angiogenesis biomarkers show wide ranges that overlap in subjects with disease and in those without it, and studies on healthy subjects are lacking. For this reason, various cutoff points should be tested for their ability to detect disease even at subclinical stage, followed by definition of threshold levels with maximized sensitivity and specificity as suggested by a recent paper providing evidences that angiogenesis biomarkers are heterogeneously expressed in patients with carotid atherosclerosis (Porcu et al. 2004). At systemic level cytokine activation, and in particular IL-6, is followed by CRP secretion from the liver which acts as ligand-binding protein for complement. Growing amounts of data have shown that CRP itself acts on monocyte/macrophages, endothelial cells, and smooth muscle cells to secrete an array of proinflammatory molecules that have been shown to be directly or indirectly associated with the progression of atherogenesis in pathological conditions (Venugopal et al. 2005). A significant positive correlation was found between pre-interventional hs-CRP level and coronary neointimal hyperplasia, consisting in smooth muscle proliferation and migration from the underlying media, suggesting that hs-CRP level may help predict the development of restenosis after stenting (Hong et al. 2005); hs-CRP has also been demonstrated to be an excellent marker for plaque instability or poststenotic inflammatory status, as shown by the local release in coronary arterial blood, sampled just distal and proximal to the culprit lesions, in patients with stable and unstable angina (Inoue et al. 2005). In a recent paper the sympathetic tone in acute coronary syndromes has been correlated to IL-6 and hs-CRP (Hamaad et al. 2005); furthermore, serum hs-CRP level, measured immediately before coronary angiography, was an independent marker of coronary vasospasm in patients who had no hemodynamically significant coronary artery disease (Hung et al. 2005). In normal subjects, elevation of hs-CRP within the conventional normal range associated with VEGF changes and PB-ECs, as found in about 40% of our subjects, suggests a possible association to local low grade inflammation processes; the pathophysiological basis for the observed association may relate to potential physiological effects such as hypoxemia and reoxygenation, increased sympathetic activation, vascular endothelial dysfunction, oxidative stress, and metabolic dysregulation that may upregulate systemic inflammation. In this regard, data confirm that CRP is not exclusively produced by the liver but also by macrophages and the stimulus for the production might be lipid peroxidation and infections triggering a proinflammatory cytokine cascade (Dong and Wright 1996). Resembling other observations, we hypothesize that the effects of hs-CRP are different depending on its relative concentration: at low concentrations, hs-CRP may upregulate systemic inflammation. In this regard, data confirm that CRP is not exclusively produced by the liver but also by macrophages and the stimulus for the production might be lipid peroxidation and infections triggering a proinflammatory cytokine cascade (Dong and Wright 1996). Repeating these observations, we hypothesize that the effects of hs-CRP are different depending on its relative concentration: at low concentrations, hs-CRP may upregulate systemic inflammation. In this regard, data confirm that CRP is not exclusively produced by the liver but also by macrophages and the stimulus for the production might be lipid peroxidation and infections triggering a proinflammatory cytokine cascade (Dong and Wright 1996). Repeating these observations, we hypothesize that the effects of hs-CRP are different depending on its relative concentration: at low concentrations, hs-CRP may upregulate systemic inflammation.
acterize a subpopulation who deserves deeper investigation. Nevertheless, we cannot exclude that this subpopulation includes subjects with very low colony frequency under the detectability threshold of EPCs clonogenic assay. On the other hand, in subjects who are able to form colonies, undislosed vascular stress could be responsible for the activation of ECs function by increasing the specific mitogen factor VEGF. In this regard, it is noteworthy that both induction and modulation of angiogenesis via VEGF modulation has been correlated to the mechanism of response to low oxygen concentration such as exercise (Laufs et al. 2004) and high altitude hypoxia (Ciulla et al. 2005). The cascade of these events are non inhibited by the TIMP-1 levels, a known suppressor of angiogenesis (Akahane et al. 2004), that in our subjects were within the normal-low range; this fact confirms that VEGF and TIMP-1 are temporally regulated as TIMP-1 activated later to prevent uncontrolled action of MMP (Stetler-Stevenson et al. 1996). Accordingly in our subjects, the clonogenic potential as assessed by PB-ECs is related to VEGF levels and does not seem to be temporally associated with TIMP-1 levels.

Technical Limitations

On the technical point of view, the main limitations of all studies based on circulating EPCs enumeration consist in the lack of a uniform immunophenotype definition of EPCs (Rafii and Lyden 2003) and therefore of an experimental method to discriminate between different populations (Khan et al. 2005). Furthermore, EPCs are extremely rare events in normal PB, representing somewhere between 0.01 and 0.0001% of peripheral mononuclear cells and the reliable enumeration EPCs by flow cytometry remains a technical challenge. Since progenitor cells are identified by their clonogenic and proliferative potential, in this study we used a multistep technique to assess this potential by isolating and culturing in vitro mononuclear cells on fibronectin substrate. Even if it could be argued that a subjective evaluation of staining may affect the reliability of our results, in our study we carefully standardized the culture procedures as demonstrated by limited intra-assay and inter-assay over-time bias.

Clinical Implications

Even in normal subjects, defined according to several selective criteria, quite an elevated number of subjects who formed PB-ECs were found suggesting an active homeostasis involving the endothelium, possibly associated to local low grade inflammation processes (denoted as microinflammation) revealed by higher hs-CRP levels. Therefore, the determination of microinflammation in apparently healthy individuals might have prognostic significance in terms of future vascular events and accelerated atherothrombotic disease; in this regard, it has been shown that atherothrombosis is associated with the presence of low grade subclinical inflammation (Libby et al. 2002) and hs-CRP assays have emerged as promising laboratory methods for the determination of the presence of this microinflammation (Ridker 2001). Finally, we cannot exclude that hs-CRP might have also a direct pathogenetic role as well (Jialal et al. 2004) since the inflammatory response is characterized by a complex cross talk between local and systemic factors acting as growth-factors-cytokines.

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