Circulating Endothelial Cells: Realities and Promises in Vascular Disorders

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Abstract
Endothelial contribution to human vascular disorders is difficult to investigate, owing to the paucity of non-invasive methods and of specific endothelial markers. Circulating endothelial cells (CECs) might be used as a surrogate non-invasive marker for the study of vascular alterations. To address this problem, we produced an antibody against the endothelial molecule CD126 (S-Endol) and developed, in the nineties, an original and sensitive immunomagnetic separation assay. Using this approach, we demonstrated elevated number of CECs in clinical diseases linked with vascular injury like heart catheterization, sickle cell anemia, bacterial infection, thrombotic thrombocytopenic purpura or acute coronary syndromes. CECs correspond to very rare cells present in blood since levels in the range of 3 cells/ml are detectable in these pathologies. Several clinical interest of CECs will be discussed including their relevance as marker of disease activity, severity or treatment efficacy, or their use in diagnostic tests. The origin of endothelial cells in peripheral blood is difficult to establish. They could correspond to endothelial cells dislodged from the vessels in response to injury. It was subsequently shown that a subset of CECs comprised a population of bone marrow-derived endothelial progenitors that participate in angiogenesis. Identification of the origin and characteristics of CECs provides fascinating insights into endothelial cell pathophysiology. Moreover, CECs constitute original and promising tools for diagnosis, prognosis and therapy of vascular disorders.

Introduction
The endothelium is one of the largest organs of the body, consisting of more than $10^{14}$ cells lining the vascular tree. Strategically located between blood and tissues, the endothelium plays a crucial role in the control of several fundamental responses such as haemostasis, blood pressure regulation and angiogenesis [1]. The structural and functional integrity of the endothelium is essential for the maintenance of vascular homeostasis, and loss of function leads to thrombosis, hypertension and oedema. Endothelial function can be assessed by the measurement of soluble markers, such as von Willebrand factor (vWF) released into the blood, and by physiological techniques such as flow mediated dilatation [2]. Although endothelial cells themselves were first demonstrated in the blood over 30 years ago (hence circulating endothelial cells (CECs)), they have been recog-
nised only recently as an additional marker for assessing vascular integrity [3].

The objective of this article is to 1) review the current status of CECs, 2) document methodological issues adapted to the concept of rare events, 3) examine pathological situations associated with CECs and their value in clinical medicine, and 4) consider the origin of CECs.

Circulating Endothelial Cells: from the Concept of Rare Events to Technical Issues

Bouvier and Hladovec were the first to report the presence of non-hematopoietic cells of possible endothelial origin in human blood. On the basis of morphology, intact nucleated cells were recovered in leukoconcentrates by Bouvier et al [4] whereas anuclear "carcasses" were detected in platelet rich plasma by Hladovec [5]. Several authors subsequently described similar cells in different models of endothelial damage such as shock by E. Coli endotoxin in animals [6], or smoking, acute myocardial infarction, immunosuppression, hypertension and homocysteinaemia in man [7-11]. However, divergent results were reported by these groups, due to the variety of cell fractions studied, cell identification by morphology, and methods of cells concentration, based on physical properties such as size or density.

Like tumour cells or trophoblast cells, CECs belong to the population of rare non-hematopoietic cells present at a very low frequency in peripheral blood. Their accurate detection has to fulfil at least two prerequisites: cell enrichment of these rare cells from whole blood using a sensitive method, and subsequent identification with a specific endothelial marker. We addressed these requirements by using an immunophysical method combining cell enrichment and specific labelling by the use of magnetic beads coupled to a monoclonal antibody (S-Endo1) directed against the endothelial antigen CD146 [12]. This adhesion molecule belonging to the Ig superfamily is largely distributed on all types of endothelial cells but is not detectable on hematopoietic cells [13,14]. CD146 is concentrated at the endothelial junction where it plays a key role in the control of cell-cell cohesion, permeability and signalisation [15,16]. Briefly, magnetic dynabeads coated with anti-CD146 monoclonal antibody are mixed with venous blood in a head over head mixer. Unbound cells are washed out with buffer, bound cells are retained by a magnet. Cells forming rosettes with the beads are recovered in two fractions, used respectively for CEC counts and subsequent phenotypic characterization. CECs are identified according to size and number of

<table>
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<tr>
<th>Cardiovascular disease</th>
<th>Number of CECs/mL</th>
<th>Immunological Markers</th>
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<tbody>
<tr>
<td>Coronary angioplasty [17,19]</td>
<td>6-10</td>
<td>&lt;3</td>
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<tr>
<td>Acute coronary syndromes [20]</td>
<td>7.5</td>
<td>0</td>
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<tr>
<td>Sickle cell anaemia [21]</td>
<td>13.2 - 22.8</td>
<td>2.6</td>
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<td>Pulmonary hypertension [22]</td>
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<td>3.5</td>
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<tr>
<td>Peripheral vascular disease [23, 42]</td>
<td>1.1 - 3.5</td>
<td>0.9</td>
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<tbody>
<tr>
<td>Rickettsial infection [24]</td>
<td>5-1,600</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Cytomegalovirus infection [18, 25]</td>
<td>45</td>
<td>4</td>
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<tr>
<td>Septic shock [26]</td>
<td>16.1</td>
<td>1.9</td>
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<td>6-220</td>
<td>&lt;3</td>
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<tr>
<td>Behcet's disease [28]</td>
<td>0-25</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Systemic lupus erythematosus [29]</td>
<td>32</td>
<td>5</td>
</tr>
<tr>
<td>Inflammatory vasculitis [31]</td>
<td>136</td>
<td>5</td>
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<tr>
<td>Kawasaki disease [32,39]</td>
<td>15</td>
<td>6</td>
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<tr>
<td>Bone marrow transplantation [35, 44]</td>
<td>16-44</td>
<td>8</td>
</tr>
<tr>
<td>Renal transplantation [32-34]</td>
<td>24-72</td>
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<tr>
<td>Breast cancer, lymphoma [36]</td>
<td>6,800-39,100</td>
<td>2,00-7,900</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD 34</td>
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<td>CD 45</td>
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beads bound (i.e. more than 10 beads bound to cells of 20-50 μm diameter). The endothelial nature of cells isolated by the beads is confirmed by vWF expression and lack of expression of leukocyte antigens such as CD45. Thus using anti-CD146 antibody and immunomagnetic separation, we unequivocally demonstrated that CECs exist and can be detectable in peripheral human blood [19,20], a technique now widely adopted in a wide variety of conditions. Other endothelial-associated markers such as endoglin [17] or vWF have been used by other groups [18]. An alternative to the immunobeads method is flow cytometry [36] were blood is labelled antiendothelial monoclonal antibodies.

Pathological Settings Associated with CECs

In humans, CECs have been detected in diverse conditions of endothelial injury (Table 1). Elevated levels were reported in various cardiovascular disorders, as a result of mechanical injury, ischemic injury or hypertension [17,19,20-23]. Among infectious diseases, high levels of CECs were also reported in pathologies in which the endothelium is the target of pathogens such as Rickettsia [24], cytomegalovirus (CMV) [18, 25] and in septic shock [26]. Increased numbers of CECs have also been described in association with immune disorders (thrombotic thrombocytopenic purpura, Behcet's disease, systemic lupus erythematosus, Kawasaki disease and inflammatory vasculitis [27-31]), but also in transplantation [32,35], and in cancer [36]. There is a great variability in the levels of CEC reported in these diseases, ranging from an average of 1.1 to 1600 cell/ml. Part of this variability can be ascribed to the diverse disease processes as CEC counts vary according to the extent of the endothelial lesion. For example, a high CEC count is found in widespread vascular damage associated with Rickettsial vasculitis, sickle cell crisis, or CMV infection. In contrast, the number of CECs found in localized vessel damage such as coronary angioplasty only slightly exceeds the normal range. Of more concern is the variety in CEC levels in healthy controls, showing good agreement in groups using immunomagnetic beads and CD146, with values lower than 10 cells/ml [17-35]. In contrast, using the same immunological markers and flow cytometric detection, greater numbers of CEC (up to 1000-fold higher than the immunobeads methods) were detected in cancer patients and their relative controls [36].

Thus the literature clearly indicates that CECs are now considered as a marker to assess (mural) endothelial injury [19-24,27-38] However, the discrepancies of data obtained using immunomagnetic separation versus flow cytometry argues in favor of standardized methodologies.

Potential Value of CECs In Clinical Medicine

Since the level of CECs is very low in normal individuals, elevated levels represent a non-invasive marker of potential use in documenting endothelial alterations on a quantitative basis. In various situations, the longitudinal quantification of CEC showed that levels vary according to the clinical evolution, with a good relationship with disease severity. Several studies in the literature demonstrated that CEC levels in patients who are acutely ill are higher than those in patients in clinical remission or in recovery phase of the disease. For example, CECs are higher in acute myocardial infarction than in angina [20], and are higher in critical limb ischaemia than in intermittent claudication [23]. The iterative monitoring of CECs in sickle cell patients showed that their level increases at the onset of painful episodes, suggesting their predictive value of acute crisis [21]. In patients with Mediterranean spotted fever, the most elevated values were detected in patients with malignant forms who developed thrombotic complications [24]. A strong correlation between CEC numbers and disease severity was reported in patients with inflammatory vasculitis, whereas CEC numbers fell when patients were in remission [32]. Apart from this prognostic value, the CEC count can also have a diagnosis value, in combination with other biological markers. In patients with non ST elevation acute coronary syndrome, a multi-marker strategy combining CEC count and troponin level increases the number of patients diagnosed within the six first hours after chest pain (Personal data).

From a pharmacological point of view, CEC level can be used to monitor the efficiency of the therapy. Renal transplant recipients who received cyclosporine had higher number of CEC than their age and creatinine matched counterparts who did not receive these drugs [32]. In the same manner, high CEC counts were reported in patients being pre-conditioned for allogenic bone marrow transplantation with total body irradiation and cytotoxic agents, cyclophosphamide and busulphan [35]. A working hypothesis is that mural endothelial cells are detached as a result of injury induced by condition regimen. In contrast, in patients with thrombocytopenic purpura, plasma exchange in combination with vincristine results in lower CEC counts [27].

In addition to the quantitative aspect, the analysis of the phenotypic and function of CEC potentially provides useful informations since CECs are submitted to the same environmental stimuli and blood born activating factors that vessel wall endothelium. Phenotypic analysis of CEC is also useful to determine whether they circulate in an activated state. For example, tissue-factor is expressed on CECs from patients with acute coronary syndromes or sickle cell anaemia [20,38] suggesting that they may participate in the activation of tissue factor pathway. Whether or not this...
property is clinically important is unknown but it is tempting to speculate that some CECs do indeed contribute adversely to the disease process by activating prothrombotic pathways. In addition, regardless of clinical status, most CECs from sickle cell patients express adhesive receptors for leukocytes (ICAM-1, VCAM-1, E selectin), suggesting that the endothelium they come from adopts a pro-inflammatory phenotype [21]. Interestingly sulfasalazine, an inhibitor of NFκ, significantly reduced the expression of adhesion molecules in CECs from patients and from sickle transgenic mice [38]. This pilot study indicates that not only the number but also the level of activation of CECs can be used to evaluate the effectiveness of drug therapy targeted to diseases of the endothelium. Therefore, more than a non-invasive marker of endothelial injury, CECs may represent an opportunity to study endothelial phenotype and functions and thus explore endothelial associated vascular disorders.

**Origin of Blood Endothelial Cells**

A key question is to know from which anatomical region CECs are detached. CD36 labelling has been used to determine whether or not CECs arise from micro- or macro-vessels. CD36 positivity indicated that CECs were mainly of micro-vessel (mesenchymal) origin in sickle cell patients [21] and cancer (36), whereas none of the CECs recovered in acute coronary syndromes stained for CD36, suggesting that they detached from large vessels [20]. It is tempting to speculate that the increased number of CECs following acute myocardial infarction arises from the coronary arteries or from the chambers of the heart, but no clear data are available. An important future development will be to analyse tissue specific markers to target the anatomical site of vessel injury. Although we have focussed up to now on CECs that may likely arise from mural endothelial cells, there is another population of endothelial-like cells found in the blood that may arise from the bone marrow [39]. Knowledge accumulated these 15 last years, indicates that the endothelium is a tissue with a high plasticity in equilibrium between 3 different compartments, vessel wall endothelium, blood endothelium cells, and bone marrow endothelium cells defining a reservoir of Progenitor Endothelial Cells (PECs) [46]. These cells may be recruited in response to angiogenic stimuli, such as VEGF, ischemia or vascular trauma. They are mobilized in the peripheral blood where they become circulating (CEPs) and home to sites of neovascularization. These CEPs can be discriminated from CECs on the basis of phenotypic and functional characteristics. Indeed, they are defined by the co-expression of immaturity markers (CD133) and endothelial markers such as CD31, VE-cadherin, and are characterized by an ability to form in vitro colonies with a high proliferative potential [39,40]. One function of these CEPs may be to replace CECs damaged or destroyed by a pathological process(es). Identification of the origin of blood endothelial cells may facilitate the use of these cells in clinical diagnosis and biomedical applications [41].

**Conclusions**

Increased number of CECs in various diseases reflects severe vascular disturbance and may contribute adversely to the disease process. In contrast, CEPs may play a key role in the regenerative response of the vessels and represent an excellent autologous biomaterial source for cell-based therapeutic application, as already demonstrated in the treatment of ischemic disorders. These different subpopulations of blood endothelial cells open fascinating new directions in the study of endothelial injury and repair.

**References**

Disorders

Circulating Endothelial Cells in Vascular Disorders

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