

Review Article

Circulating endothelial cells

Biomarker of vascular disease

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Summary

Recent research has recognised new populations of non-hematopoietic cells in the blood. One of these, circulating endothelial cells (CECs), often defined by the expression of membrane glycoprotein CD146, are rarely found in the blood in health, but raised numbers are present in a wide variety of human conditions, including inflammatory, immune, infectious, neoplastic and cardiovascular disease, and seem likely to be evidence of profound vascular insult. An additional population are endothelial progenitor cells, defined by the co-expression of endothelial and immaturity cell surface molecules and also by the ability to form colonies *in vitro*. Although increased numbers of CECs correlate with other markers of vascular disease, questions remain regarding the precise definition, cell biology and origin of CECs. For example, they may be damaged, necrotic or apoptotic, or alive, and could possess procoagulant and/or proinflammatory

properties. However, since these cells seem to be representative of *in situ* endothelium, their phenotype may provide useful information. Indeed, whatever their phenotype, there is growing evidence that CECs may well be a novel biomarker, the measurement of which will have utility in various clinical settings related to vascular injury. Despite this promise, progress is impeded by the diversity of methodologies used to detect these cells. Accordingly, results are sometimes inconclusive and even conflicting. Nevertheless, increased CECs predict adverse cardiovascular events in acute coronary syndromes, suggesting they may move from being simply a research index to having a role in the clinic. The objective of the present communication is to condense existing data on CECs, briefly compare them with progenitor cells, and summarise possible mechanism(s) by which they may contribute to vascular pathology.

Keywords

Circulating endothelial cells, von Willebrand factor, E-selectin, tissue factor, CD146

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Introduction

The last twenty years have demonstrated unequivocally that well-maintained endothelial function and integrity is of undoubted importance in numerous conditions, including infectious, neoplastic, inflammatory and cardiovascular disease and its risk factors. One approach to the assessment of endothelial functioning is changes in specific plasma markers such as von Willebrand factor, soluble thrombomodulin, tissue plasminogen

activator, soluble endothelial cell protein C receptor (sEPCR) and soluble E selectin: another is by physiological techniques such as flow mediated dilatation after reactive hyperaemia (1, 2). More recently, an additional method for assessing vascular integrity has been developed: the measurement of immunologically-defined circulating endothelial cells (CECs) in the peripheral blood (3). These cells are thought to originate from sloughing off the vessel wall following some form of pathological insult. They are defined phenotypically by the expression of endothelial

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markers (e.g. von Willebrand factor, VE-cadherin, CD146) together with the absence of the expression of leukocyte (CD45) and immaturity markers (CD133).

More recently, another population of cells in the blood (often designated as circulating endothelial progenitor cells (EPCs)) have been described that are believed to arise not from the vessel wall but from the bone marrow, and to be important in repair following vascular damage. Defined variously by the co-expression of immaturity (e.g. CD34, CD133) and 'mature' endothelial markers (von Willebrand factor, VE-cadherin), and an ability to form colonies *in vitro*, EPCs may also contribute to angiogenesis and could be physiological replacements for replace dead, effete or damaged mural endothelial cells (4, 5).

The objective of this communication is to summarise the current status of CECs in terms of cell biology and its translation to clinical medicine. Although there is clearly some degree of commonality in how different investigators define CECs, there is also diversity in technique that leads to difficulty in comparing data between such groups. To achieve this we conducted a literature study in PubMed, Medline and Ebase to identify published work with key words circulating endothelial cells. Whilst acknowledging the advances brought by work in animal models, we shall focus primarily on human data on CECs. The

Table 1: Cell surface and other glycoproteins used to prepare phenotype and quantify CECs and EPCs.

CD Designation	Endothelial associated antigen	References
CD31	PECAM-1	4, 5, 33, 37, 38, 83, 87, 88
CD34	Immature haemopoietic/stem cell marker	4, 5, 45, 82, 84, 86–88
CD36	gpIV (IIb), thrombospondin receptor	26, 37, 40
CD45	Leucocyte common antigen	33, 45, 87, 88
CD54	ICAM-1	37, 87
CD62 E	E-selectin	26, 37, 39, 40, 45, 88
CD105	Endoglin	33, 87
CD106	VCAM-1	26, 33, 37, 45, 87
CD133	AC133, primitive haemopoietic cell marker	4, 33, 39, 44, 84
CD141	Thrombomodulin	52, 77
CD146	Mel-CAM, MUC18, A32, PIH12	4, 19, 22, 26, 28, 33, 34, 36, 37–41, 45, 52, 77, 87
Flt-1	Vascular endothelial growth factor receptor-1	5, 77, 88
KDR	Vascular endothelial growth factor receptor-2	4, 32, 40, 77, 82–88
Tie-2	Angiopoietin receptor	5, 86
Ulex Europaeus lectin I		36, 38, 44, 82, 85, 86, 88
Von Willebrand factor		26, 32, 38, 40, 52, 77, 85
Fibroblast growth factor receptor		4, 87
Vascular endothelial cadherin		4, 39, 85
Tissue factor		26, 77
Endothelial cell Protein C receptor		79, 80
Nitric oxide synthase		80, 87, 88, 94

The markers are used by different groups to isolate, define and characterise, endothelial cells, CECs, EPCs, leukocytes, cells of microvascular origin, and whether or not the cells are activated.

communication will close with a short comparison of CECs and EPCs.

Historical perspective

The undoubted pioneers, Bouvier, Gaynor and Hladovec and their colleagues, initially defined CECs over 30 years ago by techniques such as vital light microscopy using morphology and Giemsa staining, and separation by Ficoll density centrifugation (6–8). Animal models showed that damage or shock by *E. Coli* endotoxin, ovalbumin or trisodium citrate results in increased numbers of CECs, which, in the case of the latter, were frequently reported to be without a nucleus (7–9). Subsequent work in humans demonstrated changes in CECs in smoking, acute myocardial infarction, immunosuppression, hypertension and homocysteinaemia after methionine load, again some reporting CECs to be anucleate carcasses (10–14). Others defined endothelial cells isolated from blood by indirect immunofluorescence with antibodies to von Willebrand factor, an intracellular molecule that is gaining acceptance as a marker of this cell (15–17). However, poor specificity of these various techniques hampered research in this area and highlighted the need for a reliable, endothelial-specific cell surface molecule that could be used as an appropriate tool. Potential candidates were either non-specific for the endothelium (e.g. adhesion molecules, integrins) or were intra-cellular (e.g. tissue plasminogen activator, von Willebrand factor) and therefore relatively inaccessible to monoclonal antibodies, without cell permeabilization.

Simultaneously, in 1991, two groups reported monoclonal antibodies to new cell surface antigens on endothelial cells (HEC 19 and S-Endo 1) and used them to quantify blood-borne CECs (18, 19). Dignat-George and colleagues subsequently characterised their antibody (20, 21) as recognizing the CD146 molecule and used it to demonstrate CECs in a variety of conditions including rickettsial infection, sickle cell disease, thrombotic thrombocytopenia and acute coronary syndromes (3, 24–26). In 1997, Solovey et al. also used a monoclonal antibody (PIH12) against CD146 (22) to detect CECs in sickle cell anaemia (23). More recently, various groups have reported increased numbers of CECs in many diverse diseases, situations and conditions including infectious and cardiovascular disease, cancer, and inflammatory connective tissue disease (24–46). However, these reports are diverse not only with regard to the disease studied but also in the precise methods of isolation and detection employed. Not surprisingly, these technical variations impact on the number of CECs detected.

Methodological issues in CEC quantification

In practice, one of the major problems in clinical studies with the quantification of CECs are the low numbers present in the blood (Gaynor et al. suggests a frequency of approximately one per million nucleated cells (47)). However, improved detection of these rare events can be achieved by combining cell enrichment techniques with labelling of the cells with a specific marker. Different groups have used various techniques in the pursuit of cell enrichment, such as the elimination of red blood cells and con-

centration by centrifugation (10–14, 28, 29), preparation of a mononuclear cell suspension by density centrifugation (Ficoll, Percoll, Lymphoprep, Histopaque etc)(17, 23, 31, 32) and capture by immunomagnetic beads bearing CD146 (19–21, 24, 26). Once prepared, CECs may be phenotyped by monoclonal antibodies to cell surface glycoproteins (Table 1) using indirect immunofluorescence, immunocytochemistry, or by flow cytometry. Table 2 shows data on CECs in clinical case/control studies of human disease.

Due to the diversity of each of these diseases and methods, there is variability in the levels of CECs reported in disease states ranging from an average of 1 to 39,000 cells/ml of whole blood. Some of this variance can clearly be ascribed to the diverse disease processes in operation. However, of more concern is the wide variation of CECs in those supposedly healthy controls, ranging from zero to 7,900 cells/ml. There is a greater degree of agreement in those using the immunobead method (19, 24–27, 36, 38, 39, 42) and density centrifugation (32, 34, 37, 40), with values in the order of 10 cells/ml, whereas flow cytometry seems more sensitive, often reporting greater numbers, up to 1000-fold higher than the immunobead method (33, 45, 46).

Numerous groups have used the immunobead capture method initially developed by Dignat-George et al. (Table 3). The method has been slightly modified by others, e.g. the addition of albumin and EDTA as a countermeasure to minimise the auto-aggregation of CECs (23), bringing CECs to a glass slide and air drying before counting (a method that allows storage at room temperature for later counting as well as second-labelling) (42), and the use of the plant lectin Ulex Europeus-1 to aid positive identification (48) with use of a F_c blocking agent to minimise non-specific binding by leucocytes (36, 38, 44). Immunobead-generated CECs can also be double-labelled for other analyses, e.g. for CD133, CD34, or adhesion molecules (Table 1).

The most common alternative to the CD146/immunobead method is flow cytometry (a method used for EPC determination, see later in this document), where whole blood is generally labelled with monoclonal antibodies tagged with fluorochromes (33, 45, 46). An advantage of this technique is rapid multiparametric analysis, and the ability to detect sub-populations, such as 'bright' versus 'dim' labelling (34), and activated (e.g. bearing CD106) or resting (45), although (as discussed) CECs purified by the immunobead method can also be double labelled. However, there are substantial differences between the two methods, as indicated by the high variation in levels of CECs of healthy controls (Table 2). These differences may be secondary to isolation procedure, where a high error of measurement may exist due to inadequate standardization of flow conditions. For example, the gating of the forward and side scatter as well as the threshold may collect measurements not only for intact circulating endothelial cells but also aggregates of leukocyte-endothelial cells as well as endothelial cell microparticles. The fluorescence measurements will be difficult to interpret since a consequence of the choice of gating is that measurements include many non-CECs.

It is clear from the above that CD146 is one of the most popular, if not *the* most popular, choice of a marker for CECs. However, this molecule has also been described on trophoblast, mesenchymal stem cells, periodontal tissue and malignant (prostatic

Table 2: Reports of CECs in case/control studies of human disease, and particular methodology.

Condition (reference)	Method ¹	Number of CECs/ml ²	
		Cases	Controls
Coronary Angioplasty (19)	IB	6–10	<3
Sickle cell anaemia (23)	IB	13.2 – 22.8 ³	2.6
Rickettsial infection (24)	IB	5–1,600	<3
Thrombotic thrombocytopenia (25)	IB	6–220	<3
Acute coronary syndromes (26)	IB	7.5	0
Behcet's disease (27)	IB	0–25	<3
Chronic venous insufficiency (28)	SC	1,001	514
Aortoarteritis (29)	SC	58	16 ⁴
Septic shock (32)	DC	16.1	1.9
Breast cancer and lymphoma (33)	FC	6,800–39,100	1,200–7,900 ⁵
Systemic lupus erythematosus (34)	DC	32	5
Renal transplantation (36)	IB	24 – 72 ⁶	6
Thalassaemia (37)	DC	45	4 ⁷
Inflammatory vasculitis (38)	IB	136	5
Kawasaki disease (39)	IB	15	6
Pulmonary hypertension (40)	DC+IB	30	3.5
Peripheral vascular disease (42)	IB	1.1–3.5 ⁸	0.9
Bone marrow transplantation (44)	IB	16 – 44 ⁹	8
Systemic sclerosis (45)	FC	243–375	77
Systemic lupus erythematosus (46)	FC	89	10

¹Methods: IB = immunobeads; DC = density centrifugation, SC = standard centrifugation, FC = flow cytometry. ²As the distribution of CECs is often non-normal, median number is appropriate although some report the mean. ³Stable or crisis sickle cell disease. ⁴Controls are patients with inactive disease. ⁵Dependent on being resting or activated. ⁶Highest in acute rejection. ⁷Raw data not supplied, therefore estimated from a figure. ⁸Highest in critical limb ischaemia. ⁹Highest after pre-transplantation conditioning.

cancer, melanoma) tissues (49–51), so that some caution is demanded, especially in cancer.

Where do CECs come from and how do they appear?

Perusal of the list of diseases and conditions in table 2 supports the conclusion that the majority are associated with a severe and injurious pathology. It seems reasonable, therefore, that increased CECs numbers in the blood are the product of a disease process that irreversibly damages the endothelium. This theory is intrinsically attractive, given the knowledge of the pathophysiology of conditions in which CECs were found, such as established vascular damage in cardiovascular disease, the 'response to injury' theory of atherosclerosis (52), and the widespread vasculopathy of inflammatory connective tissues and infectious diseases (24, 32, 34, 38).

Solovey et al. (23) reported that about half of the CECs in healthy controls expressed CD36 (i.e. were of microvascular origin (53, 54)), a figure rising to 78% in patients with sickle cell anaemia. Mutin et al. (26) found no CECs from patients with acute coronary syndromes stained for CD36, indicating a macrovascular origin. Mutunga et al. (32) were able to culture CECs from six of fifteen patients with septic shock, proving at least some CECs to be viable. Lin et al. have also showed that vessel-

Table 3: Brief protocol for estimating CECs by Immunobead Capture (according to Dignat-George et al. (19, 24).

1. The subject is venesected from an ante-cubital vein: the first 4 ml (e.g. a vacutainer) are discarded as it may contain mural ECs removed by trauma.
2. A stock solution of immunomagnetic beads (diameter 4.5 μ m) coated with anti-CD146 antibody is prepared by mixing 100 μ l Anti-CD146 mAb (Biocytex, Marseille, France) with 125 μ l Dynabead at 4×10^8 beads/ml (themselves coated with an anti-pan mouse IgG) (DynaL Biotech ASA, Oslo, Norway) at room temperature for 30 min. Unbound CD146 is washed off with three washes with PBS and the stock coated beads are stored at 4°C.
3. Fifteen μ l from a preparation of 7×10^7 immunobeads/ μ l are mixed with venous blood diluted 1 ml with 3 ml in PBS in a head-over-head mixer for 30 min, at 4°C.
4. Excess bound and unbound cells are washed out with PBS four times inside a magnet at 4°C.
5. Rosetted cells are recovered and divided into fractions allowing staining with acridine orange (5 μ g/ml PBS, Sigma, Poole, UK) for counting with fluorescence microscopy and haemocytometer, and for subsequent cell characterisation.
6. CEC are defined (a) morphologically as CD146 rosetted cells, bearing more than 10 beads AND size, i.e. diameter 20–50 μ m, or bearing less than 10 beads but with a clear nucleus in a well-delimited cytoplasm and (b) immunologically, by the expression of endothelial markers and the lack of expression of leukocytes antigens.
7. Sheets or clumps of cells can often be found. For these aggregates, the number of cells is deduced from the number of nuclei or from the number of spherical rosetted features discriminated in the aggregate.

wall derived CD146⁺ CECs can be viable, although they have limited growth capability (55). At least half the CECs from patients with cancer (33) were of microvascular origin, whilst 70–80% of CECs from patients with thalassaemia were of microvascular origin (37). There was no immunoreactivity using anti-CD36 or anti-CD34 for CECs from patients with systemic lupus erythematosus, indicating a macrovascular origin (56).

Del Papa et al. (45) used flow cytometry directly on whole venous blood to define CECs as being negative for CD45, but bearing CD34 and CD146. Therefore, assessment of the state of activation of their CECs (as defined by the expression of CD62 (precise molecule not stated by the Authors but presumed to be CD62E) or CD106) is problematical. Notably, Nakatani et al. (39) estimated at 0–18% of CD146-defined CECs from patients with Kawasaki disease were also positive for CD133-defined EPCs. Similarly, Rajagopalan et al. (46) focussed on apoptotic CECs that co-stained for both CD146 and annexin V. Again, therefore, comparisons with data of total CD146⁺ CECs are difficult. Furthermore, variable levels of apoptotic CECs have been reported in SLE although these findings have not been uniformly verified (34, 46).

The potential mechanisms of endothelial cell detachment from the vessels are multiple and not exclusive. Different experimental models have documented that denudation of the vessels can be triggered by mechanical injury, defective adhesive properties of the endothelial cells, protease or cytokine mediated detachment, or the activation of an apoptotic program

Regarding defective adhesion, particular interest has been devoted to the role of endothelial adhesive molecules belonging to the integrin family that allow adhesion to matrix components such as vitronectin and fibronectin (57, 58) and also to the cadherin family, especially VE cadherin involved in the control of monolayer integrity (59). These molecules promote the assembly of cytoskeletal proteins and mediate signals critical for cell survival. Loss of the survival signals derived from anchorage to the extracellular matrix can trigger apoptosis (60).

Excessive detachment of the endothelium during CMV infection has been linked with the absence of expression of the integrin avb3 on CEC isolated *in vivo* (61). *In vivo* experiments also showed defective binding of infected cells to anchoring matrix proteins such as fibronectin, laminin, or type IV collagen (62). These effects may result in both excessive endothelial detachment and impaired vascular repair in Herpes Simplex virus infection. In the same clinical setting proteases released by granulocytes were also found to cause more detachment of the virally infected cells reported to be especially susceptible to inflammatory cells (62). It was recently reported that the integrin avb3 is involved in tumour EC detachment induced by TNF and IFN (57). *In vitro*, exposure of EC to these cytokines results in reduced activation of integrin avb3, leading to decreased avb3-dependent EC adhesion and survival. Detachment and apoptosis of EC was also evidenced *in vivo*, in melanoma metastasis of patients treated by TNF and IFN. These results demonstrate a new mechanism by which cytokines control cell adhesion. Recently, Clancy et al. suggested that nitric oxide (NO) may introduce a defect in adhesive property of endothelial cells. NO (via peroxynitrite) acts to decrease the affinity of α 5 β 1 integrin for its matrix ligand, which results in an increased level of CEC in SLE (34, 63).

Relationships between CECs and other markers of vascular damage

Numerous epidemiological studies have shown that increased levels of plasma markers such as von Willebrand factor, tissue plasminogen activator, and, to a lesser extent, soluble E selectin, suggest some degree of disturbance of the endothelium, be it damage, dysfunction or activation, and also indicate adverse cardiovascular outcome (1, 2, 64–66). More recently, impaired flow mediated dilation (FMD) has also been used to define endothelial dysfunction (67, 68), and as predicted, there is an inverse relationship between von Willebrand factor and FMD (69). Therefore, if increased numbers of CECs do indeed reflect a damaged endothelium, such levels should correlate with plasma markers and with FMD. Indeed, Rajagopalan et al. (46) found an inverse correlation between CD146⁺/Annexin V⁺ CECs and FMD.

Kas-Deelen et al. (30) found that CECs strongly ($p < 0.001$) correlated with von Willebrand factor but not with soluble E selectin, whilst Makin et al. (42) also correlated CECs with von Willebrand factor ($p = 0.002$), and also with plasma tissue factor ($p = 0.02$). Since von Willebrand factor is essentially the gold standard plasma marker for endothelial damage (2, 64–66), this strongly indicates that CECs also reflect severe vascular disturbance. Del Papa correlated numbers of total and activated (i.e. CD62⁺ CD106⁺) CECs with soluble E selectin, whilst Rajagopalan et al. (46) correlated CD146⁺/Annexin V⁺ CECs with plasma tissue factor, although this pro-coagulant may arise from monocytes/macrophages as well as from the endothelium (70).

Moreover, if we presume that CECs appear in the blood as the result of one or more pathological processes then we would also expect further evidence of vascular perturbation, such as the release of microparticles by activated or apoptotic endothelial cells. These are known to be biologically active in promoting thrombosis (71), and apoptotic endothelial cells are procoagu-

lant (72). As yet there are scarce data on relationships between microparticles and CECs.

Relationships between CECs and activity/severity of different diseases

Numerous studies in the literature have documented that increased numbers of CECs (by almost any definition) correlate with, and thus have common ground with, disease severity, as assessed by standard clinical methods.

In cardiovascular disease, the group of Dignat-George et al. (26) concluded that the highest numbers of CECs are found in the blood of subjects with the most severe and acute coronary artery disease. More recently, they demonstrated that CEC count, combined with troponin level, can be used as an early, specific, independent diagnostic marker for non-ST elevation ACS (73). Similarly, Makin et al. (42) found the highest CECs in those patients with the most severe peripheral artery disease whilst Bull et al. (40) and Del Papa et al. (45) reported a correlation between pulmonary artery blood pressure and CEC number. This relationship may be due to the high blood pressure damaging and thus converting mural endothelial cells into CECs. Notably, it has been established that the plasma marker of endothelial damage, von Willebrand factor, is raised in hypertension (74–76).

In the field of transplantation, Popa et al. (35) concluded that the appearance of donor CECs was due to post-transplantational injury. More recently, Woywodt et al. (44) showed elevated numbers of CECs in patients being pre-conditioned for allogeneic bone marrow transplantation, with lower cell numbers after reduced-intensity conditioning. A likely explanation is that the conditioning regime is such an insult to the vascular tree that mural endothelial cells are peeled off the intima to become CECs.

In inflammatory and connective tissue diseases, Dignat-George et al. reported the highest CEC levels in patients with malignant forms of Mediterranean spotted fever (24). Woywodt et al. reported (38) a strong correlation ($r=0.704$, $p<0.001$) between CEC number and the severity of disease in 86 patients with inflammatory vasculitis. In systemic sclerosis, Del Papa et al. (45) reported that total and activated CEC counts were positively correlated with the disease activity score. In SLE, a prototypic autoimmune condition in which circulating immune complexes are likely involved in endothelial stimulation and shedding, patients with active disease expressed significantly higher levels of CECs in peripheral blood compared to patients with inactive disease or healthy controls (34). As expected, the levels of CECs correlated positively with complement activation as assessed by plasma C3a. Furthermore, CECs from these patients expressed an activated phenotype as indicated by staining for nitrotyrosine, a proxy of the inflammatory mediator, peroxynitrite (34).

In cancer, the generation of new blood vessels, angiogenesis, is crucial in tumour growth and metastasis; the endothelium is central to this process (77). Indeed, as new classes of drugs with targeted activity on angiogenic vessels have been developed to control cancer progression (78, 79), assessment of endothelial function is crucial. In breast cancer and lymphoma patients, CECs were increased 5-fold ($p<0.0008$ vs. control) and cor-

related significantly with plasma VCAM-1 and VEGF. CECs were similar to healthy controls in 7 lymphoma patients achieving complete remission after chemotherapy (33). Repeated CEC measurements in patients and controls indicated a low longitudinal CEC variation, and the count of resting and activated CECs did not correlate with the count of white cells, red cells or platelets.

To date, the origin of these CECs (tumour and/or its vessels, normal endothelium or bone marrow) is unclear but is obviously must be determined as it may have a bearing on tumour biology (e.g. reflective or not of new vessel development?). For example, the correlation between CECs and VEGF ($r=0.42$) may imply a relationship with angiogenesis, although about half of CECs expressed CD36, implying a microvascular origin. However, there was no difference in CEC counts in early versus metastatic breast cancer. Other data from patients with cancer is urgently required.

Implications of CECs for thrombosis and haemostasis

As endothelial cells are dynamic participants in the homeostasis of coagulopathy and hypertension, their loss from the intima seems likely to have important repercussions (1). It follows that CECs themselves may participate in pathology. Various groups have performed additional characterisation of the CECs in which molecules contributing to the balance of coagulation of inflammation have been evaluated. For example, Dignat-George et al. (19) reported CECs positive for thrombomodulin, but whether or not this molecule is still bio-active is unclear. Solovey et al. (80) showed in CECs in sickle cell disease the expression of tissue factor that is functionally active. Some CECs reported by Mutin et al. (26) were also tissue factor positive. Furthermore, these CECs were not apoptotic (as defined by the TUNEL assay). CECs from patients with small vessel vasculitis stained predominantly for tissue factor, suggesting a prothrombotic potential; all were TUNEL assay negative, and most (84%) stained with annexin and propidium, suggesting necrosis (38). However, although many groups have found tissue factor positivity, whether or not this is clinically important in promoting thrombosis is unknown.

With regard to molecules regulating leukocyte endothelial adhesion, half of the CECs isolated from patients with thalassaemia expressed ICAM, E-selectin and VCAM (37), whilst approximately two thirds of the CECs in sickle cell disease analysed by Solovey et al. (23) also expressed these adhesion molecules, suggesting an activated state (23). Activated CECs, defined as VCAM-1 and E-selectin positivity by Del Papa et al., were higher in patients with systemic sclerosis (45).

At the interface between coagulation and inflammation, the endothelial cell protein C receptor (EPCR) is a cell-surface protein with important roles, such as inhibition of thrombosis (81). A consequence of stimulation is the shedding of membrane EPCR which would be expected to have a negative impact on the delicate balance of coagulation and inflammation (82). Increased levels of soluble EPCR were recently noted in the plasma of patients with SLE, particularly those with renal disease, as well as a variable expression of membrane EPCR on CECs from SLE patients. However, in the controls, levels were lower (83).

Thus the absence of membrane EPCR on CECs further supports a procoagulant state as the thrombin-thrombomodulin-dependent protein C activation is attenuated and functionally involves a loss of a 'brake' to thrombosis (84).

CECs and EPCs

Endothelial progenitor cells are a circulating, bone-marrow-derived cell population of large non-leukocyte cells that appear to participate in vascular repair and homeostasis. From a theoretical point of view, the key features that separate CECs from EPCs are origin (vasculature vs. bone marrow), phenotype, morphology, and proliferative capacities. A number of characteristic properties of EPCs have been proposed, notably positivity for CD34 (marking haematopoietic progenitor cells), CD133 (a putatively more specific marker of EPCs) and KDR, the receptor for vascular endothelial growth factor (5, 52, 85–93). Other surface markers of EPCs are UEA-1, which is also present on CECs, and Tie-2. The dominant determining methods are flow cytometry and culture, whereby uptake of acetylated LDL is often used to prove endothelial lineage of outgrowing cells. The general thrust of the literature is that bone marrow derived EPCs are viable and capable of promoting endothelialisation: indeed several groups raise colonies *in vitro* from CD34⁺ cells that resemble mature endothelial cells (5, 86, 87, 91). Lin et al. generated endothelial outgrowths from peripheral blood mononuclear cells that were subsequently positive for CD146, von Willebrand factor and, crucially, CD36, supporting a microvascular origin for this population (53–55). The importance of EPCs as a new therapy in vascular disease has been emphasized elsewhere (92, 93).

However, given the variety of defining markers (Table 1), a good consensus of how to distinguish CECs from EPCs is, at best, difficult. For example, Mutunga et al. (32) use von Willebrand factor and KDR to define CECs from patients with septic shock, subsequently culturing cells from 6 of 15 patients. Between 70% and 90% of these cells were positive for von Willebrand factor, KDR (thus, according to the authors, excluding other circulating haemopoietic cells) and endoglin. There is indeed considerable scope for the dual identification of CECs and EPCs. For example, Nakatani et al. (39) used P1H12-immunobeads to isolate CECs, and AC133 to define EPCs, concluding that EPCs are a sub-population of CECs and that the two populations have different time courses. Del Papa et al. (45) used flow cytometry to define CECs as CD45⁻, CD34⁺, and P1H12⁺ (addi-

tionally defining activation by CD62 [the authors do not state the precise molecule, and although we presume CD62E, it could be CD62P] and CD106), EPCs as CD34⁺ and CD133⁺. In summary, the liberal use of definitions and antigens has caused some confusion, and progress in CEC and EPC research will not advance until a consensus has been reached.

Most groups working on CECs first capture cells with CD146-immunobeads and then perform additional phenotyping, e.g. for markers of activation. Others prepare mononuclear cells by density centrifugation then stain for CD146. These studies generally fail to address the question of the viability of CECs: one that does (from sickle cell disease, [80]) reports that two thirds are viable. Another study in vasculitis using CD146-dependent immunobead isolation demonstrated a majority of necrotic cells with no growth in culture (38). Hence, this study suggests that CD 146-dependent immunobead isolation does not isolate relevant numbers of EPCs since these cells would have proliferated well. It has been demonstrated previously that the isolation procedure itself does not affect apoptosis or necrosis in endothelial cells (38). However, it has been proposed that EPCs or even pericytes may be CD146-positive, suggesting that capture of those cells may still occur (94). Consistent with this hypothesis, Burger et al reported the expression of CD146 on CD34⁺/FGFR1⁺ circulating cells yielding small round cells with low growth potential when cultured (4). However, in another study, Woywodt et al. (44) were unable to find any CD146-immunobead prepared CECs that stained for AC133 or alpha-smooth-muscle-actin, thus minimising the likelihood that these cells are EPC or pericytes. Finally, CECs are often seen in morphologies (irregular carcasses, clumped cells or even sheets, filamentous cell remnants) that differ markedly from that of EPCs. Taken together, although until now there is no clear evidence that CD146-dependent isolation does not significantly capture EPCs, the possibility that CD146 identifies some EPCs in addition to CECs needs further investigation.

In summary, the common view is emerging that CECs and EPCs are indeed separate populations. The former represent mature endothelial cells shed from the vessel wall in response to a damaging stimulus, while the latter are progenitor cells derived from bone marrow. EPCs bear endothelial markers and (at least) CD34 and CD133 while CECs lack CD133. A formal consensus in this regard can be seen in table 4, which summarises characteristic features of CECs as opposed to EPCs.

Conclusions

It is clear that increased numbers of CECs are present in the blood of patients with a wide variety of clinical diseases linked by a history of vascular injury. Their presence implies severe vascular damage, a feature that other vascular function tests (plasma markers such as von Willebrand factor, flow mediate dilatation) cannot offer with such confidence. The enumeration of CEC may provide useful information in the monitoring of disease activity and/or treatment efficacy. Indeed, there are reports from two different groups that high levels of CECs may be a novel predictor of adverse cardiovascular events in patients with acute coronary syndromes (73, 95). In one of these studies (95), the predictive power of CECs exceeded that of plasma marker von

Table 4: Differences between CECs and EPCs.

	CECs	EPCs
Origin	Blood vessel wall	Bone Marrow
Morphology	Mature cells of diameter 20–50 μ M	Immature cells of diameter less than 20 μ M
Phenotype	CD133 –ve	CD133 +ve
Capacity to form colonies with high proliferative potential	No	Yes
Pathophysiology	Reflective of damage	Neovascularisation

CECs = circulating endothelial cells, EPCs = endothelial progenitor cells

Willebrand factor. However, the absence of a uniform measure of quantitation to ensure comparable reproducible results, especially between immunomagnetic isolation and flow cytometry, is a drawback. Although there is a growing belief that CEC can be considered an indicator of endothelial injury, it is vital to reach a general consensus regarding the most appropriate technique in order to validate the reporting of CECs in large cohorts of patients. This validation has critical implications on future clinical trials in which CECs might function as a novel biomarker and perhaps as a surrogate endpoint.

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