Review

Circulating endothelial cells: A novel marker of endothelial damage

Uta Erdbruegger, Marion Haubitz, Alexander Woywodt *

Department of Medicine, Division of Nephrology, Hannover Medical School, Carl-Neuberg-Strasse 1, D-30625 Hannover, Germany

Abstract

Circulating endothelial cells (CECs) were first described over 30 years ago in smears of peripheral blood. Since then, more sophisticated techniques for CEC isolation have become available. In particular, immunomagnetic isolation and fluorescence-activated cell sorting (FACS) have been employed with success. We provide a short historical perspective and a comprehensive review on the subject. We review isolation and enumeration of CECs with an emphasis on CD146-driven immunomagnetic isolation and FACS. We describe, in great detail, advantages and pitfalls of both approaches and compare their specificity. Moreover, we provide a comprehensive list of clinical studies in this field and describe the possible clinical use of CECs. We also describe the phenotype of these cells and list typical surface markers. In addition, we review the phenotype of CECs and discuss mechanisms of detachment. We speculate about potential interactions between CECs and other cell subsets. We also describe other serum markers of endothelial damage and compare CECs with these markers. Finally, we highlight differences between circulating endothelial cells and endothelial progenitor cells. In summary, CECs must now be regarded as a sensitive and specific marker of endothelial damage. We emphasize that use of CECs in a clinical setting is on the horizon and pathogenetic clues may also be obtained.
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Keywords: Circulating endothelial cells; Endothelial damage; Fluorescence-activated cell sorting; Immunomagnetic isolation

Contents

1. Circulating endothelial cells — a historical perspective

Circulating endothelial cells (CECs) were first described over 30 years ago by Bouvier and Hladovec. Their techniques included vital light microscopy, Giemsa staining and separation by density centrifugation ([1–4]). Increased numbers of circulating endothelial cells were also detected in animal models of shock, such as treatment with endotoxin ([3]). Between 1970 and 1980 studies in humans followed and these cells were identified in various conditions, such as smoking, acute myocardial infarction and hypertension ([2]). Finally drug effects on CECs were analyzed in patients taking prostaglandin E inhibitors ([5]) or immunosuppressive agents ([6]). It must be emphasized,

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* Corresponding author. Tel.: +49 511 532 6319.
  E-mail address: Woywodt.Alexander@MH-Hannover.de (A. Woywodt).

however, that all of these studies identified CECs on the basis of morphological criteria alone; hence their methodology must be regarded as rather crude by today's standards. In addition, some cells may have been lost during preparation of the smears.

The next technical advance was the use of immunofluorescence with antibodies against endothelial markers, such as von Willebrand factor ([7,8]). These techniques were hampered by the paucity of specific markers for endothelial cells. Antibodies were often directed against non-specific (e.g. adhesion molecules, integrins) or intra-cellular antigens (e.g. tissue plasminogen activator, von Willebrand factor). The former lacked specificity while the latter were relatively inaccessible to monoclonal antibodies without prior permeabilization. Furthermore, these studies still relied on smears.

In the early 1990s more specific surface markers for endothelial cells became available [9,10]. The S-Endo 1 monoclonal antibody targets the CD146 molecule [11,12], which is intimately involved in cytoskeleton formation [13] and signalling [14]. It must be noted that the availability of the surface marker CD 146 permitted immunomagnetic isolation as a more standardized technique to isolate CECs. With this approach, CECs were enumerated in a broad variety of vascular disorders including Rickettsial disease [15,16] and acute coronary syndrome [17]. In 2001 Solovey et al. identified another monoclonal antibody (P1H12) against CD 146 and confirmed increased CEC levels in sickle cell anaemia [18]. As of today, most groups employ immunomagnetic isolation while others use Fluorescence-activated cell sorting (FACS). Hence, both techniques need to be discussed to appreciate advantages and disadvantages of each approach.

2. Immunomagnetic isolation of CECs

In healthy individuals the endothelial layer is continuously renewed at only a low replication rate of 0–1% per day. Endothelial proliferation is clustered at sites of branching [19] while laminar flow has been reported to suppress endothelial apoptosis [20]. The turnover of endothelial cells varies greatly in different organs. In accordance with these data, detection of circulating endothelial cells in a healthy adult is a rare event and immunomagnetic isolation has consistently yielded as few as 0–10 cells/ml in healthy controls. It must be emphasized that immunomagnetic isolation was originally devised to detect rare events in peripheral blood (e.g. metastatic tumour cells) and thus performs well in detecting these rare CECs.

Immunomagnetic technique isolates endothelial cells from whole blood with paramagnetic particles (Dynabeads™), which have been coated with anti-endothelial antibodies. The technique has been reviewed in great detail elsewhere [21]. Briefly, whole blood is incubated with antibody-labelled magnetic Dynabeads™. Next, target cells with bound anti-endothelial antibody and Dynabeads™ are recovered with a magnet. CECs can then be enumerated after acridine staining. Fig. 1 displays the principle of the technique. Immunomagnetic capturing is mostly performed using the cell surface marker CD 146 [10].

Experimental data shows that there is no difference in analyzing arterial versus venous blood [10,22]. In addition, the immunomagnetic isolation avoids centrifugation thus improving recovery of CECs. To avoid false positive results caused by traumatic venipuncture resulting in dislodgement of endothelial cells from the vessel wall, it is recommended to discard the first tube of blood [10]. Adding albumin or EDTA represents another step to minimize non-specific binding of CECs and anti-CD 146-coupled beads. It is clear that such events occur via the Fc receptor. Accordingly, Fc-blocking agents are added to further reduce non-specific binding [23]. Most investigators count the cells directly after labelling with fluorescent dye (e.g. acridine). Over time, however, it became very clear that a secondary stain is needed, since activated lymphocytes and other cell subsets may, at least under certain conditions, also harbour CD 146. The CD146 molecule has been described on activated T lymphocytes, trophoblasts and mesenchymal stem cells, periodontal tissue and in malignancy (prostatic cancer, melanoma [24]). This issue may affect magnetic bead selection or RT-PCR and argues in favour of FACS studies and multiplex analysis in particular. It has been argued, though, that these effects are unlikely to play a major role in most clinical scenarios.
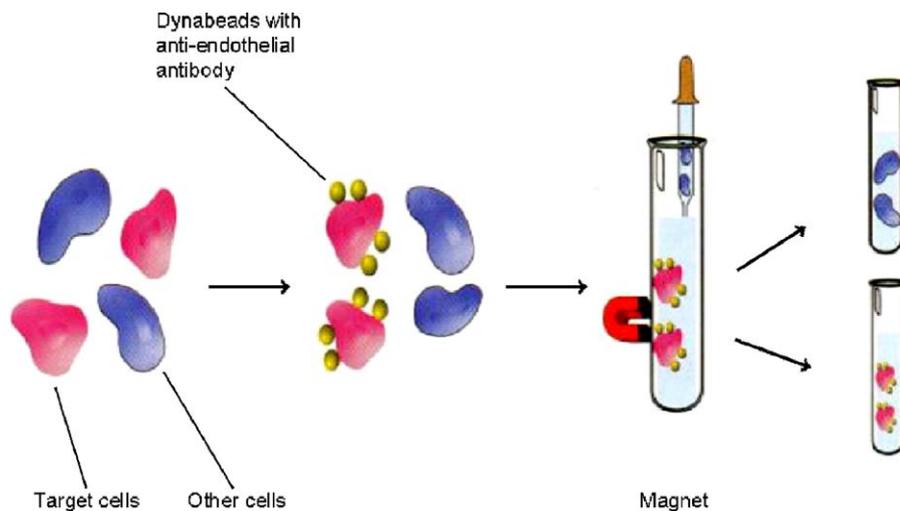

Fig. 1. Immunomagnetic isolation.

Many endothelial antigens were studied as a secondary stain and finally excluded since they lack specificity (Table 1). Most of these markers, such as thrombomodulin or vWF, necessitate a cumbersome multiple-step procedure, therefore not being a feasible approach in the clinical setting. Ulex Europaeus lectin 1 (UEA-1), one of the lectins of ULEX europaeus (gorse), has long been used to stain endothelial cells. UEA-1 positivity appears to be a stable phenomenon among endothelial cells in several disorders. For example, hepatic sinusoidal endothelial cells stain UEA-1 positive while expression of vWF may be sparse or even absent [25]. Similar findings are true for endothelial cells in thyroid neoplasms [26]. A lectin stain with Ulex Europaeus lectin 1 (UEA-1) was thus employed with good success [23] although these results still require confirmation by other groups. This approach has been included in the recent proposal of a consensus methodology [27].

Size of the CECs spans a wide spectrum, ranging from multi-nucleated cell conglomerates to entire cells and endothelial microparticles. To make matters still more complicated, the phenotype of CECs differs markedly between disorders [28]. Sheets of intact cells have been isolated in patients with acute coronary syndromes [28] whereas severely damaged and necrotic cells were detected in Rickettsial infection and vasculitis [22]. Entire cells are usually in the range of 10 to 70 μm but larger aggregates of cells are also seen. Giant cells have been reported in cytomegalovirus infection [8]. Endothelial microparticles have been recently described. They are thought to result from exocytic budding, consisting of cytoplasmic component and phospholipids and measured by annexin staining, endothelial surface marker and size using flow cytometry. Their small size may pose a particular problem because neutrophils, platelets and cell fragments, which express certain markers present on endothelial cells, may decrease the sensitivity of the analysis. These particles are too small to be isolated with immunomagnetic beads. We are left with the dilemma that consensus is required before the test can enter clinical applications. A recent consensus paper proposed the following definition: Circulating endothelial cells are nucleated or a-nuclear cellular particles that exceed 10 μm in length and bear more than 5

Table 1
Antigens of endothelial cells that are also present on non-endothelial cells

CD/ antigen name	Other names	Expression by non-endothelial cells
CD31	PECAM-1	Platelets, monocytes, neutrophils, T cell subsets
CD62e	E-selectin	Activated endothelial cells
CD54	ICAM-1	Endothelial cells, activated B and T lymphocytes, monocytes
CD105	Endoglin	Endothelial cells, activated monocytes, tissue macrophages, erythroid marrow precursors
CD106	VCAM-1	Activated endothelial cells, stromal cells
CD141	Thrombomodulin	Endothelial cells, keratinocytes, platelets, monocytes, neutrophils
CD146	P1H12, S-endo-1	Endothelial cells, activated T-lymphocytes, melanoma cells, trophoblast
Tissue factor		Endothelial cells, monocytes/macrophages

anti-CD 146-coated immunomagnetic beads attached and stains UEA-1 positive [27].

Finally, it must be borne in mind that even the secondary stain with UEA-1 does not differentiate between CECs and EPCs. This issue will be discussed below.

3. Isolation of CECs by fluorescence-activated cell sorting (FACS)

Flow cytometry represents another attractive approach to isolate and enumerate CECs [29–33]. In general, multi-parametric flow cytometry is used to detect endothelial cells and discriminates them from cells with overlapping expression of antigens. For example, CD 146 expression on activated T cells can be distinguished from CD 146 on endothelial cells by co-staining with CD45 or CD3 (or both). CD 133 may help to identify EPCs because it is not present on CECs or any mature endothelial cells. The addition of viability stains, such as propidium iodide or 7-AAD, may also help to identify EPCs. Markers of endothelial activation can be studied as well, e.g. intracellular adhesion molecule-1, E-selectin, vascular cell adhesion molecule-1 or markers of pro-coagulant activity (e.g. tissue factor). Cortelezzi et al. and Mancuso et al. developed a 4-color flow cytometry protocol to measure CECs and EPCs in cancer patients [34,35]. Resting CECs were defined as negative for the leukocyte marker CD45, positive for endothelial markers P1H12, CD31, negative for activation markers CD 105 and CE 106, and negative for the progenitor cell marker CD133. Activated CECs were defined as CD45−, P1H12+, CD31+, CD34+, CD105 and CD106+ and CD133− [35]. Recently, Fuerstenberg et al. showed that in cancer patients the quantified mRNA of CD 146 by real time PCR correlates with the number of CECs enumerated by flow cytometry [36].

It must be noted that expression of CD 146 on endothelial progenitor cells has been proposed [37]. Its differentiation from CECs is discussed later on in this review. In contrast to immunomagnetic isolation, FACS does not permit characterization of the cell phenotype. Furthermore the cell numbers obtained with FACS differ markedly from those obtained with immunomagnetic isolation. FACS Cell numbers range from 0 to 39,100/ml in patients with vascular disorders and from 0 to 7900 in healthy controls. It is remarkable that all investigators using immunomagnetic isolation enumerate in the range of 10 CECs/ml blood in healthy individuals while those using FACS report cell numbers in the thousands per ml with a broad range [21]. This discrepancy must, we believe, indicate a fundamental methodological difference. In addition, cell numbers differ between various FACS studies, presumably due to different protocols. Holmen et al. measured a mean of 50 CECs/ml in healthy controls [29], Del Papa et al. enumerated 77 CECs/ml [31] and Mancuso et al. counted 1200 CECs/ml of rested cells [35].

4. CECs in vascular disorders

Numbers of CECs also reflect the extent of the endothelial lesion. High cell counts have been observed in diseases with widespread vascular damage, such as rickettsial infection, sickle

cell disease or vasculitis [22]. In contrast, localized damage may be seen in patients undergoing coronary angioplasty and low cell numbers have been observed in this setting. In healthy subjects, renewal of the endothelial layer takes place at a low replication rate of 0–1% per day. Therefore, detection of circulating endothelial cells in a healthy adult is a rare event, about 0–12/ml blood are considered normal [21,22].

Many different groups have detected CECs in conditions associated with vascular damage using various techniques described in the previous paragraphs. These conditions included infectious and cardiovascular diseases, inflammatory and connective tissue diseases, transplantation and cancer. In cardiovascular diseases CECs have been found to be elevated in acute myocardial infarction with highest numbers in most severe disease [17]. Elevated CEC numbers were also documented after coronary angioplasty for stable angina [38]. In another study of acute coronary syndrome CEC numbers at 48 h were the only independent predictor of major cardiovascular endpoints [39]. CECs were also used in conjunction with troponin levels as an early, specific, independent diagnostic marker for non-ST elevation acute coronary syndrome [40]. Wang et al. recently demonstrated that CECs correlate with C-reactive protein in acute myocardial infarction [41]. In peripheral artery disease Bull et al. also described elevated CECs [42]. Similar findings were observed in chronic venous insufficiency [43]. More recently, CECs were enumerated in patients with acute and chronic heart failure [44]. Freestone et al. demonstrated that patients with atrial fibrillation and an acute cardiovascular or cerebrovascular event have significantly elevated CECs compared to patients with uncomplicated chronic atrial fibrillation [45]. A very recent study documented elevated CEC numbers in acute ischemic stroke [46] although relation to stroke subtypes and the change of cell numbers during the course of the disease remain unknown. It is known, however, that cell numbers are elevated in amaurosis fugax [47]. Bull et al. reported a correlation between pulmonary artery pressure and CEC numbers in pulmonary hypertension [42].

ANCA-associated small-vessel vasculitis serves as a paradigm of the clinical use of CECs. We have described markedly elevated cell numbers in active disease and noted a decline of cell numbers during successful therapy [22] (Fig. 2). Markedly elevated cell numbers distinguished active vasculitis from granulomatous di-
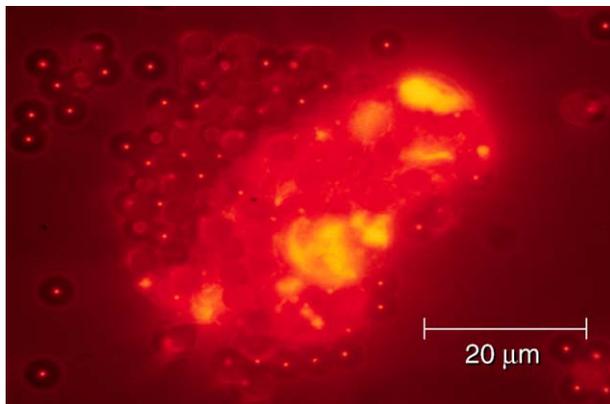

Fig. 2. CEC from a patient with ANCA-associated vasculitis (immunomagnetic isolation and subsequent UEA-1 stain).

sease without vasculitis [48]. In our experience, CECs serve as a valuable marker in the care of these patients as reviewed elsewhere [49]. Elevated CECs could be demonstrated in Behcet disease [50] and Kawasaki's syndrome [51]. A recent study confirms elevated numbers of CECs in acute coronary lesions due to Kawasaki's disease [52]. At present, there is only limited experience with large and medium size vasculitides. In systemic lupus erythematodes, patients with active disease had significantly higher levels of CECs in peripheral blood than patients with inactive disease or healthy controls [53]. Levels of CECs correlate positively with complement activation as assessed by plasma C3a. Elevated CEC numbers have also been described in other connective tissue diseases. Del Papa et al. found that total and activated CEC counts in patients with systemic sclerosis positively correlated with the disease activity score [31].

CECs in infection have been of interest, particularly because pathogens can be identified in these circulating cells. Very high numbers of CECs were detected in Rickettsial infection [16]. Moreover, the detection of Rickettsia in CECs was advocated as a diagnostic test [15]. Malaria represents another endothelial infection but only preliminary reports exist here [54]. It is known that cytomegalovirus is detectable in circulating endothelial giant cells [8,55]. Other herpes viruses may also be detectable in CECs (e.g. HHV-8) but this has not been tested so far. Mutunga et al. demonstrated in septic patients that the highest numbers of CECs were enumerated in patients with septic shock [56]. We have reported normal cell numbers in moderate infection [22].

Renal transplantation is another field on interest, since vascular damage may occur due to pre-existing atherosclerosis (e.g. .in the renal transplant) or due to drugs, such as calcineurine inhibitors. In kidney transplantation the highest numbers of CEC could be shown in patients with acute vascular rejection compared to patients without rejection, with interstitial or borderline rejection [57]. Interestingly, CEC numbers were elevated in all other renal transplant recipients. In a subsequent study, marked differences were detected between patients with calcineurine inhibitors and matched renal transplant recipients without those drugs [58]. These results confirm that calcineurine inhibitors cause endothelial damage [59]. More recently, Mohamed et al. confirmed that CEC numbers were higher in patients with a history of rejection [60]. These cells appear to be of donor origin [61], a finding that supports previous reports of endothelial chimerism in renal grafts [62]. Only preliminary data exist in bone marrow cell transplantation. CEC numbers increased after conditioning with a dose relationship [63]. It is unknown whether such rises in CEC numbers precede vascular complications [64].

Not surprisingly, CECs are elevated in haematological diseases involving the endothelial cell layer. Sickle cell disease is a classic example. Several groups have demonstrated that the endothelium is activated in crises of sickle cell disease [18]. Thalassemia is another haemoglobinopathy with elevated numbers of CECs [65]. Finally, there is one case report about elevated CECs in thrombotic microangiopathy (TMA) [66]. We studied 15 patients with TMA and detected markedly elevated cell numbers. Patients with favourable outcome hat significantly higher initial CEC levels and cell numbers decreased after successful plasma exchange [67].

Cancer is another field of interest since angiogenesis is crucial for tumour growth and metastasis. Mancuso et al. studied breast cancer and lymphoma patients and reported that CECs are increased by 5-fold and correlate with plasma VEGF [33]. Others found a correlation with cancer progression [68]. Angiogenesis has been emphasized as a crucial factor for tumour progression and response to chemotherapy [69,70]. Accordingly, agents inhibiting tumour angiogenesis cause an increase in mature CECs [71]. In summary, the situation in cancer may be somewhat different in that CECs may not reflect endothelial damage but angiogenesis. Very recently, the subject of CECs in cancer has been reviewed elsewhere in great detail [72].

There are many more clinical conditions with increased numbers of CECs. Elevated numbers have been described in renal diseases [73] such as focal segmental glomerulosclerosis [74] although we did not find such effect in patients with glomerulonephritis [22]. Accelerated atherosclerosis is a key feature of end-stage renal disease; elevated numbers of CECs were described in hemodialysis patients with clinical evidence of active atherosclerosis [75]. A subsequent study reported that elevated cell numbers convey a risk of future vascular events [76]. Surprisingly, a comparison between hemodialysis and peritoneal dialysis has not been done so far. For completeness, retroperitoneal fibrosis [30] and acute thermal injury have to be mentioned [77]. Table 2 summarizes findings of CECs in various disorders.

5. Phenotype and mechanisms of detachment of endothelial cells

The vascular network has an estimated surface area of more than $1000 \, m^2$ and maintains an anticoagulant, anti-thrombotic and anti-inflammatory state [78]. Endothelial cells can be activated by various stimuli, such as pro-inflammatory cytokines, growth factors, infectious agents, lipoproteins, or oxidative stress. Irreversible loss of integrity of the endothelial layer eventually leads to cell detachment. It must be noted that detached cells could be apoptotic or necrotic [79]. Unfortunately, little information, if any, is available regarding the cell phenotype in different disorders. Light microscopy has demonstrated that the phenotype of CECs varies considerably, ranging from sheets of intact cells to solitary cells [28] or even microparticles. These findings may also indicate that the cells originate from different vascular beds. At present, the origin of CECs remains incompletely understood. It has been reported that about half of the CECs in healthy controls express CD36 and must be regarded as microvascular. In sickle cell anemia this percentage increased to 78% [18]. Mutin et al. found no staining with CD36 in CECs from patients with acute coronary syndrome, consistent with a macro-vascular origin of these cells [17]. Another group analyzed patients with cancer. At least half the CEC from these patients were of microvascular origin, whereas 70–80% of CECs from patients with Thalassemia were of microvascular origin [32,65].

The phenotype of CECs remains ill defined. We have demonstrated that CECs in ANCA-associated small-vessel vasculitis are mainly necrotic [22]. We were unable to cultivate these cells and this finding gave us confidence in the interpretation. Others, however, describe culture of CECs that were isolated by FACS.

Viable cells have been observed temporarily in 6 of 15 patients with septic shock [56]. Lin et al. could also demonstrate that vessel-wall derived CD146+ CECs can be viable, although they have limited growth capability [80]. Another group was recently able to grow CEC for about 10 days, but no significant proliferative capacity was observed [29]. Beerepoot et al. demonstrated

Table 2
CECs in vascular disorders; cell numbers are provided as median unless described otherwise

Disorder	Method	Cell numbers in disease	Cell numbers in controls	Reference
Cardiovascular disease				
Acute myocardial infarction	IB	7.5	0	[17]
Angioplasty in stable coronary heart disease	IB	15.9 (after procedure)	n.d.	[38]
Peripheral vascular disease	IB	1.1–3.5 [a]	0.9	[102]
Congestive heart failure	IB	14	4.5	[44]
Chronic venous insufficiency	C	1001	514	[43]
Atrial fibrillation with stroke/LVF/MI	IB	15/10/9	4.5	[45]
Stroke	IB	15.5	2.7	[46]
Amaurosis fugax	C	52	n.d.	[47]
Pulmonary hypertension	C+IB	30	3.5	[42]
Type II diabetes	IB	69 (mean)	10 (mean)	[116]
Vasculitis and lupus				
Behcet's disease	IB	0–25	<3	[50]
Aortoarteritis	C	58	16 [b]	[117]
ANCA-associated small-vessel vasculitis	IB	136	5	[22]
Systemic lupus erythematosus	FC	89	10	[118]
Systemic lupus erythematosus	C	32	5	[53]
Kawasaki disease	IB	15	6	[51]
Infectious disease				
Sickle cell anaemia	IB	13.2–22.8	2.6	[18]
Rickettsial infection	IB	5–1600	<3	[16]
Septic shock	C	16.1	1.9	[56]
CMV infection	C	n.a.	0–49	[8]
Miscellaneous				
Breast cancer and lymphoma	FC	6800–39,100	1200–7900	[33]
Renal transplantation	IB	24–72	6	[57]
Thalassaemia	C	45	4 [c]	[65]
Bone marrow transplantation	IB	16–44	8	[63]
Systemic sclerosis	FC	243–375	77	[31]
Cancer	IB	399 (mean)	121 (mean)	[68]
Thrombotic thrombocytopenia	IB	6–220	<3	[66]

n.d. denotes not done. IB denotes immunobeads method. FC denotes FACS method. C denotes centrifugation and identification in smears. LVF denotes left ventricular failure. MI denotes myocardial infarction.
As the distribution of CECs is often non-normal, median number is appropriate although some report the mean.
  [a] Highest in critical limb ischaemia.
  [b] Controls are patients with inactive disease.
  [c] Raw data not supplied, therefore estimated from a figure.

viable CECs in cancer patients but this finding may well reflect a different situation of ongoing tumour angiogenesis in these patients [68]. These cells were tissue-factor negative [81] in contrast to findings in ANCA-associated small-vessel vasculitis [22]. It remains to be demonstrated that none of these proliferating cells were in fact endothelial progenitor cells rather than mature endothelial cells (for a discussion of CECs and EPCs see below). Given the severely damaged phenotype we do not believe that these CECs can proliferate [21]. In summary, the issue of viability of CECs remains unresolved.

There are various potential mechanisms for endothelial cell detachment. A simple question needs to be asked first: Is endothelial detachment the cause or the effect of cell death? A very recent study demonstrated that at least in a model of endothelial cell detachment due to integrin disruption, detachment precedes apoptosis [82]. Endothelial cell detachment can be caused by defective adhesive properties of the endothelial cells, by action of proteases and/or cytokines or, very simply, by mechanical injury. The importance of endothelial tethering to matrix has been emphasized. Endothelial adhesive molecules of the integrin and cadherin family, such as vitronectin and fibronectin and VE cadherin, respectively, promote adhesion of endothelial cells to matrix [79,83]. These proteins accelerate the assembly of cytoskeletal proteins and mediate signals for cell survival. Loss of these survival signals triggers detachment and apoptosis of endothelial cells [84]. Disturbance of these survival signals has been implicated in endothelial detachment caused by cytomegalovirus (CMV) [85]. In general, viral infections appear to inhibit interaction of endothelial cells with basement membrane proteins [86]. Protective factors have also been described. In Sickle cell disease endothelial apoptosis is impaired by vascular endothelial growth factor (VEGF). This has also been shown in vitro, where VEGF inhibits apoptosis of unanchored culture cells [87]. Finally, nitric oxide has a role in protecting endothelial cells against apoptosis [88]. Clancy et al. suggest that nitric oxide (NO) may result in

defective adhesive endothelial cell properties. NO (via peroxynitrite) decreases the affinity of $\alpha5\beta1$ integrin for its matrix ligand, which results in increased levels of CEC in SLE [89]. Release of proteases by granulocytes is another well-documented cause of endothelial cell detachment [90,91]. Serine protease inhibitors are believed to inhibit detachment under normal conditions [92]. Cytokines, such as Tumour Necrosis Factor and Interferon, induce Integrin $\alpha v\beta3$ and cause detachment of endothelial cells [93]. Finally, mechanical force can detach endothelial cells from the basement membrane as shown in patients undergoing percutaneous catheter interventions [17]. Fig. 3 summarizes presumed mechanisms of endothelial detachment.

6. Interactions of circulating endothelial cells with other cell subsets

It is unknown whether circulating endothelial cells are pro-inflammatory [79]. Several products released by necrotic cells have been found to initiate an inflammatory response. These products include high mobility group 1 protein (HMGB1) [94], cytochromes, plasma DNA or heat shock proteins. Other studies have observed that necrotic but not apoptotic cells initiate a Toll-like-receptor-2/NFκB-dependent reaction in monocytes and fibroblasts [95]. The uptake of necrotic cellular material activates macrophages. Healthy endothelial cells or circulating leukocytes could react in a similar way. Our own preliminary studies indicate that necrotic endothelial cell do interact with their healthy counterparts (unpublished data). Other in vitro experiments showed that CEC could express high levels of inducible nitric oxide synthases and neutrophil-activating chemokines such as macrophage inflammatory protein-1α, growth-related oncogene-α, epithelial neutrophil activating peptide-78, and IL-8 and induced increased neutrophil migration [29]. In these in vitro studies, CECs significantly inhibited proliferation, migration and endothelial nitric oxide synthase expression in EPC, thus possibly impairing functional capacity of
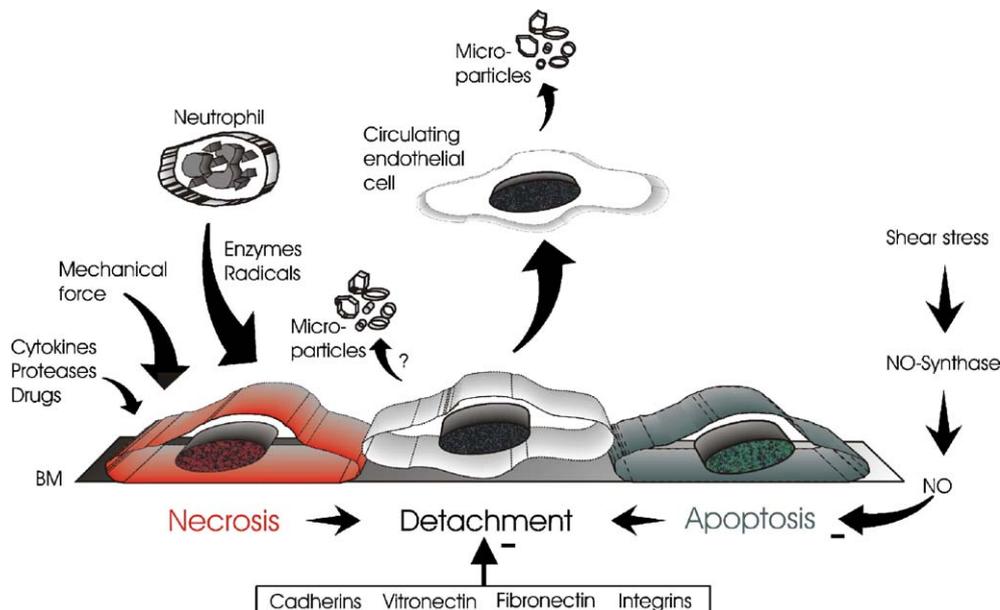

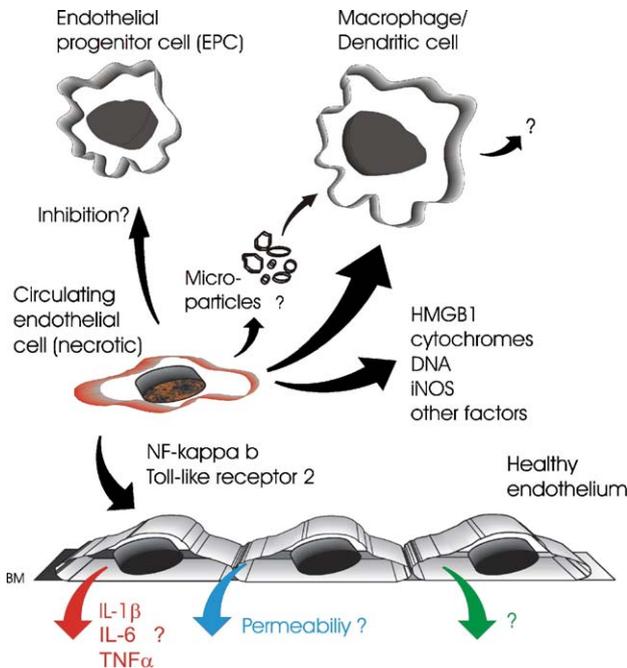Fig. 3. Mechanisms of detachment of endothelial cells.

Fig. 4. Pathogenetic implications of CECs (hypothetic).

vascular repair [29]. We reported that time kinetics of EPC in ANCA associated vasculitis mirrors those of CECs; numbers of CECs decreased with achievement of remission whereas numbers of EPCs increased [96]. Impairment of EPC function by CECs now needs to be corroborated by further studies. It must be assumed that such impairment does not occur universally. Otherwise, vascular lesions could never heal. Fig. 4 shows hypothetic pathogenetic mechanisms of CECs on other cells.

7. CECs and other circulating markers of vascular damage

Endothelial cells express a broad variety of proteins [97] but only few of these have been studied in serum or plasma in vascular disease. Currently, von Willebrand factor (vWF), thrombomodulin and soluble E-selectin are best described [98–101]. It must be noted that several factors may influence the levels of these circulating proteins. For example, thrombomodulin undergoes renal excretion. Hence, serum levels are influenced by renal function. Other confounding factors, such as liver function, clotting or fibrinolysis may also influence these proteins. Finally, these soluble markers do not distinguish between endothelial activation and damage. Some investigators compared levels of these markers with numbers of CECs. A recent study found a correlation between CECs, von Willebrand factor ($p=0.002$) and plasma tissue factor ($p=0.02$) [102]. Microparticles have recently received more attention as markers of activation in eukaryotic cells. Resulting from exocytic budding, these vesicles consist of cytoplasmic components and phospholipids. They carry markers of the parent cell, including those induced by activation or apoptosis. Platelet derived microparticles have been found to be capable of inducing a pro-coagulant state [103]. Endothelial microparticles have been found in vascular disorders [104–106]. A recent study documented endothelial microparticles in children

with vasculitis [107]. We have previously demonstrated markedly elevated numbers of CECs in this disorder [22]. To our best knowledge, however, endothelial microparticles and CECs have not been measured in the same patient. Such studies are clearly needed to understand the relation of these two markers of endothelial damage. In addition, it remains unknown whether microparticles derive from resident or circulating endothelial cells or, more likely, from both.

8. CECs and EPCs

CECs and EPCs represent two groups of non-hematopoietic cells in the blood. Agreement on the phenotypic differentiation of EPC and CEC is still lacking, not least because several markers occur on both. It is believed that CECs and EPCs have different origins. CECs derive from mature endothelium while EPCs derive from the bone marrow. Accordingly, EPCs are characterized by the expression of CD 34 and the receptor for vascular endothelial growth factor (VEGFR-2 or KDR/Flk-1) [108]. Many researchers regard a CD34+ KDR+ cell as an EPC [109]. EPCs furthermore express KIT (CD 117), a tyrosine kinase that works as a receptor for stem cell factor (SCF), and CD 133, a trans-membrane glycoprotein. No ligand has yet been identified for CD133 and it has thus been dubbed an orphan receptor. It is known that CD133 is lost when EPCs differentiate into mature endothelial cells [110]. Fibroblast growth factor (FGF) is another protein that is crucial to EPC induction and EPCs harbour the FGF receptor (FGFR). In summary, EPCs have been described as CD 34+, FGFR+, VE-Cadherin+, c-kit+, KDR+, CD133+. EPCs are also characterized by their ability to grow in culture; hence some investigators enumerate EPCs by culture assays. Another functional characteristic of EPCs is the uptake of acetylated low-density lipoprotein (ac-LDL) [111]. Unfortunately, consensus as to the definition of EPCs has not been achieved due to several issues. Some investigators have described sub-populations of EPCs, i.e. early EPCs versus late EPCs [112]. Others have described a population of CD 133-endothelial progenitor cells, which were CD 14+ in keeping with a monocytic lineage. Matters are further complicated by the fact that several populations of different progenitor cells exist (e.g.

Table 3
Characteristic properties of CECs and EPCs

	CEC	EPC
Cell type	Mature endothelium	Endothelial progenitor cell
Origin	Vessel wall	Bone marrow
Morphology	Cells, a-nuclear carcasses or sheets of multiple cells 10–100 μm	Diameter less than 20 μm
Characteristic properties	VWF	CD 133
	CD 31	CD 34
	Thrombomodulin	TIE-2
	CD 146	KDR
	UEA-1	Uptake of acetylated LDL
		UEA-1 (unclear)
Colony-forming potential	None (controversial)	Yes
Laboratory methods	Immunomagnetic isolation, FACS	FACS, culture assays

mesenchymal or lymphatic) [113,114]. Table 3 summarizes characteristic properties of CECs and EPCs.

The distinction between EPCs and CECs remains difficult to say the least. In particular, it must be appreciated that endothelial markers, such as CD-146 and UEA-1 are also present on EPCs. However, the severely damaged morphology of cells obtained by CD-146-driven immunomagnetic isolation and the inability to growth these cells in culture [22] was regarded as indication that these cells are not EPCs, at least not to a substantial degree. Our own experience shows that CD 146 positive cells were CD 133 negative [63]. Very recently, however, Delorme et al. clearly demonstrated EPCs among a population of cells isolated by CD-146-driven immunomagnetic isolation [115]. Although their findings need corroboration, new protocols of immunomagnetic isolation may be needed to exclude EPCs.

9. Conclusion

Circulating endothelial cells are a novel marker of vascular damage. Cell numbers correlate with disease activity across a variety of diseases. Their use in a clinical setting is on the horizon and pathogenetic clues may also be obtained. The phenotype and functional capacity of these cells as well as interactions with other cell subsets need to be further elucidated. Another crucial issue is standardization of the technique, a task that has been addressed by a European multi-center effort. In addition, the proportion of endothelial progenitor cells obtained by CD 146-driven immunomagnetic isolation needs to be determined. Finally, more circulating markers of endothelial damage may be characterized. A panel of markers, including circulating endothelial cells, endothelial microparticles, thrombomodulin/vWF and endothelial progenitor cells may be optimal to gauge vascular integrity and repair.

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