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Circulating Endothelial Cells, Endothelial Progenitor Cells, and Endothelial Microparticles in Cancer

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Abstract

Cancer, a proliferative disease hallmarked by abnormal cell growth and spread, is largely dependent on tumor neoangiogenesis, with evidence of vascular endothelial dysfunction. Novel ways to assess vascular function in cancer include measuring levels of circulating endothelial cells (CEC). Rare in healthy individuals, increased CEC in peripheral blood reflects significant vascular damage and dysfunction. They have been documented in many human diseases, including different types of cancers. An additional circulating cell population are endothelial progenitor cells (EPC), which have the ability to form endothelial colonies *in vitro* and may contribute toward vasculogenesis. At present, there is great interest in evaluating the role of EPC as novel markers for tumor angiogenesis and drug therapy monitoring. Recently, exocytic procoagulant endothelial microparticles (EMP) have also been identified. CEC, EPC, and EMP research works may have important clinical implications but are often impeded by methodological issues and a lack of consensus on phenotypic identification of these cells and particles. This review aims to collate existing literature and provide an overview on the current position of CEC, EPC, and EMP in cell biology terms and to identify their significance to clinical medicine, with particular emphasis on relationship with cancer.

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Introduction

Recent research has established the importance of altered vascular endothelium function to the neoplastic disease process. Studies have shown that cancer progression is largely dependent on abnormal angiogenesis, whereby new vessel formation ensures an adequate supply of nutrients, oxygen, and growth factors to the growing tumor and also facilitates tumor dissemination [1]. Vascular endothelial cells (EC) respond to numerous pathophysiological stimuli such as growth factors, cytokines, lipoproteins, and oxidative stress. Prolonged or unregulated activation of these cells often results in a loss of EC integrity and, thus, dysfunction—a process that can be assessed by the use of specific plasma markers such as von Willebrand factor (vWf), tissue plasminogen activator, soluble EC protein C receptor, soluble E selectin, and soluble thrombomodulin, as well as physiological techniques such as flow-mediated dilatation (FMD) [2]. Indeed, endothelial perturbation in cancer may well contribute to an increased risk of thrombosis in these patients [3].

The presence of circulating endothelial cells (CEC) has recently been recognized as a useful marker of vascular damage. Usually absent in the blood of healthy individuals, CEC counts are elevated in diseases hallmarked by the presence of vascular insult, such as sickle cell anemia, acute myocardial infarction, Cytomegalovirus (CMV) infection, endotoxemia, and neoplastic processes. Current opinion suggests that CEC are cells driven from the intima after vascular insult, and are thus the consequence—rather than the initiator—of a particular pathology [4].

A related circulating cell population are endothelial progenitor cells (EPC), which originate from the bone marrow, rather than from vessel walls. Seen in small numbers in healthy individuals, their numbers tend to increase following vascular injury [5]. So far, experiments have established the ability of EPC to form colonies *in vitro*, suggesting a role in both angiogenesis and in the maintenance of existing vessel walls [6]. Recent evidence has suggested the involvement of EPC in tumor vasculogenesis [7].

Recently, another endothelial marker linked with vascular dysfunction has been identified. Endothelial microparticles (EMP) are vesicles formed by the EC membrane after injury or activation, harboring cell surface proteins and cytoplasmic elements and expressing endothelial-specific surface markers reflective of parent cell status (e.g., activated, apoptotic) [8].

Due to the diversity of techniques found in CEC, EPC, and EMP research, it is often difficult to compare data between different investigating groups, frequently leading to confusion. The main objectives of this review article are: 1) to collate existing literature; 2) to provide an overview of the current

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position of these cells and particles in cell biology terms; and 3) to identify its significance in clinical medicine, with particular emphasis on relationship with cancer.

Search Strategy

We conducted a literature search using Medline, PubMed, and EMBASE to highlight published works with keywords CEC, EPC, EMP, vascular injury, angiogenesis, and cancer. Results were limited to articles published between 1980 and 2005 in English peer-reviewed journals. Abstracts and reports from meetings were included only when they related directly to previously published works.

CEC

Background CEC were first described in the 1970s using methods such as light microscopy, cell morphology, May Grünwald Giemsa staining, and density centrifugation. None of these methods identified CEC conclusively due to lack of endothelium-specific antibody markers; moreover, the methods were generally too cumbersome.

In 1991, monoclonal antibodies to two new cell surface antigens specific to EC (HEC 19 and S-Endo 1) were reported, allowing for a more accurate quantification of CEC [9]. George et al. named the antigen for their S-Endo 1 antibody CD146. Solovey et al. [10] subsequently used another antibody P1H12 against CD146 to enumerate CEC in sickle cell anemia. Mancuso et al. [11] were the first to describe CEC in cancer.

Enumeration Despite the lack of a clear consensus on phenotypic identification, CEC are generally accepted as cells expressing endothelial markers [e.g., vWf, CD146, and vascular endothelial cadherin (VE-cadherin)] in the absence of hematopoietic (CD45 and CD14) and progenitor (CD133) markers. Interestingly, the progenitor marker CD34 is also present on mature CEC. Although CD146 is widely regarded as the principal marker for CEC (mature cell form), it has also been described in trophoblasts, mesenchymal stem cells, periodontal and malignant (prostatic cancer and melanoma) tissues [4], and activated lymphocytes [12]. Consequently, caution in interpreting results with CD146 alone is demanded as cells identified by this marker may indeed be circulating tumor cells or other non-endothelial circulating cell. As such, it may be advisable to use a second identification method, such as *Ulex europaeus* lectin-1 (UEA-1), acetylated low-density lipoprotein cholesterol, or vWf.

Together with EPC, CEC only represent between 0.01% and 0.0001% of mononuclear cells in normal peripheral blood [12], making it very difficult to accurately quantify their numbers. To do this, it is often necessary to employ cell enrichment techniques combined with specific cell marker labeling.

The immunobead capture method (immunomagnetic beads bearing CD146 antibodies) developed by George et al. [9] is the most widely used. Immunobeads have been successfully employed by other investigators, albeit with modifications [e.g., addition of EDTA and albumin to minimize CEC autoaggregation; drying CEC on a glass slide before counting (this enables storage at room temperature and secondary labeling); use of UEA-1 (an EC-specific stain); addition of an F_c receptor blocking agent, and double labeling for further analyses (e.g., for CD31 and CD34)] [4]. After cell separation, either fluorescence microscopy (Figure 1), immunocytochemistry, or flow cytometry is used to confirm the endothelial phenotype of the cells. Other methods used to concentrate mononuclear cell suspensions include standard and density (Lymphoprep, Axis-Shield, Oslo, Norway; Percoll, Sigma, St. Louis, MO; Ficoll, Sigma) centrifugation and mononuclear cell culturing on fibronectin-coated plates. [4]. The main alternative to the immunobead method is flow cytometry [11,12].

In current published literature, the reported numbers of CEC vary significantly from 1 to 39,000 cells/ml in disease states, and from 0 to 7900 cells/ml in healthy controls. The variation in numbers stems partly from the diverse nature of the diseases investigated, but also from the nonstandardization of the methods used. For example, immunobead and density centrifugation methods tend to show values of around 10 cells/ml in healthy controls, whereas the numbers yielded by flow cytometry are much greater (up to a 1000-fold increase) [4,11]. The reasons for this discrepancy are unclear but would appear methodological [i.e., technical (gating) and/or different choices of cell surface markers to define CEC] [12]. Accordingly, consensus is badly needed.

Owing to significant differences in CEC enumeration depending on the technique used, a comparison of meaningful data between investigating groups is difficult. As such, the optimal method for CEC quantification remains unknown, with more research needed on correlative functional and genomic studies of separated cells to measure the accuracy of immunophenotyping [4].

Origin and pathophysiology of CEC The endothelium can be viewed as a membrane-like layer lining the circulatory system, its primary function being the maintenance of vessel wall permeability and integrity. The EC layer is relatively quiescent, with an estimated cell turnover period of between 47 and 23,000 days, as shown by labeling studies [13]. Proliferation seems to occur mainly at sites of vasculature branching and turbulent flow. As mentioned before, CEC

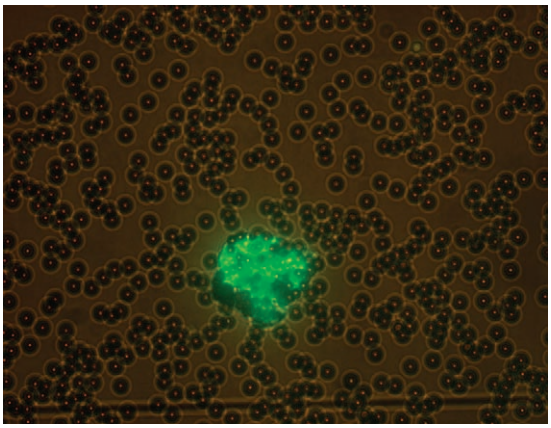

Figure 1. *Fluorescence microscopy of a large CEC rosetted by immunobeads.*

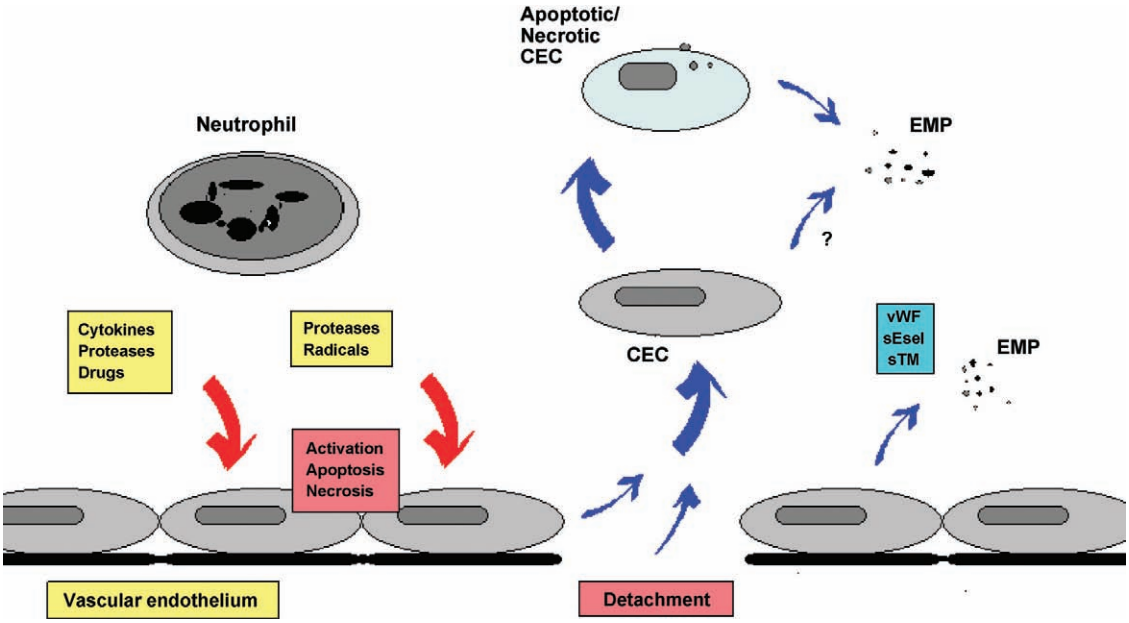

Figure 2. *Potential mechanisms for EC detachment and microparticle formation. CEC, circulating endothelial cells; EMP, endothelial microparticles; sTM, soluble thrombomodulin; sE-Sel, soluble E-selectin; vWF, von Willebrand factor.*

are thought to have "sloughed off" vessel walls, indicating severe endothelial damage [4] (Figure 2). Thus, unsurprisingly, CEC have been shown to correlate with various endothelial dysfunction and inflammatory markers (Refs. [14–19]; Table 1).

Although not fully understood, it would appear that CEC detachment from the endothelium involves multiple factors, such as mechanical injury, alteration of endothelial cellular adhesion molecules (such as integrin $\alpha_V\beta_3$), defective binding to anchoring matrix proteins (such as fibronectin, laminin, or type IV collagen), and cellular apoptosis with decreased survival of cytoskeletal proteins [4,20]. The net effect is a reduced interaction between the EC and basement membrane proteins, with subsequent cellular detachment.

Depending on the disease process, it would seem that the vessel origin of CEC can vary significantly. Researchers using specific antibody markers were able to delineate the microvascular (CD36) origin of CEC in diseases such as cancer, thalassemia, and sickle cell disease (SCD) [10,11,21]. Conversely, CEC in acute coronary syndrome and systemic lupus erythematosus (SLE) patients were from the macrovasculature [4,22]. In short, by analyzing the phenotypic expression of CEC, important knowledge on the severity and pathogenesis of vascular diseases can be obtained in a relatively noninvasive manner.

CEC in cancer Elevated numbers of CEC have been variously described in lymphoma, melanoma, and glioma patients, as well as in breast, colonic, gastric, esophageal, renal cell, ovarian, cervical, carcinoid, testicular, prostate, and head and neck cancer patients, reflecting the perturbation of vascular endothelium in cancer disease (Refs. [11,23–26]; Table 2). However, the clinical significance of CEC in cancer is still poorly understood; we do not know whether or not CEC are merely markers of altered vascular integrity, or are direct contributors to the neoplastic process and its associated complications. Particularly significant is the way in which CEC appear in the circulation of cancer patients. Are the CEC being shed from localized damaged or activated tumor vessels, or from a more generalized

Table 1. Correlation of CEC with Markers of Inflammation and Endothelial Dysfunction.

References	Disease	Marker/Measurement of Vascular Damage	Correlation with CEC Count	
			ρ (r)	P
Chong et al. [14]	CHF	FMD	−0.423	<.002
		vWf	0.29	.032
Del Papa et al. [15]	Systemic sclerosis	sE-Sel	0.594	.01
Kas-Deelen et al. [16]	CMV	vWf	_*	<.001
Lee et al. [17]	ACS	IL-6	0.55	<.001
		vWf	0.44	<.001
Makin et al. [18]	Atherosclerotic disease	vWf	0.40	<.002
		TF	0.296	<.021
Rajagopalan et al. [19]	SLE	FMD	−0.32	<.037
		TF	0.46	<.002

CHF, congestive heart failure; *ACS*, acute coronary syndromes; *sE-Sel*, soluble E-selectin.
*Data not provided.

Table 2. Studies in Human Cancer Patients and the Relationship with CEC and EPC.

References	Method	Cancer Type	Number of Patients	Mean CEC Number (ml)		Mean EPC Number (ml)	
				Controls	Patients	Controls	Patients
Beerepoot et al. [23]	IB	Variety*	95	121 ± 16	399 ± 36	NA	NA
Beerepoot et al. [24]	IB	Variety*	34	82 ± 25	157 ± 27	NA	NA
Kim et al. [25]	Culture + IC*	Gastric, breast	71	NA	NA	40.2 ± 10.2†	37.6 ± 4.2†
Mancuso et al. [11]	FC	Breast, lymphoma	76	7900	39,000	<500	<500
Zhang et al. [26]	FC	MM	31	<1000‡	>7,500‡	NA	NA

IB, immunobead; *IC*, immunocytochemistry; *FC*, flow cytometry

*A variety of cancers: head and neck, colon, prostate, gastric, esophagus, renal, breast, ovarian, cervix, and carcinoid cancers, as well as melanoma and glioma.

†Stated per unit area (mm^2).

‡Raw data not supplied; estimated from figures in the text.

systemic endothelial activation? Are they cells that have originated from bone marrow progenitor cells differentiating into mature CEC form?

Recently, Mancuso et al. [11], using cytometric analysis, demonstrated a five-fold increase ($P < .0008$) of CEC in breast cancer ($n = 46$) and lymphoma ($n = 30$) patients compared with healthy controls ($n = 20$). In this study, CEC levels in early and metastatic (advanced stage) breast cancer patients were not significantly different, whereas quadrantectomy (breast-conserving surgery) was associated with a reduction in CEC. In addition, lymphoma patients with total disease remission after chemotherapy ($n = 7$) achieved normal levels of CEC, therefore suggesting a potential role for CEC in monitoring response to anticancer treatment [11]. Beerepoot et al. [23,24] also found a significant rise in CEC in cancer patients with progressive disease ($n = 95$; $P < .001$), whereas patients with stable disease ($n = 17$; $P < .69$) yielded levels comparable to healthy controls. Different methods of assessing disease stage were employed in these studies. For example, Beerepoot et al. [23] defined "progressive disease" as a radiologic increase in tumor size, but Mancuso et al. [11] employed node and metastatic status. In addition, the immunobead method was employed by Beerepoot et al., whereas Mancuso et al. used flow cytometry; this clearly precludes a direct comparison of results between the two investigating groups.

Zhang et al. [26] characterized CEC in multiple myeloma (MM), reporting elevated numbers of both CEC ($P < .001$) and EPC. CEC also correlated well with serum markers of disease activity, namely, serum M protein and β_2-microglobulin ($r = 0.62$, $P < .001$; $r = 0.72$, $P < .001$, respectively). They also demonstrated a clinical response to treatment with thalidomide and its immunoregulatory derivative CC-5013, indicated by a parallel decline in both CEC and EPC levels with disease activity. This makes CEC/EPC measurements a potentially useful indicator of response to treatment in MM.

However, a serious confounder in clinical studies is the role of chemotherapy and radiotherapy. Indeed, it has been established that a certain cytotoxic chemotherapy is specifically designed to attack the endothelium and, as such, endothelial damage may well be a side effect of other chemotherapies [27,28]. Accordingly, as does raised plasma vWf [29], raised CEC may well reflect treatment but not necessarily the oncologic process.

Association of CEC with other markers in cancer As a surrogate marker of angiogenesis, it is not surprising to note that vascular endothelial growth factor (VEGF) has a strong association with malignant disease [7,30]. In cancer, increased VEGF released from tumor cells, as well as macrophages and platelets [31], is associated with tumor progression and poor prognosis [32]. Indeed, Mancuso et al. [11] demonstrated a positive correlation between CEC and both VEGF ($r = 0.42$, $P = .009$) and vascular adhesion molecule-1 ($r = 0.582$, $P < .0001$) in breast cancer patients. Their findings may imply a relationship with angiogenesis. In contrast, Beerepoot et al. [23] showed no such correlation (CEC *versus* VEGF) in their study subjects ($n = 95$) and postulated a number of reasons for this, such as the limited half-life of plasma cytokines and the possible variations between local and systemic levels.

Other studies also note that CEC viability appears markedly pronounced in tumor-bearing models *versus* controls [33]. Because VEGF is a mitogen and a survival factor for EC [7] possessing antiapoptotic properties, it is conceivable that it has a protective role in preventing CEC apoptosis [34]. Beerepoot et al. [23] also investigated the possible association of other cytokines [placental growth factor (PlGF), stromal-derived factor-1 (SDF-1), and stem cell factor (SCF)] with CEC and found increased PGF levels in cancer patients ($P = .01$). SDF-1 and SCF levels were no different between patients and controls.

Recently, interest has risen in the assessment of the endothelial-specific adhesion molecule VE-cadherin (cadherin-5 or CD144) specifically in the context of tumor angiogenesis [35]. Martin et al. [36] established a positive relationship between levels of VE-cadherin and microvessel density (MVD) in breast cancer specimens, relating increased VE-cadherin RNA with poorer prognosis, positive node status, and higher TNM staging. Furthermore, Rabascio et al. [37] reported elevated levels of VE-cadherin RNA expression in cancer patients ($P < .001$) compared to controls, suggesting a novel technique in assessing cancer angiogenesis. Importantly, they demonstrated a significant correlation between VE-cadherin RNA and viable CEC in patients with hematologic malignancies ($r = 0.86$, $P = .008$). The clinical significance is unclear because VE-cadherin is important for cell-to-cell interactions and the integrity of the cellular monolayer [38,39]; an increase in its expression should likewise reflect a stable vascular endothelium,

resulting in an expected decrease in CEC. In reality, the loss of VE-cadherin expression actually results in the disassembly of nascent blood vessels in murine models [40]. Further studies are warranted to establish a similar positive association in solid tumors.

CEC and coagulopathy Thrombosis is a big problem in cancer [3]. Tissue factor (TF), the primary cellular initiator of blood coagulation *in vivo*, is associated with both systemic hypercoagulability [41] and tumor angiogenesis in cancer [42]. Secreted by tumor cells [43], TF upregulation contributes to tumor progression and correlates with clinical stage, histologic grade, and poor prognosis [44,45], although direct inhibition of TF suppresses tumor growth [46]. To date, the only demonstrable positive correlation between CEC and circulating TF has been in diseases with vascular damage/dysfunction, such as atherosclerotic diseases [18], SLE [19], and sickle cell anemia [10]. In cancer, despite clear TF expression on malignant tumor EC [47], there is a distinct lack of any similar exression on CEC, as described by Beerepoot et al. [24]. The reason for this is unclear, as *in vitro* stimulation of CEC induced TF expression. To explain this conundrum, the authors speculate that local intratumoral coagulation [48] results in a "sump" for TF-positive CEC and prevents these CEC from appearing in the peripheral circulation. Of note, this has not been validated in any experiment to date.

EPC

Background and methodology Asahara et al. [49] was the first to isolate EPC in human peripheral blood, using anti-CD34 monoclonal antibodies. With the use of CD133, an antigen specifically identifying primitive stem cells, a novel means to precisely delineate mature (CEC) from immature (EPC) EC forms was possible [50], although this antigen is only present in human EPC and cannot be applied to mouse EPC [51]. To detect EPC in peripheral blood, flow cytometry and culture have become the principal methods employed. Other markers used include vWf, VE-cadherin, vascular endothelial growth factor receptor-2 (VEGFR-KDR) and binding by lectins and acetylated low-density lipoproteins [50–52].

Origin and pathophysiology of EPC EPC are potentially crucial for neovascularization and may be recruited from the bone marrow after tissue ischemia, vascular insult, or tumor growth [5,51–53]. They possess the ability to migrate, colonize, proliferate, and, ultimately, differentiate into endothelial lineage cells. These cells have yet to acquire mature EC characteristics (Figure 3) while appearing to contribute to vascular homeostasis.

EPC have been isolated previously from human umbilical cord blood, adult bone marrow, human fetal liver cells, and cytokine-mobilized peripheral blood, and an increase in circulating EPC follows *in vivo* administration of the angiogenic

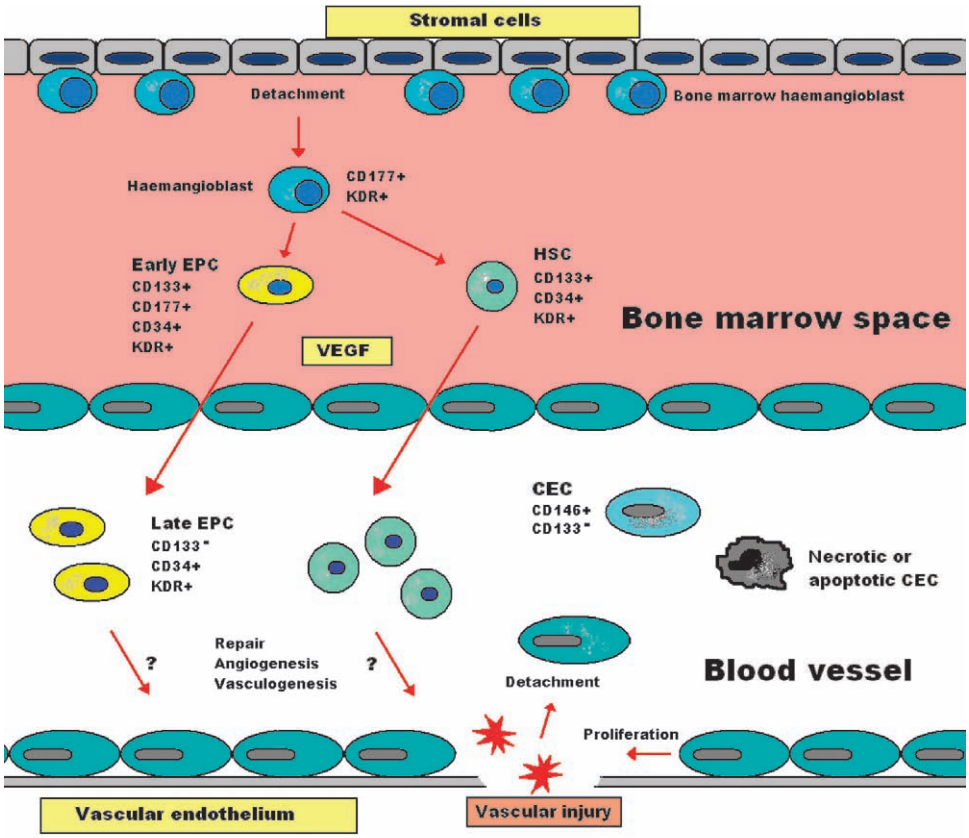

Figure 3. *Schematic representation of CEC, EPC, and HSC in vascular damage, repair, and angiogenesis. EMP, endothelial microparticles; VEGF, vascular endothelial growth factor; KDR, VEGFR-2.*

growth factor VEGF [52,53]. When incubated with VEGF, fibroblast growth factor-2 (FGF-2), and insulin-like growth factor, $CD133^+$ cells differentiated into mature-type adherent EC, expressing endothelial-specific cell markers (vWf and VE-cadherin) and abolishing CD133 expression [54]. For example, Lin et al. [55] documented the generation of endothelial outgrowths that are positive for CD146, vWf (mature endothelial markers), and CD36 (a microvascular marker) markers from circulating mononuclear cells (of donor genotype in bone marrow transplant patients), which strongly suggests the viability and proliferative potential of EPC.

EPC recruitment and mobilization have been positively correlated with increased levels of angiogenic growth factors such as VEGF [56]. VEGF induces the proliferation, differentiation, and chemotaxis of EPC, and is essential for hematopoiesis, angiogenesis, and, ultimately, survival, as evidenced by the nonviability of mouse embryos expressing only a single VEGF allele [57]. EPC influence cells mainly by interactions with VEGFR-1 and VEGFR-2, both being receptors expressed on hematopoietic stem cells (HSC) and EPC (Figure 3) [58]. In another study, granulocyte colony-stimulating factor also increased the number of $CD34^+$ cells, potentially stimulating neovascularization in areas of ischemic myocardium [59]. Other angiogenic growth factors stimulating EPC mobilization include angiopoietin-1, FGF, SDF-1, PlGF, and (in mice) macrophage colony-stimulating factor [60–64]. After mobilization, EPC appear to "home in" and become incorporated into sites of vascular injury and ischemia, with evidence of improvement in the function and viability of tissue (e.g., after acute myocardial infarction) [59]. Chemotactic agents responsible for this process include VEGF [65] and SDF-1 [62], but others may also be involved.

In the clinical setting, moderate exercise of patients with stable coronary artery diseases leads to a significant increase in circulating EPC [66]. Furthermore, EPC and HSC introduced into the circulation of acute and chronic cardiovascular disease patients through injection have shown encouraging preliminary results, with evidence of improved cardiovascular function and tissue perfusion [67]; as yet, there are no randomized control trials.

Cancer, Neovascularization, and EPC: More Questions than Answers?

Blood vessels are essentially composed of EC that align and interconnect, forming tubes for directing and maintaining blood flow. In cancer, new blood vessel formation is essential for its growth and dissemination. The traditionally accepted view is that neovascularization in adult life occurs by a process known as angiogenesis [68], whereby new capillaries sprout from existing vasculature as vessel wall–associated EC proliferate and migrate. New research has suggested an alternative means by which vessels are formed, namely, by postnatal vasculogenesis or by differentiation of primitive/progenitor EC into mature EC [49]. These cells originate from the bone marrow cell population, with subsequent mobilization and homing to sites of vascular growth and repair [68].

More controversially, in cancer, do EPC mobilize in response to cytokine release either by tumor cells or from damaged tissues/host immune cells? If so, do EPC merely perform a regulatory role in angiogenesis, or do they form part of the new tumor vasculature? The current evidence for BM-derived EC neoincorporation into tumor vasculature is conflicting. In some studies, it has been shown that bone marrow–derived EPC contribute to tumor vessel formation by incorporating into the neoendothelium [53,68]. In mice transplantation studies, donor BM-derived EC integrated into newly formed blood vessels, sometimes by as much as 50% [69], whereas other studies reported lower but significant levels between 10% and 20% [51,70]. These findings have recently been challenged by the work of De Palma et al. [71], who demonstrated BM-derived hemopoietic cells ($CD45^+/CD11b^+/CD31^-/Tie2^+$) rather than EPC ($CD31^+$), homed specifically to tumors, without any evidence of incorporation. The reason for such diametrically conflicting results remains unclear. The reason might be the use of differing experimental models and techniques to identify BM-derived endothelium [72]. Despite the controversy, De Palma et al. [71] demonstrated that inhibition of the BM-derived Tie2-expressing mononuclear cells with a "suicide" gene approach resulted in a significant reduction of tumor angiogenesis and growth. The implications may herald new targets for novel antitumor treatment [72].

Several other reports, some based on laser scanning confocal microscopy techniques (i.e., a more definitive cell detection method), have also questioned the importance and veracity of the "neointegration" concept and, instead, have implicated other hematopoietic BM-derived cells such as monocytes, macrophages, or pericyte precursors [73,74]. These cells are felt to be angiogenesis-promoting and adhere to vessel walls without actual integration. Indeed, Shaked et al. [75] strongly correlated peripheral blood $CD13^+/VEGFR\text{-}2^+/CD45^-/CD117^+$ cells (defining them as EPC) with angiogenesis but did not directly address this contentious issue. Instead, they concluded that there are measurable circulating $VEGFR\text{-}2^+$ cells contributing to angiogenesis, which might be true endothelial "progenitor" cells or perivascular adherent "support" cells; these constitute a valid surrogate marker for angiogenesis, particularly when assessing antiangiogenesis therapy.

As mentioned, VEGF-induced BM-derived EPC mobilization has been reported [61,68]. However, in human cancer patients, Mancuso et al. [11] noted no significant rise in EPC levels as compared with healthy controls, even with raised levels of VEGF. This finding was recently corroborated by Kim et al. [25] and suggests that typical VEGF levels in cancer patients might not be sufficient to mobilize EPC into the circulation (Table 3). In another study, Zhang et al. [26] (mentioned earlier) reported raised EPC levels in MM patients.

CEC and EPC: Surrogate Markers of Tumor Angiogenesis and Growth?

Because the role of tumor angiogenesis is seen as crucial in the progression of cancer, attempts have been made to accurately measure this process [e.g., relative MVD estimates

Table 3. Differences Between CEC, EPC, and EMP.

Plasma Marker	CEC	EPC	EMP
Origin	Mature endothelium	Bone marrow, cord blood, mobilized MC	EC
Morphology	Mature cells 20–50 μM in diameter	Immature cells <20 μM in diameter	Exocytic vesicles 0.5–1.5 μM in diameter
Phenotype	CD133 −ve /CD146 +ve	CD133 +ve/CD34 +ve/KDR + CD146 −ve	Endothelial markers*
High proliferative potential	No	Yes	No
Pathophysiology	Reflective of damage	Neovascularization	Reflective of damage Procoagulant Inflammatory†

MC, mononuclear cells
*Dependent on the status of parent cell (i.e., activated, apoptotic).
†Causes monocyte activation and endothelial damage (reproduced with permission and adapted from Ref. [92]).

(Chalkley count method), EC proliferation fractions, and VEGF levels] [76]. Currently, measurements of tumor angiogenesis aimed at evaluating antiangiogenic cancer therapy are based mainly on MVD; in this technique, blood vessels in tumor samples are stained with relevant endothelial antibodies and counted through light microscopy. However, it is labor-intensive and requires tissues that could potentially be unrepresentative of the whole tumor.

Animal models bearing human lymphoma show a correlation of CEC levels with tumor growth/volume and suggest its use as a potential surrogate marker of angiogenesis [32–34]. Mice xenografted with human lymphoma also show higher values of CEC compared with controls. CEC and tumor volume correlate strongly ($r = 0.942$, $P = .004$), compared with standard MVD measurements (MVD *versus* tumor volume: $r = 0.948$, $P = .05$). In addition, CEC correlated positively with tumor weight ($r = 0.885$, $P = .01$) and tumor-generated human VEGF ($r = 0.669$, $P = .02$).

In the same model, administration of cyclophosphamide (CTX) was investigated using maximum tolerable dose (MTD) and metronomic regimens. MTD provoked a vigorous EPC elevation in peripheral blood, in stark contrast to metronomic CTX, which suppressed EPC numbers and viability (increased apoptosis) with concurrent tumor inhibition. MTD CTX induced the apoptosis of circulating hematopoietic and, to a lesser extent, of CEC. The authors suggest a mechanism of direct cell death by CTX and, possibly, inhibition of EPC mobilization, causing an antivasculogenetic effect to explain the results of metronomic chemotherapy [77].

In parallel studies, continuous endostatin infusion (CEI) was compared with bolus administration. Compared with bolus chemotherapy, CEI treatment caused greater inhibition as well as differentiation of EPC, with significant tumor (human lymphoma) suppression. Unlike CTX, endostatin seemed to target cells of endothelial, rather than hematopoietic, lineage [33]. Schuch et al. [78] reported a reduction in EPC numbers along with decreased bone marrow neovascularization in mice receiving endostatin. Furthermore, endostatin was shown to target EPC mobilization (in the presence of VEGF stimulation) and increased the rate of apoptosis, thus confirming the findings of Capillo et al. [33]. Preliminary reports of phase 1 clinical trials with endostatin highlighted a >10-fold CEC reduction in patients with stable lymphoma disease, compared with no change or an increase in progressive disease patients [79].

Another report [75] demonstrated a strong correlation between tumor growth and both CEC and EPC numbers in mice using various tumor models [transplanted *versus* spontaneous, solid *versus* leukemic; syngeneic Lewis lung carcinoma LL/2, erythroleukemia, orthotopic human breast cancer MDA-MB-231, and human lymphoma (Namalwa)] and was able to effectively define the optimal antiangiogenic drug (anti–VEGFR-2) dosage based on CEC and EPC monitoring. Their results are all the more significant as they were based on different tumor types, antiangiogenic drugs, and mouse strains. In a recent phase 1 trial, VEGF-specific antibody bevacizumab (Avastin) reduced tumor MVD, tumor perfusion and vascular volume, interstitial fluid pressure, and the number of viable CEC and EPC in rectal carcinoma patients ($P < .05$, Wilcoxon signed rank), indicating that VEGF blockade has a direct antivascular effect on human tumors in the clinical setting [80].

These reports are especially encouraging as they suggest the potential of peripheral blood CEC/EPC evaluation to monitor antiangiogenic therapy efficacy and also help define the balance between cytotoxic and therapeutic thresholds of various drug regimes. This method of assessment would be a significant advantage compared with current methods (e.g., MVD or dynamic contrast-enhanced magnetic resonance imaging) [30].

EMP

One of the earliest descriptions of EMP was the shedding of membrane-derived particles from human umbilical vein endothelial cells (HUVEC) after complement-mediated lysis [81]. Combes et al. [82] subsequently induced EMP formation after the activation of HUVEC with TNF-α and also by the incubation of EC with the serum from antiphospholipid syndrome patients. Elevated EMP levels have since been demonstrated in a variety of diseases [83–91], but, as yet, there have been no adequately powered case–control studies in cancer.

Origin and enumeration The main cellular mechanisms known to induce EMP release from the endothelium are cellular activation, damage, and apoptosis [83] (Figure 2). EMP formation has been demonstrated using *in vitro* EC activation by cytokines such as TNF-α and interleukin (IL) 1 [83]. Laurence et al. [84] further reported apoptotic cell changes in EC after exposure to TTP plasma, suggesting a

potential correlation of EMP elevation with apoptosis. The phenotypic profile of EMP can vary considerably, depending on whether parent cells have undergone either activation (abundant $CD62E^+$) or apoptosis (predominantly $CD31^+$) [83]. The commonest technique used to identify EMP is high relative centrifugal force (RCF) in conjunction with flow cytometry [82]. Less well known is the solid phase capture method [85]. Unfortunately, EMP identification by these methods also lends itself to significant variations in the numbers obtained, possibly due to variations in technique—a situation reminiscent of CEC.

Pathophysiology Although their significance remains unclear, there is a growing view that EMP can function as important diffusible mediators of cytokines and adhesins, thus promoting cellular signaling and activity [86]. *In vitro* stimulation of HUVEC by angiogenic growth factors (VEGF and FGF-2) resulted in EMP formation that is rich in matrix metalloproteins and capillary cord–like structures, suggesting a potential *in vivo* role in angiogenesis [87].

It has been shown that EMP have procoagulant activity, defined by platelet factor 3 activity and TF [88]. In prothrombotic states such as SCD, Shet et al. [89] reported raised TF-positive EMP in patients compared with controls, and a strong correlation with procoagulant activity. Of note, Jimenez et al. [90] reported severe endothelial dysfunction in mice aorta after incubation with EMP, affecting the endothelial nitric oxide (NO) transduction pathway, but not NO synthase expression. EMP may also be proinflammatory as they tend to bind and activate monocytes, resulting in cytokine release (e.g., TNF-α and IL-1β), which causes further paracrine and/or autocrine activation of monocytes and endothelium [91].

These studies raise the possibility of EMP being mediators of vascular insult and inflammation in diseases, rather than just being markers of endothelial dysfunction. More data are required to answer these issues, particularly in its relationship with cancer, of which there are currently no reports to the best of our knowledge. These lead to the speculation of raised EMP in cancer and, possibly, to the hypothesis that they may have a role in the coagulopathy of this disease.

Conclusions and Future Directions

CEC elevation in the blood of patients is becoming established as a useful marker for severe vascular dysfunction [92]. The presence of CEC in significant numbers denotes a high degree of vascular damage and, in this respect, is more useful as a clinical marker than, for example, raised vWf or abnormal FMD. The significance of CEC elevation in cancer patients is less clear. Certainly, CEC reflect loss of vascular integrity to some degree (e.g., in the case of necrotic tumors or in postchemotherapy tumors) [11]. What is less clear is the functional contribution, if any, of CEC toward tumor angiogenesis. From existing research data, it is apparent that EPC have a potentially significant role to play in the evaluation of tumor angiogenesis and growth [93]. Mobilization by growth factors [56,59–65] and other agents such as statins [94] may reflect a new therapy. However, to clearly elucidate the biology of CEC, EPC, and EMP in cancer, more research is essential. Current efforts are hampered, in part, by the lack of a clear consensus on phenotypic definitions, which has led to much difficulty in comparing data. Indeed, this lack of precise definition has surely contributed to conflict.

Recent reports suggest that CEC and EPC enumeration can be used to monitor antiangiogenesis drug therapy, with some success. This exciting prospect needs to be fully corroborated in a clinical setting. In addition, CEC and EPC monitoring would need to be efficient, specific, robust, and reproducible. Therefore, it is vital to reach a general consensus regarding definitions and techniques for CEC, EPC, and EMP quantification, in order to validate further reports that have implications for future clinical trials involving these markers. Further study of CEC and EPC may represent a vital source of information in the understanding of tumor biology and would potentially be an important evaluator of tumor growth and angiogenesis, as well as a means of monitoring antiangiogenic drug activity.

References

[1] Folkman J (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* **1,** 27–31.
[2] Blann AD (2004). Assessment of endothelial dysfunction: focus on atherothrombotic disease. *Pathophysiol Haemost Thromb* **33,** 256–261.
[3] De Cicco M (2004). The prothrombotic state in cancer: pathogenic mechanisms. *Crit Rev Oncol Hematol* **50,** 187–196.
[4] Goon PKY, Boos CJ, and Lip GYH (2005). Circulating endothelial cells: markers of vascular dysfunction. *Clin Lab* **51,** 531–538 (review).
[5] Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, Girardi L, Yurt R, Himel H, and Rafii S (2001). Vascular trauma induces rapid but transient mobilisation of $VEGFR\text{-}2^+$, $AC133^+$ endothelial precursor cells. *Circ Res* **88,** 167–174.
[6] Burger PE, Coetzee S, McKeehan WL, Kan M, Cook P, Fan Y, Suda T, Hebbel RP, Novitsky N, Muller WA, et al. (2002). Fibroblast growth factor receptor-1 is expressed by endothelial progenitor cells. *Blood* **100,** 3527–3535.
[7] Rafii S, Lyden D, Benezra R, Hattori K, and Heissig B (2002). Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? *Nat Rev Cancer* **2,** 826–835.
[8] Mallat Z, Benamer H, Hugel B, Benessiano J, Steg PG, Freyssinet JM, and Tedgui A (2000). Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation* **101,** 841–843.
[9] George F, Brisson C, Poncelet P, Laurent JC, Massot O, Arnoux D, Ambrosi P, Klein-Soyer C, Cazenave JP, and Sampol J (1992). Rapid isolation of human endothelial cells from whole blood using S-Endo 1 monoclonal antibody coupled to immunomagnetic beads: demonstration of endothelial injury after angioplasty. *Thromb Haemost* **67,** 147–153.
[10] Solovey A, Lin Y, Browne P, Choong, Wayner E, and Hebble RP (1997). Circulating activated endothelial cells in sickle cell anemia. *N Engl J Med* **337,** 1584–1590.
[11] Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, and Bertolini F (2001). Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. *Blood* **97,** 3658–3661.
[12] Khan SS, Solomon MA, and McCoy JP Jr, (2005). Detection of circulating endothelial cells and endothelial progenitor cells by flow cytometry. *Cytometry B Clin Cytom* **64,** 1–8.
[13] Hobson B and Denekamp J (1984). Endothelial proliferation in tumours and normal tissues: continuous labelling studies. *Br J Cancer* **49,** 405–413.
[14] Chong AY, Blann AD, Patel J, Freestone B, Hughes E, and Lip GYH (2004). Endothelial dysfunction and damage in congestive heart failure: relation of flow-mediated dilatation to circulating endothelial cells, plasma indexes of endothelial damage, and brain natriuretic peptide. *Circulation* **110,** 1794–1798.

[15] Del Papa N, Colombo G, Fracchiolla N, Moronetti LM, Ingegnoli F, and Maglione W (2004). Circulating endothelial cells as markers of ongoing vascular disease in systemic sclerosis. *Arthritis Rheum* **50,** 1296–1304.

[16] Kas-Deelen AM, Harmsen MC, de Maar EF, Oost-Kort WW, Tervaert JW, Van der Meer J, Van Son WJ, and The TH (2000). Acute rejection before cytomegalovirus infection enhances von Willebrand factor and soluble VCAM-1 in blood. *Kidney Int* **58,** 2533–2542.

[17] Lee KW, Lip GYH, Tayebjee M, Foster W, and Blann AD (2005). Circulating endothelial cells, von Willebrand factor, interleukin-6, and prognosis in patients with acute coronary syndromes. *Blood* **105,** 526–532.

[18] Makin A, Chung NA, Silverman SH, and Lip GY (2004). Assessment of endothelial damage in atherosclerotic vascular disease by quantification of circulating endothelial cells. *Eur Heart J* **25,** 371–376.

[19] Rajagopalan S, Somers EC, Brook RD, Kehrer C, Pfenninger D, Lewis E, Chakrabarti A, Richardson BC, Shelden E, McCune EJ, et al. (2004). Endothelial cell apoptosis in systemic lupus erythematosus: a common pathway for abnormal vascular function and thrombosis propensity. *Blood* **103,** 3677–3683.

[20] Ruegg C, Yilmaz A, Bieller G, Bamat J, Chaubert P, and Lejeune FJ (1998). Evidence for the involvement of endothelial cell integrin $\alpha v\beta 3$ in the disruption of the tumour vasculature induced by TNF and IFN-gamma. *Nat Med* **4,** 408–414.

[21] Butthep P, Rummavas S, Wisedpanichkij R, Jindadamrongwech S, Fucharoen S, and Bunyaratvej A (2002). Increased circulating activated endothelial cells, vascular endothelial growth factor, and tumour necrosis factor in thalassaemia. *Am J Hematol* **70,** 100–106.

[22] Mutin M, Canavy I, Blann AD, Bory M, Sampol J, and Dignat-George F (1999). Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. *Blood* **93,** 2951–2958.

[23] Beerepoot LV, Mehra N, Vermaat JS, Zonnenberg BA, Gebbink MF, and Voest EE (2004). Increased levels of viable of circulating endothelial cells are an indicator of progressive disease in cancer patients. *Ann Oncol* **15,** 139–145.

[24] Beerepoot LV, Mehra N, Linschoten F, Jorna AS, Lisman T, Verheul HM, and Voest EE (2004). Circulating endothelial cells in cancer patients do not express tissue factor. *Cancer Lett* **213,** 241–248.

[25] Kim HK, Song KS, Kim HO, Chung JH, Lee KR, Lee YJ, Lee DH, Lee ES, Kim HK, Ryu KW, et al. (2003). Circulating numbers of endothelial progenitor cells in patients with gastric and breast cancer. *Cancer Lett* **198,** 83–88.

[26] Zhang H, Vakil V, Braunstein M, Smith EL, Maroney J, Chen L, Dai K, Berenson JR, Hussain MM, Klueppelberg U, et al. (2005). Circulating endothelial progenitor cells in multiple myeloma: implications and significance. *Blood* **105,** 3286–3294.

[27] de Vos FY, Willemse PH, de Vries EG, and Gietema JA (2004). Endothelial cell effects of cytotoxics: balance between desired and unwanted effects. *Cancer Treat Rev* **30,** 495–513.

[28] Eissner G, Multhoff G, Gerbitz A, Kirchner S, Bauer S, Haffner S, Sondermann D, Andreesen R, and Holler E (2002). Fludarabine induces apoptosis, activation, and allogenicity in human endothelial and epithelial cells: protective effect of defibrotide. *Blood* **100,** 334–340.

[29] Nicolson GL and Custead SE (1985). Effects of chemotherapeutic drugs on platelet and metastatic tumor cell–endothelial cell interactions as a model for assessing vascular endothelial integrity. *Cancer Res* **45,** 331–336.

[30] Morgan B, Thomas AL, Drevs J, Hennig J, Buchert M, Jivan A, Horsfield MA, Mross K, Ball HA, Lee L, et al. (2003). Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/ZK 222584, an inhibitor of the VEGF receptor tyrosine kinases, in patients with advanced colorectal cancer and liver metastases: results from two phase 1 studies. *J Clin Oncol* **21,** 3955–3964.

[31] Gunsilius E, Tschmelitsch J, and Petzer AL (2001). Correlation of plasma and serum vascular endothelial growth factor levels with platelet count in colorectal cancer: clinical evidence of platelet scavenging? *Clin Cancer Res* **7,** 443.

[32] Adams J, Carder PJ, Downey S, Forbes MA, McLennan K, Allgar V, Kaufman S, Hallam S, Bicknell R, Walker JJ, et al. (2000). Vascular endothelial growth factor (VEGF) in breast cancer: comparison of plasma, serum and tissue VEGF and microvessel density and effects of tamoxifen. *Cancer Res* **60,** 2898–2905.

[33] Capillo M, Mancuso P, Gobbi A, Monestiroli S, Pruneri G, Dell'Agnola C, Martinelli G, Shultz L, and Bertolini F (2003). Continuous infusion of endostatin inhibits differentiation, mobilization and clonogenic potential of endothelial cell progenitors. *Clin Cancer Res* **9,** 377–382.

[34] Mancuso P, Calleri A, Cassi C, Gobbi A, Capillo M, Pruneri G, Martinelli G, Shultz L, and Bertolini F (2003). Circulating endothelial cells as a novel marker of angiogenesis. *Adv Exp Med Biol* **522,** 83–97.

[35] Corada M, Zanetta L, Orsenigo F, Breviario F, Lampugnani MG, Bernasconi S, Liao F, Hicklin DJ, et al. (2002). A monoclonal antibody to vascular endothelial-cadherin inhibits tumor angiogenesis without side effects on endothelial permeability. *Blood* **100,** 905–911.

[36] Martin TA, Watkins G, Lane J, and Jiang WG (2005). Assessing microvessels and angiogenesis in human breast cancer, using VE-cadherin. *Histopathology* **46,** 422–430.

[37] Rabascio C, Muratori E, Mancuso P, Calleri A, Raia V, Foutz T, Cinieri S, Veronesi G, Pruneri G, Lampertico P, et al. (2004). Assessing tumor angiogenesis: increasing VE-cadherin RNA in patients with cancer indicates viability of circulating endothelial cells. *Cancer Res* **64,** 4373–4377.

[38] Re F, Zanetti A, Sironi M, Polentarutti N, Lanfrancone L, Dejana E, and Colotta F (1994). Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. *J Cell Biol* **127,** 537–546.

[39] Lampugnani MG and Dejana E (1997). Interendothelial junctions: structure, signalling and functional roles. *Curr Opin Cell Biol* **9,** 674–682.

[40] Crosby CV, Fleming PA, Argraves WS, Corada M, Zanetta L, Dejana E, and Drake CJ (2005). VE-cadherin is not required for the formation of nascent blood vessels but acts to prevent their disassembly. *Blood* **105,** 2771–2776.

[41] Kakkar AK, DeRuvo N, Chinswangwatanakul V, Tebbutt S, and Williamson RC (1995). Extrinsic-pathway activation in cancer with high factor VIIa and tissue factor. *Lancet* **346,** 1004–1005.

[42] Zhang Y, Deng Y, Luther T, Muller M, Ziegler R, Waldherr R, Stern DM, and Nawroth PP (1994). Tissue factor controls the balance of angiogenic and antiangiogenic properties of tumour cells in mice. *J Clin Invest* **94,** 1320–1327.

[43] Yu JL, May L, Lhotak V, Sharzhad S, Shirasawa S, Weitz JI, Coomber BL, Mackman N, and Rak JW (2005). Oncogenic events regulate tissue factor expression in colorectal cancer cells: implications for tumor progression and angiogenesis. *Blood* **105,** 1734–1741.

[44] Shigemori C, Wada H, Matsumoto K, Shiku H, Nakamura S, and Suzuki H (1998). Tissue factor expression and metastatic potential of colorectal cancer. *Thromb Haemost* **80,** 894–898.

[45] Seto S, Onodera H, Kaido T, Yoshikawa A, Ishigami S, Arii S, and Imamura M (2000). Tissue factor expression in human colorectal carcinoma: correlation with hepatic metastasis and impact on prognosis. *Cancer* **88,** 295–301.

[46] Hembrough TA, Ruiz JF, Swerdlow BM, Swartz GM, Hammers HJ, Zhang L, Plum SM, Williams MS, Strickland DK, and Pribluda VS (2004). Identification and characterization of a very low density lipoprotein receptor–binding peptide from tissue factor pathway inhibitor that has antitumor and antiangiogenic activity. *Blood* **103,** 3374–3380.

[47] Contrino J, Hair G, Kreutzer DL, and Rickles FR (1996). *In situ* detection of tissue factor in vascular endothelial cells: correlation with the malignant phenotype of human breast disease. *Nat Med* **2,** 209–215.

[48] Verheul HM, Hoekman K, Lupu F, Broxterman HJ, van der Valk P, Kakkar AK, and Pinedo HM (2000). Platelet and coagulation activation with vascular endothelial growth factor generation in soft tissue sarcomas. *Clin Cancer Res* **6,** 166–171.

[49] Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatterman G, and Isner JM (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275,** 964–967.

[50] Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, and Rafii S (2000). Expression of VEGFR-2 and AC133 by circulating human $CD34^+$ cells identifies a population of functional endothelial precursors. *Blood* **95,** 952–958.

[51] Rafii S and Lyden D (2003). Therapeutic stem and progenitor cell transplantation for organ vascularisation and regeneration. *Nat Med* **9,** 702–712.

[52] Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Ito Y, and Isner M (1999). VEGF contributes to postnatal neovascularisation by bone marrow derived endothelial progenitor cells. *EMBO J* **18,** 3964–3972.

[53] Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, et al. (2001). Impaired recruitment of bone marrow derived endothelial and haematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* **7,** 1194–1201.

[54] Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, Schuch G, Schafhausen P, Mende T, Kilic N, et al. (2000). *In vitro* differentiation of endothelial cells from AC 133–positive progenitor cells. *Blood* **95,** 3106–3112.

[55] Lin Y, Weisdorf DJ, Solovey A, and Hebbel RP (2000). Origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest* **105,** 71–77.

[56] Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki K, Shimada T, Oike Y, Imaizumi T (2001). Mobilisation of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* **103,** 2776–2779.

[57] Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kiekens L, Gertstenstein M, Fahrig M, Vanderhoeck A, Harpal K, Eberhardt C, et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380,** 435–439.

[58] Rafii S, Heissig B, and Hattori K (2002). Efficient mobilization and recruitment of marrow-derived endothelial and hematopoietic stem cells by adenoviral vectors expressing angiogenic factors. *Gene Ther* **9,** 631–641.

[59] Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, and Itescu S (2001). Neovascularisation of ischaemic myocardium by human bone marrow–derived angioblasts prevents cardiomyocyte apoptosis, reduces remodelling and improves cardiac function. *Nat Med* **7,** 430–436.

[60] Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, and Isner JM (1999). Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularisation. *Circ Res* **85,** 221–228.

[61] Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M, Hicklin DJ, Zhu Z, Witte L, Crystal RG, et al. (2001). Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vascular and hematopoietic stem cells. *J Exp Med* **193,** 1005–1014.

[62] Yamaguchi J, Kusano KF, Masuo O, Kawamoto A, Silver M, Murasawa S, Bosch- Marce M, Masuda H, Losordo DW, Isner JM, et al. (2003). Stromal cell–derived factor-1 effects on *ex vivo* expanded endothelial progenitor cell recruitment for ischaemic neovascularisation. *Circulation* **107,** 1322–1328.

[63] Okazaki T, Ebihara S, Takahashi H, Asada M, Kanda A, and Sasaki H (2005). Macrophage colony-stimulating factor induces vascular endothelial growth factor production in skeletal muscle and promotes tumor angiogenesis. *J Immunol* **174,** 7531–7538.

[64] Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, and Asahara T (1999). Ischaemia and cytokine-induced mobilization of bone marrow–derived endothelial progenitor cells for neovascularisation. *Nat Med* **5,** 434–438.

[65] Rafii S, Meeus S, Dias S, Hattori K, Heissig B, Shmelkov S, Rafii D, and Lyden D (2002). Contribution of marrow-derived progenitors to vascular and cardiac regeneration. *Semin Cell Dev Biol* **13,** 61–67.

[66] Laufs U, Werner N, Link A, Endres M, Wassmann S, Jurgens K, Miche E, Bohm M, and Nickenig G (2004). Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation* **109,** 220–226.

[67] Tse HF, Kwong YL, Chan JK, Lo G, Ho CL, and Lau CP (2003). Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. *Lancet* **361,** 47–49.

[68] Risau W (1997). Mechanisms of angiogenesis. *Nature* **386,** 671–674.

[69] Garcia-Barros M, Paris F, Cordon-Cardo C, Lyden D, Rafii D, Haimovitz-Friedman A, Fuks Z, and Kolesnick R (2003). Tumor response to radiotherapy regulated by endothelial cell apoptosis. *Science* **300,** 1155–1159.

[70] Crosby JR, Kaminski WE, Schatterman G, Martin PJ, Raines EW, Seifert RA, and Bowen-Pope DF (2000). Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. *Circ Res* **87,** 728–730.

[71] De Palma M, Venneri MA, Roca C, and Naldini L (2003). Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat Med* **9,** 789–795.

[72] Jain RK and Duda DG (2003). Role of bone marrow–derived cells in tumor angiogenesis and treatment. *Cancer Cell* **3,** 515–516.

[73] Göthert JR, Gustin SE, van Eekelen JA, Schmidt U, Hall MA, Jane SM, Green AR, Gottgens B, Izon DJ, and Begley CG (2004). Genetically tagging endothelial cells *in vivo*: bone marrow–derived cells do not contribute to tumor endothelium. *Blood* **104,** 1769–1777.

[74] Rehman J, Li J, Orschell CM, and March KL (2003). Peripheral blood endothelial progenitor cells are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation* **107,** 1164–1169.

[75] Shaked Y, Bertolini F, Man S, Rogers MS, Cervi D, Foutz T, Rawn K, Voskas D, Dumont DJ, Ben-David Y, et al. (2005). Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis: implications for cellular surrogate marker analysis of antiangiogenesis. *Cancer Cell* **7,** 101–111.

[76] Vermeulen PB, Gasparini G, Fox SB, Colpaert C, Marson LP, Gion M, Belien JA, de Waal RM, Van Marck E, Magnani E, et al. (2002). Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours. *Eur J Cancer* **38,** 1564–1579.

[77] Bertolini F, Paul S, Mancuso P, Monestiroli S, Gobbi A, Shaked Y, and Kerbel RS (2003). Maximum tolerable dose and low dose metronomic chemotherapy have opposite effects on the mobilization and viability of circulating progenitor cells. *Cancer Res* **63,** 4342–4346.

[78] Schuch G, Heymach JV, Nomi M, Machcluf M, Force J, Atala A, Eder JP Jr, Folkman J, and Soker S (2003). Endostatin inhibits the vascular endothelial growth factor–induced mobilization of endothelial progenitor cells. *Cancer Res* **63,** 8345–8350.

[79] Eder JP, Clark J, Supko J, Puchalski TA, Garcia-Carbonero R, Ryan DP, Schulman LN, Proper J, Kirvan M, Rattner B, et al. (2002). A phase 1 trial of recombinant human Endostatin. *J Clin Oncol* **20,** 3772–3784.

[80] Willett CG, Boucher Y, di Tomaso E, Duda DG, Munn LL, Tong RT, Chung DC, Sahani DV, Kalva SP, Kozin SV, et al. (2004). Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. *Nat Med* **10,** 145–147.

[81] Hamilton KK, Hattori R, Esmon CT, and Sims PJ (1990). Complement proteins C5b-9 induce vesiculation of the endothelial plasma membrane and expose catalytic surface for assembly of the prothrombinase enzyme complex. *J Biol Chem* **265,** 3809–3814.

[82] Combes V, Simon A, Grau GE, Arnoux D, Camoin L, Sabatier F, Mutin M, Sanmarco M, Sampol J, and Dignat-George F (1999). *In vitro* generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. *J Clin Invest* **104,** 93–102.

[83] Jimenez JJ, Jy W, Mauro LM, Soderland C, Horstmann LL, and Ahn YS (2003). Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. *Thromb Res* **109,** 175–180.

[84] Laurence J, Mitra D, Steiner M, Staiano-Coico L, and Jaffe E (1996). Plasma from patients with idiopathic and human immunodeficiency virus–associated thrombotic thrombocytopenic purpura induces apoptosis in microvascular endothelial cells. *Blood*, 3245–3254.

[85] Mallat Z, Benamer H, Hugel B, Benessiano J, Steg PG, Freyssinet JM, and Tedgui A (2000). Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation* **101,** 841–843.

[86] Freyssinet JM (2003). Cellular microparticles: what are they bad or good for? *J Thromb Haemost* **1,** 1655–1662.

[87] Taraboletti G, D'Ascenzo S, Borsotti P, Giavazzi R, Pavan A, and Dolo V (2002). Shedding of the matrix metalloproteinases MMP-2, MMP-9 and MT1-MMP as membrane vesicle–associated components by endothelial cells. *Am J Pathol* **160,** 673–680.

[88] Casciola-Rosen L, Rosen A, Petri M, Petri M, and Schlissel M (1996). Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosus. *Proc Nat Acad Sci USA* **93,** 1624–1629.

[89] Shet AS, Aras O, Gupta K, Hass MJ, Rausch DJ, Saba N, Koopmeiners L, Key NS, and Hebbel RP (2003). Sickle blood contains tissue factor–positive microparticles derived from endothelial cells and monocytes. *Blood* **102,** 2678–2683.

[90] Jimenez JJ, Jy W, Mauro LM, Horstmann LL, Soderland C, and Ahn YS (2003). Endothelial microparticles released in thrombotic thrombocytopaenic purpura express von Willebrand factor and markers of endothelial activation. *Br J Haematol* **123,** 896–902.

[91] Boulanger CM, Scoazec A, Ebrahimian T, Henry P, Mathieu E, Tedgui A, and Mallat Z (2001). Circulating microparticles from patients with myocardial infarction cause endothelial dysfunction. *Circulation* **104,** 2649–2652.

[92] Blann AD, Woywodt A, Bertolini F, Bull TM, Buyon JP, Clancy RM, Haubitz M, Hebbel RP, Lip GY, Mancuso P, et al. (2005). Circulating endothelial cells: biomarker of vascular disease. *Thromb Haemost* **93,** 1–9.

[93] Garmy-Susini B and Varner JA (2005). Circulating endothelial progenitor cells. *Br J Cancer* **93,** 855–858.

[94] Vasa M, Fichtlscherer S, Adler K, Aicher A, Martin H, Zeiher AM, and Dimmeler S (2001). Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation* **103,** 2885–2890.