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Circulating endothelial cells as biomarkers for angiogenesis in tumor progression

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1. ABSTRACT

An increased number of circulating endothelial cells (CECs) and endothelial progenitor cells (CEPs) has been reported in cancer patients. CEPs are derived from the bone marrow and will, during angiogenesis, differentiate into endothelial cells. CECs are mature endothelial cells (ECs) released from the vessel intima during physiological endothelial turnover or as a result of tumor treatment. Preclinical studies have shown that during tumor progression, the amount of circulating CECs correlates with angiogenesis. Moreover, there is growing evidence suggesting that CECs and CEPs viability and kinetics correlate with the patient responses to anti-angiogenic therapies. Thus, circulating CECs and CEPs may act as surrogate markers to test putative therapeutic efficacy. Moreover measuring CECs and CEPs may be useful to assess effects of antiangiogenic therapy.

2. INTRODUCTION

Mammalian cells require oxygen and nutrients for their survival and are located at a distance of 100 to 200 μm from blood vessels (i.e. the diffusion limit for oxygen). For cells to grow beyond this distance, they must recruit new blood vessels generated by proliferation and vascular sprouting of mature endothelial cells (ECs) from adjacent pre-existing vasculature in a process called angiogenesis. This process may also involve seeding of bone marrow-derived CEPs to the lumen of sprouting neovessels in a process called vasculogenesis (1). New vessel growth is a complicated process regulated by a balance between angiogenic factors and inhibitors, and is deranged in several diseases, including cancer. Physiological angiogenesis occurs during development and is restricted in the adult to reproduction and wound repair and is limited in time, taking days (ovulation), weeks (wound healing) or

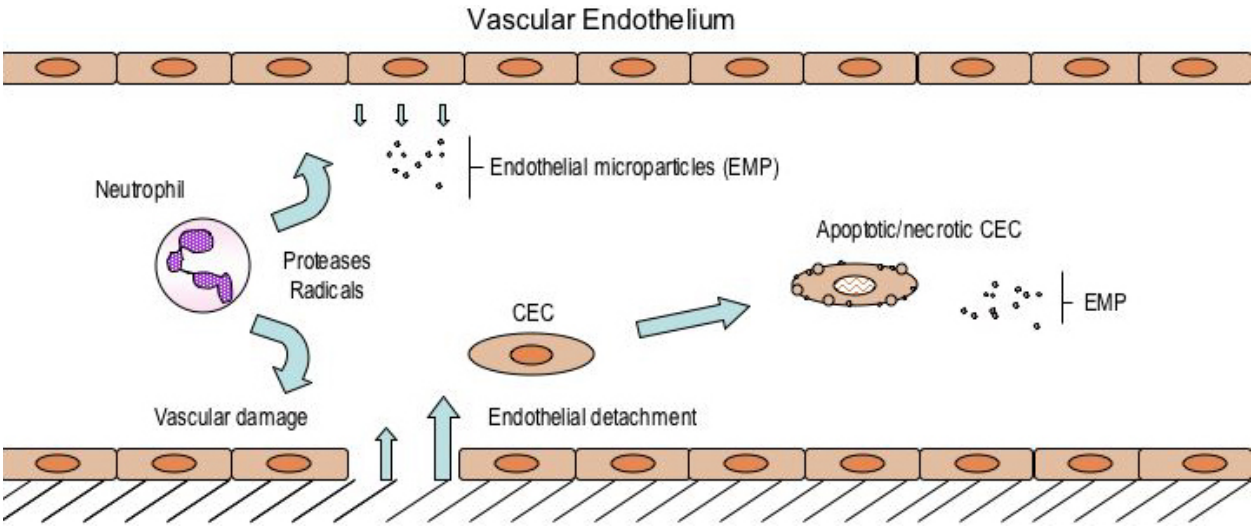

Figure 1. Mechanisms for endothelial cell detachment and microparticle formation following vascular damage. A vascular insult, such as inflammation, might induce endothelial cell detachment from the intima and shedding of endothelial microparticle (EMP) from the activated endothelial cells. Ciculating CEC, endothelial cells (CEC) undergo apoptosis by anoikis.

months (placentation). On the other hand, pathological angiogenesis can persist for years, and is necessary for tumors to grow beyond a critical size or to form solid metastases in other organs (2). At present, anti-angiogenic drugs, alone or in combination with chemotherapy, is increasingly used in cancer therapy. In many cases, however, their mechanisms of action and tailoring optimal dose/schedules are still elusive.

This review aims at providing an overview of the current knowledge of the biology behind CECs and CEPs with special reference to their phenotypes. We also discuss their role in cancer growth and their potential use as biomarkers during cancer therapy.

3. CECs, CEPs AND ENDOTHELIAL MICROPARTICLES

Endothelial turnover is very low compared to other tissues, however in vascular regions where flow turbulence and shear stress are high, ECs can detach from the basement membrane and enter into the circulation where by anoikis they become apoptotic (Figure 1). As early as in the mid 1970s it was shown that cells with endothelial characteristics circulate in the blood (3); it took two more decades to establish a procedure to quantify the CEC population.

In healthy adults, CECs can be considered as a stable population with a range of 1/1,000-100,000 of circulating blood cells (4). In contrast, the numbers of CECs are increased in diseases characterized by the presence of a vascular insult or modulation (Figure 1), such as sickle cell anemia, acute myocardial infarction, CMV infection, endotoxemia and cancer (5, 6).

Recently, another endothelial marker, endothelial microparticles (EMPs), has been linked to vascular damage. EMPs are vesicles formed by released endothelial cell membranes after injury or inflammatory activation. They contain cell surface proteins and cytoplasmic elements and can be derived from ECs present in the vessel wall or from CECs (Figure 1). They have been shown to have a pro-coagulating potential and share some specific endothelial markers, but they do not contain DNA (7).

CEPs originate from the bone marrow rather than from the vessel wall (8) and are seen in a small number in healthy individuals but their numbers tend to increase following tissue damage and cancer (9). As discussed below CEPs might have a role in both physiologic and pathologic vasculogenesis (Figure 2).

The Hebbel laboratory was the first to describe the quantitative and functional relationship between CECs and CEPs (10). Using a Y-chromosome gene marking approach in recipients of gender-mismatched bone marrow transplants, they were able to distinguish CEPs from the bone marrow (i.e. donor-derived cells), and CECs from the vessel wall (i.e. host/recipient-derived). More than 90% of endothelial cells in the blood were found to be of recipient origin (10).

As discuss below, recent studies have shown that CEPs from peripheral blood can generate mature ECs *in vitro* and *in vivo* in vascular grafts (4, 11).

3.1. Antigenic definition of CECs and CEPs: isolation and quantification

Distinguishing CEPs from CECs by means of differential expression of cell surface antigens is difficult due to the antigenic promiscuity of hematopoietic cells, mature and progenitor cells (HPC), platelets, CECs and CEPs (Figure 3). To identify the various cells, combinations of antibodies have to be used (4, 12).The first attempt to isolate CECs was developed by Dignat-George (13) using magnetic beads coupled to a CD146 (also called

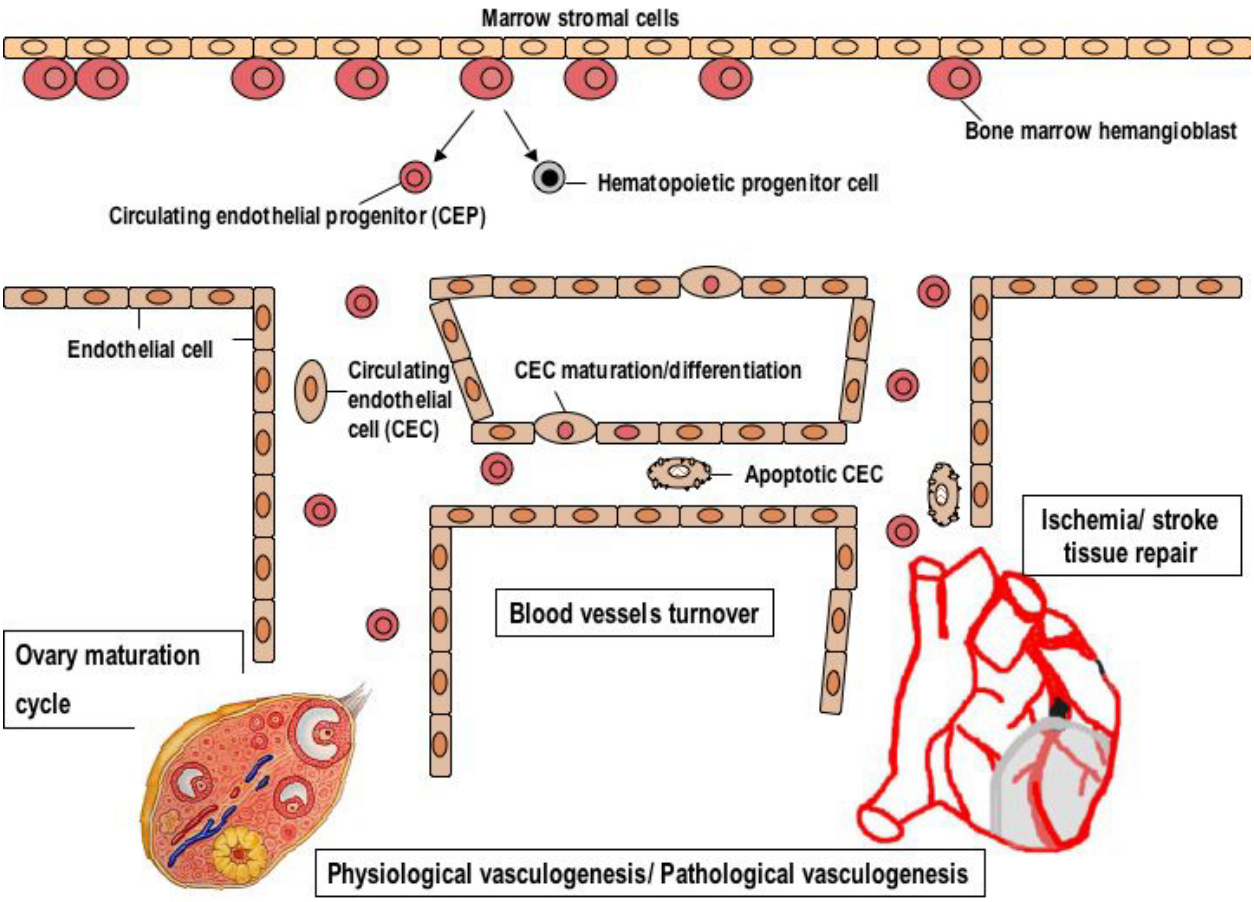

Figure 2. Role of CECs and CEPs in physiological and pathological vasculogenesis. During vascular turnover endothelial cells might be replaced by proliferation of adjacent cells or by maturation of circulating endothelial progenitors (CEPs) generated in the bone marrow. CEPs might also migrate to sites of tissue regeneration such the ovary. Ischemia and stroke provoke a tisssue damage. Homing of the CEPs is required for tissue repair.

S-endo and MUC18) monoclonal antibody. However, later it became clear that CD146 can also be expressed by activated leukocytes; a further characterization is therefore needed (14). To address this problem, a hybrid assay for CECs measurement has been developed combining pre-enrichment of $CD146^{+}$ circulating cells with multi-parametric flow cytometry (15). By this method CEC are identified by $CD45^{dim}/Cd146^{brigh}t$/PI events with highsize-related scatter characteristics. They are therefore clearly distinguished from $CD45^{bright}/CD146^{dim}$ activated T lymphocytes (15). However, it should be emphasized that pre-enrichmet procedures might result in cell loss.

Multiparameter flow-cytometry is the method of choice for counting CECs and CEPs. By simultaneous labelling with different monoclonal antibodies and by combining sequential gating and fluorescence–compensation strategies, it is possible to measure CECs and CEPs from peripheral blood. We and other groups are currently working on standardization procedures to minimize variability and increase reproducibility. Briefly, CD45 can be used to exclude hematopoietic cells from the analysis and ECs are identified by the expression of CD31, CD146 and VEGFR2. By using DNA-staining it is possible to exclude platelets and/or EMPs from the CEC fraction (4). Other markers, such us CD34, can be use to detect hematopoietic stem cells (HSC) and to exclude mature hematopoietic cells. However, CD34 is also expressed by both CECs and CEPs, and therefore this marker alone cannot be used to distinguish the two populations (4, 6). Mature ECs are frequently apoptotic when found in the circulation, consequently the use of specific apoptotic markers, such as 7AAD and SYTO16 (16) provides a discrimination between apoptotic and viable CEPs. During neoplastic disease, a high number of angiogenic factors can be released from the tumor that may lead to increased CECs survival. In the blood CD133 is known to be expressed by hematopoietic stem cells (HSCs) and by CEPs. In contrast, mature endothelial cells in the vascular wall and CEC do not express CD133 suggesting a down regulation of the epitope during endothelial differentiation (17). Thus CD133 may be a useful marker to separate CEP from CEC subpopulations.

Enumeration of murine CECs and CEPs by flow cytometry is less standardized. We and others have used the

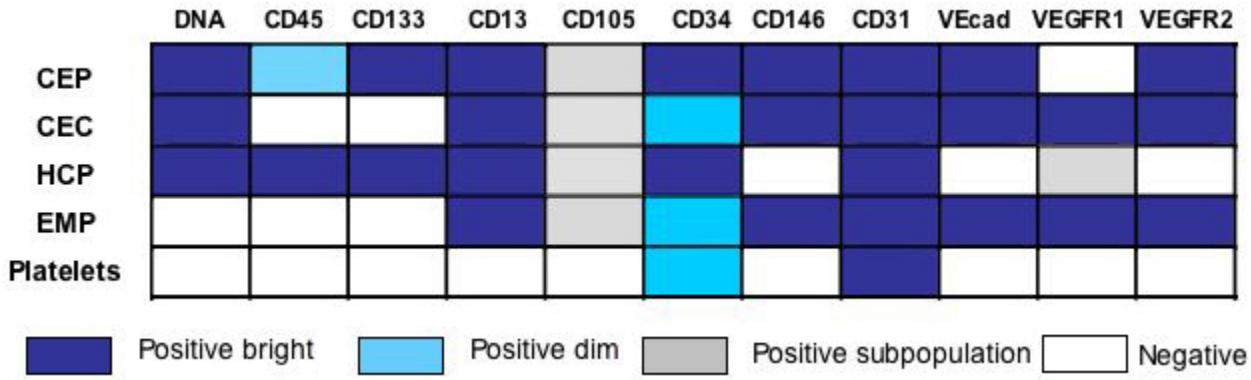

Figure 3. Flow cytometric immunophenotyping of circulating endothelial cells (CEC), circulating endothelial progenitors (CEP), endothelial microparticles (EMP), hematopoietic progenitor cells (HPC) and platelets. Positive bright and positive dim reflects high or low expression levels of the antigens, Positive subpopulations means that the antigen is only expressed in a certain fraction of the total population. CD105 (endoglin), CD146 (MUC-18 or S-endo), CD31 (PECAM).

following strategy. Briefly, a CD45 staining can be used to exclude hematopoietic cells from the analysis; $CD45^-$ $VEGFR2^+$ defines the EC population, while coexpression with CD117 (mouse c-kit) allows a delineation of the CEP fraction (18,19). Prominin-1, the mouse homolog of CD133, is still not fully characterized and it is not yet established whether it can be used to depict CEPs by flow cytometry. Recently it has been hypothesized in and *in vitro* model of endothelial differentiation that CD133 is retained longer than CD117 on CEPs (20). Thus early CEPs are $CD45^-VEFR2^+CD133^+CD117^+$, late CEPs are $CD45^-$ $VEFR2^+CD133^+CD117^-$, and mature CECs $CD45^-$ $VEFR2^+CD133^-CD117^-$ (20). Recently it was shown by Nolan *et al* (21) that the antibody E4G10 (22) specifically binds to an exposed epitope on the monomeric N-terminal domain of VE-cadherin. This specific epitope becomes masked upon transdimerization of the protein, when VE-cadherin clusters on the cell to-cell junction between endothelial cells to form a vascular structure. Thus, the E4G10 antibody recognizes specifically VE-cadherin only on CEPs but not in mature ECs. A complete characterization of this antibody in different models might be useful to make a complete definition of the CEPs phenotype.

CEPs maintain a proliferative potential that mature CECs have lost. Clonogenic assays *in vitro* show a 20-fold expansion of CECs whereas a 1000 fold expansion can be reached by CEPs (10). However, recent studies indicate that the large majority of colonies generated in commercially available kits for endothelial CEPs are of myeloid origin and have no vasculogenic potential (23) suggesting that a careful endothelial-specific phenotyping is needed when using commercially available kits.

Immunohistochemistry has also been used to determine the role of CECs and CEPs in angiogenesis and vasculogenesis. VE-cadherin and CD31 are useful endothelial markers used for a morphological recognition of the vasculature. Although VE–cadherin is the only known endothelial specific antigen in adults and CD31 is a common antigen for leukocytes, the intense CD31 staining of blood vessels (in particular when the tissue is fixed in a Zn-fixative before embedding in paraffin, , 24) is the reason of its general use to evaluate microvessel density (MVD). Evaluation of MVD in a tumor has been considered as indicator of changes in angiogenesis. However, in some instances MVD measurements may not be reliable since the vascular network in a tumor is not homogeneously distributed. Moreover, some vessels in a tumor might be tortuous and coopted, thus MVD can overestimate the functional vascularization of the tumor (25). To determine functional vessels in preclinical studies, MVD should be completed with measurements of blood flow or by perfusion with fluorescent markers (such as isolectin GS-IB4, 21).

4. OTHER BONE MARROW-DERIVED CELLS IN TUMOR ANGIOGENESIS

Adult bone marrow is a source of proangiogenic hematopoietic mural cells that are recruited to perivascular sites within the tumor bed (24-26). Several BM-derived hematopoietic cell populations have been reported to contribute to tumor angiogenesis.

Conejo-Garcia (27) identified *in vivo* a population of CD11c-expressing cells exhibiting simultaneous expression of both endothelial and dendritic cell markers, termed vascular leukocytes (VLCs). VLCs are highly abundant in human ovarian carcinomas and, depending on the microenvironment, can assemble into functional blood vessels or act as antigen-presenting cells.

Tumor-infiltrating myeloid cells, including tumor-associated macrophages, have also been implicated in tumor progression. For instance a lineage of mouse monocytes characterized by expression of the Tie2 angiopoietin receptor (Tie2-expressing monocytes, TEMs) has been shown to be required for the vascularization and growth in several tumor models (30). TEMs, are hardly detected in non-neoplastic tissues whereas within tumors,

they represent the main monocyte population distinguishable from tumor associated macrophages (31). Depletion or selective elimination of TEMs has been shown to inhibit angiogenesis and induce tumor regression (30, 32). This suggests that TEMs might participate in the angiogenic process by providing paracrine support to nascent blood vessels. Moreover, purified human TEMs, but not TEM-depleted monocytes, markedly promotes angiogenesis in xenotransplanted human tumors (31).

Adult vasculogenesis may also rely on the recruitment of bone-marrow-derived circulating cells by the secretion of VEGF from the tissue microenvironment (33). Induction of VEGF in specific organs such as the heart and liver may lead to massive infiltration of circulating cells homing to these organs. Most the recruited blood circulating cells (RBCCs) express both CD45 and VEGFR1 but not VEGFR2, indicating that the cells are predominantly of hematopoietic origin. In addition, RBCCs express the CXCR4 chemokine receptor and home to tumor perivascular sites owing to the secretion of CXCL12, which is the ligand for CXCR4.

Recently a population of tumor-associated stromal cells (TASCs), expressing CD45 and VEGFR2, was also show to promote tumor angiogenesis in a paracrine manner stimulating recruitment of ECs from neighbouring tissue (34).

De novo lymphoangiogenetic networks, provides a way for cancer cells to colonize and metastasize to other organs. For instance, information from renal tissue carcinomas of individuals with gender–mismatched transplants indicate that lymphatic progenitor cells derived from the donor can transmigrate through the connective stroma and incorporate into growing lymphatic vessels (35).

Despite the general importance of bone marrow-derived cells in tumor angiogenesis, the precise contribution of different lineages remains poorly understood. It will be of interest to study possible interactions between bone marrow-derived angiogenic cells and CECs and CEPs to determine whether they can be defined as new biomarkers to predict response to anti-angiogenic therapies.

5. ANTIANGIOGENIC THERAPIES

The first angiogenesis inhibitors were reported in the 1980s by the Folkman laboratory (2). By the mid-1990s, new drugs with anti-angiogenic activity entered clinical trials. Bevacizumab, which received FDA approval for colorectal cancer in 2004, was the first drug developed solely as an angiogenesis inhibitor.

At the present, also other anti-angiogenic compounds such as Thalidomide, Sunitinib, Sorafenib have received approval in more than 30 countries for the treatment of cancer (2). In the United States, 43 drugs are currently in clinical trials of which 17 have demonstrated some activity (Table 1).

In spite of a rapid translation from bench to bedside, our ability to monitor, or even predict, antiangiogenic efficacy has not followed the same pace.

An important question is why surrogate markers are needed to monitor antiangiogenic therapy, since one could simply administer the maximal tolerable dose. One might reason that the more vessels that are disrupted or induced to regress, the more efficacious the angiogenic therapy would be. However, a supramaximal dose of an anti-angiogenic compound may induce undesired side effects by attacking the quiescent normal vasculature. This point is highly relevant when patients are treated during early disease stages. Certain angiogenic inhibitors, such us thalidomide and bevacizumab, increase the incidence of thrombotic complications (36) and the risk of thrombosis is further increased when these angiogenic inhibitors are administrated together with conventional chemotherapy. Several angiogenesis inhibitors have been reported to follow a biphasic, U-shaped dose-efficacy curve. For example, interferon–α (37) as well as endostatin (38) are anti-angiogenic at low doses but at higher doses their efficacy decrease. It should also be pointed out that tumors might become refractory to anti-angiogenic therapy, especially if a mono-antiangiogenic therapy targets only one angiogenic protein. VEGF is expressed by up to 60% of human tumors and most tumors can also express five to eight other known angiogenic factors. When one angiogenic factor is suppressed for longer periods, the expression of other angiogenic protein may emerge (39). At present it is not clear whether this represents a "compensatory" mechanism of the tumor cells where the production of stimulating factors change or if it is due to ECs that develop resistance to the antiangiogenic therapy. It should also be emphasized that different individuals can show distinct genetic differences in their response to a given angiogenic stimulus. For example, individuals with Down syndrome, that have an extra copy of the gene for the endostatin precursor, seem to be more protected against cancer (with the exception of leukemias). In mice, Shaked *et al.* reported that animals of different strains with different genetic background show differences in tumor angiogenesis, levels CECs and CEPs and response to angiostatic therapy (18).

Even though many anti-angiogenic compounds have entered clinical trials, their exact mechanisms of action are not clear. Three major hypothesis, although not necessarily mutually exclusive, are currently used to explain how anti-angiogenic drugs reduce cancer growth and how they synergize with other anti-cancer drugs and in particular with chemotherapeutics (40).

First, it has been shown that anti-angiogenic drugs transiently reverse the chaotic and dysfunctional tumor vasculature inducing vessel maturation and restoring blood flow. As a result of such a "vessel normalization" (41, 42) there is a reduced vessel leakiness leading to reduced interstitial fluid pressure that will relieve tumor hypoxia, thus increasing tumor cell proliferation. According to this hypothesis, anti-angiogenic drugs should be administered before and along with chemotherapeutic

Table 1. Clinical Trials of antiangiogenic drugs that have shown clinical activity

Drug (Company)	Target or mechanism of action	Clinical Activity
AG-013736 (Pfizer)	VEGFR1 and VEGFR2, PDGFb receptor	Phase I: breast cancer
		Phase II: melanoma, NSCLC, breast, melanoma, thyroid, pancreatic, renal cell cancer
AMG706 (Amgen)	VEGF, PDGF, Kit and Ret receptors	Phase l: Lymphoma, NSCLC, breast and colorectal cancer
		Phase II: NSCLC, breast, thyroid, gastrointestinal stromal tumors (GIST)
AZD2171 (AstraZeneca)	VEGFR1, VEGFR2, VEGFR3 and PDGFbR	Phase I: head and neck, colorectal cancer, NSCLC, AML, CNS tumors
		Phase II: NSCLC, glyoblastoma, melanoma, mesothelioma,CLL, SCLC, breast, colorectal, ovarian, kidney and liver cancer
		Phase III: NSCLC
ZD6474 (AstraZeneca)	VEGFR2, EGFR	Phase I: Glioma
		Phase II: NSCLC, SCLC, breast, thyroid,glioma, multiple myeloma
		Phase III: NSCLC
AZD2171 (AstraZeneca)	VEGFR1, VEGFR2, VEGFR3 and PDGFbR	Phase I: head and neck, colorectal cancer, NSCLC, AML, CNS tumors
		Phase II: NSCLC
CDP-791 (Imclone)	VEGFR2	Phase I: solid tumors
IMC-1121b (Imclone)	VEGFR2	Phase I: NSCLC, gynaecologic and other solid tumors
Vatalanib (Novartis)	VEGFR1 and VEGFR2, PDGFb receptor	Phase II: NSCLC, GIST, AML, CML,VHL, Hemangioblastoma, mesothelioma, SCLC, breast, prostate, pancreatic, neuroendocrine, glyoblastoma, meningioma, myelodisplastic syndrome, multiple myoloma,
		Phase III: colorectal cancer
AP23573 (Ariad Pharmaceuticals)	mTOR	Phase l:Glioma, sarcoma, multiple myeloma and other solid tumors
		Phase ll:endometrial, protaste cancer, hematological malignancies
CCI-779 (Wyeth)	mTOR	Phase l:Prostate, CML, other solid tumors
		Phase II: NSCLC, GIST, AML, CML,NHL, glioblastoma, melanoma, CLL, SCLC, multiple myeloma, breast, pancreatic, endometrial, neoroendocrine tumors
Everolimus (Novartis)	mTOR	Phase I: breast cancer, lymphoma and other solid tumors
		Phase ll: NSCLC, melanoma, AML, ALL, CML, lymphoma, glioblastoma, prostate, colorectal, neuroendocrine, breast, kidney, endometrial,paedriatric and other solid tumors
		Phase III: Islet cell pancreas ll/lll
Enzastaurin (Eli Lilly and Company)	VEGF	Phase I: Glioma and other solid tumors
		Phase II: NSCLC, glioma, brain tumors, pancreatic, colorectal cancer
		Phase III: glioblastoma and Lymphoma prevention
VEGF Trap (Regeneron Pharmaceuticals)	VEGF	Phase I: NHL
		Phase ll: Ovarian and kidney cancer, NSCLC
		Phase lll: Ovarian cancer

drugs, because they might not only improve drug delivery within tumors, but also increase the number of proliferating tumor cells that would be expected to be more sensitive to chemotherapy. It has also been reported that induced- vessel normalization isparticularly useful for the treatment of tumors where tumor stem cells are supported in aberrant vascular niches such as malignant brain tumors (42-44).

Secondly, tumor regrowth after cytotoxic therapy (45, 46) can be slowed after treatment with anti-angiogenic compounds, i.e between successive cycles of chemotherapy (47, 48). The consequence of this hypothesis has led to the concept of metronomic chemotherapy (ie. the close, regular administration of low, non-toxic doses of chemotherapeutic drugs with no breaks, over long periods of time), and this therapeutic strategy is known to have anti-angiogenic activity (47, 48). According to this hypothesis, anti-angiogenic drugs should be administered after chemotherapeutic drugs in order to avoid tumor recurrence between chemotherapy cycles.

Thirdly, anti-angiogenic drugs may target proliferating tumor ECs or CEPs in different ways (2, 49). Anti-angiogenic drugs can directly prevent the EC response to angiogenic proteins or inhibit EC proliferation and migration. The drugs may also act indirectly by suppressing the tumor's production of pro-angiogenic factors or by neutralizing angiogenic factors. According to this concept, treatment with anti-angiogenic drugs should be done along with chemotherapeutic drugs to inhibit ECs proliferation and CEPs mobilization.

5.1. Soluble and molecular surrogate markers for angiogenesis

Several surrogate markers of angiogenesis have been considered, but few have proven to be clinically useful. In some tumors, the measurement of plasma or urinary levels of angiogenic growth factors, such as VEGF, b-FGF, HGF and IL-8 has been reported as indicators to predict patient survival (50-53). However, in renal cell carcinoma patients receiving the tyrosine kinase inhibitor sunitinib, circulating levels of VEGF-A and PlGF in the blood increase during each cycle of treatment, whereas soluble VEGFR2 decrease. Two weeks after treatment, the levels of these biomarkers returned to near basal levels but successive cycles of sunitinib induced again changes

indicating that these variations were due to the sunitinib administration and not useful to predict patient survival (54).

Soluble VEGF receptors such as VEGFR1, VEGFR2 and VEGFR3 are currently being investigated as surrogate markers in a number of patients treated with anti-angiogenic therapies. However, at present, more work is needed to determine whether these biomarkers can predict patient survival or response to anti-angiogenic therapies (55, 56). A focus has also been on the identification of genetic markers specific for cancer endothelial cells (6, 57, 58). However, the genetic profiling of the tumor vasculature and CECs needs to be fully validated in the clinical setting.

So far, only few genes are considered to be endothelial-specific. VE-cadherin, is restricted in the adult to the endothelial lineage but is also expressed by hematopoietic stem cells in the fetal liver (45). Interestingly, the number of copies of VE-cadherin transcripts in the blood of cancer patients is significantly increased compared to healthy controls (59). VE-cadherin RNA expression levels are most likely reduced (or absent) in apoptotic endothelial cells. Thus, the number of circulating VE-cadherin transcripts most likely reflects only viable CECs. Recent studies have reported an increase in circulating transcripts of CD133 in the blood of cancer patients (60, 61). However, CD133 is also expressed by hematopoietic progenitors (62) and some tumor cells (63) and further work is needed to determine the cellular source of the CD133 transcripts in patients.

5.2. CECs as biomarkers in cancer

It is known that CEC levels are increased in a number of cancer patients and that the levels return to normal values as a result of complete remission (Figure 4, 55, 64-66). Based on these observations we have chosen to compare drugs with cytotoxic or anti-angiogenic efficacy in preclinical animal models. Mice treated with cyclophosphamide (at the maximum tolerated dose) or endostatin showed different levels of CECs. After cyclophosphamide treatment, most of the circulating apoptotic cells were hematopoietic, and a relevant proportion of CECs were still viable. In contrast, in mice treated with endostatin, most CECs were dying (67). This observation indicates that CEC counts and viability are useful surrogate biomarkers in pre-clinical models involving anti-angiogenic treatment strategies.

To verify this hypothesis in clinical studies, we have analyzed circulating endothelial cell kinetics and viability in patients with metastatic breast cancer who were treated with metronomic cyclophosphamide and methotrexate therapy (68). We have shown that the CEC count after two months of continuous therapy was a good prognostic factor that was reflected in overall survival (67, 65).

To further investigate the levels of CECs during therapy, we have compared different treatments doses and regimens of cyclophosphamide in tumor bearing mice. Maximal tolerable dose (MTD) of cyclophosphamide caused a short-term suppression of viable CECs and CEPs immediately after the drug was given, which was followed by a robust increase in the number of viable CECs and CEPs (69). In contrast, metronomic chemotherapy regimens maintain low levels of viable CECs for longer periods of time because of the absence of break periods and prevent the haematopoiesis-like rebound and/or mobilization of CEPs after MTD. Similar results were reported in a clinical study (66) where CECs and CEPs were measured in patients with breast cancer who received neoadjuvant chemotherapy. The number of CECs (found to be increased in patients compared with control healthy individuals) was decreased by chemotherapy, whereas CEP mobilization was significantly increased during the drug-free break periods. These data, suggest that anti-angiogenic therapy along with MTD chemotherapy should prevent rebound and/or mobilization of CECs and CEPs after MTD.

5.3. CEPs in tumor-associated vessel growth

The first work providing evidence for that CEP contribute to the tumor vasculature was reported by Lyden and colleages (70). By using angiogenic-defective Id-mutant mice, they showed that transplantation of wild-type bone marrow or VEGF-mobilized progenitors was able to restore angiogenesis and tumor growth. However, to what extent EPCs contribute to neovessel formation remains controversial (71). Extensive variability in EPC contribution to vessel formation has been described. For instance, contributions as high as 50% (70,72) to as low as 5%–20% (73-75) and, in some cases, undetectable levels (30, 32, 76-78) have been reported. Such conflicting reports can be explained in different ways:

i) to a limited analysis of the EPC phenotype and a lack of more definitive methods to distinguish vessel incorporated bone marrow-derived ECs and intimately associated perivascular cells. In fact, in works that report 50%-90% of donor-derived vessels, incorporating cells were estimated by X-gal staining in LacZ+ bone marrow-transplants. It is possible that X-gal detection by light microscopy might, result in an over-estimation of BM-derived vessels. Since these reports were published, the use of high-resolution confocal microscopy for the accurate determination of vessel incorporated ECs has been advocated (32, 79).

ii) to the analysis of different tumor types and stages of tumor progression (80, 81). Ruzinova *et al.* reported that CEPs contribute to some, but not to all spontaneous murine tumor models. Particularly, CEP recruitment in tumor vasculature of differentiated and undifferentiated prostate adenocarcinomas were significantly different suggesting that this process might vary depending on tumor grade (80).

iii) to the tumor localization. Duda *et al.* showed that bone marrow-derived CEPs incorporate into perfused tumor vessels and this contribution varies depending on the localization site of the tumor (82). Frequency of CEPs-incorporated vessels was 58% in a model of mammary

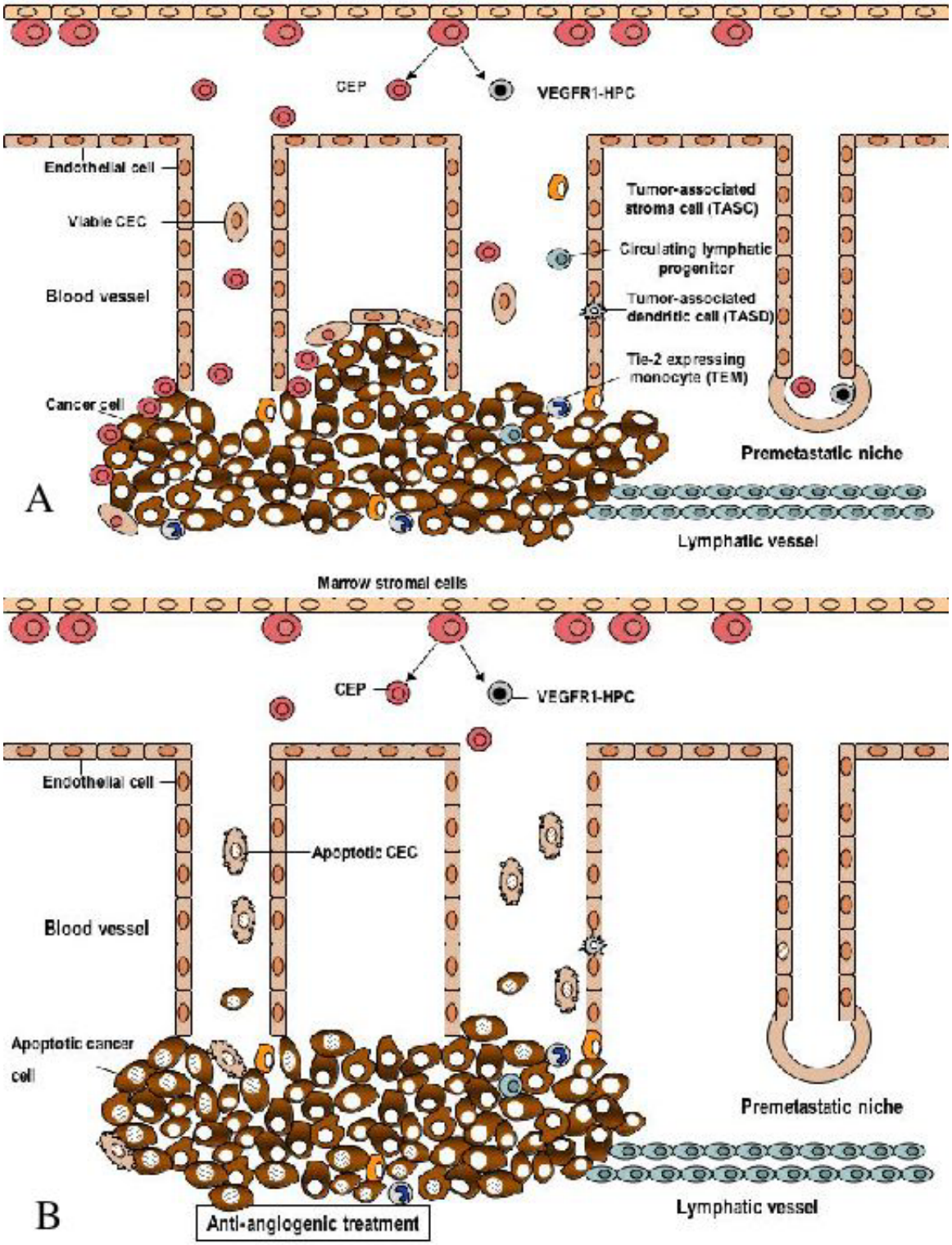

Figure 4. Role of CECs and CEPs in cancer. A. Circulating endothelial cells (CECs), with a mature phenotype, are increased during tumor progression. Endothelial progenitor cells (CEPs) home from the bone marrow to incorporate to the tumor neovessels. Other bone marrow-derived cells might participate in the process of tumor angiogenesis (see text), such as VEGFR1+-hematopoietic progenitor cells that, toghether with the CEPs, might initiate the pre-metastatic niche. B. Followig antiangiogenic treatment there is an increased of apoptotic CECs detaching from the tumor vessels. CEP mobilization is increased by high-dose chemotherapy and reduced by metronomic chemotherapy and angiogenic treatments.

metastasis of brain carcinoma whereas it accounted only to 15% in mammary fat-pad breast tumor lesions.

iv) to the kinetic of CEP measurements during tumor development. Some studies have described a time during tumor development where the CEPs contribution is more relevant. It has been shown that different EPCs are recruited to the tumor periphery preceding vessel formation, and are luminally incorporated into a subset of sprouting tumor neovessels (19). Notably, these bone marrow-derived vessels are eventually diluted with host-derived vessels, thereby explaining their low contribution as has been observed in large, established tumors.

In clinical studies, Peters *et al.* analyzed tumors of six patient that developed cancer after a bone marrow transplants (with donors of opposite sex) by FISH and immunofluorescence staining and observed that bone marrow-derived cells of the donor contributed to the 5% of the tumor neovasculature (84).

In summary, there is growing evidence that CEPs can contribute to tumor angiogenesis, but such contribution might change depending on tumor type and grade, tumor stage, organ site and timing of measurements during tumor progression.

5.4. Can CECs and CEPs be used to determine the optimal biological dose of an anti-angiogenic drug?

An important question in anti-angiogenic research is whether the quantification of CECs and CEPs might be used to determine the optimal biological dose (OBD) of anti-angiogenic drugs. Previous dose–response studies have shown that the optimal therapeutic dose of DC101 (a mAb towards mouse VEGFR2) was in the range of 800–1,200 μg/mouse, given every 3 days. We tested the effect of DC101 in escalating doses in two preclinical tumor models. 800 μg/mouse of DC101 was found to be the OBD in both models, as this dose induced the lowest level of viable CEPs, with the largest decrease in tumor volume. A higher dose did not alter the results.

Following these studies, we subsequently tested various anti-angiogenic drugs, including small molecules, antibodies and blocking peptides and we showed that in most cases a striking correlation between suppressed levels of viable CEPs and the OBD of the particular drug (4, 18, 67, 85). Finally, we have shown that CECs and CEPs counts can be used to determine OBD in tumor-bearing mice treated with low-dose metronomic chemotherapy (47).

5.5. Can CEPS be used as vehicles for anticancer treatments?

The incorporation of CEPs to sites of neovascularization during tumor progression provides a possibility of using them as vehicles for anticancer treatment. The potential of CEPs to serve as cellular vehicles to cancer targets depends on efficient and specific (*ex vivo*) gene transfer and the ability to stably carry therapeutic loads through the blood stream to the intended target. In 2003, Ferrari (87) isolated, expanded and genetically engineered *ex vivo* marrow-derived CEPs to express the β-galactosidase, green fluorescence protein or thymidine kinase (TK) genes using retrovirus-mediated gene transfer. Genetically labeled CEPs were transplanted into sublethally irradiated tumor-bearing mice, and were found to migrate to and incorporate within the angiogenic vasculature where the growing tumors maintained transgene expression. Treatment with ganciclovir resulted in significant tumor necrosis in animals that had previously been given TK-expressing CEPs, with no systemic toxicity.

Others have shown that that mouse embryonic CEPs home preferentially to hypoxic lung metastasis and this specificity is inversely related to the degree of tissue perfusion, levels of hypoxia and VEGF (86). *Ex vivo* expanded embryonic CEPs genetically modified with a suicide gene have also been shown to eradicate lung metastasis. CEPs do not express MHC I proteins and are resistant to natural killer cell-mediated cytolysis, thus they could be used in an allogeneic setting to treat hypoxic metastases which usually are resistant to conventional chemotherapy.

It has been shown by Mittal *et al.* (21) that a specific ablation of BM-derived CEPs by an anti-VE-cadherin (E4G10) antibody results in defects in angiogenesis-mediated tumor growth. The same group recently showed that CEPs have a pivotal role in controlling the angiogenic switch that determines the progression of lung micrometastasis to lethal macrometastasis (83). By suppression of Id1 after metastatic colonization they blocked EPC mobilization that causes angiogenesis inhibition, impaired pulmonary macrometastases, and increased survival of tumor-bearing animals (83). Taken together, these results suggest that CEPs manipulated *ex vivo* might be considered as a strategy for getting therapeutic vehicles to the tumor. Moreover, the use of autologous CEPs or embryonic CEPs might circumvent possible immune rejections.

6. GENETIC INSTABILITY IN ENDOTHELIAL CELLS

In contrast to cancer cells, tumor-associated vascular cells have been considered for many years to be genetically stable (88). However, recent work have described that vascular cells of a tumor bed can become genetically unstable (89), and cytogenetically abnormal ECs have recently been described in some preclinical cancer models (90). Moreover, in some non-Hodgkin's lymphoma patients with specific genetic aberrations, ECs from cancer microvasculature had the same lymphoma-specific chromosomal translocations (91). Similarly, in myeloma (92) and in some leukemias (93) circulating ECs were found to share the same genetic alterations as observed in cancer cells. There are several possible explanation for these findings. First, tumor and endothelial cells can be derived from a common cancer hemangiolblast. Second, ECs may incorporate oncogenes by take up tumor apoptotic bodies (94) or, by cell fusion events (95).

7. ENDOTHELIAL CELLS IN THE NICHE

Metastasis, the spread of invasive tumor cells to sites at a distance from the primary tumor, is responsible for the majority of cancer-related deaths (96). Over a century ago, Paget observed that circulating tumor cells would only "seed" where there was a "congenial soil" and proposed that tumor cells secrete factors that will promote microenvironmental changes that will lead to the seed of tumor cells in specific organs. In a recent work, Kaplan and colleagues demonstrated a key role of some bone marrow-derived progenitors in "primimg" distant tissues for tumor cell implantation and proliferation (97). As early as 14 days after tumor implantation and prior to tumor cell invasion, VEGFR1-HPC were observed forming clusters that dictated the contours of future metastatic sites. Then, CEPs migrate to stabilize these clusters and allowing the formation of the "pre-metastatic niche". This niche is formed before histological evidence of tumors suggesting that such processes precede the arrival of metastatic tumor cells. Targeting the cells that form the premetastatic niche with specific antibodies to VEGFR-1 for HPC or VGEFR2 for CEPs reduces micrometastasis formation and progression.

Cancer stem cells (CSCs) are thought to be critical for initiation and propagation of many types of cancer. Although glioblastomas rarely spread outside the nervous system, they infiltrate crucial structures in the brain, preventing surgical resection. Because these cells are resistant to conventional therapies, they have been very difficult to eliminate and radiation and chemotherapy offer modest benefits and remain essentially palliative. Very recent reports (43, 44, 98) presented evidence that brain tumors orchestrate vascular niches that maintain the CSC pool. Disruption of these niche microenvironments ablates the fraction of self-renewing cells in brain tumors and arrests tumor growth. These data identify a potential role for niche microenvironments in the maintenance of brain CSCs and identify a mechanism by which antiangiogenic drugs inhibit brain tumor growth targeting cancer stem cells.

8. CONCLUSIONS

There is an increasing focus on the biology of CEPs as cells that contribute and controls tumor vasculogenesis and angiogenesis. Future efforts will clearly be directed towards the development of biologically active new drugs and therapeutic strategies that target these cells. In addition, CEP and CEP levels, measured by multiparametric procedures, are shown to be useful biomarkers for monitoring anti-cancer drug activity and establishing the OBD. CEP levels are also important, for patient stratification before antiangiogenic therapy and for monitoring therapy side effects.

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