# Molecular and cellular biomarkers for angiogenesis in clinical oncology

**Francesco Bertolini[1], Patrizia Mancuso[1], Yuval Shaked[2] and Robert S. Kerbel[2]**

[1] Division of Hematology-Oncology, Department of Medicine, European Institute of Oncology, 20141 Milan, Italy
[2] The Sunnybrook Health Sciences Centre, Molecular and Cellular Biology, Department of Medical Biophysics, University of Toronto, Toronto, Ontario M4N 3M5, Canada

**Medical oncologists are increasingly using anti-angiogenic drugs, but identifying the best-suited drug and the optimal dosage and schedule for treatment of patients remain challenging issues. Circulating endothelial cells (CECs) and circulating endothelial progenitors (CEPs) are modulated in a variety of diseases including cancer, and are promising surrogate biomarkers in oncology. Molecular surrogate markers, on the other hand, are more scanty at the present time, because the identification of truly endothelial cell-restricted genes and/or antigens is complex. Here, we discuss the biological and technical facets of the search and validation of new biomarkers for angiogenesis.**

A number of 'molecularly targeted' anti-angiogenic drugs such as bevacizumab (Avastin®), ranibizumab (Lucentis), sunitinib (Sutent®), and sorafenib (Nexavar) are now available for clinical oncologists and ophtalmologists [1–4]. Their clinical use, however, suffers from several relevant limitations. Although some large clinical trials have demonstrated a benefit of these drugs in terms of prolonged survival of cancer patients or increased visual function in patients with blinding conditions such as age-related macular degeneration (AMD), there is a compelling need for determining the optimal biologic dose (OBD) of these drugs, monitoring their biologic activity, selecting and stratifying the patients who are most likely to benefit from treatment. In medical oncology, problems related to the definition of the OBD for such drugs include the low frequency of tumor responses (tumor shrinkage); the lack, in some cases, of dose limiting toxicities (DLT) normally used to define a maximum tolerated dose (MTD), observed frequently when using cytotoxics but not as frequently when using certain anti-angiogenic drugs; and (in either oncology and ophthalmology) significant (if not optimal) therapeutic activity at doses below the MTD. Most of these drugs are extremely expensive [5,6], and the escalating cost of clinical care underlines the urgent need for development and clinical validation of biomarkers of angiogenesis for patient selection and stratification and for OBD tailoring [1–4,7].

*Corresponding author:* Bertolini, F. (francesco.bertolini@ieo.it)

## Molecular and imaging strategies to measure angiogenesis and anti-angiogenic drug activity

A number of preclinical angiogenesis assays and models have been generated. Growth factor (VEGF or FGF)-induced generation and quantification of new vessels is possible in the cornea or in the skin of some animal models, for example mice or rats [4]. The chick embryo chorioallantoic membrane (CAM) assay is based on the implantation of a gelatin sponge with the agent on the top of the growing CAM on day 8 of development. In this technique, after implantation, the sponge is treated with a stimulator of blood vessel formation in the absence or presence of an angiogenesis inhibitor, and evaluated for neovessel formation [8]. These preclinical approaches have limitations: some are poorly standardized, some are difficult to reproduce and in many cases reference values are scanty. Moreover, these invasive measurements are not adaptable to patients. Thus, the evaluation of the efficacy of a given anti-angiogenic therapy so far has been mainly based on the measurement of microvessel density (MVD) in biopsy samples [9]. In this approach, blood vessels are visualized by immunohistochemistry for endothelial-associated antigens, for example CD31, CD34, CD105 or von Willebrand factor, vWf. Positive vessels are counted under the microscope in a manual


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or digital fashion. This approach has also major drawbacks. First, it is invasive, second, it is difficult to standardize, third the MVD of a biopsy does not sometimes correlate with the MVD of an entire lesion because of tumor tissue heterogeneity. In addition, changes in MVD are not necessarily induced with some antiangiogenic drugs even though the absolute numbers of blood vessels is diminished, for reasons outlined by Hlatky *et al.* [9].

### *The measurement of circulating endothelial growth factors*

Another approach is to measure circulating or urinary levels of angiogenic growth factors, such as VEGF, b-FGF, HGF and IL-8 as in some types of cancer, the pre-treatment circulating levels of one or more of these factors predict patient survival [10–13]. Endocan (also called endothelial cell-specific molecule-1), has been found to be implicated in the regulation of cell adhesion, in inflammatory disorders and tumor progression [14]. Endocan is now considered a biomarker of both inflammatory disorders and tumor progression, and has been validated as a therapeutic target in cancer.

Soluble VEGF receptors such as VEGFR1, VEGFR2 and VEGFR3 are currently being investigated in a variety of cancer indications, involving patients treated with anti-angiogenic therapies in order to understand their potential as surrogate biomarkers. For example, it was shown in renal cancer patients receiving the tyrosine kinase inhibitor sunitinib, circulating levels of VEGF-A and PlGF in the blood increased during each cycle of treatment, whereas soluble VEGFR2 decreased. Within two weeks after treatment stopped, the levels of these biomarkers returned to near basal levels but the respective changes could be induced again during the next (4 week) cycle of daily therapy [15]. More work is needed to ascertain whether these biomarkers can predict patients' survival of response to anti-angiogenic therapies [16,17].

### *Imaging angiogenesis*

Functional imaging is another promising approach for the measurement of angiogenesis and anti-angiogenic drug activity. Dynamic contrast magnetic resonance imaging (DCE-MRI), for instance, measures changes in tumor blood flow and vascular permeability [18]. This procedure has been successfully used in a small number of phase I and II clinical trails of anti-angiogenic drugs. The downside of this method is that it requires expensive instrumentation, and standardization issues are still pending. Clinical validation has not yet been shown, despite some early promising indications [18].

Some investigators are using MRI-related techniques for the imaging of the tumor vasculature. Mulder *et al.* [19] used $\alpha(v)\beta3$ targeted bimodal liposomes to quantitate angiogenesis by MRI and to evaluate the therapeutic efficacy of angiogenesis inhibitors. The authors validated their findings by fluorescence microscopy and showed an interesting positive correlation with MVD. Another innovative approach is 3D-power Doppler ultrasound, which has recently been applied to functional studies on vasculogenesis in preclinical models [20]. In this context, a new generation of protein-targeted contrast agents for multimodal imaging of VEGF receptors has recently been described. These probes are based on a single-chain recombinant VEGF with a cysteine-containing tag that allows site-specific labeling of cancer vessels with contrast agents to be depicted in a highly selective and specific way by CT or PET [21].

### *Molecular markers of angiogenesis*

The search for molecular markers of angiogenesis and thereby of anti-angiogenic drug activity has been hampered by a crucial obstacle. Endothelial cells share the large majority (if not all) of their antigens with other hematopoietic or mesenchymal cells [22]. CD31, CD34, vWf or CD105, for example, which are antigens used for MVD quantification, are not only expressed by endothelial cells but also by hematopoietic cells, platelets and some fibroblasts subpopulations. Thus, the quantification of these antigens as proteins released in circulation or their mRNA transcripts will result in information of limited clinical predictive value. Many attempts have been made to purify cancer-specific endothelial cells and to screen for genes or proteins expressed only by these cells. The transcriptome of endothelial cells purified from cancer patients has been investigated by different laboratories. Smirnov *et al.* [23] identified 61 genes overexpressed in circulating endothelial cells (CEC) from cancer patients compared to CEC from healthy volunteers. In another study [24], 17 genes were identified that were overexpressed in tumor endothelium from colorectal cancer patients compared with angiogenic endothelium of normal tissues. Another study has recently identified 25 transcripts overexpressed in tumor versus normal endothelial cells, including 13 that were not found in the endothelial cells of regenerating liver [25]. These putative markers (or genetic signatures) should still be fully validated in the clinical setting, but they are potentially important also for the development of therapeutics specifically targeting tumor vessels.

So far, only a small number of genes is considered to be truly endothelial-restricted or endothelial-specific. One of these is VE-cadherin, which has been found to be expressed outside of the endothelial lineage only by stem cells *in utero* [26]. Interestingly, the number of copies of VE-cadherin transcripts in the blood of cancer patients is significantly increased when compared to healthy controls [27]. However, caution should be applied when using this candidate biomarker to investigate the anti-angiogenic activity of a given drug or therapeutic strategy. In fact, VE-cadherin RNA expression is markedly reduced (or absent) in apoptotic endothelial cells. Thus, the number of circulating VE-cadherin transcripts most likely reflects the number of circulating endothelial cells and their viability status, and thus the number of CECs should be taken into consideration when interpreting these results.

Recent studies have reported an increase of circulating transcripts for the endothelial progenitor cell-related CD133 antigen in the blood of cancer patients [28–30]. It should be noted, however, that CD133 is expressed also by hematopoietic progenitors and other cell types [22]. Thus, further work is needed to understand the cellular source of the CD133 transcript increase in the blood of these patients.

## Cellular markers of angiogenesis and anti-angiogenic drug activity

It was first reported in the mid 1970s that cells with endothelial characteristics circulate in the blood [31]; it took two more decades to establish a procedure to quantify the CEC population. In healthy subjects, this numerically rare cell population is stable in quantity and represents 1/1000–100 000 of circulating blood cells [22,32]. In many pathological conditions, such as cancer, the number of CECs is increased (Figure 1). The majority of CECs

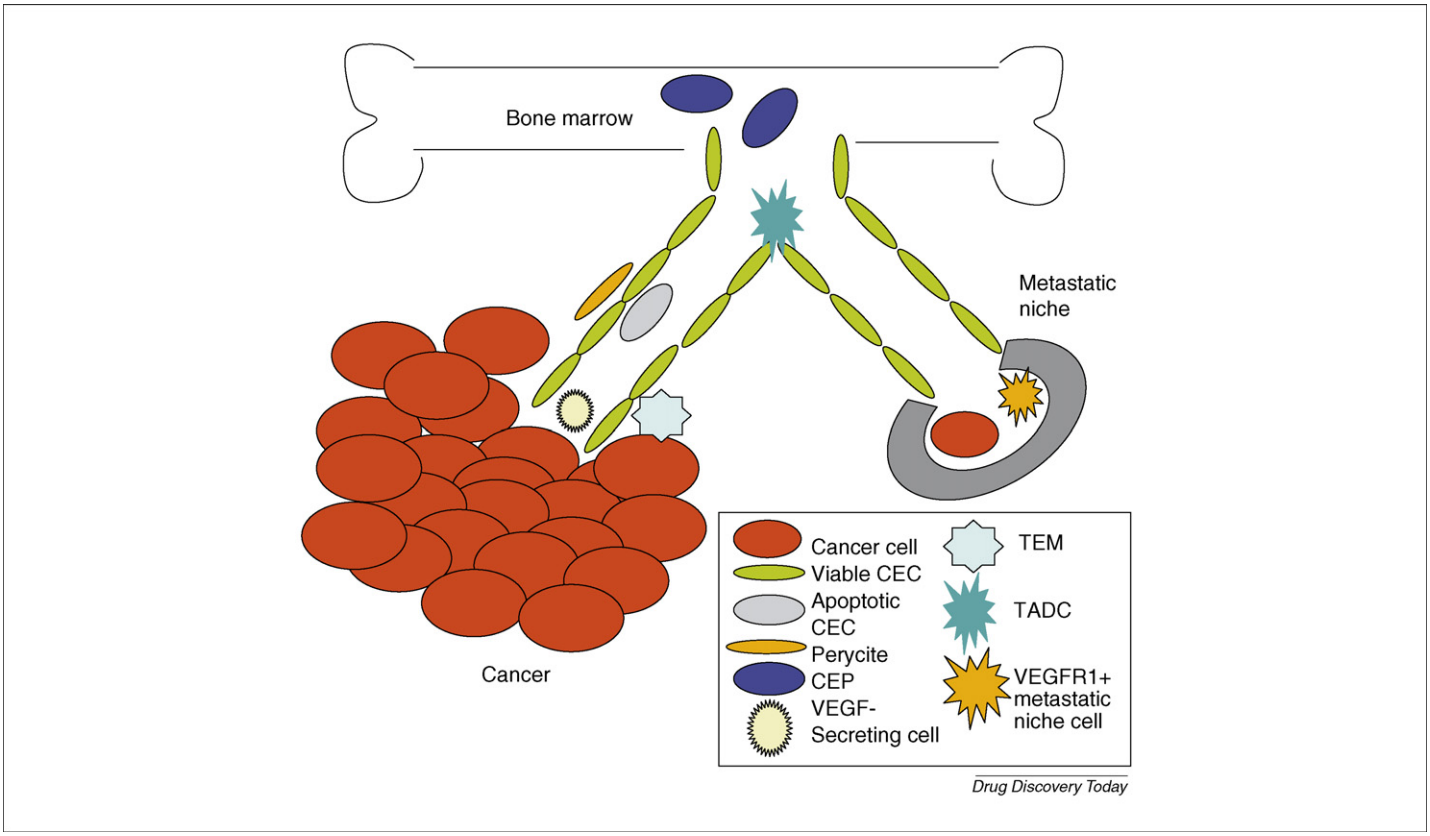


**FIGURE 1**

Some of the actors playing a role in cancer angiogenesis and vasculogenesis: Cancer vessels may release CECs with a mature phenotype, either viable (because of high levels of VEGF secreted by cancer cells), or apoptotic. Marrow-derived CEPs seem to play different parts in different cancer types and in different phases of cancer growth. Other cell populations playing a distinctive role are tumor associated dendritic cells (TADC), Tie-2 expressing monocytes promoting angiogenesis (TEM), VEGFR1-positive cells initiating metastatic niches and VEGF-secreting hematopoietic cells and perycites.

shows characteristics of mature, terminally differentiated and frequently apoptotic cells (the first morphological studies described them as 'anucleated carcasses'), only a subpopulation of which expresses antigens that suggest a stem-like or progenitor-like phenotype (Figure 2). As discussed below, these putative circulating endothelial progenitors (CEPs) might home to sites of vasculogenesis and angiogenesis and participate in new blood vessel formation by stimulating vasculogenesis [33].

The Hebbel laboratory was the first to describe the quantitative and functional relationship between CECs and CEPs [34]. Using a Y-chromosome gene marking approach in recipients of gender-mismatched bone marrow transplants, they were able to distinguish between CEPs from the bone marrow (i.e. donor-derived cells), and CECs from vessel walls (i.e. host/recipient-derived). More than 90% of endothelial cells in the blood were found to be of recipient origin. When cultured *in vitro*, these endothelial cells expanded only approximately 20-fold, in contrast to donor-derived endothelial cells, which expanded approximately 1000-fold. This indicates that most CECs probably originate from vessel walls and have limited growth capability, whereas only a rare CEC subpopulation – most likely derived from the bone marrow and defined as CEPs – is responsible for the large majority of the endothelial cell proliferative potential. This hypothesis was confirmed by studies indicating that CEPs from peripheral blood could generate mature endothelial cells *in vitro* and *in vivo* in vascular grafts [35,36].

CEPs maintain a proliferative potential that mature CECs have lost, so that clonogenic cell culture assays should be possible. However, recent studies indicate that the large majority of colonies generated in commercially available assays for endothelial CEPs are of myeloid origin and have no vasculogenic potential [37]. Therefore, a more endothelial-specific strategy is needed. In addition, subjective evaluation of colony staining is at risk for inter-operator variability.

### *Antigenic and functional definition of CECs and CEPs*

Differentiating CEPs from CECs based on different expression of surface molecules is very difficult because of the antigenic promiscuity amongst hematopoietic cells and progenitors, platelets, CECs and CEPs as mentioned above. In fact, there is no single antigen to discriminate between CECs, platelets and hematopoietic cells [22,33].

The first procedure to quantify CECs was developed by Dignat-George and Sampol [38], who used magnetic beads coupled to an anti-CD146 monoclonal antibody to isolate CECs from peripheral blood. It later became clear that CD146 is not an endothelial-specific antigen, so that the true endothelial identity of putative CECs isolated in this fashion had to be confirmed by vWf and/or lectin UEA-1 expression and by the absence of the pan-hematopoietic marker CD45 [39].

In another procedure, using multiparametric flow-cytometry, whole blood was labelled simultaneously with different

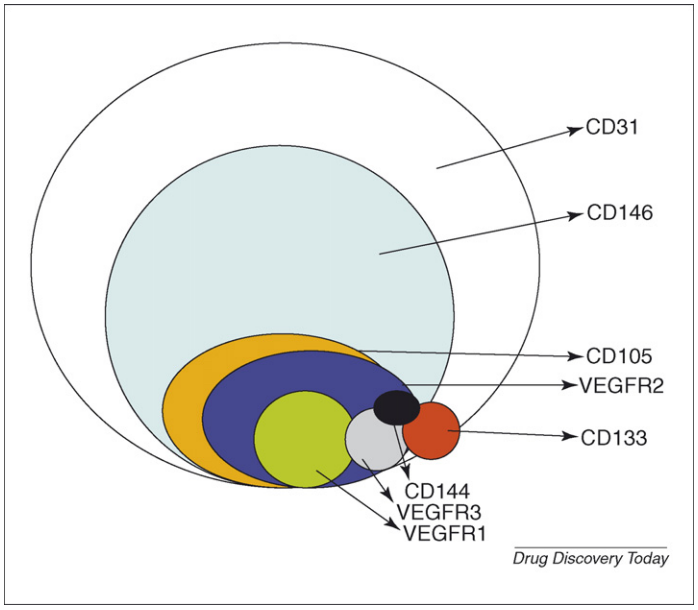


**FIGURE 2**

Representative relative distribution of some of the antigens expressed in CECs, as depicted by flow cytometry. The image indicates that virtually all CECs express CD31, but only some fractions (suggested by the size of the relative spot) express other endothelial markers such as CD146, VEGFR2, VEGFR1, CD105 or CD144. The relative sizes of other subpopulations (such as CD133 + CEPs, or VEGFR3 + CECs possibly involved in lymphangiogenesis) are also suggested by the size of the spots. The CD133 spot is designed across the CD31 border to indicate the existence of non-endothelial CD133+ cells.

monoclonal antibodies. Endothelial cells were identified by the expression of markers such as CD31, CD146 or VEGFR2; CD45 was used to exclude hematopoietic cells from the analysis. The use of a nuclear staining for DNA is crucial to exclude aggregated platelets and/or endothelial microparticles (that share surface markers with CECs and CEPs) from the CEC count [22].

Hematopoietic stem cells (HSC), but not mature hematopoietic cells, express CD34, which is why this antigen has been widely used to identify progenitor cells with a clonogenic potential. However, CD34 is also expressed by mature endothelial cells, and thus it cannot be used to differentiate CEPs from CECs [22,33,40]. At present, CD133 seems to be the sole antigen that is expressed in CEPs and subsequently downregulated in mature CECs [40]. Unfortunately, in humans CD133 is also expressed by HSCs, so that a phenotypic distinction between CEPs, HSC and (should they exist in adults) bi-potential hemangioblasts [41] is not possible.

The CD133 antigen has been well characterized in humans, but in mice it is not yet established whether it can be used to depict CEPs. In the mouse, the c-kit (CD117) antigen is expressed on HSCs, and only a subpopulation of mouse CECs express CD117. For this reason, we have proposed to use CD117 as a surrogate CEP marker in mice [42,43]. To avoid contamination with HSC, only CD117+/CD45− cells should be analyzed.

Methodological inconsistency between flow cytometry procedures, involving differences in the combinations of markers, gating strategies, and the occasional use of a pre-enrichment step, has led to different CEC values reported in the literature (summarized in Ref. [32]). Thus, there is a need for standardization of flow cytometry procedures to minimize intra- and inter-laboratory variability.

## Cellular markers and their correlation with other preclinical surrogate markers of angiogenesis

In order to validate the potential of CECs and CEPs as preclinical surrogate markers of angiogenesis, we took advantage of the genetically induced difference in angiogenic responsiveness among different mouse strains (it is currently unclear whether such a wide variability exists also in humans). We studied eight different strains and angiogenesis was assessed using the corneal micropocket assay in the mouse eye. The mouse strains differed up to 10-fold in their response to VEGF [42]. A highly significant positive correlation was found between the response after VEGF stimulation and the absolute number of CECs and CEPs in normal (unstimulated) mice. These results were confirmed using the Matrigel (subcutaneous) plug perfusion assay for angiogenesis [42] indicating that the number of CECs and CEPs is indeed representative of the angiogenic response. To investigate whether quantification of CECs and CEPs could be used to determine the OBD of targeted anti-angiogenic drugs in mice, we treated mice with DC101, a rat monoclonal antibody specifically blocking mouse VEGFR2 [44], which has an optimal anti-tumor therapeutic dose in the range of 800–1200 μg/mouse. As expected, this dose led to the greatest decrease in tumor volume and also to the lowest level of viable CEPs only one week after treatment was initiated. Increasing the dose did not cause further reductions in levels of CEPs. To rule out that CEP levels merely reflect the reduction in tumor growth and are not a consequence of the OBD of the drug, we evaluated viable CEPs in non-tumor bearing mice. Dose-escalation studies confirmed that 800 μg/mouse is the OBD of DC101 as judged by the induction of the lowest level of CEPs. A highly significant correlation between lowered levels of viable CEPs and the OBD of the tested was confirmed testing other antiangiogenic drugs including small molecules, antibodies and blocking peptides. In addition, we found that quantification of CECs and CEPs can be used to determine the OBD in cancer-bearing mice treated with low-dose 'metronomic' chemotherapy, a cancer treatment strategy thought to have an antiangiogenic basis [45]. Taken together, these findings underline the potential of CEC and CEP measurement for helping to establish OBD.

## CECs as surrogate markers of angiogenesis and anti-angiogenic drug activity in medical oncology

CEC levels are increased in the peripheral blood of patients affected by some types of cancer, and return to normal values in patients undergoing complete remission [16,22,46–49]. In preclinical models we used either MTD cyclophosphamide (CTX) or endostatin as paradigms of drugs with primarily cytotoxic versus endothelium-restricted activities. In mice given CTX at the MTD, most of the circulating apoptotic cells were hematopoietic and not endothelial, and a significant proportion of CECs were viable. After treatment with endostatin, most CECs were apoptotic, whereas hematopoietic cells were viable [50].

In metastatic breast cancer patients treated with low dose metronomic chemotherapy using CTX and methotrexate, the CEC count after two months of continuous (daily) therapy was a particularly good predictor of disease-free and overall survival after a follow-up of more than two years. Patients showing a CEC count above physiological levels after two months of therapy had a significantly improved progression-free and overall survival [47].

**TABLE 1**

**Strengths and limitations of some methods assessing cancer-related angiogenesis in patients**

| Method (Refs) | Strengths | Limitations |
|---|---|---|
| **Microvessel density** [9] | Allows morphological comparisons (e.g. branching vs. sinusoidal vessels) | Tumor tissue heterogeneity, prone to reader-dependent variability |
| **Circulating endothelial growth factor (e.g. VEGF, b-FGF) measurement by ELISA** [10–14] | Easy to standardize and reproduce | Active uptake of many growth factors from platelets and other cells may hamper the clinical relevance of these measurements |
| **Imaging (e.g. Dynamic MRI,** [18]**)** | Simultaneous study of multiple parameters (including for instance vascular permeability) | Expensive and restricted to centres with adequate instrumentation |
| **Flow cytometry CEC and CEP enumeration** [22] | Simultaneous enumeration of a variety of cell (or cell fragment) populations | Procedures require standardization |
| **CEC enumeration by Immunohistochemistry** [38,39] | Allows morphological confirmation of the endothelial nature of the cells | Subject to reader-dependent variability |
| **Quantitative enumeration of endothelial-specific or cancer endothelial-specific transcripts** [23–27] | High (potential) specificity | Subject to the activation or viability status of endothelial cells.<br>Transcripts might not be stable in the blood |

The increased number of CECs in patients with a clinical benefit was mostly because of an increased number of apoptotic CECs. This is because antiangiogenic agents are thought to reverse cancer vessel abnormalities [51] and in this scenario, the remodelling process likely involves a shedding of apoptotic endothelial cells from cancer-associated vessels. In preclinical studies, we did not observe a rise in the number of CECs in cancer-free animals treated with metronomic chemotherapy—only in tumor-bearing mice was this observed. Taken together, these findings suggest that the cancer-associated vasculature is most likely the predominant source of the rise in apoptotic CECs seen in breast cancer patients treated with metronomic therapy, thus confirming the potential of this surrogate biomarker.

The measurement of CECs, of their viability, and of CEC subpopulations (e.g. VEGFR2 + CECs, activated CD105 + CECs, or VEGFR3 + CECs, possibly involved in lymphangiogenesis) might be useful for OBD finding in phase I–II studies on antiangiogenic drugs, alone or in combination with chemotherapeutics. When considering that in undifferentiated patient pools, the number of non-responders could jeopardize a trial's endpoint, CEC-related measurements might also be of help to identify responders and non-responders to a given therapeutic regimen including anti-angiogenic drugs or strategies such as metronomic chemotherapy. This patient stratification may significantly reduce the (otherwise rampant) costs of novel antiangiogenic cancer therapies by targeting treatments to those patients most likely to benefit (Box 1).

**BOX 1**

### CECs and CEPs as potential therapeutic targets

CECs are apoptotic cells/carcasses from vessel wall turnover. CEPs are potentially contributing to vasculogenesis, and can be considered as therapeutic targets. In angiogenic-defective Id-mutant (Id1+/−Id3−/−) mice, wild-type CEPs restored tumor angiogenesis and growth [52]. Other conflicting studies showing negligible CEP contribution to tumor vessels [53,54] were later explained by the findings that CEP contribution to the tumor vasculature depends on variables such as tumor stage, size, type and/or mouse strain [55–60]. In patients' tumors [57], CEP-derived vessels were enumerated as about 5% of all cancer endothelial cells; in mice, a CEP role was found in late- [58] or early stage [59,60] cancer vascularization. CEPs might be relevant in tumor relapse after chemotherapy or anti-vascular treatment [48,61–64], and this might explain how antiangiogenic drugs can augment chemotherapy efficacy [63]. In addition to CEPs, other marrow-derived circulating cell populations might be involved in tumor angiogenesis ([65–69], Figure 1). These cells might be investigated as biomarkers, if enumeration procedures are clinically validated. Unlike cancer cells, endothelial cells have been considered to be genetically normal [70]. However, in mice [71] and in clinical studies [72–74] tumor endothelial cells were found to share the same genetic alterations observed in cancer cells. These observations may have different possible explanations. Endothelial and neoplastic cells may derive from a common hemangioblast. Genetically altered endothelial cells might be generated from 'horizontal' DNA/chromosome transfer [75]. Microenvironmental factors might de-differentiate neoplastic cells toward the endothelial phenotype [76]. Finally, cancer and endothelial cells might fuse spontaneously to create cell hybrids. These observations underline the need for a better definition of cancer-related endothelial cells.

## Conclusions

Surrogate biomarkers of angiogenesis are urgently needed to better design preclinical studies and clinical trials involving anti-angiogenic drugs, alone or in association with other therapies. Table 1 summarizes the pros and cons of different angiogenic markers currently available and under scrutiny in clinical oncology for OBD finding and patient stratification. As more anti-angiogenic agents enter the clinical arena for more indications, it is becoming clear that these drugs may induce unforeseen side effects [17,77,78]. The biomarkers described in this review might also be of help in the prediction and management of these side effects.

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## References


1 Jain, R.K. *et al.* (2006) Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nat. Clin. Pract. Oncol.* 3, 24–40
2 Bhisitkul, R.B. (2006) Vascular endothelial growth factor biology: clinical implications for ocular treatments. *Br. J. Ophthalmol.* 90, 1542–1547
3 Kerbel, R.S. and Folkman, J. (2002) Clinical translation of angiogenesis inhibitors. *Nat. Rev. Cancer* 2, 727–739
4 Folkman, J. (2007) Angiogenesis: an organizing principle for drug discovery? *Nat. Rev. Drug Discovery* 6, 273–286
5 Schrag, D. (2004) The price tag on progress – Chemotherapy for colorectal cancer. *New Engl. J. Med.* 351, 317–319
6 Berenson, A. (2006) A cancer drug shows promise, at a price that many can't pay. New York Times, Feb. 15, 2006
7 Schneider, M. *et al.* (2005) A surrogate marker to monitor angiogenesis at last. *Cancer Cell* 7, 3–4
8 Ribatti, D. *et al.* (2006) The gelatin sponge-chorioallantoic membrane assay. *Nat. Protoc.* 1, 85–91
9 Hlatky, L. *et al.* (2002) Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. *J. Natl. Cancer Inst.* 94, 883–893
10 Jain, R.K. (2002) Tumor angiogenesis and accessibility: role of vascular endothelial growth factor. *Semin. Oncol.* 29 (Suppl. 16), 3–9
11 Homsi, J. and Daud, A.I. (2007) Spectrum of activity and mechanism of action of VEGF/PDGF inhibitors. *Cancer Contr.* 14, 285–294
12 Roussidis, A.E. *et al.* (2007) The importance of c-Kit and PDGF receptors as potential targets for molecular therapy in breast cancer. *Curr. Med. Chem.* 14, 735–743
13 Bremnes, R.M. *et al.* (2006) Angiogenesis in non-small cell lung cancer: the prognostic impact of neoangiogenesis and the cytokines VEGF and bFGF in tumours and blood. *Lung Cancer* 51, 143–158
14 Sarrazin, S. *et al.* (2006) Endocan or endothelial cell specific molecule-1 (ESM-1): a potential novel endothelial cell marker and a new target for cancer therapy. *Biochim. Biophys. Acta.* 1765, 25–37
15 Motzer, R.J. *et al.* (2006) Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. *J. Clin. Oncol.* 24, 16–24
16 Norden-Zfoni, A. *et al.* (2007) Blood-based biomarkers of SU11248activity and clinical outcome in patients with metastatic imatinib-resistant gastrointestinal stromal tumor. *Clin. Cancer Res.* 13, 2643–2650
17 Feldman, D.R. *et al.* (2007) Hypothyroidism in patients with metastatic renal cell carcinoma treated with sunitinib. *J. Natl. Cancer Inst.* 99, 974–975
18 Morgan, B. *et al.* (2003) Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/ZK. 222584 an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases, in patients with advanced colorectal cancer and liver metastases: results from two phase I studies. *J. Clin. Oncol.* 2, 3955–3964
19 Mulder, W.J. *et al.* (2007) Early in vivo assessment of angiostatic therapy efficacy by molecular MRI. *FASEB J.* 21, 378–383
20 Xuan, J.W. *et al.* (2007) Functional neoangiogenesis imaging of genetically engineered mouse prostate cancer using three-dimensional power Doppler ultrasound. *Cancer Res.* 67, 2830–2839
21 Backer, M.V. *et al.* (2007) Molecular imaging of VEGF receptors in angiogenic vasculature with single-chain VEGF-based probes. *Nat. Med.* 13, 504–509
22 Bertolini, F. *et al.* (2006) The multifaceted circulating endothelial cell in cancer: towards marker and target identification. *Nat. Rev. Cancer* 6, 835–845
23 Smirnov, D.A. *et al.* (2006) Global gene expression profiling of circulating endothelial cells in patients with metastatic carcinomas. *Cancer Res.* 66, 2918–2922
24 van Beijnum, J.R. *et al.* (2006) Gene expression of tumor angiogenesis dissected: specific targeting of colon cancer angiogenic vasculature. *Blood* 108, 2339–2348
25 Seaman, S. *et al.* (2007) Genes that distinguish physiological and pathological angiogenesis. *Cancer Cell* 11, 539–554
26 Kim, I. *et al.* (2005) CD144 (VE-cadherin) is transiently expressed by fetal liver hematopoietic stem cells. *Blood* 106, 903–905
27 Rabascio, C. *et al.* (2004) Assessing tumor angiogenesis: increased circulating VE-cadherin RNA in patients with cancer indicates viability of circulating endothelial cells. *Cancer Res.* 64, 4373–4377
28 Lin, E.H. *et al.* (2007) Elevated circulating endothelial progenitor marker CD133 messenger RNA levels predict colon cancer recurrence. *Cancer* 110, 534–542
29 Yu, D. *et al.* (2007) Identification and clinical significance of mobilized endothelial progenitor cells in tumor vasculogenesis of hepatocellular carcinoma. *Clin. Cancer Res.* 13, 3814–3824
30 Mehra, N. *et al.* (2006) Progenitor marker CD133 mRNA is elevated in peripheral blood of cancer patients with bone metastases. *Clin. Cancer Res.* 12, 4859–4866
31 Hladovec, J. and Rossamn, P. (1973) Circulating endothelial cells isolated together with platelets and the experimental modification of their counts in rats. *Thromb. Res.* 3, 665–674
32 Blann, A.D. *et al.* (2005) Circulating endothelial cells. Biomarker of vascular disease. *Thromb Haemost.* 93, 228–235
33 Rafii, S. *et al.* (2002) Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? *Nat. Rev. Cancer* 2, 826–835
34 (2000) Origins of circulating endothelial cells and endothelial outgrowth from blood. *J. Clin. Invest.* 105, 71–77
35 Asahara, T. *et al.* (1997) Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275, 964–967
36 Shi, Q. *et al.* (1998) Evidence for circulating bone marrow-derived endothelial cells. *Blood* 92, 362–367
37 Yoder, M.C. *et al.* (2007) Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood* 109, 1801–1809
38 Dignat-George, F. and Sampol, J. (2000) Circulating endothelial cells in vascular disorders: new insights into an old concept. *Eur. J. Haematol.* 65, 215–220
39 Woywodt, A. *et al.* (2006) Isolation and enumeration of circulating endothelial cells by immunomagnetic isolation: proposal of a definition and a consensus protocol. *J. Thromb. Haemost.* 4, 671–677
40 Peichev, M. *et al.* (2000) Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood* 95, 952–958
41 Pelosi, E. *et al.* (2002) Identification of the hemangioblast in postnatal life. *Blood* 100, 3203–3208
42 Shaked, 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
43 Capillo, M. *et al.* (2003) Continuous infusion of endostatin inhibits differentiation, mobilization, and clonogenic potential of endothelial cell progenitors. *Clin. Cancer Res.* 9, 377–382
44 Prewett, M. *et al.* (1999) Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. *Cancer Res.* 59, 5209–5218
45 Kerbel, R.S. and Kamen, B.A. (2004) The anti-angiogenic basis of metronomic chemotherapy. *Nat. Rev. Cancer* 4, 423–436
46 Mancuso, P. *et al.* (2001) Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. *Blood* 97, 3658–3661
47 Mancuso, P. *et al.* (2006) Circulating endothelial cell kinetics and viability predict survival in breast cancer patients receiving metronomic chemotherapy. *Blood* 108, 452–459
48 Furstenberger, G. *et al.* (2006) Circulating endothelial cells and angiogenic serum factors during neoadjuvant chemotherapy of primary breast cancer. *Br. J. Cancer* 94, 524–531
49 Zhang, H. *et al.* (2005) Circulating endothelial progenitor cells in multiple myeloma: implications and significance. *Blood* 105, 3286–3294
50 Monestiroli, S. *et al.* (2001) Kinetics and viability of circulating endothelial cells as surrogate angiogenesis marker in an animal model of human lymphoma. *Cancer Res.* 61, 4341–4344
51 Jain, R.K. (2003) Molecular regulation of vessel maturation. *Nat. Med.* 9, 685–693
52 Lyden, D. *et al.* (2001) Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat. Med.* 7, 1194–1201
53 De Palma, M. *et al.* (2003) Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat. Med.* 9, 789–795
54 Gothert, J.R. *et al.* (2004) Genetically tagging endothelial cells *in vivo*: bone marrow-derived cells do not contribute to tumor endothelium. *Blood* 104, 1769–1777
55 Duda, D.G. *et al.* (2006) Evidence for incorporation of bone marrow-derived endothelial cells into perfused blood vessels in tumors. *Blood* 107, 2774–2776

56 Monsky, W.L. *et al.* (2002) Role of host microenvironment in angiogenesis and microvascular functions in human breast cancer xenografts: mammary fat pad versus cranial tumors. *Clin. Cancer Res.* 81008–81013

57 Peters, B.A. *et al.* (2005) Contribution of bone marrow-derived endothelial cells to human tumor vasculature. *Nat. Med.* 11, 261–262

58 Spring, H. *et al.* (2005) Chemokines direct endothelial progenitors into tumor neovessels. *Proc. Natl Acad. Sci. U. S. A.* 102, 18111–18116

59 Ruzinova, M.B. *et al.* (2003) Effect of angiogenesis inhibition by Id loss and the contribution of bone-marrow-derived endothelial cells in spontaneous murine tumors. *Cancer Cell* 4, 277–289

60 Nolan, D.J. *et al.* (2007) Bone marrow-derived endothelial progenitor cells are a major determinant of nascent tumor neovascularization. *Genes Dev.* 21, 1546–1558

61 Shaked, Y. *et al.* (2006) Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. *Science* 313, 1785–1787

62 Bertolini, F. *et al.* (2003) Maximum tolerable dose and low-dose metronomic chemotherapy have opposite effects on the mobilization and viability of circulating endothelial progenitor cells. *Cancer Res.* 63, 4342–4346

63 Kerbel, R.S. (2006) Antiangiogenic therapy: A universal chemosensitization strategy for cancer? *Science* 312, 1171–1175

64 Shaked, Y. and Kerbel, R.S. (2007) Antiangiogenic strategies on defense: On the possibility of blocking rebounds by the tumor vasculature after chemotherapy. *Cancer Res.* 67, 7055–7058

65 Grunewald, M. *et al.* (2006) VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell* 124, 175–189

66 De Palma, M. *et al.* (2005) Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell* 8, 211–226

67 Conejo-Garcia, J.R. *et al.* (2004) Tumor-infiltrating dendritic cell precursors recruited by a beta-defensin contribute to vasculogenesis under the influence of VEGF-A. *Nat. Med.* 10, 950–958

68 Udagawa, T. *et al.* (2006) Analysis of tumor-associated stromal cells using SCID GFP transgenic mice: contribution of local and bone marrow-derived host cells. *FASEB J.* 20, 95–102

69 Kaplan, R.N. *et al.* (2005) VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* 438, 820–827

70 Kerbel, R.S. (1991) Inhibition of tumor angiogenesis as a strategy to circumvent acquired resistance to anti-cancer therapeutic agents. *Bioessays* 13, 31–36

71 Hida, K. *et al.* (2004) Tumor-associated endothelial cells with cytogenetic abnormalities. *Cancer Res.* 64, 8249–8255

72 Streubel, B. *et al.* (2004) Lymphoma-specific genetic aberrations in microvascular endothelial cells in B-cell lymphomas. *N. Engl. J. Med.* 351, 250–259

73 Rigolin, G.M. *et al.* (2006) Neoplastic circulating endothelial cells in multiple myeloma with 13q14 deletion. *Blood* 107, 2531–2535

74 Rigolin, G.M. *et al.* (2007) Neoplastic circulating endothelial-like cells in patients with acute myeloid leukaemia. *Eur. J. Haematol.* 78, 365–373

75 Bergsmedh, A. *et al.* (2001) Horizontal transfer of oncogenes by uptake of apoptotic bodies. *Proc. Natl. Acad. Sci. U. S. A.* 98, 6407–6411

76 Hendrix, M.J. *et al.* (2007) Reprogramming metastatic tumour cells with embryonic microenvironments. *Nat. Rev. Cancer* 7, 246–255

77 van Heeckeren, W.J. *et al.* (2006) Promise of new vascular-disrupting agents balanced with cardiac toxicity: is it time for oncologists to get to know their cardiologists? *J. Clin. Oncol.* 24, 1485–1488

78 Tuma, R.S. (2006) Accrual delayed in adjuvant bevacizumab trial. *J. Natl. Cancer Inst.* 98, 439–440