Clinical value of circulating endothelial cell detection in oncology

Jaco Kraan[1], Stefan Sleijfer[1], John A. Foekens[2] and Jan W. Gratama[1]

[1] Department of Medical Oncology, Erasmus Medical Center – Daniel den Hoed Cancer Center, Groene Hilledijk 301, 3075 EA Rotterdam, The Netherlands
[2] Department of Medical Oncology, Erasmus Medical Center – Daniel den Hoed Cancer Center and Cancer Genomics Centre, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands

Given the importance of tumor vasculature in tumor biology and as a target for treatment, there is an increasing need for biomarkers that reflect effects impacting tumor vasculature accurately. Circulating endothelial cells (CECs) increase in number as a result of vascular damage in cancer and several other diseases. CEC count constitutes a promising tool for monitoring disease activity with potential to assess prognosis and response to treatment. Here, we address the current state-of-the-art of CEC enumeration as a biomarker in clinical oncology. We focus on technical issues concerning CEC detection, review results from clinical studies and explore future potential applications.

Introduction

The growth of solid tumors is dependent on their capacity to induce the growth of blood vessels that supply them with oxygen and nutrients [1,2]. There are several known mechanisms of blood vessel formation. Vessel formation can occur by: (i) sprouting of new vessels from existing ones (i.e. angiogenesis); (ii) the recruitment of bone-marrow-derived and/or vascular-wall-resident endothelial progenitor cells (EPCs) that differentiate into endothelial cells (ECs) (i.e. vasculogenesis); or by (iii) vessel splitting (intussusception); in addition (iv) tumor cells can co-opt pre-existing vessels; (v) tumor vessels can be lined by tumor cells (vascular mimicry); and (vi) putative cancer stem-like cells can differentiate into tumor endothelium. Unlike normal tissues, which use sprouting angiogenesis, vasculogenesis and intussusceptions only, tumors can use all six modes of vessel formation [3]. Another important difference between normal and tumor vasculature is that normal vasculature is arranged in a hierarchy of evenly spaced, well-differentiated arteries, arterioles, capillaries, venules and veins, whereas the tumor vasculature is composed of a chaotic mixture of abnormal and hierarchically disorganized vessels [4].

In view of the central role of the tumor vasculature in the biology of tumors, various agents have been developed and are now available or under study for the purpose of targeting the tumor vasculature. Examples include vascular endothelial growth factor (VEGF)-pathway-inhibiting agents such as monoclonal antibodies against VEGF, as well as small tyrosine kinase inhibitors targeting VEGF receptors or targeting activin receptor-like kinase1 (ALK1) [5]. Recently, a new group of anticancer drugs, vascular disrupting agents (VDAs), has been developed to target the ECs and pericytes of the already established tumor vasculature [6]. Although several clinical trials have demonstrated a benefit in terms of prolonged survival of cancer patients treated with anti-angiogenic therapies, for further development of the field of antiangiogenic treatments there is a compelling need for clinically effective biomarkers for determining the optimal dose, monitoring biologic activity and selecting and stratifying the patients most likely to benefit from treatment [7]. There is also a lack of biomarkers identifying escape pathways that underlie resistance against antiangiogenic agents and that should be targeted after tumors develop resistance to a given antiangiogenic agent [8].

Currently, there are several parameters that can serve as surrogate biomarkers to measure angiogenesis and/or antiangiogenic drug activity. These include soluble blood factors involved in angiogenesis such as VEGF, s-VEGFR-2, placenta growth factor (PlGF), soluble Tie2, E-selectin and thrombomodulin, and molecular markers of angiogenesis such as von Willebrand factor (vWF) and vascular endothelial (VE)-cadherin. Blood vessels can be visualized using vascular imaging techniques such as dynamic-contrast-enhanced

Corresponding author:. Kraan, J. (j.kraan@erasmusmc.nl)

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MRI and positron emission tomography (PET) scanning. Each of these methods has drawbacks regarding specificity, sensitivity or costs and, although several studies have revealed interesting data, none has been clinically validated [7,9].

Circulating endothelial cell (CEC) count is a relative new candidate to serve as a marker to monitor vascular effects. Current opinion states that CECs are mature ECs that have been shed from the vascular cell lining as a result of vascular damage. Because numerous diseases, including cancer, are associated with vascular damage, the enumeration of CECs has long been considered a promising tool to monitor disease activity with a potential to assess prognosis and response to treatment. Accordingly, elevated CEC numbers have been found in multiple diseases, including cardiovascular disorders [10,11], infectious diseases [12–14], immune disorders [15,16], pulmonary hypertension [17], sickle cell anemia [18,19], status after organ transplantation [20,21] and cancer [22–27].

In this review, we will address the current position of CEC enumeration as a biomarker in clinical oncology with special attention to technical issues concerning their detection, results from clinical studies obtained so far and future potential applications.

Enumeration of CECs

The first methods used to detect CECs were described in the mid-1970s and identification methods included separation by density centrifugation, vital light microscopy and May–Grünwald staining. These methods did not isolate and identify CECs reliably, and it took two more decades before procedures designed to quantify CECs were established. Because CECs occur in low frequencies in the peripheral blood of healthy donors, there is a consensus in the literature that values are less <10 CECs/ml, one of the main challenges is assay sensitivity and accuracy. As a result, assays that count CECs are prone to errors in sample preparation and analysis. Given the low frequencies, a volume of >1 ml will be needed for a precise enumeration of CECs at a frequency of <1 cell/μl blood to obtain a population of at least 100 cells fulfilling the flow cytometric criteria with a maximal coefficient of variation of 10%.

Another essential step for the proper detection and enumeration step of CECs is the use of specific markers discriminating CECs from other cells present in the peripheral circulation. Table 1 provides an overview of markers frequently used in various CEC assays. The most common marker used to define CECs is CD146 or melanoma cell-adhesion molecule (MCAM). CD146 is present on ECs and also on a subset of activated T lymphocytes, mesenchymal stem cells and some malignant cells. This implies conformation of the origin of CECs isolated through $CD146^+$ enrichment by other CEC markers and the absence of the pan-leukocyte marker CD45. A complication with CEC detection is that many endothelial surface antigens are secreted, and their interaction with platelet aggregates can result in false-positive signals [28].

During recent years, several methods have been described to enumerate CECs. However, there is no consensus between CEC phenotypes used in these enumeration techniques. Importantly, most methods have not been validated properly [28]. In combination with the relative rarity of CECs, this situation has resulted in a wide variation of reported CEC numbers. For example, reported CEC numbers in healthy humans range from 4 to 7900 cells/ml in the various CEC assays [23,25,29–31]. This large variation, probably caused by using different techniques, hampers comparisons between studies and thereby hinders further progress in the field. As a consequence, there is a need for standardization and validation of the true endothelial origin of cells designated as CECs by a particular assay. For this, several unique features of ECs can be used and these include uptake of Ulex Europaeus Lectin-1 (UEA-1) [25]. Also, immunocytochemistry or immunofluorescence can be used to demonstrate the presence of endothelial markers as well as used in the assay to isolate the cells thought to be CECs (Table 1). Another means of validation is through gene expression profiling assessing whether or not endothelial genes, such as VE-cadherin, are expressed by the population of cells designated as CECs [32].

Manual immunomagnetic isolation of CECs

Currently, most CEC enumeration tests rely on an enrichment step by immunomagnetic bead separation (IMS) using magnetic particles coupled to monoclonal antibody (mAb) targeting of an endothelial antigen such as CD146 or CD34, followed by flow cytometry or visual counting with a fluorescent microscope to identify CECs on the basis of morphological or immunophenotypic criteria [30,33,34]. A major advantage of this approach is that it enables visual identification of CECs, which leads to discrimination between CECs and endothelial microparticles or platelets with a strongly overlapping phenotype. However, all enrichment steps inevitably lead to cell loss and therefore an underestimation of the actual CEC number, whereas manual bead-based isolation procedures are labor intensive and difficult to standardize in terms of purity and recovery. Despite the differences between published IMS methods with respect to the size of the magnetic beads, antibody clones and enumeration techniques most studies are reporting a relatively narrow range of CEC levels (i.e. between 1 and 10 cells/ml) in healthy individuals [35].

Automated immunomagnetic isolation and staining of CECs

A variant of manual IMS enrichment techniques is the CellSearch® system (Veridex; http://www.veridex.com/). Initially designed to detect circulating tumor cells, this system provides a fully automated enrichment procedure that is followed by semi-automated image cytometry. The generated images are evaluated for CEC content by visual inspection, in which CECs are defined as $DAPI^+$, $CD105^+$, $CD146^+$ and $CD45^-$, and morphological criteria such as size and whole intact cells. This assay has a high yield and good reproducibility, even for low numbers of CECs as reported in healthy controls (1–20 CECs/ml). Importantly, CECs isolated in this way have been validated by morphology and global gene expression [26,32]. Drawbacks of the CellSearch® system are the costly equipment and reagents, and the assay cannot be customized. Also, the maximum number of eight samples that can be analyzed in a single run, combined with the relatively long duration of a complete run (approximately four hours), does not enable high-throughput analysis.

Flow cytometric analysis of CECs

Flow cytometric analysis of CECs has become popular for diagnostics because this approach – in comparison with cellular enrichment – is less time consuming, less costly and easier to standardize once consensus has been reached. Examples of antibodies used in cellular

identification include those against CD31, CD34, CD146, CD309 and DNA stains in combination with CD45 to exclude hematopoietic cells (Table 1). Flow cytometry has significant advantages because it is based on multi-parameter analysis, which enables a highly specific definition of a CEC. The first flow cytometry study was published in 2001 by Mancuso *et al.* [31] identifying CECs as $CD146^{+}$, $CD31^{bright}$ and $CD45^{negative}$ in a commonly applied whole-blood assay in a volume of only 100–150 μl. This approach was also applied by other investigators [36–41]. Although this approach appeared promising, the high level of CECs (>7000 ml^{-1} in healthy individuals) detected in these studies compared with those seen using IMS techniques led to doubts regarding whether or not the correct cell population had been designated as CECs. The reason for these discrepancies was unclear until it was demonstrated that the majority of the cells with the above phenotype were in fact large platelets [28]. This result underlines the need for including a DNA-specific stain such as 4′,6-diamidino-2-phenylindole (DAPI) or 1,5-bis[2-(di-methylamino) ethyl]amino-4,8-dihydroxyanthracene-9,10-dione (DRAQ5) to exclude platelets, aggregates and endothelial micro and/or macro particles, which have an overlapping phenotype with CECs.

In 2009 Mancuso *et al.* [29] presented an improved method by adding the DNA stain syto16 to their earlier described phenotype and proved the endothelial origin of the majority of these cells by the identification of Weibel–Palade bodies using electron microscopy and the presence the endothelial-specific gene VE-cadherin in the sorted CEC population. As a result, the CEC numbers found in healthy individuals were now much lower than detected by the initial approach (i.e. mean 140 ± 171 cells/ml) and approached the numbers obtained by IMS. Earlier, Goon *et al.* [25] compared IMS with a flow cytometry assay using a larger volume of 1 ml blood, which makes it possible to acquire and analyze a high number of cellular events (two to five million), and identified CECs as $CD146^{+}$, $CD34^{bright}$ and $CD45^{negative}$. They found a reasonable agreement between both methods with a median CEC count between 4 and 8 cells/ml in healthy controls. This result coincides with the values reported by two other groups using flow cytometry methods established in 1 ml blood [42,43]. Despite these convincing data, most of the recently reported studies using flow cytometry to detect CECs did not include a DNA stain and still determined CECs in a small sample volume of 50–200 μl [36–38] as a consequence of which incorrectly high CEC levels are reported.

TABLE 1

Immunophenotype of mature endothelial cells

CD antigen	Description	EC subtype association	Expression level	Co-expression	Refs
CD31	PECAM-1	Pan-endothelial	++	Platelets, leukocytes	[49,78]
CD34	Stem cell marker	Pan-endothelial	++	Stem cells	[22,49,51,79,80]
CD36	CR1	Micro vascular	+	Platelets, erythrocytes, monocytes, dendritic	[81]
CD45	Leukocyte marker	–	Neg	–	[22,49,51,79,80]
CD54	ICAM-1	Inflammation	+	Lymphocytes, monocytes	[49,82]
CD62-E	E-selectin	Inflammation	++	–	[82]
CD62-P	P-selectin	Inflammation	+	Platelets	[83]
CD90	Thy-1	EPC	Neg	Hematopoietic stem cells	[82]
CD105	Endoglin	Malignant (over expression)	++	Hematopoietic stem cells, monocyte subset	[26,49]
CD106	VCAM-1	Inflammation	+	Dendritic cell subset	[82]
CD109		Malignant		Hematopoietic stem cells	[69]
CD117	Stem cell marker	EPC	Neg	Hematopoietic stem cells	[22,49]
CD133	Stem cell marker	EPC	Neg	Hematopoietic stem cells	[22,49,51]
CD137	ILA/4	Malignant	+	Activated T cells, monocytes	[69]
CD144	VE-cadherin	Endothelial specific	++	–	[22,32,49]
CD146	MelCAM	Pan-endothelial	++	T cell subset, pericytes	[22,26,49,79,80]
CD202b	Tie2	Angiogenesis	(+)	–	[32]
CD276	B7-H3	Malignant	+	Dendritic cells, monocytes	[69]
CD309	VEGFR-2	Angiogenesis	(+)	Stem cells	[22,26,49,51,79]
NA	vWF	Pan-endothelial	++	Platelets	[22,34,79,80]
NA	UEA-1 uptake	Endothelial specific	++	–	[34,79]
NA	DNA	Pan-endothelial		Nucleated cells	[26,28,29]
NA	Ki67	Proliferation		Proliferating cells	[84,85]

Abbreviations: PECAM-1, platelet/endothelial cell adhesion molecule 1; CRI, collagen receptor I; ICAM-1, intracellular adhesion molecule 1; Thy-1, thymocyte differentiation antigen 1; VCAM-1, vascular cellular adhesion molecule 1; ILA/4, inducible by lymphocyte activation/4; NA, not assigned; MelCAM, melanoma-associated cellular adhesion molecule; Tie2, angiopoietin-1,2,4 receptor; B7-H3, B7 homologue 3; VEGFR-2, vascular endothelial growth factor receptor 2; vWF, von Willebrand factor; UEA-1, Ulex europaeus agglutinin-1; Ki67, antigen encoded by the *MKI67* gene.
Expression levels: ++ = strong; + = moderate; (+) = weak.

Flow cytometric analysis can measure CEC viability by adding markers such as Annexin V or 7-AAD. The viable [44] and necrotic/apoptotic CEC populations [45], as well as changes in the proportion of necrotic/apoptotic CECs [46], have been used to study the efficacy of antiangiogenic therapies. Excluding necrotic/apoptotic cells from analysis has the advantage that a well-known source of nonspecific binding that can cause artifacts will be deleted, but the total counts of CECs will be underestimated. Because mature CEC enumeration in particular reflects vascular damage, it is mandatory to study viable and nonviable populations.

In light of these results, CEC enumeration by flow cytometry is not yet a standardized procedure, and there is a need for validation studies demonstrating the superiority of one technique over the other regarding sensitivity and reproducibility.

Circulating endothelial progenitor cells

In addition to CECs, several studies also incorporate a strategy to enumerate circulating endothelial progenitor cells (CEPCs). In contrast to CECs, CEPCs are thought to originate in the bone marrow and contribute actively to vasculogenesis [47,48]. Usually CD34, widely used as a marker for hematopoietic progenitor cells (HPCs), is used to identify CEPCs with a clonogenic potential. However, CD34 is also expressed on mature ECs, and thus cannot be used to discriminate between CECs and CEPCs [7]. At present CD45, CD133 and CD309 (VEGFR-2) in particular are considered appropriate antigens to discriminate between CECs and CEPCs [49]. But these antigens are also expressed on HPCs rendering a distinct phenotype between CEPCs and HPCs not possible. In fact, the population of CEPCs can include a group of cells existing in a variety of stages ranging from immature HPCs to differentiated ECs [50]. Accordingly, a robust study by Ingram *et al.* [51] recently showed that EPCs reside in the $CD45^{-}CD34^{+}$ cell fractions, and do not express CD133. This further complicates the immunophenotypic discrimination of CEPCs from CECs, because CD45 and CD133 were thought to discriminate putative CEPCs from CECs. Using clonogenic assays it was also shown that $CD34^{+}CD45^{+}$ cells formed HPCs but not EPCs. Therefore, studies using $CD45^{+}$, $CD34^{+}$, $CD133^{+}$ and/or CD309 as phenotypes for CEPCs should be reexamined and the search for novel markers and parameters that accurately discriminate CECs from CEPCs with flow cytometry is mandatory [51,52].

Clinical applications of CECs in oncology

Elevated CEC levels have been described in a range of human malignancies [22–27,53,54] and mouse models [46,55]. This result implies that CEC enumeration could be useful to establish prognosis, to predict response to treatment in cancer patients and potentially to determine the most optimal drug dose in early clinical trials.

TABLE 2

A summary of the current literature reporting CEC levels at baseline in relation to outcome

Tumor type	Treatment		Patients (*n*)	Enumeration method; phenotype	Blood volume used	Baseline CEC/ml			Refs
	Antiangiogenic	Cytotoxic				Healthy controls (median)	Patients (median)	Clinical outcome	
Colorectal	Bevacizumab	5-FU-based	435	CellSearch®	4 ml	4	6.8	Not prognostic for PFS or OS	[58]
	Bevacizumab	5-FU-based	33	CellSearch®	4 ml	4	NA	CEC <65: increase of PFS and OS	[57]
	Bevacizumab	5-FU-based	40	FCM; $CD45^{-}$, $CD146^{+}$, $CD34^{+}$	0.1 ml	20	30	CEC <40: increase of PFS and OS	[39]
	Bevacizumab	5-FU-based	97	FCM; $CD45^{-}$, $CD146^{+}$, $CD31^{+}$, $7\text{-}AAD^{-}$	1 ml	6.5	16	CEC <23: increase of short PFS, not for OS	[56]
Breast	Bevacizumab	5-FU-based	46	FCM; $CD45^{-}$, $CD146^{+}$, $CD34^{+}$	0.1 ml	1300	3000 7000	- No clinical response - Clinical response	[41]
	Bevacizumab	5-FU-based	46	FCM; $CD45^{-}$, $CD146^{+}$, $CD34^{+}$	0.1 ml	1300	3500	CEC >2200: increase of PFS	[40]
	Bevacizumab	5-FU-based	67	CellSearch®	4 ml	15	17	Not prognostic for PFS	[59]
	No	Anthracycline and/or taxane based	35	CellSearch®	4 ml	4	NA	Low CEC associated with pCR	[86]
Renal	Sunitinib or sorafenib	No	55	FCM; $CD45^{-}$, $CD146^{+}$, $CD31^{+}$, $7\text{-}AAD^{-}$	1 ml	6.5	13	Not prognostic for PFS or OS	[44]
CRPC	No	Docetaxel	162	CellSearch®	4 ml	4	19 (mean)	Not prognostic for OS	[27]
NSCLC	No	Carboplatin paclitaxel	31	CellSearch®	4 ml	13 (mean)	595 (mean)	CEC >400: increase of PFS	[63]
Pancreatic	Bevacizumab, erlotinib	No	36	FCM; $CD45^{-}$, $CD31^{+}$, $CD34^{+}$	NA	NA	11 900	Low CEC associated with increase of OS	[38]

Abbreviations: CRPC, castration resistant prostate cancer; 5-FU, 5-fluorouracil; FCM, flow cytometry; NA, not available; NSCLC, non-small-cell lung carcinoma; OS, overall survival; pCR, pathological complete response; PFS, progression-free survival.

Baseline CEC levels as a prognostic marker in cancer

In colorectal cancer, three studies have reported that a low CEC level at baseline predicts a better outcome compared with a high CEC level [39,56,57]. By contrast, a large study by Simkens *et al.* [58] using the CellSearch® system suggested that CEC numbers at baseline were not prognostic for progression-free or overall survival (Table 2). A similar observation was also made in a CellSearch-based study on prostate cancer by Strijbos *et al.* [27]. By contrast, in breast cancer, high CEC levels at baseline were reported by two studies using flow cytometry to indicate a better outcome [40,41]. However, again these findings could not be confirmed in a study using the CellSearch® system [59] (Table 2). With respect to the potential prognostic value of CECs, there are great differences between the diverse tumor types. This could suggest a difference in vascular turnover according to tumor origin, but could also have resulted from differences in CEC enumeration methods and phenotype. Clearly, more studies are needed to define the value of CEC enumeration as a prognostic factor in the diverse tumor types.

Changes in CEC levels and associations with treatment outcome

To date, only a limited number of studies have been conducted that explore the value of changes in CEC numbers as a predictor of outcome to treatment. It is important to realize that outcome to treatment in cancer patients can be presented by different parameters including response according to standard criteria such a those from WHO and RECIST: progression-free survival, clinical benefit and overall survival. In 2010, a comprehensive study in prostate cancer patients treated with docetaxel-based regimens reported that an early increase in the number of CECs during treatment was associated with a worse overall survival, in particular when combined with circulating tumor cell numbers and tissue factor levels [27]. The data were supported by another study the same year [53], showing that an increase in CECs after seven days predicts response and survival in cancer patients treated with various chemotherapeutics.

Recently, several studies have investigated the association of changes in CEC numbers during treatment with outcome to anti-angiogenic-based therapy (Table 3). Several studies showed that an increase in CEC numbers in patients is associated with clinical benefit in terms of tumor response according to WHO or RECIST criteria or prolonged disease stabilization in diverse tumor types including renal cancer [60], gastrointestinal stromal tumor (GIST) [61] and breast cancer [46,59]. By contrast, studies in colon cancer [36] and glioblastoma [62] showed an increase in the number of CECs was associated with a worse clinical outcome, and was not prognostic in three other studies: two in colon cancer [56,58] and one in renal cancer [44]. These discrepancies might be explained by the timing of CEC detection, the inclusion and/or exclusion of apoptotic CECs within different assays and different antiangiogenic treatment regimens and should be further explored.

With respect to classical methods to establish antitumor effects of systemic agents, which rely on assessing changes in tumor size by radiological assessments, it is increasingly recognized that such methods do not always suffice anymore. This holds true not only when using conventional chemotherapeutic drugs but also particularly when angiogenesis inhibitors are applied. Several studies have monitored CEC levels as a biomarker for response to treatment and showed correlations between changes in CEC levels and response according to standard criteria such as those from WHO and RECIST [37,40,63–65].

Taken together, it is apparent that CECs are increased in patients with different types of malignancies and there is a growing body of evidence that suggests this cell population could evolve into a surrogate biomarker for effectiveness of conventional and targeted therapy.

CECs as biomarkers for optimal biological drug dosage

Changes in CEC counts after dose escalation might provide useful insights in establishing the optimal biological drug dose (OBD) when assessing agents targeting vasculature such as VDA or angiogenesis inhibitors. A study by Celik *et al.* reports a 50% decrease in CECs in tumor-bearing mice treated with endostatin [66]. The decrease in CECs showed a clear U-shaped dose response, suggesting a rationale for using CECs as a guideline for optimum dosing. Other preclinical studies showed a dose-dependent increase of CECs in tumor-bearing mice treated with VEGFR-2 inhibitors [55] or chemotherapy [46]. Despite these promising preclinical findings, the use of CECs as a pharmacodynamic marker in clinical studies is still limited [67,68] given the lack of a standardized assay and lack of consensus on the exact phenotype.

Identification of antigens specific for malignant angiogenesis and new targets for antiangiogenic therapies

It is important to realize that the elevated CEC numbers found in cancer patients are probably a composite of CECs from normal vasculature and from tumor vasculature. It is not clear whether or not the elevated CEC numbers in cancer patients result from the accelerated endothelial turnover of tumor vessels or from a more generalized systemic endothelial damage and/or activation. There is a strong need for assays that can discriminate CECs originating in normal vasculature from those originating in tumor vessels. Several studies have been conducted to reveal distinctive gene expression profiles of tumor-associated ECs from those of ECs from 'normal' tissues, and have attempted to identify markers in this respect. Examination of these tumor-associated endothelial markers (TEMs) on CECs can lead to a more appropriate monitoring of follow-up and response to treatment and might result in the identification of antigens specific for malignant angiogenesis.

At gene expression levels numerous TEMs could be detected with serial analysis of gene expression (SAGE) [69–71], selection with in vitro phage display libraries [72] or fluorescence in situ hybridization (FISH) [73]. Several of these TEMs were also studied for their surface expression in tumor tissue [69,70,74] showing increased expression in tumor ECs compared with normal endothelium and with a high specificity. One of these markers, CD276 (B7-H3), has already been reported as predictive for therapeutic outcome when expressed on ovarian tumor endothelium [75].

Despite the specificity of several TEMs to discriminate between normal and tumor endothelium, cautious evaluation of these TEMs for their tissue- and/or cell-specificity and biological function should be warranted before developing them as therapeutic targets clinically [76].

Another interesting area of research for TEMs will be their detection on CECs to identify the subpopulation originating from tumor vasculature, which will give a more specific marker to establish prognosis and response to treatment than counting all

TABLE 3

A summary of the current literature reporting change in CEC during treatment in relation to clinical outcome.

Tumor type	Treatment		Patients (*n*)	Enumeration method; phenotype	Blood volume used	Baseline CEC/ml		CEC number Change from baseline and clinical outcome	Refs
	Antiangiogenic	Cytotoxic				Healthy controls (median)	Patients (median)		
Colorectal	Bevacizumab	5-FU, EBRT	32	FCM; $CD45^-$, $CD31^+$, $CD34^+$, $CD133^-$	100–150,000 PBMC	0.1–6% (of PBMC)	1.02% (of PBMC)	Increased in patients with residual disease	[36]
	Bevacizumab	5-FU-based	435	CellSearch	4 ml	4	6.8	Not prognostic for PFS or OS	[58]
	Bevacizumab	5-FU-based	97	FCM; $CD45^-$, $CD146^+$, $CD31^+$,7-AAD^-	1 ml	6.5	16	Not prognostic for PFS or OS	[56]
Breast	Bevacizumab	5-FU-based	67	CellSearch	4 ml	15	17	Increase (>5): associated with improved TtP	[59]
	Thalidomide	Cyclophosphamide, methotrexate	104	FCM; $CD45^-$, $CD146^+$, $CD31^+$	0.1 ml	1300	NA	Increase (>11 000) at day 60 is associated with longer PFS and improved OS	[46]
Renal	Sunitinib	No	26	IMS (CD146); UEA-1^+	1 ml	8 (mean)	49 (mean)	Early increase is associated with an improved PFS	[60]
	Sunitinib or sorafenib	No	55	FCM; $CD45^-$, $CD146^+$, $CD31^+$, 7-AAD^-	1 ml	6.5	13	Not prognostic for PFS or OS	[44]
CRPC	No	Docetaxel	162	CellSearch	4 ml	4	19 (mean)	Increase (>3.8* baseline) is associated with shorter OS	[27]
Glioblastoma	Cediranib	No	16	FCM; $CD45^-$, $CD31^+$, $CD34^+$, $CD133^-$	100–150 000 PBMC	NA	NA	Increased at tumor progression	[62]
GIST	Sunitinib	No	73	FCM; $CD45^-$, $CD146^+$, $CD31^+$, $CD133^-$	NA (cryopreserved PBMC)	540	1090	Increased in patients with clinical benefit	[61]

Abbreviations: 5-FU, 5-fluorouracil; CRPC, castration resistant prostate cancer; EBRT, external beam radiation therapy; FCM, flow cytometry; IMS, immunomagnetic isolation; GIST, gastrointestinal stromal tumor; NA, not available; OS, overall survival; PBMC, peripheral blood mononuclear cells; PFS, progression free survival; TtP, time to progression.

CECs. In this respect, promising results have recently been reported by Matsusaka and colleagues who showed that the proportion of $CXCR4^+$ CECs at baseline was correlated with prognosis of breast cancer patients treated with bevacizumab [77].

Concluding remarks

The central role of the tumor vasculature in the biology of tumors is unquestionable and new biomarkers that accurately reflect drug effects impacting tumor vasculature are urgently needed to design clinical observational studies and trials better, eventually leading to improved individualization of cancer treatment. The serial counting of CECs appears an attractive candidate to meet this demand. Current studies have yielded promising but still inconsistent results; this situation might be caused by differences between patient populations and differences between enumeration techniques in the various studies. However, recent publications now tend to consensus regarding the phenotype and frequency of CECs in healthy individuals. CEC levels are increased in patients with different types of malignancies and there is a growing body of evidence that suggests this cell population could evolve as a suitable biomarker for antitumor therapies. The identification of new TEMs will probably advance monitoring of follow-up and response to treatment, and could result in the identification of antigens specific for malignant angiogenesis.

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