Circulating endothelial and progenitor cells during anti-angiogenic treatment in cancer patients

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CHAPTER 7

SUMMARIZING DISCUSSION
&
FUTURE PERSPECTIVES

SUMMARIZING DISCUSSION

There is a need for developing clinical blood-based biomarker assays that monitor biological effects and success of anti-angiogenesis treatment. It is a considerable challenge to select the most informative cell population(s), based on marker definition or to select the most appropriate cell population in terms of functional involvement in neovascularization. Several endothelial and hematopoietic lineage cell populations, progenitor or mature, either originating from bone marrow and, circulating in the blood or locally (permanently residing) in the microenvironment may contribute to tumor neovascularization and thereby to tumor growth. This thesis aimed to investigate a role of circulating progenitor and endothelial cell populations as potential surrogate biomarkers in the treatment of cancer patients with angiogenesis inhibitors. The research described in this thesis focused on the methodological definition and identification of cell populations by multicolour flow cytometry. Moreover, the pre-treatment cell numbers and changes in cell frequencies during treatment were investigated to evaluate their use as 'surrogate biomarkers' that predict response to treatment or prognosis. The final goal of these markers is to select only those patients for treatment who will experience benefit from anti-angiogenesis treatment.

Furthermore, the biological behavior of putative circulating endothelial progenitor cells (EPCs) was studied in hypoxia (1% O_2) as a model for tumor endothelial cell growth.

IDENTIFICATION OF CIRCULATING ENDOTHELIAL CELLS AND PROGENITORS

As described in **chapter 1** of this thesis there are several approaches to detect and enumerate circulating endothelial cells (CECs) and progenitor populations in the blood of (cancer) patients [1-4]. In the work described in this thesis, phenotypic characteristics based on cluster of differentiation numbers (CD numbers), of the cell populations of interest were used to identify and enumerate possible circulating endothelial cell populations by flow cytometry (see Box 1). During the period of this thesis most of the cell populations described have undergone one or more rounds of re-definition in the scientific literature concerning either their marker phenotype, role in participating in neovascularization or both as mentioned below.

In **chapter 2** we explored quantitative measurement of circulating (endothelial) cells and progenitor cell populations expressing vascular endothelial growth factor receptor 2 (VEGFR2) in the peripheral blood (PB) of volunteers and cancer patients as the most frequently used strategy of anti-angiogenesis is based on targeting VEGFR2. Four populations of $VEGFR2^+$-expressing cells were defined after using a specific labeling protocol and gating strategy as described in chapter 1 of this thesis.

Because the antibody used for vascular endothelial growth factor receptor 2 (VEGFR2) shows no saturation of binding upon titration (dilution) we are only confident of real VEGFR2 expression when the cells are shifted at least a order of magnitude of mean fluorescent intensity (MFI; level of expression) to the right and are visible as a separate "cloud" of cells. Basically in whole blood this was only the case in the $CD45^{-}$/ $CD34^{bright}$ cell population. We have labeled these cells: 'small size EC-like cells', because they are nucleated, are small cells and have EC markers (see below).

Among the four detected populations one cell population was defined by SSC^{low} /$FSC^{low\text{-}to\text{-}intemediate}$/ $CD45^{low}$/ $CD31^{bright}$, which was until that time referred to as CECs corresponding with measurements of Willett *et al.* and Mancuso *et al.* [5, 6]. Based on findings of Strijbos *et al.* and the results described in chapter 2 the nature of this population is now known and characterized as large platelets [7, 8].

Box 1 current "state of the art" definitions of cell populations described in this thesis.

Cell population:	***Abbreviation:***	***Descriptive definition:***	***Phenotype:***
Hematopoietic progenitor cell	→ HPC;	HPCs are multi-potent stem/progenitor cells that give rise to all the blood cell types including myeloid (monocytes and macrophages, neutrophils,basophils, eosinophils, erythrocytes, megakarycytes/ platelets, dendritic cells), and lymphoid lineages (T-cells, B-cells, NK-cells).	$CD45^{dim}$/ $CD34^{bright}$
Circulating progenitor cells	→ Pro-angiogenic CPCs → Non-angiogenic CPCs	HPC population with pro-angiogenic potential. HPC population without pro-angiogenic potential.	$CD133^{+}$/ HPCs $CD133^{-}$/ HPCs
Endothelial progenitor cell	→ "EPC";	an " EPC" is a progenitor cell which was thought to be derived from the bone-marrow able to participate in neovascularisation. Currently the origin of EPCs is not known. In culture two types of "EPCs" are known; 'early' and 'late' outgrowth cells, also called myeloid EPCs and ECFCs respectively.	not known
Circulating endothelial progenitor	→ "CEP";	"CEP" is an "EPC" in circulation.	not known
Circulating endothelial cells	→ "CECs" in general;	CECs in *general*, endothelial cells circulating in the blood.	
	→ *mature* "CECs";	*mature* CECs represent the cells in circulation that have been detached from the vessel wall, and have limited growth capability. This population of cells may come into the circulation due to vessel damage, for example upon anti-angiogenic agents.	$CD45^{-}$/ $CD34^{+}$ or $CD34^{bright}$/ $CD133^{-}$/ $CD31^{+}$ *
Endothelial colony forming cells	→ *circulating* ECFCs;	ECFCs circulating in the blood with the capability to form colonies in culture that bear endothelial markers and are negative for monocytic markers. Currently no unique marker or combination of markers is known to specificially isolate these cells from circulation.	$CD45^{-}$/ $CD34^{+}$ or $CD34^{bright}$/ $CD133^{-}$/ $CD31^{+}$ *
	→ *subcultured* ECFCs;	subcultured ECFCs have high proliferative potential, are non-hemopoietic and express robust endothelial markers. ECFCs are capable to form endothelial tubes *in vitro* and vascular structures *in vivo*. These cells are also indicated as blood outgrowth endothelial cells (BOECs) or endothelial outgrowth cells (EOCs).	$CD45^{-}$/ $CD14^{-}$/ $CD133^{-}$/ $CD105^{+}$/ $CD146^{+}$/ $CD31^{+}$ $CD144^{+}$/ Tie-2^{+}/ $VEGFR1^{+}$/ $VEGFR2^{+}$ acLDL uptake/ binding UEA and $CD34^{moderate}$

*** Accordingly, *mature* CECs and circulating ECFCs have currently an overlapping phenotype.**

The second population measured in chapter 2 was defined by $CD45^-$/ $CD34^{bright}$/ $VEGFR2^+$/ ($CD31^{bright}$/ $CD146^{low/moderate}$/ $CD133^-$) and indicated as 'small size EC-like cells'. This population is clearly different from the hematopoietic progenitor cells (HPCs) because of complete absence of leukocyte marker CD45, lack of progenitor marker CD133 and their presence in very low frequency (<100 cells/mL).

The third population of cells identified with VEGFR2 positivity is within the HPC population with the phenotype $CD45^{dim}$/ $CD34^{bright}$. HPCs have been reported to express CD34, CD133 (and VEGFR2) [9-12]. In our hands, the number of VEGFR2+ events in this population is extremely low, precluding any reliable measurement in blood volumes of 1-2 mL as used by us. However, a pilot experiment showed an increase of percentage $VEGFR2^+$ HPCs in granulocyte colony-stimulating factor treated patients suggesting that these rare events are real existing cells mobilized from the bone marrow.
There is now wide agreement on an indirect stimulating role of $CD133^+$/ $CD34^{bright}$/ $CD45^{dim}$ HPCs in neovascularization of hypoxic or tumor tissue by secreting growth factors [13, 14]. Estes *et al.* discriminated two HPC populations that differ in angiogenic potential refererred to as circulating progenitor cells (CPC). These two CPC populations, pro-angiogenic and non-angiogenic, can be distinguished by either being positive or negative for the progenitor marker CD133 [15].

The fourth population were VEGFR2-positive monocytes, mainly of interest because they have been suggested to have pro-angiogenic properties [16] and were, as described in chapter 2, elevated in cancer patients as compared with healthy volunteers. In this population, the VEGFR2 shift is small and hard to quantify in a reliable way (see earlier discussion), and is not further investigated in this thesis.

THE 'SMALL SIZE EC-LIKE CELLS' POPULATION

The 'small size EC-like cells' population mentioned above was also found by other groups [17, 18], by them called 'CECs'. These 'CECs' have a phenotype consistent with a population containing the precursor for endothelial outgrowth cells (EOCs, nowadays preferably called endothelial colony forming cells; ECFCs), namely $CD34^+$, $VEGFR2^+$ but $CD45^-$ and $CD133^-$ according to Case *et al.* and Timmermans *et al.* [9, 11]. At that time and still, it remains a mystery whether these 'CECs' contain a mixed *mature*/progenitor population or represent a single population, because it is extremely difficult to sort this rare cell population for e.g. electron microscopic identification [19].
Only very recently, in addition to the findings of Case and Timmermans et al. a cell population containing overlapping markers with our 'CECs' ($CD31^+$/ $CD34^{bright}$/ $CD45^-$/

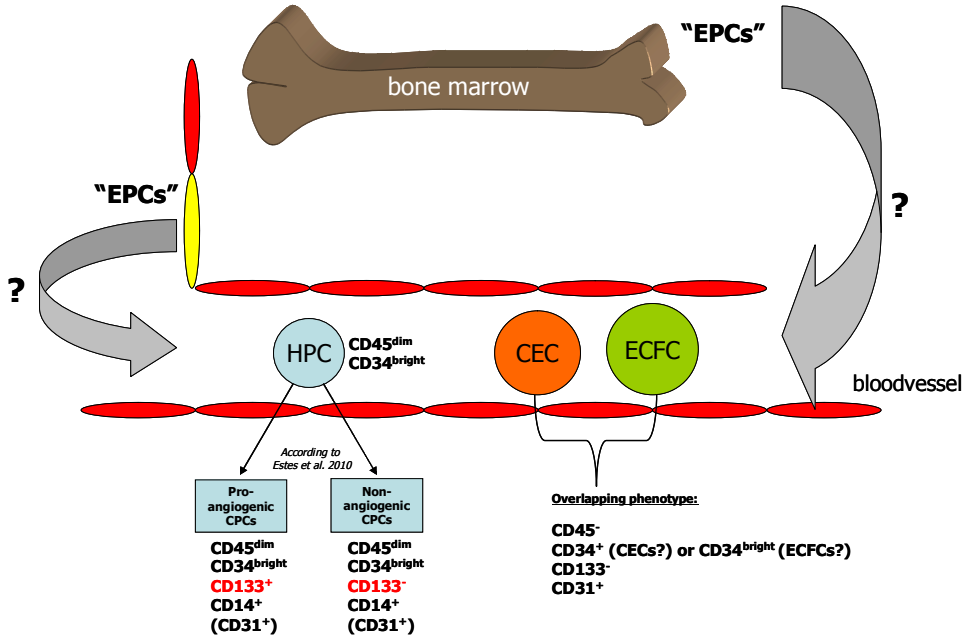

Figure 1 - Cartoon showing characteristics of circulating endothelial cells and progenitors.

The existence of "EPCs" in blood was first described by Asahara *et al.* [20] in 1997. Nowadays, the exact phenotype of EPCs in circulation is still not known. The term EPC is descriptive; an immature precursor cell that individually displays postnatal vasculogenic activity, or a cell that is capable of forming new ECs and blood vessels *in vivo* as defined by Timmermans *et al.* [21]. The origin of "EPCs" is not known. They may be bone marrow derived, residing locally in the microenvironment or originating from other tissues. HPCs are defined by $CD45^{dim}/CD34^{bright}$ and can be divided in pro-angiogenenic and non-angiogenic circulating progenitor cells (CPCs) based on progenitor marker CD133. Circulating endothelial cells (CECs) are non-leukocytes and show overlapping marker expression with a cell population containing the endothelial colony forming cells (ECFCs) defined by Estes *et al.* [15].

$CD133^-$/ $CD14^-$/ $CD41a^-$/ $CD235a^-$/ LIVE/DEAD $Violet^-$) was reported to contain progenitors with *in vivo* vessel forming capacity [15].

Thus, presently there is an overlapping phenotype between *mature* CECs and ECFCs (containing progenitor cells for EC outgrowth). Whether these 'CECs' are a mixture of *mature* CECs and ECFCs remains to be further clarified. Figure 1 summarizes the relevant cell populations with a possible role in neovascularization, which are currently being debated and described in this thesis.

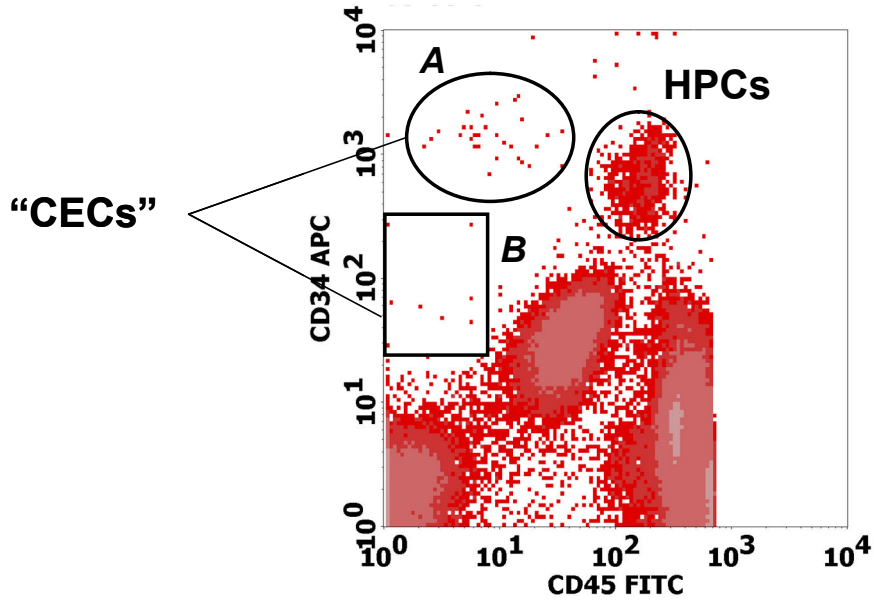

Figure 2 - Flow cytometry analysis of two circulating endothelial cell (CEC) populations and hematopoietic progenitors (HPCs) obtained from peripheral blood.
Population A, $CD45^{neg}$/ $CD34^{bright}$ or "CECs" in general, with the most endothelial proliferative potential and therefore the best candidate to include the circulating endothelial colony forming cells (ECFCs). Population B, $CD45^{neg}$/ $CD34^{+}$, might correspond with *mature* circulating endothelial cells (*mature* CECs). HPCs are $CD45^{dim}$/ $CD34^{bright}$ and clearly separated from CECs based on CD45 expression and consist of pro-angiogenic ($CD133^{+}$/HPCs) and non-angiogenic ($CD133^{-}$/HPCs) circulating progenitor cells (CPCs).

Several arguments would favor that there should be differences between the two cell populations reflecting *mature* CECs and colony forming ECFCs. First, the degree of apoptosis is likely higher in the *mature* CECs as compared to ECFCs. A characteristic of *mature* endothelial cells (EC) *in vitro* is that they rapidly become apoptotic after detachment from their matrix [22] whereas the ECFCs have high proliferative capacity. Second, the cell size (Forward Scatter; FSC) and cell granularity (Side Scatter, SSC) is presumably smaller for ECFCs in blood. *Mature* CECs are commonly characterized and defined by a heterogeneous, but rather large size and granularity, exceeding that of most mononuclear cell populations, with a cell diameter typically >20 µm [23-25]. The size of ECFCs are in range of the HPCs, which are <10 µm. In addition, phenotypic markers may

distinguish both populations. One important difference might be the intensity of the CD34 expression on 'CECs', which is depicted in figure 2, but only sorting experiments can clarify on this subject. Estes *et al.* separated these 'CECs' in two cell populations based on the intensity of CD34 expression, and found that high proliferative cell growth with cobblestone morphology was only originating from the $CD34^{bright}$ population in contrast to the cells bearing $CD34^{+}$ (personal communication with Dr J A Mund from the group of Estes and Ingram).

In summary, whether our CECs as measured in chapter 2 are reflecting the ECFCs (and not detached *mature* ECs) remains to be definitively clarified. Phenotypic markers that overlap between both populations include $CD45^{-}$, $CD31^{+}$, $CD146^{+}$, $CD105^{+}$, $CD133^{-}$ and $VEGFR2^{+}$ but several facts are in favor for this assumption:

(1) VE-cadherin is not detectable on this CEC population in our hands, while is highly expressed on *mature* ECs as shown by Strijbos *et al.* [26].

(2) Our CECs are CD13 negative as shown in chapter 2 while *mature* ECs, like human umbilical vein endothelial cells, are positive for the myeloid marker CD13;

(3) Our CECs are $CD34^{bright}$, which corresponds with the population giving highly proliferative EC outgrowth in the sorting experiments of Estes *et al.* (personal communication with Dr J A Mund);

(4) Our CECs are rather small cells based on FSC/SSC, <10 µm (within the lymphocyte-like gate); and

(5) Our CECs contain (a variable percentage of) viable cells.

CECS AND PROGENITORS AS PREDICTIVE BIOMARKER DURING ANTI-ANGIOGENESIS TREATMENT

Although anti-tumor activity is achieved by anti-angiogenic drugs either as monotherapy or in combination with chemotherapy or with targeted agents, it remains unclear which patients will benefit most from treatment [27]. A number of biomarkers that might predict treatment response are currently being investigated, in order to guide patient selection and/or to monitor early response [28, 29].

Despite that dynamic imaging techniques like computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and single photon emission computed tomography (SPECT) are *non-invasive*, they are expensive and time-consuming approaches to implement in a study design. Blood parameters on the other hand are *minimal-invasive* but are relatively easy to handle and may be processed within a couple of hours. A limitation of blood cell-based parameters is that it is not known *a priori* whether the results are influenced when measurements are done on frozen material. Validation studies have to be performed, to exclude possible experimental

complications influencing the quality of cell population measurements, due to for example Ficoll separation of the mononuclear cells (MNCs) or freezing and thawing of the MNCs. As a consequence, at present, all cellular enumerations in our studies have been performed on fresh blood within 2-4 hours after sampling.

In **chapter 3** both a number of cellular and protein candidate biomarkers were evaluated in a group of renal cell cancer (RCC) patients treated with the anti-angiogenic tysosine kinase inhibitor sunitinib. CECs ($CD45^-$/ $CD34^{bright}$ and $VEGFR2^+$) increased in parallel to plasma VEGF and erythropoietin (EPO) levels during the 4-week on and decreased during the 2-week off sunitinib period, while HPCs displayed an opposite pattern of change. Significant changes in CECs and HPCs were observed during sunitinib treatment and an increase in CECs was associated with progression free survival. no relation was found between the changes in CEC and HPCs and tumor response according to Response Evaluation Criteria in Solid Tumors (RECIST). The increased VEGF levels are in accordance with previous findings on VEGF receptor inhibitor studies in mice and man [30-32] and increased levels of EPO during sunitinib treatment were consistent with findings of Ebos *et al.* in mice [33] but functional consequences remain to be defined. The increase in CECs might be due to endothelial cell detachement and reflect sunitinib anti-vascular effects, or might include ECFCs as discussed before. It is an interesting possibility that an increase of ECFCs is a compensatory reaction to increased (tumor) hypoxia caused by sunitinib anti-angiogenic effects.
In addition, patients treated with sunitinib showed elevated hemoglobin levels related to the dosing schedule as it occurred in a cyclic pattern, which is described in **chapter 4**. We hypothesized that the cyclic kinetics of hemoglobin and erythrocytes is the result of a temporary loss of intravascular fluid caused by inhibition of VEGFR2 and subsequent reduction of nitric oxide, rather than an increase in erythropoiesis.

In **chapter 5** we further validated whether the observed increase in VEGFR2-positive CECs or decrease in HPCs was specific for angiogenesis inhibitors. To do so we had the opportunity to study non-small cell lung cancer patients treated with three different schedules all including the EGFR inhibitor erlotinib. One experimental treatment included the multi-targeted TKI sorafenib (SO/ER), the other the anti-VEGF antibody bevacizumab (BV/ER) [34], and a small group of patients treated with single erlotinib (ER) served as control. Indeed, an increase of ($VEGFR2^+$) CECs was found in the SO/ER and BV/ER, but not in monotherapy ER-treated patients which was consistent with our earlier findings in RCC patients treated with sunitinib. Therefore, the increase of this cell population is likely to be a pharmacodynamic marker for VEGF/VEGFR signaling-inhibitor treatment. Moreover, the CEC population (pre-treatment as well as changes in CECs) did not predict

for response to SO/ER or BV/ER treatment. Unlike CECs, the total HPC population decreased in SO/ER patients as also seen in sunitinib-treated RCC patients, in both cases TK inhibitors. This effect might be related to the Flt-3 signaling as this is reported to be required for HPC proliferation and mobilization into the circulation [35-38].
The pre-treatment level of the subpopulation of CD133-positive HPCs was significantly higher in the non-responders to SO/ER, and the group with higher than median CD133+ values showed a shorter time to progression. Since it has been reported that $CD133^{+}$/ $CD34^{bright}$/ $CD45^{dim}$ HPCs have a pro-angiogenic role in (tumor) adult neovascularization [14, 15, 39], it may be hypothesized that the decrease of $CD133^{+}$/HPCs resulting from suppression of the bone marrow is contributing to the anti-tumor effect of sorafenib.

EVALUATION OF CECS AND PROGENITORS (EPC/CEP, AND HPCS) IN GENERAL

Concerning CEC and progenitor evaluation in general, it is difficult to compare data from existing studies because of the lack of consensus and all the different definitions that have been used for CECs and progenitors. It is difficult to pool data from existing studies in order to arrive at a consensus opinion about these cell-populations as potential biomarkers of anti-angiogenesis treatment at the current time. Therefore, it is not unexpected that the current data are not unequivocal with regard to the role of these populations in neovascularization and their response to treatment. Several studies showing the diversity in definitions concerning CECs and/ or progenitor populations as biomarkers during anti-angiogenesis treatment are depicted in table 1.
The major inter-study variability may have multiple causes, including the application of unvalidated techniques as well as differences in sample collection, processing, cell population characterization (phenotype based on marker expression), interpretation of the level of expression of cell-surface markers (negative, low, intermediate, dim, positive, bright etcetera) and reporting. For instance, it may be that not absolute numbers or changes of CECs or progenitors will tell us how to treat or to monitor patients treated with anti-angiogenesis treatment, but rather that the ratio between two populations may be more informative. The latter has been postulated by Estes *et al.*, who detected lower ratios of pro-angiogenic $CD133^{+}$/CPCs to non-angiogenic $CD133^{-}$/CPCs in patients with vascular dysfunction (<1), while higher ratios (>2.2) were seen in several types of cancer. Additionally, they mention that anti-angiogenic therapies dramatically decreased the ratio, a shift that correlated with therapeutic response [40].
In the search to identify useful surrogate biomarkers for anti-angiogenic treatment in general there are even more challenges. The regional heterogeneous and dynamic nature of cancer (one part of the tumor will not reflect the whole tumor) implicates the need for 'dynamic biomarkers'. In addition, the inability to perform repeated biopsies hampers to assess 'dynamic biomarkers' [41].

Another concern is that the great majority of studies investigating putative biomarkers for anti-angiogenic treatment are not yet randomized trials with appropriate placebo or control groups. Furthermore, an important difficulty in defining or identifying biomarkers is the evaluation of the endpoint of the actual responses of the patients. RECIST is the most commonly used criterion of response [42]. However, it is especially problematic when evaluating anti-angiogenenic agents since these drugs may not shrink but rather stabilize the tumor size [43]. In the latter study, the authors suggest that incorporating cavitations (caused by angiogenic inhibitors) into volume assessment for target lesions maybe a better parameter for treatment outcome in NSCLC patients. In our NSCLC patient group (chapter 5), 13 out of 25 patients had cavitations because of SO/ER treatment, and incorporating these cavitations in response assessment also altered the RECIST response. Pre-treatment numbers of $CD133^+$/HPCs were only prognostic for the response if corrected for cavitations confirming the importance of adequate response assessment.

ENDOTHELIAL PROGENITORS AND THEIR (HYPOXIC) MICROENVIRONMENT IN NEO-VASCULARIZATION

Several theories about the origin and differentiation of EPCs have been put forward [21], but a major breakthrough has been the finding that two types of EPCs exist [19, 44], which can be separated on the basis of colony formation, i.e. early vs late outgrowth colonies with endothelial markers, surface markers, and hemapoietic or non-hemapoietic origin [9, 11, 45]. The large majority of "EPCs", which grow out as early EPCs are hematopoietic, myeloid/monocytic-like populations with prominent pro-angiogenenic properties but have no capacity to differentiate into endothelial cells [11, 44, 53-57].

A second only a very minor subpopulation of cells in the circulation, giving rise to the so-called late outgrowth EPCs, belong to the endothelial lineage, and are indicated as blood outgrowth endothelial cells (BOECs) [3], endothelial outgrowth cells (EOCs) [21] or – as alternatively preferred and used in this thesis - endothelial colony forming cells (ECFCs) [2].

ECFCs are circulating cells that are non-hematopoietic ($CD34^+$/ $CD45^-$/ $CD133^-$) and can form highly proliferative endothelial cell colonies [9, 11, 44]. They are present in very low numbers in human cord and peripheral blood from which they can be recovered and expanded up to 67 passages [11]. *In vivo* ECFCs are candidate progenitor cells to home to ischemic tissue or hypoxic tumor microenvironment where they may provide endothelial proliferation potential, and promote vessel repair or tumor angiogenesis. In the light of their proposed function in repair of vascular damage, the suggested role in stimulation of tumor (re)growth from hypoxic tumor areas and potential applications in tissue regeneration we have evaluated the colony forming capacity of primary circulating

Table 1 Circulating endothelial cells and progenitors as biomarker in clinical trials involving anti-angiogenic treatment

References	Treatment	Tumor type	Study size	Method of enumeration	CEC	EPC/CEP/CPC	effect on CECs and/ or progenitors (EPCs/CEPs and CPCs)
Willett *et al.* [6]	Bevacizumab[a]	Rectal carcinoma	11[b]	FC	viable $CD31^{bright}$, $CD45^-$	CPCs: $CD45^{dim}$, $CD34^+$, $CD133^+$	CECs and CPCs decreased, but no biomarker of response
Zhang *et al.* [52]	Thalidomide	Multiple myeloma	31	FC + culture	$CD34^+$, $CD146^+$, $CD105^+$, $CD11b^-$	EPC: enumerated by CFU culture assay	Elevated levels of CECs and EPCs covary with disease activity and response to thalidomide
Mancuso *et al.* [5]	Cyclophosphamide + methotrexate +/- thalidomide	Breast cancer	104	FC	$CD45^-$ $CD146^+$ $CD133^-$	CEPs: $CD45^-$, $CD146^+$, $CD133^+$	Patients with CB had an increase in apoptotic CECs, CEPs showed no correlation with clinical outcome
Norden-Zfoni *et al.* [32]	Sunitinib	Metastatic GIST[c]	73	FC	$CD45^-$ $CD31^+$ $CD146^+$ and $CD133^-$	CEPs: $CD45^-$ $CD31^+$, $CD146^+$, $CD133^+$	CECs increased; mainly in patients with CB, CEPs; no consistent change
Batchelor *et al.* [46]	Cediranib	Glioblastoma	16	FC	$CD45^-$, $CD31^{bright}$, $CD34^+$	CPCs: $CD133^+$, $CD34^+$	Viable CECs increased during tumor progression, CPCs increased during drug holiday
Glade Bender *et al.* [49]	Bevacizumab	Refractory solid tumors[d]	21	FC	resting: $CD45^-$, $P1H12^+$, $CD31^+$,$CD34^+$, $CD105^-$, $CD106^-$, $CD133^-$ activated: $CD45^-$, $P1H12^+$, $CD31^+$, $CD34^+$, CD105 or $CD106^+$, $CD133^-$	CEP: $CD45^-$, $P1H12^+$, $CD31^+$, $CD34^+$, $CD133^+$	CECs increased during treatment, no correlation of CEC or CEPs with clinical benefit
Dellapasqua *et al.* [48]	CTX+ Capecitabine + bevacizumab	Advanced breast cancer	46	FC	$CD45^-$, $CD31^+$/ $CD146^+$ (DNA^+)	$CD45^-$, $CD133^+$/ $CD34^+$ (DNA^+)	High baseline CECs correlate with OR, CB, improved PFS, and was associated with increased TTP
Zhu *et al.* [47]	Sunitinib	Advanced HCC	34	FC	$CD31^{bright}$, $CD34^+$, $CD45^-$	CPCs: $CD133^+$, $CD34^+$, $CD45^{dim}$	Significant decrease in CPCs (and not CECs) during treatment. CPCs were associated with higher hazard of immediate progression or mortality
Okamoto *et al.* [51]	BIBF 1120[e]	Various[f]	21	FC	ND	BMD-progenitors: $CD34^+$/ $CD45^{dim}$/ $CD117^+$ ($CD133^+$)	$CD117^+$ BMD progenitors decreased during treatment
Ko *et al.* [50]	Erlotinib+ bevacizumab	Metastatic pancreatic adenocarcinoma	36	FC	$CD45^-$, $CD34^+$, $CD31^+$	ND	Baseline number of CEC was inversely associated with OS

(a): in combination with chemotherapy and radiation, (b): 6 patients treated with low dose bevacizumab (5 mg/kg/day) and 5 patients with high dose bevacizumab (10 mg/kg/day), (c): patients with imatinib-resistant metastatic gastrointestinal stromal tumor (GIST), (d): in pediatric patients, (e): BIBF 1120 is an triple tyrosine kinase inhibitor that predominantly blocks VEGFR1-3, FGFR1-3, PGFRα- and β tyrosine kinases, (f): various; advanced solid tumors.

Abbreviations: CEC = circulating endothelial cell; EPC = endothelial progenito cell; CEP = circulating endothelial progenitor; CPC = circulating progenitor cell; CD = cluster differentiation; FC = flow cytometry; CB = clinical benefit; BMD = bone marrow derived; DNA = Desoxyribo Nudeic Acid; HCC = Hepatocellular Carcinoma; ND = not detected; CFU = colony forming unit; OS = overall survival; OR = overall response; PFS = progression free survival; TTP = time to progression; CTX = cyclophosphamide

ECFCs from cord blood under 1% O_2 conditions in **chapter 6**, and compared them to the commonly used *in vitro* laboratory culture conditions of 21% O_2.

We observed that primary circulating ECFCs were unable to exert their capacity to form endothelial colonies when they were initially exposed to a hypoxic condition. At least three possibilities might explain the outgrowth arrest of ECFC colonies under hypoxia: (1) direct retardation of ECFCs proliferation by oxygen deprivation; (2) differentiation arrest of ECFCs; or (3) paracrine regulation of the initial ECFC outgrowth by accessory cord blood-MNCs (CB-MNCs). Based on our results direct growth inhibition of ECFCs by hypoxia can be excluded as a cause for the hypoxic growth arrest.

The data presented are in favor of a differentiation arrest of ECFCs. Exposure of freshly isolated CB-MNCs to 21% O_2 for 3 days did overcome the inhibition of colony outgrowth under 1% O_2. There was ECFC colony growth in cultures under 21% O_2 after 2 weeks exposure of 1% O_2. In addition, the combined inhibition of HIF-1α/HIF-2α indicates that HIF activation plays a crucial role in prohibiting ECFCs from forming colonies.

In addition, the inhibition of ECFC outgrowth may be a result of an indirect inhibitory action of accessory cells. Although no direct paracrine effect on the clonal growth of ECFCs was observed, cell-cell interaction may play a role. This may occur for instance, by differentiation of the accompanying cells under normoxia, by which they lose their capacity of inhibiting outgrowth of ECFC colony formation under hypoxia.

The study data presented in chapter 6 are in favor of a putative differentiation arrest, would be in accordance with several studies showing the effect of oxygen concentration on cell differentiation [58-61]. Until it is possible to highly purify the ECFC from (cord) blood and prospectively clone these cells it will be difficult to obtain definitive answers on these suggestions. Purification of ECFCs from blood followed by producing gene expression profiles under 1% and 21% O_2 might help to come to a more definitive conclusion.

FUTURE PERSPECTIVES

The final questions are: Has there been relevant progress in the search of finding (candidate) surrogate biomarkers that reflect the response of anti-angiogenic treatment, and thereby are helpful for prediction and prognosis of clinical outcome in the treatment of cancer patients during recent years? Did this thesis contribute to it? What can be achieved in the (near) future?

Successful anti-angiogenic cancer treatment will require a more basic understanding of the molecular mechanisms of action of these type of drugs as well as about the involvement of circulating endothelial cells and/or progenitors in blood vessel formation. Currently no validated markers exist to monitor anti-angiogenic treatment. Therefore, an important task in this area of research is still to find a suitable surrogate biomarker that is easily applicable and reliable.

In recent years significant progress has been made in further clarifying the identification of circulating endothelial cells and progenitors but currently no definitive and validated method for measuring CECs and progenitors is available. Most of the circulating endothelial cell populations and progenitors have undergone several re-definitions during recent years. This has further specified both their phenotypic definitions and their functional characteristics.

Because clinical decision making in how to treat or how to continue treatment often requires high sensitivity and specificity of the biomarker, it seems unlikely at this moment that frequencies or changes in CECs or progenitors during anti-angiogenesis treatment will provide the ultimate and only solution. As postulated by Estes *et al.* [15] perhaps not absolute numbers but ratios of cell populations better reflect the response of the patient treated with angiogenesis inhibitors. Notwithstanding, CECs and progenitors might be useful in helping to define an optimal biological dose and thereby limiting the unwanted side-effects of angiogenesis inhibitors.

Further research on the functional properties of CECs and progenitors will give a better insight in their role and contribution to neovascularization. An important aspect to understand the cellular behavior of the participating cells in blood vessel formation is the hypoxic environment. In this regard, chapter 6 of this thesis may be a step forward in the understanding how endothelial cells with proliferative capacity (ECFCs) may act at the hypoxic tissue borderzone and help sprouting of oxygenated vessels.

In order for CECs and/or progenitors to serve as a biomarker in the future, further standardization of cell markers, sample preparation, reporting methods, and analytical validation will be critical for clinical implementation.

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