



ELSEVIER

Critical Reviews in Oncology/Hematology 69 (2009) 108–124

*Hematology*  
Incorporating Geriatric Oncology[www.elsevier.com/locate/critrevonc](http://www.elsevier.com/locate/critrevonc)

# Circulating endothelial cells, bone marrow-derived endothelial progenitor cells and proangiogenic hematopoietic cells in cancer: From biology to therapy

Balazs Dome<sup>a,b,\*</sup>, Jozsef Timar<sup>c</sup>, Andrea Ladanyi<sup>c</sup>, Sandor Paku<sup>d</sup>, Ferenc Renyi-Vamos<sup>a</sup>,  
Walter Klepetko<sup>e</sup>, Gyorgy Lang<sup>e</sup>, Peter Dome<sup>f</sup>, Krisztina Bogos<sup>a,b</sup>, Jozsef Tovari<sup>a,c</sup>

<sup>a</sup> Department of Tumor Biology, National Koranyi Institute of Pulmonology, Pihenó u. 1, Budapest H-1529, Hungary

<sup>b</sup> Department of Thoracic Oncology, National Koranyi Institute of Pulmonology, Budapest, Hungary

<sup>c</sup> Department of Tumor Progression, National Institute of Oncology, Budapest, Hungary

<sup>d</sup> First Institute of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary

<sup>e</sup> Department of Cardio-Thoracic Surgery, Medical University of Vienna, Vienna, Austria

<sup>f</sup> Department of Clinical and Theoretical Mental Health, Kutvolgyi Clinical Center, Semmelweis University, Budapest, Hungary

Accepted 26 June 2008

## Contents

1. Introduction	109
2. Circulating endothelial cells	109
2.1. Characterization and enumeration of circulating endothelial cells	110
2.2. Circulating endothelial cells in human malignancies (Table 2)	110
3. Endothelial progenitor cells	113
3.1. Characterization and enumeration of endothelial progenitor cells	113
3.2. Regulation of endothelial progenitor cells	113
3.3. Endothelial progenitor cells in animal tumor models (Fig. 1)	114
3.4. Endothelial progenitor cells in human tumors	115
4. Proangiogenic hematopoietic cells (Fig. 1)	116
5. Antiangiogenic and/or anticancer therapy via EPCs, proangiogenic HCs and CECs	117
6. EPCs and CECs: potential biomarkers of tumor angiogenesis?	118
7. Conclusions	119
Reviewers	119

**Abbreviations:**  $\alpha$ SMA,  $\alpha$ -smooth muscle actin; AML, acute myeloid leukemia; Ang-1, angiopoietin-1; BM, bone marrow; CAC, circulating angiogenic cell; CEC, circulating endothelial cell; CFU-EC, colony-forming unit-endothelial cells; CLL, chronic lymphocytic leukemia; CRP, C-reactive protein; CSF-1R, colony-stimulating factor-1 receptor; CT, computed tomography; DC, dendritic cell; EC, endothelial cell; ECFC, endothelial colony-forming cell; EPC, endothelial progenitor cell; EPOR, erythropoietin receptor; FGFR, fibroblast growth factor receptor; FISH, fluorescent in situ hybridization; FLT-3, FMS-like tyrosine kinase 3; FSC, forward scatter; GFP, green fluorescent protein; GIST, gastrointestinal stromal tumor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HC, hematopoietic cell; HIF-1, hypoxia-inducible factor-1; HSV-TK, herpes simplex virus thymidine kinase; IFN, interferon; IMS, immunomagnetic separation; MC, mast cell; MDSC, myeloid-derived suppressor cell; MMP, matrix metalloproteinase; MNC, mononuclear cell; MRI, magnetic resonance imaging; MTD chemotherapy, maximum tolerable dose chemotherapy; NO, nitric oxide; PB, peripheral blood; PDGF-CC, platelet-derived growth factor-CC; PET, positron emission tomography; PIGF, placental growth factor; PMN, polymorphonuclear cell; RBCC, recruited blood circulating cell; rHuEPO, recombinant human erythropoietin; SDF-1, stromal cell-derived factor-1; SPECT, single photon emission computed tomography; SSC, side scatter; TAM, tumor-associated macrophage; TASC, tumor-associated stromal cell; TEM, TIE2-expressing monocyte; TGF- $\beta$ , transforming growth factor- $\beta$ ; Tie-2/TEK, angiopoietin-1 receptor precursor or tunica intima EC kinase; TNF- $\alpha$ , tumor necrosis factor-alpha; TSP-1, thrombospondin-1; tsVEGFR2, truncated soluble VEGFR2; UEA-1, Ulex Europaeus lectin-1; VDA, vascular disrupting agent; VE-cadherin, vascular endothelial cadherin; VEGFR, vascular endothelial growth factor receptor; vWf, von Willebrand factor.

\* Corresponding author at: Department of Tumor Biology, National Koranyi Institute of Pulmonology, Pihenó u. 1, Budapest H-1529, Hungary.  
Tel.: +36 1 391 3210; fax: +36 1 391 3223.

E-mail address: [domeb@yahoo.com](mailto:domeb@yahoo.com) (B. Dome).

Conflicts of interest .....	120
Acknowledgements .....	120
References .....	120
Biographies .....	123

## Abstract

Vascularization, a hallmark of tumorigenesis, is classically thought to occur exclusively through angiogenesis (i.e. endothelial sprouting). However, there is a growing body of evidence that endothelial progenitor cells (EPCs) and proangiogenic hematopoietic cells (HCs) are able to support the vascularization of tumors and may therefore play a synergistic role with angiogenesis. An additional cell type being studied in the field of tumor vascularization is the circulating endothelial cell (CEC), whose presence in elevated numbers reflects vascular injury. Levels of EPCs and CECs are reported to correlate with tumor stage and have been evaluated as biomarkers of the efficacy of anticancer/antiangiogenic treatments. Furthermore, because EPCs and subtypes of proangiogenic HCs are actively participating in capillary growth, these cells are attractive potential vehicles for delivering therapeutic molecules. The current paper provides an update on the biology of CECs, EPCs and proangiogenic HCs, and explores the utility of these cell populations for clinical oncology.

© 2008 Elsevier Ireland Ltd. All rights reserved.

*Keywords:* Endothelial progenitor cells; Proangiogenic hematopoietic cells; Circulating endothelial cells; Vasculogenesis; Angiogenesis; Cancer

## 1. Introduction

It has been over 30 years since Judah Folkman hypothesized that neovascularization plays a significant role in tumor progression and might well be an optimal target for anti-cancer strategies [1]. Subsequent research has led to the identification of several regulators of angiogenesis, some of which represent therapeutic targets. However, it is also well established now that tumor vasculature does not necessarily derive from endothelial cell (EC) sprouting; instead, tumors can acquire their vasculature by various mechanisms including postnatal vasculogenesis, a process during which circulating bone marrow (BM)-derived endothelial progenitor cells (EPCs) home to sites of neovascularization and differentiate into ECs [2]. EPCs therefore resemble embryonic angioblasts, which are anchorage-independent cells having the capacity to proliferate, migrate and differentiate into mature ECs. Since the first description of EPCs by Asahara et al. [3], several authors have found decreased numbers and/or impaired function of EPCs in a variety of cardiovascular diseases. In contrast, blood levels of EPCs tend to increase in cancer patients and to correlate with the stage of the malignant disease [4].

Infiltration of human tumors by certain types of leukocytes, like lymphocytes and dendritic cells (DCs) is often associated with better prognosis and overall survival. However, other leukocyte subsets such as macrophages can enhance tumor angiogenesis and progression [5]. Moreover, several studies on experimental tumor models have been published suggesting that even before the onset of the so called “angiogenic switch” [6], various types of hematopoietic cells (HCs) are recruited to the tumor tissue to enhance new blood vessel formation by secreting angiogenic molecules and/or by trans-differentiation into endothelial-like cells [7]. These observations have led to the concept that certain populations of HCs are proangiogenic and co-mobilized from the BM with EPCs [8].

Another related cell type is the circulating endothelial cell (CEC). These cells are thought to be mature ECs that have detached from their basement membrane in response to some form of blood vessel injury. Accordingly, increased numbers of CECs, rare in healthy individuals, are observed in a broad range of conditions/diseases associated with vascular perturbation, including tumor-induced neovascularization [9]. Therefore, although their biology is still obscure, there is a growing belief that together with EPCs, CECs may evolve into a surrogate biomarker for monitoring tumor angiogenesis and the efficacy of anticancer/antiangiogenic therapies.

This review starts with a summary on the phenotype, enumeration strategies and clinical significance of CECs. We then discuss the characterization of EPCs and proangiogenic HCs, as well as the molecules that regulate their release from the BM and their homing to or incorporation into neovascular networks. Finally, we review the potential therapeutic and diagnostic implications of EPCs and HCs in medical oncology.

## 2. Circulating endothelial cells

Although CECs were first described over 30 years ago through methods such as vital light microscopy, May–Grünwald–Giemsa staining and separation by Ficoll density centrifugation [10,11], the development of specific monoclonal antibodies has only recently provided an opportunity to investigate the pathophysiology of these cells. In 1991, monoclonal antibodies to two novel EC specific surface antigens (HEC19 [12] and S-Endo-1 [13], later described as CD146 [14]) were developed and used to quantify CECs. More recently, these authors and others have used the immunobead technique and/or flow cytometry to investigate the significance of CECs in a variety of diseases including infections, cardiovascular, inflammatory and autoimmune syndromes and cancer (reviewed in ref. [9]).

### 2.1. Characterization and enumeration of circulating endothelial cells

In practice, the main problem in clinical studies with the quantification of CECs is their low frequency in the peripheral blood (PB). To quantify these rare cells, different techniques of cell enrichment together with immunocytochemical detection have been applied, such as density centrifugation methods, cell culture and immunomagnetic separation (IMS). The latter technique, developed by George et al., includes mixing PB with immunomagnetic beads coated with anti-CD146 antibodies, which then bind to CD146-expressing CECs and are selected by magnet retrieval [15]. However, although it is most frequently used for CEC enumeration, CD146 expression has also been reported in pericytes, bone marrow fibroblasts, cancer cells, trophoblasts, and activated lymphocytes; thus, caution in interpreting results with CD146 alone is advised [16,17]. Nevertheless, unspecific CD146 expression should not necessarily be considered as a technical limitation in the detection of CECs, since CD146-based IMS has been adapted to cope with it. IMS is best accompanied by an additional specific characterization step, such as Ulex Europaeus lectin-1 (UEA-1), CD31 or von Willebrand factor (vWf) labeling to confirm that all sorted cells are CECs. This methodology has been used successfully in a large number of studies showing altered CEC levels in various diseases. Accordingly, the authors of a recent multicentric study defined CECs as cells that exceed 10  $\mu\text{m}$  in size and have more than five immunomagnetic beads attached. The rosetted cells stain positive with at least two EC markers (for example, CD146 and UEA-1) and are negative for leukocyte markers (for example, CD14 and CD45, Table 1) [18].

A widespread alternative to the IMS technique is flow cytometry, during which whole PB is usually labeled with endothelial-specific antibodies conjugated with different fluorochromes. An advantage of flow cytometry is rapid multiparametric analysis and the ability to detect subpopulations, such as “bright” versus “dim” labeling, and activated (e.g. expressing CD106) or resting, although CECs separated by the IMS method can also be multiply labeled. For example, Duda et al. recently reported a cytometry protocol for phenotypic identification and quantification of CECs in human PB. Using four surface markers (CD31, CD34, CD133 and CD45) and multicolor flow cytometry, their group has proposed a surface phenotype of viable CECs (defined as CD31<sup>bright</sup>CD34<sup>+</sup>CD45<sup>-</sup>CD133<sup>-</sup> cells) [19]. However, there are substantial differences between IMS and flow cytometric techniques, as indicated by the high variation in reported CEC numbers. On the basis of CD31<sup>bright</sup>/CD45<sup>-</sup> staining, the amount of cells recorded per milliliter of PB is about 1000- to 100,000-fold higher than the number of CECs reported in healthy controls and in different categories of patients using CD146-based IMS. The recent results of Strijbos et al. [20] may, in part, explain the significantly higher CD146<sup>+</sup> CEC levels as reported using the single-platform flow cytometric assay and those determined by

CD146-based IMS techniques. In their study, these authors focused on confirming the widely used single-platform flow cytometric assay for CECs, as per the method of Mancuso et al. [21], using their previously reported CEC profile [forward scatter (FSC)<sup>low-to-intermediate</sup>, side scatter (SSC)<sup>low</sup>, CD31<sup>bright</sup>/CD146<sup>+</sup>/CD45<sup>-</sup>]. Interestingly, by using reverse transcription polymerase chain reaction, electron microscopy and fluorescence in situ hybridization (FISH), Strijbos et al. demonstrated that cells with the above phenotype are in fact not CECs but large platelets [20].

In light of these results, CEC enumeration is far from being a standardized procedure and the confusion in CEC numbers does raise serious questions concerning the reliability of the above techniques. Because there are no studies currently available demonstrating the superiority of one technique over the other, more research is required to measure/correlate the accuracy of the above methods.

### 2.2. Circulating endothelial cells in human malignancies (Table 2)

Elevated levels of CECs have been repeatedly found in different types of human malignancies. This observation first appeared in the literature in 2001 when Mancuso et al., using 4-color flow cytometry, found that in breast cancer and lymphoma patients, both resting and activated CECs were increased significantly [21]. In addition, CEC levels were similar to healthy controls in lymphoma patients achieving complete remission after chemotherapy, and activated CECs were found to decrease in breast cancer patients evaluated after surgery. Although they employed different methods of assessing CEC levels and disease stage, Beerepoot et al. also reported a significant CEC elevation in cancer patients with progressive disease, whereas their patients with stable disease had CEC levels comparable to those of healthy individuals [22].

Subsequent studies yielded similar results. Zhang et al. investigated CECs in multiple myeloma, demonstrating increased numbers of these cells ( $P < 0.001$  vs. healthy controls) [23]. Wierzbowska et al. evaluated CEC levels by 4-color flow cytometry in acute myeloid leukemia (AML) and reported elevated numbers of both resting and activated CECs [24]. In their study, CEC levels were correlated with disease status and response to treatment as well. In another study on breast cancer, CECs were found to be significantly elevated in cancer patients and decreased during chemotherapy [25]. More recently, Rowand et al. observed that CEC counts were significantly higher in metastatic carcinoma patients compared to healthy controls [26]. Similarly, increased CEC levels have been reported in the PB of patients with gastrointestinal stromal tumor (GIST) [27], myelodysplastic syndrome [28] and chronic lymphocytic leukemia (CLL) [29].

Taken together, it is apparent that CECs are increased in patients with different types of malignancies. Furthermore, there is a growing body of evidence that this cell population may evolve into a surrogate biomarker for measuring the

Table 1  
CECs, EPCs and proangiogenic HCs in cancer

Cell type	Source	Morphology/phenotype/molecular profile	Role/significance in tumor vascularization	Proliferative potential	Ref.
CEC	Blood vessel wall	Mature circulating cells < 50 $\mu$ m in diameter; CD146, CD31, CD34, vWf, VE-cadherin, UEA-1, acLDL	Biomarker of endothelial injury, angiogenesis and/or the efficacy of anticancer (antiangiogenic) therapy	No	[16–19]
EPC	BM	Immature circulating cells ~20 $\mu$ m in diameter; CD133, CD34, VEGFR2, CD38, c-kit, CD31, CXCR4	Support of tumor vascularization/biomarker of endothelial injury, angiogenesis and/or the efficacy of anticancer (antiangiogenic) therapy/vehicle for drug delivery	Yes	[33,43]
CFU-EC	Culture	PBMNCs growing in colonies for ~7 days	Support of tumor vascularization/biomarker of endothelial injury, angiogenesis and/or the efficacy of anticancer (antiangiogenic) therapy/vehicle for drug delivery	Yes	[39]
CAC	Culture	PBMNCs growing in the presence of angiogenic cytokines for ~4–6 days	Support of tumor vascularization/biomarker of endothelial injury, angiogenesis and/or the efficacy of anticancer (antiangiogenic) therapy/vehicle for drug delivery	Yes	[40]
ECFC	Culture	PBMNCs growing in cobblestone patterned colonies for ~21 days	Support of tumor vascularization/biomarker of endothelial injury, angiogenesis and/or the efficacy of anticancer (antiangiogenic) therapy/vehicle for drug delivery	Yes	[42]
TEM	BM	CD11b, CD45, TIE2	Support of tumor vascularization	Yes	[68]
DC	BM	MHC II <sup>+</sup> /CD11c <sup>+</sup>	Support of tumor vascularization		[119]
		conventional/myeloid DCs CD123 <sup>+</sup> /CD303 <sup>+</sup> plasmacytoid DCs CD11c <sup>+</sup> /CCR6 <sup>+</sup> /MHC II <sup>+</sup> TADCs	Support of tumor vascularization	Yes	[119] [120,121]
MDSC	BM	CD11b, Gr-1	Support of tumor vascularization	Yes	[100,106]
TASC	BM	CD45, VEGFR2	Support of tumor vascularization	Yes	[122]
RBCC	BM	CD45, CD11b, CXCR4, VEGFR1	Support of tumor vascularization	Yes	[123]
VEGFR1 <sup>+</sup> HC	BM	VEGFR1, VLA-4	Support of tumor vascularization/initiation of the pre-metastatic niche	Yes	[124]

BM, bone marrow; CAC, circulating angiogenic cell; CEC, circulating endothelial cell; CFU-EC, colony-forming unit-endothelial cells; DC, dendritic cell; ECFC, endothelial colony-forming cell; EPC, endothelial progenitor cell; MDSC, myeloid-derived suppressor cells; PBMNC, peripheral blood mononuclear cell; RBCC, recruited blood circulating cell; TADC, tumor-associated dendritic cell; TASC, tumor-associated stromal cell; TEM, TIE2-expressing monocyte; VEGFR1, vascular endothelial growth factor receptor-1.

Table 2  
Levels of CECs and EPCs in patients with malignant diseases

Tumor type	Number of cases	Enumeration	Phenotype		Mean CEC levels (patients vs. controls, n/mL of PB)	Mean EPC levels (patients vs. controls, n/mL of PB)	Ref.
			CEC	EPC			
Breast cancer, lymphoma	76	FC	CD45 <sup>-</sup> /CD146 <sup>+</sup> /CD31 <sup>+</sup> /CD34 <sup>+</sup>	CD45 <sup>-</sup> /CD31 <sup>+</sup> /CD133 <sup>+</sup>	39,100 vs. 7900	Below 500 both in patients and controls	[2]
Various <sup>a</sup>	95	CD146-IMS	CD146 <sup>+</sup> /CD31 <sup>+</sup> /vWF <sup>+</sup> /VEGFR2 <sup>+</sup>	NA	438 vs. 121 <sup>b</sup>	NA	[22]
Multiple myeloma	31	FC + culture	CD34 <sup>+</sup> /CD146 <sup>+</sup> /CD105 <sup>+</sup> /CD11b <sup>-</sup>	CFU-ECs	CFU score ~6-fold higher in patients <sup>c</sup>	NA	[23]
AML	48	FC	CD45 <sup>-</sup> /CD31 <sup>+</sup> /CD34 <sup>+</sup> CD146 <sup>+</sup>	CD45 <sup>-</sup> /CD31 <sup>+</sup> /CD34 <sup>+</sup> CD133 <sup>+</sup>	36,700 vs. 3200	700 vs. 100	[24]
Myelofibrosis	110	FC	NA	CD133 <sup>+</sup> /CD34 <sup>+</sup> /VEGFR2 <sup>+</sup>	NA	165 vs. 0	[88]
CLL	20	FC	CD45 <sup>-</sup> /CD31 <sup>+</sup> /CD146 <sup>+</sup>	NA	26.5 vs. 18.5	NA	[29]
MDS	128	FC	CD45 <sup>-</sup> /CD34 <sup>+</sup> /CD146 <sup>+</sup> /CD133 <sup>-</sup>	NA	512 vs. 153	NA	[28]
Breast cancer	16	FC	CD45 <sup>-</sup> /CD146 <sup>+</sup> /CD31 <sup>+</sup> /CD34 <sup>+</sup>	CD34 <sup>+</sup> /VEGFR2 <sup>+</sup>	5700 vs. 1300	370 vs. 140	[25]
Breast cancer	47	FC	NA	CD34 <sup>+</sup> /VEGFR2 <sup>+</sup>	NA	0.44 vs. 0.18 <sup>d</sup>	[84]
Breast cancer	25	FC	NA	CD133 <sup>+</sup> /VEGFR2 <sup>+</sup>	NA	0.032 vs. 0.023 <sup>d,e</sup>	[85]
Gastric/breast cancer	71	Culture	NA	CFU-ECs	NA	40.2 vs. 37.6 (n.s.) <sup>f</sup>	[155]
Lung cancer	53	FC	NA	CD34 <sup>+</sup> /VEGFR2 <sup>+</sup>	NA	1162 vs. 345	[80]
Lung cancer	10	FC CD34-IMS	NA	CD45 <sup>-</sup> /CD34 <sup>+</sup> /VEGFR2 <sup>+</sup> and Cytospin	NA	90 vs. 42 and 0.3 vs. 0.1 <sup>g</sup>	[83]
Liver cancer	80	Culture	NA	CD45 <sup>-</sup> /CD133 <sup>+</sup> /VEGFR2 <sup>+</sup>	NA	CFU score 10-fold higher in patients	[46]
Liver cancer	64	Culture	NA	CFU-ECs	NA	CFU score ~2-fold higher in patients	[79]
Glioma	32	FC	CD34 <sup>+</sup> /CD146 <sup>+</sup> /VEGFR2 <sup>-</sup>	CD133 <sup>+</sup> /CD34 <sup>+</sup> /VEGFR2 <sup>+</sup>	n.s.	0.18 vs. 0.01 <sup>d</sup>	[89]
Various <sup>h</sup>	206	IMS	CD45 <sup>-</sup> /CD146 <sup>+</sup> /CD105 <sup>+</sup>	NA	111 vs. 21	NA	[26]
Various <sup>i</sup>	44	FC	CD45 <sup>-</sup> /CD34 <sup>+</sup> /CD133 <sup>-</sup> /CD105 <sup>-</sup>	CD45 <sup>-</sup> /CD34 <sup>+</sup> /CD133 <sup>+</sup> /CD105 <sup>-</sup>	470 vs. 140 (n.s.)	90 vs. 30 (n.s.)	[154]
GIST	16	FC	CD45 <sup>-</sup> /CD31 <sup>+</sup> /P1H12 <sup>+</sup> /CD133 <sup>-</sup>	NA	1090 vs. 540	NA	[27]

CEC, circulating endothelial cell; CLL, chronic lymphocytic leukemia; CFU-ECs, colony-forming unit-endothelial cells; EPC, endothelial progenitor cell; FC, flow cytometry; GIST, gastrointestinal stromal tumor; IMS, immunomagnetic separation; MDS, myelodysplastic syndrome; n.s., non-significant; PB, peripheral blood.

<sup>a</sup> Different cancer patients with progressive disease. Tumor types included head and neck (10 patients), colon (13 patients), prostate (25 patients), gastric (3 patients), esophagus (3 patients), renal cell (6 patients), breast (10 patients), ovarian (5 patients), cervical cancer (2 patients), carcinoid (3 patients), melanoma (3 patients), glioma (2 patients), and 10 patients with other tumor types.

<sup>b</sup> Patients with stable disease had CEC numbers equal to that circulating in healthy subjects ( $P=0.69$ ).

<sup>c</sup> Raw data not supplied.

<sup>d</sup> % of PB mononuclear cells.

<sup>e</sup> Stage III vs. stage IV patients.

<sup>f</sup> Stated per unit area (mm<sup>2</sup>).

<sup>g</sup> % of CD34 enriched cells.

<sup>h</sup> 50 cases of breast cancer, 49 of colorectal cancer, 35 of lung cancer, 48 of prostate cancer, and a group of other carcinomas consisting of 8 ovarian/pancreatic, 3 renal, 2 bladder, 2 thyroid, 2 gastric, and 1 breast/colon, colon/prostate, esophageal, gastric, carcinoid tumor, squamous cell, tongue, and mandibular cancer.

<sup>i</sup> Patients with different refractory solid malignancies pretreated with chemotherapy. Details of the patient population not supplied.



effectiveness of conventional and targeted (antiangiogenic) anticancer therapy. What is less clear is whether or not CECs are simply biomarkers of the accelerated endothelial turnover of tumor capillaries, or are active participants of tumor progression and vascularization. However, it is also possible that CECs are not being desquamated from activated tumor vasculature. Instead, their increased number in the PB may be the result of a more generalized systemic (i.e. paraneoplastic) endothelial damage and/or activation.

### 3. Endothelial progenitor cells

#### 3.1. Characterization and enumeration of endothelial progenitor cells

EPCs were discovered and identified in 1997 by Asahara et al. [3] on the basis of vascular endothelial growth factor receptor-2 (VEGFR2) and CD34 co-expression. Since then, the emergence of specific membrane markers and molecular probes has facilitated the identification and purification of functional stem and progenitor cells. A number of researchers have set out to better characterize these cells, and EPCs were subsequently shown to express fibroblast growth factor receptor (FGFR), CD38, c-kit, CD31, CXCR4, vWf, vascular endothelial cadherin (VE-cadherin), Tie-2/TEK (angiopoietin-1 receptor precursor or tunica intima EC kinase) and CD133 [30–33]. However, it is still extremely difficult to differentiate EPCs from HCs or CECs, since the markers used to separate EPCs are expressed on subsets of HCs (CD34, VEGFR2, CD133, VE-cadherin) and mature ECs/CECs (CD34, VE-cadherin) as well. In fact, the population of EPCs may include a group of cells existing in a variety of stages ranging from immature HSCs to completely differentiated ECs.

Although to date no clear phenotype of EPCs exists and their putative precursors and the exact differentiation lineage remain to be determined, at present it is widely accepted that early EPCs (localized in the BM or immediately after migration into the bloodstream) are CD133<sup>+</sup>/CD34<sup>+</sup>/VEGFR2<sup>+</sup> cells, whereas circulating EPCs are positive for CD34 and VEGFR2, lose CD133 and begin to express membrane molecules typical to mature ECs [2]. Thus, the major candidate for a specific circulating EPC marker is the CD133, an orphan receptor specifically expressed on early EPCs, but whose expression is lost once these progenitors differentiate into mature ECs [34]. Unfortunately, because in humans CD133 is expressed by HSCs as well [35], the techniques for phenotypic differentiation between vasculogenic-restricted immature EPCs, committed HSCs and their putative common precursor (bi-potential hemangioblast) have yet to be developed further.

Results on PB EPC levels in the bloodstream are variable, ranging from 70 to 210 cells/mL of PB [36] to 3000–5000 cells/mL of PB [37], depending most likely on the isolation technique used. These relatively low numbers

of circulating EPCs as measured by flow cytometry are in sharp contrast to the high numbers of adherent cells (often confusingly defined as “EPCs” too) that are observed in PB mononuclear cell cultures ( $\sim 10^5$  from 1 mL blood) (Table 1). In general, three different methods for culturing “EPCs” have been reported [38]. In the original approach, in which the identification of EPCs is based on their clonogenic and proliferative potential, PB mononuclear cells (MNCs) are plated on fibronectin-, gelatin- or collagen-coated dishes. Discrete colonies appear in a week, containing round cells centrally and spindle-shaped cells peripherally. The cells of these colonies are usually referred to as colony-forming unit-ECs (CFU-ECs) [39]. In the second method, MNC cultures are treated with angiogenic cytokines for 4–6 days, whereupon non-adherent cells are discarded, resulting in a target adherent cell population [40]. Because these adherent cells have been reported to enhance angiogenesis in vivo [41], they have been defined as circulating angiogenic cells (CACs). Although CACs do not form colonies and are observed in cultures in larger numbers than CFU-ECs, they express endothelial markers such as CD31, vWF, VE-cadherin and Tie-2/TEK, bind *Bandeiraea simplicifolia* (BS-1) and UEA-1 lectins, and have the potential to take-up acetylated low-density lipoprotein/aLDL. Therefore, CACs appear analogous to CFU-ECs in surface molecular profile and in vitro properties. The third and least known type of “EPC” is now defined as “endothelial colony-forming cell” (ECFC). In this method, MNCs are growing in the presence of endothelial-specific growth factors. After removal of non-adherent cells, ECFC colonies displaying cobblestone pattern typical of ECs emerge from the adherent cell population. Because ECFCs emerge much later in culture than CFU-ECs or CACs, they have also been termed “late outgrowth EPCs” [42].

#### 3.2. Regulation of endothelial progenitor cells

In order for EPCs to facilitate the growth of tumor capillaries, they must respond to signals released from the BM, home to the tumor site, and differentiate into mature ECs. Although the exact molecular background of EPC mobilization remains vague, VEGF is thought to be the key cytokine in the regulation of EPC mobilization and homing [43]. In animal models, VEGF through interaction with its receptors, VEGFR2 and VEGFR1 expressed on EPCs and HSCs [44], effectively induces the mobilization of these cell populations into the circulation; EPC levels in the PB rise within 24 h following exogenous VEGF administration [45]. Accordingly, the increased circulating VEGF level triggers the release of EPCs from the BM of cancer patients [25,46].

Cytokines that induce the release of white and red blood cells may also trigger EPC mobilization. Elevated levels of EPCs were reported in mice subsequent to granulocyte-macrophage colony-stimulating factor (GM-CSF) treatment, and accelerated corneal angiogenesis with BM-derived cells was found in animals treated with GM-CSF [47]. In another animal model, granulocyte colony-stimulating factor (G-

CSF) markedly enhanced growth of a colorectal carcinoma cell line, in part mediated by EPCs incorporated into sites of active blood vessel growth, whereas it had no effect on cancer cell proliferation *in vitro* [48]. Similarly, administration of recombinant human erythropoietin (rHuEPO) increased both the level of functionally active EPCs by differentiation *in vitro* in a dose-dependent manner and also the number of functionally active EPCs in human PB [49]. In addition, serum levels of EPO were found to be significantly associated with the number and function of circulating EPCs [50]. Interestingly, although EPO has a similar potency for the stimulation of EPC mobilization as VEGF [51] and it is widely used for correction of hemoglobin level by increasing the number of red blood cells, there is no data on the effect of rHuEPO on EPC mobilization and recruitment when it is delivered to tumor-bearing animals or cancer patients. However, in addition to the potential effects of rHuEPO on cancer cell proliferation, the expression of EPO receptor (EPOR) in ECs and their progenitors raises the possibility that exogenous rHuEPO may enhance the processes of angio- and/or vasculogenesis in tumors (reviewed in ref. [52]). Nevertheless, as it has been suggested by recent studies, the overall direct effect of EPO-EPOR signaling on tumor progression and therapy is not a straightforward one. For instance, rHuEPO administration has recently been shown to be associated with decreased intratumoral hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and VEGF expression and increased sensitivity to radio- and chemotherapy of human tumor xenografts [53,54].

In addition to the above molecules, recent results indicate that PIGF (placental growth factor) [55], Ang-1 (angiopoietin-1) [56], PDGF-CC (platelet-derived growth factor-CC) [57], SDF-1 (stromal cell-derived factor-1) [58], NO (nitric oxide) [59], 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors (statins) [60], estrogens [61] and physical training [62] enhance EPC mobilization as well. In contrast, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and C-reactive protein (CRP) promote apoptosis, attenuate the function and decrease the level of EPCs [63,64].

### 3.3. Endothelial progenitor cells in animal tumor models (Fig. 1)

The observation that EPCs are capable of enhancing tumor vascularization means that although these progenitor cells are primarily programmed to support embryogenic vasculogenesis, they retain this capability within an angiogenic milieu in the adult. But what evidence is there that EPCs actually support new blood vessel growth in tumors? The first report on the role of EPCs in tumor-induced vasculogenesis dates back to 2001, when Lyden et al. [65] demonstrated that EPCs contribute about 90% to vascularization in lymphomas grown in angiogenesis-defective Id-mutant mice in which implanted tumors rapidly regress, most probably because of the weak angiogenic potential of these animals. BM transplantation from wild-type mice, not from Id-mutant mice, restored the tumor neovascularization and growth in Id-

mutant mice. However, the high contribution of EPCs in the neovessels of this tumor model almost certainly results from the fact that recipient Id-deficient mice are angiogenic-deficient as well, and therefore compensatory mechanisms (such as tumor-induced vasculogenesis) are activated to sustain tumor growth. In subsequent animal transplantation models, EPCs incorporated into neovessels, sometimes by as much as 50% [66], whereas other authors reported lower but significant levels between 10% and 20% [67]. These reports have been challenged by some other experiments in which EPCs had no significant contribution to the tumor vasculature. For example, De Palma et al. [68] found that TIE2-expressing monocytes (TEMs), rather than EPCs, homed to tumors and interacted with vascular ECs. Interestingly, these authors did not observe EPCs in the tumor vasculature. Similarly, based on their observations in a transgenic mouse model, Gothert et al. failed to observe EPCs in tumor capillaries [69]. Although possible reasons for such inconsistent results might include the use of differing experimental models/techniques to identify EPCs, recent data suggest that the involvement of EPCs in experimental tumor vascularization might also vary depending on tumor stage and/or grade. Using different mouse models, a German group reported recently that only advanced tumors recruit and incorporate EPCs into neovessels, possibly to further compensate for escalating blood supply requirements [70]. Along this line, Ruzinova et al. found that the contribution of EPCs to the tumor vasculature depends on the tumor grade, since EPCs distinguished between well- and poorly differentiated carcinoma cell lines [71]. Finally, variations in EPC levels and their involvement in the actual phase of tumor growth might also be caused by chemotherapy. The evidence for this assumption comes from another animal study in which mice were treated with the maximum tolerable dose (MTD) versus metronomic (i.e. antiangiogenic [72]) chemotherapy. Surprisingly, while animals treated with the MTD chemotherapy experienced a robust EPC mobilization a few days after the end of a cycle of drug administration, the administration of metronomic chemotherapy was associated with a consistent decrease in EPC levels [73].

In addition to the physical contribution of EPCs to newly formed capillaries, the angiogenic cytokine release of EPCs may be a supportive mechanism to improve neovascularization as well [74]. This idea is supported by a recent report by Gao et al. [75]. These authors found that although only 12% of the new blood vessels showed incorporation of EPCs, blocking EPC mobilization caused severe angiogenesis inhibition and significantly impaired tumor progression. Moreover, in the same study, gene expression analysis of EPCs revealed up-regulation of a variety of key proangiogenic genes.

In conclusion, EPCs seem to have both paracrine and structural roles in new vessel growth. However, although EPCs are obviously able to support tumor vascularization, the involvement of this cell population may vary depending on circumstances such as the experimental model or detection method used, the histology and stage of the tumor, and the type of the anticancer treatment.

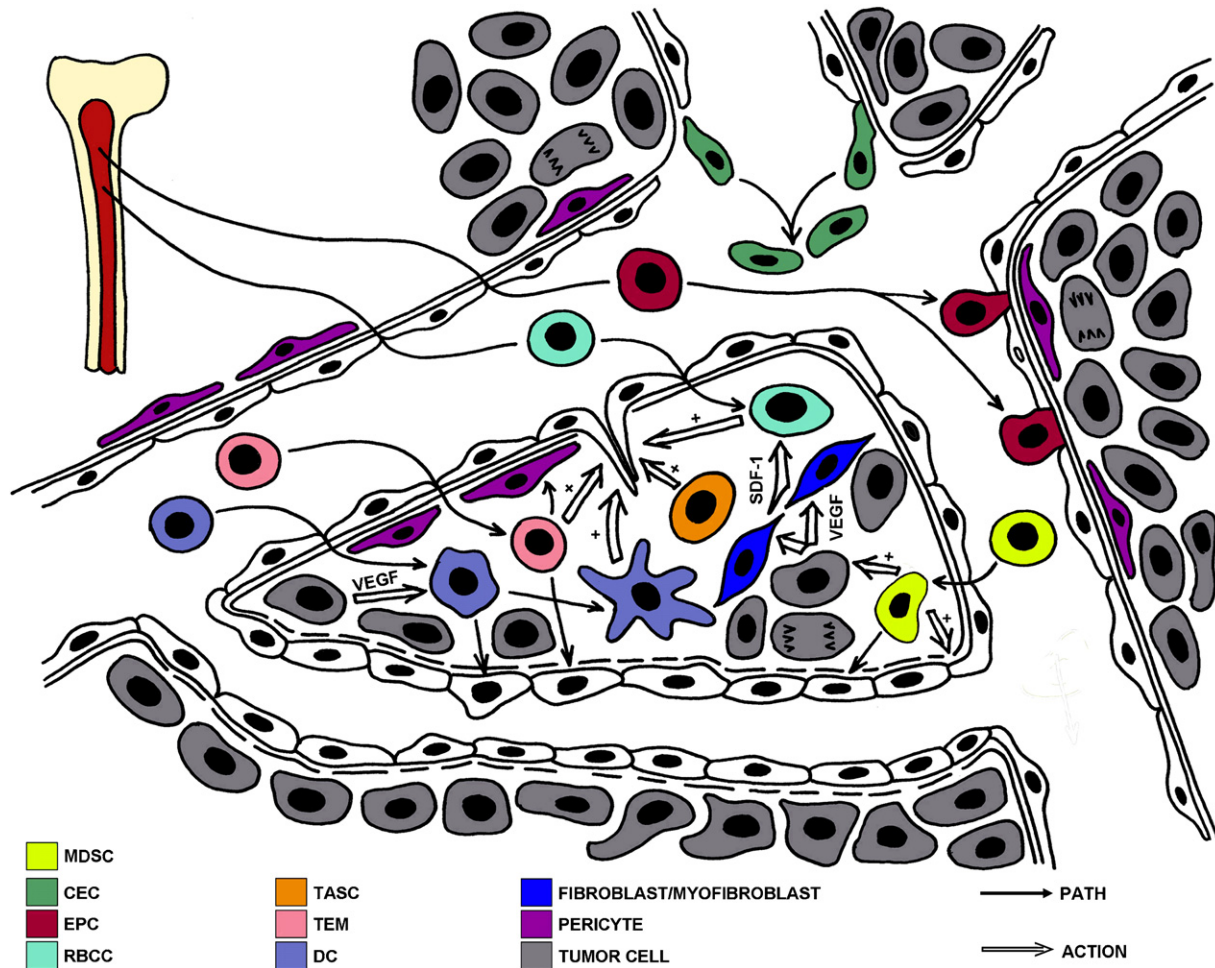


Fig. 1. Schematic representation of the roles of CECs, EPCs and HCs in cancer. CECs represent a population of mature ECs that have desquamated from their basal membrane into the circulation in response to some form of blood vessel injury [16]. CEC levels are elevated in patients with different types of malignancies and in various other conditions including ischemic, infective, autoimmune and inflammatory diseases [17]. EPCs are circulating, BM-derived cells that appear to enhance neovascularization in both physiologic and pathologic settings. These cells have been found in decreased numbers and/or with impaired function in a variety of cardiovascular diseases. In contrast, circulating levels of EPCs tend to increase in cancer patients and to correlate with the stage of the malignant disease [43]. In addition to EPCs, tumor vascularization and growth might be modulated by some other BM-derived cells including (but not limited to) RBCCs, TASCs, DCs, TEMs and MDSCs. Mediated by SDF1, a chemokine induced by tumor-derived VEGF in activated perivascular myofibroblasts, RBCCs enhance new capillary growth from a perivascular position [123]. TASCs colonize the tumor stroma and are thought to enhance tumor capillary sprouting in a paracrine manner by inducing or increasing the expression angiogenic cytokines [122]. DCs might promote angiogenesis through different mechanisms: by stimulating EC sprouting through the expression of angiogenic molecules and by differentiating into endothelial-like cells. Besides inhibiting the functional maturation of DCs, VEGF is thought to be a major player in these processes [119]. TEMs are monocytes that express the TIE2 receptor, are recruited to periendothelial tumor sites and promote angiogenesis in a paracrine manner [68]. MDSCs contribute to tumor growth and angiogenesis by producing MMP9, incorporating into the endothelial tube and differentiating into ECs [106].

#### 3.4. Endothelial progenitor cells in human tumors

The involvement of EPCs in the vascularization process of human tumors has been investigated in some recent studies as well. Peters et al. studied tumor samples from patients who developed malignancies after BM transplantation with donor cells derived from individuals of the opposite sex. By using FISH with sex chromosome-specific probes, these authors reported that the percentage of BM-derived ECs in the tumors ranged from 1% (head and neck sarcoma) to 12% (lymphoma) [76], which was closer to the numbers observed in spontaneous animal tumors than the zero

or extremely high numbers found when implanting tumor cells. Recent studies have demonstrated the presence, based on their CD133 immunoreactivity, of incorporated EPCs in the walls of human tumor blood vessels as well [77–80]. Accordingly, CD133 mRNA expression in the PB of cancer patients was shown to be an independent predictor for overall survival in patients with bone metastases [81] and for recurrence in colorectal cancer patients [82]. However, because CD133 expression is continuously decreasing on the cell surface of circulating EPCs and lost once EPCs differentiate into more mature ECs in the endothelial tube [2,33,43], it seems obvious that based on CD133 staining, the



rate of incorporated EPCs in cancer capillaries is inevitably underestimated.

Recently reported data also indicate that EPCs circulate in increased numbers in the PB of patients with various types of cancers. Elevated EPC levels have been reported in the PB of patients with lung [80,83], hepatocellular [46,79], breast [25,84,85] and colorectal [86,87] cancers, as well as multiple myeloma [23], myelofibrosis [88], non-Hodgkin's lymphoma [77], AML [24] and malignant gliomas [89] (Table 2).

#### 4. Proangiogenic hematopoietic cells (Fig. 1)

Hematopoiesis has an evolutionarily conserved relationship with vascular development [90]. HSCs adhere closely to ECs at various sites in the embryo, including the yolk sac and the dorsal aorta [91,92]. In turn, yolk sac ECs can support the proliferation of multipotent hematopoietic stem cells [93]. Furthermore, as mentioned above, HCs and ECs are believed to originate from a common precursor cell, known as the hemangioblast [94,95]. Accordingly, in the adult, substantial evidence indicates that beside EPCs, hematopoietic lineage cells also support the process of tumor vascularization, although most of them are localized to periendothelial tumor sites.

Mast cells (MCs) participate in various angiogenesis-dependent diseases/states including rheumatoid arthritis, ovulation, wound healing and tumor growth. Accordingly, several MC mediators are angiogenic and control EC proliferation and function. MCs express interleukin-8 (IL-8), MMPs, basic FGF, TNF- $\alpha$  and VEGF. Moreover, they can enhance tumor vascularization indirectly by producing MC-specific serine proteases (MCP-4 and MCP-6) that activate pro-MMPs (reviewed in ref. [96]). MCs are also able to produce histamine and heparin, which can stimulate EC sprouting (directly or indirectly by the stabilization of growth factors) and may have a role in the leakiness of immature tumor capillaries. Finally, reduction of tumor MC density/function has been shown to inhibit angiogenesis and, therefore, tumor growth [97,98].

Most cancers appear to be infiltrated by tumor-associated macrophages (TAMs), which can comprise more than 50% of the total tumor mass. Depending on forms of activation, macrophages can show two general types of polarization, M1 and M2 (described in detail in ref. [99]). Although M1-polarized macrophages have the potential to kill tumor cells, many observations indicate that TAMs more likely represent an M2-polarized macrophage population exhibiting protumor functions, including the secretion of angiogenic molecules [99,100]. Indeed, in most human studies, macrophage infiltration of the tumor was associated with poor prognosis, and generally correlated with vascular density [101–103].

A heterogeneous population of cells sharing their differentiation pathway with TAMs is designated myeloid-derived suppressor cells (MDSCs). MDSCs are myelomonocytic

cells lacking the markers of mature myeloid cells and expressing CD11b and Gr-1 in mice. They have been reported to accumulate in tumor-bearing hosts and to suppress T-cell-mediated antitumor immune responses by diverse mechanisms [100,104,105]. Furthermore, when co-injected with tumor cells, CD11b<sup>+</sup>Gr-1<sup>+</sup> cells promoted tumor vascularization by producing MMP-9, and were also found to directly incorporate into tumor capillaries [106]. However, the human equivalents of MDSCs are less well characterized, although immunosuppressive granulocyte subpopulations and immature myeloid cells have been described in several cancer types [107–109].

TIE2-expressing monocytes (TEMs, a subset of circulating and tumor-infiltrating monocytes), identified by De Palma et al. [68], are recruited to periendothelial positions and enhance tumor vascularization in a paracrine manner in mice. They are CD11b<sup>+</sup>/CD45<sup>+</sup>/TIE2<sup>+</sup> cells, but do not express VEGFR2 or any established EC or pericyte-associated markers (e.g. CD31, CD34 or  $\alpha$ SMA and NG2). In subsequent studies, the same authors demonstrated that TEMs are specifically recruited to spontaneously arising murine pancreatic carcinomas and to human glioma xenografts [110]. The surface-marker profile and angiogenic behavior of human TEMs were found to be reminiscent of those of previously described murine TEMs [111].

In contrast to TAMs, most studies on infiltrating DCs have demonstrated that high DC density in tumors is associated with good prognosis and reduced incidence of recurrent disease in various malignancies [112–114]. However, some DC subsets, such as CD123<sup>+</sup>/CD303<sup>+</sup> plasmacytoid DCs or immature or incompletely matured DCs have been suggested to mediate tolerance instead of immune activation [104,115]. It is also important to note that tumor-derived factors, such as VEGF and TGF- $\beta$ , can inhibit functional maturation of DCs [116,117] and that VEGF expression negatively correlated with DC density in tumors [112,116,118].

Recent results also suggest that different DC subtypes express and release a wide range of pro- and antiangiogenic molecules depending on their activation status and cytokine milieu. A major subset of DCs, MHC II<sup>+</sup>/CD11c<sup>+</sup> myeloid DCs, for example was shown to express the proangiogenic molecules VEGF, bFGF, TNF- $\alpha$ , IL-6 and the antiangiogenic cytokines IL-10, IL-12, IL-18 and TSP-1 (thrombospondin-1) as well. Similarly, depending on the stimulus, plasmacytoid DCs, the other major DC subtype, can also release both angiogenic (TNF- $\alpha$ , CXCL8) and angiostimulatory IFN- $\alpha$  (interferon- $\alpha$ ) molecules (reviewed in ref. [119]). Moreover, a novel DC subpopulation (CD11c<sup>+</sup>/CCR6<sup>+</sup>/MHC II<sup>+</sup> DC precursors, tumor-associated DCs, TADCs, Table 1) that supports tumor vascularization was described recently by Conejo-Garcia et al. [120,121]. In their experiments, these authors found that  $\beta$ -defensins recruited dendritic precursors through CCR6 into the tumor, where VEGF-A transformed them into endothelial-like cells. Unlike TEMs, these cells mainly migrated to the endothelial tubes, becoming true endothelial-like cells. All in all, DCs

might enhance tumor vascularization by two different but possibly interconnected mechanisms: by promoting endothelial sprouting through the expression of angiogenic cytokines and by supporting vasculogenesis via trans-differentiation into endothelial-like cells (reviewed in ref. [119]).

Further proangiogenic HCs that have been directly implicated in tumor vascularization include tumor-associated stromal cells (TASCs) [122], recruited blood circulating cells (RBCCs) [123] and VEGFR1<sup>+</sup> hematopoietic progenitors [65] (Table 1).

Tumor-associated stromal cells (TASCs) were described by Udagawa et al. [122]. These CD45<sup>+</sup>/VEGFR2<sup>+</sup> double positive cells have the ability to enhance tumor angiogenesis, although are minimally recruited into the tumor capillary walls. Instead, these authors suggested that TASCs might indirectly augment tumor vascularization in a paracrine manner by inducing or increasing the angiogenic molecules that stimulate *in situ* vessel formation (endothelial sprouting).

Like TEMs and TASCs, RBCCs [123] were shown to support new blood vessel growth via secreting proangiogenic factors from a perivascular position. RBCCs are positive for CD45, CD11b, CXCR4 and VEGFR1 but not for VEGFR2, indicating that they are recruited by VEGF and CXCL12 and are predominantly hematopoietic in nature. It is also important to note that Lyden et al. recently described VEGFR1<sup>+</sup> hematopoietic progenitors that proliferate in the BM, mobilize to the bloodstream along with VEGFR2<sup>+</sup> EPCs, and incorporate into pericapillary connective tissue, thereby stabilizing tumor vasculature [65]. More interestingly, these cells appear to home in before the metastatic tumor cells arrive to the target organ, promoting the metastatic process by forming niches where cancer cells can locate and proliferate [124]. However, to what extent these VEGFR1<sup>+</sup> progenitors overlap with RBCCs remains to be elucidated.

The aforementioned studies together with Harraz et al.'s [125] suggestion that CD34-angioblasts are a subset of CD14<sup>+</sup> monocytic cells, Rehman et al.'s [126] demonstration of the isolation of CACs from the monocyte/macrophage fraction of PB, and Yoder et al.'s [127] finding that CFU-ECs expressed colony-stimulating factor-1 receptor (CSF-1R) and actively phagocytosed *Escherichia coli* highlighted the ability of proangiogenic HCs to enhance tumor vascularization. These studies also demonstrated that hematopoietic and endothelial lineage cells share functional and phenotypical features, including the expression of common metabolic and surface molecules, as well as the capacity to shape vascular-like structures. However, these experiments with the cell populations growing in the above-described cell cultures containing the PB mononuclear cell fraction have also led to some controversy over whether CACs and CFU-ECs represent EPCs or in fact identify monocytes/macrophages. To clarify the complex nomenclature and the relationships among EPC types to mononuclear cell subtypes, an elegant working hypothesis was suggested recently by Prater et al. [38]. According to the proposal of these authors, CACs represent the largest population of cultured EPC types, comparable in

size to PB monocytes, which are hypothesized to belong to the CAC population. These authors also suggested that CD45<sup>+</sup> proangiogenic HCs overlap with CFU-ECs to an undefined degree and that ECFCs are included in the CEC population.

It is also important to note that beside the above described proangiogenic HCs that are directly implicated in tumor vascularization, other HC types such as polymorphonuclear cells (PMNs), NK cells and T and B lymphocytes may also participate in the vascularization process. Activated PMNs are reported to secrete a number of angiogenic molecules including MMPs, VEGF and IL-8. However, the PMN population was demonstrated to be a source of endogenous angiogenesis inhibitors (such as angiostatin, IL-12-inducible protein 10, and IFN- $\gamma$ ) as well [5]. To make the picture more complex, PMNs also secrete chemotactic factors to recruit other PMNs, monocytes, T cell subsets and immature DCs [5]. Regarding lymphocytes, Qin et al. found that the primary mechanism of tumor rejection by CD8<sup>+</sup> T cells in mouse models was angiostasis mediated by IFN- $\gamma$  [128]. On the other hand, production of VEGF by tumor-infiltrating T cells has been described, which could play a role in tumor angiogenesis [129]. In cutaneous melanoma, we found a correlation between peritumoral microvessel density and the infiltration by T cells [130]. In some animal models, a role of NK cells as angiogenesis inhibitors via IL-12 and IFN- $\gamma$  secretion has been suggested [131]. Few data are available on the effect of B lymphocytes on tumor vascularity; in a transgenic mouse model, transfer of B cells from HPV16 mice into T and B cell-deficient/HPV16 mice restored chronic inflammation in premalignant skin and reinstated regulatory mechanisms necessary for angiogenesis [132]. Taken together, although all these cell types have been reported to express a wide repertoire of pro- and antiangiogenic factors, their "angiogenic function" has been poorly investigated and their exact role in the blood supply of tumors remains unclear (reviewed in ref. [103]).

In summary, tumor-derived angiogenic factors do not merely trigger the release of EPCs, but also enhance the co-mobilization of proangiogenic HCs to the tumor vascular network and/or stroma. This co-recruitment of different lineages may support capillary sprouting and stabilization of immature cancer capillaries through the release of additional proangiogenic factors or by generating permissive conditions in the tumor stroma that favor the survival and/or growth of preexisting tumor vessels.

## 5. Antiangiogenic and/or anticancer therapy via EPCs, proangiogenic HCs and CECs

One of the greatest hopes for the study of EPCs and, to a lesser extent, of proangiogenic HCs and CECs, is their potential use in cancer therapy as cellular vehicles for delivering suicide genes, toxins or antiangiogenic molecules. These novel anticancer techniques, typically with the *ex vivo* manipulation of these cells, have been applied to transplantation

models and, to some extent, have reduced cancer progression. For example, Ferrari et al. transduced human EPCs with a retroviral vector expressing the herpes simplex virus thymidine kinase (HSV-TK) transgene, and then injected these cells intravenously into sublethally irradiated mice bearing subcutaneous or intracranial tumors. Ganciclovir treatment resulted in significant tumor regression in mice previously injected by TK-expressing EPCs with no systemic toxicity [133]. In a similar study, unsorted murine BM cells were transduced with a retroviral vector to express truncated soluble VEGFR2 (tsVEGFR2) together with green fluorescent protein (GFP) or GFP alone. The subsequent experiments have demonstrated that GFP-positive BM-derived cells contributed to tumor capillaries and, when modified to express the angiogenesis inhibitor tsVEGFR2, restricted tumor growth [134]. In another gene delivery approach, ex vivo expanded EPCs that were genetically modified with a suicide gene specifically and efficiently eradicated hypoxic lung metastases [135].

Differentiated endothelial cells have also been employed for experimental tumor therapy. In a murine metastatic melanoma model, the intravenous administration of genetically modified CECs expressing a human IL-2 transgene abrogated the tumor metastases and prolonged survival of the animals [136]. Similarly, co-injections of HSV-TK-expressing ECs and tumor cells reduced in vivo tumor growth and provided a statistically significant survival benefit in experimental animals [137].

Finally, the observation that proangiogenic HCs are able to support tumor growth and home to sites of active angiogenesis suggests that these cells may provide the means for selective gene delivery and targeted inhibition of tumor angiogenesis as well. Consistent with this hypothesis, De Palma et al. transduced TEMs with lentiviral vectors expressing genes from transcription-regulatory elements of TIE2/TEK gene and achieved a substantial inhibition of angiogenesis and slower tumor growth without systemic toxicity by delivering a “suicide” gene [68].

Taken together, the use of the above cells in cancer therapy as cellular vehicles for delivering suicide genes, toxins or anticancer/antiangiogenic agents opens new ways to hinder tumor growth. However, given the existence of alternative vascularization mechanisms in cancer [4], the different EPC and CEC counts reported in various tumor models/cancer types, the association of EPC levels with the histological type/stage of the tumor, and the unresolved question of whether or not CECs are active participants in tumor vascularization, the applicability of these cell populations as “Trojan horses” in anticancer therapy certainly needs further investigation.

## 6. EPCs and CECs: potential biomarkers of tumor angiogenesis?

The efficacy of conventional antitumor treatments (i.e. chemo- and radiotherapy) is typically assessed by measuring

their direct effects on tumor size and/or survival. Because antiangiogenic drugs specifically target the tumor vasculature, in case of these treatments, assessment of the above parameters is an inadequate strategy and we need to be able to evaluate the biological effects of antiangiogenic drugs on the tumor capillaries independently of their general anticancer activity.

The biomarkers used to measure the efficacy of therapeutic drugs of any type can be classified as either direct or surrogate (indirect) in nature. In case of antiangiogenic drugs, the direct biomarker is the actual capillary network in the tumor, which is generally difficult to depict and quantify [138]. Consequently, reliable surrogate markers are needed that indirectly indicate the effect of antiangiogenic therapy on tumoral blood vessels and that can help to identify patients responsive to these therapies, recognize resistance and predict the efficacy of combinations that include antiangiogenic drugs [139,140].

Although currently no single reliable biomarker is available, encouraging results from different disciplines have been reported.

One of the potential strategies is the measurement of serum/plasma angiogenic cytokine and/or soluble growth factor receptor levels in the blood and/or urine. For example, plasma concentrations of total VEGF and PlGF were observed to be significantly elevated in bevacizumab-treated colon carcinoma patients [87]. In another clinical study on patients with colorectal cancer, an elevation of plasma VEGF-A and bFGF was found following the first cycle of PTK787/ZK222584 (an angiogenesis inhibitor targeting all known VEGF receptor tyrosine kinases) treatment [141]. Similarly, a progressive increase of total VEGF levels after initiation of treatment with bevacizumab in renal cancer was reported [142]. However, the use of cytokines as biomarkers of angiogenesis is complicated by the release of angiogenic growth factors from platelets and there are several studies of angiogenic molecules as surrogate markers that have yielded inconclusive evidence of their reliability [139].

Measuring the efficacy of antivascular therapy could also be achieved by imaging the tumor capillaries themselves (i.e. direct imaging by agents targeted at cytokines or receptors involved in tumor vascularization) or investigating the result of such treatments on the anatomic features and the blood supply of tumors (indirect imaging). Currently, almost all direct techniques are available solely in murine models, whereas indirect techniques are typically used in clinical settings [138]. Accordingly, with the exceptions of a few recent studies [143,144], experience with vascular imaging in human studies has been gained primarily by indirect techniques. These include measurements of contrast enhancement, blood volume and oxygen saturation with computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and single photon emission computed tomography (SPECT) (reviewed in ref. [138]).

Circulating EPC/CEC levels as surrogate markers of angiogenesis have also been investigated recently. Based

on results from studies on murine tumor systems, there is a clear relationship between tumor burdens and EPC/CEC counts in the PB [145]. Moreover, circulating CEC and EPC counts have also been demonstrated to change with anti-cancer/antiangiogenic treatments in preclinical models. For example, as mentioned above, Bertolini et al. found that when tumorous mice are treated with MTD chemotherapy, there is a marked elevation in EPC counts in the PB during the drug-free break periods. This tendency was not observed when the same drug was administered metronomically. In fact, the opposite was reported; namely, almost total suppression of EPC and CEC numbers and viabilities [73]. In other murine studies, treatment of tumor-bearing mice with vascular disrupting agents (VDAs) led to an abrupt release of EPCs, which incorporated into the capillaries of viable peripheral tumor areas (tumor rims) that characteristically survive after such treatment. Suppression of this EPC mobilization by antiangiogenic agents resulted in marked reductions in tumor rim size and blood flow as well [146]. Moreover, endostatin decreased EPC numbers in the PB along with tumor regression [147,148], and VEGFR2 targeting caused a dose-dependent decrease in EPC counts that paralleled the anticancer activity of the experimental agent [149].

More importantly, techniques for EPC/CEC detection and counting have been tested in the clinics [9], and studies have been undertaken measuring the numbers of these cells in cancer patients treated with antiangiogenic therapies. Particularly encouraging in this regard is a phase 1 trial in which bevacizumab, an anti-VEGF antibody, decreased tumor perfusion, vascular volume, microvascular density, interstitial fluid pressure and the number of EPCs and CD31<sup>bright</sup>/CD45<sup>-</sup> viable CECs in rectal carcinoma patients [86,87]. Interestingly, in a vigorously criticized [150] subsequent study [151], the investigators of the above trial were unable to detect significant changes in CD146<sup>+</sup> CEC levels during VEGF blockade.

Subsequent studies have yielded promising but sometimes inconsistent results likely dependent on the type and stage of the malignant disease and, moreover, on the therapeutic regime and enumeration technique chosen. In a phase I/II study of patients with imatinib-resistant metastatic GIST, the authors investigated plasma and PB cellular biomarkers for sunitinib malate, a multitargeted tyrosine kinase inhibitor with activity against VEGFR1, VEGFR2, VEGFR3, PDGF receptor, KIT, and FLT-3 (FMS-like tyrosine kinase 3) and found that changes in CECs, but not the plasma markers (VEGF and soluble VEGFR2), differed between the patients with clinical benefit and those with progressive disease [27]. A phase II prospective study of low-dose cyclophosphamide given continuously (i.e. metronomically) in combination with celecoxib in adult patients with relapsed or refractory aggressive non-Hodgkin's lymphoma has recently demonstrated that CECs and EPCs declined and remained low in responders, whereas plasma VEGF tended to decline in responding patients but increase in nonresponders [152]. Similarly, CEC

and EPC counts were found to be correlated with disease activity (i.e. levels of serum M protein and  $\beta_2$  microglobulin) and response to thalidomide therapy in multiple myeloma [23]. Moreover, in a phase I trial of pediatric patients with refractory solid tumors, although not statistically significantly, CECs tended to increase with bevacizumab therapy [153]. There are other studies, however, in which no correlation between circulating levels of CECs/EPCs and tumor progression/response was found. For example, in a phase I study of patients with refractory solid malignancies, the differences in the numbers of CECs and EPCs between patients and controls were not statistically significant and, furthermore, no changes in the levels of these cells were observed during low-dose cyclophosphamide and celecoxib or low-dose etoposide and celecoxib therapy [154]. Similarly, in a phase I study of the protein kinase C  $\beta$  inhibitor enzastaurin in combination with gemcitabine and cisplatin in patients with advanced tumors, the single-agent enzastaurin had no effect on any of the angiogenesis biomarkers analyzed (circulating CEC levels and mRNA expression of CD133 and CD146 in the PB).

As antivasular therapies for cancer become increasingly integrated into routine oncology care, there is an urgent need for the proper selection of the patients most likely to benefit from these treatments. The results described above are particularly significant in this regard, as they may establish the role of EPC/CEC quantification not only in the evaluation of the efficacy of antiangiogenic treatments, but also in the definition of optimal biologic dose ranges as well. More studies, however, are needed to expand and validate these initial findings.

## 7. Conclusions

The key role of the vasculature during tumor progression is unquestionable. Moreover, it is becoming clear that BM-derived EPCs and proangiogenic HCs are involved in the process of neovascularization and that CEC and EPC levels can be biomarkers of targeted anticancer/antivasular therapies. Recent reports also suggest that BM-derived circulating cells can be used as cellular vehicles to deliver anticancer agents. Questions remain, however, regarding the precise functional and phenotypic nature of these circulating cells and whether certain HCs will have any value as biomarkers as well. Hence, further studies and consensus is required regarding the phenotype and enumeration approaches of these cell populations in order to help define their optimal role in clinical oncology.

## Reviewers

Douglas Noonan, Ph.D., Assistant Professor, University of Insubria, Department of Clinical and Biological Science, Viale Borri 57, I-21100 Varese, Italy.



Johann de Bono, Ph.D., Royal Marsden Hospital, Centre for Cancer Therapeutics, Institute for Cancer Research, Downs Road, Sutton, Surrey SM2 5PT, United Kingdom.

### Conflicts of interest

The authors indicate no potential conflicts of interest.

### Acknowledgements

Balazs Dome is a recipient of the Bolyai Janos Scholarship of the Hungarian Academy of Sciences. Jozsef Tovari is a recipient of the Eotvos Hungarian State Fellowship. Further support: GVOP-2004-3.2.1, KFIIF 2005, OTKA-F68916, OTKA-NK-73082 (B.D.); Hungarian Ministry of Education, NKFP1a-0024-05 (B.D.; J.Ti.); OTKA-D048519, OTKA-F046501 (J.To.); OTKA-NK-73119 (S.P.); OTKA-NK-72595 (J.Ti.); OTKA K72836 (A.L.).

### References

- [1] Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971;285:1182–6.
- [2] Asahara T, Kawamoto A. Endothelial progenitor cells for postnatal vasculogenesis. *Am J Physiol Cell Physiol* 2004;287:C572–9.
- [3] Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;275:964–7.
- [4] Dome B, Hendrix MJ, Paku S, Tovari J, Timar J. Alternative vascularization mechanisms in cancer: Pathology and therapeutic implications. *Am J Pathol* 2007;170:1–15.
- [5] Brigati C, Noonan DM, Albini A, Benelli R. Tumors and inflammatory infiltrates: friends or foes? *Clin Exp Metastasis* 2002;19:247–58.
- [6] Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996;86:353–64.
- [7] De Palma M, Naldini L. Role of haematopoietic cells and endothelial progenitors in tumour angiogenesis. *Biochim Biophys Acta* 2006;1766:159–66.
- [8] Schmid MC, Varner JA. Myeloid cell trafficking and tumor angiogenesis. *Cancer Lett* 2007;250:1–8.
- [9] Blann AD, Woywodt A, Bertolini F, et al. Circulating endothelial cells. Biomarker of vascular disease. *Thromb Haemost* 2005;93:228–35.
- [10] Hladovec J. Circulating endothelial cells as a sign of vessel wall lesions. *Physiol Bohemoslov* 1978;27:140–4.
- [11] Wright HP, Glacometti NJ. Circulating endothelial cells and arterial endothelial mitosis in anaphylactic shock. *Br J Exp Pathol* 1972;53:1–4.
- [12] Sbarbati R, de Boer M, Marzilli M, Scarlattini M, Rossi G, van Mourik JA. Immunologic detection of endothelial cells in human whole blood. *Blood* 1991;77:764–9.
- [13] George F, Brisson C, Poncelet P, et al. Rapid isolation of human endothelial cells from whole blood using S-Endo1 monoclonal antibody coupled to immuno-magnetic beads: demonstration of endothelial injury after angioplasty. *Thromb Haemost* 1992;67:147–53.
- [14] Bardin N, George F, Mutin M, et al. S-Endo 1, a pan-endothelial monoclonal antibody recognizing a novel human endothelial antigen. *Tissue Antigens* 1996;48:531–9.
- [15] George F, Brouqui P, Boffa MC, et al. Demonstration of Rickettsia conorii-induced endothelial injury in vivo by measuring circulating endothelial cells, thrombomodulin, and von Willebrand factor in patients with Mediterranean spotted fever. *Blood* 1993;82:2109–16.
- [16] Khan SS, Solomon MA, McCoy Jr JP. Detection of circulating endothelial cells and endothelial progenitor cells by flow cytometry. *Cytometry B Clin Cytom* 2005;64:1–8.
- [17] Goon PK, Boos CJ, Stonelake PS, Lip GY. Circulating endothelial cells in malignant disease. *Future Oncol* 2005;1:813–20.
- [18] Woywodt A, Goldberg C, Scheer J, Regelsberger H, Haller H, Haubitza M. An improved assay for enumeration of circulating endothelial cells. *Ann Hematol* 2004;83:491–4.
- [19] Duda DG, Cohen KS, Scadden DT, Jain RK. A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. *Nat Protoc* 2007;2:805–10.
- [20] Strijbos MH, Kraan J, den Bakker MA, Lambrecht BN, Sleijfer S, Gratama JW. Cells meeting our immunophenotypic criteria of endothelial cells are large platelets. *Cytometry B Clin Cytom* 2007;72:86–93.
- [21] Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, Bertolini F. Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. *Blood* 2001;97:3658–61.
- [22] Beerepoot LV, Mehra N, Vermaat JS, Zonnenberg BA, Gebbink MF, Voest EE. Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients. *Ann Oncol* 2004;15:139–45.
- [23] Zhang H, Vakil V, Braunstein M, et al. Circulating endothelial progenitor cells in multiple myeloma: implications and significance. *Blood* 2005;105:3286–94.
- [24] Wierzbowska A, Robak T, Krawczynska A, et al. Circulating endothelial cells in patients with acute myeloid leukemia. *Eur J Haematol* 2005;75:492–7.
- [25] Furstemberger G, von Moos R, Lucas R, et al. Circulating endothelial cells and angiogenic serum factors during neoadjuvant chemotherapy of primary breast cancer. *Br J Cancer* 2006;94:524–31.
- [26] Rowand JL, Martin G, Doyle GV, et al. Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. *Cytometry A* 2007;71:105–13.
- [27] Norden-Zfoni A, Desai J, Manola J, et al. Blood-based biomarkers of SU11248 activity and clinical outcome in patients with metastatic imatinib-resistant gastrointestinal stromal tumor. *Clin Cancer Res* 2007;13:2643–50.
- [28] Della Porta MG, Malcovati L, Rigolin GM, et al. Immunophenotypic, cytogenetic and functional characterization of circulating endothelial cells in myelodysplastic syndromes. *Leukemia* 2007;(December) [Epub ahead of print].
- [29] Go RS, Jobe DA, Asp KE, et al. Circulating endothelial cells in patients with chronic lymphocytic leukemia. *Ann Hematol* 2008;(January) [Epub ahead of print].
- [30] Burger PE, Coetzee S, McKeehan WL, et al. Fibroblast growth factor receptor-1 is expressed by endothelial progenitor cells. *Blood* 2002;100:3527–35.
- [31] Massa M, Rosti V, Ferrario M, et al. Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction. *Blood* 2005;105:199–206.
- [32] Heissig B, Werb Z, Rafii S, Hattori K. Role of c-kit/Kit ligand signaling in regulating vasculogenesis. *Thromb Haemost* 2003;90:570–6.
- [33] Hristov M, Erl W, Weber PC. Endothelial progenitor cells: mobilization, differentiation, and homing. *Arterioscler Thromb Vasc Biol* 2003;23:1185–9.
- [34] Rafii S, Lyden D, Benezra R, Hattori K, Heissig B. Vascular and hematopoietic stem cells: novel targets for anti-angiogenesis therapy? *Nat Rev Cancer* 2002;2:826–35.
- [35] Wognum AW, Eaves AC, Thomas TE. Identification and isolation of hematopoietic stem cells. *Arch Med Res* 2003;34:461–75.
- [36] Peichev M, Naiyer AJ, Pereira D, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood* 2000;95:952–8.

- [37] Kalka C, Masuda H, Takahashi T, et al. Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects. *Circ Res* 2000;86:1198–202.
- [38] Prater DN, Case J, Ingram DA, Yoder MC. Working hypothesis to redefine endothelial progenitor cells. *Leukemia* 2007;21:1141–9.
- [39] Hill JM, Zalos G, Halcox JP, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003;348:593–600.
- [40] Dimmeler S, Zeiher AM. Endothelial cell apoptosis in angiogenesis and vessel regression. *Circ Res* 2000;87:434–9.
- [41] Kalka C, Masuda H, Takahashi T, et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci USA* 2000;97:3422–7.
- [42] Yoon CH, Hur J, Park KW, et al. Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: the role of angiogenic cytokines and matrix metalloproteinases. *Circulation* 2005;112:1618–27.
- [43] Khakoo AY, Finkel T. Endothelial progenitor cells. *Annu Rev Med* 2005;56:79–101.
- [44] Rafii S, Heissig B, Hattori K. Efficient mobilization and recruitment of marrow-derived endothelial and hematopoietic stem cells by adenoviral vectors expressing angiogenic factors. *Gene Ther* 2002;9:631–41.
- [45] Asahara T, Takahashi T, Masuda H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J* 1999;18:3964–72.
- [46] Ho JW, Pang RW, Lau C, et al. Significance of circulating endothelial progenitor cells in hepatocellular carcinoma. *Hepatology* 2006;44:836–43.
- [47] Takahashi T, Kalka C, Masuda H, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* 1999;5:434–8.
- [48] Natori T, Sata M, Washida M, Hirata Y, Nagai R, Makuuchi M. G-CSF stimulates angiogenesis and promotes tumor growth: potential contribution of bone marrow-derived endothelial progenitor cells. *Biochem Biophys Res Commun* 2002;297:1058–61.
- [49] Bahlmann FH, De Groot K, Spandau JM, et al. Erythropoietin regulates endothelial progenitor cells. *Blood* 2004;103:921–6.
- [50] Heeschen C, Aicher A, Lehmann R, et al. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood* 2003;102:1340–6.
- [51] Aicher A, Zeiher AM, Dimmeler S. Mobilizing endothelial progenitor cells. *Hypertension* 2005;45:321–5.
- [52] Sinclair AM, Todd MD, Forsythe K, Knox SJ, Elliott S, Begley CG. Expression and function of erythropoietin receptors in tumors: implications for the use of erythropoiesis-stimulating agents in cancer patients. *Cancer* 2007;110:477–88.
- [53] Lövey J, Bereczky B, Gilly R, et al. Recombinant human erythropoietin alpha improves the efficacy of radiotherapy of a human tumor xenograft, affecting tumor cells and microvessels. *Strahlenther Onkol* 2008;184:1–7.
- [54] Tóvári J, Gilly R, Rásó E, et al. Recombinant human erythropoietin alpha targets intratumoral blood vessels, improving chemotherapy in human xenograft models. *Cancer Res* 2005;65:7186–93.
- [55] Li B, Sharpe EE, Maupin AB, et al. VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. *FASEB J* 2006;20:1495–7.
- [56] Moore MA, Hattori K, Heissig B, et al. Mobilization of endothelial and hematopoietic stem and progenitor cells by adenovector-mediated elevation of serum levels of SDF-1, VEGF, and angiopoietin-1. *Ann N Y Acad Sci* 2001;938:36–47.
- [57] Li X, Tjwa M, Moons L, et al. Revascularization of ischemic tissues by PDGF-CC via effects on endothelial cells and their progenitors. *J Clin Invest* 2005;115:118–27.
- [58] Hattori K, Dias S, Heissig B, et al. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. *J Exp Med* 2001;193:1005–14.
- [59] Aicher A, Heeschen C, Mildner-Rihm C, et al. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med* 2003;9:1370–6.
- [60] Llevadot J, Murasawa S, Kureishi Y, et al. HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells. *J Clin Invest* 2001;108:399–405.
- [61] Strehlow K, Werner N, Berweiler J, et al. Estrogen increases bone marrow-derived endothelial progenitor cell production and diminishes neointima formation. *Circulation* 2003;107:3059–65.
- [62] Laufs U, Werner N, Link A, et al. Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation* 2004;109:220–6.
- [63] Seeger FH, Haendeler J, Walter DH, et al. p38 mitogen-activated protein kinase downregulates endothelial progenitor cells. *Circulation* 2005;111:1184–91.
- [64] Verma S, Kuliszewski MA, Li SH, et al. C-reactive protein attenuates endothelial progenitor cell survival, differentiation, and function: further evidence of a mechanistic link between C-reactive protein and cardiovascular disease. *Circulation* 2004;109:2058–67.
- [65] Lyden D, Hattori K, Dias S, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* 2001;7:1194–201.
- [66] Garcia-Barros M, Paris F, Cordon-Cardo C, et al. Tumor response to radiotherapy regulated by endothelial cell apoptosis. *Science* 2003;300:1155–9.
- [67] Goon PK, Lip GY, Boos CJ, Stonelake PS, Blann AD. Circulating endothelial cells, endothelial progenitor cells, and endothelial microparticles in cancer. *Neoplasia* 2006;8:79–88.
- [68] De Palma M, Venneri MA, Roca C, Naldini L. Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat Med* 2003;9:789–95.
- [69] Gothert JR, Gustin SE, van Eekelen JA, et al. Genetically tagging endothelial cells in vivo: bone marrow-derived cells do not contribute to tumor endothelium. *Blood* 2004;104:1769–77.
- [70] Spring H, Schuler T, Arnold B, Hammerling GJ, Ganss R. Chemokines direct endothelial progenitors into tumor neovessels. *Proc Natl Acad Sci USA* 2005;102:18111–6.
- [71] Ruzinova MB, Schoer RA, Gerald W, et al. Effect of angiogenesis inhibition by Id loss and the contribution of bone-marrow-derived endothelial cells in spontaneous murine tumors. *Cancer Cell* 2003;4:277–89.
- [72] Browder T, Butterfield CE, Kraling BM, et al. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res* 2000;60:1878–86.
- [73] Bertolini F, Paul S, Mancuso P, et al. Maximum tolerable dose and low-dose metronomic chemotherapy have opposite effects on the mobilization and viability of circulating endothelial progenitor cells. *Cancer Res* 2003;63:4342–6.
- [74] Urbich C, Aicher A, Heeschen C, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. *J Mol Cell Cardiol* 2005;39:733–42.
- [75] Gao D, Nolan DJ, Mellick AS, Bambino K, McDonnell K, Mittal V. Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis. *Science* 2008;319:195–8.
- [76] Peters BA, Diaz LA, Polyak K, et al. Contribution of bone marrow-derived endothelial cells to human tumor vasculature. *Nat Med* 2005;11:261–2.
- [77] Igreja C, Courinha M, Cachaco AS, et al. Characterization and clinical relevance of circulating and biopsy-derived endothelial progenitor cells in lymphoma patients. *Haematologica* 2007;92:469–77.

- [78] Hilbe W, Dirnhofer S, Oberwasserlechner F, et al. CD133 positive endothelial progenitor cells contribute to the tumor vasculature in non-small cell lung cancer. *J Clin Pathol* 2004;57:965–9.
- [79] Yu D, Sun X, Qiu Y, et al. Identification and clinical significance of mobilized endothelial progenitor cells in tumor vasculogenesis of hepatocellular carcinoma. *Clin Cancer Res* 2007;13:3814–24.
- [80] Dome B, Timar J, Dobos J, et al. Identification and clinical significance of circulating endothelial progenitor cells in human non-small cell lung cancer. *Cancer Res* 2006;66:7341–7.
- [81] Mehra N, Penning M, Maas J, et al. Progenitor marker CD133 mRNA is elevated in peripheral blood of cancer patients with bone metastases. *Clin Cancer Res* 2006;12:4859–66.
- [82] Lin EH, Hassan M, Li Y, et al. Elevated circulating endothelial progenitor marker CD133 messenger RNA levels predict colon cancer recurrence. *Cancer* 2007;110:534–42.
- [83] Pircher A, Kähler CM, Skvortsov S, et al. Increased numbers of endothelial progenitor cells in peripheral blood and tumor specimens in non-small cell lung cancer: a methodological challenge and an ongoing debate on the clinical relevance. *Oncol Rep* 2008;19:345–52.
- [84] Richter-Ehrenstein C, Rentzsch J, Runkel S, Schneider A, Schönfelder G. Endothelial progenitor cells in breast cancer patients. *Breast Cancer Res Treat* 2007;106:343–9.
- [85] Naik RP, Jin D, Chuang E, et al. Circulating endothelial progenitor cells correlate to stage in patients with invasive breast cancer. *Breast Cancer Res Treat* 2008;107:133–8.
- [86] Willett CG, Boucher Y, di Tomaso E, et al. Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. *Nat Med* 2004;10:145–7.
- [87] Willett CG, Boucher Y, Duda DG, et al. Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients. *J Clin Oncol* 2005;23:8136–9.
- [88] Massa M, Rosti V, Ramajoli I, et al. Circulating CD34+, CD133+, and vascular endothelial growth factor receptor 2-positive endothelial progenitor cells in myelofibrosis with myeloid metaplasia. *J Clin Oncol* 2005;23:5688–95.
- [89] Zheng PP, Hop WC, Luider TM, Sillevs Smitt PA, Kros JM. Increased levels of circulating endothelial progenitor cells and circulating endothelial nitric oxide synthase in patients with gliomas. *Ann Neurol* 2007;62:40–8.
- [90] Takakura N. Role of hematopoietic lineage cells as accessory components in blood vessel formation. *Cancer Sci* 2006;97:568–74.
- [91] Moore MA, Metcalf D. Ontogeny of the haemopoietic system: yolk sac origin of *in vivo* and *in vitro* colony forming cells in the developing mouse embryo. *Br J Haematol* 1970;18:279–96.
- [92] Dieterlen-Lièvre F, Martin C. Diffuse intraembryonic hemopoiesis in normal and chimeric avian development. *Dev Biol* 1981;88:180–91.
- [93] Lu LS, Wang SJ, Auerbach R. *In vitro* and *in vivo* differentiation into B cells, T cells, and myeloid cells of primitive yolk sac hematopoietic precursor cells expanded >100-fold by coculture with a clonal yolk sac endothelial cell line. *Proc Natl Acad Sci USA* 1996;93:14782–7.
- [94] Prindull G. Hemangioblasts representing a functional endothelio-hematopoietic entity in ontogeny, postnatal life, and CML neovascularogenesis. *Stem Cell Rev* 2005;1:277–84.
- [95] Forrai A, Robb L. The hemangioblast-between blood and vessels. *Cell Cycle* 2003;2:86–90.
- [96] Nienartowicz A, Sobaniec-Lotowska ME, Jarocka-Cyrta E, Lemancewicz D. Mast cells in neoangiogenesis. *Med Sci Monit* 2006;12:RA53–6.
- [97] Zhang W, Stoica G, Tasca SI, Kelly KA, Meininger CJ. Modulation of tumor angiogenesis by stem cell factor. *Cancer Res* 2000;60:6757–62.
- [98] Coussens LM, Raymond WW, Bergers G, et al. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. *Genes Dev* 1999;13:1382–97.
- [99] Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *Eur J Cancer* 2006;42:717–27.
- [100] Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. *J Clin Invest* 2007;117:1155–66.
- [101] Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res* 1996;56:4625–9.
- [102] Takanami I, Takeuchi K, Kodaira S. Tumor-associated macrophage infiltration in pulmonary adenocarcinoma: association with angiogenesis and poor prognosis. *Oncology* 1999;57:138–42.
- [103] Dirx AE, Oude Egbrink MG, Wagstaff J, Griffioen AW. Monocyte/macrophage infiltration in tumors: modulators of angiogenesis. *J Leukoc Biol* 2006;80:1183–96.
- [104] Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* 2005;5:263–74.
- [105] Serafini P, Borrello I, Bronte V. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin Cancer Biol* 2006;16:53–65.
- [106] Yang L, DeBusk LM, Fukuda K, et al. Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* 2004;6:409–21.
- [107] Pak AS, Wright MA, Matthews JP, Collins SL, Petruzzelli GJ, Young MR. Mechanisms of immune suppression in patients with head and neck cancer: presence of CD34(+) cells which suppress immune functions within cancers that secrete granulocyte-macrophage colony-stimulating factor. *Clin Cancer Res* 1995;1:95–103.
- [108] Almand B, Clark JI, Nikitina E, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol* 2001;166:678–89.
- [109] Ochoa AC, Zea AH, Hernandez C, Rodriguez PC. Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma. *Clin Cancer Res* 2007;13(2 Pt 2):721s–6s.
- [110] De Palma M, Venneri MA, Galli R, et al. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell* 2005;8:211–26.
- [111] Venneri MA, De Palma M, Ponzoni M, et al. Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. *Blood* 2007;109:5276–85.
- [112] Inoshima N, Nakanishi Y, Minami T, et al. The influence of dendritic cell infiltration and vascular endothelial growth factor expression on the prognosis of non-small cell lung cancer. *Clin Cancer Res* 2002;8:3480–6.
- [113] Ishigami S, Natsugoe S, Matsumoto M, et al. Clinical implications of intratumoral dendritic cell infiltration in esophageal squamous cell carcinoma. *Oncol Rep* 2003;10:1237–40.
- [114] Ladanyi A, Kiss J, Somlai B, et al. Density of DC-LAMP(+) mature dendritic cells in combination with activated T lymphocytes infiltrating primary cutaneous melanoma is a strong independent prognostic factor. *Cancer Immunol Immunother* 2007;56:1459–69.
- [115] Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol* 2002;23:445–9.
- [116] Gabrilovich DI, Chen HL, Girgis KR, et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med* 1996;2:1096–103.
- [117] Geissmann F, Revy P, Regnault A, et al. TGF-beta 1 prevents the noncognate maturation of human dendritic Langerhans cells. *J Immunol* 1999;162:4567–75.
- [118] Saito H, Tsujitani S, Ikeguchi M, Maeta M, Kaibara N. Relationship between the expression of vascular endothelial growth factor and the density of dendritic cells in gastric adenocarcinoma tissue. *Br J Cancer* 1998;78:1573–7.
- [119] Sozzani S, Rusnati M, Riboldi E, Mitola S, Presta M. Dendritic cell-endothelial cell cross-talk in angiogenesis. *Trends Immunol* 2007;28:385–92.

- [120] Conejo-Garcia JR, Benencia F, Courreges MC, et al. Tumor-infiltrating dendritic cell precursors recruited by a beta-defensin contribute to vasculogenesis under the influence of Vegf-A. *Nat Med* 2004;10:950–8.
- [121] Conejo-Garcia JR, Buckanovich RJ, Benencia F, et al. Vascular leukocytes contribute to tumor vascularization. *Blood* 2005;105:679–81.
- [122] Udagawa T, Puder M, Wood M, Schaefer BC, D'Amato RJ. Analysis of tumor-associated stromal cells using SCID GFP transgenic mice: contribution of local and bone marrow-derived host cells. *FASEB J* 2006;20:95–102.
- [123] Grunewald M, Avraham I, Dor Y, et al. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell* 2006;124:175–89.
- [124] Kaplan RN, Riba RD, Zacharoulis S, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* 2005;438:820–7.
- [125] Harraz M, Jiao C, Hanlon HD, Hartley RS, Schatteman GC. CD34– blood-derived human endothelial cell progenitors. *Stem Cells* 2001;19:304–12.
- [126] Rehman J, Li J, Orschell CM, March KL. Peripheral blood “endothelial progenitor cells” are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation* 2003;107:1164–9.
- [127] Yoder MC, Mead LE, Prater D, et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood* 2007;109:1801–9.
- [128] Qin Z, Schwartzkopf J, Pradera F, et al. A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by CD8+ T cells. *Cancer Res* 2003;63:4095–100.
- [129] Freeman MR, Schneck FX, Gagnon ML, et al. Peripheral blood T lymphocytes and lymphocytes infiltrating human cancers express vascular endothelial growth factor: a potential role for T cells in angiogenesis. *Cancer Res* 1995;55:4140–5.
- [130] Kiss J, Tímár J, Somlai B, et al. Association of microvessel density with infiltrating cells in human cutaneous malignant melanoma. *Pathol Oncol Res* 2007;13:21–31.
- [131] Yao L, Sgadari C, Furuke K, Bloom ET, Teruya-Feldstein J, Tosato G. Contribution of natural killer cells to inhibition of angiogenesis by interleukin-12. *Blood* 1999;93:1612–21.
- [132] de Visser KE, Korets LV, Coussens LM. De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell* 2005;7:411–23.
- [133] Ferrari N, Glod J, Lee J, Kobiler D, Fine HA. Bone marrow-derived, endothelial progenitor-like cells as angiogenesis-selective gene-targeting vectors. *Gene Ther* 2003;10:647–56.
- [134] Davidoff AM, Ng CY, Brown P, et al. Bone marrow-derived cells contribute to tumor neovasculature and, when modified to express an angiogenesis inhibitor, can restrict tumor growth in mice. *Clin Cancer Res* 2001;7:2870–9.
- [135] Wei J, Blum S, Unger M, et al. Embryonic endothelial progenitor cells armed with a suicide gene target hypoxic lung metastases after intravenous delivery. *Cancer Cell* 2004;5:477–88.
- [136] Ojeifo JO, Lee HR, Rezza P, Su N, Zwiebel JA. Endothelial cell-based systemic gene therapy of metastatic melanoma. *Cancer Gene Ther* 2001;8:636–48.
- [137] Rancourt C, Robertson 3rd MW, Wang M, et al. Endothelial cell vehicles for delivery of cytotoxic genes as a gene therapy approach for carcinoma of the ovary. *Clin Cancer Res* 1998;4:265–70.
- [138] Provenzale JM. Imaging of angiogenesis: clinical techniques and novel imaging methods. *AJR Am J Roentgenol* 2007;188:11–23.
- [139] DePrimo SE, Bello C. Surrogate biomarkers in evaluating response to anti-angiogenic agents: focus on sunitinib. *Ann Oncol* 2007;18(Suppl. 10):x11–9.
- [140] Rüegg C, Meuwly JY, Driscoll R, Werffeli P, Zaman K, Stupp R. The quest for surrogate markers of angiogenesis: a paradigm for translational research in tumor angiogenesis and anti-angiogenesis trials. *Curr Mol Med* 2003;3:673–91.
- [141] Dreys J, Zirrgiebel U, Schmidt-Gersbach CI, et al. Soluble markers for the assessment of biological activity with PTK787/ZK 222584 (PTK/ZK), a vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor in patients with advanced colorectal cancer from two phase I trials. *Ann Oncol* 2005;16:558–65.
- [142] Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med* 2003;349:427–34.
- [143] Santimaria M, Moscatelli G, Viale GL, et al. Immunoscintigraphic detection of the ED-B domain of fibronectin, a marker of angiogenesis, in patients with cancer. *Clin Cancer Res* 2003;9:571–9.
- [144] Beer AJ, Grosu AL, Carlsen J, et al. [18F]Galacto-RGD positron emission tomography for imaging of alphavbeta3 expression on the neovasculature in patients with squamous cell carcinoma of the head and neck. *Clin Cancer Res* 2007;13(22 Pt 1):6610–6.
- [145] Bertolini F, Shaked Y, Mancuso P, Kerbel RS. The multifaceted circulating endothelial cell in cancer: towards marker and target identification. *Nat Rev Cancer* 2006;6:835–45.
- [146] Shaked Y, Ciarrocchi A, Franco M, et al. Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. *Science* 2006;313:1785–7.
- [147] Schuch G, Heymach JV, Nomi M, et al. Endostatin inhibits the vascular endothelial growth factor-induced mobilization of endothelial progenitor cells. *Cancer Res* 2003;63:8345–50.
- [148] Capillo M, Mancuso P, Gobbi A, et al. Continuous infusion of endostatin inhibits differentiation, mobilization, and clonogenic potential of endothelial cell progenitors. *Clin Cancer Res* 2003;9:377–82.
- [149] Shaked Y, Bertolini F, Man S, et al. Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis; implications for cellular surrogate marker analysis of antiangiogenesis. *Cancer Cell* 2005;7:101–11.
- [150] Dignat-George F, Sabatier F, Blann A, Woywodt A. Detection of circulating endothelial cells: CD146-based magnetic separation enrichment or flow cytometric assay? *J Clin Oncol* 2007;25:e1–2.
- [151] Duda DG, Cohen KS, di Tomaso E, et al. Differential CD146 expression on circulating versus tissue endothelial cells in rectal cancer patients: implications for circulating endothelial and progenitor cells as biomarkers for antiangiogenic therapy. *J Clin Oncol* 2006;24:1449–53.
- [152] Buckstein R, Kerbel RS, Shaked Y, et al. High-dose celecoxib and metronomic “low-dose” cyclophosphamide is an effective and safe therapy in patients with relapsed and refractory aggressive histology non-Hodgkin’s lymphoma. *Clin Cancer Res* 2006;12:5190–8.
- [153] Bender JL, Adamson PC, Reid JM, et al. Phase I trial and pharmacokinetic study of bevacizumab in pediatric patients with refractory solid tumors: a Children’s Oncology Group Study. *J Clin Oncol* 2008;26:399–405.
- [154] Twardowski PW, Smith-Powell L, Carroll M, et al. Biologic markers of angiogenesis: circulating endothelial cells in patients with advanced malignancies treated on phase I protocol with metronomic chemotherapy and celecoxib. *Cancer Invest* 2008;26:53–9.
- [155] Kim HK, Song KS, Kim HO, et al. Circulating numbers of endothelial progenitor cells in patients with gastric and breast cancer. *Cancer Lett* 2003;198:83–8.

## Biographies

*Balazs Dome*, M.D., Ph.D., is the Head of the Department of Tumor Biology at the National Koranyi Institute of Pulmonology, Budapest, Hungary



*Jozsef Timar*, M.D., Ph.D., is a professor of pathology and Head of the Department of Tumor Progression at the National Institute of Oncology, Budapest, Hungary. Dr. Timar's main areas of research interest are in tumor metastasis and angiogenesis. Dr. Timar is author and co-author of more than 150

peer-reviewed publications. He is the founding editor and editor-in-chief of the journal *Pathology Oncology Research*. Furthermore, he is the member of the editorial board of the journals *Clinical and Experimental Metastasis* and *Cancer and Metastasis Reviews*.