High VEGFR-3–positive Circulating Lymphatic/Vascular Endothelial Progenitor Cell Level Is Associated with Poor Prognosis in Human Small Cell Lung Cancer

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Abstract

Purpose: The newly identified bone marrow–derived cell population, called lymphatic/vascular endothelial progenitor cells (LVEPC), has been shown to contribute to lymph capillary growth in experimental tumor systems. The clinical significance of these cells has not yet been investigated in a human malignancy. Our aim was to study whether peripheral blood circulating LVEPCs participate in the progression of human small cell lung cancer (SCLC).

Experimental Design: A total of 88 patients with limited-stage SCLC and 32 tumor-free control subjects were included. Peripheral blood circulating LVEPC labeled with CD34 and vascular endothelial growth factor receptor-3 (VEGFR3) antibodies and the serum levels of the key lymphangiogenic molecule VEGF-C were measured by flow cytometry and ELISA, respectively.

Results: CD34-positive/VEGFR3-positive LVEPC levels were significantly increased in patients (versus controls; $P < 0.01$), and there was also a significant relationship between LVEPC counts and lymph node metastasis ($P < 0.01$). High pretreatment circulating LVEPC numbers correlated with poor overall survival ($P < 0.01$). Although we observed significantly elevated VEGF-C concentrations in patients (versus controls; $P < 0.01$), there was no significant correlation between VEGF-C and LVEPC levels. Moreover, no significant differences in peripheral blood VEGF-C levels were seen between patients subgrouped by clinicopathologic variables including tumor and lymph node stages and survival.

Conclusions: Peripheral blood levels of bone marrow–derived LVEPCs are significantly increased in patients with SCLC and correlate with lymphatic involvement and prognosis. This is the first study that shows evidence of increased numbers of circulating LVEPC in patients with a malignant tumor.

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Small cell lung cancer (SCLC) is an aggressive pulmonary malignancy that constitutes approximately 13% of lung cancers (1). Despite its sensitivity to chemotherapy and radiotherapy, SCLC is rarely curable with these treatment strategies (2). Consequently, new biological targets are needed to develop more effective therapies. Among the potential targets are hemangiogenesis and lymphangiogenesis, which are thought to be fundamental to the progression of different solid tumors (3, 4). However, because no specific markers for lymphatic endothelium were available until recently, our knowledge of the lymphatic system of malignant tumors lags far behind that of the vascular system (5), and the role of lymphangiogenesis in the growth and dissemination of SCLC remains unexplored. Nevertheless, based on recent observations, lymphangiogenesis seems to be a critical mechanism for the progression in a variety of human cancers (6). As part of the lymphangiogenic machinery, the newly identified bone marrow–derived cell population, called lymphatic/vascular endothelial progenitor cells (LVEPC; ref. 7), has been shown to contribute to *de novo* lymphangiogenesis in human renal transplants (8), and more importantly, in experimental tumor systems (9). It is still unclear, however, whether LVEPC participate in SCLC-induced

Translational Relevance

According to recent results, lymphatic vessels in tumors do not necessarily derive from capillary sprouting; instead, similar to the mechanism of vasculogenesis, they can also arise through "lymphvasculogenesis," a process by which bone marrow – derived lymphatic/vascular endothelial progenitor cells (LVEPC) are recruited and differentiate *in situ* into mature endothelial cells to form new lymphatic capillaries. The current study shows for the first time that small cell lung cancer patients have peripheral blood circulating CD34-positive/VEGFR3-positive LVEPC numbers significantly higher than those in tumor-free control subjects. Moreover, this is the first study that shows the clinical significance of these cells in a human cancer.

lymph vessel growth. Nevertheless, because an analogous cell population [vascular endothelial growth factor receptor 2 (VEGFR2)-positive hemangiogenic endothelial progenitor cells (EPC)] has been shown recently to have clinical significance in the hemangiogenic process of a wide range of human malignancies (10–14), including non-SCLC (15, 16), we hypothesized that LVEPC could be involved in the progression of human SCLC. Hence, using peripheral blood samples obtained from SCLC patients, we assessed the numbers of circulating LVEPCs by flow cytometry and investigated whether these numbers might be related to the levels of the key lymphangiogenic molecule VEGF-C and/or to the risk of lymph node metastasis and to patient survival.

Materials and Methods

Clinical data. To measure the number of circulating LVEPC, peripheral blood samples were collected in EDTA tubes through 21G needles from 88 patients with limited-disease SCLC before therapy. According to the consensus report of the International Association for the Study of Lung Cancer, limited disease was defined as disease that is limited to one hemithorax with regional lymph node metastases, including hilar, ipsilateral and/or contralateral mediastinal and/or ipsilateral and/or contralateral supraclavicular nodes (17). However, patients with limited disease with the presence of malignant pericardial and/or pleural effusions were not included in the current study. SCLC patients were free of additional malignant or inflammatory diseases, pulmonary fibrosis, wounds or ulcers, and cardiovascular risk states including diabetes mellitus, chronic renal failure, untreated hypertension, and rheumatoid arthritis that, as described in the case of the analogous bone marrow–derived cell population (VEGFR2-positive hemangiogenic EPC; ref. 28), might influence the number of LVEPC. Moreover, because Fadini et al. have found the depletion of VEGFR2-positive hemangiogenic progenitors in the peripheral blood of patients with chronic lung disease and long-lasting hypoxia recently (18), SCLC patients with GOLD (Global Initiative for Chronic Obstructive Lung Disease; ref. 19) stage III-IV (severe or very severe) and exacerbating stage I-II chronic obstructive pulmonary disease (COPD) were also excluded from the study. There were 54 male and 34 female patients with a median age of 63 y (range, 44-77 y; Table 1). Patients underwent staging work-ups consisting of physical examination, complete blood counts, spirometry tests, comprehensive chemistry panels, chest radiographs, computed tomography scans of the chest and abdomen, bone scintigraphy, and magnetic resonance image or computed tomography scans of the brain. All limited-stage SCLC patients received chemotherapy (cisplatin and etoposide) plus thoracic irradiation. In case of disease progression, patients with chemosensitive tumors (progression >3 mo after the last cycle of first-line therapy) were retreated with the cisplatin and etoposide regimen. Patients who progressed during or within 3 mo after first-line therapy received a cyclophosphamide, epirubicin, and vincristine combination as a second-line treatment. Survival was defined as the time between the date of diagnosis and the date of death. The actual median follow-up was 15 mo (range, 4-27 mo). Potential median follow-up calculated by the "reverse Kaplan-Meier" analysis (20) was 26 mo (range, 25-27 mo). By the end of the study 77 patients (87%) had died of their SCLC. The control group included 32 individuals matched for age, gender, smoking status, and spirometry test result (Table 1). Informed consent was obtained from all patients and control volunteers, and the study was done with the approval of the ethics committees of the host institutions and in accordance with the ethical standards prescribed by the Helsinki Declaration of the World Medical Association.

Enumeration of LVEPC by flow cytometry from the peripheral blood of SCLC patients. To quantify the content of circulating LVEPC by flow cytometric analysis, following erythrocyte lysis, the remaining peripheral blood mononuclear cell fraction was resuspended in 90 μL of a fluorescence-activated cell-sorting buffer containing PBS and 0.1% bovine albumin and incubated for 30 min at 4°C with phycoerythrin-Cy5-conjugated antihuman CD34 (BD Biosciences) and phycoerythrin-conjugated antihuman VEGFR3 (R&D Systems). Fluorochrom-conjugated isotype controls were used for each staining procedure. After appropriate gating, the number of CD34-positive/VEGFR3-positive double-positive cells were quantified and expressed as the number of cells per milliliter of blood using the CyFlow SL flow cytometer and the FlowMax software (both from Partec).

Measuring the levels of VEGF-C in the peripheral blood of controls and patients with SCLC. For VEGF-C measurements, serum samples from all patients and controls were prepared and stored at -80°C until further analysis. Levels of VEGF-C were quantified with the use of a commercial ELISA kit (R&D Systems) according to the manufacturer's instructions. Results were compared with standard curves, and the lower detection limit was 4 pg/mL. Measurements were done in duplicate.

Statistical analysis. Continuous variables were compared with Student's t test if the sample distribution was normal or with Mann-Whitney U test if the sample distribution was asymmetrical. Categorical data were compared using Fisher's exact probability and χ^2 tests. Correlations of LVEPC and VEGF-C levels were determined using Spearman's rank correlation test. Overall survival analyses were done using the Kaplan-Meier method. Overall survival intervals were determined as the time period from initial diagnosis to the time of death. The comparison between survival functions for different strata was assessed with the log-rank statistic. Multivariate analysis of prognostic factors was done using Cox's regression model. Differences were considered significant when $P < 0.05$. All statistical analyses were done using Statistica 7.0 (StatSoft Inc.) software program.

Results

Characterization and levels of LVEPC in peripheral blood samples of SCLC patients. Endothelial progenitor cells (both blood and lymphatic) are thought to derive from CD34-positive hematopoietic progenitor cells (7, 21, 22). Whereas hemangiogenic progenitors can be identified by the expression of the cell surface markers CD34, CD133, and VEGFR2 (3), LVEPC are characterized by the expression of CD34, CD133, and VEGFR3 (7). However, because both types of endothelial progenitor cells rapidly lose their CD133 expression after the migration into the circulation from the bone marrow and

Table 1. Characteristics and VEGF-C levels of patient and control groups

	Patients (n = 88)	Controls (n = 32)	P
Gender (male/female)	54/34 (61.4% vs. 38.6%)	19/13 (59.4% vs. 40.6%)	0.5*
Age (y)	63 (range, 44-77)	61 (range, 48-70)	0.62 †
Smoking status (current or ex-smoker/non-smoker)	75/13 (85.2% vs. 14.8%)	26/6 (81.2% vs. 18.8%)	0.39*
Lung function, spirometry (normal/mild or moderate COPD) ‡	74/14 (84% vs. 16%)	27/5 (84.4% vs. 15.6%)	0.61*
VEGF-C (pg/mL)	4,931 ± 881§	3,992 ± 462§	<0.01‖,¶

*Fischer's exact test.
† Mann-Whitney test.
‡ According to the GOLD (Global Initiative for Chronic Obstructive Lung Diseases) classification of COPD severity (ref. 19).
§ Mean ± SD.
‖ Student's t-test.
¶ Significant difference between patient and control groups.

CD133-positive LVEPC correspond to a subfraction of the total CD34-positive LVEPC population, we determined the numbers of CD34-positive/VEGFR3-positive double-positive LVEPC in the peripheral blood of 32 control subjects and 88 SCLC patients by flow cytometry (Fig. 1A). In the control group, the median value of CD34-positive/VEGFR3-positive circulating LVEPC was 455/mL (interquartile range, 370-530/mL) of peripheral blood (n = 32; Fig. 1B). In patients with SCLC, this level was significantly higher, with a median value of 1,625 (interquartile range, 600-2,750/mL; n = 88; P < 0.01; Fig. 1B).

Correlations between LVEPC levels and clinicopathologic parameters. LVEPC numbers were also evaluated according to the clinicopathologic factors of our patients. There was a statistically significant relationship between LVEPC levels and lymph node involvement (P < 0.01; Table 2). However, no significant associations with age, smoking history, gender,or tumor (T) stage were detected (Table 2).

LVEPC levels as prognostic markers in patients with SCLC. Because lymphatic involvement of SCLCs was associated with increased LVEPC counts, we next used Kaplan-Meier analysis to calculate the overall survival rates for patients with low and high peripheral blood LVEPC levels (Fig. 2). We found that patients whose peripheral blood samples were categorized by low pretreatment CD34-positive/VEGFR3-positive LVEPC levels (based on median value, <1,625/mL of peripheral blood) had significantly longer survival times than those with high levels of circulating LVEPC (median survival time was 20 versus 11.5 months; P < 0.01; Fig. 2). The median overall survival for all patients was 14 months. Multivariate analysis (including standard prognostic variables, such as age, gender, and tumor and lymph node stage) also indicated that pretreatment circulating LVEPC levels predicted outcome independent of other variables (P < 0.01; Table 3). In accordance with the latest International Association for the Study of Lung Cancer analysis of clinical staging for SCLC (23), a further independent prognostic factor related to poor survival was $N_{2\text{-}3}$ disease (versus $N_{0\text{-}1}$ stage; P = 0.014; Table 3).

Peripheral blood levels of VEGF-C in SCLC patients. Although VEGF-C serum levels of patients were significantly elevated as compared with those of control subjects (4931 ± 881 versus 3992 ± 462 pg/mL, respectively; P < 0.01; Table 1), we were unable to detect a significant relationship between the concentrations of the key lymphangiogenic molecule, VEGF-C, and circulating CD34-positive/VEGFR3-positive LVEPC counts (P = 0.74; data not shown). Moreover, when VEGF-C levels were evaluated according to the clinicopathologic factors of our patients, no significant associations with age, smoking history,

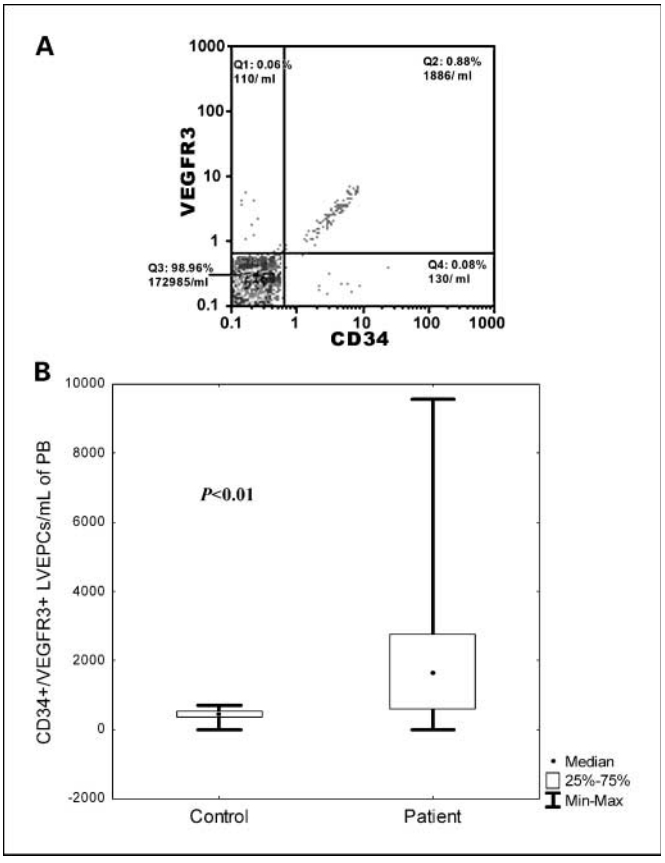

Fig. 1. Quantitative evaluation of circulating LVEPC by flow cytometric analysis (*A* to *B*). *A,* representative flow cytometric analysis for determining the number of CD34-positive/VEGFR3-positive LVEPC (Q1, CD34-negative/VEGFR3-positive; Q2, CD34-positive/VEGFR3-positive; Q3, CD34-negative/VEGFR3-negative; Q4, CD34-positive/VEGFR3-negative cells). *B,* box plots showing median (*central dots*), 25%-75% quartile ranges (*boxes*), and minimum/maximum levels (*whiskers*) of circulating CD34-positive/VEGFR3-positive LVEPC levels in control subjects (n = 32) and patients with SCLC (n = 88).

Table 2. Correlation of clinicopathologic features and circulating LVEPC numbers in 88 SCLC patients

	No. of patients (%)	CD34+/VEGFR3+ LVEPC		*P*
		Low* (%)	High* (%)	
Age (y)*				
63<	43 (48.9%)	22 (50%)	21 (47.7%)	
63>	45 (51.1%)	22 (50%)	23 (52.3%)	0.83
Smoking history				
Nonsmoker	13 (14.8%)	6 (13.7%)	7 (15.9%)	
Current or ex-smoker	75 (85.2%)	38 (86.3%)	37 (84.1%)	0.77
Gender				
Male	54 (61.4%)	25 (56.8%)	29 (65.9%)	
Female	34 (38.6%)	19 (43.2%)	15 (34.1%)	0.38
N stage				
N_{0-1}	24 (27.3%)	21 (47.3%)	3 (6.8%)	
N_{2-3}	64 (72.7%)	23 (52.7%)	41 (93.2%)	<0.01
T stage				
T_1	8 (9.1%)	6 (13.6%)	2 (4.5%)	
T_2-T_4	80 (90.9%)	38 (86.4%)	42 (95.5%)	0.14
VEGF-C level †				
High	44 (50%)	22 (50%)	22 (50%)	
Low	44 (50%)	22 (50%)	22 (50%)	1
Chemotherapy regimens				
EP	68 (77.3%)	37 (84.1%)	31 (70.5%)	
EP+CEV	20 (22.7%)	7 (15.9%)	13 (29.5%)	0.13

Abbreviations: EP, cisplatin and etoposide; CEV, cyclophosphamide, epirubicin and vincristine.
*Cutoff value is median value.
† Cut-off value is mean value. Data shown in parentheses are column percentages.

gender, or more interestingly, with lymph node status, tumor stage, or survival were detected (data not shown).

Discussion

This study presents the novel finding that patients with SCLC have peripheral blood circulating CD34-positive/VEGFR3-positive LVEPC numbers significantly higher than those in tumor-free control subjects, and that the levels of these cells correlated to lymphatic progression and to clinical behavior. Although increased levels of bone marrow–derived circulating VEGFR2-positive hemangiogenic EPC have been reported in various malignant diseases (10–16), to the best of our knowledge, this is the first study that shows evidence of high numbers of circulating lymphatic/vascular EPC in the peripheral blood of patients with a malignancy.

There is a growing body of evidence that tumor blood vessel growth not only depends on cells formerly residing within the vascular walls (i.e. endothelial sprouting) but also is considerably supported by vasculogenesis, the mechanism by which a subset of bone marrow–derived cells, EPC, enhance ongoing vascularization by providing a circulating cell population that home to the blood capillary walls and incorporate into the endothelial tube (3, 14). It is also well established now that lymphangiogenesis (i.e. *in situ* lymph vessel sprouting), facilitated by VEGFR3 signaling, contributes to tumor progression (24, 25). However, more recent evidence suggests that tumor lymphatics do not necessarily derive from endothelial sprouting; instead, similar to the mechanism of vasculogenesis, tumor lymph vessels can also arise through "lymphvasculogenesis," a process by which bone marrow–derived LVEPC are recruited and differentiate *in situ* into mature endothelial cells to form new lymphatic capillaries (9). These VEGFR3-positive LVEPC are functionally a unique population of progenitor cells expressing CD34 but not CD105, CD11b, CD14, or VEGFR1 (7). Because they have been shown to have an *in vitro* capacity to differentiate into lymphatic and/or vascular endothelial cells (7), LVEPC could contribute to both lymph and blood capillary growth of human SCLCs. The data from this current study do not allow us to measure the vasculogenic activity of LVEPC or determine the

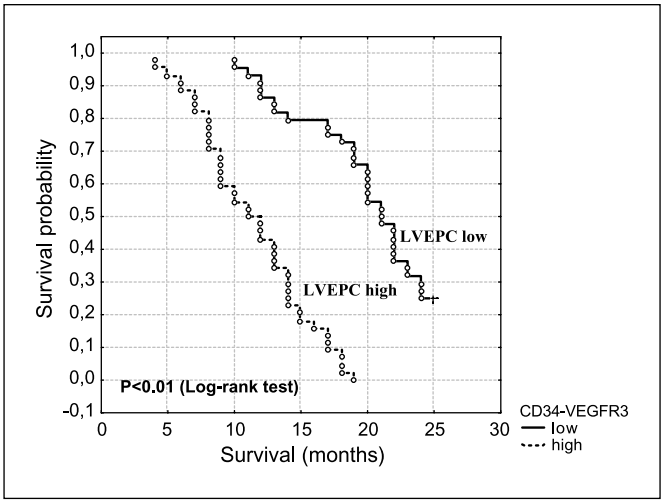

Fig. 2. Kaplan-Meier curves for the overall survival of the patient population with SCLC, according to peripheral blood circulating CD34-positive/VEGFR3-positive LVEPC numbers as determined with flow cytometry. Cutoff value between low and high pretreatment CD34-positive/VEGFR3-positive LVEPC levels was 1,625 LVEPC/mL of peripheral blood.

Table 3. Multivariate analysis of various prognostic factors in patients with SCLC

Prognostic factor	RR (95% CI)	P
Age in y (<63 versus ≥63)	1.213 (0.747-1.969)	0.434
Gender (female versus male)	1.081 (0.655-1.782)	0.761
T stage (T_1 versus T_{2-4})	2.024 (0.725-5.65)	0.178
N stage (N_{0-1} versus N_{2-3})	2.634 (1.215-5.711)	0.014
CD34+/VEGFR3+ LVEPC level (low versus high)*	5.379 (2.659-10.882)	<0.01
VEGF-C serum level (low versus high) †	1.221 (0.76-1,961)	0.408

Abbreviations: RR, relative risk; 95% CI, 95% confidence interval.
*Cutoff value is median value.
† Cutoff value is mean value.

ratio of LVEPC contributions between vasculogenesis and lymphvasculogenesis. However, given the observation that LVEPC numbers were related to the extent of lymph node metastases, one can hypothesize a potential role for these cells in the lymphangiogenic machinery, or at least the possibility that the driving force behind the lymphatic progression of SCLC and the mobilization of LVEPC from the bone marrow is similar.

Based on the above theory, one can assume that our observation on increased LVEPC numbers is the result of elevated levels of the VEGFR3 ligand VEGF-C. Recent studies in experimental animal models have shown direct evidence that this key lymphaniogenic cytokine plays a critical role in cancer progression by inducing lymphangiogenesis and enhancing metastatic spread via the lymphatics, and that these effects can be suppressed by blocking VEGFR3 signaling (reviewed in ref. 26). In a human non-SCLC xenograft model, for example, tumors overexpressing VEGF-C had higher lymph vessel densities than control tumors, and inhibition of VEGFR3 signaling suppressed tumor lymphangiogenesis and metastasis to regional lymph nodes (27). Thus, we assayed the peripheral blood levels of VEGF-C and found that although its concentrations were significantly higher in SCLC patients than in control subjects, no statistically significant relationship existed between VEGF-C levels and numbers of circulating LVEPCs. However, although the possibility of VEGF-C–induced LVEPC release from the bone marrow in SCLC is not supported by the current results, chances are that as in other (for example cardiovascular, malignant, or inflammatory) disorders in which the interaction of several inflammatory and noninflammatory cytokines controls vasculogenic EPC (reviewed in refs. 28, 29), the dynamic balance of multiple growth factors is also likely to determine the number and function of LVEPC in cancer.

In addition to the observation of significantly higher pretreatment circulating LVEPC counts in SCLC patients as compared with control subjects, this prospective study presents the novel finding that a single flow cytometric measurement of CD34-positive/VEGFR3-positive LVEPC is a useful tool to predict outcomes in patients with SCLC. During the follow-up period of 25 months, a significantly higher incidence of death from SCLC was observed in patients with high pretreatment LVEPC levels as compared with patients with low LVEPC levels, suggesting that the pretreatment levels of LVEPC, detectable by flow cytometry in the peripheral blood, correlate with the clinical behavior of human SCLC.

Besides the previous experimental findings mentioned above, several studies in various human cancers have suggested that VEGF-C expression, as assessed by immunohistochemistry in tumor tissue and/or by ELISA in peripheral blood samples, correlates with lymph node metastasis and/or patient survival (5, 30–35). Hence, we also evaluated the potential of measuring peripheral blood levels of VEGF-C as a tool for determining lymph node metastasis and/or prognosis in SCLC. However, although we showed elevated VEGF-C concentrations in SCLC patients over tumor-free controls, we failed to detect an association between VEGF-C levels and patients' survival, and analysis of the cancer patient cohort showed no differences between clinicopathologic subgroups. In particular, no difference in VEGF-C levels was seen between patients with N_{0-1} and with N_{2-3} stages. This accords with the results obtained in the only previous study on peripheral blood VEGF-C measurements in SCLC (36). In contrast, peripheral blood VEGF-C levels predicted lymph node status in a variety of tumor types including esophageal (37), gastric (38), and papillary thyroid (39) cancers, and in malignant melanoma (40) and non–small cell lung carcinoma (41). In addition to studies further investigating the regulation of LVEPC numbers/function in malignant disease, there is also a need, therefore, to better clarify and understand the biological and clinical significance of VEGF-C in SCLC.

In conclusion, in SCLC, as well as in most types of malignant tumors, lymphatic metastasis is associated with poor survival and is one of the factors associated with poor prognosis. Whether lymphatic spread is a mechanism for poor prognosis or a marker for aggressive biological behavior remains to be decided. The current study shows, for the first time, that the circulating numbers of bone marrow–derived LVEPC are significantly increased in SCLC patients and that these numbers correlate with the extent of tumor spread to regional lymph nodes and with patients' survival. Although our data suggest a participation of LVEPC in lymphatic tumor progression in SCLC patients, it is not clear yet whether LVEPC play a role only in the lymphatic spread of the tumor, or whether they also facilitate primary tumor growth and the development of blood-borne metastases via the enhancement of blood capillarization. Moreover, it has yet to be determined if LVEPC can be used as a surrogate marker to monitor the efficacy of standard or future anti(lymph)angiogenic therapies

in SCLC. Further research is also needed on whether LVEPCs can be targeted to treat patients with SCLC, or alternatively – as they are endowed with the capacity to home to the tumor lymphatic network – can be manipulated to deliver toxins or lymph vessel–targeting agents. Finally, because the above results are most likely not specific for SCLCs, they may lead to a number of novel approaches in the diagnosis and treatment of other malignant diseases as well.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing financial interest concerning this manuscript.

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ORIGINAL ARTICLE

Circulating endothelial progenitor cells and depression: a possible novel link between heart and soul

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Although depression is known to be an independent risk factor for cardiovascular disorders, the mechanisms behind this connection are not well understood. However, the reduction in the number of endothelial progenitor cells (EPCs) in patients with cardiovascular risk factors has led us to hypothesize that depression influences the number of EPCs. EPCs labeled with CD34, CD133 and vascular endothelial growth factor receptor-2 (VEGFR2) antibodies were counted by flow cytometry in the peripheral blood (PB) of 33 patients with a current episode of major depression and of 16 control subjects. Mature (CD34+/VEGFR2+) and immature (CD133+/VEGFR2+) EPC counts were decreased in patients (vs controls; $P<0.01$ for both comparisons), and there was a significant inverse relationship between EPC levels and the severity of depressive symptoms ($P<0.01$ for both EPC phenotypes). Additionally, we assayed the plasma levels of VEGF, C-reactive protein (CRP) and tumor necrosis factor (TNF)-α and observed significantly elevated TNF-α concentrations in patients (vs controls; $P<0.05$) and, moreover, a significant inverse correlation between TNF-α and EPC levels ($P<0.05$). Moreover, by means of a quantitative RT-PCR approach, we measured CD34, CD133 and VEGFR2 mRNA levels of PB samples and found a net trend toward a decrease in all the investigated EPC-specific mRNA levels in patients as compared with controls. However, statistical significance was reached only for VEGFR2 and CD133 levels ($P<0.01$ for both markers). This is the first paper that demonstrates evidence of decreased numbers of circulating EPCs in patients with a current episode of major depression.
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Keywords: cardiovascular diseases; mood disorders; depression; endothelial progenitor cells; postnatal vasculogenesis

Introduction

Mood disorders with episodes of major depression (as part of unipolar major depression and bipolar disorder) are frequent illnesses with enormous personal and society burdens worldwide. The life-time prevalence of major depressive and of bipolar disorders are 5–17 and 0.3–7.2%, respectively.[1] It is widely known that major depression or depressive symptoms (as part of dysthymia) are risk factors not only for suicide but also for non-suicide mortality as well.[2–6] Several studies with prospective design—after controlling for possible confounding factors such as hypertension, diabetes mellitus, smoking and age—have concluded that depression predicts the development of cardiovascular diseases.[4,7,8] Accordingly, depression confers a relative risk between 1.5 and 2.0 for the onset of coronary artery disease in physically healthy individuals and a relative risk between 1.5 and 2.5 for cardiac morbidity and mortality in patients with existing coronary artery disease.[9] Moreover, large-scale studies revealed an association between mood disorders and susceptibility to stroke.[10–12] However, although numerous theories have been proposed to explain the amplified risk of cardiovascular disease in patients with depression (reviewed by Everson-Rose and Lewis,[3] Lett *et al.*[9] and Holtzheimer and Nemeroff[13]), the exact biological mechanisms by which depression may increase the risk of cardiovascular events have not been completely elucidated so far.

Adult bone marrow contains a subtype of progenitor cells that has the capacity to migrate to the circulation

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and to incorporate into the endothelial layer of blood vessels. This cell population, endothelial progenitor cells (EPCs), appears to be involved in both the maintenance of vascular integrity[14] and postnatal vasculogenesis (for example, tumor vascularization).[15–18] Since their identification by Asahara *et al.*,[19] several studies have shown reduced numbers and/or impaired function of EPCs in a variety of cardiovascular risk states, including diabetes mellitus,[20] hypercholesterolemia,[21] hypertension,[22] chronic renal failure,[23] rheumatoid arthritis[24] and cigarette smoking.[25] Alternatively, cardiovascular protective factors such as exercise training,[26] statin therapy,[27] angiotensin II receptor antagonists[28] and peroxisome proliferator-activated receptor agonists[29] are known to increase EPC number and function.

Because depression is characterized by increased cardiovascular morbidity and mortality that cannot be explained by traditional cardiovascular risk factors alone and depressive disorders were found to be associated with dysfunction of the immune system and the bone marrow,[30,31] we hypothesized that depression influences the number of bone marrow-derived EPCs as well. Hence, using peripheral blood (PB) samples obtained from healthy individuals and from patients with a current episode of major depression, we assessed the numbers of circulating EPCs by flow cytometry and investigated whether these numbers may be related to the presence and severity of depression. Furthermore, we measured the levels of the key vasculogenic molecule vascular endothelial growth factor (VEGF) and the proinflammatory cytokines tumor necrosis factor (TNF)-α and C-reactive protein (CRP), and used real-time quantitative reverse transcription (RT)-PCR to study the expression of the EPC-specific markers CD34, CD133 and VEGF receptor-2 (VEGFR2) in the PB of depressed patients and healthy controls.

Materials and methods

Clinical data

Thirty-three in- and outpatients diagnosed with a major depressive episode in two psychiatric centers participated in the study (Table 1). The presence of a current major depressive episode was diagnosed by the *Diagnostic and Statistical Manual of Mental Disorders*, Fourth Edition (DSM-IV) criteria.[32] The nine-item abbreviated version of the Beck Depression Inventory (BDI) was used for the assessment of the severity of depressive symptoms.[33] During participant enrollment, complete clinical (physical examination including blood-pressure monitoring, height and body weight) and laboratory evaluations were carried out (Table 1). Patients with elevated levels of fasting blood-glucose, creatinine, urea nitrogen or liver functions, or with hypertension, comorbid psychiatric diagnoses, BMI higher than 30 $kg\,m^{-2}$, higher cholesterol or triglyceride levels than upper levels of Adult Treatment Panel III[34] defined borderline hypercholesterolemia (240 $mg\,100\,ml^{-1}$ or 6.2 $mmol\,l^{-1}$) and hypertriglyceridemia (200 $mg\,100\,ml^{-1}$ or 2.25 $mmol\,l^{-1}$), or with signs of infection (subfebrile temperature, fever, white blood cell number higher than 10 $g\,l^{-1}$, erythrocyte sedimentation rate higher than 20 $mm\,h^{-1}$) were excluded from the study. Moreover, cardiovascular

Table 1 Baseline clinical characteristics and cytokine levels of patient and control groups

	Patients (n = *33*)	*Controls* (n = *16*)	P-*value*
Gender (female/male)	29/4 (88 vs 12%)	14/2 (88 vs12%)	0.98[†]
Age (years)	40.6 ± 10.6	40.3 ± 9.5	0.93*
BUN ($mmol\,l^{-1}$)	4.26 ± 1.52	5.14 ± 1.18	0.055*
Body mass index ($kg\,m^{-2}$)	23.3 ± 3.49	22.7 ± 4.1	0.61*
White blood cells (10^9 per liter)	7.27 ± 1.83	7.94 ± 1.48	0.18*
Blood glucose (mmol per liter)	4.75 ± 0.58	4.52 ± 0.75	0.31*
Total cholesterol (mmol per liter)	4.69 ± 0.82	5.11 ± 0.93	0.14*
Triglyceride (mmol per liter)	1.17 ± 0.5	0.89 ± 0.57	0.11*
hs-CRP ($mg\,dl^{-1}$)	0.13 ± 0.06	0.11 ± 0.04	0.29*
TNF-α ($pg\,ml^{-1}$)	2.68 ± 0.8	1.5 ± 0.46	0.03*[#]
VEGF ($pg\,ml^{-1}$)	19.37 ± 3.83	17.35 ± 3.82	0.1
BDI score	38.6 ± 10.7	0.9 ± 1.44	< 0.01[§#]
Smoking status (current smoker/ nonsmoker)	19/14 (58 vs 42%)	10/6 (62.5 vs 37.5%)	0.74[‖]
Smoking amount in smoker subgroups (no. of cigarettes per day)	23.1 ± 11.7	15.3 ± 9.3	0.064*

Abbreviations: BDI, beck depression inventory; BUN, blood urea nitrogen; hs-CRP, high sensitivity C-reactive protein; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.
Data are expressed as mean + s.d.
*Independent-samples *t*-test
[†]Fischer's exact test.
[§]Mann–Whitney's test.
[‖]χ^2-test.
[#]Significant difference between patient and control groups.

risk factors (diabetes mellitus, hypertension, hypercholesterolemia, hypertriglyceridemia, renal failure) and cardiovascular diseases (acute myocardial infarction, arterial obstructive syndromes of limbs, ischemic stroke) in the medical history of the patients were also criteria for exclusion. Accordingly, no participants received medications with known effects on EPC numbers (Table 2). Data about the smoking habits (smoking status and intensity, defined as self-reported average number of cigarettes smoked daily) of patients and healthy controls were collected as well (Table 1). Based on smoking behavior, the following categories were used: 'non-smokers' (ex- and never smokers) and 'current smokers.' Based on the observations of Kondo *et al.*[25] on the effects of smoking cessation on EPC levels, ex-smokers were defined as those who had quit smoking at least 1 month before taking the blood sample.

The control group included 16 individuals matched for age, gender and smoking status (Table 1). Smoker and non-smoker subgroups of patients and controls were also matched for age. Exclusion criteria for control persons were the same as those for patients with depression. Healthy controls had no previous or current episode(s) of major depression. The study was approved by the Local Ethical Committees of the National Institute of Psychiatry and Neurology, Budapest, and of the Central Hospital of the Hungarian Army, Budapest. All subjects gave their informed consent.

Table 2 Medications of patients and control persons

Psychotropics	*No. of patients receiving*
Anxiolytics[a]	29
Second-generation antipsychotics	4
Mood stabilizers	10
Hypnotics	8
SSRIs	19
SNRIs	7
Other antidepressants	13
Other medications	*No. of patients (P) or control persons (C) receiving*
Tiotropium bromide	1 (P)
Calcitonin	1 (P)
Piroxicam	1 (P)
Propranolol[b]	2 (P)
L-Thyroxine	1 (P)
Calcium dobesilate	1 (P)
Diclofenac	1 (C)

Abbreviations: SNRI, serotonin norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor.
No participants received medications with known effects on EPCs.
[a]Benzodiazepines were given to all except one patient, who received hydroxyzine hydrochloride.
[b]For tachycardia.

Enumeration of EPCs by flow cytometry from the peripheral blood of controls and patients with depression

To quantify the content of circulating EPCs by flow cytometric analysis, following erythrocyte lysis, the remaining PB mononuclear cell fraction was resuspended in 90 μl of a fluorescence-activated cell-sorting buffer containing phosphate-buffered saline and 0.1% bovine albumin and incubated for 30 min at 4 °C with R-Phycoerythrin (PE)-Cy5-conjugated anti-human CD34 (BD Biosciences, San Jose, CA, USA) and allophycocyanin (APC)-conjugated anti-human VEGFR2 (R&D Systems, Minneapolis, MN, USA) or with PE-conjugated anti-human CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany) and APC-conjugated anti-human VEGFR2. Appropriate fluorochrom-conjugated isotype controls were used for each staining procedure. After appropriate gating, the number of CD34+/VEGFR2+ and CD133+/VEGFR2+ cells were quantified and expressed as the number of cells per milliliter of blood using the CyFlow SL flow cytometer and the FlowMax software (both from Partec, Münster, Germany).

Measuring the level of EPC markers by quantitative real-time RT-PCR in the peripheral blood of controls and patients with depression

PB was incubated for 10 min with Red Blood Cell Lysing Buffer (Sigma-Aldrich, Budapest, Hungary), and centrifuged for 20 s at full speed in a microcentrifuge. Total RNA was extracted from the remaining PB mononuclear cell fraction after lysis using Qiagen RNeasy Mini Kit (Hilden, Germany) and digested with RNase-free DNase Set according to the manufacturer's protocol. Total RNA (3 μg) was reverse transcribed from each sample using deoxy-NTPs (0.5 mM each), a mixture of random primer and oligo dT (final concentration 3 μM), RNasin ribonuclease inhibitor (20 U per reaction, Promega, Madison, WI, USA), reverse transcriptase buffer and M-MLV reverse transcriptase (200 U per reaction, Sigma-Aldrich). Samples (30 μl) were incubated for 50 min at 37 °C and then at 85 °C for 10 min. The sequences of CD34 primers[35] were 5′-TTGACAACAACGGTACTGC TAC-3′ and 5′-TGGTGAACACTGTGCTGATTAC-3′. The sequences of CD133 primers[35] were 5′-TGGATGC AGAACTTGACAACGT-3′ and 5′-ATACCTGCTACGA CAGTCGTGGT-3′. The sequences of VEGFR2 primers[35] were 5′-CACCACTCAAACGCTGACATGTA-3′ and 5′-GCTCGTTGGCGCACTCTT-3′. The real-time PCR analysis was standardized by co-amplifying the genes of interest with the housekeeping gene β-actin (primers: 5′-TCTGGCACCACACCTTCTAC-3′ and 5′-CTCC TTAATGTCACGCACGATTTC-3′). The real-time PCR reaction was run on the iCycler iQ (Bio-Rad, Richmond, CA, USA) using standard conditions, namely, an optimized concentration of primers (final concentration 200 nM), iQ SYBR Green Supermix and 2 μl cDNA. A no-template control (containing water) was used as a negative control for every different primer-pair. The cycling parameters were 95 °C

(3 min), 50 cycles of 95 °C (30 s), 64 °C (30 s) and 72 °C (1 min). The starting quantity of gene expression in the sample was determined by comparison of an unknown to a standard curve generated from a dilution series of template DNA of known concentration, and normalized to its own β-actin expression.

Measuring the levels of CRP, VEGF and TNF-α in the peripheral blood of controls and patients with depression

For CRP, VEGF and TNF-α measurements, plasma samples from all patients and controls were prepared and stored at −80 °C until further analysis. Levels of VEGF and TNF-α were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (both from R&D Systems) according to the manufacturer's instructions. Results were compared with standard curves, and the lower detection limits were VEGF, 5 pg ml^{-1}; TNF-α, 0.2 pg ml^{-1}. Measurements were performed in duplicate. Concentrations of CRP were determined by turbidimetric immunoassay (Olympus CRP Latex assay, Hamburg, Germany).

Statistical analysis

Continuous variables were compared with Student's *t*-test. The differences among more than two groups were analyzed with analysis of variance (ANOVA) and Scheffe's *post hoc* method. Continuous data were compared with Mann–Whitney *U*-test if the sample distribution was asymmetrical. Categorical data were compared using Fishers' exact probability and χ^2-tests. Linear regressions were analyzed using the simple regression model. Correlations of EPC and cytokine levels were determined using Spearman's rank correlation test. Differences were considered significant when $P < 0.05$. All statistical analyses were carried out using Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA) software program.

Results

Characterization and numbers of EPCs in peripheral blood samples of controls and patients with depression

EPCs are thought to derive from CD34+ hematopoietic progenitor cells and can be identified by the expression of the cell surface markers CD34, CD133 and VEGFR2.[36] However, these surface molecules are differentially expressed in late/mature and in early/immature phenotypes and none of them by itself is specific for EPCs. The lack of a special and exclusive marker truly specific for EPCs dictates that combinations of the above markers must be used to best identify this cell population.[14,18,37–39] Therefore, we determined the numbers of both CD34+/VEGFR2+ (late/mature phenotype; Figure 1a, cells in Q2) and CD133+/VEGFR2+ (early/immature phenotype; Figure 1b, cells in Q2) double-positive EPCs in the PB of healthy individuals and depressed patients by flow cytometry. By the same token, cell populations positive for only one marker (Figures 1a and b, cells in Q1 or Q4) were not considered in our experiments.

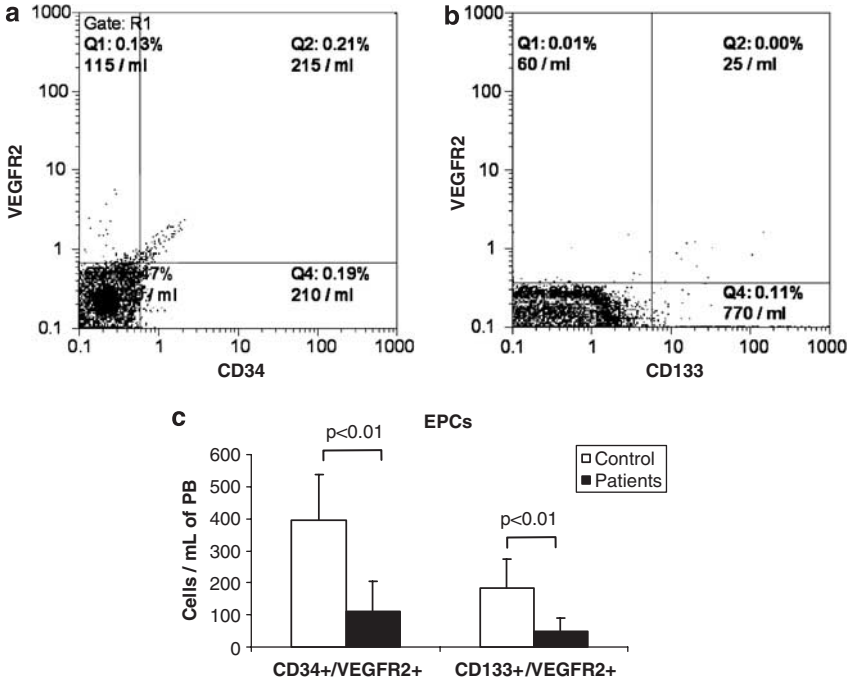

Figure 1 Quantitative evaluation of circulating endothelial progenitor cells (EPCs) by flow cytometric analysis. (**a**, **b**) Representative flow cytometric analyses for determining the number of CD34+/ vascular endothelial growth factor receptor-2 (VEGFR2+) (**a**) and CD133+/VEGFR2+ (**b**) double-positive cells. (**c**) Circulating EPC levels in healthy controls ($n = 16$) and patients with depression ($n = 33$). Data are mean EPC numbers per milliliter of peripheral blood (PB) ± s.d. In (**a**), Q1 = CD34−/VEGFR2+, Q2 = CD34+/VEGFR2+, Q3 = CD34−/VEGFR2−, Q4 = CD34+/VEGFR2− cells. In (**b**), Q1 = CD133−/VEGFR2+, Q2 = CD133+/VEGFR2+, Q3 = CD133−/VEGFR2−, Q4 = CD133+/VEGFR2− cells.

In the patient population, both the levels of mature (CD34+/VEGFR2+) and immature (CD133+/VEGFR2+) EPCs per milliliter of PB were significantly lower than those in the group of healthy controls ($P<0.01$ for both comparisons; Figure 1c). There was no statistically significant correlation between age and EPC levels either in the patient or in the control group (data not shown). Nevertheless, it is important to note that the patient and control populations investigated in the current study included mostly middle-aged individuals (Table 1). EPC numbers of our participants were also evaluated according to the severity of depressive symptoms (as assessed by the BDI[33]). There was a statistically significant inverse relationship between EPC levels and BDI scores ($P<0.01$ in cases of both EPC phenotypes, data not shown) independent of the phenotypes of EPCs.

Because cigarette smoking has recently been demonstrated to decrease circulating EPC numbers,[25,40] patients and controls were matched for smoking habits, as shown in Table 1. In addition, smoker and non-smoker subgroups of patient and control populations were also matched for age. In smoker subgroups, the mean ages of patients and controls were 39.5 ± 11.1 years and 39.7 ± 7.71 years, respectively (mean ± s.d.; $P=0.95$). In non-smoker subgroups, the mean ages of patients and controls were 42.1 ± 10.2 years and 41.3 ± 12.65 years, respectively (mean ± s.d.; $P=0.9$).

Assessment of EPC numbers using CD34/VEGFR2 labeling indicated a significant decrease among smokers compared to non-smokers in both control and patient groups (P-values are <0.01 and <0.001, respectively; Figure 2a). Quantification of EPCs by CD133/VEGFR2 labeling also revealed that the EPC level in the control population was significantly lower in smokers as compared with non-smokers ($P<0.01$; Figure 2b). Although a similar tendency was observed, the difference in CD133+/VEGFR2+ EPC levels between smokers and non-smokers remained statistically insignificant in the patient population ($P=0.34$; Figure 2b). When smoker controls were compared with smoker patients and non-smoker controls with non-smoker patients, both the CD34+/VEGFR2+ (Figure 2a) and the CD133+/VEGFR2+ (Figure 2b) EPC levels were significantly lower in the patient groups.

Evaluation of EPC markers in peripheral blood samples of controls and patients with depression by quantitative real-time RT-PCR

CD34, CD133 and VEGFR2 mRNA levels in healthy controls and in 33 patients with major depression were determined by quantitative real-time RT-PCR (Figure 3). Levels of VEGFR2 and CD133 were significantly lower in the PB of patients when compared with the levels of healthy controls ($P<0.01$ for both comparisons, (Figures 3b and c), whereas CD34 level was not significantly decreased in patients with depression ($P=0.08$, Figure 3a). Accordingly,

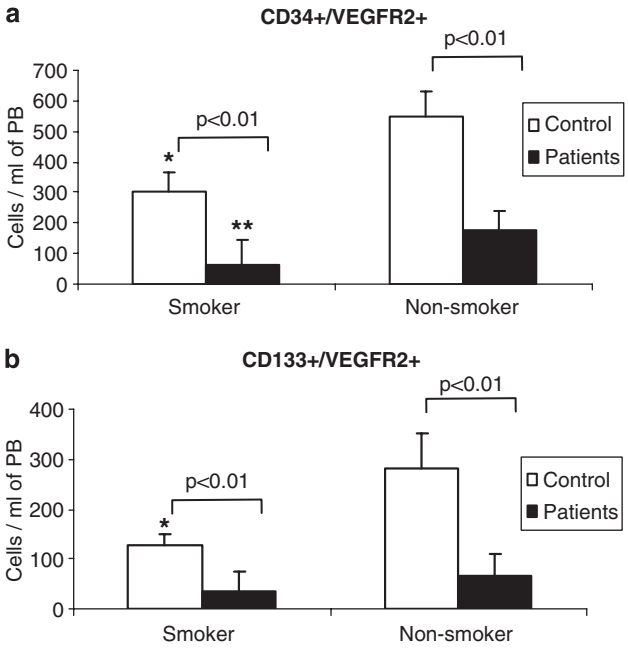

Figure 2 Quantitative evaluation of circulating endothelial progenitor cells (EPCs) by flow cytometric analysis based on both CD34+/vascular endothelial growth factor receptor-2 (VEGFR2+) (**a**, **b**) CD133+/VEGFR2+ double labeling in smoker and non-smoker subgroups of controls and patients. Data are mean EPC numbers per milliliter of peripheral blood (PB) ± s.d. In (**a**), the single asterisk (*) marks significant difference ($P<0.01$) between smoker and non-smoker controls. The double asterisks (**) mark significant difference ($P<0.001$) between smoker and non-smoker patients. In (**b**), the single asterisk (*) marks significant difference ($P<0.01$) between smoker and non-smoker controls.

although there was a statistically significant inverse relationship between VEGFR2 and CD133 mRNA levels and BDI scores ($P<0.01$ in cases of both markers, data not shown), no such correlation was present in the case of CD34. Furthermore, as in the results of flow cytometric analyses, there was no statistically significant association between age and EPC marker levels either in the patient or in the control group (data not shown). Finally, although we also investigated the relationship between EPC numbers and the levels of EPC-specific mRNA levels, statistically significant results were found only in the case of CD133 ($P<0.01$ in cases of both mature and immature EPC phenotypes, data not shown).

When subjects in the control and the patient groups were classified according to their smoking status (Table 1), no significant differences in CD34, CD133 and VEGFR2 mRNA levels between smokers and non-smokers were demonstrated, either within the control or within the patient population ($P>0.05$ for all analyses, Figure 4).

Comparisons of both smoker controls to smoker patients and non-smoker controls to non-smoker patients with respect to all the investigated EPC markers showed higher levels of mRNA in the PB of

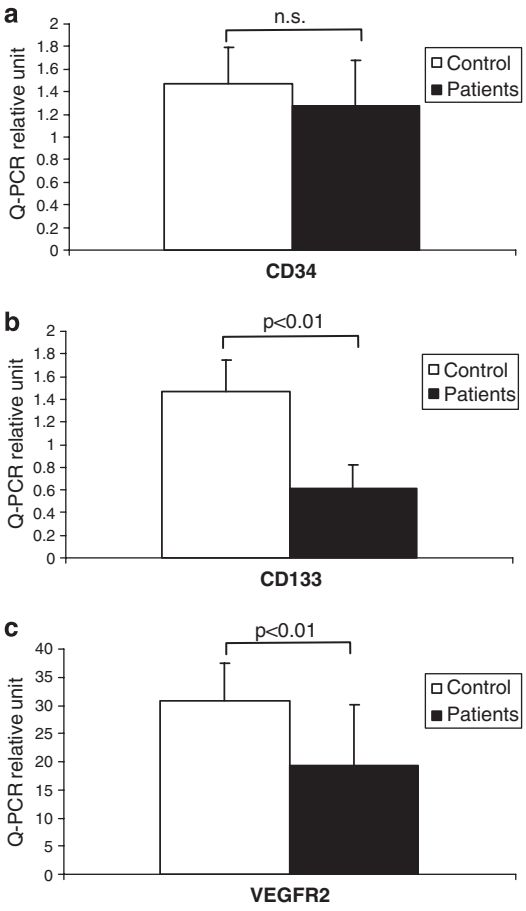

Figure 3 Relative quantification of CD34 (**a**), CD133 (**b**) and vascular endothelial growth factor receptor-2 (VEGFR2) (**c**) mRNAs by real-time quantitative reverse transcriptase (RT)-PCR in the peripheral blood of healthy controls and patients with depression. Results are expressed as means ± s.d. In (**a**), NS is non-significant.

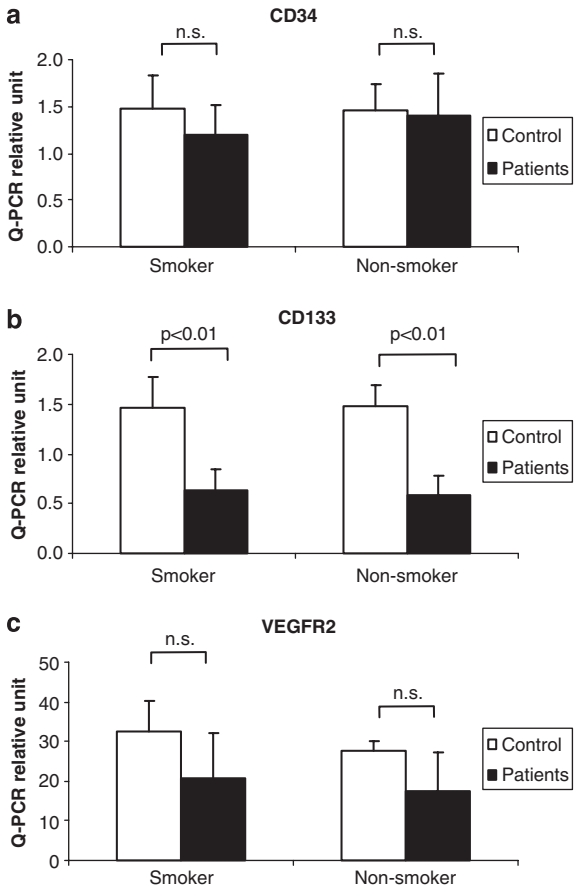

Figure 4 Relative quantification of CD34 (**a**), CD133 (**b**) and vascular endothelial growth factor receptor-2 (VEGFR2) (**c**) mRNAs by real-time quantitative reverse transcriptase (RT)-PCR in the peripheral blood of smoker and non-smoker subgroups of healthy controls and patients with depression. In (**a**, **c**), NS = non-significant.

the healthy controls versus that of the depressed patients. However, this tendency proved to be statistically significant only in the case of CD133 ($P < 0.01$ for both comparisons, Figure 4b).

Peripheral blood levels of VEGF and the proinflammatory cytokines TNF-α and CRP

Although patients with depression tended to have higher CRP levels than healthy controls, the difference between the two groups remained insignificant ($P = 0.29$, Table 1). Furthermore, we were unable to detect a significant relationship between CRP concentrations and circulating CD34+/VEGFR2+ or CD133+/VEGFR2+ EPC counts (*P*-values are 0.55 and 0.39, respectively, data not shown). However, TNF-α levels of patients were significantly elevated as compared with those of healthy controls ($P = 0.03$, Table 1) and, moreover, a statistically significant inverse correlation was observed between TNF concentrations and EPC numbers ($P < 0.05$, data not shown).

No significant difference was detected in the plasma levels of the key vasculogenic molecule, VEGF, between controls and patients ($P = 0.1$, Table 1). Furthermore, there was no statistically significant correlation between VEGF concentrations and circulating CD34+/VEGFR2+ or CD133+/VEGFR2+ EPC counts (both *P*-values are 0.08, data not shown).

Discussion

There is a growing body of evidence that blood vessel integrity not only depends on cells formerly residing within the vascular walls, but also is considerably controlled by bone marrow derived cells. Recent studies suggest that a subset of these cells, EPCs, may contribute to ongoing vascular repair by providing a circulating cell population that can home to the blood vessel walls and incorporate into the injured endothelial tube to replace dysfunctional endothelial cells.[14,36] Consequently, impairment of this EPC pool is considered to have negative effects on the cardiovascular system and patients with reduced numbers of EPCs are at increased risk for endothelial injury and for arteriosclerotic plaque development.[20–25]

A further established risk factor recognized today in the pathogenesis of arteriosclerosis and cardiovascular disorders is depression. However, although an association between mood disorders and susceptibility to cardiovascular events has been discovered by several researchers,[2,3,7–12,41–44] and altered circulating EPC levels have been reported in various conditions associated with vascular diseases,[20–25] to date no studies have attempted to evaluate the significance of EPCs in patients with major depression. Therefore, we investigated the significance of EPC numbers in our study sample by using flow cytometry and found a significant decrease in both mature (CD34+/VEGFR2+) and immature (CD133+/VEGFR2+) circulating EPC numbers in depressed patients versus healthy controls, and moreover, a statistically significant inverse relationship between EPC counts and the severity of depression independent of EPC phenotypes (as assessed by BDI scores[33]). Accordingly, although statistical significance was reached only for VEGFR2 and CD133 mRNA levels, there was a net trend toward a decrease in all the investigated EPC-specific mRNA (CD34, CD133 and VEGFR2) levels in patients with depression as compared with healthy controls. Interestingly, however, with the exception of CD133 mRNA levels, we failed to show a significant association between EPC-specific mRNA levels and EPC numbers. The reasons for this discrepancy between the results of flow cytometric analysis and PCR technique are not fully understood. However, it could be attributed to a complication that arises in using real-time quantitative RT-PCR to analyze mRNA levels in PB specimens. Although the PCR method offers the potential to rapidly and quantitatively analyze a number of gene products with limited material, the diversity of cellular populations present make it difficult to identify cell-specific gene expression patterns. In other words, possible reasons for the inconsistent results using two different techniques may include variations in EPC marker expression intensity at the stage of development in which they were studied, namely in the peripheral circulation following release from the bone marrow but prior to homing at the blood vessel site.

The finding that EPC levels are decreased in patients with depression may be secondary to a variety of mechanisms: depletion of the pool of EPCs in the bone marrow, reduced mobilization of the EPC population, or reduced survival and/or differentiation in the circulation. However, because VEGF is believed to be the most important among the molecules that participate in the regulation of EPCs,[14,18] one can assume that our observation on EPC numbers is the result of the reduced levels of this cytokine. Hence, we tested this hypothesis, but found normal VEGF levels in our patient population. Consequently, the possibility of insufficient bone marrow stimulation by VEGF in depression is not supported by the current results.

Recent experimental and clinical data also suggest that a variety of inflammatory mediators could be involved in the pathogenesis of low circulating EPC counts in depression.[42] Patients with depression who are otherwise medically healthy have been observed to have increased concentrations of proinflammatory cytokines and acute phase proteins. Elevated serum and/or plasma levels of CRP and/or interleukin-6 have been most frequently reported, although an increase in TNF-α plasma concentrations have also been described.[31] Combined with data demonstrating that CRP promotes apoptosis and attenuates the function and differentiation of EPCs[45] and TNF-α reduces the number of EPCs,[46] these findings suggested to us that CRP and/or TNF-α might promote EPC number reduction in depressed patients. Thus, we assayed the plasma levels of both cytokines and found that although CRP concentrations were, on average, higher in patients than in the control subjects, no statistically significant relationship existed between CRP concentrations and circulating progenitors. Nevertheless, the levels of TNF-α were significantly higher in the PB of patients when compared with the levels of healthy controls and, moreover, TNF-α levels inversely correlated with EPC counts. Therefore, the results of a recent study on rheumatoid arthritis reporting a significant increase in EPC levels after a single dose of infliximab, a monoclonal anti-TNF-α antibody,[47] might be interesting in this context. However, although the current data suggest a possible link between TNF-α and a decrease in circulating progenitors, chances are that as in other (for example, cardiovascular, malignant or inflammatory) disorders in which the interaction of multiple growth factors controls EPCs[14,18,36] the dynamic balance of several inflammatory and non-inflammatory cytokines is also likely to determine the number and function of these cells in depression. Therefore, additional studies are necessary to confirm the role of inflammation in modulating EPC numbers/function in depression.

The patient population analyzed in the current study had reduced numbers of EPCs but was free from confounding factors known to alter circulating EPC numbers, with the exception of cigarette smoking. Using flow cytometry, we found a significant decrease in CD34+/VEGFR2+ EPC counts among smokers, regardless of the population category (patients or controls). A similar tendency was observed among smokers in the case of CD133+/VEGFR2+ cells, although statistically significant reduction of EPCs with this phenotype was reached only in the control population. Nevertheless, these findings accord with the results of previous papers, in which smoking was demonstrated to decrease circulating EPC numbers.[25,40] Surprisingly, there were no significant correlations between the smoking status and the EPC-specific mRNA levels as evaluated by real-time quantitative RT-PCR. It is important to note, however, that because our patients and controls were carefully matched for smoking habits and because patients had always significantly lower EPC counts (both in the case of immature and mature EPCs, independent

of their smoking status), the significant decrease of EPC numbers and the tendency toward lower EPC-specific marker levels in the patient group are presumably attributable only to depression in the current study.

In addition to the relatively low number of participants, another limitation to this study was that most members of the patient group received some kind of psychotropic medicine while those in the control group were medication free (except for one person receiving diclofenac). However, the following observations, taken together, allow us to conclude that the observation of reduced EPC counts in our patients is significant, even in the presence of adequate pharmacological treatment. Firstly, none of the psychotropic drugs used in the current study has been reported to alter the number and/or function of EPCs. Secondly, the main categories of the psychotropic drugs used in the current study (benzodiazepines and selective serotonin reuptake inhibitor antidepressants) have been only very rarely observed to cause disturbances in the function of bone marrow (as a source of EPCs). Finally, and most importantly, because CRP and TNF-α levels are in an inverse relationship with the circulating numbers of EPCs[45,46] and because antidepressant agents were reported to decrease the elevated concentrations of these molecules,[48,49] one can assume that antidepressants rather increase than decrease the levels of EPCs.

In summary, EPCs are obviously involved in the regeneration of injured endothelium, and their number is thought to be a surrogate marker of vascular function.[36] The current study demonstrates for the first time that EPCs circulate in decreased numbers in the PB of patients with depression and, furthermore, opens a number of new perspectives in the pathogenesis of cardiovascular disorders in depression. However, although our results could be related to the EPC suppressive role of proinflammatory cytokines, further studies are needed to investigate the exact pathomolecular background of the connection between mood disorders and altered EPC counts. Moreover, it remains to be determined whether the EPC number abnormalities are state- or trait-dependent phenomena in affective disorders. Finally, our results also suggest that individuals should be screened for depression in future studies investigating EPC number and function in patient populations with cardiovascular risk factors (diabetes mellitus,[50] chronic renal failure,[51] smoking,[52] rheumatoid arthritis[53]) or with definitive cardiovascular diseases (myocardial infarction,[9] stroke[10]). It is especially crucial that the depression be considered an independent variable in such studies because depression is so highly comorbid with these cardiovascular conditions and diseases.

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Apelin Expression in Human Non-small Cell Lung Cancer

Role in Angiogenesis and Prognosis

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Introduction: The recently discovered bioactive peptide, apelin, has been demonstrated to stimulate angiogenesis in various experimental systems. However, its clinical significance and role in tumor vascularization have not yet been investigated in a human malignancy. Therefore, our aim was to study whether apelin expression is associated with angiogenesis and/or tumor growth/behavior in human non-small cell lung cancer (NSCLC).

Methods: A total of 94 patients with stage I–IIIA NSCLC and complete follow-up information were included. Apelin expression in human NSCLC samples and cell lines was measured by quantitative reverse-transcriptase polymerase chain reaction, enzyme-linked immunosorbent assay, and immunohistochemistry. Effects of exogenous apelin and apelin transfection were studied on NSCLC cell lines in vitro. In vivo growth of tumors expressing apelin or control vectors were also assessed. Morphometric variables of human and mouse tumor capillaries were determined by anti-CD31 labeling.

Results: Apelin was expressed in all of the six investigated NSCLC cell lines both at the mRNA and protein levels. Although apelin overexpression or apelin treatments did not increase NSCLC cell proliferation in vitro, increasing apelin levels by gene transfer to NSCLC cells significantly stimulated tumor growth and microvessel densities and perimeters in vivo. Apelin mRNA levels were significantly increased in human NSCLC samples compared with normal lung tissue, and high apelin protein levels were associated with elevated microvessel densities and poor overall survival.

Conclusions: This study reveals apelin as a novel angiogenic factor in human NSCLC. Moreover, it also provides the first evidence for a direct association of apelin expression with clinical outcome in a human cancer.

Key Words: Non-small cell lung cancer, Apelin, Angiogenesis, Prognosis.

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Lung cancer, the leading cause of cancer-related deaths in the Western world,[1] is currently classified into two major groups: small-cell lung cancer (~20% of all cases) and non-small cell lung cancer (NSCLC, ~80%). Even with improving efficacy of surgery, irradiation, and chemotherapy, poor prognosis for patients with NSCLC remains. For this very reason, the identification of novel targets for NSCLC treatment is needed.[2]

Apelin, initially isolated from bovine stomach homogenates, has been recognized as the endogenous ligand of the human orphan G protein-coupled receptor APJ, a member of the seven-transmembrane-receptor family.[3,4] In mammals, the apelin gene encodes a secreted prepropeptide of 77 amino acids with a signal peptide, a prodomain, and a C-terminal peptide, which is proteolytically cleaved to yield bioactive apelin peptides, 36, 17, and 13 amino acids in size. However, each of these predominant and most active isoforms contains the extreme C-terminal region of the precursor protein, and all bioactivity is thought to reside in the terminal 13-amino acid fragment.[5,6] During embryonic development, APJ expression is largely restricted to the endothelial cells (ECs) of the developing vascular system,[7] and apelin is essential for regular vascular patterning of the embryo.[8] Apelin and its receptor are also highly expressed in the adult vessel walls, especially in blood ECs.[9] Apelin was reported to stimulate the in vitro growth of human umbilical[10] and mouse brain mi-

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crovasculature-derived ECs[8] and the in vitro migration, proliferation, and capillary-like tube formation of monkey retinal ECs.[11] In line with these findings, apelin was reported to stimulate in vivo angiogenesis in the chicken chorioallantoic membrane[8] and in the mouse subcutaneous matrigel plug[11] assay systems. Furthermore, apelin overexpression was observed to enhance the vascularization and in vivo growth of different murine tumors.[12,13] Although the molecular mechanisms behind the regulation of apelin expression are not yet completely understood, it has been reported recently that increased apelin expression by hypoxia is mediated by the binding of hypoxia-inducible factor-1α to a hypoxia-responsive element located within the first intron of the apelin gene.[14]

Given the biologic and clinical significance of angiogenesis in the progression of human NSCLC[15] and the finding that apelin expression is up-regulated in some human cancers,[16–18] our aim was to examine the association between apelin expression and tumor growth, angiogenesis, and clinical behavior in human NSCLC.

PATIENTS AND METHODS

Human Lung Tissue Samples, Clinicopathologic Data, and Cell Lines

For immunohistochemical analysis, a total of 94 patients with histopathologically defined NSCLC treated during January 1997 to December 2001 were included in the study. There were 68 male and 26 female patients with a median age of 63 years (range, 44–81 years; Table 1). Formalin-fixed, paraffin-embedded NSCLC samples were retrieved from the files of our pathology department, with the approval of the ethics committee of the host institutes and in accordance with the ethical standards prescribed by the Helsinki Declaration of the World Medical Association. Histologic diagnosis and N stage were determined on hematoxylin and eosin-stained sections. There were 35 squamous-cell carcinomas, 54 adenocarcinomas, and 5 large-cell carcinomas. Samples of tumor-free lung parenchyma were also obtained from lung tissue distant from the tumor site. The cases were staged according to operative and pathologic findings based on the American Joint Committee on Cancer/Union Internationale Contre le Cancer TNM classification.[19]

For polymerase chain reaction (PCR) analyses, fresh surgical tumor and normal lung specimens of 46 NSCLC patients were used. The tumor samples were obtained immediately after surgical removal and cut in half. One half of each specimen was embedded in paraffin and processed for routine histologic examination, and the percentage of apelin-positive tumor cells was determined by immunohistochemistry. The other half was frozen in liquid nitrogen and stored at −80°C until mRNA isolation.

All the tumor and normal lung samples were obtained after elective surgery. None of the patients included in the current study were treated with neoadjuvant therapy.

H358, H1650, H1975, and A549 cell lines were obtained from American Type Culture Collection (Manassas, VA). The LCLC-I03H and HCC15 cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). All cell lines were maintained in RPMI 1640 with 10% fetal bovine serum and 1% penicillin/streptomycin (all from Sigma Chemical Co., St. Louis, MO) at 37°C in a humidified incubator with 5% CO_2.

TABLE 1. Correlation of Clinicopathologic Features and Expression of Apelin in Patients with NSCLC (n = 94)

		Apelin Expression		
	No. Patients (%)	Low (%)	High (%)	p
All patients	94 (100)	30 (31.91)	64 (68.09)	
Age (yr)[a]				
<63	45 (47.87)	11 (36.67)	34 (53.13)	
>63	49 (52.13)	19 (63.33)	30 (46.87)	0.14
Smoking				
Nonsmoker	21 (22.34)	7 (23.33)	14 (21.88)	
Current or ex-smoker	73 (77.66)	23 (76.67)	50 (78.12)	0.87
Gender				
Male	68 (72.34)	21 (70)	47 (73.44)	
Female	26 (27.66)	9 (30)	17 (26.56)	0.73
N stage				
N0	35 (37.23)	12 (40)	23 (35.94)	
N1	30 (31.92)	10 (33.33)	20 (31.25)	
N2	29 (30.85)	8 (26.67)	21 (32.81)	0.83
T stage				
T1	37 (39.36)	13 (43.33)	24 (37.5)	
T2	32 (34.04)	8 (26.67)	24 (37.5)	
T3	25 (26.6)	9 (30)	16 (25)	0.58
Histology				
AC	54 (57.45)	18 (60)	36 (56.25)	
SCC	35 (37.23)	10 (33.33)	25 (39.06)	
LCC	5 (5.32)	2 (6.67)	3 (4.69)	0.62
MVD[b]				
Low	51 (54.26)	21 (70)	30 (46.87)	
High	43 (45.74)	9 (30)	34 (53.13)	0.04

[a] Cutoff value is median value.
[b] Cutoff value is mean value. Data shown in parentheses are column percentages.
NSCLC, non-small cell lung cancer; AC, adenocarcinoma; SCC, squamous-cell carcinoma; LCC, large-cell carcinoma; MVD, microvessel density (n/mm^2).

Development of Stable Transfectant Cell Lines

Human apelin complementary DNA was purchased from OriGene (Rockville, MD). The coding region was amplified with the following primers: 5′-CGCGAATTCGGCATGAATCTGCGGCTCTG and 5′-GCGCTCGAGTCAGAAAGGCATGGGTCC. PCR products were subcloned into the pcDNA 3.1. vector using *Eco*RI and *Xho*I restriction enzymes (Invitrogen, Carlsbad, CA). The nucleotide sequence of the expression vector was confirmed by DNA sequencing. H358 and H1975 cells, which had a relatively low endogenous expression of apelin, were transfected with a control or an apelin-encoding pcDNA 3.1 vector using the FuGENE 6 transfection reagent (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's guidelines. Stable transfectants were selected by their resistance to geneticin (400 μg/ml; GIBCO, Paisley, UK).

In Vitro Cell Proliferation Studies

For in vitro cell growth studies, 1 to 2 $\times$ 10^4 cells per well were cultured in triplicate in flat-bottomed 96-well plates in serum-containing or serum-free medium with 0 to 10^{-6} M apelin-36 (Phoenix Pharmaceuticals, Karlsruhe, Germany) for 72 hours. Cell growth was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich Co.) as previously described.[20] Each assay was done with six measurements for each data point. Absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) Microplate Reader (Bio-Rad, Hercules, CA). Experiments were repeated three times.

Determination of Apelin Protein Expression and Microvessel Density in Human and Murine Tumors

Immunohistochemical stainings were performed on samples fixed in 10% neutral buffered formalin and embedded in paraffin. Five-micrometer paraffin sections were dewaxed and rehydrated. For light microscopy, peroxidase was quenched with methanol and 3% H_2O_2 for 15 minutes. Antigen retrieval was performed in 0.1 M citrate buffer (pH 6) in an 800-W microwave for 15 minutes. Following sequential 15-minutes incubations with 0.1% avidin and 0.01% biotin (Vector Laboratories, Inc., Burlingame, CA) to block endogenous avidin and biotin, sections were incubated in a mixture of 0.05% casein (Sigma Chemical Co.), 0.05% Tween-20, and phosphate-buffered saline for 30 minutes to block nonspecific protein binding. Slides were then incubated with antibodies to mouse anti-human CD31 (DakoCytomation, Carpinteria, CA) and rabbit anti-human apelin-36 (Phoenix Pharmaceuticals). The specificity of this antiapelin antibody, which recognizes all C-terminal fragments, has been confirmed repeatedly.[9,17,21–23] Normal mouse and rabbit immunoglobulin G's (IgGs) were substituted for primary antibodies as a negative control. Biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) and F(ab′)2 fragment of swine anti-rabbit IgGs (DakoCytomation) served as the secondary antibodies and were detected by streptavidin-horseradish peroxidase, and colorized by 3-amino-9-ethylcarbazole (both from DakoCytomation). Finally, counterstaining was performed using Mayer's modified hematoxylin.

In the case of apelin staining, all samples were analyzed by two investigators who had no information on the clinical data. Disagreement in the evaluation was found in less than 10% of the samples examined, and consensus was reached on further review. The expression of apelin was scored as a fraction of positive NSCLC cells in the tumor area. The degree of positivity was initially classified according to the percentage of positive tumor cells as follows: (−) no tumor cells positive, (1+) 1 to 10% cells positive, (2+) 10 to 50% cells positive, and (3+) more than 50% cells positive. Sections were examined using a Nikon Eclipse 80i microscope and digital images were captured using a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Microvessel densities (MVDs) and perimeters were determined by labeling of blood vessels with mouse anti-human CD31 or rat anti-mouse CD31 antibodies (purchased from DakoCytomation and Pharmingen, San Diego, CA, respectively). Three sections per tumor were analyzed using the CUE-2 computerized image analysis system (consisting of special software, image processor, digital camera, and video monitor; Olympus, Tokyo, Japan) as described previously.[20,24]

Cellular Apelin Secretion

Cell culture supernatants of wild-type human NSCLC cell lines (including H358, H1650, HCC15, LCLC-I03H, H1975, and A549), and H358 and H1975 cells stably transfected with a control or an apelin expression vector were removed after 72 hours of culture. The concentration of apelin in each sample was determined using a commercially available ELISA directed against apelin-36, following the manufacturer's (Phoenix Pharmaceuticals) suggested protocol. This ELISA has 100% cross-reactivity to human apelin-36, -13, and -12. Results were compared with standard curves, and the lower detection limit was 0.08 ng/ml. Measurements were performed in triplicate.

RNA Isolation and Reverse Transcription-PCR

Total RNA was extracted from human NSCLC cell lines and from fresh frozen tumor and normal lung specimens of 46 NSCLC patients using Qiagen RNeasy Mini Kit and digested with RNase-free DNase Set according to the manufacturer's protocol. Three micrograms of total RNA were reverse transcribed from each sample using deoxy-NTPs (0.5 mM each), a mixture of random primer and oligo dT (final concentration 3 μM), RNasin ribonuclease inhibitor (Promega, 20 U/reaction), reverse transcription buffer, and M-MLV reverse transcription (Sigma, 200 U/reaction). Thirty microliters of the samples were incubated for 50 minutes at 37°C and then at 85°C for 10 minutes. The success of reverse transcription and the purity of the samples were validated using primers to β-actin (sense, 5′-GTGGGGCGCCCCAGGCACCA-3′; and antisense, 5′-CTCCTTAATGTCACGCACGATTTC-3′).

Qualitative PCR was performed for apelin (sense, 5′-CTGCTCTGGCTCTCCTTGAC-3′; and antisense, 5′-GAATTTCCTCCGACCTCCCTG-3′) and β-actin. Amplification was done using the AmpliTaq Gold kit (Applied Biosystems, Foster City, CA). The PCR cycling conditions were as follows: 42 cycles of 1 minute at 94°C, 1 minute at 55°C, and 2 minutes at 72°C. PCR products were separated by electrophoresis in a 2% agarose gel, visualized by ethidium bromide staining and analyzed using the GelDoc 2000 (Bio-Rad) system. PCR products were isolated from the agarose gel using the MEGA-spin Agarose Gel Extraction Kit (iNtRON Biotechnology, Gyeonggi-do, Republic of Korea) and were sequenced by the ABI-PRISM 310 DNA Sequencer (AME Bioscience, Bedfordshire, UK) at the Laborigo Ltd. (Budapest, Hungary) sequencing core.

The real-time PCR reaction was run on the iCycler iQ (Bio-Rad) using standard conditions, namely, an optimized concentration of primers (final concentration 200 nM), iQ SYBR Green Supermix (BioRad), and 2 μl of complementary DNA. No-template controls were included for each primer pair. The cycling parameters were 95°C (10 minutes),

50 cycles of 95°C (30 seconds), 64°C (30 seconds), and 72°C (1 minute). Apelin expression level was determined after normalization to β-actin expression.

Xenograft Tumors

Growth of the apelin-transfected human NSCLC cells were compared with those of control vector-expressing cells in xenograft tumors formed in 6-week-old female athymic (nu/nu) mice from Charles River Laboratories (Wilmington, MA). According to the institutional animal welfare guidelines, all mice were maintained on a daily 12:12-hour light-dark cycle and were housed under pathogen-free conditions in microisolator cages with laboratory chow and water ad libitum. H358 and H1975 cells stably expressing apelin or transfected with control vector were grown to 80% confluence, harvested by trypsinization, and washed twice. NSCLC xenografts were established by injecting mice subcutaneously with 1×10^7 H358 or H1975 cells in Matrigel (Collaborative Research Biochemicals, Bedford, MA). Tumor size was measured every 4 days with a caliper and expressed in mm^3 by the formula for the volume of a prolate ellipsoid (length $\times$ width^2 $\pi/6$). Tumors were removed from mice after 32 days of growth and were fresh-frozen in liquid nitrogen for further analysis.

Statistical Analysis

Continuous variables were compared with Student *t* test if the sample distribution was normal or with Mann-Whitney *U* test if the sample distribution was asymmetric. Categorical data were compared using Fisher's exact probability and χ^2 tests. Correlations of apelin protein and mRNA expressions were determined using Spearman's rank correlation test. Overall survival analyses were done using the Kaplan-Meier method. Overall survival intervals were determined as the time period from initial diagnosis to the time of death. The comparison between survival functions for different strata was assessed with the log-rank statistic. Multivariate analysis of prognostic factors was done using Cox regression model. Differences were considered significant if the *p*-value is less than 0.05. All statistical analyses were done using Statistica 8.0 (StatSoft Inc. Tulsa, OK) software program.

RESULTS

Apelin mRNA and Protein Expression in Tumor and Normal Lung Tissue Samples of NSCLC Patients and in Human NSCLC Cell Lines

Apelin protein was expressed by the human bronchial glands (Figure 1*A*) and epithelium adjacent to tumors, exhibiting a cytoplasmic pattern of expression. Alveolar cells were negative. Typical patterns of apelin staining in human NSCLC samples are shown in Figure 1*B* (low expression) and Figure 1*C* (high expression).

Reverse-transcriptase (RT)-PCR demonstrated the presence of apelin mRNA in six of six human NSCLC cell lines (Figure 1*D*). An ELISA for apelin indicated that all examined cell lines secreted the protein into their cell culture medium (Figure 1*E*).

Quantitative RT-PCR analysis of 46 paired nontumor and tumor mRNA extracts revealed that apelin levels were significantly higher in the tumor samples of patients compared with the levels of their normal lung tissue specimens ($p < 0.01$; Figure 1*F*). Moreover, when apelin mRNA levels of human NSCLC specimens were compared with expression levels detected by immunohistochemistry, a significant correlation was found ($p < 0.01$; Figure 1*G*).

Effect of Exogenous Apelin and Apelin Overexpression on In Vitro and In Vivo NSCLC Growth

To confirm or rule out that apelin influences NSCLC cell growth in an autocrine manner, the effect of treatments with different doses of apelin was studied on six different human NSCLC cell lines. Importantly, exogenous apelin did not stimulate the in vitro proliferation rate of these cells, compared with untreated cells after 72 hours (Figure 2*A–F*).

To further investigate the influence of apelin on NSCLC growth, H358 and H1975 cells with relatively low levels of endogenous apelin were stably transfected with an apelin expression vector to increase apelin levels. ELISA was used to verify the elevated cellular secretion of apelin after transfections. Although genetic modification of H358 and H1975 cell lines resulted in a significant elevation in secreted levels of apelin ($p < 0.05$ in case of both cell lines, Figure 3*A*), when the possible effect of this increase in apelin protein expression was evaluated on cell growth in vitro, neither H358 nor H1975 cells overexpressing apelin showed an increased proliferation rate compared with control vector–transfected cells ($p > 0.05$, in case of both cell lines, Figure 3*B*).

Next, we sought to test the effect of apelin on tumor growth in vivo using xenografted human NSCLC cells in immunodeficient mice. Therefore, in our next set of experiments, the in vivo growth rates of tumors after subcutaneous injection of H358 or H1975 cells expressing apelin or control vectors were assessed. Tumor growth was significantly accelerated in mice injected with apelin-overexpressing cells compared with mice carrying cells transfected with the empty control vector (Figure 4*A*, *B*).

Association of Apelin Expression with Angiogenesis

As mentioned above, exogenous apelin (Figure 2*A–F*) or overexpression of apelin by transfected NSCLC cells (Figure 3*B*) did not seem to affect the growth rate of tumor cells in vitro, but implantation of apelin-overexpressing tumor cells into mice resulted in accelerated tumor growth in vivo (Figure 4*A*, *B*). This observation, taken together with the results of other studies demonstrating the angiogenic potential of apelin in different in vitro and in vivo experimental models,[8–13] prompted us to investigate whether apelin might also induce angiogenesis in xenotransplanted human NSCLC tumors in vivo. As expected, morphometrical analysis using CD31 as an EC marker (Figure 5*A–D*) revealed a significantly higher MVD present in the apelin-overexpressing tumors compared with controls ($p < 0.05$ in case of both H358 and H1975 cell lines; Figure 5*E*). Moreover, assessment of tumor capillary perimeters indicated a significant

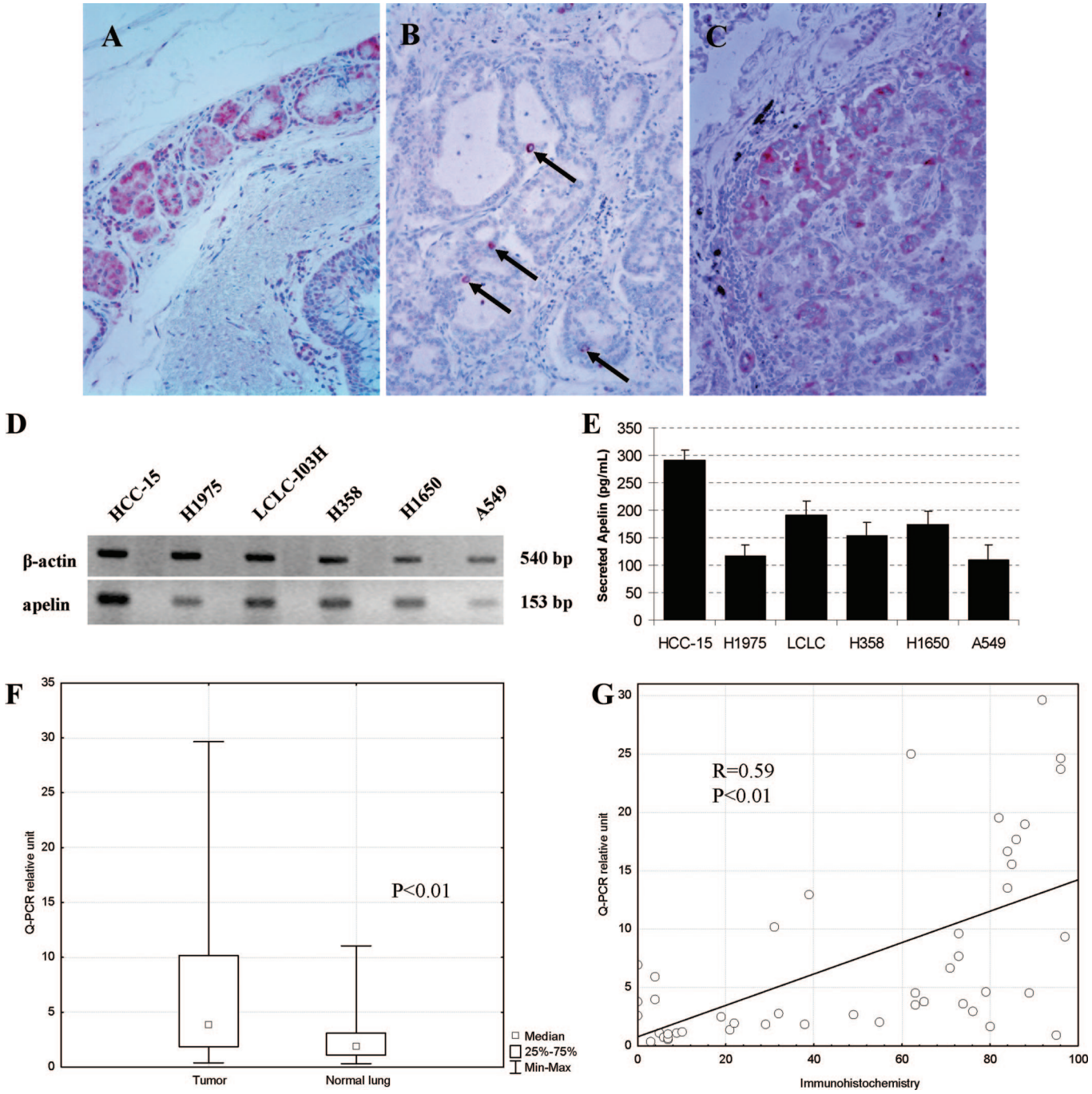

FIGURE 1. Apelin is expressed in normal human lung and non-small cell lung cancer (NSCLC) tissues and cell lines. *A*, Immunostaining of apelin in bronchial glands in normal human lung tissue. (*B*, *C*), Immunohistochemistry showing examples for focal (*B*) and diffuse (*C*) apelin staining patterns (corresponding to low and high apelin expression, respectively) in human NSCLC. (*B*) Arrows point at single apelin-expressing tumor cells within the tumor. Magnification, ×200 (*A*–*C*). *D*, Reverse-transcriptase polymerase chain reaction (RT-PCR) demonstrating the expression of apelin mRNA in human NSCLC cell lines and β-actin as control. *E*, Enzyme-linked immunosorbent assay detection of secreted apelin in conditioned medium from NSCLC cell cultures. Columns, mean for three experiments; bars, standard deviation. *F*, Box plots showing significant a difference between apelin mRNA levels of tumor and normal lung specimens of patients with NSCLC as depicted by median (central dots), 25 to 75% quartile ranges (boxes), and minimum/maximum levels (whiskers) ($n = 46$). *G*, Correlation between apelin mRNA and protein expressions of human NSCLC specimens as determined by quantitative real-time RT-PCR and immunohistochemistry, respectively ($n = 46$, $R = 0.59$, $p < 0.01$).

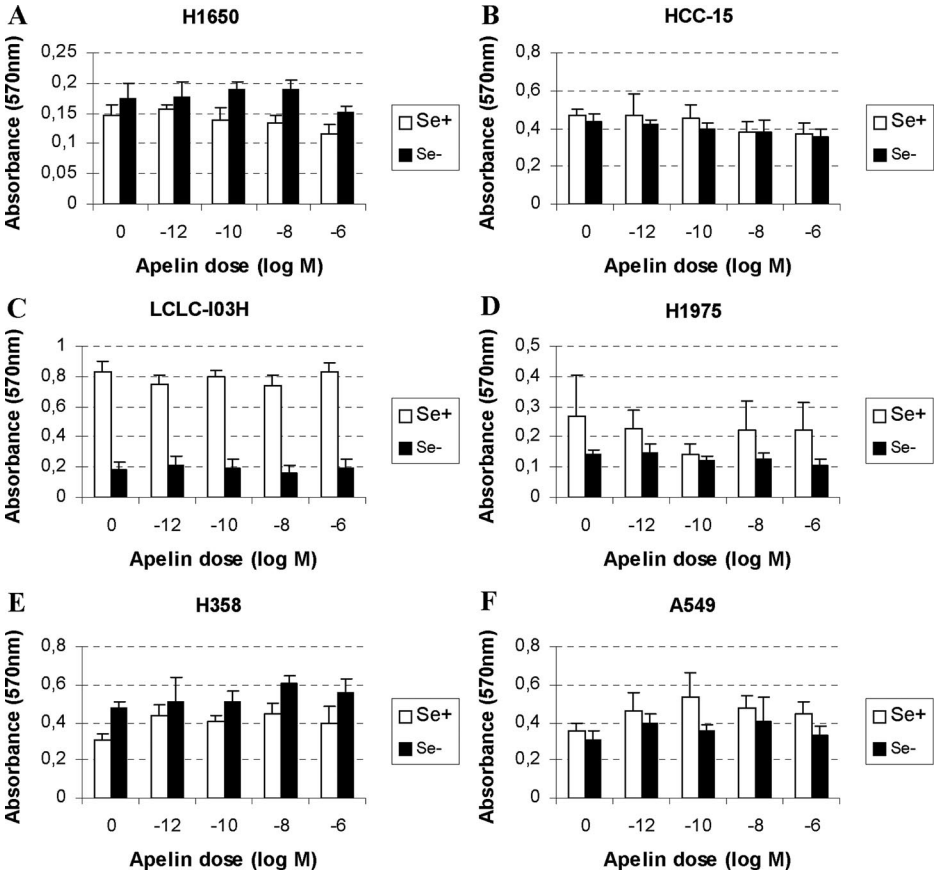

FIGURE 2. Exogenous apelin does not stimulate non-small cell lung cancer (NSCLC) cell proliferation in vitro. Wild-type H1650 (*A*), HCC15 (*B*), LCLC-I03H (*C*), H1975 (*D*), H358 (*E*), and A549 (*F*) cells were cultured in serum-containing (Se+) or serum-free (Se−) medium. Apelin-36 (0 to 10^{-6} M) was added to the cells, and cell numbers were estimated at 72 hours by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. No significant effect of apelin on in vitro NSCLC proliferation was found. Columns, mean for three experiments; bars, standard deviation.

increase in the cases of both H358 and H1975 cell lines in the apelin-transfected compared with the control vector–transfected tumors ($p < 0.05$; Figure 5*F*).

To determine the clinical relevance of apelin expression, we performed comparative statistical analysis of apelin expression and clinicopathologic variables (summarized in Table 1). No significant associations with age, smoking history, gender, tumor (T) status, lymph node (N) status, or histologic type were detected (Table 1). However, corroborating the angiogenic potential of apelin seen in human NSCLC xenograft tumors, high apelin expression was found significantly more frequently in human NSCLC samples with high MVDs compared with tumors with low MVDs ($p = 0.04$, Table 1). Accordingly, the mean number of blood capillaries counted in high apelin-expressing tumors was significantly greater than that in tumors with low apelin protein expression, as determined by anti-CD31 immunohistochemistry ($p = 0.002$; Figure 6*A*).

The association of apelin expression with the angiogenic activity of human NSCLCs, as determined by capillary perimeters, was also studied. Although a tendency toward an increased microvessel perimeter in the cases with high apelin expression was observed, the difference in blood capillary perimeters between tumors with high and low apelin protein expression remained statistically insignificant ($p = 0.14$; Figure 6*B*).

Prognostic Significance of the Increased Expression of the Apelin Protein

When initial apelin immunolabeling categories (−, 1+, 2+, and 3+) were tested for discriminating power in predicting disease outcome, we found that patients whose tumor samples were categorized by an apelin score − or 1+ (i.e., ≤10%; defined as "apelin low") had significantly longer survival times than those with "high" (>10% of positive cells) apelin expression. The 5-year survival rates of patients with high apelin expression and patients with low apelin expression were 29.9% and 63.3%, respectively ($p = 0.002$, log-rank test, Figure 6*C*).

Multivariate analysis (including standard prognostic parameters, such as tumor extension, lymph node status, and patient age) also showed that apelin expressions predicted outcome independent of other variables ($p = 0.021$; Table 2). A further prognostic factor related to poor survival was lymph node stage ($p = <0.01$; Table 2).

DISCUSSION

Apelin has been reported to stimulate endothelial growth in various in vitro and in vivo experimental systems,[8–11] and recent studies have provided evidence for its expression in human[13] and angiogenic potential in murine tumors.[12,13] However, to date, no studies have attempted to evaluate the role of apelin in human cancer vascularization,

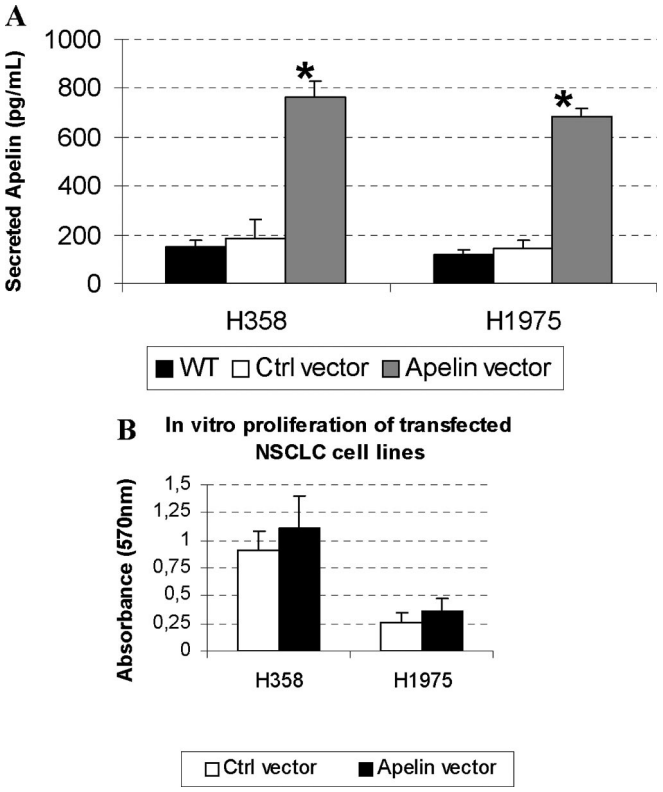

FIGURE 3. Genetic manipulation increases cellular expression of apelin but does not stimulate in vitro proliferation of non-small cell lung cancer cell lines. *A*, Enzyme-linked immunosorbent assay demonstrating the effects of apelin overexpression on apelin levels in conditioned medium of H358 and H1975 cells stably transfected with either apelin or control vectors. Columns, mean for three experiments; bars, standard deviation; *$p < 0.05$ versus controls. *B*, No significant difference in proliferation was found by a 72-hour 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay when comparing H358 and H1975 cells stably transfected with control or apelin expression vectors. Columns, mean for three experiments; bars, standard deviation.

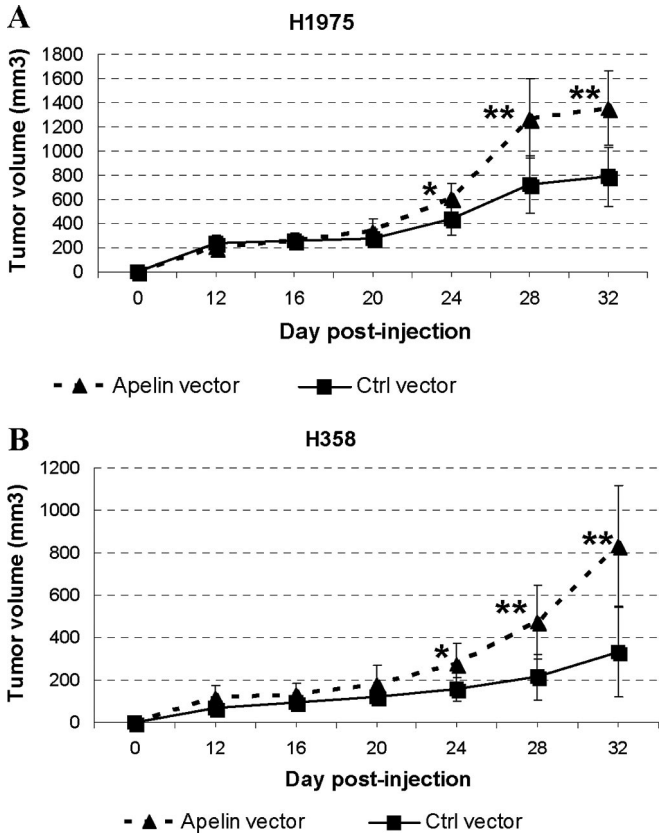

FIGURE 4. Overexpression of apelin through genetic manipulation-stimulated in vivo growth of human non-small cell lung cancer cells in nude mice. Growth curves of control vector- and apelin-transfected H1975 (*A*) and H358 (*B*) cells. (■) and (▲), means for eight mice per group; bars, standard deviation; *$p < 0.05$ and **$p < 0.01$ versus controls.

and no studies have been reported on its clinicopathologic significance. To the best of our knowledge, our results presented here demonstrate for the first time a direct association of apelin expression with angiogenesis and clinical outcome in a human malignancy.

NSCLC remains the leading cause of cancer death in both sexes and is one of the major challenges of medical oncology.[1] Even in surgically resectable cases, more than 50% of patients develop metastases within 5 years. The mechanisms responsible for the aggressive behavior of this cancer are not fully understood. A large body of preclinical evidence has confirmed angiogenesis as a key process of NSCLC progression, and several agents targeting the tumor vasculature have been evaluated. However, because recent clinical trials investigating these drugs have been both encouraging and disappointing,[25,26] success with antivascular strategies undoubtedly requires a deeper knowledge of the clinical significance of the different angiogenic factors that control NSCLC.

Apelin has been shown to be highly expressed in normal lung tissue,[27] and the up-regulation of apelin gene expression in various human solid tumors (including lung cancer) was also demonstrated recently.[13,28] In the present study, we further investigated the expression of apelin in normal and tumorous human lung tissues, and found that apelin, as measured by quantitative RT-PCR, is overexpressed in fresh frozen NSCLC samples at the mRNA level and that these levels are commensurate with protein levels detected by immunohistochemistry in corresponding formalin-fixed, paraffin-embedded pathologic specimens. Apelin was also present in all the investigated human NSCLC cell lines both at the mRNA and the protein levels. Although genetic manipulation to increase apelin expression in NSCLC cells resulted in increased tumor growth in mice, neither apelin transfection nor exogenous apelin stimulated NSCLC cell proliferation in vitro. Thus, in agreement with the previous results of Sorli et al. in mouse melanoma[12] and mammary carcinoma,[13] these findings suggested that apelin plays a role in promoting NSCLC progression in vivo but did not indicate the presence of an autocrine component to this effect. Given

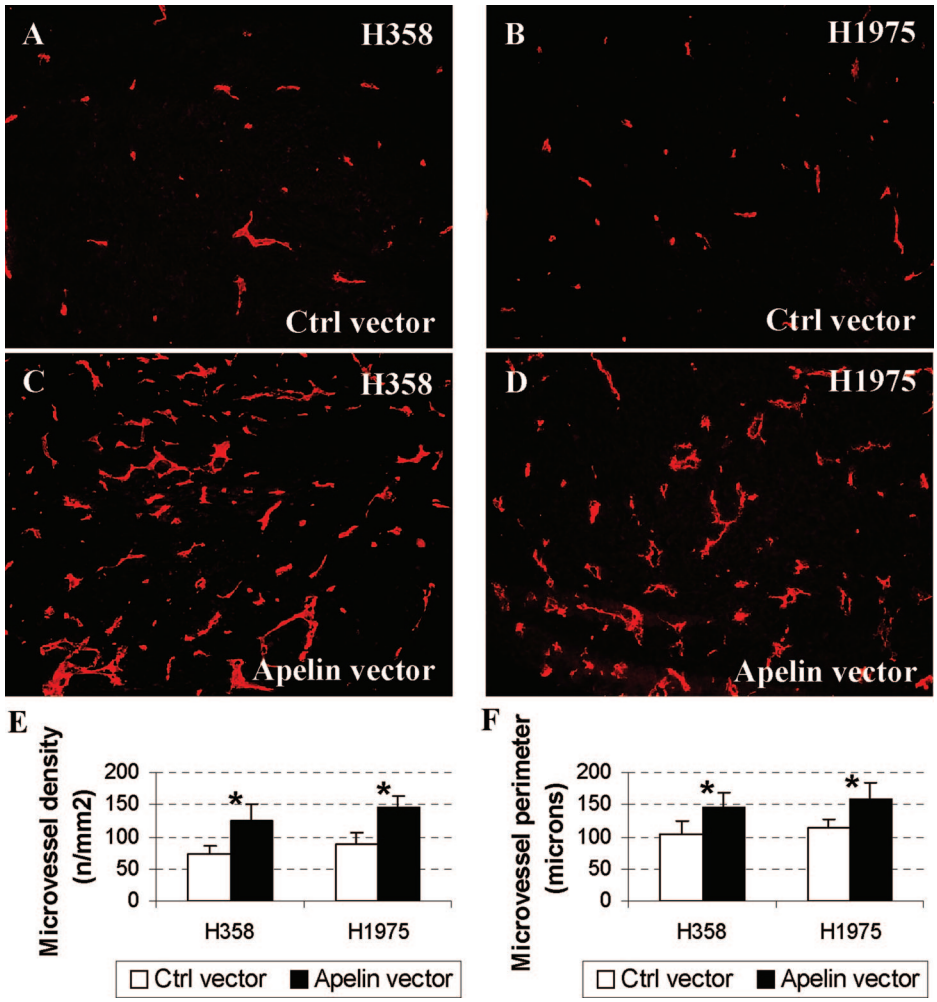

FIGURE 5. Apelin overexpression increases non-small cell lung cancer angiogenesis in vivo. Frozen sections of 32-day-old apelin-overexpressing H358 (*C*) and H1975 (*D*) tumors or of control H358 (*A*) and H1975 (*B*) tumors were stained for the endothelial cell marker CD31 (red fluorescence). Magnification, ×200 (*A–D*). *E*, Microvessel densities (MVDs) of 32-day-old apelin-overexpressing or of control H358 and H1975 tumors. MVDs are mean blood vessel counts per square millimeter. *F*, Microvessel perimeters of 32-day-old apelin-overexpressing or of control H358 and H1975 tumors. Microvessel perimeters are expressed in micrometers. Columns, means for eight mice per group; bars, standard deviation; $*p < 0.05$ versus controls.

its well-documented angiogenic functions,[8–13] we hypothesized, therefore, that apelin might enhance NSCLC growth by exerting a stimulatory effect on blood capillaries. Investigating the association of apelin protein expression with the angiogenic activity in tumor samples of NSCLC patients, we found that the microvessel counts of high apelin expressors were significantly higher than that of low apelin expressors. This result was further corroborated by the observation that apelin overexpression of transfected human NSCLC cells significantly increased intratumoral MVDs of tumors growing subcutaneously in nude mice.

Apelin overexpression in experimental tumors was associated not only with a high number of intratumoral capillaries but with significantly increased vessel perimeters. Although NSCLC patients who had tumor samples with high apelin protein expression also tended to have larger intratumoral blood capillaries than those who had tumors with low apelin expression, this tendency remained nonsignificant, which may reflect the differences in tumor microenvironment (such as extracellular matrix composition of the host tissue or mechanical forces) between subcutaneously growing experimental and human tumors. However, it is difficult to conclude, based on our results in clinical samples presented here, that enlargement of blood vessels during the vascularization of human NSCLC is definitely a result of apelin action, as other recent studies investigating apelin-induced angiogenesis in developmental[29] and experimental tumor[13] models have also found. Based on our above observations in human NSCLC samples, one can rather hypothesize that apelin predominantly acts as a branching factor during the development of the vascular network of human NSCLCs. This assumption is supported by recent results of chorioallantoic membrane experiments in which, for example, apelin was demonstrated to stimulate capillary branching 2.5-fold relative to controls approximately equivalent to the degree of branching induced by vascular endothelial growth factor.[5]

The observed association between apelin expression and angiogenic activity in NSCLC samples, taken together with experimental and clinical evidence accumulated thus far on the role of angiogenesis in NSCLC progression[15,25,26] prompted our decision to investigate the associations between apelin expression and the clinicopathologic parameters of our patients. During the follow-up period of 5 years, a significantly higher incidence of death from NSCLC was observed in patients with high apelin protein expression compared with patients with low apelin expression, and high apelin protein

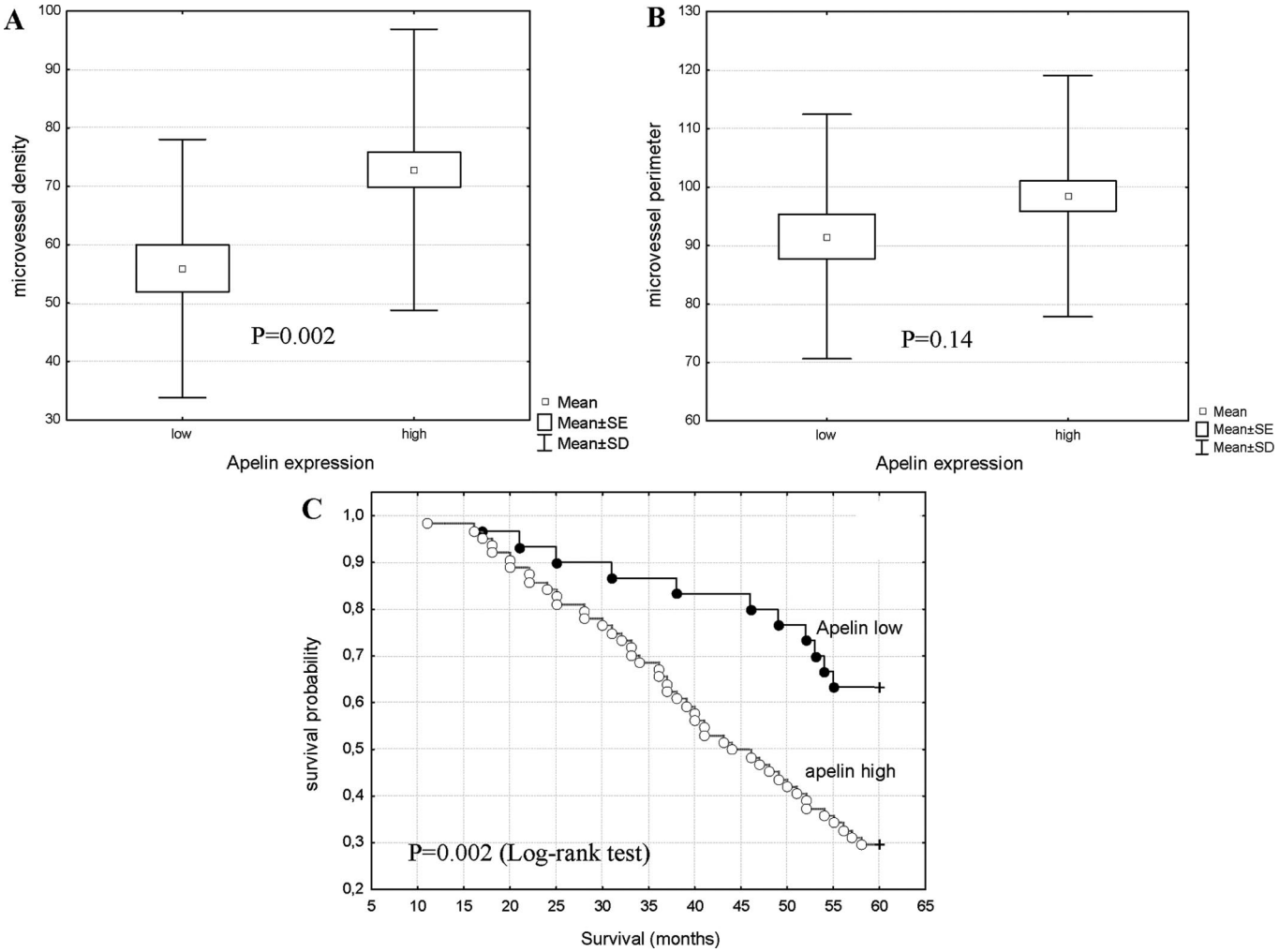

FIGURE 6. Apelin expression is associated with angiogenesis and prognosis in human non-small cell lung cancer (NSCLC). *A*, Box plots showing median (central dots), 25 to 75% quartile ranges (boxes), and minimum/maximum levels (whiskers) of microvessel densities (MVDs), as determined by CD31 immunolabeling, in human NSCLC specimens with low versus high apelin protein expression. MVDs are mean blood vessel counts per square millimeter; $n = 94$. *B*, Box plots showing median (central dots), 25 to 75% quartile ranges (boxes), and minimum/maximum levels (whiskers) of microvessel perimeters, as determined by CD31 immunostaining, in human NSCLC specimens with low versus high apelin protein expression. Microvessel perimeters are expressed in micrometers; $n = 94$. *C*, Kaplan-Meier curves for the overall survival of the patient population with NSCLC, according to apelin expression as determined by immunohistochemistry. High apelin expression in the tumors was a significant prognostic factor for reduced overall survival; $n = 94$.

TABLE 2. Multivariate Analysis of Various Prognostic Factors in Patients with NSCLC ($n = 94$)

Prognostic Factor	RR	95% CI	*p*
Age in years (<63 vs. ≥63)	1.577	0.9–2.761	0.111
Gender (male vs. female)	0.547	0.289–1.036	0.064
Histology (AC vs. SCC/LCC)	1.015	0.65–1.585	0.946
Pathologic T-stage (T_1 vs. T_{2-3})	0.94	0.711–1.243	0.664
Pathologic N-stage (N_0 vs. N_{1-2})	5.974	2.888–12.357	<0.01
Apelin expression (low vs. high)	2.354	1.135–4.883	0.021

RR, relative risk; CI, confidence interval; AC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma.

expression was found to be a significant independent factor for predicting poor prognosis. Thus, this study reports, for the first time, the presence and important prognostic role of apelin as an angiogenic factor that increases the aggressive behavior of human NSCLC. It is important to note, however, that the vascularization process of solid tumors is tightly regulated by the opposing effects of proangiogenic factors and inhibitors, the balance of which may change at different stages during tumor progression.[30] The observation that apelin signaling was demonstrated to regulate pathologic retinal vascularization in a cooperative manner with vascular endothelial growth factor or fibroblast growth factor[31] further supports this idea. Therefore, additional studies to reveal the interrelationships between apelin and other angiogenic mol-

ecules not only in NSCLC but also in other solid human tumors are warranted. Nevertheless, our novel observations add apelin to the growing list of angiogenic molecules in NSCLC and open the door for the development of potential future therapies targeting apelin signaling to treat human NSCLC.

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Expert Opinion

Antivascular agents for non-small-cell lung cancer: current status and future directions

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Background: Despite improvements in surgery and chemo(radio)therapy which have allowed for modest advances in the treatment of patients with non-small-cell lung cancer (NSCLC), survival remains poor and further improvements are needed. Attention over recent years has focused, therefore, on targeted therapies, with notable success in the development of antivascular drugs. *Objective*: To summarize the current knowledge on antivascular therapy in patients with NSCLC. *Method*: Review of randomized controlled trials exploring treatment of NSCLC patients with antivascular drugs. *Results/conclusion*: Bevacizumab, a humanized monoclonal antibody against the vascular endothelial growth factor (VEGF), when added to cytotoxic chemotherapy, was the first treatment to prolong the overall survival of patients with advanced NSCLC beyond 12 months, a significant breakthrough in the management of advanced NSCLC. Small-molecule tyrosine kinase inhibitors and alternative antivascular strategies such as VEGF-trap and vascular disrupting agents are also being investigated and have shown promise in clinical trials. This review summarizes the most recent and important findings in antivascular agents in NSCLC.

Keywords: angiogenesis, antivascular drug, clinical trial, non-small-cell lung cancer

Expert Opin. Investig. Drugs [Early Online]

1. Introduction

Lung cancer, a serious public health problem and the leading cause of cancer-related death worldwide [1,2], is classified in two major groups: small-cell and non-small-cell cancer (SCLC and NSCLC, respectively). NSCLC accounts for the majority (~ 85%) of all lung cancer cases. Unfortunately, more than 60% of patients with NSCLC present with locally advanced, unresectable or metastatic (stage III/IV) cancer at initial diagnosis and die from the disease.

Standard first-line chemotherapy for patients with advanced NSCLC is a platinum-based doublet regimen incorporating a third-generation cytotoxic drug such as gemcitabine (Gemzar®; Eli Lilly and Company, Indianapolis, IN), paclitaxel (Taxol®; Bristol-Myers Squibb, Princeton, NJ), docetaxel (Taxotere®; Sanofi-Aventis, Bridgewater, NJ) or vinorelbine (Navelbin®; Pierre Fabre Medicament, Albi Area, France). However, although these combinations have improved survival compared with best supportive care, the response of NSCLC to first-line treatments is usually transitory, with progression occurring 4 – 6 months after chemotherapy is completed. Furthermore, the median overall survival (OS) is still only 8 – 11 months [3-5]. The benefit of first-line cytotoxic regimens for advanced NSCLC therefore seems to have reached a plateau, and more effective treatments are clearly required. The need for more successful anti-NSCLC treatments has led to the development of

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new therapeutic approaches targeting molecular pathways involved in tumor progression. Targeted antivascular drugs have shown particular promise in preclinical NSCLC models, demonstrated notable success in clinical studies and are the focus of this review.

2. Antivascular strategies for NSCLC

Any classification of antivascular treatments is difficult, with overlap in several features. However, the main categories of these drugs that have been developed are angiosuppressive (antiangiogenic) and vascular-disrupting agents (VDAs) (**Table 1**) [6]. As described in this review, many of these agents are at an early phase of development, but a number have reached later phases of testing (**Table 2**).

2.1 Antiangiogenic (angiosuppressive) therapy

This approach is based on the observation that new blood vessel growth in tumors requires the induction of endothelial cell proliferation by specific or nonspecific mitogens. By inhibiting the production of endothelial mitogens, the mitogens themselves, their endothelial receptors, the associated signaling pathways, the endothelial integrins and the matrix metalloproteinases (MMPs), these agents specifically target endothelial sprouting (defined as the *in situ* proliferation and budding of endothelial cells [7]) and postnatal vasculogenesis (i.e., bone-marrow-derived endothelial progenitor cells, reviewed in [8]) in cancer.

2.1.1 *Inhibitors of VEGF signaling*

In order to develop and metastasize, growing tumors require an adequate blood supply. Similar to other solid tumors, NSCLCs achieve this by secreting various angiogenic cytokines that regulate the complex series of events of blood vessel growth. Among these angiogenic molecules, vascular endothelial growth factor (VEGF) has been identified as the key cytokine for endothelial sprouting in NSCLC [9]. The VEGF protein has four isoforms, VEGF 121, 165, 189 and 206 and is encoded on chromosome 6p21.3. At present, the VEGF family members (including placental growth factor 1 and 2, and VEGF A, B, C, D and E) act as ligands for three tyrosine kinase receptors: VEGFR-1 (flt1), VEGFR-2 (KDR) and VEGFR-3 (flk4). VEGFR-2 is the principal receptor for VEGF (VEGF-A) signaling, whereas VEGFR-1 functions as a decoy receptor to control the availability of VEGF. VEGF-C and D are ligands for VEGFR-3, which is predominantly expressed by lymphatic endothelial cells [10].

2.1.1.1 *Bevacizumab*

Bevacizumab (Avastin®; Genentech/Roche, South San Francisco, CA, USA) is a humanized monoclonal antibody that acts by binding and neutralizing the VEGF-A isoform, thus preventing VEGF ligand-receptor binding. This agent has been studied in a variety of solid tumors both as a single agent and in combination with cytotoxic chemotherapy or with other targeted therapies. This is so far the first and only antivascular drug to be licensed for the treatment of NSCLC. Bevacizumab has also been approved for the treatment of breast [11], renal cell [12] and metastatic colorectal cancers [13] in Europe, while in the USA the FDA has approved bevacizumab in breast and colorectal cancers in addition to NSCLC.

2.1.1.1.1 *First-line clinical development of bevacizumab*

In lung cancer, the first evidence for the benefit of bevacizumab came from a Phase II randomized trial for patients with advanced or recurrent NSCLC (**Table 2**) [14]. Results showed that those treated with higher bevacizumab doses (15 mg/kg) had a significantly increased time to progression (TTP) compared with chemotherapy alone. In addition, there was a trend towards improved OS. However, this improvement was not statistically significant, possibly because of the large number of patients who crossed over from receiving chemotherapy only to receiving chemotherapy plus bevacizumab upon showing signs of progression. In general, bevacizumab was well tolerated, although six patients on the bevacizumab arm developed severe pulmonary hemorrhage [14]. In a *post hoc* multivariate analysis, squamous cell histology was identified as an independent risk factor for bleeding [15]. Consequently, patients with squamous cell histology were excluded from most of the clinical trials of bevacizumab in NCSLC.

Subsequent to the above Phase II study, the Eastern Cooperative Oncology Group (ECOG) E4599 trial was initiated (**Table 2**) [16]. This study, which is the first published Phase III randomized trial of an antiangiogenesis agent in combination with chemotherapy in patients with advanced NSCLC, randomized chemotherapy-naive patients with predominantly non-squamous cell histology. In the bevacizumab treatment arm, following completion of chemotherapy, single-agent bevacizumab was continued until disease progression. The primary study end point was OS. Results showed that the addition of bevacizumab was associated with a significant improvement in the median OS compared with chemotherapy alone. Progression-free survival (PFS) was also significantly improved (**Table 2**).

As a result of this study, the US FDA approved the use of bevacizumab in combination with carboplatin and paclitaxel for the initial systemic treatment of patients with unresectable, locally advanced, recurrent or metastatic, non-squamous NSCLC, and this treatment has become the standard of care for these patients in the USA.

A retrospective analysis of treatment outcomes in the E4599 trial based on different histologic subtypes was also presented by Sandler *et al*. [17]. For evaluable patients, the predominant tumor histology was adenocarcinoma (602 of 878 patients; 68.6%). There were 165 patients (18.8%) with tumors of not otherwise specified histology, and 48 patients (5.5%) with large cell undifferentiated tumors. Fewer than 5% of patients had tumors of other histological subtypes. Baseline demographics and patient characteristics by histology type

Table 1. Antivascular agents under development in human NSCLC.

Drug name	Developer	Phase of development	Study status
Inhibitors of VEGF signaling			
Bevacizumab, Avastin	Genentech, Inc.	Approved	
IMC-1121b	ImCloneSystems, Inc.	Phase II	Ongoing
IMC-18F1	ImCloneSystems, Inc.	Phase I	Ongoing
Aflibercept, VEGF-Trap	Regeneron Pharmaceuticals	Phase III	Ongoing
Tyrosine kinase receptor inhibitors			
Vandetanib, ZD-6474, Zactima	Astra-Zeneca	Phase III	Completed
Cediranib, AZD2171, Recentin	Astra-Zeneca	Phase II	Ongoing
Axitinib, AG-013736	Pfizer	Phase II	Completed
Sorafenib, BAY 43-9006, Nexavar	Bayer	Phase III	Ongoing
Sunitinib, SU011248, Sutent	Pfizer	Phase III	Ongoing
Motesanib, AMG-706	Amgen	Phase III	Ongoing
Vatalanib, (PTK787/ZK 222584)	Bayer	Phase II	Completed
Pazopanib, GW786034	GlaxoSmithKline	Phase II/III	Ongoing
BIBF 1120	Boehringer Ingelheim	Phase III	Ongoing
ABT-869	Abbott Laboratories	Phase II	Ongoing
BMS-690514	Bristol Myers Squibb	Phase II	Ongoing
OSI-930	OSI Pharmaceuticals	Phase I	Ongoing
AEE788	Novartis International AG	Phase II	Completed
XL647	Exelixis	Phase II	Ongoing
XL999	Exelixis	Phase I	terminated
CP-547,632	Pfizer	Phase II	Completed
AV-951	Aveo Pharmaceuticals, Inc.	Phase Ib/IIa	Ongoing
Thalidomide and analogs			
Thalidomide	Celgene	Phase III	Ongoing
Lenalidomide, CC-5013, Revlimid	Celgene	Phase II	Completed
Matrix metalloproteinases			
BMS-275291	Bristol Myers Squibb	Phase III	Completed
Other antiangiogenic agents			
cilengitide, EMD 121974	Merck	Phase II	Ongoing
volociximab, M200	PDL BioPharma, Biogen Idec	Phase I	Ongoing
TNP-470	Takeda Chemical Industries, Inc.	Phase I	Completed
AMG-386	Amgen	Phase I	Completed
Vascular disrupting agents			
DMXAA, ASA-404, AS1404	Novartis International AG	Phase III	Ongoing
Zybrestat, combrestatin, CA4P	OxiGene, Inc.	Phase II	Ongoing
NPI-2358	Nereus Pharmaceuticals, Inc.	Phase I	Ongoing
ABT-751	Abbott Laboratories	Phase II	Completed

Table 2. Results of recent Phase II – III clinical trials of antivascular agents in the treatment of NSCLC.

Ref.	Phase	Setting	Treatment	No. of patients	DCR (%)	OS (months)	TTP (months)	PFS (months)
[14]	II (r)	1st line	paclitaxel + carboplatin vs paclitaxel + carboplatin + bevacizumab (7.5 mg/kg) vs paclitaxel + carboplatin + bevacizumab (15 mg/kg)	32 32 35	31* 22* 40*	14.9 11.6 17.7	4.2 4.3 7.4	NR
[17]	III (r)	1st line	paclitaxel + carboplatin vs paclitaxel + carboplatin + bevacizumab	444 434	15* 35*	10.3 12.3	NR	4.5 6.2
[19]	III (r)	1st line	cisplatin + gemcitabine vs cisplatin + gemcitabine + bevacizumab (7.5 mg/kg) vs cisplatin + gemcitabine + bevacizumab (15 mg/kg)	347 345 351	20.1* 34.1* 30.4*	13.1 13.6 13.4	NR	6.1 6.7 6.5
[20]	II	1st line	pemetrexed + carboblatin + bevacizumab	50	55*	13.5	NR	9.3
[27]	II (r)	2nd line	chemo vs chemo + bevacizumab vs erlotinib + bevacizumab	41 40 39	39.0 52.2 51.3	8.6 12.6 13.7	NR	3.0 4.8 4.4
[28]	III (r)	2nd line	erlotinib vs erlotinib + bevacizumab	317 319	34.0 45.2	9.2 9.3	NR	1.7 3.4
[40]	II (r) (2 parts)	2nd, 3rd line	gefitinib vs vandetanib	85 83	34‡ 45‡	7.4§ 6.1¶	NR	1.9‡ 2.5‡
[41]	II (r)	2nd line	docetaxel vs docetaxel + vandetanib (100 mg) vs docetaxel + vandetanib (300 mg) vs	41 42 44	56 83 63	13.4 13.1 7.9	NR	2.8 4.3 3.9
[42]	II (r)	1st line	paclitaxel + carboplatin vs paclitaxel + carboplatin + vandetanib vs vandetanib	52 56 73	58 57 NR	12.6 10.2 NR	NR	5.3 5.5 NR
[52]	II	All lines	axitinib	32	9.4*	12.8	9.4	NR
[55]	II	2nd line	sorafenib	52	59	6.8	NR	2.7
[56]	II	2nd line	sorafenib	6	4/5	NR	NR	NR
[62]	II	1st line maintenance	carboplatin + paclitaxel followed by sunitinib	87	NR	10.5	2.6	NR
[63]	II	2nd, 3rd line	sunitinib (continuous daily)	47	19	NR	NR	2.8
[69]	II	2nd line	vatalanib (qd) vatalanib (b.i.d.)	54 58	35 39	7.0 6.8	NR NR	2.4 3.7
[70]	II	neoadjuvant	pazopanib	35	86#	NR	NR	NR
[74]	II	2nd, 3rd line	BIBF 1120 (250 b.i.d.) BIBF 1120 (150 b.i.d.)	73	48	NR	NR	1.6
[93]	II	2nd, 3rd line	XL999	9	3/9	NR	NR	NR

*Stable disease not reported.

‡Part A.

§Part B (switched to vandetanib from gefitinib).

¶Part B (switched to gefitinib from vandetanib).

#Primary end point = tumor reduction.

b.i.d.: Bis in die (twice daily); DCR: Disease control rate; NR: Not reported; OS: Overall survival; PFS: Progression-free survival; qd: Quaque die (every day); r: Randomized; TTP: Time to progression.

Table 2. Results of recent Phase II – III clinical trials of antivascular agents in the treatment of NSCLC (continued).

Ref.	Phase	Setting	Treatment	No. of patients	DCR (%)	OS (months)	TTP (months)	PFS (months)
[94]	II (r)	1st line	paclitaxel + carboplatin vs paclitaxel + carboplatin + CP-547,632	17 14	25 28.6	NR	7.7 4.7	NR
[98]	II	1st line	carboplatin + irinothecan + thalidomide	36	74	7.3	3.6	NR
[99]	II	1st line	gemcitabine + irinothecan + thalidomide	11	50	NR	4.3	NR
[105]	III (r)		paclitaxel + carboplatin vs paclitaxel + carboplatin + BMS-275291	387 387	81.9 78.6	9.2 8.6	NR	5.3 4.9
[118]	II (r)	1st line	paclitaxel + carboplatin vs paclitaxel + carboplatin + ASA404	36 37	61.3 78.2	8.8 14.0	4.4 5.4	NR

*Stable disease not reported.
‡Part A.
§Part B (switched to vandetanib from gefitinib).
¶Part B (switched to gefitinib from vandetanib).
#Primary end point = tumor reduction.
b.i.d.: Bis in die (twice daily); DCR: Disease control rate; NR: Not reported; OS: Overall survival; PFS: Progression-free survival; qd: Quaque die (every day); r: Randomized; TTP: Time to progression.

were comparable among the paclitaxel/carboplatin (PC) and paclitaxel/carboplatin/bevacizumab (PCB) treatment arms. For patients with adenocarcinoma histology, median OS was 10.3 months for PC alone and 14.2 months for PCB. Median PFS was 5.0 months for PC alone and 6.6 months for PCB.

A second Phase III trial, Avastin® in Lung (AVAiL), evaluating bevacizumab in combination with cisplatin and gemcitabine [18] (another commonly used and efficacious regimen in NSCLC) was originally initiated with a primary end point of OS. However, after the positive OS results of E4599, the study design was amended to change the primary end point from OS to PFS (this decision was made to ensure completion of the trial, to accelerate reporting of the efficacy data and thus expedite the availability of a potentially active treatment option for patients, and to avoid the risk of the OS end point being confounded by the increasing use of second-line therapies, including non-protocol crossover of patients in the placebo arm to bevacizumab). Patients were randomly assigned to receive cisplatin 80 mg/m^2 and gemcitabine 1250 mg/m^2 for up to six cycles plus low-dose bevacizumab (7.5 mg/kg), high-dose bevacizumab (15 mg/kg) or placebo every 3 weeks until disease progression. Between February 2005 and August 2006, 1043 patients were randomly assigned (placebo, n = 347; low dose, n = 345; high dose, n = 351). PFS was significantly prolonged with bevacizumab (**Table 2**).

Interestingly, according to the final efficacy analysis, OS was > 13 months in all treatment groups (**Table 2**), which is the longest OS reported for advanced non-squamous NSCLC in a clinical trial setting, although it did not yield a statistically significant prolongation with either bevacizumab dose [19]. Among the possible explanations for these results are the good performance status of the placebo arm and the unprecedentedly high proportion of second-line and third-line line therapies administered to these patients. To support this assumption further, there was a trend towards improved OS in patients not receiving post-protocol therapies (HR = 0.84; bevacizumab to placebo).

As a result of the above trials, bevacizumab in combination with platinum-based chemotherapy was approved for the first-line treatment of patients with advanced NSCLC by the European Medicines Agency (EMEA), in August 2007.

2.1.1.1.2 New combinations with bevacizumab in the first-line setting

Ongoing Phase II – III trials are evaluating bevacizumab with other platinum-based chemotherapy regimens.

A recent study of pemetrexed and carboplatin plus bevacizumab with maintenance pemetrexed and bevacizumab was designed to evaluate toxicities and estimate median TTP of this new regimen in advanced non-squamous NSCLC patients [20]. Results were based on 50 patients who received pemetrexed 500 mg/m^2, carboplatin AUC 6, and bevacizumab 15 mg/kg repeated every 21 days for six cycles. For patients with CR (complete response), PR (partial response), or SD (stable disease), pemetrexed 500 mg/m^2 and bevacizumab 15 mg/kg were continued as maintenance every 21 days until disease progression or toxicity (**Table 2**). There were no grade > 3 hemorrhagic events, nor did any patients experience grade 3/4 hypertension.

A Phase II study has also evaluated the efficacy and safety of oxaliplatin and gemcitabine with bevacizumab

in non-squamous NSCLC patients [21]. Nineteen of 44 eligible patients had PR for an objective response rate (RR) of 43%. Sixteen patients had SD for a disease control rate of 80%. Median TTP was 5.5 months. Median OS was 13.7 months. The most common grade 3 or 4 adverse events were hypertension (11%), neutropenia (9%), diarrhea (7%), dyspnea (7%) and thromboembolic events (7%). Pulmonary hemorrhage was not observed.

2.1.1.1.3 *Bevacizumab in combination with epidermal growth factor receptor (EGFR) inhibitors*

2.1.1.1.3.1 *Bevacizumab and EGFR inhibition: first-line therapy*

The safety and effectiveness of bevacizumab and erlotinib (an EGFR tyrosine kinase inhibitor) in the first-line setting is now being investigated in several Phase II studies. The SAKK 19/05 trial evaluates bevacizumab and erlotinib, followed by platinum-based chemotherapy at disease progression [22]. The INNOVATIONS study [23] compares bevacizumab and erlotinib with gemcitabine, cisplatin and bevacizumab. Both studies are actively recruiting patients.

In a Phase II study, the combination of bevacizumab, erlotinib and chemotherapy for first-line treatment was evaluated [24]. Patients received either up to four cycles of bevacizumab (15 mg/kg) and erlotinib (150 mg qd) or up to four cycles of carboplatin (AUC = 6), paclitaxel (200 mg/m^2) and bevacizumab (15 mg/kg). Patients treated with erlotinib and bevacizumab who had achieved at least SD or any response continued treatment with bevacizumab and erlotinib until progression. The primary objective was to determine the rate of disease stabilization of the combination of bevacizumab and erlotinib. With 48 patients enrolled, the median OS for all patients was 12.9 months. Patients treated with erlotinib and bevacizumab who had disease stabilization or response had a median survival of 23.2 months (vs 6.6 months, $p < 0.001$).

Another interesting approach is the four-drug regimen, which is subsequently being tested in the SWOG S0536 trial [25]. Eligibility included patients with treatment-naive stage IIIb or IV incurable non-squamous cell NSCLC, and these patients were treated with carboplatin, paclitaxel, bevacizumab and cetuximab for up to six cycles, followed by bevacizumab and cetuximab until progression. The primary end point was feasibility defined by the frequency and severity of hemorrhagic toxicities. One hundred and ten patients were enrolled. Grade 4 adverse events occurred in 40 patients. The estimated rate of grade 4 or worse hemorrhage was 2%. Disease control rate was reported as 74%, while PFS is 7 months and OS is 14 months. The combination of carboplatin, paclitaxel, bevacizumab and cetuximab demonstrated safety, tolerability and efficacy in patients with advanced NSCLC and was found to be the most active NSCLC regimen studied to date within SWOG (Southwest Oncology Group).

2.1.1.1.3.2 *Bevacizumab and EGFR inhibition: first-line maintenance therapy*

AVF3671g (ATLAS) is a Phase IIIb, placebo-controlled, double-blind, randomized trial to evaluate the efficacy of bevacizumab in combination with erlotinib for the first-line maintenance treatment of advanced non-squamous or peripherally located squamous NSCLC following four cycles of bevacizumab and chemotherapy. According to a press release [26], the study has met its primary end point, post-chemotherapy PFS, without new safety signals. Although final survival data have not yet been published, the results further indicate the notion that the dual inhibition of VEGF and EGFR pathways in the treatment of NSCLC may confer additional clinical benefit.

2.1.1.1.3.3 *Bevacizumab and EGFR inhibition: second-line setting*

A multicenter, randomized Phase II trial evaluated the safety and efficacy of combining bevacizumab with either chemotherapy (docetaxel or pemetrexed) or erlotinib [27]. All patients had histologically confirmed non-squamous NSCLC that had progressed during or after one platinum-based regimen and were randomly assigned on a 1:1:1 basis to bevacizumab in combination with chemotherapy (docetaxel or pemetrexed), bevacizumab in combination with erlotinib, or chemotherapy alone (docetaxel or pemetrexed + placebo). The primary efficacy end point of the trial was PFS (**Table 2**). No unexpected adverse events were noted. Fewer patients (13%) in the bevacizumab-erlotinib arm discontinued treatment as a result of adverse events than in the chemotherapy alone (24%) or bevacizumab-chemotherapy (28%) arms.

BeTa Lung, a randomized Phase III trial comparing erlotinib monotherapy with erlotinib plus bevacizumab in the second-line setting has also recently published its findings (**Table 2**). The primary end point of prolonging OS was not met (HR = 0.97; $p = 0.7583$). There was, however, clear evidence of clinical activity with improvements in secondary end points such as PFS (HR = 0.62, $p < 0.0001$) and RR (6.2 vs 12.6%) when bevacizumab was added to erlotinib compared with erlotinib monotherapy. As in the AVF3671g, no new safety signals were identified [28].

2.1.1.1.4 *Current objectives and future perspectives with bevacizumab*

Based on positive data from two completed Phase III trials (E4599 and AVAiL), bevacizumab has become the standard of care in the bevacizumab-eligible patient population in many western countries. Moreover, a wide range of clinical trial activities are underway to examine the potential role of bevacizumab in combination with cytotoxic chemotherapy or other biological agents not only in the first-line treatment of NSCLC but also at subsequent stages. It should be emphasized, however, that defining the eligible patient pool based on an evaluation of safety issues is of the greatest importance.

2.1.1.1.4.1 *Combination of bevacizumab with (chemo) irradiation*

Several Phase I studies are ongoing to investigate concurrent bevacizumab with radiotherapy, or concomitant chemoradiation in combination with bevacizumab. A Phase I/II trial investigating the maximum tolerated dose (MTD) of bevacizumab and erlotinib when given together with carboplatin, paclitaxel and thoracic conformal radiotherapy in stage IIIa/b NSCLC is now underway [29].

2.1.1.1.4.2 *Neoadjuvant and adjuvant settings*

Based on the unique mode of action of angiogenesis inhibitors, there is a strong rationale for investigating these drugs in the neoadjuvant and adjuvant settings.

The Phase II BEACON study, among other Phase II studies, is evaluating the role of neoadjuvant bevacizumab. Its primary goal is to show that the addition of bevacizumab to a cisplatin-based chemotherapy in the neoadjuvant setting for non-squamous NSCLC improves the rate of pathologic downstaging. The study is now recruiting participants [30].

Bevacizumab is also now being tested in the adjuvant setting in the ECOG E1505 trial. This trial aims to recruit 1500 patients with completely resected stage Ib – IIIa tumors and compare postoperative chemotherapy using four cycles of cisplatin with vinorelbine, docetaxel or gemcitabine and with or without bevacizumab [31].

2.1.1.1.5 *Safety and tolerability of bevacizumab in combination with chemotherapy*

Since the publication of the AVF0757g trial [14], the risk of severe pulmonary hemorrhage has been an issue in patients with NSCLC treated with bevacizumab. Owing to the exclusion of predominantly squamous cell cancer and clinically significant hemoptysis at baseline, the 9.1% incidence of severe pulmonary hemorrhage observed in the AVF0757g trial was reduced to 2.3% in the E4599 trial [16]. With the additional exclusion of patients with tumors invading or abutting major blood vessels, the incidence of this adverse event decreased to 1.5% in the AVAiL trial. By adopting essentially the same exclusion criteria in a large Phase IV trial (SAiL), based on 1699 patients as the safety population, Dansin *et al*. reported grade 3 – 5 hemoptysis in four cases (0.2%) only, underscoring the importance of patient selection in the improvement of patient safety [32].

The AVAiL data also indicate that the incidence of grade ≥ 3 hypertension observed in bevacizumab-treated patients is likely to be dose-dependent (2%, 6% and 9% for placebo, low-dose bevacizumab and high-dose bevacizumab, respectively). Patients with controlled hypertension at baseline are eligible for bevacizumab treatment. The incidence of bevacizumab-associated grade ≥ 3 hypertension was 2.6% in the SAiL study [32].

Although the number of grade ≥ 3 ischemic and venous thromboembolic events were not considerably different between the bevacizumab-treated patients and the control group in the Phase III AVAiL study, caution should be exercised when treating patients with a history of thromboembolic events or those aged over 65 years. Grade ≥ 3 bevacizumab-associated thromboembolic event was found in 2.2% in the SAiL study.

While there was a significantly higher frequency of febrile neutropenia in the bevacizumab arm of the Phase III E4599 trial [16] compared with the control arm, the incidences of febrile neutropenia was only modestly higher in the bevacizumab arms of the Phase III AVAiL study [19] than in the placebo arm.

Higher incidence of proteinuria has been observed during bevacizumab treatment with the majority being grade 1. In the SAiL study, grade ≥ 3 bevacizumab-associated proteinuria was reported in 0.5% [32]. Monitoring of proteinuria by dipstick urinalysis is therefore recommended before starting and during therapy.

All in all, bevacizumab-based therapy until progression has a well-characterized safety profile. This was demonstrated in the E4599, the AVAiL and the Phase IV SAiL trials. Across all indications, the most frequently observed adverse drug reactions in patients receiving bevacizumab were hypertension, fatigue or asthenia, diarrhea and abdominal pain. However, based on the results of the above trials with advanced NSCLC, hemorrhage (including pulmonary hemorrhage/hemoptysis), hypertension and arterial and venous thromboembolism, febrile neutropenia and proteinuria are the most relevant adverse events. Thus, according to the bevacizumab summary of product characteristics, therapy should be permanently discontinued in patients who develop any of the following symptoms: gastrointestinal perforation, grade 4 fistula, grade 4 proteinuria (nephrotic syndrome), arterial thromboembolic events, grade 4 pulmonary embolism, grade 3/4 bleeding, uncontrollable hypertension, hypertensive crisis or hypertensive encephalopathy [33]. However, additional studies are also in progress to evaluate the safety and efficacy of bevacizumab in subjects with squamous NSCLC or in patients with intracranial metastases. It is hoped that these studies will help to refine the role for bevacizumab in NSCLC.

AVF3744g (BRIDGE), an open-label, single-arm, multicenter pilot study of bevacizumab plus carboplatin and paclitaxel in subjects with advanced, previously untreated, squamous NSCLC aimed to determine whether delayed bevacizumab administration could improve safety in patients with squamous cell NSCLC [34]. Patients received two cycles of carboplatin and paclitaxel, followed by four cycles of chemotherapy plus bevacizumab (15 mg/kg every 3 weeks), and then bevacizumab until disease progression. Eligible patients had stage IIIb/IV or recurrent squamous NSCLC. The primary end point of this study was the incidence of grade 3 or higher pulmonary hemorrhage. Altogether, 44 patients were enrolled; 27 patients had received at least one dose of bevacizumab. So far, there have been three reports of hemorrhage in two patients.

PASSPORT, a Phase II study, has assessed the efficacy and safety of bevacizumab combined with first- or second-line

systemic therapy in patients with non-squamous NSCLC and treated brain metastases [35]. Treatment for brain metastases included radiosurgery, neurosurgery or whole brain radiation therapy. Eighty-five subjects with treated brain metastases received bevacizumab without symptomatic grade > 2 hemorrhages observed during the main treatment (pre-progression) phase; no additional safety signals were identified.

Based on the safety data from clinical trials as well as the global safety database, the EMEA has recently removed a restriction in the product label which prevented the use of bevacizumab in patients with brain metastases.

2.1.1.2 *IMC-1121B*

IMC-1121B (ImClone Systems, Inc.) is a fully humanized monoclonal antibody that blocks binding of the VEGF ligand to the extracellular domain of VEGFR-2. A Phase II study to evaluate the PFS rate at 6 months of IMC-1121B administered in combination with paclitaxel and carboplatin as first-line therapy for stage IIIb/IV NSCLC is ongoing [36].

2.1.1.3 *IMC-18F1*

IMC-18F1 (ImClone Systems, Inc.) is a humanized monoclonal antibody against VEGFR-1. A Phase I study of weekly IMC-18F1 in patients with advanced and refractory solid tumors is ongoing [37].

2.1.1.4 *Aflibercept (VEGF trap)*

Aflibercept is a recombinantly produced, fully human, soluble VEGF receptor fusion protein that scavenges both VEGF and placental growth factor removing important ligands for the VEGF receptors expressed on tumor endothelium. In a Phase II study of aflibercept in advanced NSCLC resistant or refractory to platinum-based chemotherapy and erlotinib, patients were given 4 mg/kg VEGF-trap every 2 weeks. Two patients (3.7%) achieved a PR and 34 (67%) maintained SD for more than 60 days [38]. A Phase III second-line study (VITAL) of aflibercept in combination with docetaxel for advanced NSCLC is now recruiting patients [39].

2.1.2 *Tyrosine kinase inhibitors with antiangiogenic effects*

2.1.2.1 *Vandetanib*

Vandetanib (Zactima®, ZD6474; AstraZeneca, Macclesfield, UK) selectively inhibits the tyrosine kinase activity of both VEGFR-2 and EGFR. In a comparative two-part study of vandetanib and gefitinib, 168 patients with previously treated stage IIIb – IV NSCLC were randomized to receive either vandetanib (300 mg p.o. o.d.) or gefitinib (250 mg p.o. o.d.) in part A of the study. At disease progression or development of unacceptable toxicity, subjects were switched to the alternative treatment (part B) after a washout period of 4 weeks. Response rate was 8% in the vandetanib arm compared with 1% in the gefitinib arm, and a longer PFS time for vandetanib followed by gefitinib was observed (**Table 2**). Overall survival, a secondary assessment, was not significantly different between patients initially randomly assigned to either vandetanib or gefitinib [40].

The efficacy of vandetanib in combination with standard chemotherapy regimens compared with chemotherapy alone has been investigated in two subsequent randomized trials.

The results of the first, a randomized, placebo-controlled study of vandetanib (100 mg/day or 300 mg/day) plus docetaxel in 127 patients with NSCLC who have progressed after first-line platinum-based chemotherapy, have been published (**Table 2**). The combination of vandetanib and docetaxel was generally well tolerated and adverse events manageable, with the incidence of vandetanib-associated toxicities increased at the higher dose level (diarrhea (grade 3/4), 50%; rash (grade 3), 46%; QTc-related events (grade 3/4), 16%) [41].

Another trial investigated vandetanib alone or with paclitaxel and carboplatin as first-line treatment for advanced NSCLC (**Table 2**) [42]. With a total of 181 subjects, the primary objective was met, with vandetanib + carboplatin-paclitaxel significantly prolonging PFS compared with carboplatin-paclitaxel alone (HR = 0.76; p = 0.098). Given this encouraging Phase II data, Phase III studies evaluating vandetanib both as monotherapy and in combination regimens for NSCLC were started.

The developer of vandetanib also announced results from three Phase III studies of vandetanib in combination with the chemotherapy agents docetaxel (ZODIAC) and pemetrexed (ZEAL) and as monotherapy (ZEST) in relapsed NSCLC compared with erlotinib [43].

ZODIAC is a randomized, double-blind, placebo-controlled Phase III study evaluating the combination of vandetanib 100 mg with docetaxel versus docetaxel alone. The study enrolled 1391 patients previously treated with one previous anticancer therapy for advanced NSCLC.

ZEAL is a randomized, double-blind, placebo-controlled Phase III study evaluating the combination of vandetanib 100 mg with pemetrexed versus pemetrexed alone. The study enrolled 534 previously treated, advanced NSCLC patients. In both studies the addition of vandetanib to chemotherapy prolonged PFS, the primary endpoint, which achieved statistical significance in the larger ZODIAC study, but not in the smaller ZEAL study. Clinical benefits were seen in secondary end points. Both studies showed that adding vandetanib to chemotherapy significantly improved overall RR. Additionally, positive trends in prolongation of OS were seen, although these did not reach statistical significance.

ZEST, a randomized, double-blind Phase III study evaluating the efficacy of vandetanib 300 mg versus erlotinib 150 mg, did not meet the primary objective of demonstrating a statistically significant prolongation of PFS for vandetanib. However, vandetanib and erlotinib showed equivalent efficacy for PFS and OS in a pre-planned noninferiority analysis. The study enrolled 1240 patients with locally advanced or metastatic NSCLC after failure of at least one previous therapy.

The observed safety profile in these three Phase III studies was consistent with previous studies with vandetanib in

NSCLC. The most common adverse events associated with vandetanib included rash, diarrhea and hypertension.

ZEPHYR is a Phase III, randomized, double-blind, parallel-group, multicenter study evaluating the efficacy of vandetanib 300 mg plus best supportive care versus best supportive care in patients with locally advanced or metastatic (stage IIIb – IV) NSCLC after previous therapy with an EGFR inhibitor. The study is running in approximately 170 centers across 23 countries [43].

Vandetanib is also being tested in the first-line maintenance setting. A multicenter, randomized, double-blind, placebo-controlled Phase II study is comparing vandetanib (300 mg daily) plus best supportive care (BSC) to placebo plus BSC as maintenance treatment in patients with locally advanced or metastatic NSCLC, who have received and responded to prevoius platinum-doublet systemic chemotherapy. The primary objective of the study is to compare the PFS rate at 3 months in locally advanced or metastatic NSCLC patients with or without vandetanib maintenance. The study is actively recruiting patients [44].

A feasibility and safety study of the addition of vandetanib to carboplatin and paclitaxel administered neoadjuvantly in stage Ib, II and T3, N1 NSCLC was recently completed. Results have not yet been presented [45].

A Phase I/II dose-escalation and safety study of vandetanib in combination with irradiation is ongoing. The goal of the study is to assess the safety of vandetanib by evaluating the frequency, severity and duration of treatment-emergent adverse events in patients with poor prognosis lung cancer [46].

2.1.2.2 *Cediranib*

Cediranib (Recentin, AZD2171, Astra-Zeneca, Macclesfield, UK) is an oral tyrosine kinase inhibitor (TKI) targeting VEGFR-1,2, and VEGFR-3. Phase I trials evaluated AZD2171 with carboplatin and paclitaxel, and also with gemcitabine and cisplatin [47,48]. In both studies toxicities were manageable and antitumor activity was observed.

BR.24, a Phase II/III, double-blind, randomized trial of cediranib at 30 mg versus placebo in patients receiving paclitaxel and carboplatin chemotherapy for the first-line treatment of advanced or metastatic NSCLC was conducted by the National Cancer Institute of Canada Clinical Trials Group (NCIC-CTG). All NSCLC histologic subtypes were allowed, but patients with a central thoracic lesion with cavitation or clinically relevant hemoptysis within the preceding 4 weeks were ineligible. The NCIC-CTG has informed the developer that the study will not continue into Phase III following the planned end of Phase II efficacy and tolerability analysis by the study's Data Safety Monitoring Committee. Although evidence of clinical activity was seen, there seemed to be an imbalance in toxicity and, therefore, the study was considered not to have met the predefined criteria for automatic continuation into Phase III [49].

N0528 is a randomized, Phase II, first-line trial run by the North Central Cancer Treatment Group investigating how the administration of gemcitabine and carboplatin together with cediranib works compared with gemcitabine and carboplatin without cediranib as first-line therapy in treating patients with stage IIIb or stage IV NSCLC [50].

The combination of cediranib with pemetrexed is also undergoing Phase II evaluation for relapsed NSCLC [51].

2.1.2.3 *Axitinib*

Axitinib (AG-013736, Pfizer, New York, USA) is an oral TKI that inhibits all VEGF receptors, PDGFR-β (platelet-derived growth factor-β), and c-Kit. Axitinib as monotherapy has been evaluated in an open-label, multicenter, Phase II study of 32 patients (adenocarcinoma histology in 75%) with advanced NSCLC (**Table 2**) [52]. Adverse events were generally manageable, with grade 3/4 toxicities consisting mainly of fatigue (22%), diarrhea (6%) and hypertension (6%). Furthermore, a randomized, open-label, Phase II study comparing axitinib versus bevacizumab in association with carboplatin-paclitaxel in chemotherapy-naive patients with advanced, non-squamous NSCLC [53] has been recently approved. The enrollment of 108 patients is expected and the main end point is PFS. Axitinib is also being tested in combination with cisplatin and gemcitabine in squamous cell lung cancer in a Phase II trial [54].

2.1.2.4 *Sorafenib*

Sorafenib (Nexavar, BAY43–9006, Bayer Pharmaceuticals Corporation, West Haven, CT, USA) is an oral multitargeted TKI against B-RAF, C-RAF, VEGFR-2,3, PDGFR-β, and c-Kit. Tumor responses were observed in two Phase II trials investigating sorafenib monotherapy in relapsed advanced disease (**Table 2**) [55,56]. Sorafenib was well tolerated, with rash, diarrhea and fatigue as the most commonly cited toxicities.

Three Phase III first-line trials are randomizing patients to standard chemotherapy with sorafenib or placebo.

The Phase III ESCAPE study was stopped early after a planned interim analysis demonstrated that the trial would not meet its primary end point of an improvement in OS. In this study, 926 previously untreated NSCLC patients were randomized to receive either sorafenib or a placebo in combination with carboplatin and paclitaxel. Median OS was similar in the two treatment groups. However, in the subset of patients with squamous cell histology, there was significantly greater mortality in the sorafenib group [57].

The Phase III NEXUS trial is comparing the efficacy of gemcitabine, cisplatin and sorafenib to gemcitabine, cisplatin and placebo. When the ESCAPE trial was halted, the developer approached the data-monitoring committee of the NEXUS study and asked to see the results obtained so far, particularly for the subset of patients with squamous cell carcinoma. Although there was no signal in this subset of patients, the committee recommended that squamous cell patients taking part in the NEXUS study withdraw and that no further squamous cell patients be recruited. The study was formally amended and is now is continuing in non-squamous NSCLC [58].

In a dose-escalation trial, 31 patients received oral sorafenib (200 – 400 mg) twice daily with gefitinib (250 mg orally) once daily. One patient had PR; 20 patients had SD ≥ 4 months. Most adverse events were grade 1/2. The most frequent grade 3/4 events included diarrhea and elevated alanine aminotransferase [59].

Sorafenib is also being tested for recurrent/refractory NSCLC in several Phase II trials either in monotherapy or in combination with erlotinib. A Phase I/II trial evaluates sorafenib with concurrent thoracic radiotherapy for poor prognosis NSCLC [60].

2.1.2.5 *Sunitinib*

Sunitinib (Sutent, SU11248, Pfizer, New York, USA) is also an orally available multitargeted TKI against VEGFR-1,2, PDGFR and c-Kit. A randomized Phase II trial (SABRE-L) evaluated the safety and efficacy of combining sunitinib with bevacizumab + paclitaxel/carboplatin as first-line treatment for metastatic non-squamous NSCLC. Treatment in Phase I of this three-phase study: bevacizumab (15 mg/kg) + paclitaxel/carboplatin q3w, +/- sunitinib (25 mg) qd 2 weeks on, 1 week off. If tolerated, Phase II would include a patient arm with sunitinib at 37.5 mg qd; Phase III would include sunitinib at the highest tolerable dose (25 or 37.5 mg qd). Owing to poor tolerability, patients were never escalated to 37.5 mg sunitinib and the study was closed. At the time of analysis, estimated mean treatment duration was 9.5 weeks for the bevacizumab + paclitaxel/carboplatin arm and 7.6 weeks for the bevacizumab + paclitaxel/carboplatin + sunitinib arm [61].

A Phase II study assessed the clinical activity and safety of sunitinib when used as maintenance therapy following standard first-line chemotherapy in patients with locally advanced or metastatic NSCLC (**Table 2**) [62].

The efficacy and safety of continuous daily sunitinib dosing in previously treated advanced NSCLC was evaluated in a Phase II trial [63]. Forty-seven patients received oral sunitinib 37.5 mg/day continuously in 4-week cycles (**Table 2**). Sunitinib was generally well tolerated; most adverse events were grade 1/2 and included fatigue/asthenia, pain/myalgia, nausea/vomiting, diarrhea, dyspnea and stomatitis/mucosal inflammation.

Sunitinib + erlotinib is a new VEGFR-PDGFR-EGFR triple inhibition treatment strategy that may confer additive or synergistic antitumor effects and increase clinical benefit in patients with advanced NSCLC. Under the umbrella of the SUN program, two ongoing studies will assess the efficacy and tolerability of this combination. Both studies included NSCLC patients with 1/2 previous chemotherapy regimens. SUN 1058 is a randomized, multicenter, Phase II study evaluating the safety/tolerability of sunitinib at 37.5 mg as a continuous daily dose (CDD) + erlotinib 150 mg/day versus placebo + erlotinib 150 mg/day (4-week cycles); a nonrandomized lead-in cohort was used to evaluate safety/tolerability. SUN 1087 is a randomized, multicenter, pivotal Phase III study: 956 patients will be randomized to sunitinib 37.5 mg CDD + erlotinib 150 mg/day or to placebo + erlotinib 150 mg/day (4-week cycles). The primary end point for SUN 1058 and SUN 1087 is PFS and OS, respectively; secondary end points include OS and PFS, respectively. Both studies will evaluate 1-year survival, duration of confirmed response, safety and patient-reported outcomes. Sunitinib + erlotinib was well tolerated in the lead-in cohort of the SUN 1058 study, and 2/12 patients had durable PR. Enrollment is ongoing in the randomized Phase II portion of SUN 1058 and SUN 1087 [64].

2.1.2.6 *Motesanib*

Motesanib diphosphate (AMG706, Amgen, Inc., Thousand Oaks, CA, USA) is an orally bioavailable small molecule inhibiting VEGFR-1,3, PDGFR and c-Kit. Preliminary results indicate that motesanib can be combined safely with paclitaxel/carboplatin and/or the investigational EGFR-targeted agent, panitumumab, in patients with advanced NSCLC. Treatment-related adverse events were generally mild to moderate in severity [65].

A Phase II randomized trial compares the objective tumor RR as primary end point between paclitaxel/carboplatin plus motesanib and paclitaxel/carboplatin plus bevacizumab in subjects with advanced non-squamous NSCLC [66].

A Phase III multicenter, randomized, placebo-controlled, double-blind trial of motesanib in combination with paclitaxel and carboplatin for advanced NSCLC (MONET1) recruited 1240 patients. The primary end point was OS. Patients were randomized 1:1 to receive paclitaxel and carboplatin administered every 3 weeks with or without 125 mg motesanib taken daily. In November 2008 an independent data monitoring committee (DMC) recommended treatment discontinuation in subjects with squamous histology and enrollment suspension in subjects with non-squamous histology. This recommendation was based on an observation of higher early mortality rates in the motesanib group compared with the placebo group and a higher incidence of hemoptysis in the squamous population. Patients with non-squamous NSCLC receiving motesanib were allowed to continue treatment during the temporary suspension. In February 2009, the DMC recommended the trial resume enrollment of patients with non-squamous NSCLC following a 3-month enrollment suspension [67].

2.1.2.7 *Vatalanib*

Vatalanib (PTK787/ZK222584, Novartis International AG, Basel, Switzerland) also inhibits VEGFR-1,2,3, PDGFR-β, and c-Kit. Clinical evaluation is ongoing in NSCLC in a Phase II trial (GOAL) of vatalanib monotherapy at 1250 mg in relapsed or refractory NSCLC patients (**Table 2**) [68,69]. No additional study is ongoing with this agent.

2.1.2.8 *Pazopanib*

Pazopanib (GW786034, GlaxoSmithKline, Middlesex, UK) inhibits VEGFR-1,2,3, PDGFR-α, PDGFR-β and c-Kit. Preliminary results of a Phase II trial to evaluate the safety

and efficacy of pazopanib as neoadjuvant treatment-naive subjects with stage Ia or Ib, resectable NSCLC have been recently presented (**Table 2**) [70]. Before surgery, patients received pazopanib 800 mg qd for 2 – 6 weeks followed by a 7-day washout period before surgery. The primary end point was tumor volume change. Pazopanib treatment was associated with reduction of sVEGFR-2. A significant correlation between baseline levels of 11 cytokines/angiogenic factors and tumor reduction was observed. Multivariate classification analysis identified hepatocyte growth factor (HGF) and IL-12 as predictive of response in 81% of cases.

The effect of adjuvant pazopanib versus placebo on post-surgical disease-free survival in patients with stage I NSCLC is now being investigated in a Phase II/III trial [71].

A Phase II, nonrandomized, multicenter study to evaluate the efficacy and safety of Pazopanib (GW786034) monotherapy in patients with advanced stage IIIb/IV NSCLC has just finished recruitment [72].

2.1.2.9 *BIBF 1120*

BIBF 1120 (Boehringer Ingelheim, Ingelheim, Germany) is a potent inhibitor of VEGF, PDGF and FGF receptors. Thirty-nine patients with a variety of advanced solid malignancies, including NSCLC, were enrolled in a Phase I study. Ten patients had SD. Overall, BIBF 1120 was well tolerated, and 400 mg once-daily was defined as the MTD [73].

BIBF 1120 has also been investigated in a randomized Phase II trial as second-line or third-line treatment of NSCLC. Of 73 treated patients, there were no objective responses but SD was observed in 48% (**Table 2**) [74].

The effect of BIBF 1120 in advanced/recurrent NSCLC is being evaluated in two parallel Phase III trials. LUME Lung 1 and LUME Lung 2 studies are comparing BIBF 1120 with placebo in addition to standard second-line docetaxel or pemetrexed, respectively [75,76].

2.1.2.10 *ABT-869*

ABT-869 (Abbott Laboratories, Abbott Park, IL, USA) is an oral and structurally novel multitargeted TKI that potently inhibits all members of the VEGF and PDGF receptor families. A Phase II study of efficacy and tolerability of ABT-869 has enrolled 139 NSCLC patients who have received at least one line of therapy in the metastatic setting [77].

Another Phase II study evaluating carboplatin/paclitaxel in combination with ABT-869 versus carboplatin/paclitaxel alone in subjects with advanced or metastatic NSCLC as first-line treatment is ongoing [78].

2.1.2.11 *BMS-690514*

BMS-690514 (Bristol Myers Squibb, New York, USA) is a multitargeted TKI that inhibits all members of the EGF and VEGF receptor families. A Phase I study to determine the safety, pharmacokinetics and pharmacodynamics of BMS-690514 in combination with paclitaxel/carboplatin in solid tumors is ongoing [79].

The rationale behind the Phase II randomized trial of BMS-690514 versus erlotinib in previously treated NSCLC patients [80] is that preclinical studies have demonstrated activity of BMS-690514 in erlotinib-resistant NSCLC cell lines [81].

2.1.2.12 *OSI-930*

OSI-930 (OSI Pharmaceuticals, Melville, NY, USA) is a potent oral multitargeted TKI that inhibits c-Kit and kinase insert domain receptors. It is now in Phase I clinical trials. Preclinically, OSI-930 demonstrated significant antitumor activity as a monotherapy in a variety of tumor xenograft models. In xenograft models showing initial sensitivity to erlotinib, after tumor progression on erlotinib monotherapy, the addition of OSI-930 with the continuation of erlotinib treatment resulted in significantly delayed tumor growth [82].

A Phase I study was performed to determine the MTD and evaluate safety, pharmacokinetics/pharmacodynamics and efficacy of OSI-930 with two dosing schedules [83]. Altogether 27 patients have been treated. Common grade 1/2 toxicities (per patient) were limited to fatigue (7), diarrhea (5), nausea (5) and rash, lymphopenia and anorexia (2). OSI-930 was well tolerated with promising antitumor activity in previously treated patients.

A Phase I dose-escalation study of daily oral OSI-930 and erlotinib in patients with advanced and refractory solid tumors is ongoing [84].

2.1.2.13 *AEE788*

AEE788 (Novartis International AG), a multitargeted TKI, inhibits EGFR, HER2 and VEGFR-2. Dual tyrosine kinase inhibition with AEE788 in combination with the mTOR inhibitor RAD001 of human NSCLC cell-lines resistant to gefitinib resulted in effective growth inhibition [85]. AEE788 has been investigated in Phase I trials including patients with NSCLC [86,87]. A Phase I/II, dose-escalation study of oral AEE788 on intermittent dosing schedules in patients with advanced cancer to assess the safety, pharmacokinetic/pharmacodynamic profiles and clinical activity of AEE788 has recently been completed [88].

2.1.2.14 *XL647*

XL647 (Exelixis, Inc., South San Francisco, CA, USA) is an oral multitargeted TKI with activity against EGFR, HER2, EphB4 and VEGFR-2. In Phase I trials, XL647 has been studied using two different dosing schemes, an intermittent schedule with 5 days of treatment every 14 days and a continuous schedule [89,90]. In the first reported Phase II trial of this agent in an NSCLC patient population enriched for likelihood of having an EGFR mutation, XL647 seemed to have activity and to be well tolerated. The preliminary data from this Phase II trial show a RR of 29% (10/34) [91].

Recruitment has recently stopped in a Phase II trial in which patients with NSCLC who have developed progressive

disease after 3 months of previous treatment with erlotinib or gefitinib are being treated with continuous XL647 [92].

2.1.2.15 XL999

XL999 (Exelixis, Inc., South San Francisco, CA, USA) is an oral multikinase inhibitor. A Phase II study determined the efficacy, safety, and tolerability of XL999 [93]. Eligible patients had previously treated, stage IIIb/IV NSCLC (**Table 2**). Two tumor responses and one SD were reported.

2.1.2.16 CP-547,632

CP-547,632 (Pfizer, New York, USA) is an inhibitor of VEGFR-2 and PDGFR. The combination of CP-547,632 and paclitaxel and carboplatin was assessed in a Phase I/ randomized Phase II study (**Table 2**) [94]. Patients with stage IIIb/IV or recurrent NSCLC receiving first-line chemotherapy were treated with oral daily CP-547,632 in combination with paclitaxel and carboplatin. Sixty-eight patients were treated, 37 in Phase I and 31 in Phase II. Dose-limiting toxicity at 250 mg was grade 3 rash and grade 3 diarrhea. In Phase I, seven subjects (22.6%) had a confirmed PR. In Phase II, four subjects in each arm (chemotherapy + CP-547,632: 28.6%; chemotherapy alone: 25%) had a confirmed PR.

2.1.2.17 AV-951

AV-951 (Aveo Pharmaceuticals, Cambridge, MA, USA) is a new, highly potent and specific inhibitor of VEGF receptors 1, 2 and 3. Strong activity was observed in a Phase I study consisting of 40 patients with advanced solid tumors; AV-951 was also found to be well tolerated [95]. A Phase Ib and a Phase IIa trial will examine the safety, tolerability and MTD of AV-951 with a once-daily oral dosing schedule, as well as overall RR of AV-951 administration in NSCLC [96].

2.1.3 Thalidomide and its analogs

2.1.3.1 Thalidomide

Thalidomide (Celgene, Summit, NJ, USA) is thought to exert antiangiogenesis activity partially by antagonizing basic fibroblast growth factor (bFGF)-induced angiogenesis [97]. Phase II studies of thalidomide in patients with advanced NSCLC have shown promising activity (**Table 2**) [98,99]. Several ongoing trials are examining the potential role of thalidomide in the treatment of advanced NSCLC. A Phase II trial will determine the RR of 37 patients with stage III or IV NSCLC treated with second-line docetaxel and thalidomide [100]; another Phase II trial will determine the RR of 21 patients with stage II or IIIa NSCLC receiving neoadjuvant carboplatin, gemcitabine and thalidomide [101]. Recruitment has stopped for both studies; final data collection is in progress.

A Phase III study to evaluate the survival and TTP of patients with stage III NSCLC when treated with carboplatin-paclitaxel and radiotherapy with or without thalidomide is ongoing (ECOG3598) [102].

2.1.3.2 Lenalidomide

Lenalidomide (Revlimid, CC-5013, Celgene; Summit, NJ, USA) is a thalidomide analog. A Phase I study that investigated lenalidomide monotherapy in 55 pretreated patients with advanced solid tumors demonstrated three radiologic responses. The drug was well tolerated, although grade 3 or 4 neutropenia was observed in four patients [103]. A Phase II study to evaluate lenalidomide monotherapy in 40 patients with recurrent NSCLC has been completed [104]. The final results have not yet been published.

2.1.4 Matrix metalloproteinase inhibitors

2.1.4.1 BMS-275291

BMS-275291 (Bristol Myers Squibb, New York, USA) is a new broad-spectrum sulfhydryl-based second-generation matrix metalloproteinase (MMP) inhibitor rationally designed to spare a class of closely related metalloproteinases known as sheddases. The interim analysis of a large, randomized, Phase III study of the MMP inhibitor, BMS-275291 conducted in advanced lung cancer in 774 patients demonstrated no survival benefit from addition of this MMP inhibitor to chemotherapy with carboplatin and paclitaxel (**Table 2**) [105]. Moreover, the combination of the MMPI with chemotherapy caused an increased toxicity in the experimental arm, and study treatment was stopped.

2.1.5 Other antiangiogenic agents

2.1.5.1 Cilengitide

Cilengitide (EMD 121974, Merck, Darmstadt, Germany) binds to and inhibits the activities of the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, thereby inhibiting endothelial cell–cell interactions, endothelial cell-matrix interactions and angiogenesis. After Phase I studies proved the tolerability of cilengitide [106,107], a randomized Phase II study with a safety run-in part was started to investigate cilengitide in combination with cetuximab and platinum-based chemotherapy compared with cetuximab and platinum-based chemotherapy alone as first-line treatment for patients with advanced NSCLC (CERTO) [108].

2.1.5.2 Volociximab (M200)

Volociximab (M200, PDL Biopharma, Incline Village, NV, USA) is a chimeric monoclonal antibody jointly developed by PDL BioPharma and Biogen Idec for treatment of a variety of advanced solid tumors. It binds to and inhibits the functional activity of $\alpha 5\beta 1$ integrin. The results of a Phase I, pharmacokinetic, and biological correlative study of volociximab have recently been published [109]. Twenty-one patients with advanced solid malignancies, including one NSCLC patient, were treated with escalating doses of volociximab. Treatment was well tolerated, and dose-limiting toxicity was not identified over the range examined. One minor response (renal, 7 months) and one durable SD (melanoma, 14 months) were reported.

Two Phase Ib studies investigating the effect of volociximab in combination with carboplatin and paclitaxel [110], and

carboplatin, paclitaxel and bevacizumab [111] in previously untreated advanced (stage IIIb/IV) NSCLC patients are now recruiting.

The effect of dual inhibition with volociximab and erlotinib in advanced refractory NSCLC is also being evaluated in a Phase II study [112].

2.1.5.3 *TNP-470*

TNP-470 (Takeda Chemical Industries Ltd, Osaka, Japan) blocks angiogenesis by inhibiting methionine aminopeptidase, an enzyme critical for endothelial cell proliferation. The combination of TNP-470 administered at 60 mg/m^2 three times a week and paclitaxel 225 mg/m^2 administered every 3 weeks was defined as both the MTD and the optimal dose in a Phase I study involving 32 patients with solid tumors, including 16 NSCLC [113]. TNP-470, at present, is not going forward in development.

2.1.5.4 *AMG 386*

AMG-386 (Amgen, Inc.) is an intravenously administered recombinant Fc-peptide fusion protein (peptibody) that inhibits angiogenesis by preventing interaction between angiopoietins and Tie2 receptors. The safety and pharmacokinetics of AMG 386 either in monotherapy [114] or in combination with paclitaxel [115] in advanced solid tumors were examined in two Phase I trials. The treatments were well tolerated. Efficacy data are limited. Phase I and II studies are ongoing with solid tumors other than NSCLC.

2.2 Vascular disrupting agents (VDAs)

Targeting of VEGF has been shown to result in apoptosis only in newly formed, immature tumor vessels and in the developing vasculature of the neonatal mouse, but not in the tumor vessels of adult mice or in quiescent tumor vascular networks [116]. Vascular targeting therapy recognizes that clinical diagnosis of cancer commonly occurs when the tumor tissue has already established its vasculature. VDAs specifically target pre-existing tumor capillaries, resulting in rapid cancer tissue ischemia and secondary tumor cell death in the central regions of tumors, although they leave the perfusion in peripheral tumor regions relatively intact. The two major categories of VDA that are in clinical development are the small-molecule VDAs and ligand-directed VDAs [117]. While small-molecule VDAs achieve selective occlusion of tumor vessels by exploiting phenotypic differences between tumor and host tissue ECs (i.e., increased reliance on the tubulin cytoskeleton to maintain cell shape and accelerated proliferation), ligand-directed VDAs use toxins and pro-coagulant agents coupled to peptides or antibodies that selectively bind to the endothelial tube. Although animal studies with ligand-directed VDAs have certainly been elegant, and, furthermore, several potential target molecules exist that are upregulated on tumor versus host tissue capillaries, testing of ligand-based agents are still in the preclinical phase. For that reason, and because they are at a much more advanced stage of clinical development, only representatives of small molecule VDAs are discussed here.

2.2.1 *DMXAA (5,6 dimethylxanthenone-4-acetic acid)*

The combination of DMXAA (AS1404 or ASA404, Antisoma, London, UK) with carboplatin and paclitaxel was evaluated in a Phase II trial in previously untreated IIIb or IV NSCLC (**Table 2**) [118]. Safety profiles were similar and manageable in both groups, with most adverse effects attributed to standard therapy.

Two large Phase III trials are ongoing to investigate ASA404 in the treatment of NSCLC. ATTRACT-1 evaluates ASA404 in combination with paclitaxel and carboplatin as first-line treatment [119], whereas ATTRACT-2 compares ASA404 with placebo in combination with docetaxel in the second-line treatment of patients with stage IIIb/IV NSCLC [120].

2.2.2 *Zybrestat*

Zybrestat (CA4P, Combretastatin A4 Phosphate, Oxigene, Inc., Waltham, MA, USA) is a phosphate prodrug of the tubulin-binding agent combrestatin A4. Because the CA4P-bevacizumab combination has appeared safe, resulted in significantly decreased tumor blood-flows and shown clinical activity without simultaneous chemotherapy, the developer initiated a controlled Phase II study to assess the safety and efficacy of the combination of carboplatin, paclitaxel and bevacizumab ± CA4P in chemotherapy-naive, stage IIIb/IV non-squamous NSCLC histology. Patients who complete the first six cycles of therapy and have not experienced disease progression will receive maintenance therapy with bevacizumab alone or with bevacizumab plus CA4P [121].

2.2.3 *NPI-2358*

NPI-2358 is a synthetic analog of diketopiperazine phenylahistin (halimide), a natural product that was isolated from a marine and a terrestrial fungus *Aspergillus* sp. Dose escalation of NPI-2358 was conducted in a Phase I trial that enrolled patients with advanced solid tumors and lymphomas [122]. Twenty-five subjects were enrolled. A recommended Phase II dose of 30 mg/m^2 was selected based on toxicities of nausea, vomiting, fatigue, fever, tumor pain and transient elevations in blood pressure.

A Phase I/II study of NPI-2358 in combination with docetaxel in patients with advanced NSCLC that has progressed after treatment with at least one chemotherapy regimen is ongoing [123].

2.2.4 *ABT-751*

ABT-751 (Abbott Laboratories, Abbott Park, IL, USA) is an orally bioavailable antimitotic sulfonamide. ABT-751 binds to the colchicine-binding site on beta-tubulin and inhibits the polymerization of microtubules. There have been three published Phase I trials of ABT-751 in patients with hematological malignancies and in adult and pediatric solid tumors [124-126]. In advanced NSCLC, a Phase I/II trial is evaluating ABT-751

in combination with pemetrexed. The recruitment period for this study has been closed [127]. Another Phase I/II study evaluating the safety and efficacy of ABT-751 in combination with docetaxel versus docetaxel alone in advanced NSCLC has been terminated [128]. A Phase II study of ABT-751 in patients with NSCLC refractory to taxane regimens has been completed, but the results have not yet been released [129].

3. Potential biomarkers for monitoring antivascular therapy in NSCLC

Tumor response to antivascular treatments is frequently associated with cavitation rather than shrinkage. Antivascular therapy represents an exciting advance in the management of NSCLC, although even massive cavitation in the absence of shrinkage may not be defined as a response using the Response Evaluation Criteria in Solid Tumors [130]. Therefore, there is an urgent need in this field to identify patients responsive to these therapies, to predict the efficacy of combinations that include antivascular drugs and, moreover, to identify biomarkers that can help recognize tumor resistance.

Some of the most promising biomarkers for antivascular treatments are the circulating populations of endothelial progenitor cells (EPCs) and mature, desquamated ECs (circulating endothelial cells; CECs) [131]. Accordingly, the clinical value of peripheral blood EPC and CEC measurements is being studied in many ongoing clinical trials, including those investigating patients with NSCLC [132-134].

Although the use of cytokines as biomarkers of angiogenesis can be complicated by the release of angiogenic growth factors from platelets, there are also several studies of angiogenic molecules as surrogate markers of response to antivascular treatments in solid malignancies, including NSCLC [135,136]. However, the practical utility of using changes induced by antivascular agents in circulating EPC, CEC or angiogenic cytokine levels as diagnostic or surrogate biomarkers remains to be confirmed, and their use might be confounded by cytokine concentration and circulating EPC/CEC level changes associated with tumor resistance or progression [137].

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). Although the impact of vascular imaging techniques on decisions during anticancer drug development is still modest, their clinical value is under investigation in many ongoing clinical studies, and angiogenesis imaging will certainly play a key role in shaping the next decade's cancer management [138].

In brief, because antivascular treatments are developing at a rapid pace, there is an urgent need to identify reliable biomarkers for the efficacy of these therapies. Even though some pieces of the puzzle are already in place, monitoring techniques should be explored further to understand fully their possible implications in solid tumors, as well as in NSCLC. At present, the optimal technique for evaluating the effects of antivascular treatments in cancer patients remains a matter of active discussion among experts.

4. Expert opinion

Agents that target tumor capillaries, in particular bevacizumab, a humanized anti-VEGF monoclonal antibody, have become an important option for many patients with NSCLC and have opened a clear path for further research on the role of antivascular drugs in NSCLC. However, clinical experience in this field is still limited and several questions remain unanswered. Most important are the problems in elucidating the optimal biologic dose as well as the best possible combination treatment strategy of these agents in the setting of chemoradiotherapy. Furthermore, routine assessment of tumor response may be inadequate for antivascular agents. Tumor shrinkages characterized by cavitation have been observed and these do not meet the usual standard radiologic criteria for response. An additional relevant clinical challenge is, therefore, to find the best techniques for monitoring the effects of antivascular drugs. In line with that, efforts have been made to assess angiogenesis during antivascular treatments and to identify patients responsive to these therapies, recognize tumor resistance and predict the efficacy of antivascular agents. In fact, identifying biomarkers for individualized antivascular strategies may not only improve efficacy, but through better patient selection, could also decrease the unnecessary use of these expensive agents and result in improvements in the cost-effectiveness of these agents.

Hypertension is the most frequent adverse event reported with the antivascular agents, although it is usually reversible with a standard management algorithm. However, because the long-term side effects of antivascular agents are not known, establishing their toxicity profile over the long term remains essential.

In summary, although antivascular drugs have changed the landscape of the clinical management of NSCLC and the results of ongoing studies are eagerly awaited, additional studies are required to define the precise and optimal role of these agents in the NSCLC treatment paradigm.

Declaration of interest

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

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Contents

Abbreviations: αSMA, α-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 metalloprotease; 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; PlGF, 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-β, transforming growth factor-β; Tie-2/TEK, angiopoietin-1 receptor precursor or tunica intima EC kinase; TNF-α, 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.

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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.

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 neovasculature plays a significant role in tumor progression and might well be an optimal target for anticancer 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 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 μm 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 $CD31^{bright}CD34^{+}CD45^{-}CD133^{-}$ 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 $CD31^{bright}/CD45^{-}$ 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 $CD146^{+}$ 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 $(FSC)^{low\text{-}to\text{-}intermediate}$, side scatter $(SSC)^{low}$, $CD31^{bright}/CD146^{+}/CD45^{-}$]. 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 μm 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 μm 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^+/$CD11c^+$ conventional/myeloid DCs	Support of tumor vascularization		[119]
		$CD123^+$/$CD303^+$ plasmacytoid DCs	Support of tumor vascularization		[119]
		$CD11c^+$/$CCR6^+$/MHC II^+ TADCs	Support of tumor vascularization	Yes	[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]
$VEGFR1^+$ 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	$CD45^-/CD146^+/CD31^+/CD34^+$	$CD45^-/CD31^+/CD133^+$	39,100 vs. 7900	Below 500 both in patients and controls	[2]
Various[a]	95	CD146-IMS	$CD146^+/CD31^+/vWF^+/VEGFR2^+$	NA	438 vs. 121[b]	NA	[22]
Multiple myeloma	31	FC + culture	$CD34^+/CD146^+/CD105^+/CD11b^-$	CFU-ECs	CFU score ∼6-fold higher in patients[c]	NA	[23]
AML	48	FC	$CD45^-/CD31^+/CD34^+CD146^+$	$CD45^-/CD31^+/CD34^+CD133^+$	36,700 vs. 3200	700 vs. 100	[24]
Myelofibrosis	110	FC	NA	$CD133^+/CD34^+/VEGFR2^+$	NA	165 vs. 0	[88]
CLL	20	FC	$CD45^-/CD31^+/CD146^+$	NA	26.5 vs. 18.5	NA	[29]
MDS	128	FC	$CD45^-/CD34^+/CD146^+/CD133^-$	NA	512 vs. 153	NA	[28]
Breast cancer	16	FC	$CD45^-/CD146^+/CD31^+/CD34^+$	$CD34^+/VEGFR2^+$	5700 vs. 1300	370 vs. 140	[25]
Breast cancer	47	FC	NA	$CD34^+/VEGFR2^+$	NA	0.44 vs. 0.18[d]	[84]
Breast cancer	25	FC	NA	$CD133^+/VEGFR2^+$	NA	0.032 vs. 0.023[d,e]	[85]
Gastric/breast cancer	71	Culture	NA	CFU-ECs	NA	40.2 vs. 37.6 (n.s.)[f]	[155]
Lung cancer	53	FC	NA	$CD34^+/VEGFR2^+$	NA	1162 vs. 345	[80]
Lung cancer	10	FC CD34-IMS Cytospin	NA	$CD45^-/CD34^+/VEGFR2^+$ and $CD45^-/CD133^+/VEGFR2^+$	NA	90 vs. 42 and 0.3 vs. 0.1[g]	[83]
Liver cancer	80	Culture	NA	CFU-ECs	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	$CD34^+/CD146^+/VEGFR2^-$	$CD133^+/CD34^+/VEGFR2^+$	n.s.	0.18 vs. 0.01[d]	[89]
Various[h]	206	IMS	$CD45^-/CD146^+/CD105^+$	NA	111 vs. 21	NA	[26]
Various[i]	44	FC	$CD45^-/CD34^+/CD133^-/CD105^-$	$CD45^-/CD34^+/CD133^+/CD105^-$	470 vs. 140 (n.s.)	90 vs. 30 (n.s.)	[154]
GIST	16	FC	$CD45^-/CD31^+/P1H12^+/CD133^-$	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.

[a] 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.

[b] Patients with stable disease had CEC numbers equal to that circulating in healthy subjects ($P = 0.69$).

[c] Raw data not supplied.

[d] % of PB mononuclear cells.

[e] Stage III vs. stage IV patients.

[f] Stated per unit area (mm^2).

[g] % of CD34 enriched cells.

[h] 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.

[i] 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 $CD133^+/CD34^+/VEGFR2^+$ 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 liproprotein/acLDL. 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-1α (HIF-1α) 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 PlGF (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-α) 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.

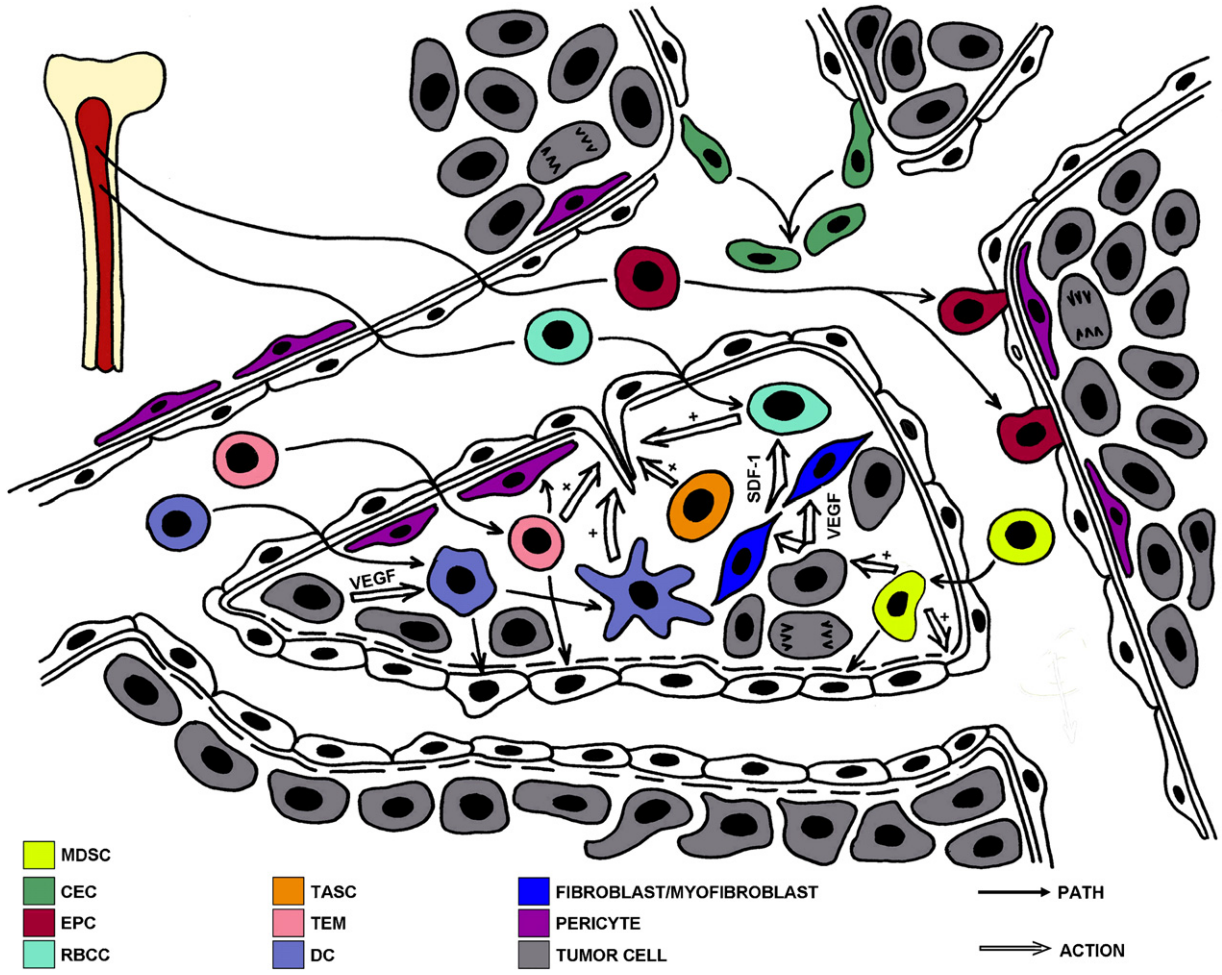

Fig. 1. Schematic representation of the roles of CECs, EPCs and HCs in cancer. CECs represent a population of mature ECs that have desquamated from their basal membrane into the circulation in response to some form of blood vessel injury [16]. CEC levels are elevated in patients with different types 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-α 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, $CD11b^{+}Gr\text{-}1^{+}$ 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 $CD11b^{+}/CD45^{+}/TIE2^{+}$ cells, but do not express VEGFR2 or any established EC or pericyte-associated markers (e.g. CD31, CD34 or αSMA 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 $CD123^{+}/CD303^{+}$ 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-β, 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, $MHC\ II^{+}/CD11c^{+}$ myeloid DCs, for example was shown to express the proangiogenic molecules VEGF, bFGF, TNF-α, 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-α, CXCL8) and angiosuppressive IFN-α (interferon-α) molecules (reviewed in ref. [119]). Moreover, a novel DC subpopulation ($CD11c^{+}/CCR6^{+}/MHC\ II^{+}$ 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 β-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$^+$ hematopoietic progenitors [65] (Table 1).

Tumor-associated stromal cells (TASCs) were described by Udagawa et al. [122]. These CD45$^+$/VEGFR2$^+$ 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$^+$ hematopoietic progenitors that proliferate in the BM, mobilize to the bloodstream along with VEGFR2$^+$ 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$^+$ 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$^+$ 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$^+$ 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-γ) 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$^+$ T cells in mouse models was angiostasis mediated by IFN-γ [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-γ 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 anticancer/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 $CD31^{bright}/CD45^{-}$ 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 $CD146^{+}$ 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 β_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 β 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 antivascular 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/antivascular 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.

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Conflicts of interest

The authors indicate no potential conflicts of interest.

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REVIEW ARTICLE

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Circulating Bone Marrow-Derived Endothelial Progenitor Cells: Characterization, Mobilization, and Therapeutic Considerations in Malignant Disease

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Abstract

Until recently, tumor vascularization was thought to occur exclusively through angiogenesis. However, recent studies using different animal models of cancer suggested the importance of bone marrow-derived endothelial progenitor cells (EPCs) (i.e. postnatal vasculogenesis) in tumor vascularization and growth. EPCs are present in the peripheral blood, their levels are increased in response to certain signals/cytokines, and they home into the neovascular bed of malignant tissues. Furthermore, at the clinical level, evidence is emerging that changes in EPC levels might predict the efficacy of anticancer drug combinations that include antiangiogenic agents. On the basis of these observations, EPCs have attractive potential diagnostic and therapeutic applications for malignant diseases. In this paper, we review biological features of EPCs and speculate on the utility of these progenitor cells for medical oncology. © 2007 International Society for Analytical Cytology

Key terms

endothelial progenitor cells; vasculogenesis; angiogenesis; cancer

UNTIL recently, it was generally accepted that in adults the formation of new blood vessels results exclusively from the proliferation and migration of preexisting, completely differentiated endothelial cells (ECs) (a process referred to as angiogenesis). Vasculogenesis (defined as the in situ differentiation of vascular ECs from primitive precursor cells) was thought to occur only in the embryonic phases of vascular development. Recent studies have shown, however, that circulating bone marrow (BM)-derived endothelial progenitor cells (EPCs) home to sites of neovascularization and differentiate into ECs (1). EPCs therefore resemble embryonic angioblasts, which are anchorage-independent cells having the ability to proliferate, migrate, and differentiate into mature ECs. Since the identification of this cell population by Asahara et al. (2), several studies have shown reduced numbers and/or impaired function of EPCs in a variety of cardiovascular risk states, including diabetes mellitus (3), hypercholesterolaemia (4), hypertension (5), chronic renal failure (6), rheumatoid arthritis (7), and cigarette smoking (8). Alternatively, cardiovascular protective factors such as exercise training (9), statin therapy (10), angiotensin II receptor antagonists (11), and peroxisome proliferator-activated receptor agonists (12) are known to increase EPC number and function. However, in addition to their role in the maintenance of vascular integrity, EPCs (i.e. postnatal vasculogenesis) are thought to participate in the process of tumor vascularization as well (13). This review focuses on the phenotype of EPCs, as well as the molecules that control their mobilization from the BM and their recruitment to sites of tumor vessel formation. In addition, we discuss the clinical significance of EPCs and the potential therapeutic implications in anticancer treatments.

Characterization of Endothelial Progenitor Cells and the Comobilized Haematopoietic Precursors

EPCs were initially identified and isolated in 1997 by Asahara et al. (2) on the basis of vascular endothelial growth factor receptor-2 (VEGFR2) and CD34 coexpression of these cells. However, in the past few years the emergence of specific surface markers and molecular probes has facilitated the identification and purification of functional stem and progenitor cells. EPCs, ECs, and haematopoetic stem cells share not only the aforementioned but many other surface markers (Table 1). As a result, to date no simple definition of EPC exists. Since the initial report, a number of groups have set out to better define this cell population, and EPCs were subsequently shown to express fibroblast growth factor receptor, CD38, c-kit, CD31, CD146, CXCR4, von Willebrand factor (vWF), vascular endothelial cadherin (VE-cadherin), Tie-2/TEK (angiopoietin-1 receptor precursor or tunica intima EC kinase), and CD133 (14,27–29). The term "EPC" may therefore encompass a group of cells existing in a variety of stages ranging from primitive haemangioblasts to fully differentiated ECs. Although their putative precursors and the exact differentiation lineage of EPCs remain to be determined, at present it is widely accepted that early EPCs (localized in the BM or immediately after migration into the circulation) are CD133+/CD34+/VEGFR2+ cells, whereas circulating EPCs are positive for CD34 and VEGFR2, lose CD133 and begin to express cell surface markers typical to mature ECs (14). Thus, the major candidate for a specific EPC marker is the CD133, an orphan receptor specifically expressed on early EPCs, but whose expression is lost once these progenitors differentiate into more mature ECs (30). Unfortunately, because in humans CD133 is expressed by haematopoietic stem cells as well (31), the methods for phenotypic differentiation between vasculogenic-restricted immature EPCs, committed haematopoietic progenitors, and their putative common precursor (bipotential haemangioblast) have yet to be developed further.

Reports on the number of EPCs in peripheral circulation are variable, ranging from 70–210 cells/mL of blood (32) to 3,000–5,000 cells/mL of blood (33), depending most likely on the isolation procedure used. These relatively low levels of circulating EPCs as assessed by flow cytometry are in sharp contrast to the high numbers of attached cells (often confusingly referred to as "EPCs" too) that are obtained ($\sim 10^5$ from 1 mL blood) from cell cultures containing the blood mononuclear cell fraction. In general, three different methods for culturing "EPCs" have been described (18). In the original method, peripheral blood mononuclear cells (PBMNCs) are plated on fibronectin-, gelatin-, or collagen-coated dishes. After the preplating step to reduce the numbers of differentiated ECs and adherent macrophages, the nonadherent cells are removed and replated on additional dishes. Discrete colonies appear in a week, containing round cells in the center with spindle-shaped attaching cells proliferating peripherally. These colonies are usually defined as colony-forming unit-ECs (CFU-ECs) (16). In the second commonly used technique, PBMNCs are cultured in the presence of angiogenic cytokines for 4–6 days, whereupon nonadherent cells are discarded, leading to a target adherent cell fraction (34). Because these adherent cells have been demonstrated to support angiogenesis in animal models of myocardial or limb ischemia (17), they have been defined as circulating angiogenic cells (CACs). Although CACs do not exhibit the colony morphology of CFU-ECs and can be assembled from culture in larger numbers than CFU-ECs, they have an endothelial phenotype (they bind *Bandeiraea simplicifolia*/BS-1 and *Ulex europeus* Agglutinin-1/UEA-1 lectins, express CD31, vWF, VE-cadherin, and Tie-2/TEK, and have the potential to take-up acetylated low-density lipoprotein/acLDL) and thus appear analogous to CFU-ECs in surface molecular profile and in vitro properties. Consequently, both cell populations have often been termed in the literature as "EPCs" (18). The third and least studied type of "EPCs" is now termed "endothelial colony-forming cells" (ECFCs). In this method, PBMNCs are cultured in the presence of endothelial-specific growth media. After removal of nonadherent cells, ECFC colonies displaying cobblestone appearance typical of ECs emerge from the adherent cell population. Given that ECFCs emerge much later in culture when compared with both CFU-ECs and CACs, they have also been named "late outgrowth EPCs" (19).

Harraz et al.'s (35) suggestion that CD34− angioblasts are a subset of CD14+ monocytic cells, Rehman et al.'s (36) demonstration of the isolation of CACs from the monocyte/macrophage fraction of PB, and Yoder et al.'s (37) finding that CFU-ECs expressed colony-stimulating factor-1 receptor and actively phagocytosed *Escherichia coli* have all led to some controversy over whether CAC and CFU-EC 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. (18). 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. The aforementioned authors also suggested that CD45+ haematopoietic progenitor cells overlap with CFU-ECs to an undefined degree, and that ECFCs are included in the circulating EC (CEC) population.

To make the picture more complex, recently various authors have described different CD45+ (sub)types of BM-derived circulating cell populations that contribute to tumor angiogenesis (38), although most of them are localized in periendothelial tumor sites and some are presumably included in the aforedescribed cell populations growing in cultures.

TIE2-expressing monocytes (TEMs), discovered by De Palma and coworkers (20,21), are recruited to periendothelial positions and promote angiogenesis in a paracrine manner. They express CD11b, CD45, and TIE2, but not VEGFR2 or any established EC or pericyte-associated markers (e.g. CD31, CD34 or α-smooth muscle actin, and NG2).

Tumor-associated stroma cells (TASCs) were described by Udagawa et al. (23). These CD45+/VEGFR2+ double positive cells have the ability to promote tumor angiogenesis, although are minimally incorporated into the endothelial tubes of tumor vasculature. Instead, these authors found that TASCs

Table I. Bone marrow-derived angiogenic progenitors in malignant disease

CELL TYPE	ANTIGEN PROFILE	ORIGIN	MORPHOLOGY	FUNCTION IN CANCER	REF.
EPC	CD31, CD34, CD38, CD133, c-kit, CXCR4, VEGFR2	BM	Immature PB cells, ~20 μm in diameter	Enhancing angiogenesis/biomarker of vascular damage, angiogenesis and the efficacy of antitumor–antivascular treatment/vehicle for drug delivery	(13–15)
CFU-EC	CD31, CD34, Tie-2, VEGFR2	Culture	PBMNCs growing in fibronectin-coated dishes. Discrete colonies emerge in ~7 days, comprised of round cells centrally with spindle-shaped cells growing at the periphery	Enhancing angiogenesis/biomarker of vascular damage, angiogenesis, and the efficacy of antitumor–antivascular treatment/vehicle for drug delivery	(16)
CAC	CD31, vWF, Tie-2, VE-cadherin	Culture	Adherent PBMNCs following 4–7-day culturing/CACs do not display colony formation	Enhancing angiogenesis/biomarker of vascular damage, angiogenesis, and the efficacy of antitumor–antivascular treatment/vehicle for drug delivery	(17)
ECFC	CD31, CD36, Tie-2, VEGFR2, VE-cadherin, vWF	Culture	PBMNCs growing in cobblestone-patterned colonies for ~21 days; tube formation on Matrigel	Enhancing angiogenesis/biomarker of vascular damage, angiogenesis, and the efficacy of antitumor–antivascular treatment/vehicle for drug delivery	(18,19)
TEM	CD11b, CD11c CD16, CD45, CD133, CD115, CCR5, Tie-2	BM	Roundish cytoplasmic outline and small nuclei, 10–30 μm in diameter	Enhancing angiogenesis	(20,21)
TADC	CD11c, CCR6, MHC-II	BM	Leukocyte precursors exhibiting properties of dendritic and endothelial-like cells	Enhancing angiogenesis	(22)
TASC	CD45, VEGFR2, c-kit, Sca-1	BM	Small (<10 μm), stellate-shaped cells in perivascular position	Enhancing angiogenesis	(23)
RBCC	CD11b, CD45, CXCR4, VEGFR1	BM	Stellate-shaped cells clustered around blood vessels in response to SDF-1	Enhancing angiogenesis	(24)
VEGFR1 + HC	VEGFR1, VLA-4	BM	Immature PB cells, forming cellular clusters in metastatic target organs	Enhancing angiogenesis/generating and maintaining the "premetastatic niche"	(25,26)

BM, bone marrow; CAC, circulating angiogenic cell; CFU-EC, colony-forming unit-endothelial cells; c-kit, stem cell factor; ECFC, endothelial colony-forming cell; EPC, endothelial progenitor cell; HC, haematopoietic cell; PB, peripheral blood; PBMNC, peripheral blood mononuclear cell; RBCC, recruited blood circulating cell; Sca-1, stem cell antigen 1; SDF-1, stromal cell-derived factor-1; TADC, tumor-associated dendritic cell; TASC, tumor-associated stromal cell; TEM, Tie-2-expressing monocyte; VEGFR1, vascular endothelial growth factor receptor-1.

indirectly facilitated tumor vascularization in a paracrine manner by inducing or increasing the angiogenic factors that stimulate in situ vessel formation (endothelial sprouting).

Like TEMs and TASCs, recruited bone marrow-derived circulating cells (RBCCs) (24) were demonstrated to augment proliferation of preexisting ECs cells via secreting proangiogenic factors from a perivascular position. RBCCs express CD45, CD11b, CXCR4, and VEGFR1, but not VEGFR2, indicating that they are recruited by VEGF and CXCL12 and are predominantly haematopoietic in nature. It is also important to note that Lyden et al. recently identified VEGFR1+ haematopoietic progenitors that proliferate in the BM, mobilize to the circulation along with VEGFR2+ EPCs, and incorporate into pericapillary connective tissue, thereby stabilizing tumor vasculature (25). More interestingly, these cells appear to home in before the metastatic tumor cells arrive to the target organ, promoting cancer growth by forming niches where tumor cells can locate and proliferate (26). However, to what extent these VEGFR1+ progenitors overlap with RBCCs remains unclear.

A further novel leukocyte progenitor population (CD11c+CCR6+ dendritic cell precursors, tumor-associated dendritic cells) that enhances tumor vascularization was described recently by Conejo-Garcia et al. (22). In their experiments, these authors found that β-defensins recruited dendritic precursors through CCR6 into the tumor, where VEGF-A transformed them into endothelial-like cells. Unlike TEMs and TASCs, these cells mainly migrate to the capillary walls, becoming true endothelial-like cells.

In conclusion, tumor-derived angiogenic cytokines do not merely induce the mobilization of EPCs, but also enhance the corecruitment of haematopoietic precursors to the tumor vascular bed and/or stroma. This comobilization of different lineages may promote sprouting and stabilization of ECs through the release of additional proangiogenic cytokines or by generating permissive conditions in the tumor stroma that support the in situ growth of resident blood vessels.

Mobilization of EPCs

To support tumor vascularization, EPCs must respond to signals released from the BM, home to the tumor site, and differentiate into mature ECs. Although the molecular pathways involved in EPC mobilization are in the early stage of definition, VEGF is thought to be the most significant of the other molecules (15). VEGF can activate matrix metalloproteinase-9 (MMP-9) that cleaves the membrane-bound stem cell cytokine mKitL in BM stromal cells to liberate soluble sKitL, which then stimulates cKit-positive EPCs to migrate from a quiescent BM niche to a permissive BM microenvironment, the so called vascular zone. This translocation activates EPCs from a quiescent to a proliferative state (39). Furthermore, VEGF has been found to upregulate stromal cell-derived factor-1 (SDF-1, also known as CXCL12) and CXCR4 (the SDF-1 receptor) (40,41). SDF-1 is chemotactic for EPCs and recruits EPCs to sites of neovascularization (42). Accordingly, in a recent animal study, CXCR4 blockade abrogated progenitor homing, whereas local injection of SDF-1 into the target organ increased their homing (43). However, in the same study, SDF-1 in the absence of VEGF failed to enhance BM-derived cell recruitment, whereas blocking of CXCR4 activity reduced BM-derived cells in the target organ even in the presence of high levels of VEGF. Therefore, it appears that SDF-1 is not sufficient to recruit EPCs to tumors without an additional signal, such as VEGF. On the other hand, because additional studies have demonstrated that SDF-1 is essential for the adhesion of BM-derived cells, it may significantly help to sequester EPCs at the site of vessel formation (41). Taken together, VEGF, through interaction with MMP-9 and SDF-1, rapidly triggers the release of EPCs into the bloodstream; EPC levels in the circulation rise within 24 h following VEGF treatment (44). Accordingly, the increased circulating VEGF induces the mobilization of EPCs from the BM of cancer patients (45,46).

Molecules that induce leukocyte or erythrocyte mobilization may similarly influence EPC mobilization. Increased numbers of EPCs were found in animals following exogenous granulocyte macrophage colony stimulating factor (GM-CSF) administration, and accelerated corneal blood vessel growth with BM-derived cells was observed in animals treated with GM-CSF (47). In another murine model, granulocyte colony-stimulating factor markedly promoted growth of colon cancer cells inoculated subcutaneously in mice, in part mediated by BM-derived cells incorporated into new blood vessels (48). Similarly, administration of recombinant human erythropoietin (rHuEPO) increased both the number 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 elicits a similar potency for the improvement of EPC mobilization as VEGF (51), there are no data on the effect of rHuEPO on EPC mobilization and recruitment when it is delivered to tumor bearing animals or cancer patients.

In addition to the above factors, recently collected data indicate that placental growth factor (52), angiopoietin-1 (53), platelet-derived growth factor-CC (54), nitric oxide (55), 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors (statins) (56), physical training (57), and estrogens (58) stimulate EPC mobilization as well. In contrast, C-reactive protein and tumor necrosis factor-α promote apoptosis, attenuate the function, and reduce the number of EPCs (59,60).

Contribution of EPCs to Tumor Vascularization

The fact that EPCs are able to facilitate tumor-induced vasculogenesis means that although they are primarily programmed to support blood vessel growth during embryogenesis, this progenitor population retains this capability within an angiogenic milieu in the adult. But what evidence is there that EPCs actually facilitate tumor vascularization? The first description of tumor-induced vasculogenesis was reported in 2001 by Lyden et al. (25). These authors demonstrated that EPCs contribute about 90% to vascularization in lymphomas grown in angiogenesis-defective Id-mutant mice in which implanted tumors rapidly regress in association with poor de-

velopment of tumor neovessels. BM transplantation from wild-type mice, not from Id-mutant mice, restored the tumor neovascularization and growth in Id-mutant mice. However, this high EPC contribution in the tumor vasculature is most probably due to the fact that recipient Id-deficient mice are unable to sustain endothelial sprouting to support tumor growth, and therefore, alternative vascularization mechanisms will be activated. In subsequent animal transplantation models, EPCs were incorporated into neovessels, sometimes by as much as 50% (61), whereas other authors reported lower but significant levels between 10 and 20% (62). These observations have been challenged by some other studies in which EPCs had no measurable contribution to tumor neovessels. For example, De Palma et al. (63) reported that TEMs rather than EPCs homed to tumors and interacted with vascular ECs. Interestingly, these authors did not find EPCs in tumor vessels. Similarly, based on their observations in a transgenic mouse model, Gothert et al. suggested that EPCs might not contribute to tumor endothelium (64). Although possible reasons for such conflicting results might include the use of differing experimental models/techniques to identify EPCs, recent data suggest that their involvement in experimental tumor vascularization might also vary depending on tumor stage (65).

The contribution of EPCs in the vasculature of human malignancies has been assessed in some recent studies as well. Peters et al. investigated patients who developed malignancies after BM transplantation with donor cells derived from individuals of the opposite sex. By using fluorescence in situ hybridization with sex chromosome-specific probes, these authors found that the percentage of BM-derived ECs in the tumor vasculatures ranged from 1% (head and neck sarcoma) to 12% (lymphoma) (66), which was closer to the numbers observed in spontaneous mouse tumors than the zero or extremely high numbers observed when implanting tumor lines. Recent studies demonstrated the presence of CD133+ EPCs in the endothelial tubes of human tumor capillaries as well (67–69) (Fig. 1). Moreover, EPCs have been detected at increased frequency in the PB of patients with various malignancies including lung (69), hepatocellular (46), breast (43) and colorectal (70) cancers, and myeloma multiplex (71), myelofibrosis (72), non-Hodgkin's lymphoma (67), acute myeloid leukemia (73), and malignant gliomas (74).

In conclusion, although it seems obvious that EPCs are able to support tumor vascularization, the involvement of these cells may vary depending on circumstances such as the experimental model or detection technique used, the histological type and stage of the tumor, and whether anticancer treatment has been started.

Endothelial Progenitor Cells as Potential Biomarkers of Human Tumor Angiogenesis

Because vascularization is seen as fundamental in tumor progression, efforts have been made to assess angiogenesis (75,76) and to identify patients responsive to antivascular therapies, recognize tumor resistance, and predict the efficacy of combinations that include antiangiogenic drugs (77). However, currently there are no proven biomarkers of tumor angiogenesis. Thus, development of noninvasive biomarkers of tumor response/relapse is a crucial objective to help in the management of patients treated with antivascular agents.

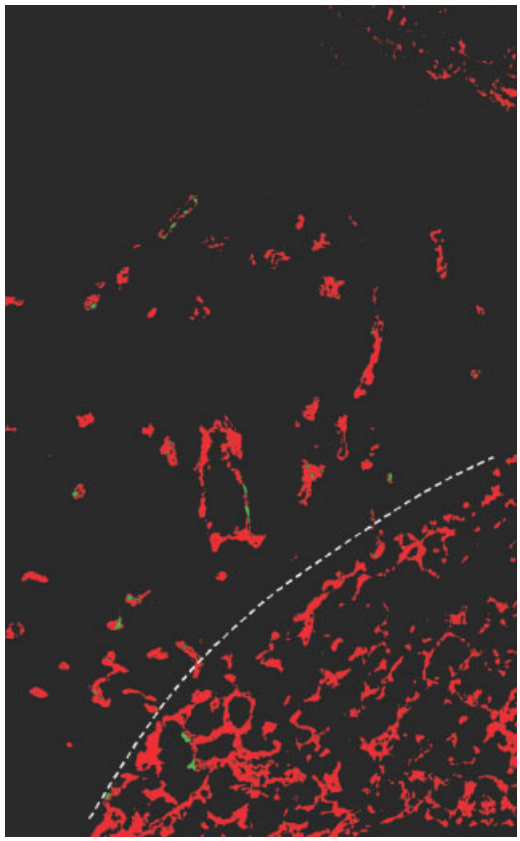

Figure 1. Example for the identification of EPCs by using confocal laser scanning microscopy. In mouse Lewis lung carcinoma, CD133+ EPCs (green fluorescence) were arrested mainly in small CD31+ intratumoral capillaries (red fluorescence), or much less frequently, in the alveolar capillaries of the peritumoral lung tissue. White broken line represents the border between tumor and host tissues. The tumor is present at the upper left.

As mentioned, mouse models demonstrated a correlation between circulating EPC levels and tumor volume (25,61,62). However, other researchers have found that the number of EPCs also changes with anticancer/antiangiogenic therapy. For example, maximum tolerable dose chemotherapy was reported to provoke an EPC elevation, in contrast to metronomic chemotherapy [targeting tumor ECs (13)], which suppressed EPC numbers/viability (78). In additional studies, the mobilization of EPCs by vascular disrupting agents was disrupted by the administration of antiangiogenic agents (79), and endostatin was shown to reduce circulating EPC numbers along with tumor regression (80,81). In addition, treatment with a targeted VEGFR2 antibody caused a dose-dependent reduction in EPC levels that paralleled the antitumor activity of the experimental drug (82). More importantly, methods for EPC measurements have been tested in cancer patients (45,46,69,71–73), and studies have been undertaken assessing EPC levels in individuals treated with antiangiogenic drugs.

Particularly encouraging in this regard are two recent clinical trials. In a Phase 1 trial, bevacizumab, an anti-VEGF antibody, reduced the tumor vascular density and the number of EPCs in rectal carcinoma patients (70). In a subsequent Phase 2 trial on AZD2171 therapy in glioblastoma (83), progression on treatment with this pan-VEGF receptor tyrosine kinase inhibitor was associated with an increase in CEC (84), SDF-1, and FGF-2 levels, whereas progression after drug interruptions correlated with elevations in EPC counts and FGF-2 levels. Moreover, the elevation in the levels of these circulating biomarkers correlated with the magnetic resonance imaging measurements, demonstrating an increase in the relative capillary density and perimeters.

With the rapid increase in the number of the cancer patients treated with antivascular agents, there is an urgent need to define biomarker algorithms for the follow up. These studies are especially important in this regard, as they suggest the potential of EPC quantification not only to assess antiangiogenic therapy efficacy, but to help define optimal biologic dose ranges, establishment of appropriate tumor response criteria, and, hopefully, reduction of the adverse effects.

Endothelial Progenitor Cells as Cellular Vehicles for Anticancer Therapy

The finding that circulating BM-derived EPCs are recruited to tumor capillaries suggests novel strategies to halt tumor growth. This might be achieved by using ex vivo manipulated EPCs as cellular vehicles to deliver suicide genes, toxins, or antiangiogenic drugs. These novel approaches have been applied to transplantation models and, to some extent, reduced cancer progression (85–87). However, given the existence of different vascularization mechanisms in cancer (13), the variability in EPC levels reported in different experimental models, and the association of vasculogenesis with the histological type and stage of the tumor, the use of EPCs as "Trojan horses" in an antiangiogenic gene therapy-mediated anticancer strategy certainly deserves further investigation.

Conclusions

In summary, EPCs obviously contribute to the vascularization of malignant tumors. It is not clear yet, however, whether they are indispensable for this process or what the relative contribution of vasculogenesis (i.e. BM-derived EPCs) is compared with that of in situ angiogenesis (i.e. endothelial sprouting). Moreover, it still remains to be determined whether EPCs can only be used as surrogate biomarkers for monitoring anticancer/antiangiogenic drug efficacy or can be targeted to treat certain types of malignancies, or alternatively—as they are endowed with the capacity to home to the tumor vasculature—can be applied to deliver therapeutic genes, toxins, or vascular targeting agents.

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Erythropoietin in Cancer: An Update

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Abstract: Erythropoietin (EPO) has long been recognized as the major hematopoietic cytokine regulating normal erythropoiesis. Moreover, there is a growing interest in the non-erythropoietic, tissue-protective effects of EPO. Because of its potential to correct anemia, EPO has been increasingly prescribed to cancer patients. However, although recombinant human Epo (rHuEPO) significantly reduces the risk for red blood cell transfusions in cancer patients, recent clinical studies have reported decreased survival and disease control following rHuEPO treatment in patients with different cancer types. The issue of EPOR expression in tumor cells is critical in this respect. The expression of EPOR in tumor cells raises the possibility that exogenous rHuEPO may directly influence tumor growth or sensitivity to chemo-radiation therapy. In addition, EPOR expression in endothelial cells suggests what potential effects EPO may have on tumor capillaries, such as the stimulation of angiogenesis. However, as experimental studies reveal, the overall direct effect of EPO-EPOR signaling on cancer progression and therapy is not a straightforward one. The current paper provides an update on the biology of EPO, and discusses its utility in the treatment of cancer patients.

Keywords: Erythropoietin, hypoxia, anemia, cancer.

INTRODUCTION

Anemia frequently occurs in patients with cancer [1, 2]. The degree of anemia depends on cancer type, tumor stage, duration of the disease, treatment status, patient age and bone marrow reserve. In the European Cancer Anemia Survey, the prevalence of anemia was 39% at enrollment and increased to 67% during the survey [3]. The incidence of anemia was 63% in patients receiving chemotherapy, 42% in those receiving concurrent chemoradiotherapy, and only 19.5% in those receiving radiotherapy alone [3]. The incidences were highest in patients receiving platinum- or anthracycline-based chemotherapy to treat lung cancer, gynecological malignancies, genitourinary cancer and lymphomas [1, 3].

The etiology of cancer-related anemia includes myelosuppressive therapy, tumor infiltration of the bone marrow, bleeding, hemolysis, relative deficiency of erythropoietin, inappropriate response of the bone marrow, functional iron deficiency, and anemia associated with chronic disease, the most common type of non-treatment-induced anemia in patients with cancer.

Anemia has been shown to be a prognostic factor in many cancers with an overall 65% increase in the relative risk of death [4], owing perhaps to anemia's possible impact on the outcome of both chemotherapy and radiotherapy as a result of hypoxia-induced treatment resistance [5]. The systemic hypoxia caused by anemia in cancer patients contributes significantly to tissue hypoxia, a common feature of growing cancer. However, the correction of anemia, and the increased oxygen level inside the tumor not only result in the improvement of quality of life but also enhance the success of cancer therapy, leading to improved survival of patients [6]. Recombinant human erythropoietin (rHuEPO) is widely used for correction of hemoglobin level by increasing the number of red blood cells [7, 8]. Recent studies initiate that, besides hematopoietic progenitor cells, numerous other cell types (endothelial- and cancer cells) express erythropoietin receptor (EPOR) and rHuEPO may affect their functions [9, 10]. However, new clinical and experimental data are contradictory on the way by which exogenous rHuEPO treatment can influence cancer growth and therapy [11-17]. Below, we will discuss the molecular consequences of cancer hypoxia, providing a solid basis for correction efforts in anemic cancer patients. We will also analyze the recent controversies over erythropoietins, pre-clinical findings and current usage guidelines. We firmly believe that only scientific and clinical evidences can resolve the controversies over clinical use of rHuEPOs.

EPO IN THE HEMATOPOIETIC SYSTEM

The glycoprotein hormone erythropoietin (EPO, MW 30.4 kDa) belongs to the family of class I cytokines [18]. The peptide core, containing 165 amino acids, has a globular structure with 4 α-helix bundles and two structure-stabilizing disulfide bonds and is attached by four carbohydrate chains including one O-linked acidic and three N-inked oligosaccharides [19]. The protein is

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responsible for cytokine binding and the stimulation of erythropoiesis, while the carbohydrate parts are required for the production and secretion of the mature EPO and ensure the survival of the cytokine in the blood stream [20]. During embryogenesis, EPO is produced mainly by the liver, but after birth production is taken over by the peritubular fibroblast-like cells of the kidney cortex [21, 22]. However, in adults, several other tissues (i.e. liver, brain, spleen, testis, lung) and cell types (i.e. peripheral endothelial cells, vascular smooth muscle cells, pancreatic islets) express EPO mRNA, although these EPOs have no role in normal erythropoiesis [23-26].

The EPO gene is located on the long arm of chromosome 7, exists as a single copy in a 5.4-kb region of the genomic DNA, is composed of five exons and four introns, and is under the control of the hypoxia-inducible factor-1 (HIF-1) pathway [27-30]. Interestingly, although EPO is the main modulator of erythropoiesis, the decreasing red blood cell number does not induce EPO production; only the diminished tissue oxygen concentration turns on gene expression and protein secretion [31]. The O_2-sensor controls several hypoxia-related genes through the hypoxia-responsible elements (HRE) sequence located in the regulatory site of these genes [32], which bind the hypoxia-inducible transcription factors (HIFs). HIFs are dimers containing one α- and one β-subunit and belong to the family of basic helix-loop-helix (bHLH) proteins. Three types of α-subunits are known (1α, 2α, 3α), but only the HIF-1α/β is described as the regulator of hypoxia-related genes [33]. Both subunits are constitutively produced and in normoxic conditions HIF-1α is hydroxylated at two proline residues by an enzyme containing specific prolyl-hydroxylase domain (PHD) [34]. Prolyl-

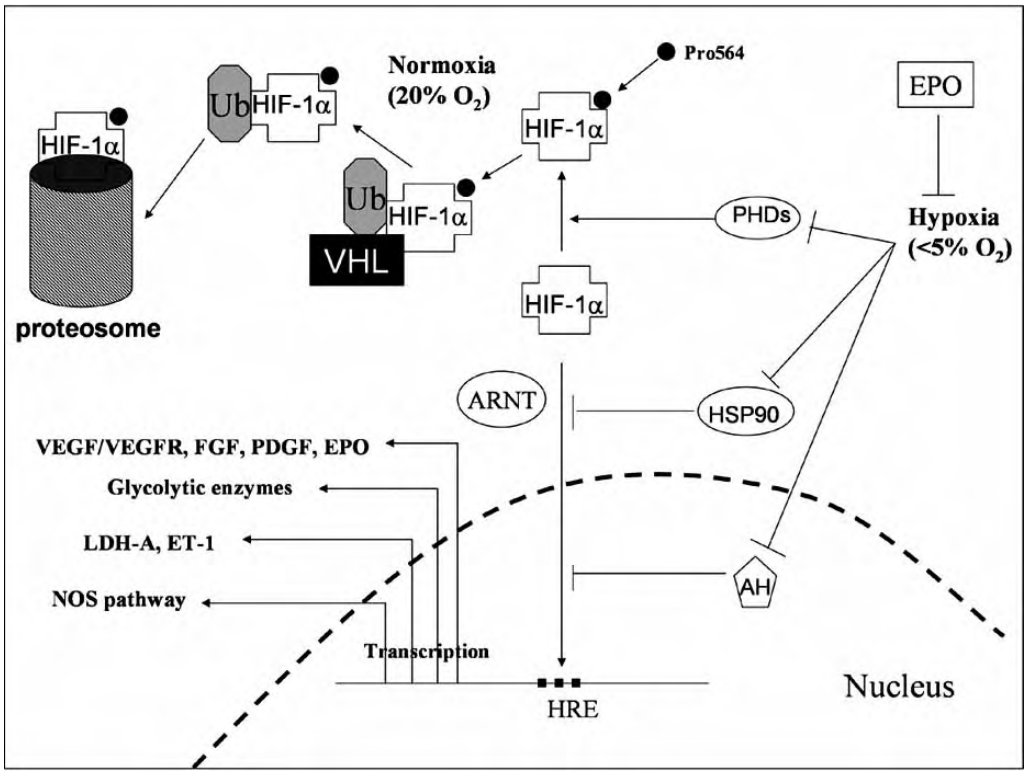

Fig. (1). Theoretical effect of EPO treatment on hypoxia signaling in cancer.

HIF-1α levels are mainly regulated post-transcriptionally, by the rate of HIF-1α production and degradation. Under normoxia, HIF-1α is hydroxylated at specific proline residues (proline 402 and 564) *via* prolyl hydroxylases (PHDs 1–3). This HIF-α modification allows binding to the tumor-suppressor protein von Hippel–Lindau (VHL), a recognition component of an E3 ubiquitin ligase complex. Subsequently, HIF-1α subunits become ubiquitylated that drive them to degradation in the proteasomal system. Under hypoxia, HIF-1α remains unhydroxylated, slowing the HIF-1α destruction rate and allowing large HIF-1α quantities to accumulate in the cytoplasm. The hypoxic stabilization of HIF-1α also allows its translocation to the nucleus where it heterodimerizes with HIF-1ß to bind to the "hypoxia-response elements" (HREs) in the promoter and enhancer regions of target genes. Because, as demonstrated in various anemic animal models, the oxygen tension of tumors tends to rise with increasing Hb levels, and EPO treatment has recently demonstrated to be associated with increased tumor perfusion, EPO may have the potential to block HIF signaling and to improve sensitivity of tumors to chemo- and/or radiotherapy.

AH= asparagine hydroxylase; ARNT= aryl hydrocarbon receptor nuclear translocator; bFGF= basic fibroblast growth factor; EPO= erythropoietin; ET-1= endothelin-1; HRE= hypoxia response element; HSP90= heat shock protein 90; LDH-A= lactate dehydrogenase-A; NOS= nitric-oxide synthase; PDGF= platelet derived growth factor; PRH= prolyl hydroxylase; U= ubiquitin; VEGF= vascular endothelial growth factor; VEGFR= vascular endothelial growth factor receptor; VHL= von Hippel-Lindau protein.

hydroxylated HIF-1α is the target of the von Hippel-Lindau protein (pVHL) and the E3 ubiqutin ligase complex. Polyubiquitinated HIF-1α is degraded at the cellular proteosomes [35, 36]. Under hypoxic conditions, HIF-1α escapes from the degradation mechanism and forms a functionally active transcription factor complex with HIF-1β at the nucleus (Fig. **1**).

The main targets of endogenous EPO are the "burst-forming unit erythroid" (BFU-E) and the "colony-forming unit erythroid" (CFU-E) stem cells in the bone marrow [37-39]. These stem cells produce erythroblast colonies whose number correlates with EPO concentrations [40]. It is well documented that the primary effects of EPO on the production of erythrocytes is the prevention of stem cell apoptosis [41, 42]. BFU-Es and even more CFU-Es express GATA-1 transcription factor [43], which can modulate the caspase system through activation of the bcl-X_L anti-apoptotic protein [44]. The erythroid progenitors can produce a small amount of EPO to maintain a basal level of proliferation and erytropoiesis [45]. However, as the level of erythropoietin in the blood increases, a growing number of erythroid progenitors can survive, proliferate and differentiate into proerythroblasts and normoblasts.

EPO regulates the stem cell proliferation and apoptosis through the specific erythropoietin receptor (EPOR). This glycosylated cell surface protein consists of 484 amino acids in a single chain and is a member of the cytokine class I receptor superfamily [46]. One EPO molecule crosslinks two EPORs forming a functionally active dimer through the extracellular ligand-binding domains, which induce conformational change and start signal transduction. Since the intracellular domain has no catalytic activity, the signal is mediated by the tyrosine kinase JAK2 that is constitutively associated to the receptor [47, 48]. After receptor activation by EPO, JAK2 phosphorylates several proteins (including EPOR) and these tyrosine-phosphorylated molecules can bind signal peptides containing Src homology 2 domains (SH2) [49]. Accordingly, several signal transduction pathways can be activated, such as phosphatidyl-inositole 3-kinase (PI3K)/protein kinase B (Akt), Grb2/Ras/MAPK, protein-kinase C, Src-homology phosphatases (SHP1,2) and STAT5 [50] (Fig. **2**). The main downstream signal is the JAK2/STAT5 pathway: phosphorylated STAT5 molecules form stable and functionally active dimers as transcription factors, which regulate target gene expression after translocation to the nucleus, resulting in erythroid differentiation [51]. EPO prevents apoptotic cell death by maintaining the mitochondrial membrane potential, which prevents the cellular release of cytochrome C and modulates caspase activity using these different signaling pathways. Signaling terminates with the dephosphorylation of the JAK2 and the EPOR following EPO/EPOR internalization and degradation in proteosomes [52]. In addition to these full-length forms of EPOR (F-EPOR), there are two other isoformes expressed by different erythroid cells. The truncated form

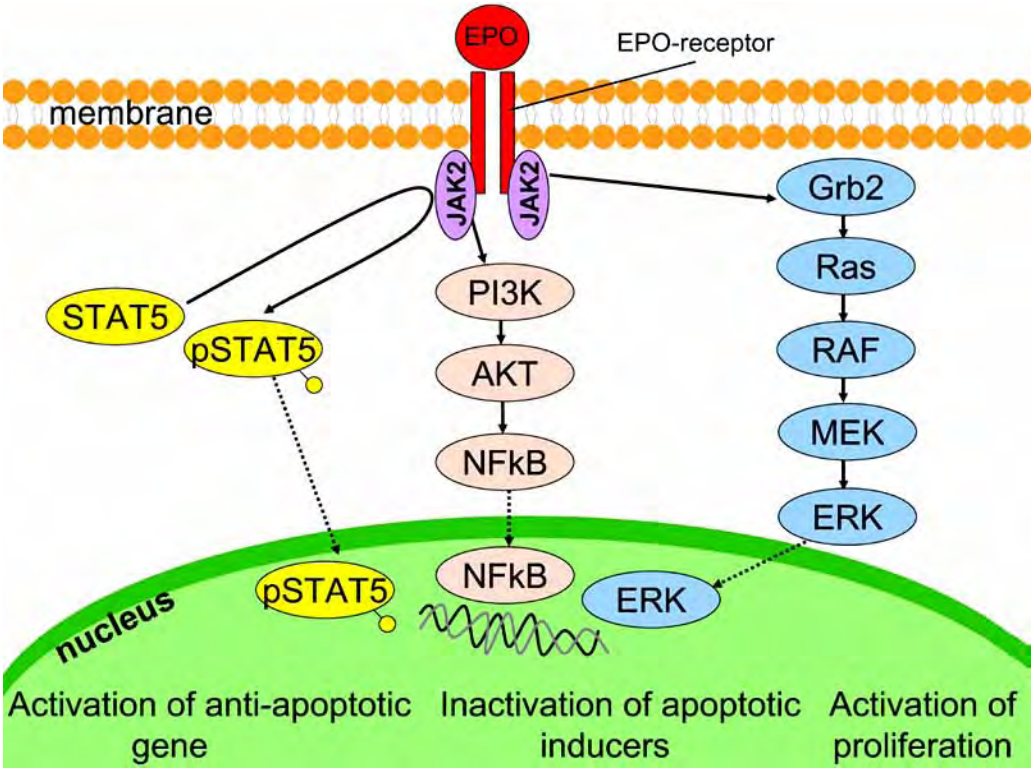

Fig. (2). EPOR signaling pathways.

ERK= extracellular signal-related kinase; Grb2= Growth factor receptor-bound protein 2; JAK2= Janus kinase-2; MEK= mitogen extracellular kinase; NFkB= nuclear factor-kappa B; Raf= MAP3K= mitogen-activated protein kinase kinase kinase; Ras= Rat sarcoma viral oncogen homolog; STAT5= Signal transducer and activator of transcription-5.

(T-EPOR) plays a role in the survival of erythroid progenitors in low EPO concentration, while the soluble form (S-EPOR) is an antagonist of F-EPOR in neuronal tissues [53, 54].

EPOR EXPRESSION ON NON-HEMATOPOIETIC CELLS

Until recently it was generally accepted that EPO plays a role solely in erythropoiesis. However, several data indicate that the EPO-receptor is expressed by a variety of non-hematopoietic cells. EPOR can be detected on neurons, microglias, astrocytes, megakariocytes, cardiomyocytes, epithelial-, insulin producing-, vascular smooth muscle- and endothelial cells (reviewed in ref. [55]). Molecular (RT-PCR, nested PCR) and immuntechniques (Western-blot, histochemistry) can be used to detect EPOR, but it should be emphasized that using antibodies to detect EPO receptor protein often has questionable results. Elliot *et al*. [56] tested four commercially available rabbit polyclonal antipeptide anti-EPOR antibodies, including C-20 (sc-695; anti-human EPOR), M-20 (sc-697; anti-mouse EpoR), H-194 (sc-5624; anti-human EPOR) from Santa Cruz Biotechnology (Santa Cruz, CA), and 07-311 (anti-mouse EPOR) from Upstate Biotech (Waltham, MA) using immunoblotting and immunohistochemistry techniques. They found that none of the investigated antibodies were suitable for detecting EPOR by immunohistochemistry (because of a cross-reaction with other proteins), and only M-20 gave an adequate result in detecting EPOR by immunoblotting.

TUMOR HYPOXIA AND ADAPTATION MECHANISMS

Hypoxia arises due to a critical imbalance between the tissue's supply of O_2 and its consumption rate. In solid tumors, there are several basic mechanisms leading to hypoxia: structural/functional abnormalities of the tumoral blood vessels [57] (perfusion caused decreased O_2 delivery), diffusion abnormalities (poor diffusion of O_2) and tumor- or therapy-induced anemia (anemic hypoxia).

Poor perfusion of cancer tissue usually leads to acute ischemic hypoxia. In malignant tumor tissues, the diffusion distance of O_2 usually increases above 70 μm from the supplying blood vessel, which results in chronic hypoxia in the supplied area (Table **1**). The irregular development and structure of tumoral vessels causes this condition to further deteriorate.

Systemic anemia, a common phenomenon in cancer, significantly reinforces the negative consequences of both the perfusion- and diffusion-limited hypoxia in cancer tissues. Because it is increasingly clear that tumor hypoxia fundamentally affects not only the prognosis of the disease but the therapeutic responses as well, it is important to summarize here our growing knowledge on the molecular and physiological consequences of hypoxia in cancer cells.

There are two oxygen sensors in mammalian (and cancer) cells: the PHD (prolyly-hydroxylase domain) proteins, belonging to the family of non-haem oxidizing enzymes, and the asparaginyl hydroxylase FIH (factor inhibiting HIF [58]). Both proteins target (and regulate) the transcription factor HIF-1α [59]. In a well-oxygenated milieu (~20% O_2) the half life of HIF-1α is less than 5 min due to proteasomal degradation caused by VHL protein and E3 ubiquitin ligase complex. Although there are three isoforms of PHD, in cancer cells PHD1 function predominates in regulating HIF-1α degradation. On the other hand, in normoxic conditions, FIH hydroxylates the C-terminal asparagin-containing transcriptional activation domain of HIF-1α inhibiting its interaction with its co-activators p300 and CBP. This results in repression of the transcriptional activity of HIF-1α already associated with the DNA [58-60].

HIF1 is a family of transcription factors, where HIF-1β is constitutively expressed and HIF1α, HIF2α and HIF3α are regulated by the level of O_2 [58, 59, 61]. The α and β subunits must heterodimerize in order to recognize the HRE elements (RCGTG) in the promoter regions of several genes. HIF is activated at 5% O_2 (40 mmHg) level *in vitro* with a progressive increase in its activity to near anoxia (0.1-0.2%). This activation process is initiated by stabilization of the cytoplasmic protein (escape from proteasomal degradation), nuclear translocation, heterodimerization and transcriptional activation. Knowing the approximately 100 genes controlled by HIF under hypoxic conditions is critical. According to our current knowledge these genes can be divided into the following categories: genes involved in angio- and erythrogenesis, glucose metabolism, and metastasis.

Promoters of the key angiogenic cytokines VEGFs, bFGF, HGF as well as EPO all contain HRE and their gene expression are regulated by hypoxia through HIF. Furthermore, the expression of the receptors of VEGFs as well as of HGF (c-met) is also regulated by the HIF

Table 1. Oxygenation Status and Expression of HIF1α in Human Cancers. [See in Ref: 122]

Cancer type	Hypoxic fraction in % (pO_2<2.5 mmHg)	Incidence of HIF1α protein (%)
Breast cancer	25-40	40-100
Cervical cancer (uterine)	11-47	72-100
Head and neck cancer	0-25	64-94
Various cancers	NA	53 [Ref. 61]

system. HIF controls the expression of (i)NOS, a survival enzyme of the endothelial cells, too [62].

HIF may also control enzymes involved in glucose metabolism under hypoxia. These include glucose transporters (GLUT1/GLUT3), glycolytic enzymes, LDH-A, pyruvate dehydrogenase kinase-1 and cytochrome C oxidase subunit COX4-2. HIF regulates enzymes involved in intracellular pH regulation as well, such as MCT (monocarboxylate transporter), hexokinase-1/2 and carbonic anhydrase-9 (CAIX [63, 64]).

Hypoxia (and HIF) has been shown to be capable of modulating the expression of several genes involved in tumor progression, while HIF2α is known to regulate the expression of OCT4 and ID2 stem cell marker genes, as well as the expression of MYC, TGFα and CCND1 responsible for the regulation of cell proliferation [65]. HIF1 may interact with β-catenin as well. HIF (1α in particular) is also involved in regulating the expression of several genes involved in tumor metastasis, including intermediate filaments, vimentin, cytokeratins (14,18,19), the matrix protein fibronectin, proteases such as MMP2, uPA, cathepsin D, lysyloxydase, the autocrine motility factor (AMF, phosphoglucose isomerase), the AMF receptor gp78 and the chemokine receptor CXCR4 [66, 67]. One of the hallmarks of the invasive phenotype of epithelial cancer cells is epithelial-mesenchymal transition characterized by decreased expression of E-cadherin and cytokeratins and increased expression of vimentin and c-met. It was found that through LOX (and LOXL2 in particular), HIF can modulate the activity of Snail/slug resulting in the repression of E-cadherin. On the other hand HIF also targets E-cadherin repressors resulting in the repression of E-cadherin gene expression too [65, 66].

As summarized above, cellular hypoxia in tumor cells initiates a cascade of events which leads to the reprogramming of hypoxic cancer cells and promotes the development of aggressive cancer cell clones with a more aggressive, irradiation-[68] and therapy-resistant geno-/phenotype. This angiogenic phenotype of cancer cells is characterized by the overexpression of various angiogenic cytokines, which initiates and maintains the increased activity of neoangiogenesis in tumors, stimulates postnatal vasculogenesis in the bone marrow, and promotes vascular remodeling [57].

Hypoxia in tumor tissue acts as a double-edged sword. On the one hand, hypoxia induces cell cycle arrest and apoptosis of cancer cells through a p53- and BCL-2-dependent mechanism [59]. On the other hand, hypoxia triggers adaptation mechanisms to overcome nutrient deprivation and lack of O_2 resulting in a tumor cell population chronically adapted to a hypoxic environment. By virtue of clonal selection, hypoxia promotes the accumulation of cancer cells that are genetically resistant to hypoxic conditions. Furthermore, hypoxia through HIF-activation induces genetic mechanisms that help cancer cells to escape the hypoxic en-

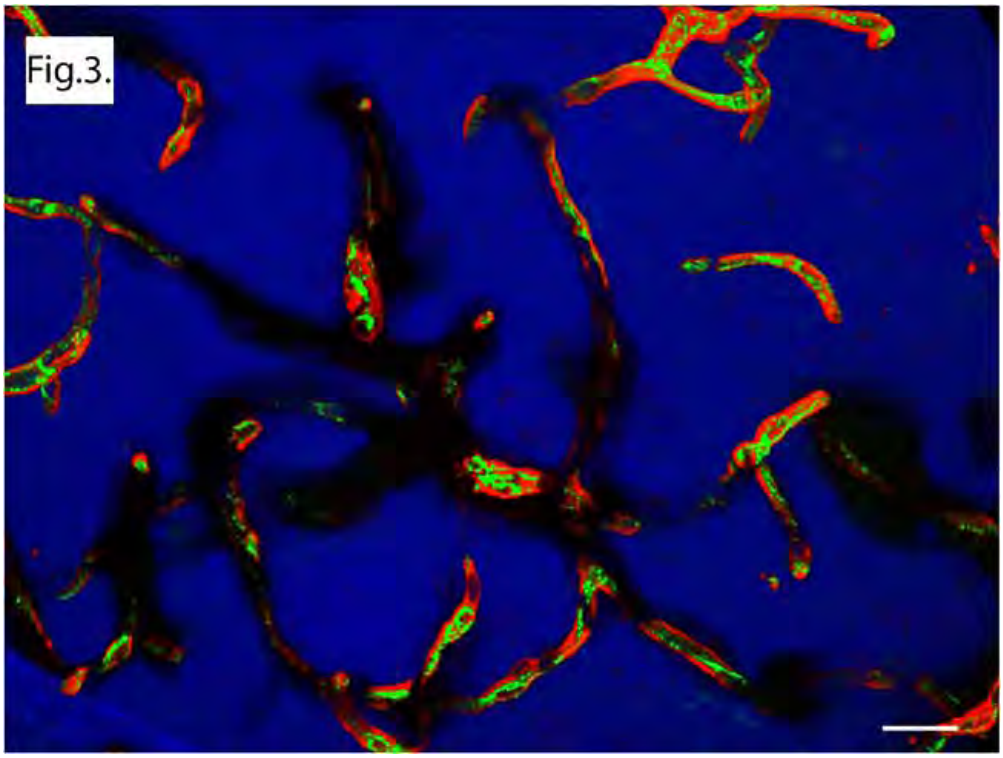

Fig. (3). Example for perivascular tumor growth.

Confocal image of mouse brain microvessels labeled for the endothelial cell marker CD31 (green) and the basal membrane component laminin (red). Hypoxia, through the activation of genetic mechanisms that help pigment containing B16 melanoma cells to escape the hypoxic environment, forces tumor cells to migrate toward host vasculature without the onset of angiogenesis.

vironment (through migration to better perfused areas [66, 67], usually to an area around the intratumoral blood vessels (Fig. **3**). Unfortunately, these activated genes are just what are needed for local invasion and intravasation of cancer cells, leading to systemic dissemination and metastasis.

Hypoxia results in a relative resistance to irradiation caused by the oxygen enhancement effect (increased DNA-damage caused by irradiation in the presence of O_2 [68]. However, it seems that acute and chronic hypoxia differentially affects radiosensitivity: while acute hypoxia/anoxia results in radioresistance, chronic hypoxia seems to increase the radiosensitivity of cancer cells. However, tumor stroma may respond differently to hypoxia than cancer cells do, which may also influence radiosensitivity of tumor tissue. The effectiveness of irradiating tumor tissue critically depends on the effect on tumoral vasculature, where the sensitivity of the blood vessels depends on angiogenic survival factors (cytokines) produced by hypoxic cancer cells [69-72]. At this point it is difficult to calculate the net outcome of those processes, but more data suggest that hypoxia of tumor tissue decreases the sensitivity to irradiation.

Novel data has revealed the molecular background, showing how hypoxia induces genetic instability in the tumor cell population, leading to clonal selection for therapy resistance and progression. Hypoxia represses the expression of miss-matched repair genes and homologue recombination repair as well. This is due to HIF-independent mechanisms that involve the phosphorylation of the E2F4/p130 repressor complex resulting in a decrease in BRCA1 and RAD51, the posttranslational modulation of H3K9, and the activation of Mnt/Max resulting in a decrease in MLH1 and activation of Mad1/max to depress MSH2 expressions [73]. All these genetic alterations can also be regarded as paving the path for a more chemotherapy-resistant genotype.

These molecular scenarios can be significantly altered in cancer cells. In various cancers, HIF genes can be constitutively active for various reasons independent of oxygenation status [61]. One rare possibility is the activating mutation of HIF genes themselves. More frequently, mutations of other regulating genes, such as of the p53 or VHL, result in HIF activation. Last but not least, activation of various oncogenic signaling pathways characteristic of various cancers such as breast, lung or pancreas and involving HER-2, EGFR or K-RAS maintains the sustained activity of HIFs [57, 59, 65]. In such conditions hypoxia of cancer tissue induces adaptive molecular responses in stromal cells exclusively.

DIRECT EFFECTS OF EPO ON TUMOR CELLS: FACTS AND UNANSWERED QUESTIONS

EPOR has been detected in growing numbers on non-hematopoietic cell types including different tumor cell lines and primary tumors, raising the question of weather EPO can modulate the biology of these cells. At the same time, it is well documented that anemia and tissue hypoxia negatively influence the tumor therapy and the patient's quality of life [74, 75]. Hence, various rHuEPOs are frequently used for correction of the decreased hemoglobin level in cancer patients, potentially increasing progression of the EPOR-expressing tumors [76].

Several *in vitro* and *in vivo* preclinical experiments have demonstrated that rHuEPO treatment modulates tumor growth and the efficacy of cancer therapy; however, these data are controversial and unexplained. A broad variety of tumor types express EPO and EPOR mRNA, particularly under hypoxic conditions [77]. Moreover, research has demonstrated that different splice variants of EPOR are expressed in tumor cell lines [14]. Administration of exogenous rHuEPO increased the *in vitro* proliferation of breast [77] and renal cell carcinoma cells [78]. Similar results, but lower induction effects of proliferation and invasion, were reported in head and neck squamous cell carcinoma cell lines [79]. The inhibition of erythropoietin signaling system by soluble EPOR or anti-EPO antibody destroyed xenografts of ovarian and uterine cancers in nude mice, as a result of increased apoptosis of tumor cells [80]. In other studies, administration of EPOR antagonist inhibited melanoma and stomach choriocarcinoma tumor cell survival [81] and rat mammary adenocarcinoma tumor growth [82].

In contrast, however, Berdel *et al*. showed that exogenous rHuEPO has no growth-modulating effect on 22 non-hematopoietic tumor cell lines [83]. Other research has similarly found that EPOR+ melanoma [84] and breast carcinoma cell lines [85], as well as six other EPOR-positive tumor cell lines [86] did not proliferate in the presence of rHuEPO. Moreover, rHuEPO enhanced the antitumor efficacy of photodynamic therapy in mice [87] and restored the anemia-induced reduction of cyclophosphamide cytotoxicity in rat tumors [88] and the radiosensitivity of experimental tumors [89]. rHuEPO alone induced tumor regression and antitumor immune response in murine myeloma models [90].

Worth mentioning is another interesting side effect of EPO that can influence exogenous rHuEPO administration in cancer patients. It is well documented that endothelial cells also express EPO receptors, and several data indicated that EPO inhibits apoptosis and induces proliferation and migration of these cells [91, 92]. EPO is a potent angiogenic factor comparable in its effect to VEGF [93], while rHuEPO has been shown to stimulate the *in vitro* endothelial tube formation and *in vivo* angiogenesis in chicken chorioallantois membrane assay [94]. Furthermore, it is also known that normoxic conditions (i.e. appropriate level of tissue oxygen) have a positive effect on the efficacy of chemo- and/or radiotherapy, verifying the important role of the tumor vasculature. Blackwell *et al*. demonstrated that administration of rHuEPOα improved the oxygenation inside the living tumor mass independent of the hemoglobin level [95], suggesting that tumor oxygenation may be increased

by enhanced tumor perfusion. In human squamous cell and colorectal carcinoma xenograft models, rHuEPO administration significantly increased the proliferation index of tumor-associated endothelial cells, but without stimulation of tumor cell proliferation, leading to larger intratumoral blood vessels. The increased vessel surface resulted in improved drug delivery to tumor cells and augmented its antitumor effects [96].

On the other hand, as described previously, one of the key factors in the antitumor action of irradiation is its anti-angiogenic effect [69, 70]. Exogenous rHuEPOs enhanced the radiation sensitivity of intratumoral endothelial cells increasing their apoptosis inducing capacity [71, 72] resulting in effective tumor control. Exogenous EPO decreased both the host- and tumor-derived VEGF expression suggesting the proliferation-promoting effect of rHuEPO on tumoral endothelial cells is independent of VEGF production [95]. Data indicate that HIF-1α could be responsible for decreased VEGF expression, since HIF-1α gene expression was also decreased in rHuEPO-treated animals before radiotherapy [97]. The underlying molecular consequences of hypoxia correction are illustrated on Fig. (**1**). Some data suggest there is a tight correlation between HIF-1α expression and tumor progression [98], and HIF-1-regulated cytokines enhance the radioresistance of endothelial cells. Moreover, inhibition of postirradiation HIF-1 activation significantly increased tumor radiosensitivity through vessel destruction [70].

These results suggest that rHuEPOα treatment has at least two different effects on tumors: first, it can decrease hypoxia, which is well-known as one of the markers of poor prognosis; second, it increases endothelial cell proliferation, causing enhanced radiosensitivity of the vessels and tumor perfusion by oxygen and chemotherapeutic agents.

rHuEPOs IN CANCER PATIENTS

Cancer-related anemia leads to cancer-related fatigue and many other symptoms [99, 100]. Patients with fatigue complain of generalized weakness, easy tiring, diminished concentration, sleep disturbances, memory loss and emotional instability. Fatigue, often associated with anxiety and depression, has a major impact on the social and occupational life of these patients. Absolute Hb levels, rapid onset of anemia, compensatory mechanisms and co-morbidity all influence anemia-related symptoms. Diagnosis of anemia should focus on symptoms, ability to perform daily activities, blood cell counts and laboratory parameters (e.g. iron status, vitamin levels). Determining the possible causes of the anemia is also essential. In making the diagnosis, quality of life instruments should be used, as they allow for a more detailed evaluation [101].

TREATMENT OF TUMOR- OR THERAPY-INDUCED ANEMIA

Treatment of cancer-related anemia should primarily aim at correcting potential causes. Red blood cell transfusion and the administration of recombinant human erythropoietins are important symptomatic treatments.

Red Blood Cell Transfusions

Red blood cell transfusions are usually given only to patients with Hb levels approaching 8 g/dl and/or highly symptomatic patients. Approximately one third of cancer patients require at least one transfusion and 16% need multiple transfusions [102]. Patients at risk for transfusions are those with low baseline Hb levels, advanced age, advanced disease, reduced performance status, weight loss, and platinum- or anthracycline-based chemotherapy. Patients with lung cancer, ovarian cancer or genitourinary cancer have the highest transfusion rates. Transfusions result in immediate but often only transient relief. Side effects of transfusions are hemolysis, iron overload and transmission of infectious agents [103, 104].

Erythropoietic Proteins

The erythropoietic proteins in clinical use are epoetin alfa, epoetin beta and darbepoetin alfa. Continuous erythropoiesis receptor activator (CERA) is in clinical development and biosimilars are expected to be available in the future.

Erythropoietic proteins increase Hb levels and decrease RBC transfusions in patients either receiving chemotherapy, chemoradiotherapy, radiotherapy or not receiving chemotherapy [105-108], for review see ref. [2]). Erythropoietic proteins decrease the relative odds of receiving a RBC transfusion by an average of 62% [109]. Erythropoietic proteins improve anemia-related symptoms and quality of life and these improvements correlate with Hb increases [107].

PRACTICE GUIDELINES

Practice guidelines on the use of erythropoietic proteins are available from the American Society of Clinical Oncology and the American Society of Hematology [109, 110], the EORTC [111] and other organizations. Currently, erythropoietic proteins are only recom-

Table 2. Guidelines for Chemotherapy-Induced Anemia [119]

Trigger Hb	<10 g/dl	
	>10 but < 12 g/dl	elderly patients with limited cardiopulmonary reserve coronary artery disease reduced ability to carry out daily life
Target Hb	near to 12 g/dl	

mended for patients with chemotherapy-induced anemia (Table **2**).

ASCO/ASH Guidelines

The previously published guidelines were recently updated [109, 110]. Epoetins are recommended for patients with chemotherapy-induced anemia and a Hb level of 10 g/dl or less. For patients with less severe anemia epoetins might be considered dependent on clinical circumstances. A target level of 12 g/dl is recommended.

Starting dose and dose modifications should be prescribed according to the package insert (Table **3**). Non-responders should be considered for a dose escalation for an additional 4-6 weeks. Continuing treatment beyond 6-8 weeks in the absence of response is not recommended. Iron stores should be monitored. However, more studies on optimal iron supplementation are warranted.

The prophylactic use of erythropoietic proteins is not recommended. The guidelines also caution against the use of erythropoietic proteins in patients not receiving chemotherapy.

EORTC Guidelines

Initiation of treatment with erythropoietic proteins is recommended at Hb levels of 9-11 g/dl based on anemia-related symptoms in patients with chemotherapy-induced anemia [111]. Based on individual factors and clinical circumstances, the erythropoietic proteins may also be considered in asymptomatic patients with anemia and in patients with only mild anemia. A target Hb level of about 12 g/dl is recommended.

SAFETY OF ERYTHROPOIETIC PROTEINS

Erythropoietic proteins are usually well tolerated. Clinically relevant side effects are hypertension and thrombo-embolic events (transient ischemic attacks, stroke, pulmonary emboli, deep vein thrombosis and myocardial infarction). The risks are increased by 1.25-fold for hypertension and by 1.67-fold for thrombo-embolic events [112,113]. Skin reactions, cephalea and influenza-like symptoms are also occasionally seen.

ERYTHROPOIETIC PROTEINS AND SURVIVAL OF CANCER PATIENTS

Because anemia contributes to tumor hypoxia, which is associated with resistance to cytotoxic drugs and radiotherapy, control of anemia by erythropoietic proteins could improve response to anticancer therapy and, thereby, also prolong survival.

Initial trials suggested that epoetins administered to patients with chemotherapy-induced anemia might improve survival in patients undergoing non-platinum chemotherapy for breast cancer [105] or platinum-based chemotherapy for small cell lung cancer, [106] but both trials lacked statistical power to exactly determine the association between treatment with erythropoietic proteins and survival. A potentially positive impact of erythropoietic proteins on survival was further supported by a meta-analysis of controlled trials, which found a survival benefit with an adjusted HR of 0.81 (95% CI 0,67-0,99) and an unadjusted HR of 0,84 (95% CI 0,69-1,02) [112].

However, two subsequent trials raised concerns about the safety of erythropoietic proteins with regard to survival [17, 114, 115]. In patients undergoing chemotherapy for metastatic breast cancer, the 1-year survival rate was 70% in the epoetin alfa group but 76% in the placebo group [17, 114]. In patients undergoing radiotherapy for head and neck cancer, survival was shorter in the epoetin group in the intent-to-treat analysis but no survival difference was seen in the per protocol analysis [115]. The percentage of patients achieving Hb levels greater than 14 g/dl (women) or 15 g/dl (men) was much higher in the epoetin beta group than in the placebo group (82% versus 15%). This high target Hb level achieved in most epoetin-treated patients could have had a detrimental impact on oxygen delivery to the tumor and, thereby, could have decreased the efficacy of radiotherapy in these patients. Other shortcomings of the trial were a heterogeneous patient population, imbalances between the groups, inappropriate treatment (radiotherapy instead of radiochemotherapy), protocol violations, and poor overall outcome.

A renewed meta-analysis on 8167 patients from 42 trials revealed a HR of 1.08 (95% CI 0.99-1.18) [113]. This meta-analysis did not include two recent trials which demonstrated a neutral effect of erythropoietic proteins on survival in patients undergoing chemotherapy for either breast cancer [116] or small-cell lung cancer [117]. A prematurely closed trial in small cell lung cancer also found no effect of epoetin alfa on survival [118]. Thus erythropoietic proteins appear to be safe in patients with chemotherapy-associated anemia.

The situation might be different for patients with cancer-related anemia in the absence of chemother-

Table 3. Recommendations for Dose Modifications

Initial dose	Epoetin alfa	150 U/kg subcutaneous 3-times weekly
	Epoetin beta	30 000 U subcutaneously weekly
	Darbepoetin alfa	2.25 µg/kg weekly or 500 µg every 3 weeks
Dose reduction	decrease dose by 25% when Hb approaches 12 g/dl or increase >1 g/dl in 2 weeks	
Dose withholding	if Hb exceeds 12 g/dl and withhold until Hb <11 g/dl, restart dose at 25% below	

apy. Three trials in which patients received either radiotherapy or best supportive care only reported a detrimental impact of erythropoietic proteins on survival. One of these trials was prematurely closed due to an unplanned safety analysis and included only 70 patients with non-small cell lung cancer [119]. The second trial was in patients with head and neck cancer undergoing radiotherapy [120]. The third trial included patients with cancer-related anemia without chemotherapy [121]. All these trials were criticized for epoetin use outside the approved indications, heterogeneous patient populations, and target Hb levels above the approved target levels. Based on the results from these trials, the up-dated ASCO guidelines caution against the use of erythropoietic proteins in these patients.

SUMMARY AND FUTURE DIRECTIONS

Cancer hypoxia has emerged as one of the key issues in tumor progression, angiogenesis and therapy resistance because of its profound effect on gene expressions of cancer cells. At the same time, cancer-related anemia is one of the most common complications of advanced disease and chemotherapy resulting in systemic (as well as local) hypoxia. Accordingly, attempts are justified to correct both the systemic as well as local (intratumoral) oxygen status of cancer patients. Although erythropoietins are powerful drugs for correcting anemia (and systemic hypoxia), they may have direct or indirect side effects due to their effects on bone marrow, tumor stroma, or the tumor cells themselves. Although preclinical data are available for the broad range of effects of EPOs on various cell types, including cancer cells, clinical data are lacking on whether this has detrimental effects in cancer patients. Most of the preclinical data have indicated that EPOs are at least neutral in relation of the efficacy of various anticancer modalities. Furthermore, most of the clinical trial data suggest that EPOs do not decrease the efficacy of anticancer therapies, although recently there have been a few disturbing exceptions. Therefore, further preclinical and clinical research on EPO biology and the association between erythropoietic proteins and disease outcome, including survival, are warranted. These trials should be based on homogeneous patient populations with homogeneous treatment and should have sufficient statistical power, otherwise they cannot provide enough strong clinical evidence to support any changes in the guideline for the use erythropoietins in cancer patients.

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Review

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Role of retinoic receptors in lung carcinogenesis

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Abstract

Several in vitro and in vivo studies have examined the positive and negative effects of retinoids (vitamin A analogs) in premalignant and malignant lesions. Retinoids have been used as chemopreventive and anticancer agents because of their pleiotropic regulator function in cell differentiation, growth, proliferation and apoptosis through interaction with two types of nuclear receptors: retinoic acid receptors and retinoid X receptors. Recent investigations have gradually elucidated the function of retinoids and their signaling pathways and may explain the failure of earlier chemopreventive studies.

In this review we have compiled basic and recent knowledge regarding the role of retinoid receptors in lung carcinogenesis. Sensitive and appropriate biological tools are necessary for screening the risk population and monitoring the efficacy of chemoprevention. Investigation of retinoid receptors is important and may contribute to the establishment of new strategies in chemoprevention for high-risk patients and in the treatment of lung cancer.

Background

Despite antismoking efforts and advances in therapy, lung cancer remains the leading cause of cancer-related death worldwide [1].

Multi-step carcinogenesis has been described as "a gradual accumulation of genetic and epigenetic aberrations resulting in the deregulation of cellular homeostasis" [2]. There is a similarity between bronchial lesions found in carcinogen-treated animals (squamous metaplasia) and the histological changes affecting the bronchial epithelia of humans or animals deficient in vitamin A. Restoration to a normal histological state occurs after vitamin A repletion, which in experimental models has also been shown to confer protection against pro-carcinogens.

The term retinoid (first coined by Sporn in 1976) generally refers to naturally occurring and synthetic vitamin A (retinol) metabolites and analogs [3].

Several studies have shown that vitamin A/retinoids are physiological regulators of embryonic development, vision, reproduction, bone formation, haematopoesis, differentiation, proliferation and apoptosis. Pharmacologically, they have been recognized as modulators of cell growth, differentiation and apoptosis. Furthermore they

have been shown to suppress carcinogenesis in various organs (e.g. oral cancer, skin, bladder, lung, prostate and breast cancers) in experimental animals [4]. Clinically, retinoids reverse premalignant human epithelial lesions and prevent lung, liver and breast cancer and second primary tumors in the head and neck [5].

It is now generally thought that the effects of retinoids are mainly mediated by the nuclear retinoid receptors, which are members of the steroid and thyroid hormone receptor superfamily [6,7]. Two families of retinoic receptors have been identified, namely RARs and RXRs with three subtypes for each (α; β, γ) and several isoforms arising from promoter usage and alternate splicing. The retinoid receptors are ligand-activated, DNA binding trans-acting, transcription-modulating proteins. The three RAR types have a strong affinity for all-trans and 9-cis isomers of retinoic acid. The three RXR types, on the other hand, have demonstrated an especially strong specificity for only the 9-cis isomers. Studies have shown that RXR/RAR heterodimers are responsible for transducing the retinoid signal *in vivo* [8]. These heterodimers bind to retinoic acid response elements found in the promoter region of retinoic acid-inducible target genes thereby activating transcription [8,9]. Without ligand RAR-RXR, heterodimers bind to co-repressors, which play an active role in repressing the transcription of targeted genes. The recruitment of histone deacetylases (HDACs) brings about transcriptional repression by preventing the opening of chromatin, which is linked to deacetylation of nucleosomes [10]. Several of the co-activators and co-repressors are shared by multiple signaling pathways, e.g. CBP (cAMP response element binding protein) has been implicated in AP-1 (activator protein 1) and p53 signaling. Meanwhile STAT signaling, Sin3 and HDAC-1 seem to have a role in what Ayer, et. al. call "Mad-Max signaling" [4].

Dawson lists a series of nuclear receptors such as thyroid hormone receptors, vitamin D3 receptors (VDRs), peroxisome proliferator activated receptors (PPARs), and several orphan receptors in which RXR is important as a "heterodimeric partner" [11].

The RARs and RXRs exhibit the conserved module structure of nuclear receptors and their amino acid sequence can be divided into six regions (A-F) based on homology among themselves and with other members of the nuclear superfamily.

The central region C consists of 66 amino acids and has two zinc-binding motifs very much like the core of the DNA binding domain (DBD) which enables cognate response elements to be recognized specifically. Both this central C region and the functionally complex E region are highly conserved between RARs and RXRs. Region E gains its complexity from the ligand binding domain (LBD), the ligand-dependent transcriptional activation function AF-2, and a dimerization surface contained within it. The name AF-1 has been given to a second transcriptional activation function found in both the amino-terminal A/B regions.

In humans, the genes encoding RAR α, β and γ are respectively located on chromosomes 17q21.1, 3p24 and 12q13. Those for RXR α, β and γ lie on chromosomes 9q34.3, 6p21.3, and 1q22. The physiological importance of the multiple isoforms of RARs is not known precisely, but these isoforms may explain why RARs have pleiotropic biological effects.

There are two major isoforms for RARα (α1 and α2) and for RAR γ (γ1 and γ2), and four major isoforms for RARβ (β1–β4) and the recently described RARβ1' [12], whose absence seems to be responsible for retinoid resistance in lung carcinognesis. RAR isoforms can be classified as those which are transcribed from either the P1 (class I: RAR α1, β1, and β3, γ1) or P2 (class II: RARα2, β2 and β4, γ2) promoter. All class II isoform P2 promoters contain an RA response element and are RA-inducible to varying degrees [13].

Similarly, several isoforms differing from one another in their amino-terminal region have been identified for RXRα (α1, α2), RXRβ (β1 and β2), and RXRγ (γ1, γ2) [14].

Epigenetic and genetic changes

Respiratory epithelium carcinogenesis is a multifactorial process which includes inherited and acquired genetic changes, chromosomal rearrangements, epigenetic phenomena and chemical carcinogenesis.

Vitamin A deficiency has been associated with bronchial metaplasia and increased lung cancer development. Many other factors contribute to dysfunction of retinoids and their cognate receptors [2].

The first cytogenetic reports connecting chromosome 3 to lung cancers were those of Whang-Peng et al. [15,16], who reported that 100% of small cell lung cancer (SCLC) cases examined showed specific 3p deletions by Giemsa banding. These changes were observed in 12/12 cell lines and three fresh tumors after a two-day culture period. The minimal region of common deletion was 3p14-p25. A number of studies have since been undertaken that obtained similar results that were extended to non small cell lung cancer (NSCLC).

Houle et al. mapped the RARβ2 at 3p24 and demonstrated that expression was decreased or even suppressed in lung cancer cell lines, suggesting that its re-expression

could suppress malignity [17]. Frequent loss of RARβ mRNA expression has been described in both primary NSCLCs and bronchial biopsy specimens from heavy smokers [18,19]. Furthermore, in addition to lung cancer [18,20], decreased RARβ2 mRNA expression has been demonstrated in a variety of solid tumors including head and neck [21]and breast carcinomas [22]. Xu et al. [18] also reported that all RARs and RXRs were expressed in at least 89% of control normal bronchial tissue specimens from patients without a primary lung cancer and that in distant normal bronchus specimens from patients with NSCLC RARα, RXRα and γ were expressed in more than 95% of the tumor-free specimens. In contrast, RARβ, RARγ and RXRβ expression was decreased, detected in only 76% of NSCLC specimens. Picard et. al similarly showed diminished or absent RARβ protein expression in ~50% of resected NSCLCs [23]. Furthermore, these authors observed normal or elevated RARα and RXRα expression in NSCLCs. The expression of RARβ, RARγ, and RXRβ was found to be decreased, however, in many tumors, while LOH at 3p24 occurred at a high frequency. This phenomenon was also seen in non-neoplastic lesions. The authors concluded that altered retinoid receptor expression might be involved in lung carcinogenesis. Martinet et al. extended the above study investigating RARs and RXRs alteration in lung cancer precursor lesions. They performed allelotyping for microsatellites located near the RAR/RXR gene loci and immunohistochemistry was additionally carried out to evaluate P53 and RARβ expression. Microsatellite changes occurred frequently in all samples, but without specificity for any group. RARβ marker losses were found in all examined groups, with a concomitant RARβ protein expression [24].

Aberrant methylation of the promoter regions of genes is a major mechanism of gene silencing in tumors [25]. Virmany et al. [26] identified hypermethylation as the underlying mechanism for this frequent loss of RARβ expression. Twenty-one of 49 (43%) primary resected NSCLC samples showed RARβ hypermethylation. In addition, it was demonstrated that RARβ hypermethylation was also important in the pathogenesis of SCLCs, with 62% of SCLCs methylated for RARβ. In the same study, it was also demonstrated that treatment of lung cancer cell lines with the demethylation agent 5-aza-2'-deoxycytidine (5-AZA-CdR) can restore RARβ expression. Moreover, a phase I-II trial in patients with stage IV NSCLC suggests that 5-AZA-CdR may have some clinical activity against metastatic NSCLC [27]. The loss of RARβ mRNA expression has been observed in many lung cancer cell lines also suggesting that to function as a tumor suppressor gene, RARβ expression is contingent on the intracellular concentration of retinoids [28]. The effects of retinol (vitamin A) depend on its intracellular metabolism including its transport by specialized proteins such as Cellular Retinol Binding Proteins (CRBP) and on its binding as retinoic acid to specific nuclear receptors: the Retinoic Acid Receptors (RARs) and the Retinoid X Receptors (RXRs) [7]. The CRBP I and II transport retinol in the cell and serve as chaperon proteins to prevent unscheduled retinol catabolism. It is the first building block in retinoic acid synthesis.

Retinoid signaling

The mechanisms through which retinoids suppress carcinogenesis, although complex, are gradually being elucidated. Their complexity results from the large number of genes involved in tumor cell differentiation and proliferation that include retinoic acid response elements in their promoters. Retinoids also inhibit tumorigenesis and tumor growth through their ability to induce either apoptosis (programmed cell death) or terminal differentiation. Interestingly, it has been established that the apoptotic process triggered by Retinoid Related Molecules is independent of p53 activation and proceeds through a novel pathway in which the mitochondrion seems to play a pivotal role [29].

As Karamouzis et al. stated in a recent publication [2], a 'switch on/off' model determines the relationship between retinoid receptors and other signaling pathways during bronchial carcinogenesis. According to this model, RXR selective compounds specifically inhibit AP-1 (activator protein 1) activity resulting in inhibited cell proliferation in normal respiratory epithelium, RARβ and RXRα AP-1-dependent interaction with other nuclear receptors, such as PPARγ with contribution cofactors (CBP/p300cAMP response element binding protein), ensures cyclin D1 mediated cell cycle inhibition, hence favoring apoptosis or differentiation. Down-regulation of the RARβ mechanism (as detailed above) combined with CBP and AP-1 up-regulation triggers tumor progression and proliferation. Concurrently the inability of RXRα to form heterodimers with PPARγ enables an AP1/CBP-dependent up-regulation of Cox2, resulting in the inhibition of apoptosis. This crucial role of RXRs may explain the observation of Brabender et. al as well. They observed suppressed mRNA expression of all subtypes of RXRs in curatively resected NSCLC that is followed by statistically worse overall survival [30].

In addition, retinoids play a central role in tumor stroma production and thus in the control of tumor progression and invasion through their ability to regulate the expression of matrix metalloproteinases, transforming growth factor-β, and cell cycle regulator proteins, such as cyclin dependent kinase I, such as p16, or p21 [31,32].

Up to now, the use of retinoids in clinical trials has been limited because of their pharmacologic effects and side effects. Furthermore a majority of human or experimental

NSCLCs are resistant to all *trans*-retinoic acid, and the mechanism of retinoic acid resistance has not been totally elucidated. The absence of the newly recognized RARβ1' (alternatively spliced from RARβ1 isoform) could be one reason for retinoid resistance in lung carcinogenesis [12]. In that study RARβ1' expression was repressed in RA-resistant BEAS-2B-R1 cells in lung cancer, compared with adjacent normal lung tissues. In H358 lung cancer cells that were transiently transfected with RARβ1', RA treatment was able to restore target gene expression. In order to better understand the mechanism of RARβ1' repression more studies are needed, and the authors note that "potential reexpression in lung cancer may be important to future approaches to lung cancer chemoprevention" [33].

Chemoprevention

Chemoprevention has been defined as: the application of natural or synthetic molecules to prevent, inhibit or reverse the carcinogenic machinery [34].

For the respiratory tract there are two major classes of agents which appear to prevent damage induced by inhaled carcinogens: retinoids and antioxidants. (In addition to those mentioned above, new classes of chemopreventive agents are under investigation, such as EGFR inhibitors, farnesyl transferase inhibitors, Cyclooxygenase-2 inhibitors etc., but presently we are focused on retinoids and synthetic Retinoid Related Molecules (RRMs).

Clinical trials have shown how complex the chemoprevention approach is. Nevertheless, large primary prevention trials in volunteers (physicians and nurses in the Physicians Health Study) and in high-risk populations (smokers, ex smokers and asbestos workers in the CARET and ATBC studies, and in the more recent EUROSCAN trial) using either beta-carotene, or the combination of beta-carotene and retinyl palmitate and the combination of beta-carotene and alpha-tocopherol have documented a higher incidence of lung cancer among smoking participants who received beta-carotene [35,36]. Interestingly a negative effect of beta-carotene supplementation has also been observed in experimental animals exposed to cigarette smoke [37]. In the EUROSCAN study, where retinyl palmitate and/or N-acetylcisteine supplementation were used, no beneficial effects on the incidence of second primary cancer and survival were observed. There was one exception for retinal given to workers exposed to asbestosis, which seemed almost protective against mesothelioma development [38]. One possible explanation for the failure and harm seen in the chemoprevention trials could be the procarcinogenic effect of the toxic oxidative carotene metabolites. The oxidative metabolites induce cytochrome P450 enzymes, lowering the serum levels of retinoid acid and down regulating RXR and RARβ. Nicotine by itself inhibits RARβ expression via methylation.

Further randomized, controlled chemoprevention trials designed to test retinoids, β-carotenes or α-tocopherol defined their target population based on smoking history, preneoplastic changes of the bronchial epithelium, or cancer history [39] (Table 1).

In a recent study Lam S. et al. observed that retinol was not effective in the up-regulation of RARβ in lesions with bronchial dysplasia among individuals who continued to smoke [40].

In addition Khurie FR. et al. reported worse prognosis in stage I. lung cancer, which indicated maintenance of RARβ expression and overexpression of RARβ correlated with increased expression of cyclooxygenase-2, an enzyme that contributes to progressive carcinogenesis and is a marker of poor prognosis [41,42].

The gene promoter hypermethylation is a leading cause of gene silencing. The loss of RARβ expression due to hypermethylation is of interest as are bronchial premalignant

Table 1: Lung cancer chemoprevention trials on retinoic acid analogs

Study	Drug	Number of patients	End point	Result	Reference
ATBC	β-Carotene α-Tocoferol	29,133	Lung cancer	Negative/harmful	[57]
CARET	β-Carotene Retinol	18,314	Lung cancer	Negative/harmful	[58]
EUROSCAN	Retinyl-palmitate N-Acetycystein	2,592	Second primary tumor	Negative	[38]
Physician's Health Study	β-Carotene	22,071	Lung cancer	Negative	[59]
Pastorino et al.	Retinyl-palmitate	307	Second primary tumor	Positive	[35]
Lam et al.	ADT	112	Bronchial dysplasia	Positive	[40]
Kurie et al.	Fenretinide	82	Metaplasia	Negative	[60]
Lippman et al.	13cRA	1,304	Second primary tumor	Negative	[61]

ATBC, α-Tocoferol, β-Carotene Cancer Prevention Study; CARET, Carotene and Retinol Efficacy Test; ADT, anethole dithioethione (5-[p-methoxyphenyl]-1,2-dithiole-3-thione; 13cRA, 13 cis retinoic acid

lesions in developed lung cancer. Recent publications show that hypermethylation of RARβ2 genes has a different effect on the development of second primary lung cancers (SPLCs) in NSCLCs depending on smoking status. In current smokers, SPLCs developed more frequently when RARβ was unmethylated than when it was hypermethylated. In the case of former smokers it was the opposite. SPLCs were more prevalent in patients with hypermethylated RARβ. Thus, in active smokers, silencing RARβ expression by hypermethylation has a protective effect against the development of SPLCs, whereas in former smokers RARβ expression (unmethylated) appears to be protective. The authors suggested that in current smokers, the continuous high oxygen tension and free radicals induce apoptosis and offer protection from the SPLCs. This apoptosis may be inhibited by retinoic acid if RARβ is expressed on a normal level [43]. These findings explain in part the previous observation that RARβ expression is associated with poor prognosis among patients who are active smokers [44].

Now available are stronger synthetic retinoids that select for an RAR and RXR type without exposing patients to the kind of retinoid toxicity that had previously been observed [45,46]. Aerosolized early on site, these retinoids have been able to reverse the RR deficiency in stabilizing RAR/RXR expression for increased ligand binding to restore normal cellular differentiation [47]. The authors, collaborating with a French research group, conceived and designed an appropriate RR assay in order to measure efficiently the normal bronchial mucosa level of each Retinoid Receptor's mRNA by real time quantitative relative RT-PCR. This method could be useful for screening the RR's status in the damaged bronchial epithelia of the high-risk patient and for monitoring the efficacy of the different Retinoids used as chemopreventive agents [48].

Summary

There is a large body of literature on clinical and preclinical studies using natural retinoids and related compounds for the prevention and the treatment of cancer [49]. The field of lung cancer chemoprevention has been controversial until now. However, there has also been disappointment in extending the therapeutic use of bexarotene (selective RXR agonist) to patients with NSCLC. Although preclinical data and a phase II clinical trial suggested that bexarotene added to platinum based chemotherapy may improve overall survival [50], a subsequent Phase III clinical trial did not bear this out [51,52]. One possible reason is that solid tumors can acquire and develop intrinsic resistance to retinoids during carcinogenesis. The effects of receptor selective retinoids on NSCLC cell lines were examined by Sun et al. According to their findings 8 of the 37 retinoids showed growth-inhibitory activity (IC_{50}, <10 μM) against at least two of the eight NSCLC cell lines [53]. CD437, a retinoid with some selectivity toward RARγ, was highly effective [54]. The RXR selective compounds did show growth inhibitory effects when combined with the RAR retinoids. These results indicated that human lung cancer cell lines have a high degree of resistance to synthetic retinoids [55]. Freemantle et al. have summarized the potential mechanisms of Retinoic Acid resistance. Increased P450 catabolism, drug export (P glycoprotein mediated), sequestration of retinoids by CRABs or other proteins, decreased expression of RARs through promoter methylation, persistent histone deacethylation, RAR rearrangement or mutation in the RAR ligand binding domain, and coactivator alteration or alterations downstream of target gene expression may lead to cellular retinoid resistance. This knowledge should aid in predicting those most likely to benefit from retinoid therapy and in developing strategies to optimize single agent or combination retinoid regimens to overcome resistance [56]. The generation of retinoids and rexinoids with restricted selectivity has opened new possibilities for cancer therapy and chemoprevention. It is probable that demethylating and chromatin remodeling agents currently under clinical investigation could be combined with these new retinoids for a better restoration of RR expression.

Authors' contributions

KB and BD conceived of the study and co-wrote the manuscript. FR, GK and JT provided helpful comments and helped write the paper. All authors read and approved the final manuscript.

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Targeting Blood Vessels for the Treatment of Non-Small Cell Lung Cancer

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Absract: Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related mortality worldwide. Although modest survival benefit has been observed with surgery, radiotherapy and platinum-based chemotherapy, an efficacy plateau has been reached. It has become obvious, therefore, that additional treatments are needed in order to provide an improved survival benefit for these patients. The use of molecular targeted therapies, particularly those against tumor capillaries, has the potential to improve outcomes for NSCLC patients. Bevacizumab, a recombinant humanized monoclonal antibody against vascular endothelial growth factor (VEGF), is the first targeted drug that has shown survival advantage when combined with chemotherapy in NSCLC. Other antivascular agents, including vascular disrupting agents (VDAs) and different small-molecule receptor tyrosine kinase inhibitors, have also shown promise in phase I and II trials in NSCLC. The aim of this study is to describe the clinical properties of these drugs and to discuss the evidence that supports their use in the treatment of NSCLC. Furthermore, we plan to review the main pitfalls of antivascular strategies in NSCLC cancer therapy as well as assess the future direction of these treatment methods with an emphasis on clarifying the molecular background of the effects of these drugs and defining the biomarkers.

INTRODUCTION

Lung cancer remains a major worldwide health problem accounting for 12.4% of cancer diagnoses and 17.6% of cancer deaths worldwide [1]. For treatment purposes, lung cancer is currently classified into two major groups: small-cell and non-small-cell cancer (NSCLC). The latter includes squamous-cell carcinoma, adenocarcinoma, and large-cell carcinoma. Bronchioloalveolar carcinoma is generally subclassified under adenocarcinoma. Overall, approximately 85% of lung cancer patients have non-small-cell histology [2], and unfortunately, of these, a large proportion present with locally advanced or metastatic disease for which there is no curative treatment.

The current standard palliative treatment for patients with NSCLC and a good performance status is double-agent cytotoxic chemotherapy consisting of a platinum combined with a third generation agent such as gemcitabine, vinorelbine or a taxane [3]. This treatment has been shown to provide palliation of symptoms, improved quality of life and an increase in life expectancy from a median survival of approximately 4-5 months to 8–10 months; and a corresponding increase in the 1-year survival rate from 10% to 30-40% [4]. Despite multiple studies to evaluate different schedules, doses and combinations of cytotoxic agents, it appears that a therapeutic plateau has now been reached with cytotoxic therapy [5]. Consequently, attention over recent years has focused on targeted and novel therapies with notable success in the development of antivascular drugs [6, 7]. These agents have shown particular promise in preclinical NSCLC models and in clinical studies and are the focus of this review.

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VASCULARIZATION OF NON-SMALL CELL LUNG CANCER

In order to develop and metastasize, growing tumors require an adequate blood supply. Similar to other solid tumors, NSCLCs achieve this by secreting a range of angiogenic factors that coordinate the complex series of events of new capillary growth. Among these angiogenic molecules, vascular endothelial growth factor (VEGF) has been identified as the key cytokine for endothelial sprouting (defined as the in situ proliferation and budding of endothelial cells) in NSCLC [8-10]. Although the hypoxia inducible factor (HIF) - von Hippel–Lindau (VHL) protein system has a major role in the regulation of VEGF expression, a variety of oncogenes can also enhance VEGF production, including activated epidermal growth factor receptor (EGFR), mutant Ras and erbB-2/Her2. All are known to have influence on NSCLC progression. In addition to VEGF, however, many other molecules have been implicated as positive regulators of NSCLC induced endothelial sprouting, including platelet-derived growth factor (PDGF) [11], basic fibroblast growth factor (bFGF) [12], matrix metalloproteinases (MMPs) [13], EGF [14], placental growth factor (PlGF) [15], interleukin-8 (IL-8) [16], hepatocyte growth factor (HGF) [17] and angiopoietins [18]. Interactions between these molecules initiate signaling cascades that regulate further processes such as expression of additional growth factors, alterations in cell-cell and cell-matrix interactions, and activation of the members of endothelial cell (EC) adhesion molecule families. These families of cell surface adhesion receptors include integrins, cadherins, cell membrane proteoglycans, selectins and the members of the immunoglobulin superfamily. Perhaps the key adhesion molecules during EC sprouting are the members of the integrin family. A variety of integrins have been found to be expressed in migrating ECs, including $\alpha_1ß_1$, $\alpha_2ß_1$, $\alpha_3ß_1$, $\alpha_5ß_1$, $\alpha_vß_5$, and $\alpha_vß_3$. The most important among them is, perhaps, $\alpha_vß_3$, which mediates the migration of ECs

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in the fibrin-containing tumor stroma and maintains the sol state of the basement membrane because of its ability to bind to MMP-2 [19]. During the later steps of new capillary growth, PDGF-BB recruits pericytes and smooth muscle cells, whereas TGF-ß1 (transforming growth factor beta-1) and angiopoietin-1/Tie-2 stabilize the interaction between mural and ECs [20].

Although the central role of EC sprouting in lung cancer vascularization makes it an attractive target for anticancer therapy, it is well established now that tumor capillaries are not necessarily derived from EC sprouting; instead, solid tumors can acquire their vasculature by intussusceptive microvascular growth, co-option of pre-existing vessels, postnatal vasculogenesis, glomeruloid angiogenesis, or vasculogenic mimicry [20]. It could be argued that the most important non-sprouting mechanism in NSCLC is vessel co-option. During "nonangiogenic" or "alveolar type" of growth, NSCLC cells fill the alveoli, entrapping, but not destroying, the alveolar walls with the co-opted capillaries. In the tumor cell nests circumscribed by the alveolar walls no neoangiogenesis (i.e. EC proliferation or sprouting) is present [21]. Analysis of the vasculature of nonangiogenic NSCLCs has demonstrated that their vascular phenotype is the same as that of normal alveolar capillaries [22] and, furthermore, that nonangiogenic tumors can be distinguished by their gene expression profiles from angiogenic ones [23].

Another known vascularization mechanism in NSCLC is postnatal vasculogenesis, a process during which circulating bone marrow (BM)-derived endothelial progenitor cells (EPCs) incorporate into sites of new vessel growth and differentiate into ECs [24]. EPCs therefore resemble embryonic angioblasts, which are anchorage-independent cells having the capacity to proliferate, migrate and differentiate into mature ECs. Although the molecular pathways involved in EPC release and homing are in the early stage of definition, VEGF and molecules that induce leukocyte or erythrocyte mobilization (i.e. granulocyte macrophage colony stimulating factor and erythropoietin) are thought to be the most significant of the other molecules [24]. However, recent results indicate that PDGF-CC [25], PlGF [26], nitric oxide (NO) [27], angiopoietin-1 [28], 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors (statins) [29] and estrogens [30] stimulate EPC mobilization as well. In contrast, tumor necrosis factor-alpha (TNF-α) and C-reactive protein (CRP) induce apoptosis, inhibit the function and decrease the levels of EPCs [31, 32]. Since the first description of EPCs by Asahara *et al.* [33], these progenitors have been detected at increased frequency in the peripheral blood of patients with various malignancies including hepatocellular [34], breast [35] and colorectal [36] cancers, and myeloma multiplex [37], myelofibrosis [38], non-Hodgkin's lymphoma [39], acute myeloid leukaemia [40] and malignant gliomas [41]. In NSCLC, our group demonstrated recently that the levels of EPCs are significantly increased in the peripheral blood of patients, and that these levels correlate to tumor burden and to clinical behavior [42]. Accordingly, Hilbe and colleagues found a significant increase in incorporated CD133+ EPCs in tumor samples of NSCLC patients [43].

In summary, as in other malignant tumors, vascularization of NSCLC is an extremely complex process that requires a perfectly organized interaction between a host of different molecular and morphological events.

ANTIVASCULAR THERAPY FOR NSCLC

Any categorization of antivascular strategies is difficult, with overlap in several features. However, the main classes of these therapies that have been developed are angiosuppressive (anti-angiogenic) and vascular-disrupting agents (VDAs) [44]. Because it is beyond the scope of this paper to review all agents that target the vasculature of NSCLC, we focus here on the drugs that are at a more advanced stage of clinical testing.

Antiangiogenic (Angiosuppressive) Therapy

This approach is motivated by the fact that new capillary growth in cancer requires the induction of EC proliferation by specific or nonspecific mitogens. By inhibiting the production of endothelial mitogens, the mitogens themselves, their endothelial receptors, the associated signalling pathways, the endothelial integrins and the matrix metalloproteinases (MMPs), these agents specifically target endothelial sprouting and postnatal vasculogenesis (i.e. endothelial progenitors) in cancer.

Matrix Metalloproteinase Inhibitors (MMPIs)

The MMPs are a family of zinc-containing proteolytic enzymes which have been shown to facilitate tumor angiogenesis, invasion and the establishment of metastases in preclinical studies [45]. However, the results from a large, randomised, phase III study of the MMPI, BMS-275291 conducted in advanced lung cancer in 774 patients demonstrated no benefit from addition of this MMPI to chemotherapy with carboplatin and paclitaxel. Moreover, the combination of the MMPI with chemotherapy did not cause the severe musculoskeletal toxicity that has been seen with other MMPIs; it did however, increase the rate of hypersensitivity reactions, rash and febrile neutropenia, and patients in the experimental arm were more likely to stop therapy because of toxicity [46].

Bevacizumab

Bevacizumab (Avastin®; Genentech/Roche, South San Francisco, California, USA), is a humanized monoclonal antibody that acts by binding and neutralizing the VEGF-A ligand. This agent has been studied in a variety of solid tumors both as a single agent and in combination with cytotoxic chemotherapy. It is the first of the antiangiogenic class of drugs to be licensed for the treatment of NSCLC as well as metastatic cancer of the colon or rectum.

Bevacizumab Monotherapy

As a single agent, bevacizumab appears to be cytostatic. In a clinical phase I trial in patients with a variety of solid tumors, treatment with bevacizumab was associated with no responses in the 23 patients studied, although 12 of these patients achieved stable disease over the duration of the study [47]. When combined with cytotoxic chemotherapy, however, bevacizumab appears to have a synergistic effect. Studies carried out in patients with metastatic colorectal and lung cancers have all shown improvements in both progres-

sion-free and overall survival with the addition of bevacizumab to chemotherapy [48, 49].

Bevacizumab in Combination with Cytotoxic Chemotherapy

In lung cancer, the first evidence showing a benefit from administration of bevacizumab came in a phase II randomized trial for patients with advanced or recurrent NSCLC [50]. In this trial subjects were randomized to either chemotherapy alone or chemotherapy with bevacizumab. Patients received up to six cycles of 3-weekly paclitaxel 200mg/m^2 and Carboplatin (area under the curve of 6) alone or in combination with bevacizumab (7.5 or 15mg/m^2). Patients who did not progress during chemotherapy were continued on bevacizumab alone for up to 18 cycles. Results showed that those treated with bevacizumab at a dose of 15mg/m^2 had a significantly increased time to progression, (7.4 versus 4.2 months, P = 0.023), compared with chemotherapy alone. Furthermore, there was a non-significant trend to improved overall survival (17.7 versus 14.9 months, P = 0.63). It should be stressed, however, that this trial lacked sufficient statistical power to detect between-arm differences in survival outcomes. When given at a dose of 7.5mg/m^2, bevacizumab was no different than chemotherapy alone with a time to progression of 4.3 months. In general, bevacizumab was well tolerated, although one unusual and unexpected toxic effect was the development of life-threatening haemoptysis, mainly in patients with central tumors and squamous cell histology. Subsequently, a multivariate analysis identified squamous cell histology as an independent risk factor for unexpected massive and life-threatening bleeding.

Subsequent to this study, the Eastern Cooperative Oncology Group (ECOG) E4599 trial was initiated [51]. This study, which is to date the only published phase III randomized trial of an anti-angiogenesis agent in combination with chemotherapy in patients with lung cancer, randomized 878 chemotherapy-naive patients with predominantly non-squamous cell histology and advanced NSCLC to receive paclitaxel (200 mg/m^2) and carboplatin (area under the curve of 6) with or without bevacizumab (15 mg/kg). Treatment was administered every 3 weeks for six cycles. In the bevacizumab treatment arm, following completion of chemotherapy, single-agent bevacizumab was continued until disease progression. The primary study end point was overall survival. Results showed that the addition of bevacizumab was associated with a significant improvement in the median overall survival (12.3 versus 10.3months, P=0.003) compared to chemotherapy alone. Progression-free survival was also significantly improved (6.2 versus 4.5 months), corresponding to a hazard ratio for progression of 0.66 (P<0.001). However, it is important to note that the total benefit in median survival was a modest 2 months and that an unplanned subgroup analysis demonstrated that the survival improvement was confined to male patients.

Treatment with bevacizumab was however, associated with higher rates of toxicities. There was a significantly higher frequency of National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) grade 4 neutropenia (25.5 versus 16.8%, respectively; P=0.002), thrombocytopenia (1.6 versus 0.2%, respectively; P=0.04), and febrile neutropenia (5.2 versus 2%, respectively; P=0.02). The reasons for such hematological toxicity are unclear, although various mechanisms could be involved, such as enhanced delivery of the chemotherapeutic agents to the bone marrow as a result of vascular changes or direct interaction of bevacizumab with chemotherapy at the stem cell level. Non-haematological toxicities were also much higher including grade 3-5 hypertension (7.7 versus 0.7%, respectively; P<0.001), hemorrhage (4.4 versus 0.7%, respectively; P<0.001) and proteinuria (3.1 versus 0%, respectively; P<0.001). It was noted that the observed hypertension and proteinuria were generally manageable and that permanent discontinuation of bevacizumab was not required. In total, there were 17 treatment-related deaths: 2 deaths (gastrointestinal hemorrhage and febrile neutropenia) occurred with chemotherapy alone (0.5%) and 15 deaths occurred on the bevacizumab arm (3.5%; P=0.001). Of the 15 bevacizumab-related deaths, 5 were attributed to pulmonary hemorrhage, 5 to complications of neutropenic fever, two each to a cerebrovascular event or gastrointestinal hemorrhage, and 1 due to a probable pulmonary embolus.

As a result of the this study the United States (US) Food and Drug Administration (FDA) approved the use of bevacizumab in combination with carboplatin and paclitaxel for the initial systemic treatment of patients with unresectable, locally advanced, recurrent or metastatic, non-squamous, NSCLC and this treatment has become the standard of care for these patients in the US.

More recently, preliminary results from the phase III Avastin in Lung ("AVAiL", BO17704) trial were presented in abstract form at the 2007 American Society of Clinical Oncology meeting[1]. In this European trial, 1,044 patients with previously untreated, advanced, non-squamous, NSCLC were randomized to receive cisplatin (80 mg/m^2 on Day 1) and gemcitabine (1,250 mg/m^2 on Day 1 and Day 8) chemotherapy every three weeks for up to six cycles together with either bevacizumab 15 mg/kg, bevacizumab 7.5 mg/kg or placebo every three weeks until disease progression. Results showed a median progression free survival of 6.5 months in the 15 mg/kg bevacizumab plus chemotherapy arm, 6.7 months in the 7.5 mg/kg bevacizumab plus chemotherapy arm and 6.1 months in the chemotherapy alone arm. This equated to 22 and 33 percent improvements in progression free survival respectively in the 15 mg/kg and 7.5 mg/kg bevacizumab treatment arms. Toxicities were similar to previous studies of bevacizumab in NSCLC. Although the exact overall survival data has not yet been published, in a recent press release, the developer of bevacizumab announced that it did not yield a statistically significant prolongation of overall survival, a secondary endpoint, with either dose [52].

Bevacizumab is also currently being tested in the adjuvant setting in the ECOG E1505 trial. This trial aims to recruit 1,500 patients with completely resected stage IB-IIIA tumors and compare adjuvant chemotherapy using four cycles of three-weekly cisplatin with vinorelbine, docetaxel or gemcitabine with or without bevacizumab.

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A further study is currently in progress to evaluate the safety and efficacy of carboplatin and paclitaxel chemotherapy with bevacizumab in subjects with locally advanced or metastatic squamous NSCLC. These studies will help to further define the role for bevacizumab in NSCLC.

Bevacizumab in Combination with EGFR Inhibiting Agents

VEGFR and EGFR signaling is interrelated, as VEGF was demonstrated to be downregulated by anti-EGFR drugs through both HIF1α-dependent and -independent mechanisms [53, 54]. Furthermore, acquired resistance to EGFR inhibitors is associated with increased levels of VEGF [55]. Recent data also suggest that EGFR inhibition could have antitumor effects even in EGFR negative tumors, but when the cancer capillary ECs express the receptor [56]. Combining anti-EGFR and anti-VEGFR drugs might therefore provide more enhanced anti-cancer effects than either therapy alone. Accordingly, both pre-clinical and early phase clinical trials have been carried out to explore the role of simultaneous inhibition of the VEGF and EGFR cascades. The latest of these, a multicentre, randomized, phase II trial compared the use of bevacizumab in combination with either chemotherapy or erlotinib against chemotherapy alone in a cohort of pre-treated patients with non-squamous NSCLC [57]. Results showed that when compared with chemotherapy alone, the combination of bevacizumab with chemotherapy was associated with a 34% reduction in the risk of disease progression or death whilst there was a 28% risk reduction from chemotherapy and Erlotinib. Of note, however, neither of these risk reductions met statistical significance. A phase III trial comparing erlotinib alone versus erlotinib plus bevacizumab is underway to clarify the benefit of the combination of these two agents. Due to the successful clinical development of bevacizumab and erlotinib in NSCLC, a number of other agents that inhibit both VEGF and EGFR pathways are currently in development as outlined below.

In summary, the recent success of bevacizumab in combination with paclitaxel/carboplatin chemotherapy for first-line treatment of advanced, nonsquamous NSCLC in the E4599 trial [51] has confirmed that targeting of angiogenesis in NSCLC might be a reasonable therapeutic approach. However, notwithstanding the considerable progress made by the above study, a number of questions and future challenges relating to the use of bevacizumab in NSCLC linger. The clinical use of bevacizumab is restricted to patients with non-squamous histology and to those without central nervous system metastases. Moreover, the absolute improvement in median survival from the addition of bevacizumab to paclitaxel/carboplatin was modest and bevacizumab failed to improve the overall survival of NSCLC patients in combination with gemcitabine/cisplatin chemotherapy in the AVAil trial [52]. Thus, the pharmacological interactions of bevacizumab with other anticancer agents are still not fully understood. Furthermore, it is still uncertain what the optimal biological dose is and the most favourable schedule and duration of therapy as well as the ideal clinical setting have not yet been defined.

Vandetanib

Vandetanib (Zactima®, ZD6474; AstraZeneca, Macclesfield, UK), is a potent small molecule inhibitor of the tyrosine kinase domain of the VEGF receptor-2 (VEGFR-2). It also has moderate anti-EGFR activity, with an inhibitory concentration (IC50) of 500nM, compared with 40nM for VEGFR-2 [58]. At this time it is unclear how much of its anti-tumor activity can be attributed to its action at EGFR. In addition, vandetanib potently inhibits ligand dependent RET kinase activity. Constitutive RET activity is associated with certain thyroid tumors [59]. Pre-clinical studies have shown that vandetanib inhibits tumor angiogenesis, growth and metastasis in a wide range of models, across several tumor types, including lung, prostate, colon, breast and ovary [58].

Two phase I clinical trials of vandetanib in patients with refractory advanced solid tumors have been conducted in the USA, Australia and Japan. With the primary objective of evaluating the safety and tolerability of the agent, these studies have shown vandetanib to be well tolerated at a daily oral dose ≤ 300mg, with common adverse events including rash, diarrhoea and asymptomatic QTc prolongation. The Japanese study yielded tumor responses in 4 of 9 patients with NSCLC [60], prompting a series of randomised phase II studies.

In a comparative two-part study of vandetanib and gefitinib, 168 patients with previously treated stage IIIB-IV NSCLC were randomised to receive either vandetanib (300mg po od) or gefitinib (250mg po od) in part A of the study. At disease progression or development of unacceptable toxicity, subjects were switched to the alternative treatment (part B) after a washout period of four weeks. The response rate was 8% in the vandetanib arm compared with 1% in the gefitinib arm, and a longer progression-free survival (PFS) time for vandetanib followed by gefitinib (11.9 weeks versus 8.1 weeks; HR=0.63, 95% CI: 0.44-0.90; P=0.011). No survival difference was seen[2].

The efficacy of vandetanib in combination with standard chemotherapy regimes, compared with chemotherapy alone, has been investigated in two further randomized trials. The first of these, a randomized, placebo-controlled study of vandetanib (100mg/day or 300mg/day) plus docetaxel in 127 patients with NSCLC who have progressed after first-line platinum-based chemotherapy, has recently been published [61]. Median PFS was 18.7 weeks for vandetanib 100mg + docetaxel (HR versus docetaxel =0.64, 95% CI:0.38-1.05; 1-sided P=0.037); 17 weeks for vandetanib 300mg + docetaxel (HR versus docetaxel = 0.83, 95% CI:0.5-1.36; 1-sided P=0.231); and 12 weeks for docetaxel. The combination of vandetanib and docetaxel was generally well tolerated and adverse events manageable, with the incidence of vandetanib-associated toxicities increased at the higher dose level (diarrhoea (grade3/4) – 50%; rash (grade 3) – 46%; QTc-related events (grade 3/4) – 16%). Again, there was no statistically significant difference in overall survival between the three treatment arms. It is not clear why the addition of the 100 mg dose of vandetanib to docetaxel seemed to be more effective that the addition of the 300 mg dose in this trial. One possible explanation is that the anti-EGFR activity of

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vandetanib at higher doses may cause G_1 cell cycle arrest in tumor cells, thereby reducing the sensitivity of tumors to the cell cycle phase-dependent activity of chemotherapy. As discussed below, a further possibility is that high levels of vandetanib might reduce tumor blood flow and impede the delivery of chemotherapy.

For first-line treatment, a trial investigating vandetanib alone or in combination with carboplatin-paclitaxel, versus chemotherapy alone has completed, with results presented recently[3]. The primary objective was to determine whether vandetanib +/- carboplatin-paclitaxel prolonged PFS compared with chemotherapy alone. With a total of 181 subjects, the primary objective was met, with vandetanib + carboplatin-paclitaxel significantly prolonging PFS compared with carboplatin-paclitaxel alone (HR=0.76, 95%CI 0.50-1.15; P=0.098). Given this encouraging phase II data, vandetanib is currently undergoing phase III development, both as monotherapy and in combination regimes for NSCLC. The potential for efficacy in squamous cell NSCLC, in addition to non-squamous NSCLC, is a definite advantage over bevacizumab.

A number of other angiosuppressive agents are at a less advanced stage in clinical development compared to bevacizumab and vandetanib.

AMG 706 (AMGEN, USA) is a small organic molecule with potent kinase-inhibiting activity against all known VEGFRs, PDGF receptor (PDGFR) and Kit. Preliminary integrated analyses of clinical data on AMG 706 monotherapy have shown that the most common treatment-related adverse events are hypertension (42%), diarrhea (41%), fatigue (32%), headache (21%), nausea (21%), anorexia (13%) and vomiting (10%). It is currently undergoing phase III evaluation as first-line treatment in combination with paclitaxel and carboplatin for advanced NSCLC. This randomized, placebo-controlled study aims to recruit 1240 subjects, with the primary objective to determine if treatment with AMG706 in combination with chemotherapy improves overall survival compared with chemotherapy alone.

Aflibercept (VEGF Trap; Sanofi-Aventis, France) is an engineered soluble receptor, made from the extracellular domains of VEGFR-1 and VEGFR-2. It binds all isoforms of VEGF, as well as placental growth factor. Hypertension and proteinuria were the main toxicities seen in phase I trials of aflibercept, administered subcutaneously and intravenously [62]. A phase III second-line study of aflibercept in combination with docetaxel for advanced NSCLC has recently commenced recruitment.

AZD2171 (Recentin™; AstraZeneca, UK) is an orally active tyrosine kinase inhibitor (TKI) of all VEGFR subtypes. A phase I study of AZD2171 in combination with gemcitabine and cisplatin in patients with advanced NSCLC has recently reported the toxicities of this combination to be manageable and predictable (hypertension, fatigue and diarrhea)[4]. In addition, encouraging anti-tumor activity was observed, which did not appear dose dependent at the doses tested. The combination of AZD2171 with pemetrexed is currently undergoing phase II evaluation for relapsed NSCLC, and for first-line therapy, a phase III randomized study of carboplatin and paclitaxel with or without AZD2171 for stage IIIB/IV NSCLC is currently in progress.

Axitinib (AG-013736; Pfizer, USA) is a small molecule TKI of VEGFR 1,2 and 3, with activity also against PDGFR-β and Kit. Single agent activity in patients with advanced NSCLC has been demonstrated in a phase II study, with three (9.4%) confirmed responses and median duration of response 9.4 months[5]. Treatment was well tolerated with manageable toxicity in this population. Most common grade 3/4 toxicities were fatigue (22%), diarrhea (6%), hypertension (6%) and hyponatraemia (6%).

Sorafenib (Nexavar®, BAY 43-9006; Bayer, Germany), an oral multitargeted TKI, inhibits the kinase activity of both C-RAF and B-RAF, VEGFR-2 and 3 and PDGFR-β and Kit [63]. It was thought particularly active in NSCLC because the proliferation signaling of the Ras/Raf/MEK/ERK pathway is increased in NSCLC due to an increase in K-ras mutations [64]. However, the Phase III "ESCAPE" (Evaluation of Sorafenib, Carboplatin And Paclitaxel Efficacy in NSCLC) study was stopped early after a planned interim analysis demonstrated that the trial would not meet its primary endpoint of an improvement in overall survival. In this study, more than 900 therapy naïve NSCLC patients were randomized to receive either sorafenib or a placebo in combination with carboplatin and paclitaxel. The interim analysis showed more deaths among squamous cell carcinoma patients who received Nexavar, carboplatin and paclitaxel, compared to those who received placebo, carboplatin and paclitaxel [65].

Sunitinib (Sutent®, SU11248; Pfizer, USA), an oral, small-molecule, multi-targeted TKI that was approved by the FDA for the treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor (GIST) in 2006, inhibits signaling through PDGFR, Kit, FLT-3 and VEGFR-2 [66, 67].

Phase II studies of both agents as single-agent therapy in relapsed/refractory NSCLC have demonstrated good tolerability, and level of activity similar to currently approved agents[6, 7, 8].

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Vascular Disrupting Agents (VDAs)

Targeting of VEGF has been shown to result in apoptosis only in newly formed, immature tumor vessels and in the developing vasculature of the neonatal mouse, but not in the tumor vessels of adult mice or in quiescent tumor vascular networks [68]. Vascular targeting therapy (i.e. VDAs) recognizes the fact that clinical diagnosis of cancer commonly occurs when the tumor tissue has already established its vasculature. VDAs specifically target pre-existing tumor capillaries, resulting in rapid cancer tissue ischemia and secondary tumor cell death in the central regions of tumors, although they leave the perfusion in peripheral tumor regions relatively intact. An additional advantage of VDAs is that compared to angiosuppressive drugs, their effects do not depend on the type of vascularization [20] occurring in a given cancer. The two major categories of VDAs that are in clinical development are the small-molecule VDAs and ligand-directed VDAs [69]. While small-molecule VDAs achieve selective occlusion of tumor vessels by exploiting phenotypic differences between tumor and host tissue ECs (i.e. increased reliance on the tubulin cytoskeleton to maintain cell shape and accelerated proliferation), ligand-directed VDAs use toxins and pro-coagulant agents coupled to peptides or antibodies that selectively bind to the endothelial tube. Although animal studies with ligand-directed VDAs have certainly been elegant, and, furthermore, several potential target molecules (such as integrin $\alpha_v ß_3$ [70] or endoglin, an auxiliary receptor for TGFβ signaling which is controlled by the HIF-1 complex [71]) exist that are up-regulated on tumor *versus* host tissue capillaries, testing of ligand-based agents are still in the preclinical phase. For that reason, and because they are at a much more advanced stage of clinical development, only representatives of small molecule VDAs will be discussed here.

5,6 dimethylxanthenone-4-acetic acid (DMXAA) (Novartis, Switzerland) is a flavonoid VDA that has been demonstrated to augment the anticancer effects of radiation [72], paclitaxel [73] and cisplatin [74]. The combination of DMXAA with carboplatin and paclitaxel is currently undergoing phase II evaluation for IIIb or IV NSCLC previously untreated with chemotherapy. Preliminary results suggest that this combination could provide additional benefit compared with carboplatin/paclitaxel alone[9].

Combrestatin A4 phosphate (CA4P) (Zybrestat, CA-4-P, OxiGene, USA) is a phosphate prodrug of the tubulin-binding agent combrestatin A4. By inhibiting tubulin polymerization, CA4P caused selective endothelial damage in human tumor xenografts [75]. Based on the promising results of phase I studies, CA4P is currently being evaluated in several phase II trials. For example, an ongoing study is evaluating the safety and effectiveness of CA4P combined with the chemotherapy drugs carboplatin and paclitaxel in advanced solid tumors, including NSCLC [76]. More interestingly, because the CA4P-bevacizumab combination has appeared safe, resulted in significantly decreased tumor blood-flows, and shown clinical activity without simultaneous chemotherapy, OxiGene plans to initiate a controlled Phase II study of Zybrestat plus bevacizumab and standard chemotherapy in NSCLC patients by the first quarter of 2008 [77].

ABT-751 (Abbott Laboratories, Abbott Park, IL, USA) is a novel oral sulfonamide that binds to the colchicine binding site of ß-tubulin, inhibits polymerization of microtubules and has demonstrated significant antitumor activity in a variety of preclinical xenograft models [78]. Interestingly, however, it has not been demonstrated to provoke the typical tumor vascular shutdown of other VDAs. There have been three published phase I trials of ABT-751 in patients with hematological malignancies and in adult and pediatric solid tumors [79-81]. In advanced NSCLC, two phase II trials evaluating ABT-751 in combination with chemotherapy are currently in progress [82, 83]. Another phase II Study of ABT-751 in patients with NSCLC refractory to Taxane regimens has been completed, but the results have not yet been released [84].

POTENTIAL BIOMARKERS OF RESPONSE TO ANTIVASCULAR AGENTS IN NSCLC

Antivascular treatment represents a novel and exciting type of molecular-based antitumor therapies. Accordingly, there is a clinical need in this field to identify biomarkers that can help to recognize patients responsive to these therapies, detect tumor resistance and predict the efficacy of antivascular drugs cost-effectively.

Some of the most promising biomarkers for antivascular treatments are the circulating populations of EPCs and mature, desquamated ECs (circulating endothelial cells; CECs). In murine tumor models, EPC/CEC levels have been demonstrated to correlate with tumor burdens and, moreover, with the efficacy of anticancer/antiangiogenic therapies [24]. In other murine tumors systems, VDA treatments led to a rapid increase in the levels of circulating EPCs, which incorporated into the microvessels of viable peripheral tumor areas that characteristically survive after such treatment. Suppression of this EPC mobilization by antiangiogenic agents resulted in marked reductions in viable tumor rim size and blood flow as well [85]. More importantly, techniques for EPC/CEC enumeration have also been tested clinically. Particularly encouraging in this regard is a phase 1 trial in which bevacizumab decreased the number of EPCs and CECs in colorectal cancer patients [86]. Although no studies have been undertaken to determine the levels of these cells in NSCLC patients treated with antivascular drugs, in a recent study, we assessed the number of circulating EPCs by flow cytometry from the peripheral blood of NSCLC patients before and after chemoradiotherapy and found significantly lower posttreatment levels of circulating EPCs in patients who achieved a partial/complete remission than in patients with stable or progressive disease [42].

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) [87]. Currently, almost all direct techniques are available solely in murine models, whereas indirect techniques are typically used in clinical settings. Accordingly, with a few exceptions [88], experience

[9]McKeage, M. J. Phase Ib/II study of DMXAA combined with carboplatin and paclitaxel in non-small cell lung cancer (NSCLC). *J Clin Oncol.* **2006**, ASCO Annual Meeting Proceedings Part I. Vol *24*, (Suppl. 18), Abstract 7102.

with vascular imaging in human NSCLC has been gained primarily by indirect techniques. These include measurements of contrast enhancement, blood volume and oxygen saturation with computed tomography (CT) [89, 90], magnetic resonance imaging (MRI) [91], positron emission tomography (PET) [92] and singlephoton emission computed tomography (SPECT) [93].

No single predictive angiogenic molecule has been identified to date and the use of cytokines as biomarkers of angiogenesis can be complicated by the release of angiogenic growth factors from platelets. Nevertheless, because of the relative simplicity of performing protein analyses, the clinical value of peripheral blood angiogenic biomarker measurements has also been investigated recently in many human studies. For example, plasma concentrations of total VEGF and PlGF were observed to be significantly elevated in bevacizumab-treated colon carcinoma patients [86]. In another clinical study on patients with colorectal cancer, a dose-dependent elevation of plasma VEGF-A and bFGF was found following the first cycle of PTK787/ZK222584 (an angiogenesis inhibitor targeting all known VEGFR tyrosine kinases) treatment [94]. Similarly, the authors of a phase II trial study reported a progressive increase of total VEGF levels after initiation of treatment with bevacizumab in renal cancer [95]. In a phase II/III trial in which 878 patients with advanced NSCLC were randomized to receive carboplatin + paclitaxel or carboplatin + paclitaxel + bevacizumab, E-Selectin and bFGF, but not VEGF, levels showed significant decreases from the baseline in both arms[10]. Because investigators of the above trials did not separate the VEGF bound to bevacizumab from free VEGF, it is important to note that in a recent study, free VEGF levels assessed after immunodepletion of plasma samples were found to be significantly reduced following bevacizumab treatment confirming that this anti-VEGF antibody effectively decreases the plasma level of the biologically active VEGF [96].

In summary, because antivascular treatments are developing at a rapid pace, there is an urgent need to identify reliable biomarkers for the efficacy of these therapies. However, although some pieces of the puzzle are already in place, monitoring techniques should be explored further to fully understand their possible implications in solid tumors, as well as in NSCLC. Accordingly, at present, the optimal technique for evaluating effects of antivascular treatments in cancer patients is a matter of active discussion among experts.

CHALLENGES OF ANTIVASCULAR THERAPY OF NSCLC: LESSONS FROM PRECLINICAL DATA AND FROM CLINICAL TRIALS ON ANTIVASCULAR AGENTS

Improved overall survival demonstrated in the ECOG E4599 trial has confirmed the clinical value of bevacizumab (i.e. anti-VEGF) treatment in human NSCLC [51]. However, the absolute increase in overall survival was only ~2 months in the above study and, more notably, bevacizumab failed to show a statistically significant improvement in overall survival in the AVAil trial [52]. Thus, the exact mechanisms of different antivascular drugs (in combination with different chemotherapeutic agents) in NSCLC patients are still not completely understood.

One of the key unresolved questions is how antiangiogenic agents can be combined with chemo- and/or radiotherapy. There has been some concern that a strategy developed primarily to reduce tumor blood flow could impede the delivery of chemotherapeutic agents and reduce the level of intratumoral oxygen essential for effective radiotherapy. However, in different preclinical models, a combination of cytotoxic agents with angiogenesis inhibitors resulted at least additive but in certain cases synergistic antitumoral effects [97-99]. More importantly, there are now clinical examples of the improved efficacy of chemotherapy in combination with an angiosuppressive agent. Bevacizumab in combination with chemotherapy improved progression-free survival in breast cancer patients [100] and, as mentioned above, overall survival in colorectal [49] and NSCLC patients [51].

Among the several possible explanations for the improved efficacy, one called "normalization of tumor vasculature" was put forth by Jain and colleagues recently [101]. According to their hypothesis, blood vessel leakiness and tortuosity as well as increased interstitial pressure in cancer tissue may render cancer capillaries insufficient to provide adequate blood supply for tumor cells. By blockade of VEGF signaling, bevacizumab could potentially help in the "normalization" of tumor vasculature and in the improvement of the delivery of chemotherapeutic agents. Accordingly, recent animal studies demonstrated that anti-VEGF treatment induces rapid alterations in blood capillaries. Within a few hours, EC proliferation is halted, luminal stability vanishes, and blood flow ceases in microvessels. Some ECs undergo apoptosis and disappear. Remaining capillaries lack endothelial fenestrations and have reduced VEGFR-2 and VEGFR-3 expression (reviewed in ref. [102]). Thus, inhibition of VEGF signaling devastates some tumor microvessels and transforms others into a more normal phenotype.

In vitro experiments have shown that a variety of human cancer cell lines express both VEGF and VEGFRs and that inhibition of VEGF–VEGFR pathways inhibited the proliferation of these cell lines. VEGF can, therefore, serve as an autocrine growth factor for cancer cells that express VEGFRs [103]. Along that line, human studies have demonstrated that in NSCLCs expressing VEGFRs, VEGF is a part of autocrine loops that enhance the malignant potential of NSCLC cells [104, 105]. A further mechanism for the additional benefits experienced with combined chemo- and antiangiogenic treatments might therefore be the direct cytotoxic effects of VEGF blockers on (lung) cancer cells that aberrantly express VEGF receptors and depend to some extent on VEGF for their survival.

Alternatively, because VEGF is a potent survival factor for activated ECs and, as demonstrated in experimental tumor models [106, 107], because cytotoxic drugs could kill proliferating ECs during capillary sprouting, antiangiogenic and chemotherapeutic drugs might also be synergistic against proliferating tumor ECs.

[10]Dowlati, A.; Gray, R.; Johnson, D. H.; Schiller, J. H.; Brahmer, J.; Sandler, A.B. Prospective correlative assessment of biomarkers in E4599 randomized phase II/III trial of carboplatin and paclitaxel + bevacizumab in advanced non-small cell lung cancer (NSCLC). *J. Clin. Oncol.* **2006**, ASCO Annual Meeting Proceedings Part I. Vol *24*, (Suppl. 18), Abstract 7027.

Given the well-known myelosuppressive side effects of cytotoxic agents and because bone marrow-derived EPCs are mobilized into the circulation by angiogenic cytokines such as VEGF [24], an additional, indirect way for chemotherapeutic and antiangiogenic treatment approaches to synergistically control vascularization of NSCLCs might be the impairment of the release and function of EPCs. This assumption is supported by recent studies in which we and others have found an association between circulating EPC numbers and clinical behavior of NSCLCs [42, 108].

Finally, a possible explanation for the synergistic effects of cytotoxic and angiosuppressive drugs on the tumor vasculature could also be the observation that antiangiogenic agents prevent cancer cell repopulation during the break periods between courses of chemotherapy [109].

However, recent experimental and clinical studies have suggested, the overall direct effect of combined chemo(radio)- and antivascular therapy on tumor blood supply and progression is not straightforward. For instance, although it is tempting to increase the dose of angiosuppressive drugs, doing so might transform the cancer capillaries to the point where cytotoxic drug perfusion is impaired. Examples of this can be seen in a phase II trial that tested the combination of leucovorin and 5-fluorouracil with two different doses of bevacizumab in metastatic colorectal cancer patients [110] and in a phase II study of vandetanib (100 or 300 mg/day) plus docetaxel in previously treated NSCLC [61]. In these two studies, the lower doses of antiangiogenic agents were more effective than the higher doses.

In the context of the above discussed vessel normalization theory, the combination strategy of multitargeted TKIs and chemotherapy could also be challenging in the clinical practice. It has been hypothesized that because PDGF receptors expressed by pericytes have an important role in the normalization process, blocking perivascular cell recruitment with TKIs might impede vessel normalization and consequently reduce the efficacy of simultaneous chemotherapy [111, 112]. This hypothesis may provide explanation for the clinical failure of certain multitargeted TKIs to augment the efficacy of chemotherapy. However, the advantages and disadvantages of targeting pericytes in addition to ECs with TKIs during chemotherapy remain to be elucidated.

A further problem is that VEGF is not the only angiogenic factor produced by human cancers; in contrast, during progression, tumors secrete concurrently many angiogenic factors: bFGF, TGF-ß1, PIGF, PDGF, and pleiotrophin [113]. Therefore, antiangiogenic therapy should be tailored depending on the angiogenic phenotype and expression of endothelial growth factors in each single tumor. In line with that, combinations of different antivascular agents to target multiple pathways simultaneously may lead to additional survival benefit for patients with progressive tumors with alternating angiogenic profiles. However, chances are the simultaneous inhibition of all key angiogenic pathways might interfere with the "vessel normalization" process (i.e. the activity of chemoradiotherapy), just as an overdose of an antiangiogenic agent with a single target results in the excessive pruning of the vessel network and thus impedes the delivery and efficacy of cytotoxic treatment.

Another important issue is how to cope with the problem of nonangiogenic NSCLC growth. Because, as discussed above, nonangiogenic tumors can obtain sufficient blood supply from pre-existing alveolar vasculature to grow without angiogenesis (i.e. endothelial proliferation) [21-23], this subtype of NSCLCs will probably be refractory to therapy with antiangiogenic drugs (angiosuppressive agents). Moreover, it can be speculated that this resistance will arise irrespective of the proportion of nonangiogenic parts in the tumor: nonangiogenic cell clones might be selected by angiosuppressive drugs. Therefore, when nonangiogenic growth plays a role in NSCLC progression, the use of VDAs against ECs of the pre-existing intratumoral vasculature seems to be an appropriate strategy.

SUMMARY

There are a number of antivascular agents progressing through clinical trial evaluation for the treatment of advanced NSCLC. However, clinical experience in this field is still limited and several questions remain to be answered. Most important are the problems of the optimal biologic dose and the best possible combination treatment strategy with chemotherapy. A further relevant clinical problem is to find the best techniques for determining efficacy of antivascular agents. In line with that, biomarkers including imaging techniques, peripheral blood counts of EPCs and plasma levels of different angiogenic cytokines have been tested preclinically and in clinical trials as markers of activity for these agents. Establishing the toxicity profile of novel antivascular drugs over long-term periods will also be essential. Like bevacizumab, many agents display the on-target effect of hypertension in their toxicity profile, although this is usually manageable with a standard management algorithm. However, unlike bevacizumab, the majority of these agents are orally available, and therefore more convenient, less likely to cause significant hemorrhage, and thus far have not been shown to be more hazardous in those with squamous histology NSCLC.

All in all, although a large body of preclinical evidence has confirmed angiogenesis as a key process of NSCLC progression and the results of ongoing trials of antivascular agents in NSCLC are eagerly awaited, it will likely be several years before their precise role is clarified in pulmonary oncology.

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ABBREVIATIONS

BM	=	bone marrow
CA4P	=	combrestatin A4 phosphate
CEC	=	circulating endothelial cell
CRP	=	C-reactive protein
CT	=	computed tomography
CTCAE	=	common terminology criteria for adverse events
DMXAA	=	5,6 dimethylxanthenone-4-acetic acid
EC	=	endothelial cell
ECOG	=	Eastern Cooperative Oncology Group
EGFR	=	epidermal growth factor receptor
EPC	=	endothelial progenitor cell
FDA	=	Food and Drug Administration
HGF	=	hepatocyte growth factor
IL-8	=	interleukin-8
MMPI	=	matrix metalloproteinase inhibitor
MMP	=	matrix metalloproteinases
MRI	=	magnetic resonance imaging
NO	=	nitric oxide
NSCLC	=	Non-small cell lung cancer
PDGFR	=	platelet derived growth factor receptor
PET	=	positron emission tomography
PFS	=	progression-free survival
PlGF	=	placental growth factor
SPECT	=	singlephoton emission computed tomography
TGF-ß1	=	transforming growth factor beta-1
TKI	=	Tyrosine kinase inhibitor
TNF-α	=	tumor necrosis factor-alpha
VDA	=	vascular-disrupting agent
VEGF	=	vascular endothelial growth factor
VEGFR	=	vascular endothelial growth factor receptor

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