Identification and Clinical Significance of Circulating Endothelial Progenitor Cells in Human Non–Small Cell Lung Cancer

Balazs Dome,[1,2,3] Jozsef Timar,[1,3] Judit Dobos,[1,3] Livia Meszaros,[3] Erzsebet Raso,[3] Sandor Paku,[4] Istvan Kenessey,[3] Gyula Ostoros,[2] Melinda Magyar,[1] Andrea Ladanyi,[3] Krisztina Bogos,[1,2] and Jozsef Tovari[3]

Departments of [1]Tumor Biology and [2]Thoracic Oncology, National Koranyi Institute of Pulmonology; [3]Tumor Progression, National Institute of Oncology; and [4]Department of Molecular Pathology, Joint Research Organization of the Hungarian Academy of Sciences and Semmelweis University, Budapest, Hungary

Abstract

Until recently, it was generally accepted that vascularization of tumors arises exclusively from endothelial sprouting. Whether circulating bone marrow–derived endothelial progenitor cells (EPC) participate in the progression of non–small cell lung cancer (NSCLC) has not yet been evaluated. EPCs labeled with CD34, CD133, and vascular endothelial growth factor receptor-2 (VEGFR2) antibodies were counted by flow cytometry in the peripheral blood of 53 NSCLC patients. Furthermore, by means of a quantitative reverse transcription-PCR approach, we measured VEGFR2, CD133, CD34, and VE-cadherin mRNA in the peripheral blood samples of the same patient population. EPCs in tumor samples were identified by confocal microscopy using CD31, CD34, CD133, and VEGFR2 antibodies. Although immunofluorescent labeling of microvessels made clear that incorporation of EPCs is a rare phenomenon in NSCLC tissue (9 of 22 cases), circulating EPC levels before therapeutic intervention were increased in NSCLC patients ($P < 0.002$, versus healthy controls), and high pretreatment circulating EPC numbers correlated with poor overall survival ($P < 0.001$). Furthermore, in the subgroup of responders to treatment, the posttreatment EPC numbers in the peripheral blood were significantly lower compared with nonresponding patients. Interestingly, pretreatment mRNA levels of CD133, VE-cadherin, and CD34 were not significantly increased in NSCLC patients, whereas VEGFR2 expression was increased by 80-fold. Moreover, posttreatment VEGFR2 mRNA level in the peripheral blood was significantly higher in the subgroup of nonresponding patients when compared with posttreatment level of patients responding to antitumor therapy. Circulating levels of bone marrow–derived EPCs are significantly increased in NSCLC patients and correlate with clinical behavior. (Cancer Res 2006; 66(14): 7341-7)

Introduction

Non–small cell lung cancer (NSCLC) accounts for >80% of all lung cancers and is responsible for more deaths from cancer than any other tumor type in the Western world (1). Despite surgical resection and the development of new chemotherapy regimens, many NSCLC patients relapse and become fatal (2). Consequently, treatment for NSCLC is now moving beyond conventional chemotherapy with the advent of molecular-targeted therapies, and a key therapeutic strategy is inhibition of specific cytokines essential for tumor vascularization (3, 4). Currently, angiogenesis quantification to assess and predict the efficacy of antiangiogenic drugs is mainly based on the evaluation of microvascular density. However, this procedure is highly invasive, and its association with the clinical outcome is uncertain in many tumor types, including NSCLC (5–7). Accordingly, clinical markers characterizing the angiogenic profile of a tumor and able to reflect the response to antiangiogenic drugs are still scanty (8).

Until recently, malignant tumors were thought to acquire their vasculature solely through angiogenesis, the mechanism by which new capillaries arise from preexisting ones (9). However, recent evidence suggests that tumor vasculature can also arise through vasculogenesis, a process by which bone marrow–derived endothelial precursor cells (EPC) are recruited and differentiate *in situ* into mature endothelial cells to form new blood vessels (10, 11). Moreover, recent studies have provided evidence that in addition to the physical contribution of EPCs to newly formed microvessels (12), the angiogenic cytokine release of EPCs may be a supportive mechanism to improve neovascularization (13, 14). Although these data suggest that there is a close interplay between EPCs and tumor vascularization, the exact role of these cells in the pathogenesis of NSCLC remains to be determined. Hence, we assessed the number of circulating EPCs by flow cytometry from the peripheral blood of NSCLC patients and studied the incidence and contribution of EPCs in the vasculature of surgically removed NSCLCs. Furthermore, we used real-time quantitative reverse transcription-PCR (RT-PCR) to study the expression of the EPC-specific markers CD34, vascular endothelial growth factor receptor-2 (VEGFR2), VE-cadherin, and CD133 in the peripheral blood of healthy controls and NSCLC patients before and after therapy.

Materials and Methods

Clinical data. To measure the number of circulating EPCs and the level of EPC specific genes at the time of diagnosis and following the appropriate anticancer therapy, peripheral blood was collected in EDTA tubes through 21-gauge needles in 53 NSCLC patients. Newly diagnosed NSCLC patients were free of additional malignant, inflammatory or ischemic disease, pulmonary fibrosis, wounds, or ulcers that might influence the number of EPCs (15). Accordingly, to avoid the direct effects of chemotherapy or surgical wound healing on EPC numbers, second blood samples were collected 21 days after the last cytotoxic infusion and 3 months after thoracotomy. There were 28 male and 25 female patients with a median age of 58 years (range, 45-67 years; Table 1). NSCLC cases were staged according to radiological and pathologic findings based on the American Joint Committee on Cancer/Unio Internationale Contra Cancrum tumor-node-metastasis classification (16).

Note: The authors declare that they have no competing financial interest concerning this article.

Requests for reprints: Balazs Dome, Department of Tumorbiology, National Institute of Pulmonology, Piheno. u. 1., Budapest H-1529, Hungary. Phone: 36-1-391-3210; Fax: 36-1-391-3223; E-mail: domeb@yahoo.com.

©2006 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-05-4654

Table 1. Correlation of clinicopathologic features and circulating EPC number in patients with NSCLC

All patients	No. patients (%)	EPC low* (%)	EPC high* (%)	*P*
	53	36	17	
Age (y) †				
<58	26 (49.1)	18 (50)	8 (47)	NS
≥58	27 (50.9)	18 (50)	9 (53)	
Smoking history				
Nonsmoker	19 (35.8)	13 (36.1)	6 (35.3)	NS
Current or ex smoker	34 (64.2)	23 (63.9)	11 (64.7)	
Gender				
Male	28 (52.8)	18 (50)	10 (58.8)	NS
Female	25 (47.2)	18 (50)	7 (41.2)	
Histologic type				
Squamous cell	23 (43.4)	15 (41.7)	8 (47.1)	NS
Adenocarcinoma	26 (49.1)	18 (50)	8 (47.1)	
Adenosquamous	4 (7.5)	3 (8.3)	1 (11.8)	
Pathologic stage				
I	17 (32.1)	10 (27.8)	7 (41.2)	NS
II	9 (17)	8 (22.2)	1 (5.9)	
III	22 (41.5)	15 (41.7)	7 (41.2)	
IV	5 (9.4)	3 (8.3)	2 (11.7)	
Therapy				
Chemotherapy	18 (34)	12 (33.3)	6 (35.3)	NS
Chemoradiotherapy	10 (18.9)	6 (16.7)	4 (23.5)	
Surgery	22 (41.5)	15 (41.7)	7 (41.2)	
Palliative therapy	3 (5.6)	3 (8.3)	0 (0)	

NOTE: Data shown in parentheses are column percentages.
Abbreviation: NS, not significant.
*Cutoff value between low and high pretreatment EPC levels was defined as 1,000 EPCs/mL of peripheral blood.
†Cutoff value is median value.

Control blood samples were also obtained from 14 healthy individuals. There were 23 squamous cell carcinomas, 26 adenocarcinomas, and 4 adenosquamous carcinomas (Table 1). Twenty-two patients underwent surgery. Twenty-eight patients with inoperable NSCLC received chemotherapy with gemcitabine and cisplatin, of whom 10 patients with locally advanced disease received radiotherapy in combination with chemotherapy. Informed consent was obtained from all patients and healthy volunteers, and the study was done with the approval of the Ethics Committee of the National Institute of Pulmonology, Hungary and in accordance with the ethical standards prescribed by the Helsinki Declaration of the World Medical Association. To investigate the contribution of EPCs in tumor vasculature, frozen tumor samples of 22 patients with surgically removable NSCLC were also included.

Identification of EPCs by confocal laser scanning microscopy in human NSCLC tissue. Immunofluorescent stainings were done on fresh frozen NSCLC samples; 10-μm sections were fixed in −20°C methanol for 10 minutes. After washing in PBS, the following primary antibodies were used: rabbit polyclonal anti-human CD31 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), FITC-conjugated anti-human CD34 (1:50; BD Biosciences, San Jose, CA), mouse anti-human VEGFR2 (R&D Systems, Minneapolis, MN), and biotin-conjugated anti-human CD133 (1:50; Miltenyi Biotec, Bergisch Gladbach, Germany). Normal mouse and rabbit IgGs were substituted for primary antibodies as negative control (same concentration as the test antibody). After washing in PBS, slices were incubated simultaneously with the appropriate secondary antibodies or streptavidin conjugates (FITC-conjugated goat anti-rabbit IgG, rhodamine-conjugated goat anti-mouse IgG, streptavidin-conjugated rhodamine, and streptavidin-conjugated Cy5; 1:100; all from Jackson ImmunoResearch, Inc., West Grove, PA) with or without nuclear staining with TOTO-3 (1:1,000; Molecular Probes, Eugene, OR). Morphometric analysis was done by counting the number of $CD133^+$ and $VEGFR2^+CD133^+$ double-positive cells in ten 1-mm^2 fields selected at random. Sections were examined using a Nikon Eclipse 80i microscope, and digital images were captured using either a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI) or the Bio-Rad MRC-1024 confocal laser-scanning microscopy system (Bio-Rad, Richmond, CA).

Enumeration of EPCs by flow cytometry from the peripheral blood of NSCLC patients. To quantify the content of circulating EPCs by fluorescence-activated cell sorting (FACS) analysis, a volume of 90 μL peripheral blood was incubated for 30 minutes at 4°C with PE-Cy5–conjugated anti-human CD34 (BD Biosciences) and PE-conjugated anti-human VEGFR2 (R&D Systems) or with biotin-conjugated anti-human CD133 (Miltenyi Biotec) and PE-conjugated anti-human VEGFR2. Biotin conjugated anti-CD133 was revealed using streptavidin-PE-Cy5 (BD Biosciences). Appropriate fluorochrome-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 number of cells per milliliter of blood using the CyFlow SL flow cytomoter and the FlowMax software (both from Partec, Munster, Germany).

Measuring the level of EPC markers by quantitative real-time RT-PCR in the peripheral blood of NSCLC patients. Peripheral blood was incubated for 10 minutes with Red Blood Cell Lysing Buffer (Sigma, Munich, Germany) and centrifuged for 20 seconds at full speed in a microcentrifuge. Total RNA was extracted from the cells remaining after lysis using Qiagen RNeasy Mini kit and digested with RNase-free DNase Set according to the

manufacturer's protocol; 3 μg of total RNA were reverse transcribed from each sample using deoxynucleotide triphosphates (0.5 mmol/L each), a mixture of random primer and oligo dT (final concentration = 3 μmol/L), RNasin RNase inhibitor (Promega, Madison, WI; 20 units/reaction), and reverse transcriptase buffer and MMLV reverse transcriptase (Sigma; 200 units/reaction). Samples (30 μL) were incubated for 50 minutes at 37°C and then at 85°C for 10 minutes. The sequences of AC133 primers (17) were 5′-TGGATGCAGAACTTGACAACGT-3′ and 5′-ATACCTGCTACGACAG-TCGTGGT-3′. The sequences of VEGFR2 primers (17) were 5′-CACCACT-CAAACGCTGACATGTA-3′ and 5′-GCTCGTTGGCGCACTCTT-3′. The sequences of VE-cadherin primers (17) were 5′-TTTCCAGCAGCCTTTC-TACCA-3′ and 5′-GGAAGAACTGGCCCTTGTCA-3′. The sequences of CD34 primers were 5′-TTGACAACAACGGTACTGCTAC-3′ and 5′-TGGTGAA-CACTGTGCTGATTAC-3′. The real-time PCR analysis was standardized by coamplifying the genes of interest with the housekeeping gene *β-actin* (primers, 5′-TCTGGCACCACACCTTCTAC-3′ and 5′-CTCCTTAATGTCACG-CACGATTTC-3′). The real-time PCR reaction was run on the iCycler iQ (Bio-Rad) using standard conditions [i.e., an optimized concentration of primers (final concentration = 200 nmol/L), 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 variables were 95°C (3 minutes), 50 cycles of 95°C (30 seconds), 64°C (30 seconds), and 72°C (1 minute). 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.

Statistical analysis. Categorical data were compared using Fisher's exact probability test. Continuous data 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. 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 6.0 (StatSoft, Inc., Tulsa, OK) software program.

Results

Characterization and numbers of EPCs in peripheral blood and tissue samples of NSCLC patients. Although to date no clear definition of EPC exists, based on recent studies (18–21) using flow cytometry, we determined the numbers of $CD34^+VEGFR2^+$ double-positive cells in the peripheral blood of NSCLC patients (Fig. 1*A*). Additionally, in 10 patients and 14 healthy controls, we measured the number of $VEGFR2^+CD133^+$ cells, corresponding to a subfraction of immature EPCs (18). However, because cell counts of $VEGFR2^+CD133^+$ and $CD34^+VEGFR2^+$ EPCs did not differ from each other significantly ($P > 0.1$ for all analyses; data not shown), in further experiments, levels of EPCs with the latter phenotype were evaluated, in accordance with previous studies (18–21). In healthy controls, the mean value of circulating EPCs was 345 ± 54.8/mL of peripheral blood (mean ± SE; $n = 14$; Fig. 1*B*). In NSCLC patients before anticancer treatment, the number of $CD34^+VEGFR2^+$ EPCs/mL of peripheral blood was significantly higher, with a mean value of 1,162.4 ± 242.4 (mean ± SE; $n = 53$; $P < 0.002$; Fig. 1*B*).

To characterize EPCs in the vasculature of NSCLC, we carried out immunostaining with the EPC-specific markers CD133 and VEGFR2 and the panvascular marker CD31 in serial sections of tumor tissues. Confocal microscopy revealed that CD31 antibody marks the vasculature intensively. Because numbers of $CD133^+$ and $CD133^+VEGFR2^+$ cells did not differ from each other significantly, EPC-positive and EPC-negative groups were established, based on CD133 labeling. Of 22 cases with surgically removed NSCLCs, 9 cases were positive for EPCs. No EPCs were observed in the normal lung tissue. In NSCLCs, EPCs were arrested mainly in small intratumoral capillaries, or, less frequently, were adhered to the endothelium of larger vessels or were located in the capillary walls

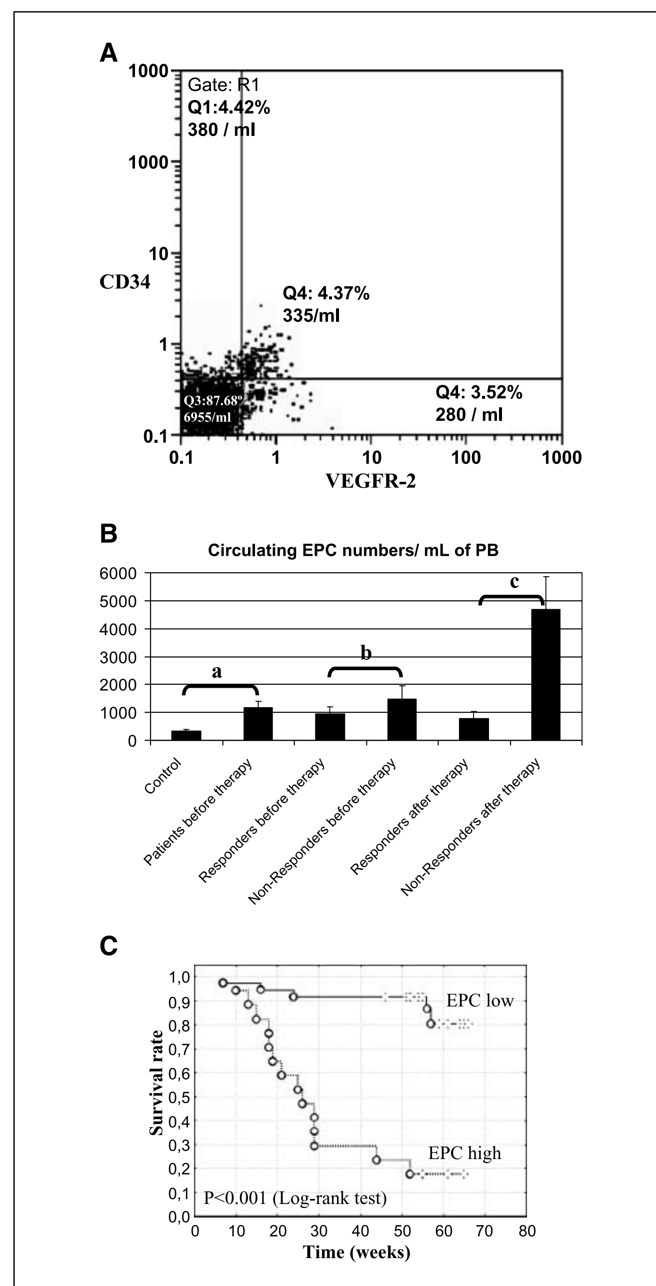

Figure 1. Quantitative evaluation of circulating EPCs by flow cytometric analysis. *A,* representative flow cytometric analysis for determining the number of CD34/VEGFR2 double-positive cells. Q1 = $CD34^+$, Q2 = $CD34^+/VEGFR2^+$, Q3 = $CD34^-/VEGFR2^-$, Q4 = $VEGFR2^+$ cells. *B,* circulating EPC levels in healthy controls and different categories of patients. *Columns,* mean EPC numbers/mL of peripheral blood; *bars,* SE. a, $P < 0.002$ (healthy controls versus all NSCLC patients before therapy); b, $P = 0.12$ (responders versus nonresponders to treatment before therapy); c, $P < 0.005$ (responders versus nonresponders to treatment after therapy). *C,* Kaplan-Meier curves for the overall survival of the entire patient population with NSCLC, according to pretreatment circulating EPC numbers as determined with CD34/VEGFR2 double labeling and flow cytometry. Cutoff value between low and high pretreatment EPC levels was defined as 1,000 EPCs/mL of peripheral blood.

(Fig. 2*A-B*). The mean number of EPCs within the tumor specimens of EPC-positive patients was 2.4 ± 1.1/mm^2 (mean ± SD; n = 9). However, there was no significant correlation between the presence or the number of EPCs identified in NSCLC vasculature and the circulating EPC levels as evaluated by FACS (data not shown).

Evaluation of EPC markers in blood samples of NSCLC patients by quantitative real-time RT-PCR. CD34, CD133, VE-cadherin, and VEGFR2 mRNA levels in healthy controls and in 53 therapy-naive NSCLC patients were determined by quantitative real-time RT-PCR (Fig. 3). Pretreatment levels of CD34, CD133, and VE-cadherin (Fig. 3*B-D*) were not significantly altered in NSCLC patients, whereas VEGFR2 expression was increased 80-fold (P < 0.05, versus healthy controls; Fig. 3*A*).

Correlation of circulating EPC numbers and EPC-specific mRNA levels with therapy. NSCLC patients were also evaluated after surgery or completing standard chemoradiotherapy. Independently of the type of therapy, nonresponding patients (patients with local recurrence or stable/progressive disease) tended to have higher pretreatment EPC numbers and VEGFR-2 and VE-cadherin mRNA levels than those who responded to therapy (patients who achieved a tumor-free status with surgery and patients with complete or partial response to chemotherapy or chemoradiotherapy). Despite this finding, there was no statistically significant difference between responders and nonresponders in the case of either pretreatment EPC numbers (Fig. 1*B*) or pretreatment EPC marker levels (Fig. 3). However, in the subgroup of responders to treatment, the mean number of posttreatment EPCs/mL of blood was 776.1 ± 265 (mean ± SE; n = 36), which was significantly lower than in nonresponder patients with a posttreatment value of 4,687.9 ± 1,178.6 (mean ± SE; n = 17; P < 0.005; Fig. 1*B*). It is also important to note, however, that although responder and nonresponder patients received the same therapy, circulating EPC numbers decreased in 74% of the responder population, whereas it increased in 93% of nonresponders during anticancer treatment in case of all treated patients (P < 0.001). Considering chemo-irradiated patients, a decrease in 89% versus increase in 88% of patients was found in responders and nonresponders, respectively (P < 0.001). These results suggest that the alterations in circulating EPC numbers were independent of the therapeutic intervention and were significantly associated with clinical behavior.

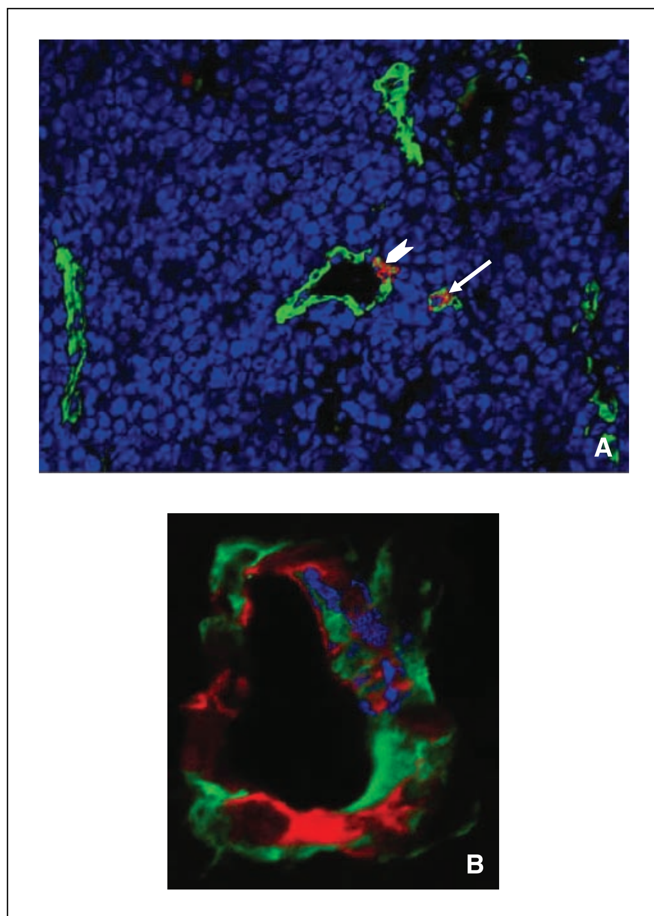

Figure 2. Identification of EPCs in human NSCLC tissue. *A,* CD133$^+$ EPCs (*red fluorescence*) were arrested mainly in small intratumoral capillaries (*arrow*) or were adhered to the endothelium (*green fluorescence*) of larger capillaries (*arrowhead*). Nuclear staining was applied with TOTO-3 iodide (*blue fluorescence*). *B,* less frequently, EPCs (CD133, *blue fluorescence*; VEGFR2, *red fluorescence*) were incorporated into the capillary walls (CD31, *green fluorescence*).

Moreover, posttreatment VEGFR2 mRNA level in the peripheral blood was significantly higher in the subgroup of nonresponding patients when compared with the level of patients responding to antitumor therapy (P < 0.05; Fig. 3*A*). On the contrary, mRNA levels of CD34, CD133, and VE-cadherin were not altered significantly either in pretreatment or in posttreatment samples of newly diagnosed or treated NSCLC patients compared with healthy controls or to each other (Fig. 3*B-D*).

EPC number as progression marker in NSCLC. Because 16 of the 53 NSCLC patients died within 30 weeks, and because the circulating EPC level of these patients at the time of diagnosis was significantly higher than in those who survived (2,206.8 ± 552 versus 735.6 ± 222.3; P < 0.02), we established pretreatment EPC cutoff values (500, 750, 1,000, and 1,250/mL of peripheral blood), which were tested for discriminating power in predicting disease outcome. This classification indicated that patients whose blood samples were categorized by a pretreatment EPC level < 1,000/mL (EPC low) had significantly longer survival times than those with high levels of circulating EPCs (median survival time, 55.5 versus 26 weeks; P < 0.001; Fig. 1*C*). However, no significant associations with age, gender, histologic type, smoking history, disease stage, or therapy were detected (Table 1). Multivariate analysis (including standard prognostic variables, such as tumor stage, smoking history, and histologic type) also indicated that pretreatment circulating EPC numbers predicted outcome independent of other variables (P < 0.001; Table 2).

Discussion

Although increased circulating EPC levels have been reported in various conditions associated with vascular diseases in humans (18–21), and several studies in different rodent models have shown that circulating EPCs derived from the bone marrow contribute to tumor vascularization (10, 11, 22), to date, only a few studies have attempted to evaluate the significance of EPCs in human tumor vascularization (23–25), and no articles have been reported on circulating EPCs in human NSCLC. Thus, we analyzed the level of circulating EPCs by flow cytometry; studied the expression of the EPC-specific markers CD34, VEGFR2, VE-cadherin, and CD133 by real-time quantitative RT-PCR from the peripheral blood of patients with NSCLC; and investigated the frequency and contribution of EPCs in the vasculature of surgically removed

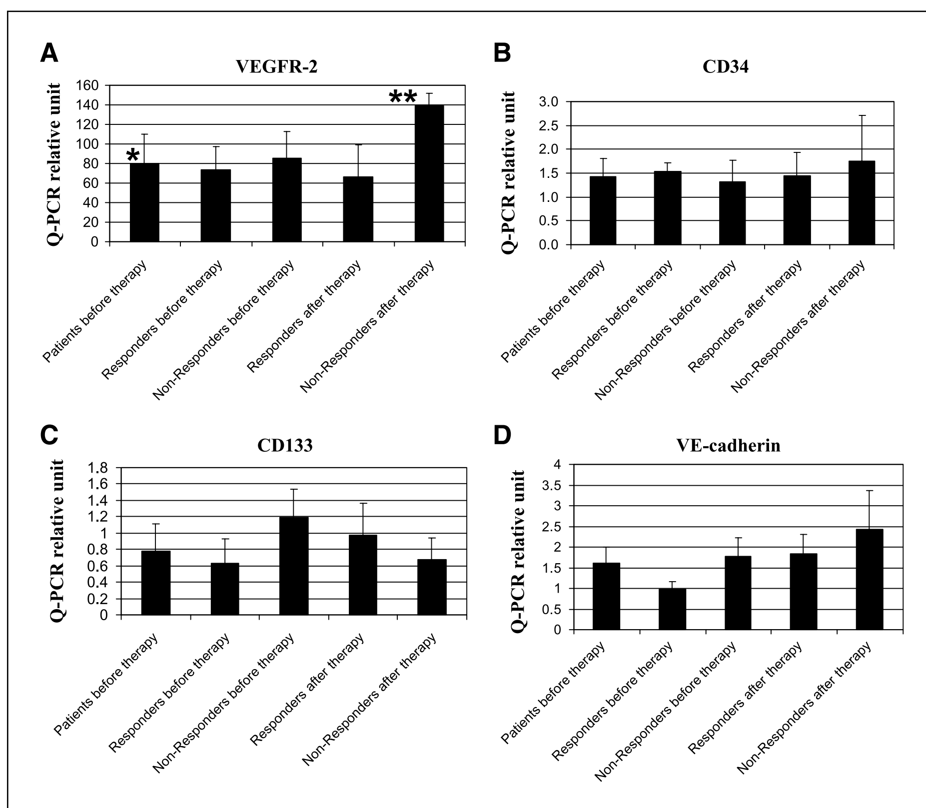

Figure 3. Relative quantification of VEGFR2 (*A*), CD34 (*B*), CD133 (*C*), and VE-C (*D*) mRNA in different categories of cancer patients. Relative to healthy controls. *Columns,* means; *bars,* SE. *, $P < 0.05$ (versus healthy controls); **, $P < 0.05$ (versus responders after therapy).

NSCLCs. To the best of our knowledge, this is the first article that shows evidence of an increased number of EPCs in the peripheral blood of patients with NSCLC.

The *VEGF* gene was cloned just over 15 years ago, and already, bevacizumab (a humanized monoclonal antibody against VEGF) has been approved as a first-line anticancer treatment (26). Despite this rapid progress from bench to bedside, our facilities to monitor tumor angiogenic status or the response to angiostatic agents have not improved at the same pace (27). Currently, the activity of angiogenesis and its influence on patients' prognosis or the efficacy of antiangiogenic drugs are measured by counting the microvascular density or by determining the pretreatment and posttreatment levels of angiogenic cytokines in the serum. Unfortunately, these approaches are highly invasive and not always reliable (5–7, 28). It is noteworthy, therefore, that the circulating EPC level was found to be a sensitive surrogate marker of the antiangiogenic activity of low-dose metronomic chemotherapy in a murine model (29), and that the numbers of circulating EPCs rapidly decline in rectal cancer patients receiving bevacizumab (30). Based on these data, in addition to EPC levels before therapy, we measured EPC numbers after the adequate anticancer treatment. Posttreatment levels of circulating EPCs proved to be significantly lower in patients who achieved a partial/complete remission (responders to treatment) than in patients with stable or progressive disease (nonresponders). Given the background described above, and because the addition of the novel antiangiogenic agent bevacizumab to the standard chemotherapy regimen significantly improves survival in NSCLC patients (31), the finding that EPC levels in NSCLC correlate to tumor burden may have particular importance in the future.

In addition to the observation of significantly higher pretreatment numbers of circulating EPCs in NSCLC patients compared with healthy controls, this prospective study presents the novel finding that a single measurement of $CD34^{+}VEGFR2^{+}$ EPCs by flow cytometry is a useful tool to predict outcomes in patients with NSCLC. Although it did not reach statistical significance, responders tended to have lower pretreatment EPC numbers than those who did not respond to therapy. More importantly, during the follow-up period of 15 months, a significantly higher incidence of death from NSCLC was observed in patients with high

Table 2. Multivariate analysis of various prognostic factors in patients with NSCLC

Prognostic factor	Relative risk (95% confidence interval)	*P*
Gender (female vs male)	0.719 (0.289-1.789)	0.478
Smoking history (nonsmoker vs smoker)	1.602 (0.576-4.453)	0.366
Histologic type (non-adenocarcinoma vs adenocarcinoma)	0.781 (0.367-1.665)	0.523
Stage (I-II vs III-IV)	1.277 (0.811-2.013)	0.291
Circulating pretreatment EPC number (low vs high)*	8.41 (3.333-26.610)	<0.001

*Cutoff value between low and high pretreatment EPC levels was defined as 1,000 EPCs/mL of peripheral blood.

pretreatment EPC levels compared with patients with low EPC levels, suggesting that the pretreatment levels of EPCs, detectable by flow cytometry in the peripheral blood, correlate with the clinical behavior of human NSCLC. This assumption corresponds to the "vessel normalization" hypothesis described by Jain et al. (32, 33) regarding the clinical effects of anti-VEGF therapy. Our data suggest that those patients with lower pretreatment EPC numbers, presumably having more "normal" tumor vessels, respond better, whereas those with higher EPC numbers (with tortuous intratumoral capillaries) do not respond well. Consequently, patients with high pretreatment EPC numbers could be treated with anti-VEGF therapy to lower EPCs (normalizing the vasculature) before chemotherapy, thus potentially improving therapeutic responses.

Although in a previous study on other cancer types the level of VE-cadherin mRNA in the peripheral blood was reported to be significantly correlated with tumor progression (34), in the current study, there were no obvious associations between CD34, CD133, or VE-cadherin mRNA levels and malignancy. However, we found that pretreatment VEGFR2 mRNA expression was increased in NSCLC patients compared with healthy controls, and moreover, that the posttreatment level of this marker was significantly higher in the subgroup of nonresponding patients when compared with patients responding to anticancer therapy. Possible reasons for the isolated VEGFR2 elevation may include variations in EPC marker expression intensity at the stage of development in which they were studied (i.e., in the peripheral circulation following release from the bone marrow but before homing at the tumor site). Nevertheless, the host (35) or tumor capillary (36) source for some of this VEGFR2 mRNA level increase cannot be excluded, because this cell surface receptor can be expressed on mature endothelial cells as well (37). However, because we failed to detect such a remarkable change in the mRNA level of VE-cadherin (expressed also on mature endothelial cells), it is more probable that the high level of VEGFR2 mRNA, measured from the peripheral blood, is the result of intravasated NSCLC cells positive for VEGFR-2 (36).

The association between pretreatment EPC levels and death from tumoral causes was independent of standard prognostic variables, such as tumor extension and hystologic type, and, more interestingly, of the presence of incorporated EPCs in NSCLC vasculature. In fact, the observed rate of EPCs in the tumor tissue of NSCLC patients was lower than anticipated from our flow cytometric results. Assuming that the major proportion of circulating EPCs reaches and incorporates into the tumor vasculature, the reasons for the discrepancy between circulating and tumor tissue EPC numbers are not completely clear. Because CD34 and VEGFR2 are expressed on EPCs and on mature endothelial cells lining the tumor vasculature, and because CD133 expression is continuously decreasing on the cell surface of circulating EPCs and lost once EPCs differentiate into more mature endothelial cells in the endothelial tube (38), it seems obvious that the rate of incorporated EPCs in NSCLC tissue is inevitably underestimated. However, it is difficult to conclude that vascularization in human NSCLC is exclusively the result of EPC incorporation into the preexisting endothelial tube, as recent studies using experimental tumor models have found (10, 11, 22). It is more likely that, as described in nontumorous ischemic tissue models (13, 14, 39), in addition to their physical contribution to the tumor vasculature, EPCs are able to induce endothelial sprouting through the paracrine release of angiogenic growth factors. This assumption, however, would need further experimental and clinical support.

In conclusion, vascularization is a key mechanism in NSCLC progression and is frequently used as a prognostic factor. Our study shows, for the first time, that the levels of bone marrow–derived EPCs are significantly increased in patients with NSCLC, and that these levels are correlated to tumor burden and to clinical behavior. Although our data suggest a participation of EPCs in tumor growth and vascularization in NSCLC patients, it is not clear yet whether EPCs are essential for these processes or what the relative contribution of EPCs is compared with that of *in situ* proliferating endothelial cells. Moreover, it has yet to be determined whether EPCs can be targeted to treat NSCLC, or alternatively their levels can be used as a surrogate marker to monitor the efficacy of standard or antiangiogenic therapies in NSCLC.

Acknowledgments

Received 12/29/2005; revised 4/16/2006; accepted 5/9/2006.

Grant support: Ministry of Education, Hungary grant NKFP1a-0024-05 and National Research Foundation grants OTKA-D048519, OTKA-F046501, OTKA-TS49887, GVOP-2004-3.2.1., and KFIIF 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Piroska Horvath for the excellent technical assistance.

References

1. Jemal A, Murray T, Ward E, et al. Cancer statistics, 2005. CA Cancer J Clin 2005;55:10–30.
2. Gridelli C, Rossi A, Maione P. Treatment of non-small-cell lung cancer: state of the art and development of new biologic agents. Oncogene 2003;22:6629–38.
3. Herbst RS, Onn A, Sandler A. Angiogenesis and lung cancer: prognostic and therapeutic implications. J Clin Oncol 2005;23:3243–56.
4. Sandler AB, Johnson DH, Herbst RS. Anti-vascular endothelial growth factor monoclonals in non-small cell lung cancer. Clin Cancer Res 2004;10:4258–62s.
5. Pastorino U, Andreola S, Tagliabue E, et al. Immunocytochemical markers in stage I lung cancer: relevance to prognosis. J Clin Oncol 1997;15:2858–65.
6. Apolinario RM, van der Valk P, de Jong JS, et al. Prognostic value of the expression of p53, bcl-2, and bax oncoproteins, and neovascularization in patients with radically resected non-small-cell lung cancer. J Clin Oncol 1997;15:2456–66.
7. Chandrachud LM, Pendleton N, Chisholm DM, et al. Relationship between vascularity, age and survival in non-small-cell lung cancer. Br J Cancer 1997;76:1367–75.
8. Vermeulen PB, Gasparini G, Fox SB, et al. Second international consensus on the methodology and criteria of valuation of angiogenesis quantification in solid human tumours. Eur J Cancer 2002;38:1564–79.
9. Timar J, Dome B, Fazekas K, et al. Angiogenesis-dependent diseases and angiogenesis therapy. Pathol Oncol Res 2001;7:85–94.
10. Davidoff AM, Ng CY, Brown P, et al. Bone marrow-derived cells contribute to tumor neovasculature and, when modified to express an angiogenesis inhibitor, can restrict tumor growth in mice. Clin Cancer Res 2001;7:2870–9.
11. Bolontrade MF, Zhou RR, Kleinerman ES. Vasculogenesis plays a role in the growth of Ewing's sarcoma *in vivo*. Clin Cancer Res 2002;8:3622–7.
12. Hilbe W, Dirnhofer S, Oberwasserlechner F, et al. CD133 positive endothelial progenitor cells contribute to the tumour vasculature in non-small cell lung cancer. J Clin Pathol 2004;57:965–9.
13. Urbich C, Aicher A, Heeschen C, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol 2005;39:733–42.
14. Yoon CH, Hur J, Park KW, et al. Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: the role of angiogenic cytokines and matrix metalloproteinases. Circulation 2005;112:1618–27.
15. Hristov M, Erl W, Weber PC. Endothelial progenitor cells: isolation and characterization. Trends Cardiovasc Med 2003;13:201–6.

16. Mountain FC. Revisions in the international system for staging lung cancer. Chest 1997;111:1710–7.
17. Sussman LK, Upalakalin JN, Roberts MJ, et al. Blood markers for vasculogenesis increase with tumor progression in patients with breast carcinoma. Cancer Biol Ther 2003;2:255–6.
18. Werner N, Kosiol S, Schiegl T, et al. Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med 2005;353:999–1007.
19. Fadini GP, Miorin M, Facco M, et al. Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. J Am Coll Cardiol 2005;45:1449–57.
20. Adams V, Lenk K, Linke A, et al. Increase of circulating endothelial progenitor cells in patients with coronary artery disease after exercise-induced ischemia. Arterioscler Thromb Vasc Biol 2004;24:684–90.
21. Laufs U, Werner N, Link A, et al. Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. Circulation 2004;109:220–6.
22. Natori T, Sata M, Washida M, et al. G-CSF stimulates angiogenesis and promotes tumor growth: potential contribution of bone marrow-derived endothelial progenitor cells. Biochem Biophys Res Commun 2002;297:1058–61.
23. Massa M, Rosti V, Ramajoli I, et al. Circulating $CD34^+$, $CD133^+$, and vascular endothelial growth factor receptor 2-positive endothelial progenitor cells in myelofibrosis with myeloid metaplasia. J Clin Oncol 2005;23:5688–95.
24. Cortelezzi A, Fracchiolla NS, Mazzeo LM, et al. Endothelial precursors and mature endothelial cells are increased in the peripheral blood of myelodysplastic syndromes. Leuk Lymphoma 2005;46:1345–51.
25. Peters BA, Diaz LA, Polyak K, et al. Contribution of bone marrow-derived endothelial cells to human tumor vasculature. Nat Med 2005;11:261–2.
26. Ellis LM. Bevacizumab. Nat Rev Drug Discov 2005;Suppl:S8–9.
27. Schneider M, Tjwa M, Carmeliet P. A surrogate marker to monitor angiogenesis at last. Cancer Cell 2005;7:3–4.
28. Miller JC, Pien HH, Sahani D, et al. Imaging angiogenesis: applications and potential for drug development. J Natl Cancer Inst 2005;97:172–87.
29. Shaked Y, Emmenegger U, Man S, et al. Optimal biologic dose of metronomic chemotherapy regimens is associated with maximum antiangiogenic activity. Blood 2005;106:3058–61.
30. Willett CG, Boucher Y, Duda DG, et al. Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients. J Clin Oncol 2005;23:8136–9.
31. Treat J. Incorporating novel agents with gemcitabine-based treatment of NSCLC. Lung Cancer 2005; 50S1:S8–9.
32. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 2005;307:58–62.
33. Tong RT, Boucher Y, Kozin SV, et al. Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer Res 2004;64:3731–6.
34. Rabascio C, Muratori E, Mancuso P, et al. Assessing tumor angiogenesis: increased circulating VE-cadherin RNA in patients with cancer indicates viability of circulating endothelial cells. Cancer Res 2004;64:4373–7.
35. Lin Y, Weisdorf DJ, Solovey A, et al. Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 2000;105:71–7.
36. Decaussin M, Sartelet H, Robert C, et al. Expression of vascular endothelial growth factor (VEGF) and its two receptors (VEGF-R1–1 and VEGF-R2–1/KDR) in non-small cell lung carcinomas (NSCLCs): correlation with angiogenesis and survival. J Pathol 1999;188: 369–77.
37. Tammela T, Enholm B, Alitalo K, Paavonen K. The biology of vascular endothelial growth factors. Cardiovasc Res 2005;65:550–63.
38. Rafii S, Lyden D, Benezra R, et al. Vascular and haematopoietic stem cells: novel targets for antiangiogenesis therapy? Nat Rev Cancer 2002;2:826–35.
39. O'Neill TJ IV, Wamhoff BR, Owens GK, et al. Mobilization of bone marrow-derived cells enhances the angiogenic response to hypoxia without transdifferentiation into endothelial cells. Circ Res 2005;97: 1027–35.