VEGFR2 expressing circulating (progenitor) cell populations in volunteers and cancer patients

Laura Vroling1, Yuana Yuana1, Gerrit Jan Schuurhuis2, Victor W. M. van Hinsbergh3, Chad Gundy4, Richard de Haas1, Hester van Cruijsen1, Epie Boven1, Klaas Hoekman1, Henk J. Broxterman1

Departments of 1Medical Oncology, 2Hematology, 3Physiology, 4Clinical Epidemiology and Biostatistics, VU University Medical Center, Amsterdam, The Netherlands

Summary
Circulating cells of several lineages are thought to participate in angiogenesis and tumor growth. Experimental studies in tumor-bearing mice have pointed to the potential importance of VEGF-responding circulating (endothelial) progenitor cells in tumor growth. We have studied circulating CD31- and/or CD34-positive cell populations with a low to moderate VEGFR2 expression in human volunteers and cancer patients. We recognized four cell populations, which were further characterized by their content of major hematopoetic progenitor, monocytic, endothelial and platelet markers. After establishing the test-retest stability of the measurements in nine patients, we determined the frequencies of the various cell populations in a group of 20 volunteers and 14 cancer patients. Two populations were markedly increased in cancer patients. Small CD45\textsuperscript{dim}/CD34\textsuperscript{bright}/VEGFR2\textsuperscript{+} cells amounted to 12 and 64 cells/ml (P<0.0001), respectively, and 246/ml and 578/ml VEGFR2\textsuperscript{+}/CD45\textsuperscript{bright} (/CD14\textsuperscript{+}) monocytic cells were present in controls and cancer patients, respectively (P=0.017). A third population of CD45\textsuperscript{dim}/CD34\textsuperscript{bright}/VEGFR2\textsuperscript{low} cells amounted to 25 and 30 cells/ml (P=0.38). Unexpectedly, a population of mainly anucleated CD45\textsuperscript{low}/CD34\textsuperscript{bright}/CD4\textsuperscript{+} cells was present in numbers of 9,076 and 16,697/ml (P=0.04) in volunteers and cancer patients, which contained a VEGFR2\textsuperscript{low} (compared to IgG isotype control) expressing population amounting to 1,142 and 1,642 cells/ml (P=0.12). This fourth population probably reflects large platelets. The role of the herein identified VEGFR2\textsuperscript{+} circulating cell populations deserves further investigation in cancer patients treated with VEGF(R)-targeted therapies. Quantification of such cell populations in the blood of tumor patients may be valuable to monitor the efficacy of anti-angiogenic treatment.

Keywords
Circulating progenitor cells, circulating endothelial cells, VEGFR2,KDR, CD31, peripheral blood, large platelets, cancer

Introduction
Adult bone marrow is a rich reservoir of stem cells and tissue-specific progenitor cells. Among these, a scarce population of cells known as endothelial progenitor cells (EPCs) exists, which can be mobilised into the circulation and accumulate in areas of neangiogenesis (reviewed in [1]). A supportive role of such so-called endothelial progenitor cells in angiogenesis has been postulated (2), but this issue is still controversial (3). First, it is still uncertain whether these cells actually participate in the formation of new vascular structures, or whether they only play an orchestrating role in angiogenesis (4). Second, the definition of the population of these bone marrow-derived cells with acquired endothelial properties is incomplete (5), and it is likely that various types of cells including vascular leukocytes (6), tie-2 containing monocytes (7), circulating –shed or damaged- endothelial cells (CECs) (8) and a scarce population of cells with real EPC characteristics (1) participate. As several studies pointed to the potential importance of enumerating EPCs/CECs in the blood of tumor patients to monitor vascular injury and the efficacy of anti-angiogenic treatment (9–14), a further definition of the specific cell populations with endothelial-like characteristics that circulate in man is desired to optimise such monitoring.
Bone marrow-derived EPCs are presently characterised by expression of CD34, and/or CD133 (AC133) and VEGFR2 (vascular endothelial growth factor receptor 2, also known as KDR or Flk-1) (1). These cells when mobilised to the peripheral blood compartment (CEPs) may then lose their expression of CD133 while upregulating mature endothelial markers (1). However, recent studies indicate that markers previously thought to be exclusively endothelial, such as VE-cadherin and tie-2, can also be encountered on specific subtypes of leukocytes, such as vascular leukocytes and tie-2 expressing monocytes (6, 7).

A second population of cells with an endothelial nature are CECs, which may be derived either from the existing peripheral vasculature or newly forming vessels. Mature endothelial cells derived from the peripheral vasculature express combinations of typical endothelial cell markers such as CD31, CD146 (S-endo or P1H12), CD34, von Willebrand factor (vWF) and CD144 (VE-Cadherin). It is likely that the number of CECs will increase after inducing damage to the vascular endothelium and by anti-angiogenesis treatment.

Based on recent animal studies, assessment of the kinetics of both mature VEGFR2+ CECs and VEGFR2+ CEPs has been proposed to monitor pharmacodynamic effects of anti-angiogenic therapy in cancer patients (9–11). Willett et al. have reported that treatment with the anti-VEGF antibody bevacizumab, decreased the number of viable CECs, defined as 7-AAD- /CD45hi/CD31hi cells in patients with rectal cancer (12, 13). In another study CECs, defined as CD45hi/CD31- /CD146- cells, were monitored in cancer patients receiving low-dose chemotherapy (14). In studying the characteristics of CECs and CEPs it is important that various characteristics are studied simultaneously in mutual connection (9). This has yet not been done for VEGFR2 in humans and in particular in cancer patients, in part because a suitable directly labeled anti-VEGFR2 antibody was not available until recently and in part, because there is no consensus on which cell populations should be studied.

In this study we determined the feasibility and reproducibility of concurrent enumeration of VEGFR2+ cell populations in the blood of cancer patients by four-color flow cytometry using a novel mouse anti-human VEGFR2 antibody labeled with phycoerythrin (PE) and allophycocyanin (APC) in combination with other CD markers to further define the lineage of the cells, such as CD45, CD31, CD34, CD41 and CD105. Four populations of cells were analyzed and quantified in volunteers and cancer patients.

Patients and methods

Reagents

FACS lysing solution was purchased from Becton-Dickinson (San Jose, CA, USA). CD45-FITC or CD45-perCP Cy5.5 (clone j.33), CD38-FITC were from Beckman-Coulter. CD34-APC (clone 8G12), CD31-PE, CD31-FITC (clone L133.1), CD3-FITC, CD14-FITC, CD19-FITC and CD61-FITC were from Becton-Dickinson. VEGFR2-PE and VEGFR2-APC (clone 89106), IgG1-PE and IgG1-APC (clone 11711) were from R&D systems (Minneapolis, MN, USA). CD146-PE (clone P1H12) was from BD or Chemicon (Temecula, CA, USA), CD105-PE (Clone SN6, Serotec), CD144-PE was from Pharmingen, CD13-PE (clone L138, BD), CD133 (clone AC133, Miltenyi Biotec). CD41a-FITC and CD42b-FITC were from DAKO (Glostrup, Denmark). In all experiments, appropriate combinations of IgG isotype were used to set analysis gates. Saponin was from Sigma Aldrich (St. Louis, MO, USA) and was used in a concentration of 0.1% to permeabilise cells, LDS-751 was from Extron chemicals. 7-aminoactinomycin D (7-AAD) from Pharmingen (San Diego, CA, USA) was used to gate-out dead cells and in general 98–100% of the cells were viable as defined by a negative 7-AAD staining, reflecting the good quality of these fresh blood samples. In addition 7-AAD was used after saponin permeabilisation to reveal nuclear staining. DAPI (4'-6-Diamidino-2-phenylindole) was from Vector Laboratories and was used as nuclear stain for sorted cells. FACS buffer was prepared from PBS supplemented with 0.1% BSA (Sigma, The Netherlands) and 0.05% sodium azide (Sigma). Antibody details are listed in Table 1.

VEGFR2-PE and -APC expression on human umbilical cord vein endothelial cell (HUVECs)

New batches of the VEGFR2 antibody labeled with PE or APC were always tested for positive staining on primary HUVEC cultures, because HUVECs have been suggested to have a similar VEGFR2 expression levels as EPCs (15). From a titration experiment, a concentration of 50 µg/ml for VEGFR2-PE and 25 µg/ml for VEGFR2-APC was shown to give optimal staining of HUVECs. The expression level expressed as MFI (mean fluorescence intensity) in a representative staining was 22 for VEGFR2-PE and 8.4 for VEGFR2-APC (Fig. 1). In addition the labeling of HUVECs with VEGFR2-PE and VEGFR2-APC was done after resuspension in whole blood, which showed a similar VEGFR2 expression (data not shown). Thus the VEGFR2 antibody seems to perform appropriately in a whole blood matrix. Subsequently, we tested the VEGFR2 antibody on blood samples mobilised with granulocyte-colony stimulating factor (G-CSF), which contains increased numbers of immature progenitor cells, including CEPs (1). In the mobilised blood we clearly detected increased VEGFR2+ subpopulations of cells in the CD34hi/CD133+ hematopoietic progenitor cell fraction (not shown).

Patients and volunteers

Patients visiting the hospital, but not receiving anti-cancer therapy, were selected for both studies according to a protocol approved by the local medical ethical committee. They were 18 years of age or older, had a confirmed histological and/or cytological diagnosis of advanced solid tumor and had adequate liver and bone marrow function. They had recovered from myelosuppression of prior treatment (at least 4 weeks interval). Exclusion criteria were use of recombinant human erythropoietin, significant cardiovascular co-morbidities and any active infection or inflammatory disease. The patients for the test-retest stability study had six different tumor types (3 renal cell, 2 pancreas, 2 rectum, 1 endometrium, 1 breast and 1 prostate cancer), they had a mean age of 64 (range 46–72) years, four were female and six male, and blood was collected on two days in the morning from these patients, who were selected based on the protocol criteria and availability on two separate days in one week. The samples from one patient were excluded from the analysis because the
total white blood cell count of this patient had decreased from 7.5 x 10^6 to 3.7 x 10^6 cells/ml within two days for unknown reasons. The 14 patients in the second study had a mean age of 63 years (range 52–76), four were females and ten males, and they had nine different tumor types (3 rectum, 2 colon, 2 stomach, 2 prostate, 2 non-small cell lung cancer, 1 bladder, 1 hypopharynx and 1 unknown primary. The 20 volunteers had a mean age of 37 years (range 21–57). Seven were females and 13 males.

### Identification of VEGFR2-expressing cell populations by flow cytometry

In order to first determine the reproducibility of the measurements of these cell populations in the blood of cancer patients, 7 ml of blood was withdrawn in heparinised tubes on two separate days within the same week (second sample 2 or 3 days later). The first 2 ml of blood was discarded. Blood cell count was done with a Sysmex K-4500 (Sysmex corporation, Japan), which was

<table>
<thead>
<tr>
<th>Population identified</th>
<th>Cluster differentiation (CD)/reagents</th>
<th>Antibody clone</th>
<th>Manufacturer</th>
<th>Conjugation</th>
<th>Concentration</th>
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<tr>
<td>T-lymphocyte</td>
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<td>N/A</td>
<td>N/A</td>
<td>PBS+ 0.1% BSA+0.05% sodium azide</td>
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**Table 1: Reagents and target antigens; related cluster of differentiation (CD), antibody clone, manufacturer, conjugation of MoAb and concentration used in the study.**

BD; BD Biosciences, DAKO; DAKO-Cytomation, BC; Beckman-Coulter, MB; Miltenyi Biotec, R&D; R&D systems, VL; Vector Laboratories. All MoAb’s are mouse IgG1 isotype except CD3, which was IgG2a and CD14 which was IgG2b. N/A; Not applicable.
used to calculate the numbers of cells in a certain population per ml of blood, and then the blood was directly incubated with the antibodies. All processing and labeling was done within 6 hours (h) (often within 2 h) after drawing the blood, and all incubations were done in triplicate. For the immunophenotyping, 100–400 µl of blood was incubated for 30 minutes (min) at room temperature with one of the following antibody combinations: Combination one for CD45 /CD34-based analysis: CD45-FITC (1:20 diluted), CD34-APC (1:50 diluted), VEGFR2-PE (1:5 diluted) and 7-AAD (20 µl/10⁶ cells). Either of two combinations was used for CD45 /CD31-based analysis: CD45-FITC (1:20 diluted) with VEGFR2-APC (1:5 diluted), CD31-PE (1:20 diluted) and 7-AAD (20 µl/10⁶ cells) or CD45-PerCP-Cy5 (1:20 diluted) with VEGFR2-APC (1:5 diluted), CD31-FITC (1:20 diluted) and CD146-PE (1:200 diluted). Thereafter, red blood cells were lysed for 10 min at room temperature with 3–4 ml FACS lysing solution (1x concentration) and the cells were washed two times in FACS buffer and centrifuged at 1,500 rpm for 5 min. The cells were resuspended in 300–500 µl ice-cold FACS buffer and kept cold until analysis.

**Statistical comparisons for the test-retest study**

Table 2 shows the combined stability (test-retest over days) and equivalence (internal consistency over replicates) scores for two VEGFR²⁺ cell populations, which is calculated as the generalizability score Φ (see Results below and Fig. 2 for detailed gating strategy). Φ was highest (> 0.9) for the CD45bright CD34bright, and > 0.75 for the CD45low/CD31bright cell population. The very rare VEGFR²⁺/CD45dim CD34bright cell population was acquired in a too low number of events. Still the internal consistency (over triplicates) was high, but the stability (over days) was low. Therefore, the number of patients in which accurate monitoring of the VEGFR²⁺/CD45dim CD34bright is possible will be determined by the blood volume analyzed. Still, the assessment of these values, including those cases with VEGFR²⁺/CD45dim CD34bright close to zero /ml may be very useful (see Discussion).

After showing in principle the feasibility of measuring consistently several VEGFR²⁺ cell populations in full blood in cancer patients, we set out to quantitate the cell populations in a group of 20 volunteers and 14 cancer patients. In this study we also included two additional low-frequency populations, noted in the first study, namely CD45neg/CD34bright/VEGFR²⁺ cells and CD45bright/VEGFR²⁺ cells with FSC/SSC characteristics of monocytes. In addition, the cell populations were further characterised by measuring additional CD markers. In this study EDTA blood was used after it had been checked in several samples that no differences were found with heparinised blood.
Flow cytometry

Samples were measured on a FACS Calibur (Becton Dickinson) equipped with a 488 nm Argon laser and a 635 nm red diode laser and about $1 \times 10^6$ events (or about $2 \times 10^6$ for rare cell populations) were acquired per sample. Data acquisition and analysis were performed using Cell Quest software.

Sorting of the CD45$^{low}$/CD31$^{bright}$ population was done on a FACS Aria after labeling of 2 ml of blood as described above. Cytospins were prepared from flow-sorted cells and stained with the nuclear stain DAPI.

Statistical analysis

The variance components for the cell populations of interest were computed by maximum likelihood methods, and, in order to study the reproducibility of the processing and gating procedure, the equivalence (over triplicates) and the test-retest stability (over days) reliability coefficients were simultaneously calculated using the generalizability theory of Brennan (16). This results in a generalisability score ($\Phi$), reflecting the proportion of the total variance in the measurements due to differences between patients. For the comparison of the cell populations between cancer patients and healthy volunteers a Mann-Whitney U test (SPSS software) was performed.
Results

Gating strategy of VEGFR2-positive cell populations

To study the CD31− and/or CD34-positive VEGFR2 populations in the blood of healthy donors and cancer patients we performed for each sample two parallel four-color flow cytometric cell characterizations. In both samples CD45, VEGFR2–antibodies and viability marker 7-AAD were present. The initial gating strategy (Fig. 2A-C) was identical for both samples in order to include the CECs as defined by Mancuso et al. (14, 17). It was based on gating of viable cells using FSC and SSC (Fig. 2A) in combination with the degree of expression of the hematopoietic marker CD45 (Fig. 2B). The initial broad gate was chosen to include CD45neg, CD45low and CD45dim cell populations, and was further refined based on FSC/ SSC (population R3 in Fig. 2C). The CD31 and/or CD34 positive VEGFR2-positive cells were analysed from R3 (Fig. 2C). In addition to these cells, VEGFR2-positive monocytes were recognised in the blood (see below).

CD45min CD34bright /VEGFR2 positive cells

Further analysis of the tube based on CD34/CD45 showed a small population of CD45min CD34bright /VEGFR2 positive cells (Fig. 2D1) as part of the CD45dim CD34bright population (R4 in Fig. 2D). These cells were viable as shown by 7-AAD exclusion. In addition VEGFR2-positive cells were found in the CD45neg R5 population (as discussed below; see Fig. 6) and in the CD45dim R6 population. The latter CD45dim cells were further analyzed in the parallel tube, as these VEGFR2 positive cells appeared CD45low/CD31bright cells.

SSC low/FSC low-to-intermediate / CD45low / CD31 bright cells

In further analyzing the parallel tube based on CD31/CD45, we focused on the combination of CD45low with CD31 bright cells in accordance with Willett et al. (12). Indeed, using the appropriate IgG controls as cut-off level, the CD45low/CD31 bright cell population (R6 in Fig. 2E) showed a low level of VEGFR2 expression (Fig. 2E1). Moreover, these cells partially overlap with the CD45low/CD45dim population (R6 in Fig. 2D), as can be inferred from their similar SSC/FSC and CD45 staining. These cells as well as the VEGFR2neg/CD31bright cells (R9 in Fig. 2E1) were apparently viable as revealed by negative staining for 7-AAD (Fig. 2E3, 2E4), which was, however, analysed in more detail (see below). When CD146 was assayed in additional parallel tubes, the CD146 expression in the CD45low/CD31bright cell population was usually low or absent, and always much lower than that of control HUVECs.

Nature of the SSClow/FSClow-to-intermediate / CD45low / CD31bright cells

When the CD45dim/CD31bright cells were isolated by flow-sorting and stained on glass-slides with the nuclear staining agent DAPI, we observed by microscopy small CD31-positive cellular elements, but no nuclear staining, reminiscent to platelets (Fig. 3). The presence of nuclei was quantitatively analyzed by FACS analysis using LDS-751 and 7-AAD as nuclear staining and saponin to permeabilise the cells. While lymphocytes, granulocytes and monocytes clearly accumulated the dye 7-AAD after permeabilization, only few or none of the CD45low/CD31bright cells became positive for 7-AAD after permeabilization (Fig. 4, lower part). The number CD45low/CD31bright events in this gate remaining 7-AAD negative after cellular permeabilisation was 97.5 ± 2.4% (mean ± SD of N = 3), indicating that very few nucleated cells are present in this population. The LDS-751 analyses showed positivity in CD45low/CD31bright cells (Fig. 4, upper part), but this staining was consistently lower than in the typically nucleated cell populations.

To verify whether these anuclear elements reflect big platelets, the CD45low/CD31bright cells were further analyzed by FACS. As shown for several markers in Figure 5, the cells were CD13neg/low (myeloid marker), CD3neg/low and CD19neg/low (lymphocyte markers) and CD105neg (mesenchymal/endothelial marker). In addition they were CD133neg (progenitor marker) and differentiation marker CD38neg. Importantly, they were CD41a/CD61pos (megakaryocyte lineage markers) and platelet marker CD42bpos (glycoprotein Ibα) and CD144negative and therefore most likely are “large platelets” in accordance with recent data of Strijbos et al. (40).

CD45neg/CD34bright /VEGFR2+ cells

In addition to the CD45dim/CD34bright cells, a second population of CD34bright cells, clearly distinct from the first population,
based on the CD45<sup>neg</sup> marker (R5 in Fig. 2D) was consistently observed. We further analyzed this small population with additional markers and found that the majority of cells in this population (75%-100%) were positive for the markers VEGFR2, CD146 and the mesenchymal marker / activated endothelial marker CD105 (Fig. 6) and CD31 (not shown), but were negative for the myeloid marker CD13 (Fig. 6) and the progenitor marker CD133 (not shown).

**CD45<sup>bright</sup>/VEGFR2<sup>+</sup> cells**

A fourth VEGFR2-positive population was identified in the CD45<sup>bright</sup> monocytic cell fraction (Fig. 7). In order to confirm that this cell fraction belonged to the monocytes, we analyzed the blood of five individuals, and compared the VEGFR2<sup>+</sup>/CD45 gated population with the VEGFR2<sup>+</sup>/CD14 population with all antibodies in the same sample. Indeed, we found an excellent agreement using either of both markers. A mean of 359,671 and 347,780 CD45<sup>bright</sup> and CD14-positive cells/ml were found, respectively, and the VEGFR2<sup>+</sup> subpopulation did not differ significantly between either gates (P=0.89; paired t-test) measured in this population (Fig. 7).

**Comparison of VEGFR2-expressing subpopulations of cells in cancer patients and volunteers**

We subsequently compared the four VEGFR2-expressing subpopulations in healthy volunteers and cancer patients (Table 3). The population of CD45<sup>neg</sup> / CD34<sup>bright</sup> was significantly higher in the group of cancer patients (mean of 82 vs. 35; P=0.001) with a significant difference in the CD45<sup>neg</sup>/CD34<sup>bright</sup>/VEGFR2<sup>+</sup> subpopulations (12 vs. 64/ml; P < 00001). The CD45<sup>dim</sup> / CD34<sup>bright</sup> cells showed a not-significant trend towards a higher level in cancer patients (1801 vs. 3813; P=0.88) and the CD45<sup>dim</sup>/

Figure 6: Additional marker expression in CD45\textsuperscript{-}/CD31\textsuperscript{bright} cells. CD45\textsuperscript{-}/CD34\textsuperscript{bright} cells staining for VEGFR2, CD146, CD105 and CD13.

Discussion

In this study we describe a flow cytometric procedure for the quantitative measurement of circulating cells populations expressing the endothelial marker VEGFR2\textsuperscript{+} in the peripheral blood of volunteers and patients with advanced solid tumors. We defined four populations of VEGFR2\textsuperscript{+}-expressing cells, which may be of interest in the monitoring of clinical studies of VEGF-targeted therapy.

The rationale for choosing an approach which quantifies the numbers of circulating VEGFR2\textsuperscript{+} cells was three-fold. First, the anti-VEGF antibody bevacizumab (20) and VEGF tyrosine kinase inhibitors, like SU11248 (21), are among the most successful anti-angiogenic agents with other classes of anti-vascular drugs having less antitumor effect (22–24). Therefore it is of clinical interest to monitor cells, which express the target receptor. Second, recent preclinical studies have provided compelling evidence for a role of VEGFR2\textsuperscript{+}-circulating cell populations in tumor angiogenesis and in particular for their use as biological marker to monitor effects of anti-angiogenic therapies (9, 10, 20, 25). Third, recent studies have provided evidence that the pro-angiogenic effects of blood monocytic cells may be limited to VEGFR2\textsuperscript{+} subpopulations of monocytes (26–28). In our study, the cell populations were defined by combinations of CD45\textsuperscript{neg}, low or dim, CD34\textsuperscript{dim} or bright, CD31\textsuperscript{bright}, CD146\textsuperscript{low/moderate} and VEGFR2\textsuperscript{low/moderate} expression and a first estimation of the numbers of these subpop-

Since the mean age of the group of cancer patients in this study was higher than that of the healthy volunteers (63 vs. 37 years) we performed a subgroup analysis of the healthy volunteers by making two more homogeneous groups, one of the six oldest subjects and the second group consisting of the 14 others: the ages of these groups were 55 ± 2 (range 51–57) and 30 ± 6 (21–42), respectively. We compared all the cell populations in both groups. There were no significant differences or a trend towards a lower value in the older subgroup (for VEGFR2\textsuperscript{+} monocytes and CD45\textsuperscript{neg}/CD31\textsuperscript{bright} cells). Therefore it seems highly unlikely that any differences in frequencies of cell populations between the (older-aged) cancer patients and volunteers, which are higher in the patient group (see Table 3), would be age-related.
Table 3: Frequencies of VEGFR2 expressing cell populations in the blood of healthy volunteers and cancer patients.

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Subpopulation</th>
<th>Volunteers Cells/ml blood</th>
<th>Cancer patients Cells/ml blood</th>
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<td>WBC</td>
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<td>CD45&lt;sup&gt;dim&lt;/sup&gt;/CD34&lt;sup&gt;bright&lt;/sup&gt;</td>
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<td>1801 ± 1060</td>
<td>3813 ± 7349</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>/VEGFR2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>25 ± 22</td>
<td>30 ± 44</td>
<td>0.38</td>
</tr>
<tr>
<td>CD45&lt;sup&gt;dim&lt;/sup&gt;/CD34&lt;sup&gt;bright&lt;/sup&gt;</td>
<td>total</td>
<td>35 ± 23</td>
<td>82 ± 40</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>/VEGFR2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>12 ± 12</td>
<td>64 ± 39</td>
<td>0.0001</td>
</tr>
<tr>
<td>CD45 monocytes</td>
<td>total</td>
<td>3.6 ± 0.9x10^5</td>
<td>4.9 ± 1.6x10^5</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>/VEGFR2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>246 ± 226</td>
<td>578 ± 534</td>
<td>0.017</td>
</tr>
<tr>
<td>CD45&lt;sup&gt;low&lt;/sup&gt;/CD31&lt;sup&gt;bright&lt;/sup&gt;</td>
<td>total</td>
<td>9076 ± 3654</td>
<td>16697 ± 12551</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>/VEGFR2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1142 ± 815</td>
<td>1642 ± 924</td>
<td>0.12</td>
</tr>
<tr>
<td>CD45&lt;sup&gt;neg&lt;/sup&gt;/CD31&lt;sup&gt;bright&lt;/sup&gt;</td>
<td>total</td>
<td>9775 ± 5005</td>
<td>14362 ± 10566</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>/VEGFR2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1011 ± 892</td>
<td>1275 ± 711</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Data are from 14 cancer patients and 20 healthy volunteers. Statistics and cell analysis occurred by flow cytometry as described in the materials and methods section. CD45<sup>low</sup>/CD34<sup>neg</sup> represent hematopoietic progenitor cells. CD45<sup>low</sup>/CD34<sup>bright</sup> and CD34<sup>neg</sup>/CD45<sup>bright</sup> are from the analysis with CD45-PerCP-Cy5. Both populations are qualitatively and quantitatively comparable and reflect a population of cells with a low VEGFR2 expression (based on IgG isotype gating as negative control), which were identified as "large platelets", in agreement with recent observations by Strijbos et al. (40).

The most frequent population expressing a combination of the selected markers was designated here as SSC<sup>low</sup>/FSC<sup>low-intermediate</sup> / CD45<sup>low</sup> / CD31<sup>bright</sup> cells. The most frequent population expressing a combination of the selected markers was designated here as SSC<sup>low</sup>/FSC<sup>low-intermediate</sup> / CD45<sup>low</sup> / CD31<sup>bright</sup> cells. Based on scatter properties and marker expression this population does not represent lymphocyte or monocyte subpopulations, but most closely corresponds to the CECs as reported by Willett et al. (12, 13), which correlated with anti-tumor effects of bevacizumab co-administered with 5-fluorouracil and radiation therapy in rectal carcinoma patients and with the population of cells described by Mancuso et al. (17), who reported a frequency of 15.8 CECs/µl in breast cancer patients. In those studies no VEGFR2 expression was reported.

The mean frequency of this cell population in our two study groups is in the order of 10,000 cells/ml peripheral blood (see Tables 2 and 3) with an inter-individual range of as low as 155 up to more than 25,000 cells/ml.

What can be concluded, however, for a number of reasons, is that it seems unlikely that a cell population with this frequency represents bona fide CECs, which are shed from damaged (tumor) vessel walls. Foremost are the data from a very recent study by Strijbos et al. (40), who performed an electron microscopic study on a population of cells with very similar scatter and marker characteristics and reported that these cells were anucleated and had no endothelial cell specific Weibel-Palade bodies. In addition, the expression of the endothelial markers CD146 (P1H12) in this population is low or absent (40) and does not seem to be of help in defining ECs, even within the CD31<sup>bright</sup> subpopulation. Of note, a subset of activated T cells with prominent CD146 expression could also be also identified by us as shown before by others (29, 30), indicating that the antibody did work.

In addition, numerous studies using anti-CD146-coupled paramagnetic bead isolation for CEC identification suggest that ECs are present in frequencies of less than 10–100 /ml of peripheral blood in healthy volunteers with values up to several hundreds per ml at most in several vascular diseases (29–36). Although the bead isolation technique does not allow an easy and straightforward identification and accurate quantification of CECs, it likely identifies CECs, which originate from vascular damage (see recent review by Woywodt et al. [37]).

Finally, following the calculations by Segal et al. (38) a number of 15 CECs/ml shed acutely from damaged vessels in acute myocardial infarction would mean about 70,000 CECs (in 4.7 liters of blood) reflecting a minimum of 3 cm² vessel surface released, which is already far larger than the expected surface of such an acute injury. Then, although no values for the half-life of shed ECs in the circulation are known, it seems highly unlikely that a 1,000-fold higher steady-state number of cells, in the order of 10–15 /µl of PB will reflect cells that are shed from damaged vasculature, even from extensive, instable tumor vasculature.

In conclusion, the CD45<sup>low</sup>/CD31<sup>bright</sup> cell population as identified here as well as similar populations in other studies (10, 12, 17, 39) are unlikely mature CECs shed from (tumor) vasculature and are more likely to contain in majority a population of cells consistent with “large platelets”, because of the presence of the CD41a/CD61 marker [40] and this study. A low VEGFR2 expression would be consistent with earlier data, suggesting the presence of functionally active VEGF receptors on human platelets (41). Also, a low re-expression of VEGFR2 in megakaryocytic cells cultured in vitro for 12 days from CD34+ hematopoietic progenitor cells has been described (42). Whether this low VEGFR2 expression in cells of megakaryocytic lineage has a biological function is unknown.
CD45neg/CD34bright/VEGFR2+ cells
Despite the intense interest in the role of CECs in human disease and possible implications for treatment international consensus definitions and analytical procedures are not yet available. Given the fact that this is now recognised widely to hamper further progress in the clinical development and implementation of CEC analysis attempts to formulate unifying definitions such as those by Woywodt et al. (37) are a necessary first step. A particularly insightful step forward might be the comparison of paramagnetic bead technology with flow cytometry-based analysis of CECs (36). In our present study we have consistently identified a population of CD45neg/CD34bright CD31bright CD146low/moderate and VEGFR2+ cells, clearly different from the CD45dim/CD34bright cells, because of complete absence of CD45, lack of CD133 expression and by their low frequency (Fig. 6). A very similar CD133+/CD45low/moderate population referred to as CECs has been recently reported by flow cytometric analysis (43, 44). The frequency of this population as found by us is in the order of what is generally reported for CECs (a mean of 35 in volunteers and 82 in cancer patients; see Table 2). Yet, because of their small size based on SSC/FSC, these cells may not represent the same heterogeneous population of CECs as defined by bead isolation (29–32, 44) and might be better referred to as “small size EC-like cells”. In addition, we also further search with multicolor flow cytometry for other very low frequency populations with heterogeneous light scatter and EC markers combinations, such as CD31bright CD146+ or CD105+, which previously have gone undetected by flow cytometry (30, 37), but were reported very recently by Farace et al. (45) as detected by flow cytometry in healthy subjects in a range of 0–15/ml and in metastatic cancer patients of 0–179/ml of blood.

CD45dim/CD34bright/VEGFR2+ cells
The enumeration of CD34bright progenitor (with or without CD133+) VEGFR2+ cells can be done in a flow-cytometrically well-defined CD45dim/CD34bright gating procedure. For a good separation from other populations, expressing CD45low/CD34+ (see e.g. R6 in Fig. 2D), the use of CD34-APC is an important advantage. In our study, a frequency of CD45dim/CD34bright/ VEGFR2+ in the order of 20 (0–100) cells/ml blood was found, consistent with a published value for this rare progenitor population in the order of 0.0002% of mononuclear cells (about 20/ ml) (5) and allowing to calculate the volume of blood that has to be analysed to obtain meaningful results for the monitoring of therapy effects in cancer patients. However, by itself, the fact that certain patients may have very low numbers of VEGFR2+ progenitor cells (i.e. undetectable in even a few ml of blood) might be very informative or predictive for anti-tumor effects (9). Data on the possible role this subpopulation in anti-angiogenic cancer therapies in human cancer patients are still to be awaited (11, 20).

Little is known yet about the cell biology of circulating endothelial progenitors, but recently the identification of ECs with varying proliferative potential originating from blood as well as mature endothelial cell sources has led to a novel model incorporating several subpopulations of ECs with low proliferative and high proliferative capacity (5). Further studies will be necessary to determine the relationship of cell populations, that we and others have identified with populations of cells described in terms of proliferative and/or angiogenic capacity.

VEGFR2+ monocytes
Circulating cells with angiogenic properties, originating from myeloid lineages, have been implicated in adult angiogenic processes in many studies during the last few years (e.g. [26–28, 47]). In this study we recognised a VEGFR2+ subpopulation of (CD45+/CD14+) monocytes. The combination of CD45+/CD14+/VEGFR2 seems to be the best suited in cases where the study of this population is an important goal. Since in particular VEGFR2+ monocytes have been suggested to have pro-angiogenic properties (26), it will be interesting to sort this population of cells from the blood of selected cancer patients and study them in a functional way.

In summary, we demonstrate here the accurate monitoring of populations of circulating cells with a low/moderate VEGFR2 expression in cancer patients. This analysis shows the versatility and power of flow cytometry which allows the use of multiple markers. Thus, while flow cytometry with its increasingly versatile and improved instruments available has increasing potential to analyse multiple small cell subsets, we realise that there is too early for routine implementation of the current protocol. Multilaboratory standardization studies will have to be performed in order to define optimal parameters, such as blood handling, antibody clones, concentrations and gating procedures. For the detection of very-low-frequency cell populations also a combination with immunobead-enriched methodology (46, 47) may be a useful option. Addition of multiple markers (e.g. CD133, CD14, CD105) to the protocol, in particular when the analysis is done on a flow cytometer allowing 6– or more color analysis also seems to be a useful future option. The simultaneous accurate monitoring of CD45+/CD34bright/VEGFR2+ (“small EC-like cells”) and CD45dim/CD34bright/VEGFR2+ is possible, but in a number of patients these values will be basically “zero” or undetectable. Still it may hypothesised based on animal experiments (1, 9) that groups of patients with “zero” or relatively high numbers of these cells would show different responses to anti-angiogenic therapy. This type of analysis will be of particular interest in clinical trials with anti-VEGF or anti-VEGFR directed (combination) therapies.

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Note added in proof
A very recent paper shows that a CD45neg/CD34pos/CD133neg cell population from cord blood is the actual source of endothelial outgrowth cells in vitro (see F. Timmermans et al., Arterioscler Thromb Vasc Biol 2007; doi: 10.1161/ATVBAHA.107.144972).
References