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CHAPTER 2

VEGFR2 EXPRESSING CIRCULATING (PROGENITOR) CELL POPULATIONS IN VOLUNTEERS AND CANCER PATIENTS

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ABSTRACT
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. In this study 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 hematopoietic progenitor, monocytic, endothelial and platelet markers. After establishing the test-retest stability of the measurements in 9 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{neg}/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 25 and 30 cells/ml (P=0.38). Unexpectedly, a population of mainly anucleated CD45\textsuperscript{low}/CD31\textsuperscript{bright}/CD41\textsuperscript{bright} cells was present in numbers of 9076 and 16697/ml (P=0.04) in volunteers and cancer patients, which contained a VEGFR2\textsuperscript{low} (compared to IgG isotype control) expressing population amounting to 1142 and 1642 cells/ml (P=0.12). This fourth population probably reflects large platelets.

The role of the herein identified VEGFR2\textsuperscript{+} circulating cell populations deserve 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.
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 mobilized into the circulation and accumulate in areas of neoangiogenesis [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 that they only play an orchestrating role in angiogenesis [4]. Furthermore, 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 optimize such monitoring.

Bone marrow derived EPCs are presently characterized 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 mobilized 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⁻ / CD45neg / CD31bright cells in patients with rectal cancer [12,13]. In another study CECs, defined as CD45neg / 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 not yet 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 4-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). 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 (San Jose, CA). VEGFR2-PE and VEGFR2-APC (clone 89106), IgG1-PE and IgG1-APC (clone 11711) were from R&D systems (Minneapolis, Minnesota). CD146-PE (clone P1H12) was from Chemicon (Temecula, CA), CD105-PE (Clone SN6, Serotec), CD13-PE (clone L138, BD), CD133 (clone AC133, Miltenyi Biotec). CD41a-FITC was from DAKO. In all experiments, appropriate combinations of IgG isotype were used to set analysis gates. Saponin was from Sigma Aldrich and was used in a concentration of 0.1% to permeabilise cells, LDS-751 was from Exitron chemicals. 7-aminoactinomycin D (7-AAD) from Pharmingen (San Diego, CA) 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, The Netherlands). All antibodies used are listed in Table 1.

**VEGFR2-PE and -APC Expression on HUVECs**

New batches of the VEGFR2 antibody labeled with PE or APC were always tested for positive staining on primary human umbilical cord vein endothelial cell (HUVECs) cultures, because HUVECs have been suggested to have a similar VEGFR2 expression levels as
<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>HIT3a</td>
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<td>L138</td>
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<td></td>
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<td>WM-47</td>
<td>DAKO</td>
<td>FITC</td>
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<tr>
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<td>DAKO</td>
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<td>RUU-PL 7F12</td>
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<td>FITC</td>
<td>1:10</td>
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<td>FITC</td>
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<tr>
<td></td>
<td>CD45</td>
<td>2D1</td>
<td>BD</td>
<td>PerCP</td>
<td>1:20</td>
</tr>
<tr>
<td></td>
<td>CD45</td>
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<td>BD</td>
<td>APC</td>
<td>1:20</td>
</tr>
<tr>
<td></td>
<td>CD45</td>
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<td>Immunotech</td>
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<td>AC133</td>
<td>MB</td>
<td>APC</td>
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<td>AC133</td>
<td>CLB</td>
<td>PE</td>
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<td>VE-cadherin</td>
<td>CD144</td>
<td>TEA1/13</td>
<td>Pharmingen</td>
<td>PE</td>
<td>1:6</td>
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<td>Endothelial</td>
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<td>P1H12</td>
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<td>PE</td>
<td>1:200</td>
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<tr>
<td></td>
<td>CD146</td>
<td>P1H12</td>
<td>BD</td>
<td>PE</td>
<td>1:10</td>
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<td>VEGFR2/KDR</td>
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<td>89106</td>
<td>R&amp;D</td>
<td>PE</td>
<td>1:5</td>
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<td></td>
<td>CD309</td>
<td>89106</td>
<td>R&amp;D</td>
<td>APC</td>
<td>1:5</td>
</tr>
<tr>
<td>Viability marker</td>
<td>7-AAD</td>
<td>N/A</td>
<td>Pharmingen</td>
<td>N/A</td>
<td>20 µl/1x10⁶ cells</td>
</tr>
<tr>
<td>DNA and RNA stain</td>
<td>LDS-751</td>
<td>N/A</td>
<td>Exciton</td>
<td>N/A</td>
<td>1:500</td>
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<td>DNA stain</td>
<td>DAPI</td>
<td>N/A</td>
<td>VL</td>
<td>N/A</td>
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<td>Permeability</td>
<td>Saponin</td>
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<td>Sigma Alldrich</td>
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<tr>
<td>Lysing solution</td>
<td>FACS lyse</td>
<td>N/A</td>
<td>BD</td>
<td>N/A</td>
<td>1x concentration</td>
</tr>
<tr>
<td>Washing solution</td>
<td>FACS buffer</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>PBS+ 0,1% BSA+0,05% sodium azide</td>
</tr>
</tbody>
</table>

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; DAKOCytomation, 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.
VEGFR2-EXPRESSING CELL POPULATIONS

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 mobilized with granulocyte-colony stimulating factor (G-CSF), which contains increased numbers of immature progenitor cells, including CEPs [1]. In the mobilized blood we clearly detected increased VEGFR2⁺ subpopulations of cells in the CD34bright/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 and 4 were female and 6 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 10⁶ to 3.7 x 10⁶ 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 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 twenty volunteers had a mean age of 37 year (range 21-57) and were 7 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, seven ml of blood was withdrawn in heparinized tubes on 2 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-
**Figure 1 - VEGFR2 staining of HUVECs.**

VEGFR2-PE (left; black), VEGFR2-APC (right; black) and isotype control (open) staining of HUVECS.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>WBC</th>
<th>CD45dim/CD34bright</th>
<th>CD45dim/CD34bright/VEGFR2+</th>
<th>CD45low/CD31bright/(VEGFR2+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>9.4 x10⁶</td>
<td>2397</td>
<td>20</td>
<td>8888 (1324)</td>
</tr>
<tr>
<td>Minimum</td>
<td>4.7 x10⁶</td>
<td>161</td>
<td>0</td>
<td>985 (155)</td>
</tr>
<tr>
<td>Maximum</td>
<td>22.1 x10⁶</td>
<td>7726</td>
<td>119</td>
<td>25252 (2882)</td>
</tr>
<tr>
<td>Stability (Φ)</td>
<td>0.9423</td>
<td>0.3506</td>
<td>0.7513</td>
<td></td>
</tr>
<tr>
<td>Equivalence(Φ)</td>
<td>0.9954</td>
<td>0.8391</td>
<td>0.8352</td>
<td></td>
</tr>
<tr>
<td>S &amp; E (Φ)</td>
<td>0.9423</td>
<td>0.3183</td>
<td>0.6823</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2 - Frequencies of some cell populations and statistics of reproducibility in blood samples taken on two different days.**

Data (in number of cells per ml peripheral blood) are from 9 patients sampled on two different days. The test-retest stability over days and the equivalence (over triplicates) were calculated according to Generalizability theory (Brennan, 2001). The generalizability coefficient (Φ) may be interpreted as the variance between individuals divided by the sum of the variance between individuals plus other sources of (error) variance. A frequency of 2397 (100-7700) cells/ml blood is consistent with published values for hematopoietic progenitor cells in non-cancer patients [18]. (CD45dim/CD34bright/VEGFR2+ = R10 in Fig. 2D1) were detected at low numbers of 20 cells/ml blood consistent with published frequencies of CD34bright / VEGFR2+ cells in human blood [1, 18].
VEGFR2-EXPRESSING CELL POPULATIONS

4500 (Sysmex corporation, Japan), which was 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 (often within 2 hours) after drawing the blood and all incubations were done in triplicate. For the immunophenotyping, 100-400 µl of blood was incubated for 30 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/10^6 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/10^6 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 2 times in FACS buffer and centrifuged at 1500 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 triplicates) scores for two VEGFR2^+ cell populations, which is calculated as the generalizability score $\Phi$ (See Results below and Figure 2 for detailed gating strategy). $\Phi$ was highest (> 0.9) for the CD45^{dim}CD34^{bright}, and > 0.75 for the CD45^{low}/CD31^{bright} cell population. The very rare VEGFR2^+/CD45^{dim}CD34^{bright} 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^+/CD45^{dim}CD34^{bright} is possible will be determined by the blood volume analyzed. Still, the assessment of these values, including those cases with VEGFR2^+/CD45^{dim}CD34^{bright} 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 CD45^{neg}/CD34^{bright}/VEGFR2^+ cells and CD45^{bright}/VEGFR2^+ cells with FSC/SSC characteristics of monocytes. In addition, the cell populations were further characterized 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 heparinized blood.
Figure 2 - Gating strategy.
(A-C): Viable gating is based on forward and side scatter including the lymphocyte gate, eliminating most debris and platelets (R1). (B) CD45\textsuperscript{reg/dim} gating to exclude monocytes, granulocytes and lymphocytes (R2). (C) Refined gating of the CD45\textsuperscript{reg/dim} population (R3) in more detail: Selection of cells with FSC in the 200-400 region to further exclude possible cell debris. The populations of...
interest were identified based on expression of CD45 / CD34 / VEGFR2 (Fig. 2D and 2D1-D3) or CD45 / CD31 / VEGFR2 (Fig.2E and E1-4).

(D): analysis of samples based on CD45 /CD34 /VEGFR2 /7-AAD combination.

D: Identification of CD45$^{\text{dim}}$/CD34$^{\text{bright}}$ (R4), CD45$^{\text{neg}}$/CD34$^{\text{bright}}$ population (R5); and CD45$^{\text{low}}$/CD34$^{\text{+}}$ (R6); the CD45$^{\text{neg}}$/CD34$^{\text{neg}}$ cells (R7) were not further quantitated, because they were CD31$^{\text{neg}}$. (D1-3): analysis of VEGFR2 positive cells. Their viability defined by 7-AAD staining (D3).

(E): analysis of samples based on CD45 /CD31 /VEGFR2 /7-AAD combination.

(E1-4) Analysis of CD31$^{\text{bright}}$ staining population (R9) and CD31$^{\text{bright}}$/VEGFR2$^{+}$ (R8) in CD45$^{\text{low}}$ cells (R6). 7-AAD staining of CD31$^{\text{bright}}$/VEGFR2$^{+}$ and CD31$^{\text{bright}}$.

FLOW CYTOMETRY

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

Sorting of the CD45$^{\text{low}}$/CD31$^{\text{bright}}$ 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 generalizability 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
CHAPTER 2

Figure 3 - DAPI-staining of cells sorted based on FSC/SSC, CD45^{low} / CD31^{bright} staining.
Blood was stained with CD45-APC and CD31-FITC (green)- labeled antibody and two fractions of cells were flow sorted and stained for nuclei with DAPI (blue). Left: SSC^{low} / FSC^{low-moderate} / CD45^{neg-low} / CD31^{bright} = “large platelets”. Right: lymphocytes (SSC/ CD45^{bright}). Lymphocytes show a low, barely visible CD31 staining. No nucleated CD31^{bright} cells are seen compared to the positive control lymphocytes (magnification 63x).

expression of the hematopoietic marker CD45 (Fig 2B). The initial broad gate was chosen to include CD45^{neg}, CD45^{low} and CD45^{dim} 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 recognized in the blood (see below).
Figure 4 - LDS-751 and saponin/7-AAD staining of CD45<sup>low</sup> / CD31<sup>bright</sup> cells.
Staining of CD45<sup>low</sup>/CD31<sup>bright</sup> cells, lymphocytes, granulocytes and monocytes for LDS-751 positivity (upper) and 7-AAD after permeabilisation of the plasma-membranes to allow nuclear staining of viable cells (lower).

Figure 5 - Additional marker expression in CD45<sup>low</sup>/CD31<sup>bright</sup> cells.
CD45<sup>low</sup>/CD31<sup>bright</sup> cells staining for hematopoietic and megakaryocytic markers.
**CD45**<sup>dim</sup> **CD34**<sup>bright</sup> /VEGFR2 POSITIVE CELLS

Further analysis of the tube based on CD34/CD45 showed a small population of CD45<sup>dim</sup> CD34<sup>bright</sup> /VEGFR2 positive cells (Fig. 2D1) as part of the CD45<sup>dim</sup> CD34<sup>bright</sup> population (R4 in Fig. 2D). These cells were viable as shown by 7-AAD exclusion. In addition VEGFR2 positive cells were found in the CD45<sup>neg</sup> R5 population (as discussed below; see Fig. 6) and in the CD45<sup>low</sup> R6 population. The latter CD45<sup>low</sup> cells were further analyzed in the parallel tube, as these VEGFR2 positive cells appeared CD45<sup>low</sup>/CD31<sup>bright</sup> cells.

**SSC**<sup>low</sup> /FSC<sup>low-to-intermediate</sup> / CD45<sup>low</sup> / CD31<sup>bright</sup> CELLS

In further analyzing the parallel tube based on CD31/CD45, we focused on the combination of CD45<sup>low</sup> with CD31<sup>bright</sup> cells in accordance with Willett et al (12). Indeed, using the appropriate IgG controls as cut-off level, the CD45<sup>low</sup> / CD31<sup>bright</sup> cell population (R6 in Fig. 2E) showed a low level of VEGFR2 expression (Fig. 2E1). Moreover, these cells partially overlap with the CD34<sup>low</sup> / CD45<sup>low</sup> population (R6 in Fig. 2D), as can be inferred from their similar SSC /FSC and CD45 staining. These cells as well as the VEGFR2<sup>neg</sup> / CD31<sup>bright</sup> 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 CD45<sup>low</sup> /CD31<sup>bright</sup> cell population was usually low or absent, and always much lower than that of control HUVECs.

**NATURE OF THE SSC**<sup>low</sup> /FSC<sup>low-to-intermediate</sup> / CD45<sup>low</sup> / CD31<sup>bright</sup> CELLS

When the CD45<sup>low</sup>/ CD31<sup>bright</sup> 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 permeabilize the cells. While lymphocytes, granulocytes and monocytes clearly accumulated the dye 7-AAD after permeabilization, only few or none of the CD45<sup>low</sup>/ CD31<sup>bright</sup> cells became positive for 7-AAD after permeabilization (Fig. 4, lower part). The number CD45<sup>low</sup>/ CD31<sup>bright</sup> 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 CD45<sup>low</sup>/CD31<sup>bright</sup> 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-sized platelets, the CD45<sup>low</sup>/ CD31<sup>bright</sup> cells were further analyzed by FACS. As shown for several markers in Figure 5, the cells were CD13<sup>neg/low</sup> (myeloid marker), CD3<sup>neg/low</sup> and CD19<sup>neg/low</sup> (lymphocyte markers) and CD105<sup>neg</sup> (mesenchymal/endothelial marker). In addition they were
VEGFR2-EXPRESSING CELL POPULATIONS

CD133\textsuperscript{neg} (progenitor marker) and differentiation marker CD38\textsuperscript{neg}. Importantly, they were CD41a/CD61\textsuperscript{pos} (megakaryocyte lineage markers) and platelet marker CD42b\textsuperscript{pos} (glycoprotein Ib\(\alpha\)) and CD144\textsuperscript{neg} and therefore most likely are “large platelets” in accordance with recent data of Strijbos \textit{et al} (40).

**CD45\textsuperscript{NEG} / CD34\textsuperscript{BRIGHT} / VEGFR2\textsuperscript{+} CELLS**

In addition to the CD45\textsuperscript{dim}/CD34\textsuperscript{bright} cells, a second population of CD34\textsuperscript{bright} cells, clearly distinct from the first population, based on the CD45\textsuperscript{neg} 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\textsuperscript{BRIGHT} / VEGFR2\textsuperscript{+} CELLS**

A fourth VEGFR2 positive population was identified in the CD45\textsuperscript{bright} monocytic cell fraction (Fig. 7). In order to confirm that this cell fraction belonged to the monocytes, we analyzed the blood of 5 individuals, and compared the VEGFR2\textsuperscript{+}/CD45 gated population with the VEGFR2\textsuperscript{+}/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\textsuperscript{bright} and CD14 positive cells/ml were found, respectively, and the VEGFR2\textsuperscript{+} subpopulation did not differ significantly between either gates (P=0.89; paired t-test) in this population were measured (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\textsuperscript{neg} / CD34\textsuperscript{bright} was significantly higher in the group of cancer patients (mean of 82 versus 35; P=0.001) with a significant difference in the CD45\textsuperscript{neg}/CD34\textsuperscript{bright}/VEGFR2\textsuperscript{+} subpopulations (12 versus 64/ml; P < 0.0001). The CD45\textsuperscript{dim} / CD34\textsuperscript{bright} cells showed a not-significant trend towards a higher level in cancer patients (1801 versus 3813; P=0.88) and the CD45\textsuperscript{dim} / CD34\textsuperscript{bright}/VEGFR2\textsuperscript{+} subpopulations were similar (25 versus 30/mL; P = 0.38).

In addition, in this study we recorded the number of VEGFR2\textsuperscript{+} monocytes (gated on SSC/CD45), which were elevated in cancer patients (578 cells/ml) as compared with healthy volunteers (245/ml; P =0.02). No significant differences were observed in the CD45\textsuperscript{(neg+)}\textsuperscript{low}/CD31\textsuperscript{bright} populations.
Figure 6 - Additional marker expression in CD45\(^{\text{neg}}\)/CD34\(^{\text{bright}}\) cells.

CD45\(^{\text{neg}}\)/CD34\(^{\text{bright}}\) cells staining for VEGFR2, CD146, CD105 and CD13.
Figure 7 - Gating of monocytes according to SSC/CD45 or SSC/CD14 and VEGFR2-staining.

Gating of monocytes according to CD45 or CD14 and VEGFR2+ cell populations within the monocytic cell population.

Since the mean age of the group of cancer patients in this study was higher than that of the healthy volunteers (63 versus 37 year) we have done a subgroup analysis of the healthy volunteers by making two more homogeneous groups, one of the 6 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+ monocytes and CD45^{low}/CD31^{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.

**DISCUSSION**

In this study we describe a flow cytometric procedure for the quantitative measurement of circulating cells populations expressing the endothelial marker VEGFR2+ in the peripheral blood of volunteers and patients with advanced solid tumors. We defined four populations of VEGFR2+ - expressing cells, which may of interest in the monitoring of
Table 3 - Frequencies of VEGFR2 expressing cell populations in the blood of healthy volunteers and cancer patients.

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\textsuperscript{dim}/CD34\textsuperscript{bright} represent hematopoietic progenitor cells.

\[^{(*)}\): CD45\textsuperscript{low} / CD31\textsuperscript{bright} cells are from the analysis using CD45-FITC antibody, while CD45\textsuperscript{neg+low} / CD31\textsuperscript{bright} 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).

Clinical studies of VEGF-targeted therapy. The rationale for choosing an approach, which quantifies the numbers of circulating VEGFR2 expressing cells was three-fold. First, the anti-VEGF antibody bevacizumab [20] and VEGFR 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, 23, 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+ subpopulations of monocytes [26, 27, 28]. In our study, the cell populations were defined by combinations of CD45neg, low or dim, CD34dim or bright, CD31bright, CD146low/moderate and VEGFR2low/moderate expression and a first estimation of the numbers of these subpopulations of cells per ml of blood in cancer patients and volunteers are given.

**SSC<sub>LOW</sub> / FSC<sub>LOW</sub> / CD45<sub>LOW</sub> / CD31<sub>BRIGHT</sub> CELLS**

The most frequent population expressing a combination of the selected markers was designated here are SSC<sub>low</sub> / FSC<sub>low-to-intermediate</sub> / CD45<sub>low</sub> / CD31<sub>bright</sub> 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 of 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 hundred 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 litres 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 1000-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 CEC 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<sup>+</sup> hematopoietic progenitor cells has been described [42]. Whether this low VEGFR2 expression in cells of megakaryocytic lineage has a biological function is unknown.

**CD45<sup>NEG</sup> / CD34<sup>BRIGHT</sup> /VEGFR2<sup>+</sup> 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 recognized 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 particular 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 CD45<sup>neg</sup> CD34<sup>bright</sup> CD31<sup>bright</sup> CD146<sup>low/moderate</sup> and VEGFR2<sup>+</sup> cells, clearly different from the CD45<sup>dim</sup> CD34<sup>bright</sup> cells, because of complete absence of CD45, lack of CD133 expression and by their low frequency (Fig. 6). A very similar CD133<sup>neg</sup> / CD146<sup>low/moderate</sup> population referred to as CECs has been reported by flow cytometric analysis recently [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). Still, because of their small size based on SSC/FSC, these cells may not represent the same heterogenous 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 CD31<sup>bright</sup> CD146<sup>+</sup> or CD105<sup>+</sup>, 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.

**CD45\textsuperscript{dim} / CD34\textsuperscript{bright} / VEGFR2\textsuperscript{+} CELLS**

The enumeration of CD34\textsuperscript{bright} progenitor (with or without CD133\textsuperscript{+}) VEGFR2\textsuperscript{+} cells can be done in a flow-cytometrically well-defined CD45\textsuperscript{dim} / CD34\textsuperscript{bright} gating procedure. For a good separation from other populations, expressing CD45\textsuperscript{low} / CD34\textsuperscript{+} (see e.g. R6 in Fig. 2D), the use of CD34-APC is an important advantage. In our study, a frequency of CD45\textsuperscript{dim}/CD34\textsuperscript{bright}/VEGFR2\textsuperscript{+} 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 analyzed to obtain meaningful results for the monitoring of therapy effects in cancer patients. However, on itself, the fact that certain patients may have very low numbers of VEGFR2\textsuperscript{+} 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\textsuperscript{+} 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 recognized a VEGFR2\textsuperscript{+} subpopulation of (CD45\textsuperscript{+} / CD14\textsuperscript{+}) 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\textsuperscript{+} 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 realize that it is too early for routine implementation of the current protocol. Multi-laboratory 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<sup>-</sup> / CD34<sup>bright</sup> / VEGFR2<sup>+</sup> ("small EC-like cells") and CD45<sup>dim</sup> /CD34<sup>bright</sup> /VEGFR2<sup>+</sup> is possible, but in a number of patients these values will be basically "zero" or undetectable. Still it may hypothesized 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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