Circulating endothelial and progenitor cells during anti-angiogenic treatment in cancer patients
Vroling, L.

2011

document version
Publisher's PDF, also known as Version of record

Link to publication in VU Research Portal

citation for published version (APA)
Vroling, L. (2011). Circulating endothelial and progenitor cells during anti-angiogenic treatment in cancer patients.

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain.
• You may freely distribute the URL identifying the publication in the public portal.

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

E-mail address:
vuresearchportal.ub@vu.nl
INCREASED NUMBERS OF SMALL CIRCULATING ENDOTHELIAL CELLS IN RENAL CELL CANCER PATIENTS TREATED WITH SUNITINIB

Laura Vroling
Astrid A.M. van der Veldt
Richard de Haas
John B.A.G. Haanen
Gerrit Jan Schuurhuis
Dirk J. Kuik
Hester van Cruijsen
Henk M.W. Verheul
Alfons J.M. van den Eertwegh
Klaas Hoekman
Epie Boven
Victor W.M. van Hinsbergh
Henk J. Broxterman

Angiogenesis, 2009;12(1):69-79
ABSTRACT

Mature circulating endothelial cell (CEC) as well as endothelial progenitor (CEP) populations may reflect the activity of anti-angiogenic agents on tumor neovasculature or even constitute a target for anti-angiogenic therapy. We investigated the behavior of circulating endothelial cells in parallel with hematopoietic progenitor cells (HPCs) in the blood of renal cell cancer patients during sunitinib treatment.

We analyzed the kinetics of a specific population of small VEGFR2-expressing CECs (CD45\(^{-}\)/CD34\(^{\text{bright}}\)), HPCs (CD45\(^{\text{dim}}\)/CD34\(^{\text{bright}}\)) and monocytes in the blood of 24 renal cell cancer (RCC) patients receiving 50 mg/day of the multitargeted VEGF inhibitor sunitinib, on a 4 week-on/2-week-off schedule. Blood was taken before treatment (C1D1), on C1D14, C1D28 and on C2D1 before the start of cycle 2. Also plasma VEGF and erythropoietin (EPO) were determined.

Remarkably, while CD34\(^{\text{bright}}\) HPCs and monocytes decreased during treatment, CD34\(^{\text{bright}}\) CECs increased from 69 cells/mL (C1D1) to 180 cells/mL (C1D14; \(p=0.001\)) and remained high on C1D28. All cell populations recovered to near pre-treatment levels on C2D1. Plasma VEGF and EPO levels were increased on C1D14 and partly normalized to pre-treatment levels on C2D1.

In conclusion, opposite kinetics of two circulating CD34\(^{\text{bright}}\) cell populations, HPCs and small CECs, were observed in sunitinib-treated RCC patients. The increase in CECs is likely caused by sunitinib targeting of immature tumor vessels.
INTRODUCTION

Anti-angiogenic compounds have shown efficacy in the clinic during recent years. In particular, the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab [1] and the receptor tyrosine kinase inhibitors (TKIs) of the VEGF receptor family [2], sunitinib [3, 4] and sorafenib [5], have proven activity in a number of tumor types [6]. Sunitinib is an oral TKI of the VEGF receptors, platelet-derived growth factor (PDGF) receptors, Flt-3 and c-Kit and has been approved for treatment of advanced renal cell cancer (RCC) and imatinib-resistant gastrointestinal stromal tumors (GISTs). In a phase III trial in RCC patients, sunitinib has proven to be effective, albeit that a subset of RCC patients did not benefit from it [4]. Therefore, there is still a need for better understanding which conditions, factors and cells facilitate or limit the beneficial effects of sunitinib on tumors.

In addition to immunohistochemical staining of tumor biopsies and imaging techniques that quantify tumor growth and perfusion [7], measurement of plasma circulating proteins, such as VEGF [8] or soluble VEGFRs [9], may reflect responsiveness to treatment. However, VEGF or sVEGFR2 plasma levels have not been shown to be predictive of response to sunitinib in GIST patients [10]. Alternatively, changes in the levels of circulating cells, such as newly recruited progenitor cells and monocytes or detached endothelial cells may be induced by anti-angiogenic treatment [7, 11].

Circulating endothelial progenitors (CEPs) cells have been suggested as potential pharmacodynamic or predictive biomarker in tumor patients [11]. CEPs were first described by Asahara et al, who introduced the concept of circulating, bone-marrow derived endothelial progenitor cells, contributing to adult vasculogenesis [12]. Later, Lyden et al [13] have demonstrated that both VEGFR2pos-circulating endothelial cells as well as VEGFR1pos-myeloid, monocytic cells contributed to tumor vascularization. Recently, the source of highly proliferative endothelial outgrowth cells has been identified in CD34pos/CD45neg/CD133neg circulating cell populations [14, 15]. Besides CEPs, circulating endothelial cells (CECs) as thought to be shed from mature blood vessels may reflect the efficacy of anti-vascular treatment as suggested in a number of studies [10, 16-18]. At present, no studies have reported on changes in frequencies of CECs or CEPs in combination with hematopoietic progenitor cells (HPCs) during sunitinib treatment of RCC patients.

Previously we have identified a rare population of small CD45neg/CD34bright/CD133neg/VEGFR2pos cells in the peripheral blood (PB) of healthy volunteers, with increased numbers in cancer patients [19]. On the basis of endothelial marker expression these cells were indicated as “small-size EC-like cells” or CECs [20], because they are relatively small (<10 µm) as compared to mature CECs [21-23]. Also, their marker profile is the same as that of the source of highly proliferative late outgrowth endothelial cells present
in umbilical cord blood or PB [15] and is clearly distinct from CD45\textsuperscript{dim}/CD34\textsuperscript{bright}/CD133\textsuperscript{+} hematopoietic progenitors. Here, we demonstrate that these CECs increase during sunitinib treatment of RCC patients in parallel to plasma VEGF and erythropoietin (EPO) levels, while HPCs and monocytes show the opposite changes, i.e. a decrease. In addition, a preliminary evaluation of the relation of CECs with clinical response is discussed.

**Patients and Methods**

**Patients and Study Design**

From January 2006 to March 2007 24 patients treated with sunitinib for advanced RCC in an expanded access program were included. Sunitinib was administered orally as monotherapy at the currently recommended dose of 50 mg daily in cycles of 6 weeks, consisting of 4 weeks on treatment followed by 2 weeks of rest (4/2 schedule). Before study entry, each participant signed an institutional review board-approved protocol-specific informed consent in accordance with national and institutional guidelines which strictly adhere to the principles of the Declaration of Helsinki and its subsequent amendments. During cycle one, peripheral blood (PB) was taken on 4 occasions: C1D1 before receiving the first dose of sunitinib, C1D14, C1D28 and C2D1 (=C1D42) before administration of sunitinib of cycle 2. Computed Tomography (CT) or Magnetic Resonance Imaging (MRI) was performed before treatment and after every two to three cycles to assess clinical response according to Response Evaluation Criteria in Solid Tumors (RECIST) [24]. RECIST is based on the sum of the largest diameters of appointed target tumor lesions at baseline and compared to the sum calculated in follow-up scans. Progression was defined based on an increase of 20% of the sum of the target lesions or clear clinical evidence of progressive disease (PD), and a 20% decrease of the sum of the target lesions was considered as partial response (PR). Responses not fitting these criteria were considered as stable disease (SD). Tumor response, PFS and overall survival (OS) were used as parameters of treatment outcome. The PFS was the time between the first day of sunitinib and the date of PD on CT or MRI or clear clinical evidence of PD. OS was the time between the first day of treatment and the date of death or the date on which patients were last known to be alive. Data collection was closed on January 1\textsuperscript{st}, 2008.

**HPCs, CECs and Plasma Monitoring**

At the time of blood sampling the first 2 ml of blood were discarded and blood for flow cytometric enumerations was processed within 2-4 hours. At each time-point 7 ml of EDTA blood and 7 ml of citrate blood in a CPT tube (Becton Dickinson) were collected for
measurement of circulating cell populations. One ml of full blood was used for the measurement of CECs and HPCs based on CD45 and CD34 marker expression and expressed as number/mL, as published in detail [19]. Analysis of the subsets of cells was performed with the antibodies CD45-FITC, CD34-APC, and IgG isotypes as has been described in detail [19]. For additional measurements of cell populations in patients VEGFR2-APC and –PE antibodies were used. The viability marker 7-AAD was used to gate viable cells and annexin-V staining was used to determine early stages of apoptosis. To assure the gating of nucleated small CD34\textsuperscript{bright} cells only, in a number of patients we added extra analysis tubes using the dye styril-751 (LDS-751). Furthermore, we added tubes with 7-AAD plus 0.1% saponin to permeabilize the cells and allow access of the dye to nuclei of viable cells as described before [19, 25]. Flow cytometry was performed on a FACSCalibur (BD Biosciences) and data were analyzed using CellQuest Pro software. Subfractions of white blood cells (WBC) were calculated as number/mL of blood by using standard total WBC count on Sysmex [19]. The remaining EDTA blood was used for the preparation of plasma and stored at –80°C. Plasma VEGF levels and EPO were measured in duplicate with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis). Albumin was determined with conventional methods in the department of clinical chemistry.

Human umbilical cord blood was obtained from full-term deliveries and was processed for flow cytometry according to the patients peripheral blood samples and used as a reference to identify the CD45\textsuperscript{neg}/CD34\textsuperscript{bright}/CD133\textsuperscript{neg} CEC population [15].

**Statistics**

Frequencies of circulating cell populations (numbers/mL), plasma levels of VEGF (pg/mL) and EPO (mIU/mL) were enumerated and expressed as median (range). Wilcoxon Signed Ranks test (SPSS for Windows 14.0, SPSS, Inc., Chicago, IL) was used to compare the biomarkers at pre-treatment and during treatment on C1D14, C1D28 and C2D1. Clinical benefit (CB) was defined as stable disease (SD) plus partial response (PR). PFS and OS were calculated with the Kaplan-Meier method and tested with the log rank test. Values of p ≤ 0.05 (two-sided) were considered statistically significant.

**Results**

**Patient Characteristics and Response to Treatment**

Twenty-four RCC patients treated with sunitinib were enrolled in the study. One patient died on C1D14 due to early progression and was excluded from the analysis. The remaining patients (17 males and 6 females) had a median age of 63 years (range 40-84) at the start of treatment. For further patients characteristics see Table 1 and 2.
Table 1. Patient Characteristics and Best Response to sunitinib Treatment

| Characteristic                                | No. | %   |
|-----------------------------------------------|-----|-----|
| **Total**                                     | 23  | 100 |
| **Sex**                                       |     |     |
| Male                                          | 17  | 74  |
| Female                                        | 6   | 26  |
| **Median age, years (range)**                 | 63  | (40-84) |
| **Histology**                                 |     |     |
| Clear cell                                    | 19  | 83  |
| Papillary                                     | 3   | 13  |
| Other                                         | 1   | 4   |
| **Prior treatment**                           |     |     |
| Prior nephrectomy                             | 17  | 74  |
| Prior cytokine-based therapy                  | 15  | 65  |
| **Site of metastatic disease**                |     |     |
| Lung                                          | 20  | 87  |
| Liver                                         | 9   | 39  |
| Bone                                          | 4   | 17  |
| **No. of disease sites**                      |     |     |
| 1                                             | 3   | 13  |
| 2                                             | 5   | 22  |
| ≥ 3                                           | 15  | 65  |
| **MSKCC risk groups**                         |     |     |
| Favorable risk                                | 3   | 13  |
| Intermediate risk                             | 15  | 65  |
| Poor risk                                     | 5   | 22  |
| **Best response to sunitinib treatment**      |     |     |
| Partial response                              | 4   | 17 (19) |
| Stable disease                                | 11  | 48 (52) |
| Progressive disease                           | 6   | 26 (29) |
| No evaluation§                                | 2   | 7 (-) |
| **Progression-free survival**§                 | 8.0 | (1.1-19.3) |
| **Survival**§                                 | 12.7| (1.4-23.2) |

* Abbreviations: MSKCC; Memorial Sloan-Kettering Cancer Center

* CT or MRI was performed before treatment and after every two to three cycles to assess clinical response according to Response Evaluation Criteria in Solid Tumors (RECIST)24.
§ Two out of 23 patients could not be evaluated for treatment response because of early discontinuation due to sunitinib related side-effects.
ψ The PFS was the time between the first day of sunitinib and the date of progressive disease (PD) on CT or MRI or clear clinical evidence of PD.
φ Survival was the time between the first day of treatment and the date of death or the date on which patients were last known to be alive.
Two out of 23 patients could not be evaluated for treatment response because of early discontinuation due to sunitinib related side-effects. Of the 21 evaluable patients, 4 patients (19%) achieved a PR as best response, 11 patients (52%) had SD, and 6 patients (29%) had PD. The median PFS of these 23 patients was 8.0 months (range: 1.1-19.3) and the median OS was 12.7 months (range: 1.4-23.2).

**Blood cell counts during the first cycle of sunitinib**

The median WBC count of the patients showed a decrease from $7.9 \times 10^6$ to $6.9 \times 10^6$ cells/ml on C1D14 ($n = 23$; $p = 0.002$) and a further decrease on C1D28, (from median pre-treatment $7.9 \times 10^6$ to $4.4 \times 10^6$ cells/ml, $n = 15$; $p = 0.001$), which partly reverted after 2 weeks of rest (from median pre-treatment $7.7 \times 10^6$ to $4.8 \times 10^6$, $n = 15$; $p = 0.001$). A similar pattern was seen for thrombocytes, neutrophils and monocytes. The reduction of circulating monocytes and their partial recovery proceeded faster than the total WBC change, whereas the neutrophil decrease showed a more delayed effect.

### Table 2. Patients Characteristics and Best Response to sunitinib

| Patient No. | Age (years) | Sex | RCC Type        | Prior Treatment | Response* | PFS (months) | Survival (months) |
|-------------|-------------|-----|-----------------|-----------------|-----------|--------------|-------------------|
| 1           | 68          | F   | Clear cell      | Second-line     | PR        | 18.4         | 23.2              |
| 2           | 48          | M   | Clear cell      | Second-line     | PD        | 3.0          | 8.2               |
| 3           | 40          | M   | Papillary ca    | Second-line     | SD        | 10.1         | 11.0              |
| 4           | 76          | M   | Clear cell      | Second-line     | SD        | 7.0          | 12.3              |
| 5           | 57          | M   | Clear cell      | Second-line     | SD        | 10.6         | 22.2              |
| 6           | 62          | F   | Clear cell      | Second-line     | PR        | 5.8          | 20.7              |
| 7           | 66          | M   | Papillary ca    | Second-line     | PD        | 1.2          | 1.4               |
| 8           | 60          | M   | Clear cell      | Second-line     | PR        | 19.4         | 19.3              |
| 9           | 81          | M   | Clear cell      | Second-line     | SD        | 11.0         | 11.2              |
| 10          | 45          | M   | Papillary ca    | Second-line     | PD        | 2.6          | 4.6               |
| 11          | 70          | M   | Clear cell      | First-line      | SD        | 8.4          | 9.1               |
| 12          | 59          | M   | Clear cell      | First-line      | SD        | 9.3          | 15.5              |
| 13          | 59          | M   | Clear cell      | First-line      | PD        | 2.6          | 10.9              |
| 14          | 73          | F   | Clear cell      | First-line      | SD        | 3.6          | 4.6               |
| 15          | 74          | M   | Clear cell      | First-line      | SD        | 2.0          | 14.9              |
| 16          | 59          | M   | Clear cell      | Second-line     | PR        | 8.9          | 12.7              |
| 17          | 57          | F   | Clear cell      | First-line      | -         | -            | 9.7               |
| 18          | 69          | F   | Clear cell      | First-line      | SD        | 16.5         | 16.5              |
| 19          | 84          | F   | Clear cell      | Second-line     | -         | -            | 16.6              |
| 20          | 60          | M   | Clear cell      | Second-line     | SD        | 8.0          | 15.3              |
| 21          | 57          | M   | Clear cell      | Second-line     | PD        | 2.3          | 13.8              |
| 22          | 48          | M   | Chromophobe ca  | First-line      | PD        | 1.1          | 2.0               |
| 23          | 64          | M   | Clear cell      | Second-line     | SD        | 5.1          | 9.0               |

Abbreviations: RCC; Renal Cell Cancer. F; Female. M; Male. PR; Partial Response. SD; Stable Disease. PD; Progressive Disease. *According to Response Evaluation Criteria in Solid Tumors; PFS, progression-free survival
Erythrocytes and hemoglobin showed the reverse, i.e. a significant increase after 14 and 28 days while the number of lymphocytes and basophils did not change during sunitinib treatment (Fig. 1).

**Marker profile of two CD34<sup>bright</sup> populations: CECs and HPCs**

Two populations of CD34<sup>bright</sup> circulating cells were evaluated, CECs and HPCs. The definitions of CECs and HPCs according to CD45 and CD34 expression are visualized for a representative RCC patient (Fig. 2A) and for comparison from cord blood (Fig 2B). CECs are CD45<sup>neg</sup> and CD133<sup>neg</sup>; HPCs are CD45<sup>dim</sup> and are largely CD133<sup>pos</sup> (Fig. 2A and B). Moreover, CECs having a slightly higher CD34 brightness than the majority of HPCs [19]. CECs are small in size being comparable with HPCs. CECs are viable cells, because they all exclude 7-AAD. We also checked in separate analysis tubes that both the CD34<sup>bright</sup> CD45<sup>neg</sup> and CD34<sup>bright</sup> CD45<sup>dim</sup> population had a similar positive 7-AAD/saponin staining as well as LDS-751 staining, confirming that both populations are nucleated cells. Other markers for which CECs are positive are CD31, CD105, CD146 and VEGFR2 as previously reported [19]. To confirm the VEGFR2 expression on CECs we have measured VEGFR2 in parallel in additional cancer patients. VEGFR2 positivity in CECs was high (median 65%), in contrast to the CD45<sup>dim</sup>/CD34<sup>bright</sup> HPCs (< 1%). In addition, the EPO receptor was evaluated on CECs of five sunitinib-treated patients and was found largely present in 83.3% of the CECs (median, range: 66.7- 93.3%). Plasma membrane VE-cadherin was undetectable in CECs in five treated patients (data not shown).

**Kinetics of CECs and HPCs during the first cycle of sunitinib**

A distinct difference in the kinetics of CECs (CD45<sup>neg</sup>/CD34<sup>bright</sup>/7-AAD<sup>neg</sup>) and HPCs (CD45<sup>dim</sup>/CD34<sup>bright</sup>) was observed during the first cycle of sunitinib (Fig. 3A). The median number of viable CECs before treatment (C1D1) was 69 cells/ml (range 8-472), much lower than the number of HPCs (1350 cells/ml, range 305-5351). The median of CECs increased from 69 on C1D1 to 180 cells/ml on C1D14 (n = 23; p = 0.001) and from pre-treatment 76 to 229 cells/mL (n = 14; p = 0.013) on C1D28, while the HPCs displayed an opposite kinetic pattern and decreased from 1350 to 372 cells/ml on C1D14 (n = 23; p < 0.001) and from pre-treatment 1567 to 409 cells/mL on C1D28 (n = 14; p = 0.001). Both cell populations returned to values close to the pretreatment levels after the 2-week period of rest (C2D1) (Fig. 3A). In a group of non-small cell lung cancer patients not treated with a VEGFR inhibitor, but with the EGF receptor inhibitor erlotinib, the CECs did not change significantly over a 3-week period (data not shown). When the kinetic changes in circulating cells were expressed as percentage of pre-treatment values within individual patients, an increase of 102% in CECs numbers was observed after 2 weeks of
Figure 1 - Blood cell count and hemoglobin during treatment with sunitinib.

Median (range) values are shown. Timepoints of measurement: C1D1, cycle 1 day 1 (n=23); C1D14, cycle 1 day 14 (n=23); C1D28, cycle 1 day 28 (n=15); C2D1, cycle 2 day 1 before start of the second cycle (n=15). Wilcoxon Signed rank test, * p < 0.01, ** p < 0.05.
treatment, whereas the HPCs showed a decrease of 65% (Fig. 3B). A similar change was found on C1D28 (n = 14).

**Plasma VEGF and EPO Levels during the First Cycle of Sunitinib**

Plasma levels of VEGF before treatment of sunitinib varied more than 10-fold among individual patients and had a median value of 82 pg/mL (range: 29-348, n = 19). These median levels increased from 82 to 185 pg/mL on C1D14 (n = 19; \( p = 0.001 \)), from median pre-treatment 79 to 198 pg/mL on C1D28 (n = 12; \( p = 0.028 \)) and returned to near pre-treatment levels on C2D1 (from 79 to 75 pg/mL; n = 12, \( p = 0.875 \)) (Fig. 3B). In a subgroup of patients we assessed EPO levels and the median plasma EPO level on C1D1 was 12 mIU/mL, which increased with 63% after 14 days (median, n = 20, Fig. 3C). In six patients, EPO was measured during the complete cycle (Fig. 3D) showing increases of 60% and 216% at days C1D14 and C1D28, respectively, which partly normalized (to 115% increase) at C2D1. Albumin concentrations determined in a larger group of RCC patients treated with sunitinib were unaltered at C1D28 (n=67) in comparison with the initial values at C1D0, n=81 (median of 38 μmol/L range: 17-50 μmol/L and median of 41 μmol/L range: 22-52 μmol/L, respectively).

**Biomarkers and Treatment Outcome**

Clinical benefit was observed in 15 out of 21 RCC patients. 17 of all patients had clear cell RCC, of which 14 showed CB. Progressive disease was observed in 6 patients; 3 clear cell RCC patients, 2 papillary carcinoma and 1 chromophobe carcinoma indicating that the patients with a clear cell carcinoma had a good response to sunitinib. In the CB group the change in CECs after 14 days was increased in 14 out of 15 patients and in the PD group 4 out of 6 patients showed an increase, while 2 had a decrease. An increased number of CECs (n=18) after 14 days of sunitinib treatment, was associated with a longer PFS as compared to patients (n=3) with a decreased number of CECs (log rank test; \( p= 0.034 \)).

**Discussion**

We have investigated the changes in the frequency of circulating cells with specific emphasis on a population of small CD45<sup>neg</sup>/ CD34<sup>bright</sup> CECs, previously shown to be CD31<sup>pos</sup>/ CD105<sup>pos</sup>/ CD146<sup>pos</sup>/ VEGFR2<sup>pos</sup>/ CD133<sup>neg</sup> [19], in advanced RCC patients during the first cycle of sunitinib treatment. CECs increased in parallel to plasma VEGF and EPO levels during the 4-week on and decreased during the 2-week off sunitinib period, while monocytes and HPCs displayed an opposite pattern of change.
Figure 2 - Detection of hematopoietic progenitor cells (HPCs) and circulating endothelial cells (CECs) using four-color flow cytometry.

The mononuclear (MNC)-fraction of a renal cell cancer (RCC) patient on C1D1 and C1D14 (A) and human umbilical cord blood (B). In the upper panel CD45, CD34 expression, size and granularity is shown for HPCs and CECs. HPCs measured as CD45$^{dim}$/CD34$^{bright}$ and CECs measured as CD45$^{neg}$/CD34$^{bright}$ (see box). Second and third panel is showing CD133 expression for both HPCs and CECs as compared to the isotype control.
Figure 3 - Circulating endothelial cells (CECs), total hematopoietic progenitor cells (HPCs), erythropoietin (EPO) levels and changes of VEGF levels in comparison to changes of blood cells during treatment with sunitinib.

A, frequencies of CECs/mL and viable HPCs/mL are shown before (C1D1, n=23), and on C1D14 (n=23), C1D28 (n=14) and C2D1 (n=14) after start of sunitinib treatment. B, percentage change in circulating cells and plasma VEGF levels on different time-points during sunitinib treatment are given. C and D, plasma EPO levels are shown. In Fig. A, C, and D individual data and the median values are shown, while in Fig. 3B pre-treatment levels (C1D1) were used as starting-point and percentage of change on C1D14 (and other timepoints) was calculated for each individual patient. Wilcoxon Signed rank test, * p < 0.01, ** p < 0.05, ns; not significant.
Blood cell-based biomarker analysis related to sunitinib activity and clinical outcome has been studied only in GIST patients with the main conclusion that a smaller decrease in monocyte levels was seen in patients with clinical benefit compared to those with progressive disease [10]. We observed a decrease in circulating monocyte number after sunitinib treatment in RCC patients in agreement with the GIST study; a correlation with response was not seen in our population, possibly related to the limited number of patients with PD.

The number of HPCs decreased already maximally at C1D14 in our patient group, in parallel to the monocytes, while the overall WBC count dropped more slowly, due to a more delayed change in circulating neutrophils (Fig. 1). The decrease in HPCs might be partly related to bone marrow suppression associated with the Flt3-inhibitory action of sunitinib, since Flt3-signaling is required for HPC proliferation [26, 27].

Despite intense interest in developing biomarker tests for response prediction [7, 28, 29], levels of CECs during sunitinib treatment of RCC patients have not yet been reported. Therefore, the most interesting and novel finding of our study was the increase of CD45neg/CD34bright CECs during sunitinib treatment. The CEC population in peripheral blood is a rare cell population [20], which is increased 2- to 3-fold in cancer patients [19]. In the present patient group the median pre-treatment (C1D1) frequency of the CECs was 69 cells/mL (n = 23), which is well comparable to the median of 81 cells/mL (range: 32-132) in a mixed group of cancer patients [19]. The number of CECs approximately doubled in the RCC patient group by sunitinib treatment. Since we found a similar two-fold increase of CEC levels (without decrease in HPC numbers) in a group of bevacizumab plus erlotinib, but not erlotinib-single agent treated NSCLC patients (Vroling et al, unpublished) [30], this increase is more likely related to inhibition of VEGFR signaling by sunitinib, rather than to inhibition of other targets or off-target effects of sunitinib. Being a most likely specific target-related effect of sunitinib, this increase of CECs remains an interesting cell population to be further investigated.

An important question regards the precise nature and function of the CEC population that is elevated after sunitinib treatment, in particular in the light of the current controversies on the identification and role in tumor angiogenesis of CECs or CEPs [11, 14, 25, 31]. A plausible explanation for the increased number of CECs is that they reflect endothelial cells, which became detached or shed from sunitinib-targeted immature (tumor) blood vessels. Although we have defined this population by the marker combination of CD45neg and CD34bright, which are both essential for discriminating these cells from the HPCs and all other MNCs, in theory it may still be heterogeneous with regard to other EC markers. Importantly, we have assessed that this population has the highest VEGFR2 positivity (median 65%) of all by us defined cell populations in the PB, further supporting their endothelial nature. CECs are commonly characterized and defined by a heterogeneous,
but rather large size and granularity, exceeding that of most mononuclear cell populations, typically > 20 µm [22, 32, 33] and a high CD146 expression allowing selective extraction with immunobeads [34]. The median diameter of CD146+ PBMCs has been estimated 6.8 µm versus that of CD146+ CECs as 21.5 µm [22]. Our CECs are in the FSC/SSC range of the HPCs, which are < 10 µm. This fits with the idea that these small CECs originate from a rather immature vasculature and/or are mobilized bone marrow or vascular wall resident EPCs (see below). In support of this explanation, several data suggest that sunitinib might selectively prune immature nascent tumor neovessels not yet adequately stabilized by pericycle coverage [35, 36], while relatively saving mature vessels leading to vessel normalization [37].

A characteristic of endothelial cells in vitro is that they rapidly become apoptotic after detachment from their matrix [38]. However, in studies that measure CEC frequencies in PB, cell viability was either not assessed, or the viability marker dye 7-AAD has been used to exclude dead cells, as in most flow cytometric approaches. While our CEC values are intact CECs by the definition of exclusion of 7-AAD, more sensitive markers, such as annexin-V staining or the dye SYTO-16, can detect early stages of apoptosis in cells that still exclude 7-AAD [39]. We are not aware of studies reporting apoptotic CECs using annexin-V labeling, probably because this technique is not readily incorporated in most CEC protocols and also the use of frozen-thawed samples as used by some [10] precludes the reliable assessment of apoptotic cells [39]. Therefore, we have assessed the percentage of apoptotic CECs with annexin-V (with ammonium chloride) protocol in several RCC patients, separately from the main study protocol and found that the number of early apoptotic CECs was considerable (range: 50-80% of CECs).

It should be noted that the endothelial cell marker VE-cadherin was virtually absent in most of our CEC subpopulations, while others reported it to be present on mature CECs circulating in PB [40]. The lack of overt surface VE-cadherin expression may reflect the immature nature of these small CECs, or might also be explained by internalization during or after loss of endothelial junctions and detachment of the cells [41, 42].

An alternative possibility may be that our CD45neg/CD34bright CECs have endothelial progenitor (CEP) characteristics, such as those recently ascribed to CD45neg/CD34bright/CD133neg cells [14, 15, 43, 44]. A disturbed homing of VEGFR positive CEPs into the tumor vasculature caused by sunitinib might also contribute to the increase in CD45neg/CD34bright CEC population. It is important to note that the presence of a fraction of early apoptotic cells in the population of CECs does not exclude a priori the presence of endothelial progenitor cells, capable of highly proliferative outgrowth, since the CD34bright/CD45neg/CD133neg cell population from cord blood, which is the source of late endothelial outgrowth cells (EOCs), also contained up to 60% apoptotic cells (Timmermans, personal communication). This lends support to the idea that the EPCs or
EOC precursors circulating in human peripheral blood might be in majority rather resident
cells from peripheral sites than from the bone-marrow [45] and might exist in multiple
states of differentiation [46].
In addition to the increase in CECs, the soluble growth factor VEGF increased during
sunitinib exposure and partly normalized 2 weeks after cessation of drug intake. This
finding is in accordance with previous findings on VEGF receptor inhibition studies in mice
and man [10, 47, 48]. The mechanism for the VEGF increase is not known, but according
to the study of Ebos et al. (2007) may reflect a direct or indirect physiological response
to receptor inhibition by sunitinib. Indeed we found also a prominent increase of EPO
during the first cycle of sunitinib, consistent with the findings of Ebos et al (2007) in
sunitinib-treated mice [49]. Functional consequences of increased plasma EPO levels in
sunitinib-treated patients remain to be defined.
The rapid return of VEGF and CECs to the pre-treatment levels during the 2-weeks rest
period is remarkable. Studies by McDonald et al [50] have pointed to the rapid
repopulation of vascular casts after cessation of anti-angiogenic treatment of tumor-
bearing animals. The occurrence of a similar rapid resumption of vessel repair in the RCC
patients might contribute to the rapid normalization of VEGF during the drug-free period.
The primary goal of this study was to investigate the presence and pattern of change of
CD34\textsuperscript{bright}/CD45\textsuperscript{neg} CECs, separated from CD34\textsuperscript{bright}/CD45\textsuperscript{pos} HPCs in a cohort of sunitinib
treated RCC patients. The question whether the observed changes in CECs or other
circulating subsets of cells are just a pharmacodynamic marker of sunitinib activity or
might have a predictive value needs to be addressed in a larger cohort of patients [51, 52].
In conclusion, this study shows that CD34\textsuperscript{bright} CECs and CD34\textsuperscript{bright} HPCs counts change in
opposite directions by sunitinib: monocytes and HPC decrease and CECs increase.
CD34\textsuperscript{bright}/CD133\textsuperscript{neg} CECs might be detached ECs and reflect sunitinib antivascular effects
or might include CEPs which are potential targets.

**ACKNOWLEDGMENTS**

We thank Corinne N. Tillier and Henk A. Mallo for their help with patient care. Grant
support: Supported by the European Union (FP6) Integrated project “Angiotargeting”
(contract no. 504743); This study has been partly presented at the 99\textsuperscript{th} AACR Annual
Meeting in San Diego, April 2008.
1. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004;350:2335-42.

2. Choueiri TK, Rini B, Garcia JA, et al. Prognostic factors associated with long-term survival in previously untreated metastatic renal cell carcinoma. Ann Oncol 2007;18:249-55.

3. Demetri GD, van Oosterom AT, Garrett CR, et al. Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomised controlled trial. Lancet 2006;368:1329-38.

4. Motzer RJ, Hutson TE, Tomczak P, et al. Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med 2007;356:115-24.

5. Escudier B, Eisen T, Stadler WM, et al. Sorafenib in advanced clear-cell renal-cell carcinoma. N Engl J Med 2007;356:125-34.

6. Choueiri TK, Plantade A, Elson P, et al. Efficacy of sunitinib and sorafenib in metastatic papillary and chromophobe renal cell carcinoma. J Clin Oncol 2008;26:127-31.

7. Jain RK, Duda DG, Clark JW, Loeffler JS. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nat Clin Pract Oncol 2006;3:24-40.

8. Drevs J, Zirrgiebel U, Schmidt-Gersbach CI, et al. Soluble markers for the assessment of biological activity with PTK787/ZK 222584 (PTK/ZK), a vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor in patients with advanced colorectal cancer from two phase I trials. Ann Oncol 2005;16:558-65.

9. Bocci G, Man S, Green SK, et al. Increased plasma vascular endothelial growth factor (VEGF) as a surrogate marker for optimal therapeutic dosing of VEGF receptor-2 monoclonal antibodies. Cancer Res 2004;64:6616-25.

10. Norden-Zfoni A, Desai J, Manola J, et al. Blood-based biomarkers of SU11248 activity and clinical outcome in patients with metastatic imatinib-resistant gastrointestinal stromal tumor. Clin Cancer Res 2007;13:2643-50.

11. Le Tourneau C, Vidal L, Siu LL. Progress and challenges in the identification of biomarkers for EGFR and VEGFR targeting anticancer agents. Drug Resist Updat 2008;11:99-109.

12. Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85:221-8.

13. Lyden D, Hattori K, Dias S, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001;7:1194-201.

14. Hirschi KK, Ingram DA, Yoder MC. Assessing identity, phenotype, and fate of endothelial progenitor cells. Arterioscler Thromb Vasc Biol 2008;28:1584-95.

15. Timmermans F, Van Hauwermeiren F, De Smedt M, et al. Endothelial outgrowth cells are not derived from CD133+ cells or CD45+ hematopoietic precursors. Arterioscler Thromb Vasc Biol 2007;27:1572-9.

16. Batchelor TT, Sorensen AG, di Tomaso E, et al. AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. Cancer Cell 2007;11:83-95.

17. Duda DG, Cohen KS, di Tomaso E, et al. Differential CD146 expression on circulating versus tissue endothelial cells in rectal cancer patients: implications for circulating endothelial and progenitor cells as biomarkers for antiangiogenic therapy. J Clin Oncol 2006;24:1449-53.
18. Willett CG, Boucher Y, di Tomaso E, et al. Direct evidence that the VEGF-specific antibody bevacizumab has antivasular effects in human rectal cancer. Nat Med 2004;10:145-7.

19. Vroling L, Yuana Y, Schuurhuis GJ, et al. VEGFR2 expressing circulating (progenitor) cell populations in volunteers and cancer patients. Thromb Haemost 2007;98:440-50.

20. Delorme B, Basire A, Gentile C, et al. Presence of endothelial progenitor cells, distinct from mature endothelial cells, within human CD146+ blood cells. Thromb Haemost 2005;94:1270-9.

21. Blann AD, Woywodt A, Bertolini F, et al. Circulating endothelial cells. Biomarker of vascular disease. Thromb Haemost 2005;93:228-35.

22. Clarke LA, Shah V, Arrigoni F, et al. Quantitative detection of circulating endothelial cells in vasculitis: comparison of flow cytometry and immunomagnetic bead extraction. J Thromb Haemost 2008;6:1025-32.

23. Wu H, Chen H, Hu PC. Circulating endothelial cells and endothelial progenitors as surrogate biomarkers in vascular dysfunction. Clin Lab 2007;53:285-95.

24. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205-16.

25. Strijbos MH, Kraan J, den Bakker MA, et al. Cells meeting our immunophenotypic criteria of endothelial cells are large platelets. Cytometry B Clin Cytom 2007;72:86-93.

26. Faivre S, Demetri G, Sargent W, Raymond E. Molecular basis for sunitinib efficacy and future clinical development. Nat Rev Drug Discov 2007;6:734-45.

27. Gabbianelli M, Pelosi E, Montesoro E, et al. Multi-level effects of flt3 ligand on human hematopoiesis: expansion of putative stem cells and proliferation of granulomonocytic progenitors/monocytic precursors. Blood 1995;86:1661-70.

28. Broxterman HJ, Georgopapadakou NH. Anticancer therapeutics: a surge of new developments increasingly target tumor and stroma. Drug Resist Updat 2007;10:182-93.

29. Shaked Y, Ciarrocchi A, Franco M, et al. Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. Science 2006;313:1785-7.

30. Vroling L, van der Veldt AM, de haas RR, et al. CD34bright/CD133neg candidate circulating endothelial progenitor cells (ccEPCs) are a potential biomarker during treatment with sunitinib or bevacizumab. AACR Meeting Abstracts 2008;2008:4956.

31. Purhonen S, Palm J, Rossi D, et al. Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor growth. Proc Natl Acad Sci U S A 2008;105:6620-5.

32. Goon PK, Boos CJ, Lip GY. Circulating endothelial cells: markers of vascular dysfunction. Clin Lab 2005;51:531-8.

33. Woywodt A, Blann AD, Kirsch T, et al. Isolation and enumeration of circulating endothelial cells by immunomagnetic isolation: proposal of a definition and a consensus protocol. J Thromb Haemost 2006;4:671-7.

34. Widemann A, Sabatier F, Arnaud L, et al. CD146-based immunomagnetic enrichment followed by multiparameter flow cytometry: a new approach to counting circulating endothelial cells. J Thromb Haemost 2008;6:869-76.

35. Palmowski M, Huppert J, Hauff P, et al. Vessel fractions in tumor xenografts depicted by flow- or contrast-sensitive three-dimensional high-frequency Doppler ultrasound respond differently to antiangiogenic treatment. Cancer Res 2008;68:7042-9.
36. Zhou Q, Guo P, Gallo JM. Impact of angiogenesis inhibition by sunitinib on tumor distribution of temozolomide. Clin Cancer Res 2008;14:1540-9.

37. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 2005;307:58-62.

38. Re F, Zanetti A, Sironi M, et al. Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. J Cell Biol 1994;127:537-46.

39. Schuurhuis GJ, Muijen MM, Oberink JW, et al. Large populations of non-clonogenic early apoptotic CD34-positive cells are present in frozen-thawed peripheral blood stem cell transplants. Bone Marrow Transplant 2001;27:487-98.

40. Jacques N, Vimond N, Conforti R, et al. Quantification of circulating mature endothelial cells using a whole blood four-color flow cytometric assay. J Immunol Methods 2008;337:132-43.

41. Gavard J, Gutkind JS. VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. Nat Cell Biol 2006;8:1223-34.

42. Xiao K, Garner J, Buckley KM, et al. p120-Catenin regulates clathrin-dependent endocytosis of VE-cadherin. Mol Biol Cell 2005;16:5141-51.

43. Case J, Mead LE, Bessler WK, et al. Human CD34(+)AC133(+)VEGFR-2(+) cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors. Exp Hematol 2007;35:1109-18.

44. Timmermans F, Plum J, Yoder MC, et al. Endothelial progenitor cells: Identity defined? J Cell Mol Med 2008;In press.

45. Aicher A, Rentsch M, Sasaki K, et al. Nonbone marrow-derived circulating progenitor cells contribute to postnatal neovascularization following tissue ischemia. Circ Res 2007;100:581-9.

46. Barber CL, Iruela-Arispe ML. The ever-elusive endothelial progenitor cell: identities, functions and clinical implications. Pediatr Res 2006;59:26R-32R.

47. DePrimo SE, Bello CL, Smeraglia J, et al. Circulating protein biomarkers of pharmacodynamic activity of sunitinib in patients with metastatic renal cell carcinoma: modulation of VEGF and VEGF-related proteins. J Transl Med 2007;5:32.

48. Motzer RJ, Michaelson MD, Redman BG, et al. Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. J Clin Oncol 2006;24:16-24.

49. Ebos JM, Lee CR, Christensen JG, Mutsaers AJ, Kerbel RS. Multiple circulating proangiogenic factors induced by sunitinib malate are tumor-independent and correlate with antitumor efficacy. Proc Natl Acad Sci U S A 2007;104:17069-74.

50. Mancuso MR, Davis R, Norberg SM, et al. Rapid vascular regrowth in tumors after reversal of VEGF inhibition. J Clin Invest 2006;116:2610-21.

51. Shojaei F, Ferrara N. Role of the microenvironment in tumor growth and in refractoriness/resistance to anti-angiogenic therapies. Drug Resist Updat 2008;11:219-30.

52. van Cuijisen H, van der Veldt AM, Vroling L, et al. Sunitinib-induced myeloid lineage redistribution in renal cell cancer patients: CD11c+ dendritic cell frequency predicts progression-free survival. Clin Cancer Res 2008;14:5884-92.

53. Motzer RJ, Bacik J, Murphy BA, Russo P, Mazumdar M. Interferon-alfa as a comparative treatment for clinical trials of new therapies against advanced renal cell carcinoma. J Clin Oncol 2002;20:289-96.
