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ORIGINAL PAPER

Increased numbers of small circulating endothelial cells in renal cell cancer patients treated with sunitinib

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Abstract Mature circulating endothelial cell (CEC) as well as endothelial progenitor 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 CECs 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 ($CD45^{neg}/CD34^{bright}$), HPCs ($CD45^{dim}/CD34^{bright}$), and monocytes in the blood of 24 renal cell cancer (RCC) patients receiving 50 mg/day of the multi-targeted 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 $CD34^{bright}$ HPCs and monocytes decreased during treatment, $CD34^{bright}$ 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 $CD34^{bright}$ 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.

Keywords Circulating endothelial (progenitor) cells · Renal cell cancer patients · Sunitinib · VEGF · Erythropoietin

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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 progenitor (CEPs) cells have been suggested as potential pharmacodynamic or predictive biomarker in tumor patients [11]. CEPs were first described by Asahara et al. [12], who introduced the concept of circulating, bone marrow-derived endothelial progenitor cells, contributing to adult vasculogenesis. Later, Lyden et al. [13] have demonstrated that both $VEGFR2^{pos}$-circulating endothelial cells as well as $VEGFR1^{pos}$-myeloid, monocytic cells contributed to tumor vascularization. Recently, the source of highly proliferative endothelial outgrowth cells (EOCs) has been identified in $CD34^{pos}/CD45^{neg}/CD133^{neg}$ 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 $CD45^{neg}/CD34^{bright}/CD133^{neg}/VEGFR2^{pos}$ 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) when compared with 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 $CD45^{dim}/CD34^{bright}/CD133^{+}$ 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 1, PB was taken on four 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 with the sum calculated in follow-up scans. Progression was defined based on 20% increase in the sum of the target lesions or clear clinical evidence of progressive disease (PD), and a 20% decrease in 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 1st, 2008.

HPCs, CECs, and plasma monitoring

At the time of blood sampling, the first 2 ml of blood was discarded and blood for flow cytometric enumerations was processed within 2–4 h. 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 milliliter of full blood was used for the measurement of CECs and HPCs, based on CD45 and

CD34 marker expression and expressed as number per milliliter, 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 $CD34^{bright}$ 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 per milliliter 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 using 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 PB samples and used as a reference to identify the $CD45^{neg}/CD34^{bright}/CD133^{neg}$ 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 SD plus PR. PFS and OS were calculated with the Kaplan–Meier method and tested with the log rank test. Values of $P \leq 0.05$ (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 Tables 1 and 2.

Two out of twenty-three patients could not be evaluated for treatment response because of early discontinuation due to sunitinib-related side-effects. Of the 21 evaluable

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 [53]		
Favorable risk	3	13
Intermediate risk	15	65
Poor risk	5	22
Best response to sunitinib treatment[a]		
Partial response	4	17 (19)
Stable disease	11	48 (52)
Progressive disease	6	26 (29)
No evaluation[b]	2	7 (–)
Progression-free survival[c]	8.0 (1.1–19.3)	–
Survival[d]	12.7 (1.4–23.2)	–

MSKCC, Memorial Sloan-Kettering Cancer Center

[a] 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]

[b] Two out of 23 patients could not be evaluated for treatment response because of early discontinuation due to sunitinib related side-effects

[c] 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

[d] 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

Table 2 Patients characteristics and best response to sunitinib

Patient no.	Age (years)	Sex	RCC type	Prior treatment	Response[a]	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

RCC, renal cell cancer; F, female; M, male; PR, partial response; SD, stable disease; PD, progressive disease; PFS, progression-free survival

[a] According to response evaluation criteria in solid tumors

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 7.9×10^6 to 6.9×10^6 cells/ml on C1D14 ($n = 23$; $P = 0.002$) and a further decrease on C1D28 (from median pre-treatment 7.9×10^6 to 4.4×10^6 cells/ml, $n = 15$; $P = 0.001$), which partly reverted after 2 weeks of rest (from median pre-treatment 7.7×10^6 to 4.8×10^6, $n = 15$; $P = 0.001$). 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. 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^{bright} populations: CECs and HPCs

Two populations of CD34^{bright} 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^{neg} and CD133^{neg}; HPCs are CD45^{dim} and are largely CD133^{pos} (Fig. 2a, b). Moreover, CECs have 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^{bright} CD45^{neg} and CD34^{bright} CD45^{dim} 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

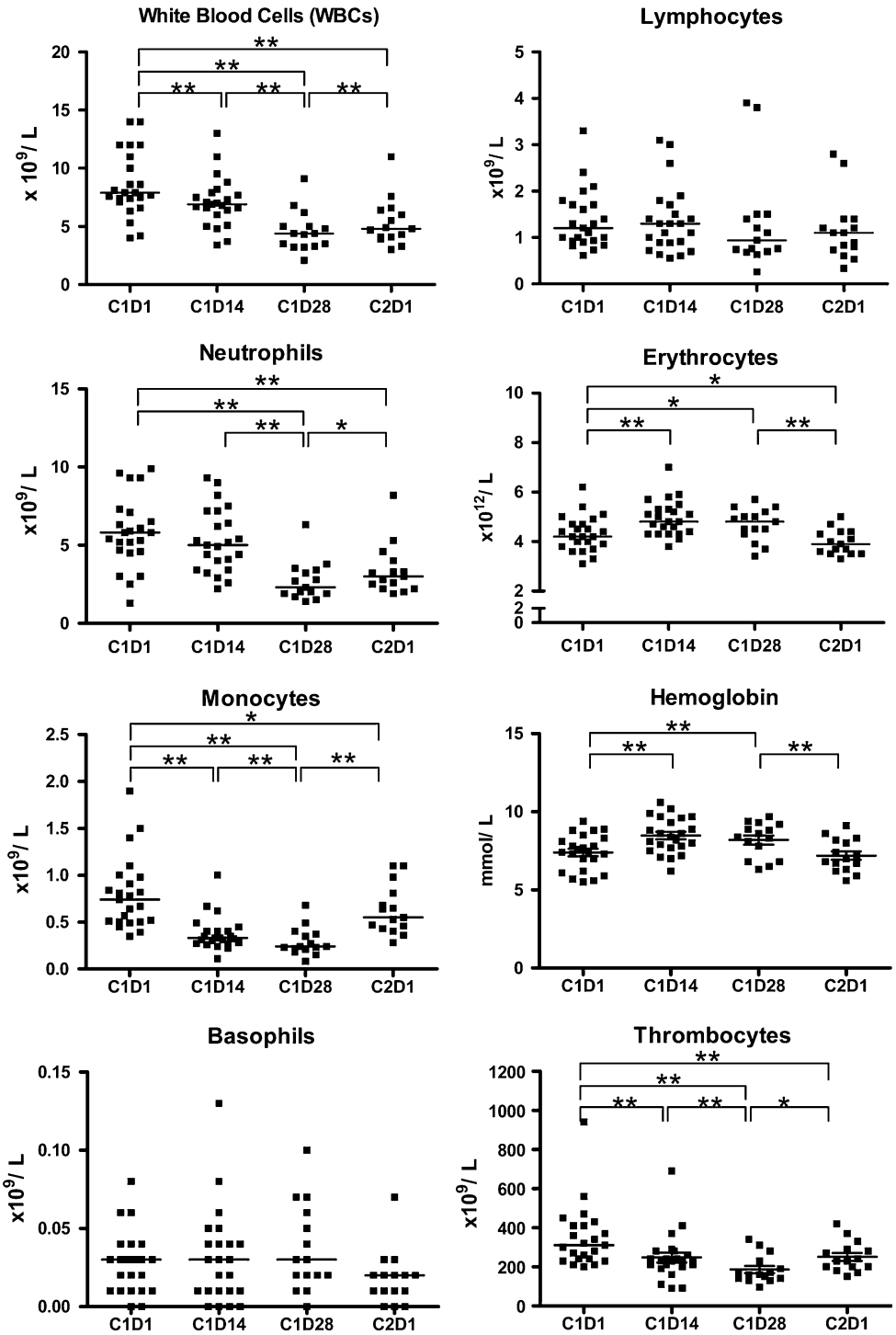

Fig. 1 Blood cell count and hemoglobin during treatment with sunitinib. Median (range) values are shown. Timepoints of measurement: *C1D1*, cycle 1 day 1 ($n = 23$); *C1D14*, cycle 1 day 14 ($n = 23$); *C1D28*, cycle 1 day 28 ($n = 15$); *C2D1*, cycle 2 day 1 before start of the second cycle ($n = 15$). Wilcoxon Signed rank test, * $P < 0.01$, ** $P < 0.05$

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 $CD45^{dim}/CD34^{bright}$ HPCs (<1%). In addition, the EPO receptor was evaluated on CECs of five sunitinib-treated patients and was found 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 ($CD45^{neg}/CD34^{bright}/7\text{-}AAD^{neg}$) and HPCs ($CD45^{dim}/CD34^{bright}/$

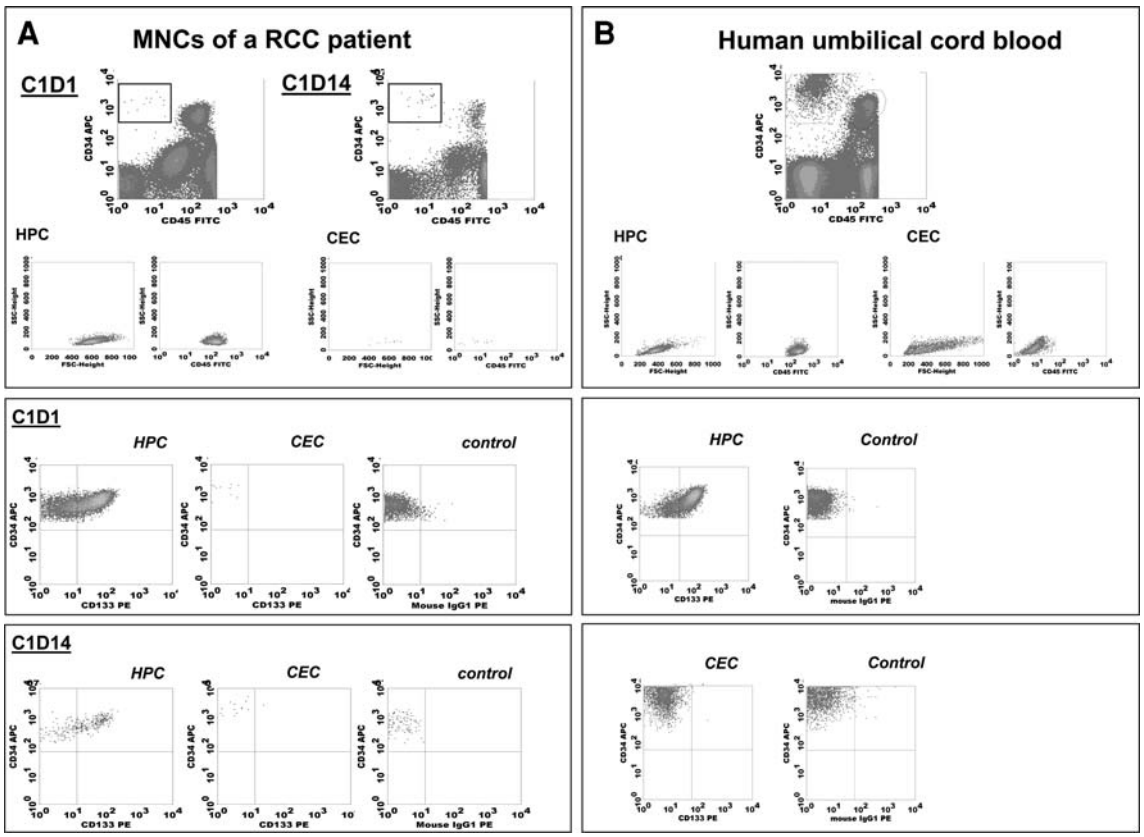

Fig. 2 Detection of hematopoietic progenitor cells (*HPCs*) and circulating endothelial cells (*CECs*) using four-color flow cytometry. The mononuclear(MNC)-fraction of a renal cell cancer (RCC) patient on *C1D1* and *C1D14* (**a**) and human umbilical cord blood (**b**). In the *upper panel CD45*, *CD34* expression, size and granularity is shown for *HPCs* and *CECs*. *HPCs* measured as $CD45^{dim}/CD34^{bright}$ and *CEC*s measured as $CD45^{neg}/CD34^{bright}$ (*see box*). *Second and third panel* is showing *CD133* expression for both *HPCs* and *CECs* when compared with the isotype control

7-AAD^{neg}) 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 (1,350 cells/ml, range 305–5,351). 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 1,350 to 372 cells/ml on C1D14 ($n = 23$; $P < 0.001$) and from pre-treatment 1,567 to 409 cells/ml on C1D28 ($n = 14$; $P = 0.001$). Both cell populations returned to values close to the pre-treatment 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 treated 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, 102% increase in CECs numbers was observed after 2 weeks of treatment, whereas the HPCs showed a 65% decrease (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 tenfold 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 remained above baseline level at C2D1. Albumin concentrations determined in a larger group of

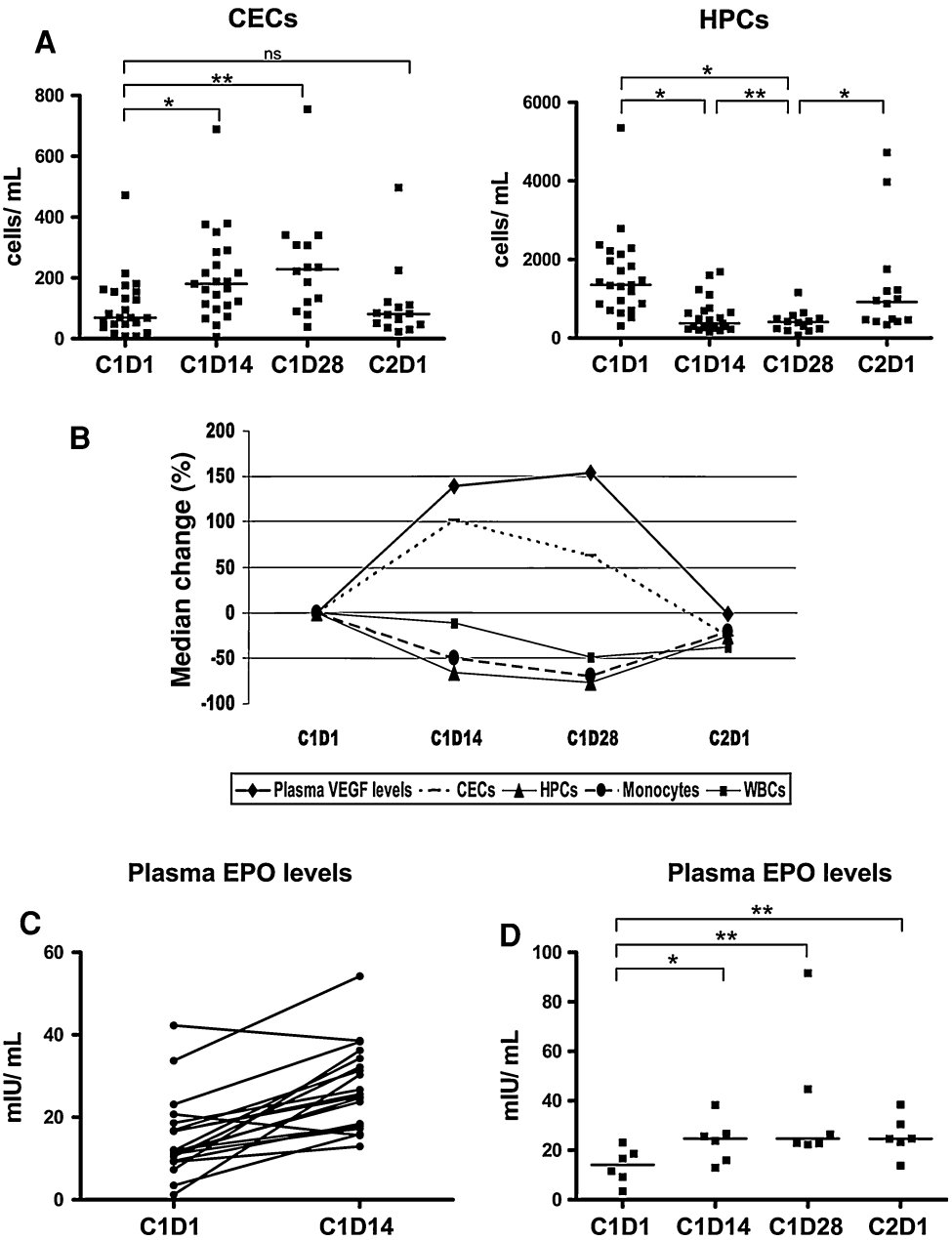

Fig. 3 Circulating endothelial cells (*CECs*), total hematopoietic progenitor cells (*HPCs*), erythropoietin (*EPO*) levels, and changes of *VEGF* levels in comparison with changes of blood cells during treatment with sunitinib. **a** Frequencies of CECs/ml and viable HPCs/ml are shown before (*C1D1*, $n = 23$), 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 (**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

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. Seventeen of all patients had clear cell RCC, of which 14 showed CB. PD 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 when compared with 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 $CD45^{neg}/CD34^{bright}$ CECs, previously shown to be $CD31^{pos}/CD105^{pos}/CD146^{pos}/VEGFR2^{pos}/CD133^{neg}$ [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.

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 PD [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 in $CD45^{neg}/CD34^{bright}$ CECs during sunitinib treatment. The CEC population in PB is a rare cell population [20], which is increased two to three-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 twofold increase in CEC levels (without decrease in HPC numbers) in a group of bevacizumab plus erlotinib, but not erlotinib-single agent treated NSCLC patients (L. 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 in 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 $CD45^{neg}$ and $CD34^{bright}$, 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. In support of this explanation, several data suggest that sunitinib might selectively prune immature nascent tumor neovessels not yet adequately stabilized by pericyte 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 $CD45^{neg}$/$CD34^{bright}$ CECs have endothelial progenitor (CEP) characteristics, such as those recently ascribed to $CD45^{neg}$/$CD34^{bright}$/$CD133^{neg}$ 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 $CD45^{neg}$/$CD34^{bright}$ 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 priory the presence of endothelial progenitor cells, capable of highly proliferative outgrowth, since the $CD34^{bright}$/$CD45^{neg}$/$CD133^{neg}$ cell population from cord blood, which is the source of late EOCs, also contained up to 60% apoptotic cells (F. Timmermans, personal communication). This lends support to the idea that the EPCs or EOC precursors circulating in human PB 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. [49], may reflect a direct or indirect physiological response to receptor inhibition by sunitinib. Indeed, we found also a prominent increase in EPO during the first cycle of sunitinib, consistent with the findings of Ebos et al. [49] in sunitinib-treated mice. 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 $CD34^{bright}$/$CD45^{neg}$ CECs, separated from $CD34^{bright}$/$CD45^{pos}$ 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 $CD34^{bright}$ CECs and $CD34^{bright}$ HPCs counts change in opposite directions by sunitinib; monocytes and HPC decrease and CECs increase. $CD34^{bright}$/$CD133^{neg}$ CECs might be detached ECs and reflect sunitinib anti-vascular effects or might include CEPs, which are potential targets.

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