Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients

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Background: There is accumulating evidence from preclinical studies that circulating endothelial cells (CECs) play an important role in neovascularization and tumor growth. The role of CECs in human cancer progression is sparsely investigated. We therefore analyzed CECs in peripheral blood of cancer patients. In addition, we correlated CEC levels in these patients with plasma levels of cytokines that are known to mobilize CECs in experimental models.

Patients and methods: Viable CECs were isolated, quantified and cultured from cancer patients’ whole blood by using magnetic beads coupled to an antibody directed against CD146, a pan-endothelial marker. Viable cells were visualized by calceinAM staining. Positive staining for specific endothelial cell markers [i.e. von Willebrand factor, CD31, vascular endothelial cell growth factor (VEGF) receptor-2] was used to confirm the endothelial phenotype.

Results: Cancer patients with progressive disease (95 patients) had on average 3.6-fold more CECs than healthy subjects (46 patients, \( P < 0.001 \)). Patients (17) with stable disease had CEC numbers equal to that circulating in healthy subjects (\( P = 0.69 \)). A subset of in vitro cultured CECs incorporated into endothelial layers and formed colonies. Plasma levels of cytokines that are thought to mobilize CECs from the bone marrow [VEGF, placental growth factor, stromal cell derived factor 1\( \alpha \) and stem cell factor (71 patients)] did not correlate with CEC amounts. The levels of viable CECs in cancer patients were modified by granulocyte colony-stimulating factor treatment and chemotherapy.

Conclusion: In progressive cancer patients, the amount of CECs is increased. These CECs are viable and may contribute to vessel formation. The number of CECs is influenced by anticancer treatment.

Key words: angiogenesis, cancer, circulating endothelial cells

Introduction

The role of angiogenesis in tumor growth is well-established [1]. New blood vessels are formed by endothelial cells (ECs) derived from pre-existing vessels in the tumor’s microenvironment. Recently it has been shown that bone marrow derived endothelial progenitor cells (EPCs) and hematopoietic stem cells (HSCs) mobilized by tumor- or ischemia-induced signals may also contribute to neovascularization through a process called vasculogenesis [2, 3]. In animal models EPCs were shown to be incorporated in areas of new vessel formation [4] and the combination of HSCs and EPCs was essential for tumor growth [5].

In patients, an increase in circulating endothelial cells (CECs) has been associated with several clinical syndromes (myocardial infarction [6], infectious vasculitis [7], ANCA-associated vasculitis [8], sickle cell crisis [9], kidney transplant rejection [10]) or interventions (CABG, burn injury [11], VEGF165 gene therapy [12], statin treatment [13]). In contrast, a decrease in CEC numbers is found in patients with increasing cardiovascular risk profile [14], in patients at risk for coronary artery disease [15] or in patients with type 2 diabetes mellitus [16]. In cancer patients, Mancuso et al. [17] detected increased amounts of CECs using flow cytometry. CEC numbers decreased in patients with lymphoma after complete remission following chemotherapy, and after mastectomy in breast cancer patients.

It is generally believed that ECs appear in the peripheral blood either due to release from the bone marrow in response to tissue ischemia regulated through cytokines [5, 18], or due to shedding from activated or damaged (tumor) vessels [19]. It has yet to be elucidated whether CECs are mobilized in a similar fashion in human subjects.

Here we report a quantitative and functional analysis of viable CECs obtained from the peripheral blood of cancer patients. We show that levels of CECs are increased in patients with progressive disease. CEC subpopulations are able to incorporate into endothelial monolayers. In addition, we show that CEC levels are modulated by chemotherapy and granulocyte colony-stimulating factor (G-CSF). We did not find a correlation between the number of CECs and the levels of vascular endothelial cell growth factor (VEGF), placental growth factor (PIGF), stromal cell derived factor
1α (SDF-1α) or stem cell factor (SCF), cytokines that mobilize EPCs. Whether CECs may serve as a target for anticancer therapy warrants further study.

Patients and methods

Patients

After obtaining informed consent, blood samples were collected from patients visiting the outpatient clinic of the Department of Medical Oncology or patients hospitalized in the University Medical Center in Utrecht, The Netherlands. Unless otherwise stated, patients did not receive concurrent anticancer therapy. Progressive disease (95 patients) was defined as radiologically documented progressive cancer (>25% increase in tumor size comparing tumor size prior to and after the blood sampling date), or as clear clinical evidence of disease progression. Tumor types included head and neck cancer (10 patients), colon (13 patients), prostate (25 patients), gastric (three patients), esophagus (three patients), ren cell (six patients), breast (10 patients), melanoma (three patients), ovarian (five patients), cervix (two patients), carcinoid (three patients), glioma (two patients), and 10 patients with other tumor types. Thirty of the progressive cancer patients had received prior cytotoxic chemotherapy; mean chemotherapy to sampling was 335 days, median 113 days, range 1–1718 days. Tumor types from patients with stable disease (17 patients) included renal cell (three patients), prostate (three patients), colon (two patients), adenoid cystic (two patients), thyroid (three patients) and four patients with other tumor types. Stable disease was defined as no increase in tumor size on radiological follow-up when comparing tumor size prior to and after the blood sampling date. In prostate cancer patients, tumor stratification of stable or progressive disease was also guided by prostate-specific antigen level. Ten of the stable cancer patients had received prior cytotoxic chemotherapy; mean time from chemotherapy to sampling was 397 days, median 103 days, range 7–1722 days. Forty-six healthy volunteers served as controls.

Isolation of CECs from whole blood

The isolation of CECs was performed according to a previously reported and validated methodology [6–9, 20]. Briefly, to isolate endothelial cells, peripheral blood was incubated with magnetic beads (Dynal M450 IgG, Dynal AS, Oslo, Norway) that had been previously conjugated to a monoclonal antibody directed against CD146, Sendol (Kordia Life Sciences, Leiden, The Netherlands). Sendol specifically recognizes all lineages of endothelial cells (not hematopoetic or epithelial cells) in peripheral blood of human subjects [21]. CD146 is a cell surface receptor involved in cell migration and maintenance of tissue organization [22]. When human blood samples were spiked with human umbilical vein endothelial cells (HUVECs) these cells could be retrieved in excess of 90% with a clear preservation of cell morphology. Dynabeads without secondary antibody did not bind cells. Peripheral blood from subjects was drawn in a siliconized tube containing EDTA 1 mg/ml by venipuncture. The first 3–5 ml were discarded to avoid contamination of ECs traumatically released from the punctured vessel wall. Whole blood (1 ml) was diluted 1:3 with NaCl 0.9% and incubated for 30 min with 20 µl (2.8 × 10⁴) antibody-coupled magnetic beads at 4°C on a roller bench. Unbound cells were removed by magnetic separation using a MPC-L magnet (Dynal AS). The beads-bound cell fraction was rinsed with phosphate-buffered saline–bovine serum albumin (PBS–BSA) 0.1%. To quantify viable ECs the isolated cells (in a final volume of 100 µl medium containing 20% fetal bovine serum) were fluorescently stained with CalceinAM (Molecular Probes, Eugene, OR, USA), and quantified in a Nageotte hemocytometer using a fluorescence microscope (Leica, Rijswijk, the Netherlands). CECs were clearly distinguishable from other cells by their distinct fluorescence, the binding of ≥10 beads, and a size of 20–50 µm. Reported CEC numbers are the mean of a duplicate quantification. Selected aliquots of beads-bound CECs were cytopsin (for 5 min at 500 r.p.m.) on glass slides, fixed with acetone and dried for >4 h. Subsequent phenotypic analysis was carried out with the primary antibodies against CD31 (PECAM, Santa Cruz Biotechnology, Santa Cruz, CA, USA), von Willebrand factor (Dako, Glostrup, Denmark), and VEGF-receptor 2 (Santa Cruz Biotechnology). For fluorescent staining, secondary antibodies conjugated to TRITC (Dako) or FITC (Southern Biotechnology Ass. Inc., Birmingham, AL, USA) were used. Slides were mounted with vectashield (Vector Laboratories, Burlingame, CA, USA) for maintenance of fluorescence. Beads-bound HUVECs were used as positive control.

Outgrowth of CECs

When attempting to monoculture CD146⁺ cells, no growth was seen. Therefore, an endothelial feeder layer was used for co-culture of CECs to provide a permissive environment for attachment and outgrowth. To be able to distinguish between the CECs and the feeders, CECs were fluorescently labeled with CFDA-SE (carboxy-fluorescein diacetate succinimidyl ester; Molecular Probes, Leiden, The Netherlands) prior to adding them to the feeders. CFDA-SE is a marker that is used for cell tracing [23, 24]. This dye is retained within cells after cell division, making it an excellent marker for follow-up of mixed cell cultures. Labeling was performed according to the manufacturer’s protocol. To provide a feeder layer for the isolated CECs, HUVECs or HMEC₁ (human dermal microvascular endothelial cells [25]) were used. The CD146⁺ cells isolated from peripheral blood 1 ml (typically 100–1000 cells) were co-cultured with HUVECs or HMEC₁ (7 × 10⁴/ml) in a six-well plate (Costar, Cambridge, MA, USA) pre-coated with fibronectin (12.5 µg/ml; Sigma-Aldrich BV, Zwijndrecht, The Netherlands). All EC cultures were performed in EGM-2 endothelial growth medium (Bio Whittaker, Walkersville, MD, USA). The CFDA-SE positive fluorescent CECs attached on the fibronectin-coated surface within a few hours between the feeder-layer endothelial cells and spread out. After 4–7 days, colonies of isolated cells were formed within the sparse feeder layer. Colonies were defined as clusters consisting of three or more green fluorescent attached spindle-shaped cells. Co-cultured isolated ECs and feeder ECs stained uniformly positive for Dil-ac-LDL (1,1’dioctadecyl-3,3’,3’-, tetramethylindo-carboxyamine-labeled acetylated low-density lipoprotein; Sanbio BV, Uden, The Netherlands), confirming the endothelial phenotype of expanding isolated cells.

Analysis of cytokine levels in plasma

In platelet-poor citrated plasma of patients and volunteers we measured levels of VEGF, PIGF, SCF and SDF-1α using commercially available ELISA kits (R&D Systems, Abingdon, Oxon, UK), following manufacturer’s guidelines. Samples were measured in duplicate. Obtained optical density (OD) values were plotted against standard curves generated on the ELISA plate with correlation coefficient of >0.99. Samples with an OD value below the lowest value on the standard curve were set at zero.

Statistical analysis

All results were analyzed by applying SPSS software (version 10.0.5). Statistical differences with a P value <0.05 were considered significant. Analysis was carried out, when appropriate, by Mann–Whitney test, (un)paired Student’s t-test and Pearson’s correlation analysis. Data are presented as mean ± standard error of the mean (SEM).

Results

Quantification of CECs

CECs isolated from peripheral blood of cancer patients using an endothelium-specific antibody (Sendol, antiCD146) coupled to Dynabeads were easily recognized and quantified (Figure 1B, inset). Characterization of the primary isolated CECs after cytopsin
preparation by immunofluorescence revealed that subsets of the CECs were positive for expression of the EC markers von Willebrand factor, VEGF receptor-2 and CD31 (Figure 1A). Mean levels of viable CECs were 3.3-fold higher in cancer patients (112 patients, mean 399 ± 36 CEC/ml) than in healthy volunteers (46 patients, mean 121 ± 16 CEC/ml; P = 0.001 by Mann–Whitney test). Because cancer patients had variable levels of CECs we determined whether disease progression was associated with an increase in CECs. Patients with progressive disease (95 patients, 438 ± 65 CEC/ml) had more CECs as compared to healthy volunteers (46 patients, P <0.001 by Mann–Whitney test; Figure 1B). Patients with stable disease (17 patients, 179 ± 61 CEC/ml; Figure 1B) showed amounts of CECs comparable to healthy volunteers (P = 0.69 by Mann–Whitney test). Longitudinal follow-up of patients not receiving anticancer treatment yielded a variation of ~30% in CEC levels. No difference in CEC amounts was found between male (319 ± 51 CEC/ml) and female subjects (241 ± 28 CEC/ml, P = 0.18 by t-test). There was a significant difference in age between volunteers and cancer patients (average 38 years in volunteers versus 57 years in patients; P <0.001 by t-test), but there was no change in CEC number with increasing age (Pearson’s correlation coefficient r = 0.089, P = 0.182). When using 250 CEC/ml as a cut-off value, CECs had a predictive value for progressive cancer of 92%. Sensitivity of the test was 44%, and specificity 91%. In the studied progressive cancer patients CECs were increased in all different tumor types (Figure 1C), reaching statistical significance in five out of six reported tumor types (tested by Mann–Whitney test).

Cytokines and CECs

We next addressed the question whether levels of cytokines that are known to be involved in the mobilization of progenitor cells from the bone marrow correlated with CECs. From 71 subjects (51 cancer patients and 20 healthy volunteers) plasma levels were measured for VEGF, PIGF, SDF-1α and SCF. Cancer patients had increased plasma levels of PIGF compared to healthy volunteers (cancer patients: 31.2 ± 7.5 pg/ml versus volunteers: 5.2 ± 2.9 pg/ml, P = 0.01 by Mann–Whitney test). Other tested cytokines showed no differences between cancer patients and volunteers. Levels of VEGF, PIGF, SDF-1α and SCF did not significantly correlate with CEC levels in tested individuals.

Outgrowth of CECs in culture

After quantification we assessed whether these viable CECs were functional. CECs adhered within 4–6 h to fibronectin-coated

Figure 1. (A) Average percentage of circulating endothelial cells (CECs) that stain positive by immunofluorescence for endothelial markers after isolation with antiCD146-coupled magnetic bead isolation. vWf, von Willebrand factor; VEGF-R2, vascular endothelial growth factor receptor-2. (B) CEC amounts in peripheral blood of cancer patients with progressive disease (PD) or stable disease (SD) and healthy volunteers (volunteer). (Inset) Appearance of CECs under fluorescence microscope. Small particles attached to cells are immunomagnetic beads used for isolation. A colour version of this figure is available as supplementary data at Annals of Oncology on-line. (C) CEC amounts are increased in progressive cancer patients irrespective of primary tumor.
culture dishes in co-cultures with feeder-layer ECs. Four to 7 days after seeding, between 5 and 70% of the isolated CECs (recognized through CFDA-SE positivity) formed colonies (Figure 2A, B). Fluorescence was less bright in cells at the periphery of the colony, consistent with the property of fluorescent CFDA-SE to divide equally to two daughter cells after division. All colonies formed in culture were acLDL-DiI-positive (data not shown). This procedure was performed with CECs from 30 patients with similar results. The percentage of isolated CECs that formed colonies was strongly dependent on the donor. No increase in colony formation per seeded CEC could be assessed in patients with progressive disease (data not shown).

Chemotherapy and growth factor treatment affect the levels of CECs

To address the question of whether CEC levels could be modulated by chemotherapy, cancer patients were monitored for levels of CECs during leukopenia in the weeks after the chemotherapy (defined as \(<1 \times 10^9\) leukocytes/l). The CEC levels became almost undetectable in leukopenic patients (Figure 3A; seven patients, \(11 \pm 8\) CEC/ml, \(P=0.0001\) by Mann–Whitney for difference with healthy volunteers). Cytotoxic chemotherapy with taxanes (seven patients, 175 mg/m\(^2\), and one patient, 200 mg/m\(^2\)) resulted in rapid (2–4 h after infusion) increase in CEC numbers for seven out of eight tested patients. The average CEC number prior to therapy was \(24 \pm 6\) CEC/ml. After treatment, numbers increased to \(104 \pm 27\) CEC/ml (\(P=0.011\), by paired \(t\)-test).

We also analyzed CECs in four patients receiving chemotherapy with the support of G-CSF. Patients’ primary tumors were seminoma testis (one patient), non-seminoma testis (two patients) and small cell lung cancer (one patient). Prior to, and 3–8 days after, initiation of the G-CSF treatment CECs were quantified in the peripheral blood of these patients (Figure 3B). In three out of four patients CEC amounts increased 8- to 9-fold. The fourth patient manifested only a minor increase in the number of CECs after G-CSF treatment. G-CSF treatment of healthy individuals, treated to mobilize stem cells for allogeneic bone marrow transplantation, resulted in similar high CEC numbers (15 patients, mean \(1736 \pm 631\) CEC/ml, \(P<0.0001\) for difference with untreated healthy volunteers, by Mann–Whitney test).

**Discussion**

We show here that patients with progressive cancer display increased levels of ECs present in the peripheral blood when compared to patients with stable disease or healthy volunteers. These CECs were viable, with a subpopulation able to adhere to an endothelial layer and proliferate in a coloniform fashion. No correlation was found between CEC levels and plasma levels of cytokines that are thought to mobilize CECs from the bone marrow.

Isolated CECs probably originated from three sources: the mature blood vessels, the tumor vasculature and the bone marrow. Not all viable CD146-positive cells express detectable amounts of generally accepted endothelial markers. The putative mixed population of mature and progenitor cells may account for a variable expression of mature endothelial markers. A heterogeneous EC-specific antigen expression is also seen in freshly isolated endothelial precursor cells [26, 27]. However, the expression of CD146 in circulating cells is restricted to cells of endothelial origin [21], whereas other markers (CD31, acLDL-DiI, Ulex-lectin) may crossreact with other cells, such as monocytes [28]. To distinguish between mature and precursor ECs, CD133, a progenitor cell marker, can be used [3]. Interpretation of CD133 expression should be carried out with caution, because the marker is lost rapidly upon differentiation from an anchor-independent EPC released from the bone marrow to a CEC that is able to incorporate into a vessel. The viability and growth capacity of the CECs in our patients and the low expression of mature EC markers are compliant with the assumption that at least a subset is derived from a progenitor pool in the bone marrow.

Some of the CECs found in peripheral blood from cancer patients may be shed from the (tumor) vessel wall, as observed in vascular injury syndromes [6–8]. Tumor (endothelial) cells may be blood-borne by mechanical stress caused by chaotic blood flow and proteolysis of the subendothelial matrix to facilitate EC migration during tumor angiogenesis. This leads to exposure of tumor cells...
to the flowing blood, resulting in so-called mosaic vessels, which consist of both endothelial and tumor cells [19]. CEC shedding may also occur in distant non-tumor-derived vessels, e.g. due to activation of the quiescent ECs by tumor-induced increases of cytokine levels. The large variation in CEC outgrowth we observed on feeder endothelium may reflect the variable contribution of shed, non-proliferating versus mobilized, proliferating CECs in different cancer patients.

Not all progressive cancer patients have increased CEC numbers in their peripheral blood. The growth speed of the tumor and its vasculature, the number, location and size of potential metastases, and the residence time of CECs in the circulation may influence the presence of CECs in peripheral blood. Our data suggest that factors other than primary tumor type determine CEC numbers. Finally, risk factors for cardiovascular co-morbidity may also confound our results [14].

An important question is whether CECs may serve as a target for the treatment of cancer. The absolute number of CECs in the peripheral blood is low (about 1–10 ECs per 10⁶ mononuclear cells from full blood). Moreover, the contribution of bone marrow derived EPCs to tumor progression in humans is still controversial. However, preclinical models provide solid evidence that bone marrow derived CECs play a role in the maintenance and formation of blood vessels. EPCs can differentiate into mature ECs in response to a variety of angiogenic growth factors produced either by the tumor cells, vessel wall ECs, vascular non-ECs (pericytes, smooth muscle cells), inflammatory cells, bone marrow stromal cells, hematopoietic stem cells, or the EPCs themselves. A hallmark of EPCs is that they have a high proliferative potential [29]. EPCs have the potential to incorporate into regenerating vessels during ischemia [4, 30]. They have clinical significance in restoring circulation [27, 31] and in improving cardiac function after myocardial infarction [32]. Bone marrow derived endothelial and hematopoietic progenitor cells can fully support tumor growth, as shown in a bone marrow transplant model with Id1+/– Id3–/– compound mutant mice that lack stress angiogenesis and are tumor-resistant [5]. Id proteins are helix-loop-helix DNA binding proteins interfering with transcription factor binding. After receiving wild-type bone marrow or VEGF-mobilized stem cells, tumor (vessel) growth was completely restored in Id1+/– Id3–/– compound mutant mice, resulting in the death of the host. Because CECs home to sites of angiogenesis they make excellent vehicles for anticancer treatment. Mice transplanted with modified bone marrow cells (containing ECs) loaded with truncated soluble VEGF receptor-2 exhibited diminished tumor growth [33]. Furthermore, injection of human CD34+ cells (including EPCs) into NOD/SCID mice with non-Hodgkin’s lymphoma significantly increased tumor growth [34]. In a different model system selective tumor homing and subsequent anti-melanoma effect could be established by intravenous injection of ECs over-expressing interleukin-2 [35]. Promising reports with therapeutic injection of bone marrow cells or bone marrow stem cells in patients with critical limb ischemia [36] or myocardial ischemia [37, 38] provide initial evidence that also in human subjects therapeutic vasculogenesis may play a role. Our correlative data of CEC increase with tumor growth and CEC disappearance during leukopenia support a possible role for vasculogenesis in human cancer.

Reported absolute numbers of CECs in the literature vary considerably, ranging from 1 to 10 000 CECs per milliliter of peripheral blood [6, 8, 9, 17]. This is probably due to different definitions of CECs and the various techniques applied for the quantification of CECs. Consensus of definitions and quantification techniques is essential to allow comparisons.

In a murine model, Heissig et al. showed mechanisms by which endothelial progenitor cells and hematopoietic stem cells (HSCs) are mobilized into the peripheral blood [39]. Several plasma factors (VEGF, PIGF and SDF-1α) induce MMP-9-mediated SCF/KitL shedding from bone marrow stroma, essential for the migration of progenitor cells of endothelial and hematopoietic origin to the vascular niche of the bone marrow, subsequent differentiation, and mobilization into the circulation. In humans,

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**Figure 3.** (A) Leukopenia after chemotherapy results in almost complete disappearance of circulating endothelial cells (CECs), in comparison to healthy volunteers. (B) Granulocyte colony stimulating factor (filgrastim) treatment induces increased amounts of CECs in cancer patients receiving chemotherapy.
increased plasma levels of VEGF are reported to correlate with CEC numbers in peripheral blood (after CABG and burn injury [11], myocardial infarction [40] and in cancer patients [17]) but the other factors in plasma did not correlate with CEC mobilization [40] or were not tested. To see if this mobilization model could be applicable to our patient group we measured cytokines in the plasma. Although one of the factors that is known to mobilize stem/progenitor cells, PIGF, was elevated in cancer patients, no correlation was found between PIGF or any of the other factors tested and CECs. Several previously observed factors may account for these findings, such as a limited half-life of cytokines in the plasma [41] or variable correlations between local and systemic cytokine levels, resulting in absent [40, 42] or inverse [43] correlation between stimulus (cytokine) and effect (i.e. CECs) in peripheral blood.

CEC levels increased in the hour following infusion of cytotoxic taxane-based chemotherapy. One possible explanation for this phenomenon would be that taxanes interact with cytoskeletal microtubules in mitotic angiogenic ECs in the vessel wall [44], resulting in detachment of tumor ECs into the circulation. Several vascular targeting agents act through interference with the tubulin cytoskeleton [45]. These effects are apparent within 24 h. We are currently investigating the use of CECs as markers of vascular damage and treatment efficacy during treatment with tubulin interacting vascular targeting agents [46].

We found that in patients with active cancer, G-CSF treatment induced CEC increase, probably by mobilization from the bone marrow. These cells may home to sites of active angiogenesis, such as the tumor microenvironment. That colony-stimulating factor-induced EC homing is a biologically relevant phenomenon was shown in models for ischemia [30], tumor growth [4, 47] and graft fallout endothelialization [48], in which growth factor treatment resulted in increased vessel growth. Our findings indicate that the use of G-CSF in cancer patients may have biological effects beyond reducing neutropenia and its complications. Further studies are warranted to investigate the effect of G-CSF and subsequent mobilization of CECs, which may enhance tumor angiogenesis in cancer patients, potentially leading to worse prognosis.

In conclusion, our findings demonstrate that CECs are increased in progressive cancer patients. The CECs are viable and subsets have a capacity to proliferate. The level of CECs can be modified both positively and negatively depending on the agents used. Further studies are warranted to determine whether CECs may serve as a target for anticancer therapy or as a biosensor of anti-(vascular) cancer treatment response.

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