Assessing Tumor Angiogenesis: Increased Circulating VE-Cadherin RNA in Patients with Cancer Indicates Viability of Circulating Endothelial Cells

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ABSTRACT

No markers are currently available to indicate the angiogenic profile of a specific malignant disease nor to predict response to antiangiogenic therapies. Nevertheless, many different antiangiogenic drugs are presently being tested in many clinical trials, with an obvious scarcity of useful endpoints for treatment outcome beside survival. By means of a quantitative reverse transcription-PCR approach, we measured VE-cadherin (VE-C), Tie-2, vascular endothelial growth factor receptor 2 and CD133 RNA in the blood of 14 healthy controls, 3 pregnant women, and 84 newly diagnosed (or relapsed) cancer patients. Circulating VE-C RNA was increased in pregnant women and cancer patients (P = 0.0002). VE-C RNA was particularly increased in patients affected by hematological malignancies and decreased to normal values in patients achieving complete remission. Conversely, circulating RNA levels of other endothelial or progenitor cell-specific markers Tie-2, vascular endothelial growth factor receptor 2, and CD133 were not significantly increased in either pregnant women or cancer patients. Comparison of various surrogate angiogenesis markers indicated a switch toward increased plasma vascular endothelial growth factor (VEGF) levels, viable circulating endothelial cells, and circulating VE-C RNA levels in patients affected by hematological malignancies. Taken together, our data indicate that the quantitative evaluation of circulating VE-C RNA is a specific and highly promising tool with which to investigate the angiogenic phenotype of cancer patients.

INTRODUCTION

The addition of the antiangiogenic drug bevacizumab to chemotherapy has been recently associated with increased survival in patients affected by advanced colorectal cancer. In addition, this and other antiangiogenic drugs have demonstrated activity and are currently under clinical investigation in various cancer types (1–3). This notwithstanding, clinical markers describing the angiogenic profile of a tumor type and able to predict the response to antiangiogenic drugs are still scanty (3–5). Using flow cytometry, we have recently reported a significant increase of circulating endothelial cells (CEC) and circulating endothelial cell progenitors (CEP) in preclinical cancer models (6) and in breast cancer and lymphoma patients (7). However, flow cytometric CEC and CEP evaluation requires a complex four-color approach. To further investigate the relevance of circulating surrogate angiogenesis markers in cancer, we used real-time quantitative reverse transcription-PCR to study the expression of the endothelial-specific genes VE-cadherin (VE-C), Tie-2, and vascular endothelial growth factor receptor 2 (VEGFR2) and of the CEP-associated gene CD133 (8) in the peripheral blood of healthy controls, pregnant women, and newly diagnosed (or relapsed) cancer patients affected by hematological malignancies or solid tumors.

MATERIALS AND METHODS

Sample Collection and Patient Population. Following a protocol similar to that used for flow cytometric CEC evaluation (7), peripheral blood was collected in EDTA tubes through 21G needles in 14 healthy controls, 3 first-trimester pregnant women, and 84 newly diagnosed cancer patients. Among cancer patients, 32 had B-cell lymphoproliferative malignant diseases (18 high-grade, 14 low-grade), 11 had acute leukemia (7 acute myelogenous leukemia, 4 acute lymphocytic leukemia), 19 had stage III-IV breast cancer, 8 had hepatocellular carcinoma, 5 had advanced ovarian cancer, 4 had neuroendocrine cancer, and 5 had lung cancer. Patients bearing intravascular instrumentation were excluded from the study. Patients with lymphoproliferative malignant diseases (including a subset of 14 patients who achieved a complete response) were also evaluated after completing standard chemotherapy or chemotherapy plus anti-CD20 antibody therapy.

CECs and CEPs Evaluation by Flow Cytometry, CECs, CEPs, and plasma VEGF levels were evaluated in all patients as described previously (7). In brief, monoclonal antibodies including anti-CD34 to exclude hematopoietic cells, anti-CD31, -CD133, -PIH12, the apoptosis marker 7-aminoactinomycin D (9), and appropriate analysis gates were used to enumerate viable and apoptotic CECs and CEPs. Cell suspensions were evaluated after red cell lysis by a FACS-Calibur equipped with a second red-diode laser (BD Biosciences, San Jose, CA). After acquisition of at least 100,000 cells/blood sample, analyses were considered as informative when adequate numbers of events (i.e., >100, typically 3–400) were collected in the CECs enumeration gates. CECs were defined as negative for hematopoietic marker CD45, positive for endothelial markers PIH12 and CD31, and negative for the progenitor marker CD133. CEPs were depicted by expression of CD133.

Culture Assays. CECs and CEPs were also evaluated by culture assays in a subset of 12 healthy controls and 28 patients with lymphoproliferative malignancies. Blood samples were processed by ficoll and mononucleated cell preparations were cultured in Medium 200 (Cascade Biologicals, Portland, OR), supplemented by 20 ng/ml VEGF, 8 ng/ml epidermal growth factor, 2.4 ng/ml basic fibroblast growth factor, 8 μg/ml heparin, 0.8 μg/ml hydrocortisone, and 20% FBS (StemCell Technologies, Vancouver, BC) in plates precoated with fibronectin (Sigma, St. Louis, MO). Medium was partially changed on day 7 and 12, and nonadherent cell discarded. After 14-day culture in duplicate, in one plate endothelial cell (EC), colonies were scored by microscopy after low-density lipoprotein and lectin staining (10), and in the other plate, EC colonies were trypsinized and evaluated for CECs and CEPs by flow cytometry as we described previously (11). Comparative studies using scrapers, EDTA, or trypsin indicated that the expression of antigens used in these studies to depict EC (CD45, PIH12, and CD31) was not modified by trypsinization.

RNA Isolation and cDNA Preparation. Blood samples were lysed by NH4Cl to remove red cells and stored with guanidine isothiocyanate at −80°C for RNA analysis. Total RNA was extracted by the QIAamp RNA blood extraction kit (Qiagen, Chatsworth, CA). To generate cDNA, total RNA was treated with RNase-Free Dnase set (Qiagen) to remove any contaminating genomic DNA. The Dnase-treated RNA (100 ng) was then converted into cDNA by murine leukemia virus reverse transcriptase (Life Technologies, Inc., Bethesda, MD).

Quantitative Real-Time Reverse Transcription-PCR. Primers are reported in Table 1. VE-C and β-actin primers (6-carboxyfluorescein-VIC) were originally designed by the Primer Express software (Applied Biosystems, Applied Biosystems, Inc., Foster City, CA; www.appliedbiosystems.com). Primers were synthesized as previously described (12) and, in brief, targeted 117 to 120 bp fragments of the respective genes. Amplification reactions were set up as in Table 1. Comparative CT method was used to calculate relative gene expression levels (13). The expression level of an unknown sample was calculated as: 2−ΔΔCT, where ΔΔCT = ΔCTunknown sample – ΔCTcalibrator sample (the calibrator sample was from healthy controls), and ΔCT = CTgene of interest−CTβ-actin of the same sample. To normalize the results, we found that neitherTNFRSF1A nor CD34 could be used as internal controls.

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Primers for Tie-2, CD133, and VEGFR2 (SYBR green) were published elsewhere (12). For each PCR evaluation, 9 μl of cDNA (diluted 1:3 in nuclease-free water) or plasmid product (serial dilutions), 10 μl of Universal PCR Master Mix (Applied BioSystems), 250 nM forward and reverse primer, and 200 nM probe were added to a final volume of 20 μl. Amplification and detection were performed with the ABI Prism 7000 Sequence Detection Systems (Applied BioSystems). The thermal cycle used was 2 min at 50°C, 10 min at 95°C, and 50 cycles of 15 s denaturation at 95°C with 1 min annealing at 60°C. To assure the specificity of each primer set, amplicons generated from PCR reactions were evaluated for specific melting point temperatures using the first derivative primer melting curve software supplied by Applied BioSystems (Fig. 1).

RNA Quantification. The level of target gene expression was calculated after normalization of the β-actin level and presented as relative quantification (ΔΔCT value). Specificity of quantitative reverse transcription-PCR was confirmed by evaluation of human umbilical vein endothelial cells and microvascular endothelial cells and of 8 nonendothelial cancer cell lines representative of myeloid (U937) and lymphoid (Namalwa, Granta-519, MOLT-4, Jurkat, CEM, RAP1-EIO, Karpas 299). For the quantitative evaluation of VE-C (the sole gene found to be significantly enhanced in cancer patients) versus the reporter β-actin gene, we then used a calibration curve with plasmids containing the respective target sequences. Standard curves were obtained by serial dilution ranging from 10^6 to 10 molecules of a linearized plasmid obtained by cloning the target sequences into a PCR II TOPO vector (Invitrogen, Groningen, the Netherlands). The sensitivity of the assays was 25 and 10 copies for VE-C and β-actin, respectively.

Table 1. Primers used in the study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe Primer</th>
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<tbody>
<tr>
<td>VE-cadherin</td>
<td>5'-aagccctaccagcccaaagt</td>
<td>5'-ttgcggagatctgcaggac</td>
<td>5'-FAM-tgtgagaacgctgtccatggccag-TAMRA</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-tacccacatcgcaggtgctgct</td>
<td>5'-cagcggaaccgctcattgcca</td>
<td>5'-VIC-atgccctccccatgccatcct-TAMRA</td>
</tr>
<tr>
<td>CD133</td>
<td>5'-tgtagctgaaagcagctgact</td>
<td>5'-atatttcggctgactgtggt</td>
<td>5'-VIC-atgccctccccatgccatcct-TAMRA</td>
</tr>
</tbody>
</table>

FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; VEGFR2, vascular endothelial growth factor receptor 2.

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Results. Statistical comparisons were performed using the t test, ANOVA and linear regression when data were normally distributed and the nonparametric analyses of Spearman and Mann-Whitney when data were not normally distributed. Values of P < 0.05 were considered as statistically significant.

RESULTS

Circulating VE-C RNA Is Increased during Pregnancy and in Cancer Patients. Human umbilical vein endothelial cells and human umbilical vein endothelial cells and microvascular endothelial cells, but not cancer cell lines, expressed the EC genes VE-C, Tie-2, and VEGFR2. Circulating RNA levels of CD133, Tie-2, and VEGFR2 were not significantly increased in pregnant women or cancer patients, whereas circulating VE-C RNA was significantly increased in pregnant women and cancer patients before therapy (P < 0.001; Fig. 2).

The mean % VE-C/β-actin ratio was 0.2 ± 0.1 in controls and
10-fold increased (i.e., 2.1 ± 1.3) in cancer-bearing patients evaluated before therapy (*P = 0.0003; Fig. 3). In the subgroup of 18 patients who achieved a partial remission, the mean ratio was 0.9 ± 0.3 (*P = 0.01 versus healthy controls). In the subgroup of 14 patients who achieved a complete remission, the mean ratio was 0.5 ± 0.3 [i.e., similar to healthy controls (*P = 0.14) and significantly lower than cancer-bearing patients (*P = 0.0004)].

When evaluated before therapy, patients affected by lymphoproliferative malignancies or acute leukemia had mean ratios of 2.0 ± 1.1 and 3.1 ± 1.7, respectively (*P < 0.001 versus healthy controls; Fig. 4). The mean ratio of solid cancer patients evaluated before therapy was 0.7 ± 0.2, i.e., lower than that of patients affected by hematological diseases, albeit still significantly higher than healthy controls (*P = 0.03).

**VE-C RNA Levels Correlate with VEGF and CEC Viability.**

As shown by a three-dimensional plot, a switch to increased CEC, VEGF levels, and VE-C ratio was observed in patients affected by lymphoproliferative malignant diseases (Fig. 5). As shown in Fig. 6, the flow cytometric evaluation of the apoptosis marker 7-aminoactinomycin D indicated that CEC viability was significantly increased in cancer patients compared with healthy controls (*P < 0.01).

When culture assays were performed in 12 healthy controls and 28 cancer patients, a significant increase in EC colonies (and in EC colonies including CD133 + CEPs) was found in cancer patients compared with healthy controls and patients in complete remission (Fig. 7). A significant correlation was found between endothelial colonies and the number of viable (7-aminoactinomycin D-negative) CEC evaluated by flow cytometry (r = 0.43, *P = 0.04).

In apoptotic (starved) human umbilical vein endothelial cells, we observed a 2- to 300-fold reduction of VE-C RNA compared with viable cells. Thus, we correlated VE-C RNA and the number of viable CEC (enumerated by flow cytometry) in patients affected by hematological malignancies. This correlation was highly significant (r = 0.86, *P = 0.008). Along this line, in this subgroup of patients evaluated before therapy, a three-dimensional plot indicated a switch toward increased CEC numbers, increased CEC viability, and increased circulating VE-C RNA (Fig. 8).

**DISCUSSION**

Surrogate markers able to predict response to antiangiogenic drugs, alone or associated with other therapeutic strategies, would allow a more rationale design of clinical trials exploring this new class of antineoplastic drugs (3–4). We and others (6–7, 11–15) have reported that flow cytometric CEC evaluation is a promising tool to investigate the antiangiogenic activity of a given drug. However, antigen promiscuity between CEC and other circulating hematopoietic cells (8) requires a complex four-color approach for CEC enumeration. Moreover, flow cytometric quantification of this tiny cell population requires fresh samples and the acquisition of a many events. Culture assays of clonogenic CEP have also been proposed as a tool for the enumeration of vasculogenic endothelial progenitors involved in a neoplastic and non-neoplastic diseases (14, 16–17). However, this culture approach is cumbersome, less reproducible, and possibly more difficult to standardize than flow cytometry.

Using a quantitative reverse transcription-PCR approach for VE-C RNA evaluation, we have found the circulating levels of this endothelial-specific transcript to be significantly increased during pregnancy (a physiological condition associated with increased angiogenesis) and in cancer patients evaluated before therapy. The absence of VE-C expression in cancer cell lines suggests that the increase of circulating VE-C RNA in cancer patients is not attributable to aberrant expression by cancer cells. Circulating VE-C levels are significantly reduced (but still higher than healthy controls) in patients achieving a partial remission, and levels return to control values in patients...
Fig. 6. Different expression of the apoptosis marker 7AAD in CEC from healthy controls and cancer patients. A–F, a representative flow cytometry evaluation of CEC enumeration and viability. CEC, circulating endothelial cells; 7AAD, 7-aminoactinomycin D. A, the gate used to exclude platelets, dead cells, and debris. B, the negative controls. C and D, the gate used to exclude CD45+ hematopoietic cells. E, the gate (on CD45− cells) used to enumerate CEC (CD45− CD31+ P1H12+). F, the gate (on CD45− cells) used to investigate CEC viability according to 7AAD expression. G, the frequency of apoptotic CECs. Results are expressed as means ± 1SD; * means $P < 0.01$; ** means $P < 0.001$ versus controls. EC, endothelial cell; CEP, circulating endothelial cell progenitor.

Fig. 7. Evaluation of endothelial cell culture assays in healthy controls and cancer patients at diagnosis and after achieving complete remission. A–D, a representative flow cytometry evaluation of low-density lipoprotein staining (B), lectin staining (C), merged image (D), and debris (A). E–H, the flow cytometric evaluation of trypan blue-stained colonies. E, the FSC-SSC gate. F, the gate used to exclude hematopoietic cells expressing CD45. G and H, the frequency of cells expressing the EC markers CD31 and P1H12 and the CEP marker CD133. I, the frequency of endothelial colonies (including or not including ≥0.1% of CD133+ CEPs in healthy controls and cancer patients). Results are expressed as means ± 1SD; * means $P < 0.01$; ** means $P < 0.001$ versus controls. EC, endothelial cell; CEP, circulating endothelial cell progenitor.
achieving a complete remission. Circulating VE-C RNA levels were found to be significantly higher in patients affected by lymphoproliferative disorders (or acute leukemia) compared with solid tumors. This finding was not completely unexpected, because a number of recent observations have indicated that some lymphoproliferative disorders seem to be, at least in part, vasculogenesis-dependent (18–21). When other endothelial (Tie-2, VEGFR2) or CEP-specific (CD133) transcripts were evaluated, we did not find any significant increase in pregnant women or cancer patients compared with controls. It should be considered that Tie-2, VEGFR2, and CD133 (but not VE-C) are expressed at various levels by nonendothelial cell populations (e.g., megakaryocytes, myeloid cells, stem cells in marrow and brain) thus possibly masking changes in the endothelial cell population. Also, our findings might be attributable to characteristics inherent to our patient population, because a correlation between circulating CD133 transcripts and breast cancer progression has been reported recently by another group (12).

Quantitative VE-C RNA evaluation offers some distinct advantages over flow cytometric CEC enumeration and culture assays. VE-C RNA enumeration can be performed in a large series of frozen samples. Inter-laboratory standardization seems to be more easily achievable, and our data indicate an interesting correlation between VE-C RNA and CEC viability. On the other hand, flow cytometric CEC enumeration and culture assays can probably discriminate more accurately than VE-C RNA evaluation between cancer patients with a prevalent angiogenic (i.e., mature endothelial cell-driven) phenotype and patients with a prevalent vasculogenic (i.e., endothelial progenitor cell-driven) phenotype (8, 13, 19, 22–24). Thus, VE-C RNA, flow cytometric CEC and CEP enumeration, and culture assays will offer nonoverlapping clinical information, and a multifaceted evaluation of these surrogate markers will be of help to design patient-tailored therapies for neoplastic and non-neoplastic patients.

REFERENCES


Fig. 8. Three-dimensional plot indicating a switch toward increased CEC numbers and viability and increased circulating VE-cadherin RNA levels in patients affected by hematological malignancies. CEC, circulating endothelial cells.
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