Coagulation, angiogenesis and cancer

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Circulating vascular endothelial growth factor in cancer patients, is it a relevant biomarker?

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Chapter 10

Abstract

Background
Circulating vascular endothelial growth factor (VEGF) has widely been used as biomarker for angiogenic activity in cancer, based on unstandardized measurements in plasma and serum. We hypothesize that in most cancer patients ‘true’ circulating VEGF levels are low and unrelated to cancer load.

Methods
We determined VEGF levels in PECT medium, citrate plasma and in platelets in 16 healthy subjects, 18 patients with metastatic non-renal cancer (non-RCC) and 12 patients with renal cell carcinoma (RCC).

Findings
Circulating plasma VEGF levels were not different in non-RCC patients compared to controls if platelet activation was minimized during the harvest procedure. Elevated VEGF in citrate plasma samples in non-RCC patients were explained by platelet activation during blood harvesting, and by a two-fold increase in VEGF content of individual platelets (controls: 3.4 IU/10^6; non-RCC: 6.2 IU/10^6 platelets). Only in RCC patients true circulating VEGF levels were elevated (PECT plasma: 64 pg/ml vs. 21 pg/ml, RCC vs. non-RCC, respectively), while VEGF content of individual platelets was not higher in RCC than in non-RCC patients (RCC: 6.0 IU/10^6 platelets).

Interpretation
Our findings suggest that ‘true’ freely circulating VEGF levels are not elevated in most cancer patients. The previously reported elevated plasma VEGF levels in cancer appear to be due to release from activated platelets with increased VEGF content during the blood harvest procedure. Only in patients with renal cell cancer, a cancer type characterized by excessive VEGF production due to a specific genetic defect, circulating VEGF levels was truly elevated.
**Introduction**

In 1995, Folkman reviewed the emerging clinical applications of research on angiogenesis, which had taken two main directions in cancer patients; the quantification of angiogenesis for the use in diagnosis and prognosis, and the inhibition of angiogenesis to halt tumor growth. Vascular endothelial growth factor (VEGF) is an important angiogenic factor and is the prime regulator of both physiological and pathological angiogenesis. It is released by cancer cells, hypoxic cells and activated platelets and leukocytes. The target cells of VEGF are primarily vascular endothelial cells on which it has powerful mitogenic effects via high affinity receptors Flt-1 and KDR/Flk-1. As VEGF is soluble and a diffusible peptide secreted by tumors, the circulating level of angiogenic factors were proposed to reflect the overall angiogenic activity of malignancies. Hence in the past few years, there has been tremendous interest among researchers in studying the role of circulating levels of VEGF and other angiogenic factors as a reflection of the angiogenic activity, prognosis in cancer patients, for monitoring treatment response and detection of early relapse.

In relation to the source of VEGF in the circulation, it has been a matter of debate whether serum or plasma should be used for measuring circulating VEGF. Serum contains high levels of VEGF due to release by activated platelets during clotting. Therefore VEGF levels in serum, which correlate closely with blood platelet count, do not reflect the true circulating concentration of VEGF in vivo. In citrate or EDTA plasma, where less platelet activation and subsequent VEGF release is expected than in serum, VEGF levels were found to be higher in cancer patients than in controls and this was interpreted as a reflection of higher levels of VEGF in the circulation. But also under these conditions release of VEGF from platelets was not excluded as a source of increased VEGF levels in the plasma samples.

The aim of this study was to determine the effect of different blood collection protocols on the measurement of circulating VEGF, and of the impact of VEGF release from platelets by in vitro platelet activation. In addition to citrate plasma collection, we employed a method to (maximally) avoid artificial ex vivo platelet activation by blood collection without tourniquet and harvest in a medium containing a mixture of anticoagulants to which prostaglandin E, and theophylline was added (e.g. PECT medium). In addition, an important tool to discriminate between in vivo platelet activation and artificial in vitro platelet activation, during plasma harvest was used, i.e. measurements of β-thromboglobulin (β-TG) and platelet factor 4 (PF 4) levels. PF 4 and β-TG concentrations within platelets are similar and upon platelet activation they are released in similar quantities. Because PF 4 clearance form plasma is much faster than β-TG clearance (t ½ for PF 4 several minutes and for β-TG > 100 minutes) a normal or only slightly elevated PF 4 level and high β-TG level suggest in vivo platelet activation, whereas a high β-TG level in combination with a high PF 4 level suggest in vitro (ex-vivo) platelet activation. We assumed that this collection method in PECT plasma would provide a more accurate estimation of the circulating levels of VEGF in vivo.
We investigated the role of in vivo and in vitro platelet activation on plasma VEGF levels in healthy controls and cancer patients. The cancer patients were divided into two groups, metastatic non-renal cell carcinoma (non-RCC) patients and patients with metastatic renal cell carcinoma (RCC; all clear cell carcinoma). The latter group is characterized by high intra-tumor VEGF production by mutations in the von Hippel-Lindau tumor suppressor gene with consequent unlimited activity of the hypoxia inducible factor 1α (HIF1α) causing high VEGF transcription\textsuperscript{17-19}.

**Subjects, Materials and Methods**

**Patients and volunteers**
We obtained venous blood from 16 healthy volunteers (A) and from 30 cancer patients, separated in two patients groups: 18 patients with metastatic non-RCC (B) and 12 patients with metastatic RCC (C). We investigated VEGF levels in citrate plasma, PECT plasma and in platelets. To discriminate between in vivo platelet activation and artificial in vitro platelet activation during plasma harvest, β-TG and PF 4 were measured in all samples. These studies were approved by the institutional ethics committee, and informed consent was obtained from each patient and volunteer.

**Plasma preparations**
From each patient or volunteer venous blood was taken with a microperfuser (diameter 1 mm, Microflex, Vycon, Ecouen, France) and divided into different tubes. Plastic (polypropylene) blood collection tubes were filled with 400 μL of a solution containing: prostaglandin E\textsubscript{1} (94 nmol/l), Na\textsubscript{2}CO\textsubscript{3} (0.63 mmol/l), EDTA (90 mM) and theophylin (10 mM) (PECT-medium). Blood samples (4 ml) were collected in these PECT tubes in an open system, drop by drop without using a tourniquet to (maximally) avoid platelet activation ex vivo. Blood was also collected in tubes filled with citrate and in tubes filled with EDTA using a tourniquet (Becton Dickinson Vacutainers Systems, Breda, The Netherlands). Blood collected in the PECT tubes was immediately placed on ice in contrast to the citrate and EDTA blood samples which were kept at room temperature. Platelet-depleted PECT plasma was prepared by spinning for 60 min at 1700 g at 4°C within 1 hour after collection. The citrate blood samples were collected in a standard fashion with tourniquet, and centrifuged within 30 minutes for 15 minutes at 100 g to obtain plasma. EDTA blood drawn in the same manner was used to measure total number of platelets. To measure VEGF, PF 4 and β-TG within the platelets, EDTA blood was used in which platelets were destroyed by a combination of Triton (2% Triton X-100), sonication during 15 seconds on ice (microtip, Branson, amplitude 50%) and centrifuging during 5 minutes at 14.000 rpm in a micro-centrifuge. Platelet-depleted PECT plasma was used to measure VEGF, PF 4 and β-TG levels. Citrate plasma was used to measure VEGF and PF 4 levels. In the RCC patient group (C) we measured VEGF levels in plasma and platelets.
Measurements of platelet activation markers and VEGF

All samples were tested for VEGF, β-TG and PF 4 using commercially available sandwich enzyme–linked immunosorbent assays (ELISAs) from Roche (Asserachrom β-TG and Asserachrom PF 4; Roche, Mannheim, Germany) and R&D Systems (Quantikine VEGF; R&D Systems, Abingdon, UK).

Recovery of VEGF

Serum, PECT plasma and citrate plasma samples were supplied with known concentrations of recombinant human VEGF (standard provided in the assay kit) to produce VEGF concentrations of 250, 62.5, 31.2, 7.8 and 3.9 pg/ml. The samples were diluted with assay buffer and the concentration of VEGF was determined. Recovery of VEGF was 100-107% in PECT plasma, 73%-107% in citrate plasma and 78-100% in serum.

Statistical analysis

Statistical analysis was performed with the computer program SPSS version 12.0 (SPSS, Gorinchem, The Netherlands) and with GraphPad Prism software (GraphPad Prism, San Diego, CA, USA). VEGF, PF 4 and β-TG levels in healthy volunteers and cancer patients were compared using the Mann-Whitney U-test (unpaired, non-normally distributed groups). Values are presented as median and interquartile range [IQR].

Results

Patients and volunteers

The characteristics of volunteers and patients are as follows: healthy controls (groups A) median age, 53 [48-60]; cancer patients (group B); median age, 62 [57-72]; with cancer of the pancreas (7), cholangio (3), Papilli of Vater (2), esophagus (1), colon and rectum (2), melanoma (1), ovarium (1), breast (1). RCC (group C) median age, 60 [56-65]; with the following prognostic groups: 5 poor-risks, 5 intermediate risks and 2 favorable risks, according to Motzer20. All cancer patients had metastatic disease.

Plasma and platelet VEGF levels

VEGF levels in citrate plasma, PECT plasma, and platelets in the patients group are shown in Table 1 and Figure 1. In the three groups, the circulating VEGF levels in citrate plasma (Figure 1A) were significantly higher compared to PECT plasma (Figure 1B). VEGF levels in citrate plasma were significantly higher in both groups of cancer patients compared to healthy controls, in PECT plasma this was only the case for patients with RCC. The VEGF content in platelets, a well known reservoir for VEGF, was twice higher in both patient groups than in healthy volunteers (Table 1 and Figure 1C). The VEGF levels in plasma were considerably higher in group C compared to group B whereas the platelet VEGF content was not different between group B and C.
Table 1: VEGF levels measured in healthy volunteers and cancer patients. Group A, healthy volunteers; group B, non-renal carcinoma patients; group C, renal cell carcinoma patients. VEGF levels in median [interquartile range]

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF – citrate (pg/ml)</td>
<td>30.8 [23.5-44.3]</td>
<td>37.8 [31.2-52.8]</td>
<td>166 [84-238]</td>
<td>0.03</td>
</tr>
<tr>
<td>VEGF – PECT (pg/ml)</td>
<td>17.4 [15.2-22.2]</td>
<td>21.0 [16.0-31.5]</td>
<td>64 [52-134]</td>
<td>NS (0.15)</td>
</tr>
<tr>
<td>VEGF in platelets (pg/10^6 platelets)</td>
<td>3.4 [2.8-4.4]</td>
<td>6.2 [4.1-7.4]</td>
<td>6.0 [3.8-7.1]</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Figure 1: VEGF levels in the circulation. A) VEGF-levels measured in citrate plasma of cancer patients compared to healthy persons. B) VEGF-levels measured in PECT plasma of cancer patients compared to healthy persons. C) VEGF levels measured in platelets of cancer patients versus healthy persons. Group A represents healthy volunteers; group B represents non renal cell carcinoma patients; group C represents renal cell carcinoma patients. Bars represent the medians.
In vitro platelet activation

In both healthy controls (A) and cancer patients (B) the PF 4 concentration was 50-100 times higher in standard citrate plasma (collected with tourniquet) compared to PECT plasma (collected without tourniquet) (Table 2). These data demonstrate that the degree of platelet activation is highly dependent on the method of harvest in both healthy

Table 2: Platelet and platelet activation parameters. Group A, healthy volunteers; group B, non-renal carcinoma patients. VEGF levels in median [interquartile range].

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Group A</th>
<th>Group B</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of platelets (10⁹/l)</td>
<td>259 [237-278]</td>
<td>278 [225-382]</td>
<td>NS (0.13)</td>
</tr>
<tr>
<td>PF4 - PECT (IU/ml)</td>
<td>7.7 [5.6-10.3]</td>
<td>14.2 [11.1-87.5]</td>
<td>0.0002</td>
</tr>
<tr>
<td>PF4 - citrate (IU/ml)</td>
<td>523 [298-738]</td>
<td>657 [283-806]</td>
<td>NS (0.81)</td>
</tr>
<tr>
<td>PF4 - in platelets (IU/10⁶ platelets)</td>
<td>11·3 [9.8-12.5]</td>
<td>15.2 [13.3-17.2]</td>
<td>0.0005</td>
</tr>
<tr>
<td>β-TG - PECT (IU/ml)</td>
<td>37.4 [24.1-46.4]</td>
<td>74.7 [51.4-151.4]</td>
<td>0.0005</td>
</tr>
<tr>
<td>β-TG - in platelets (IU/10⁶ platelets)</td>
<td>34.3 [29.6-39.3]</td>
<td>36.9 [30.6-41.4]</td>
<td>NS (0.52)</td>
</tr>
</tbody>
</table>

Figure 2: Correlation between VEGF and PF 4 in citrate plasma in cancer patients and controls. VEGF and PF 4 measured in citrate plasma correlated significantly (r=0.457, p=0.008)
controls and cancer patients. The contribution of in vitro platelet VEGF release to plasma VEGF levels is further supported by the significant correlation between PF 4 and VEGF levels in citrate plasma (Figure 2). The twice higher PF 4 values in PECT plasma from cancer patients compared to healthy controls suggest that even under optimal platelet protecting conditions, platelets from cancer patients are more vulnerable. However, the increased PF 4 concentration in platelets of cancer patients may also have contributed to the increased PF 4 concentration in PECT plasma (Figure 3).
We were unable to demonstrate in vivo increased platelet activation in patients with cancer. Different half lives of PF 4 and β–TG after in vivo release from platelets enabled us to discriminate between in vitro and in vivo platelet activation. A comparison of PF 4 and β–TG values in PECT plasma from controls and cancer patients revealed that both PF 4 and β–TG increased by a factor two. Although we were not able to demonstrate in vivo platelet activation, this does not exclude the role of local platelet activation during the process of metastasis, as demonstrated in several pre-clinical studies. The total amount of platelets was not significant different between the different groups (Figure 4).

**Discussion**

Our study demonstrates that ‘true’ circulating levels of VEGF, as determined by measurements in PECT plasma, are low in non-RCC and that they do not differ significantly from circulating VEGF levels in controls. In addition, the elevated VEGF levels in citrate plasma correlate with PF 4, a marker of ex vivo platelet activation, suggesting a release of VEGF from platelets during the harvest procedure. Our findings show that VEGF levels in blood samples are highly dependent of the method of collection and question the relevance of ‘circulating’ VEGF as a biomarker.

Although a marked increase in plasma VEGF levels has been observed in various types of cancer, interpretation of these data and comparison between studies is often difficult because of the heterogeneity in collection and determination methods. A number of
studies report a correlation between platelet counts and serum VEGF\textsuperscript{21,22}, and higher serum VEGF levels per platelet in cancer patients\textsuperscript{23,24}. This is in line with our findings and the known function of platelets as an important reservoir for VEGF. Platelet-derived VEGF may have an important pathological role in cancer due to thrombin induced platelet activation and local release of VEGF, inducing vascular permeability, promoting coagulation and cancer dissemination\textsuperscript{4,25-27}.

In the present study, we evaluated two different methods of plasma collection for VEGF measurement in healthy controls and cancer patients. The impact of the use of a tourniquet and collection medium (citrate versus PECT) on VEGF levels was demonstrated in healthy controls and cancer patients. No significant differences were detected in PECT VEGF levels between healthy controls and non-RCC cancer patients. Only in RCC patients, a cancer type characterized by excessive VEGF production due to a specific gene defect, PECT VEGF levels were significantly higher compared to controls. In vitro platelet activation during the collection procedure contributes to higher plasma VEGF levels as demonstrated by the release of PF 4 from platelets, which was much higher in citrate compared to PECT plasma, and the significant positive correlation between PF 4 and VEGF levels. The twice higher levels of PF 4 in PECT plasma in cancer patients compared to controls suggest that also with the optimal collection method platelet activation still occurs. These findings question the relevance of VEGF levels measured in regular plasma samples as a test for VEGF levels in vivo. These findings suggest that levels of freely circulating VEGF are not elevated in most cancer patients but are results of heterogeneity in collection and determination methods.

In line with other studies we demonstrated that platelet VEGF content is higher in cancer patients\textsuperscript{23,34}. In the light of our findings in PECT plasma, this suggests that ex vivo release of VEGF by platelets is altered in cancer patients, due to either alterations in platelet VEGF content or differences in degree of ex vivo platelet activation, or both. Higher platelet VEGF content may originate from increased loading in the bone marrow or due to the VEGF scavenging functions of platelets from the circulation\textsuperscript{5,29}. Remarkable is the observation that in both our patient groups the increase in platelet VEGF content was about two fold compared to controls, whereas the PECT VEGF levels in RCC patients were much higher. This may argue against a role for platelets as VEGF scavengers, because maximal platelet VEGF content is already achieved with relatively low VEGF plasma levels. Therefore, VEGF content within platelets may also not be a useful biomarker for angiogenesis in cancer patients.

In conclusion, VEGF levels are low or absent in the circulation in most cancer patients, with the exception of RCC, a cancer type with excessive VEGF production due to a genetic defect. Citrate VEGF levels do not reflect true circulating VEGF levels but are the result of ex vivo platelet activation and subsequent VEGF release from platelets with increased VEGF content. Elevated citrate VEGF levels in cancer patients, which have widely been used as a
biomarker of tumor angiogenesis, are caused by altered platelet behavior associated with the systemic disease.

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References


