Chapter 3

PLATELETS AND GRANYLOCYTES, IN PARTICULAR THE NEUTROPHILS, FORM IMPORTANT COMPARTMENTS FOR CIRCULATING VASCULAR ENDOTHELIAL GROWTH FACTOR

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Vascular Endothelial Growth Factor

ABSTRACT

The measurement of circulating VEGF levels as a prognostic factor will gain increasing relevance in the diagnosis and evaluation of treatment in cancer patients. Angiogenesis is an absolute requirement in tumor growth and metastatic disease. In the present study data are presented which indicate that circulating VEGF mainly resides in peripheral blood cells. In 13 healthy volunteers we demonstrated that approximately 34% of the circulating VEGF resides in platelets and approximately 11% in patients with cancer (n=4). An important part namely 58% in healthy volunteers and 69% in patients with cancer of the total circulating VEGF is contained in granulocytes, particular in the neutrophils, as confirmed by Fluorescence-Activated Cell Sorting (FACS). Also an increased VEGF level per granulocyte is found in patients with cancer (164 µg VEGF/l) compared with the healthy volunteers (77 µg VEGF/l). In contrast only 2% was present in plasma. The biological significance of platelet- or granulocyte derived VEGF is not yet known. Liberation of VEGF from these compartments could well be of importance for tumor angiogenesis. Therefore, future studies on the clinical value of circulating VEGF as a prognostic factor in cancer patients should include measurements of VEGF in peripheral blood cells.

List of abbreviations:

VEGF = Vascular Endothelial Growth Factor
PPP = Platelet Poor Plasma
PBMNC = Peripheral Blood Mononuclear Cells
FACS = Fluorescence-Activated Cell Sorting
TNFα = Tumor Necrosis Factor α
INTRODUCTION

Among the angiogenic growth factors Vascular Endothelial Growth Factor (VEGF) is generally considered to play a key role in physiological as well as in pathological angiogenesis because of its biological properties. VEGF was originally recognized to induce vascular permeability which is one of the first steps in angiogenesis that allows endothelial cells to migrate and eventually proliferate into capillary tubes. Its pivotal role in tumor angiogenesis has been indicated by the inhibitory effects of anti-VEGF antibodies on tumor growth in vivo. VEGF expression is increased in tumor cells of numerous human cancers.

Elevated circulating VEGF levels have been described in patients with various types of cancer such as breast cancer, ovarian, colorectal carcinoma, melanoma and lung malignancies. As the absolute requirement for angiogenesis in tumor growth and metastasis is evident, the measurement of circulating VEGF levels as a prognostic factor will gain increasing application in the diagnosis and evaluation of treatment in cancer patients. Implicated in the use of this concentration is the concept that circulating VEGF reflects localized tumor-angiogenesis. From studies published so far it can be concluded that peripheral blood cells contain VEGF. These studies found that variations of VEGF in standard serum samples reflect the release of VEGF from peripheral blood cells, such as platelets and leukocytes, into the serum due to blood cell activation during sample handling. It was therefore suggested that preferably VEGF should be measured in plasma processed with minimal activation of peripheral blood cells. However, the biological significance of VEGF in peripheral blood cells in pathological processes such as tumor growth is not yet known. In order to improve the clinical value of measurements of circulating VEGF we evaluated circulating VEGF in several blood fractions and peripheral blood cells. Here we present data which indicate that in healthy volunteers and in patients with cancer, platelets and granulocytes, in particular the neutrophils, are the major cell fractions that contain circulating VEGF.

MATERIALS AND METHODS

Venous blood sampling

Venous blood samples were obtained from 13 healthy volunteers aged between 20 and 41 years of age, and in 4 patients with extensive metastatic malignancies, aged between 39 and 54 years. Three patients were diagnosed with metastatic breast cancer and 1 patient with a cloacogenic anal carcinoma. Peripheral venous blood samples were collected in sterile CTAD (sodium Citrate Theophilline Adenosine Dypiridamole) tubes (Becton Dickinson Vacutainer Systems Europe, France). Informed consent was obtained, and all sampling had been approved by the local medical ethical committee.
CTAD tubes contain four anticoagulants: sodium citrate, theophylline, adenosine and dipyridamole to obtain maximal stabilization of platelets. After blood sampling, whole blood, platelet poor plasma (PPP), peripheral blood mononuclear cells (PBMNC), platelets, and in addition in 4/13 volunteers, also granulocytes were isolated using gradient centrifugation (described below). In 2/13 volunteers FACS analysis was performed to determine the leukocyte fraction containing VEGF (see FACS analysis).

**Whole blood and PPP**

After sampling, whole blood samples were diluted with 2 volumes of PBS and subsequently lysed by freezing and thawing twice. To obtain PPP, CTAD blood was centrifuged at 3000 x g for 15 minutes at 18°C and PPP was subsequently stored in aliquots in –80°C.

**Isolation of peripheral blood platelets**

CTAD blood was centrifuged at 200 x g for 15 minutes at 18°C without brake to obtain platelet rich plasma (PRP). PRP was then collected and centrifuged at 1000 x g for 20 minutes at 18°C subsequently to obtain a platelet pellet. The platelet pellet was resuspended in PBS and aliquots were stored at –80°C after cells were counted using a differentiated cell counter (Coulter STKS).

**Isolation of PBMNCs and granulocytes**

A PBMNC suspension and a suspension containing erythrocytes and granulocytes were obtained using density gradient centrifugation on a Ficoll gradient (density 1,077 g/ml, Pharmacia Biotech Uppsala, Sweden) and Optiprep gradient (density 1,085 g/ml). After collection of PRP the remaining blood suspension was separated from the PRP and diluted with 3 equal volumes of PBS, layered on top of the Ficoll and Optiprep gradient layers and centrifuged at 200 x g for 30 minutes at 20°C. Thereafter the PBMNC layer was collected, washed with cold PBS and centrifuged at 300 x g for 10 minutes at 4°C. The pellet was resuspended in sterile PBS. Identification of PBMNCs was confirmed by counting the cells using a differentiated cell counter. Aliquots were stored at –80°C. After collection of the PBMNC layer the remaining suspension was centrifuged at 1000 x g for 15 minutes at 200°C. For the isolation of the erythrocytes, the suspension was washed with cold PBS and centrifuged at 300 x g for 10 minutes at 4°C. After counting the erythrocytes the pellet was stored in aliquots at –80°C.

For the isolation of the granulocyte fraction, contaminating erythrocytes were hemolyzed with cold ammonium chloride solution (Merck, Germany). Subsequently granulocytes were
collected and washed once with PBS. The granulocyte pellet was stored in aliquots at –80°C after cell counting.

Cellular concentration of VEGF was calculated by dividing the VEGF content per cell by the cell volume. The cell volume of platelets is 6.5 µm³, for granulocytes 925 µm³.

**FACS analysis**

Fluorescence-Activated Cell Sorting (FACS) was performed to identify the leukocyte fraction(s) containing VEGF. Leukocytes were obtained from whole blood by hemolyzing erythrocytes using cold ammonium chloride solution (Merck, Germany). The leukocyte pellet was washed with PBS containing 0.3% BSA and 1 mM NaEDTA to prevent clumping of monocytes. Subsequently the cell pellet was incubated with CD45-FITC and CD14-PE for 30 minutes at 40°C. After washing, cells were sorted on the basis of differences in light scatter (Forward and Side Scatter) and expression of CD45 and CD14 on a MoFlo high speed flowcytometer. For antibody detection CD14 (PE-labeled) and CD45 (FITC-labeled) antibodies were used. Lymphocytes are CD45+++ and CD14-, monocytes are CD45++ and CD14+, neutrophils are CD45+ and CD14+, whereas eosinophils are CD45++ and CD14dim. After sorting of cells the cell fractions were centrifuged for 10 minutes at 300 g and suspended in PBS. After cell counting the cell fractions were lysed by freeze and thawing twice and VEGF was measured in the different leukocyte fractions.

**VEGF immunoassay**

VEGF concentrations were determined using the Quantikine Human VEGF enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc. Minneapolis, MN). This is a solid phase ELISA designed to measure VEGF₁₆₅ levels in cell lysates, serum, whole blood, and plasma. Optical densities were measured with a Bio-Tek automated microplate reader (Type) at 450 nm with correction at 575 nm. The blank was subtracted from the duplicate readings of each standard and sample. A standard curve, performed for each microplate, was created by plotting the logarithm of the mean absorbency of each standard against the logarithm of the VEGF concentration and the line best fit was determined by regression analysis. PBMNC, platelets, erythrocytes and granulocytes were lysed by freeze (minimal duration of 4 hours) and thawing twice before performing the assay. All samples were assayed in duplicate. The minimum detectable dose was 9.0 pg/ml as quoted by the manufacturer.

**Statistical analysis**

Differences between data from healthy volunteers and patients with cancer were statistically analyzed by the two-sided unpaired Students-t-test. Differences were considered significant if \( P<0.05 \).
RESULTS

**VEGF levels in PPP, whole blood, platelets, PBMNC, erythrocytes and granulocyte fractions**

VEGF levels in PPP were measured in samples of 13 healthy volunteers. In healthy volunteers VEGF levels in PPP ranged from undetectable to 22.8 pg/ml (median: 9.1 pg/ml). In contrast, levels in lysed whole blood of the same individuals drawn at the same time ranged from 212 to 727 pg/ml (median 301 pg/ml). Platelets contained 0.07 to 1.14 pg VEGF per 106 cells (median 0.48 pg/106 cells). We measured VEGF in the granulocyte fraction in 4 volunteers. Granulocytes contained 44 - 95 pg VEGF per 106 cells (median 72 pg/106 cells; Table 1). PBMNC-VEGF ranged form 2.4 to 23.2 pg/106 cells and levels correlated with the contamination of granulocytes (data not shown). In erythrocytes no VEGF was detected.

<table>
<thead>
<tr>
<th></th>
<th>WHOLE BLOOD</th>
<th>PPP</th>
<th>PLATELETS</th>
<th>GRANULOCYTES</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Healthy volunteers</strong></td>
<td>301</td>
<td>9.1</td>
<td>0.48</td>
<td>72</td>
</tr>
<tr>
<td>(n=13)</td>
<td>(212 - 727)</td>
<td>(0.0 - 22.8)</td>
<td>(0.07 - 1.14)</td>
<td>(44 - 95)</td>
</tr>
<tr>
<td><strong>Cancer patients</strong></td>
<td>1434.5</td>
<td>22.5</td>
<td>0.7</td>
<td>152.5</td>
</tr>
<tr>
<td>(n=4)</td>
<td>(811-2039)</td>
<td>(22-27)</td>
<td>(0.4-1.9)</td>
<td>(114-158)</td>
</tr>
<tr>
<td><strong>P-value</strong></td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
<td>0.052</td>
<td>&lt; 0.005</td>
</tr>
</tbody>
</table>

Data are presented as median (range).

In the four cancer patients VEGF levels in PPP (22.5 pg/ml), whole blood (1434.5 pg/ml), and granulocytes (152.5 pg/106 cells) were significantly higher compared to the VEGF levels in healthy volunteers (respectively $P<0.05$, $P<0.05$, $P<0.05$, Table 1). PPP in cancer patients ranged from 22 to 27 pg/ml (median 22.5 pg/ml). VEGF in whole blood of cancer patients ranged from 811 - 2039 pg/ml (median 1434.5 pg/ml). In cancer patients platelets and granulocytes VEGF levels per 106 ranged from 0.4 - 1.9 pg/ml (median 0.7 pg/ml) and from 114 - 158 (median 152.5) respectively. The amount of VEGF in platelets in the cancer patients was elevated relative to the VEGF levels in platelets of healthy volunteers, however this did not reach significance ($P=0.052$, Table 1).

The VEGF content per cell fraction in 1 liter whole blood in 4 healthy volunteers was calculated to indicate the relative contribution of the different cell fractions to the total amount of the circulating VEGF in 1 liter whole blood. Platelets contain approximately 34% of the total circulating VEGF, whereas in cancer patients platelets contain approximately 11% of the
total circulating VEGF in 1 liter whole blood. Only 2% was present in plasma of healthy volunteers and 1 % in cancer patients. In healthy volunteers and cancer patients respectively approximately 58 % and 69% of the total circulating VEGF resides in the granulocyte fraction (Table 2).

The median amount of granulocytes per liter whole blood in healthy volunteers (n=13) and cancer patients (n=4) is respectively $3.0 \times 10^9$/L (range: 2.4 - 5.9 $\times 10^9$/L) and 8.0 $\times 10^9$/L (range: 7.4 - 8.9 $\times 10^9$/L, data not shown).

The range of the VEGF concentration per cell for platelets and granulocytes was 10.8 - 175.4 µg/L (median: 75.4 µg/L, n=13) and 47.6 - 103.8 µg/L (median: 77.0 µg/L, n=4) in healthy volunteers. In cancer patients VEGF concentration per cell for platelets was 61.7 - 292.3 µg/L (median 107.7 µg/L, n=4), and from 123.2 - 170.8 µg/L (median 164 µg/L, n=4) for granulocytes (data not shown).

Table 2. VEGF levels in healthy volunteers and cancer patients: relative contribution

<table>
<thead>
<tr>
<th></th>
<th>WHOLE BLOOD (PG/ML)</th>
<th>PPP1 (PG/ML)</th>
<th>PLATELETS (PG/10⁶ CELLS)</th>
<th>GRANULOCYTES (PG/10⁶ CELLS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy volunteers</td>
<td>100%</td>
<td>1.7%</td>
<td>34%</td>
<td>58%</td>
</tr>
<tr>
<td>(n=13)</td>
<td></td>
<td>(1%– 2%)</td>
<td>(25% – 61%)</td>
<td>(25% – 71%)</td>
</tr>
<tr>
<td>Cancer patients</td>
<td>100%</td>
<td>1%</td>
<td>11%</td>
<td>69%</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td>(0 – 1%)</td>
<td>(5% - 55%)</td>
<td>(34%-78%)</td>
</tr>
</tbody>
</table>

Data are presented as median (range).

**FACS analysis**

FACS analysis was performed to confirm the VEGF content in the different leukocyte cell fractions. The neutrophil, lymphocyte and monocyte fraction were separated by FACS for VEGF measurement. The granulocyte fraction containing VEGF revealed to be the neutrophil fraction (99% neutrophils). Lymphocytes (99% lymphocytes) and monocytes (95% monocytes) contain negligible levels of VEGF.

**DISCUSSION**

Earlier studies showed that VEGF constitutes VEGF in plasma and VEGF in circulating blood cells, namely in platelets, peripheral mononuclear cells and neutrophils (8-12). We have quantified the contribution of the various cell fractions to the total amount of circulating VEGF. We found that the major part, namely 58% in healthy volunteers and 69% in patients with cancer of the total circulating VEGF, is contained by neutrophils. This relatively large
contribution of neutrophils to circulating VEGF has not been reported before. Platelets contain approximately 34% in healthy volunteers and approximately 11% in patients with cancer. Comparing the calculated cell concentrations of VEGF and the platelet poor plasma concentration, we found very low plasma levels of VEGF suggesting that circulating VEGF resides mainly in peripheral blood cells, in particular in platelets and neutrophils.

VEGF is produced in the megakaryocyte and is only partially released during maturation and platelet shedding but largely remains intra-cytoplasmic in the mature platelet. It has been shown that platelets transport VEGF and secrete VEGF upon activation with thrombin. Platelets in cancer patients have been shown to contain more VEGF per corpuscle than in normal subjects and is comparable with the findings in our study. From these studies it was inferred that platelets are scavengers of, presumptively, tumor-derived VEGF.

In neutrophils VEGF also is a pre-formed cytokine which is stored in the specific granule. We found an increase in the amount of VEGF per neutrophil in cancer patients compared with healthy volunteers. Release of VEGF from neutrophils typically occurs upon activation with pro-inflammatory cytokines such as TNF-α. In breast cancer tissue a heterogeneity of varying cellular infiltration is seen such as infiltration of lymphocytes, neutrophils and macrophages. It was suggested that the finding of VEGF in neutrophils might indicate an important role for neutrophil derived VEGF in angiogenesis by controlling vessel permeability. Increased vascular permeability is recognized to be a crucial step in tumor angiogenesis.

The present study provides evidence for a physiological pool of VEGF in human platelets and neutrophils. Platelet and neutrophil VEGF might be readily released from this pool for local induction of angiogenesis, for example in the acute response to a wound. However, not only healing processes will be influenced in this way. In this study even more VEGF per granulocyte in patients with cancer is found, next to an overall increase in granulocytes per liter whole blood compared to healthy volunteers. Physiological events in and near a tumor process may involve activation, degranulation, lysis and death of normal blood cells. Liberation of VEGF from these cell compartments into the tumor environment could well be of importance for subsequent tumor angiogenesis.

In addition to serving as a pool for VEGF, blood cells have been shown to constitutively produce VEGF. This has been demonstrated in T lymphocytes, monocytes, and peripheral mononuclear blood cells. This production was demonstrated by the detection of mRNA and increased release of the protein by cytokines that can play a role in malignancy such as epidermal growth factor, platelet derived growth factor, transforming growth factor α and β and IL-6. The exact role of this cellular compartment of VEGF in the growth of a tumor is not known at present. However future measurements of VEGF aimed at prognostic or intervention targets, should take these various compartments into account.
REFERENCES


