Chapter 6

SYSTEMIC VEGF LEVELS AFTER CORONARY ARTERY BYPASS GRAFT SURGERY REFLECTS THE EXTENT OF INFLAMMATORY RESPONSE

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ABSTRACT

Background: Circulating Vascular Endothelial Growth Factor (VEGF) is increasingly studied as a substitute endpoint for treatment response after VEGF plasmid therapy. The effect of coronary artery bypass surgery (CABG) with cardiopulmonary bypass (CPB) on systemic VEGF levels are however largely unknown, therefore we studied the effects of this procedure to measure VEGF levels after surgery alone.

Methods and Results: Fourteen patients requiring CABG were included. VEGF$_{165}$ levels, ischemic markers and hematology were measured before and directly after to 6 days after surgery, respectively: 249.6 ± 50.4 to 451.7 ± 56.4 (day 6, $P=0.008$) and 581.9 ± 105.1 to 783.4 ± 97.7 (day 6, $P=0.016$). There was a close correlation of circulating VEGF$_{165}$ levels with leukocyte and platelet counts and not with ischemic markers.

Conclusion: Following surgery and in case of activated leukocyte and platelet counts care must be taken in the interpretation of systemic VEGF$_{165}$ levels.

respectively: 249.6 ± 50.
INTRODUCTION

ASCULAR Endothelial Growth Factor is a highly conserved 46 kDa homodimeric protein with multiple isoforms. The most abundant isoform, VEGF$_{165}$ (or VEGF-A), was discovered in the early 80's as a protein that enhances vascular permeability, which is one of the first steps in angiogenesis allowing endothelial cells to migrate and eventually proliferate into capillary tubes.$^1$ In addition, VEGF$_{165}$ induces endothelial cell migration and replication.$^{2,5}$

Amongst the angiogenic factors VEGF$_{165}$ has been in the spotlight of scientific interest in experimental and clinical studies because of its key role in angiogenesis. In vivo, VEGF$_{165}$ has been found to be upregulated in ischemic areas of solid tumors.$^6$-$^9$ Studies have supported the importance of VEGF$_{165}$ in tumor growth and metastasis. Increased circulating VEGF$_{165}$ levels have been found to correlate with tumor stage and poor overall survival in various types of cancer such as breast, colon, and esophageal cancer.$^{10}$-$^{12}$ In addition VEGF$_{165}$ is involved in pathologic inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease and psoriasis.$^{13}$

Early clinical studies in limb ischemia and coronary artery disease have supported the therapeutic potential of VEGF$_{165}$ to induce clinically relevant neovascularization in limb ischemia and in coronary artery disease.$^{14}$-$^{21}$ The significance of VEGF in the natural history of ischemic disease has only been explored recently. Initial findings show that VEGF expression is upregulated by ischemia and that serum VEGF$_{165}$ levels increase following acute myocardial infarction.$^8,$$^{22}$-$^{24}$

Circulating VEGF$_{165}$ is increasingly perceived as a marker for disease activity or as a marker for treatment response in a wide variety of diseases.$^{10}$-$^{24}$ Circulating VEGF$_{165}$ is known to reflect mainly VEGF$_{165}$ in peripheral blood cells, namely in platelets and neutrophils, and the exact role of VEGF$_{165}$ in the different systemically circulating cell compartments still has to be further clarified.$^{25}$ VEGF is readily released from these intracellular sources by a wide variety of cytokines. As we have initiated studies in patients with coronary heart disease and peripheral artery disease$^{26}$,$^{27}$ we are interested in the systemic VEGF protein as endpoint. To address this issue we evaluated the effects of conventional CABG surgery using cardiopulmonary bypass (CPB) on systemic VEGF$_{165}$ levels. In addition the effect of ischemia and platelet and leukocyte counts (as these cell compartments are important sources of systemic VEGF in the circulation) in relation to the VEGF level was determined.

MATERIALS AND METHODS

Patients

Patients subjected to CABG surgery with CPB were included. Exclusion criteria included the presence of a positive history for cancer or diabetes mellitus. Patients who received blood
transfusion in the postoperative period or who experienced a complicated postoperative course were also excluded. The institutional medical ethics committee approved the study protocol and informed consent was obtained from all patients.

Prior to surgery, patients received pre-medication with diazepam 10-15 mg. Induction of anesthesia was performed with sufentanil and midazolam, and tracheal intubation was achieved with pancuronium 0.1 mg/kg. All procedures during CABG were performed under moderate hypothermia (32°C). Cold St.- Thomas solution (40°C) was infused into the aortic root to maintain cardioplegia. During 400 seconds an intravenous loading dose of 300 U/kg heparin was administered before starting CPB. After weaning from CPB heparin was reversed with protamine chloride (3 mg/kg) cardiopulmonary bypass times and aortic clamp times were recorded.

**Venous blood sampling and serum and whole blood sample handling**

Peripheral venous blood samples were collected in sterile CTAD (sodium Citrate Theophilline Adenosine Dypiridamole) tubes (Becton Dickinson Vacutainer Systems Europe, France). After coagulation and centrifugation serum was stored in aliquots at –80°C. Whole blood samples were diluted with 2 volumes of PBS and subsequently lysed by freezing and thawing twice. Blood samples were collected 1 day before surgery, directly after surgery (4 hours post-CABG), and at day one, two and 6 days after CABG, counting the day of surgery as day 0.

**Troponin I and Creatine Phosphokinase MB analysis**

Troponin I concentration was measured according to Wu et al. A level below 0.5 µg/l indicated the absence of myocardial damage. Creatine Phosphokinase MB is measured according to Moss et al. The normal value of CPK-MB is <10 U/l.

**VEGF and hormone assays**

VEGF concentrations were determined using the Quantikine Human VEGF solid phase enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc. Minneapolis, MN). 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. VEGF concentration was reported as pg/ml.

**Statistical analysis**

Data are presented as mean ±SEM. VEGF levels on the subsequent post-operative days were compared with the baseline levels using the Friedman-test, followed by paired wise Wilcoxon
rank test. To investigate the relation between peri-operative Troponin and post-operative CPK-MB values and VEGF in the post-operative period, Troponin and CK-MB levels were related with the maximal post-operative change in VEGF and calculated using the Pearson’s correlation test. To evaluate the relation between the thrombocyte, white blood cell counts and VEGF, Spearman’s rank correlation coefficients were calculated in each patient. Using z-transformation, the mean of the correlation coefficients and the 95% CI were calculated. A P-value of less than 0.05 was considered statistically significant.

RESULTS

Fourteen patients (10 male, 4 female) requiring coronary bypass grafting with at least 2 anastomoses were included. Median age was 68 years (range 42 – 84). Post-operative course in all patients was uncomplicated and no myocardial infarction occurred based on clinical symptoms and electrocardiogram (ECG). Blood samples were obtained 1 day prior to CABG, 4 hours post-operative and at day 1, 2 and 6 following CABG. VEGF levels as measured in serum significantly decreased directly post-operatively to increase significantly at the sixth day post-CABG: 249.6 ± 50.4 pg/ml prior to surgery vs. 451.7 ± 56.4 pg/ml (P=0.008) on day 6 (Table 1). In whole blood post-operative levels were higher immediately after surgery and remained elevated on the 1st, 2nd and 6th day post-CABG (Table 2).

Table 1. Serum VEGF levels: comparison of post-operative days with pre-operative levels

<table>
<thead>
<tr>
<th></th>
<th>Day −1</th>
<th>4 Hour Post CABG</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum-VEGF (pg/ml)</td>
<td>249.6 ± 50.4</td>
<td>113.6 ± 26.2</td>
<td>173.8 ± 56.3</td>
<td>199.45 ± 28.2</td>
<td>451.7 ± 56.4</td>
</tr>
<tr>
<td>P-value</td>
<td>0.005</td>
<td>0.203</td>
<td>0.657</td>
<td>0.008</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. P-value by Wilcoxon rank test; not corrected for multiple comparisons.

Table 2. Whole blood VEGF levels: comparison of post-operative days with pre-treatment levels

<table>
<thead>
<tr>
<th></th>
<th>Day −1</th>
<th>4 Hour Post CABG</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood-VEGF (pg/ml)</td>
<td>581.9 ± 105.1</td>
<td>957.8 ± 139.2</td>
<td>1319.2 ± 314.3</td>
<td>1366.0 ± 258.5</td>
<td>783.4 ± 97.7</td>
</tr>
<tr>
<td>P-value</td>
<td>0.007</td>
<td>0.007</td>
<td>0.017</td>
<td>0.016</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. P-value by Wilcoxon rank test; not corrected for multiple comparisons.
There was a close correlation between whole blood VEGF and leukocyte count \((r=0.69, P<0.001)\). Serum and whole blood VEGF correlated moderately but significantly with the platelet count \((r=0.45, P=0.003\) for serum VEGF and \(r = 0.28, P=0.07\) for whole blood respectively; Table 3).

Mean cardiopulmonary bypass times ranged from 120.5 \((\pm 16.2)\) minutes. Mean aortic clamping time was 97.6 \((\pm 12.6)\) minutes. The mean peri-operative troponin I level was 34.88 \(\mu\)g/l \((\pm 8.9)\). Mean peri-operative CK – MB level was 12.46 U/l \((\pm 3.9)\). Mean CK – MB level at day 1 post-CABG was 11.36 U/l \((\pm 4.7)\). VEGF in serum and whole blood did not correlate with cardiopulmonary bypass time, aortic clamping time, troponin I and CK-MB levels.

### Table 3. Relationship between VEGF levels and platelet and leukocyte counts

<table>
<thead>
<tr>
<th></th>
<th>(r)</th>
<th>95% CI</th>
<th>(P) - VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum-VEGF - leukocyte count</td>
<td>-0.078</td>
<td>-0.370 to 0.228</td>
<td>0.62</td>
</tr>
<tr>
<td>Serum-VEGF - platelet count</td>
<td>0.45</td>
<td>0.17 to 0.67</td>
<td>0.003</td>
</tr>
<tr>
<td>Whole blood-VEGF - leukocyte count</td>
<td>0.7</td>
<td>0.5 to 0.8</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Whole blood-VEGF - platelet count</td>
<td>0.28</td>
<td>-0.023 to 0.53</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Data are correlation coefficient with 95\% confidence interval (CI). \(P\)-value according to Spearman’s rank correlation using Z-transformation.

### DISCUSSION

We measured VEGF in serum and whole blood and found circulating VEGF to increase following CABG. After CABG the systemic VEGF\(_{165}\) levels remained elevated for at least 6 days. VEGF is upregulated in ischemic areas, however, no relationship was found between VEGF levels and the ischemia markers troponin I and creatin phosphokinase. Alterations in platelet count and leukocyte count correlated close with changes in serum VEGF and whole blood VEGF respectively. This finding limits the applicability of VEGF as a substitute endpoint.

Enhanced circulating VEGF levels found after surgery are in accordance with other studies in humans.\(^{30-32}\) Bondestam et al also demonstrated an effect of the extent of surgery; VEGF levels only increased after major surgery such as coronary or peripheral vascular bypass surgery but not in minor surgery such as tonsillectomy or nasal surgery.\(^{30}\) There is little information about the trigger for the systemic elevation of the VEGF level after (CABG) surgery.

Ischemia (as occurs during CABG surgery) directly induces up-regulation of VEGF expression via the transcription factor HIF-1\(\alpha\). Regulation of VEGF expression shows similarities with the oxygen-sensing mechanisms of erythropoietin, indicating an important role for VEGF in the biological responses of all higher life forms that compensate for hypoxic...
During conventional CABG surgery, using cardiopulmonary bypass, the heart is arrested with an elevated potassium-based cardioplegic solution and subjected to a relatively prolonged period of global myocardial ischemia. However, we found no relationship between the cardiac ischemia markers troponin and creatin phosphokinase and circulating VEGF levels, suggesting that CABG associated ischemia is not the main cause of the VEGF increase in the systemic circulation.

Another explanation for an increase of circulating VEGF following CABG may be induction of a surgical wound and the systemic inflammatory response to this event. We found the strongest correlation between circulating VEGF in whole blood and leukocytes. To the best of our knowledge this correlation with leukocyte counts has not been described before. One study reported a correlation of circulating VEGF with platelet counts in the post-operative period. The present data also demonstrate that circulating VEGF, as measured in whole blood and serum, correlates to some extent with platelet counts. VEGF expression and secretion by a variety of adherent cell types including endothelial and human smooth muscle cells are well known. Strikingly, Khoene et al demonstrated that low oxygen, the known key stimulus for VEGF expression in adherent cells, did not stimulate VEGF mRNA expression and release from polymorphonuclear neutrophils and platelets. In vitro release of VEGF from circulating blood cells, such as platelets and neutrophils has been demonstrated more recently. In contrast neutrophil activation and platelet aggregation by protein kinase C and thrombin respectively resulted in rapid release of cellular VEGF. These findings suggest that leukocyte and platelet derived VEGF is involved in the early onset of inflammatory processes.

Conventional coronary artery bypass grafting is, in addition to the surgery, also associated with the development of a systemic inflammatory response caused by the use of heparin and cardioplegic solution. It is known to induce a systemic inflammatory response via complement activation, release of cytokines and the use of heparin-coated circuits. In this context it is therefore important to note that in CABG, due to the technical procedure, leukocytes and platelets are activated at two fronts. CABG induced leukocyte and platelet activation may be an explanation of VEGF release from these circulating blood cell compartments. A study in piglets showed that in particular neutrophils and platelets increase and accumulate in the heart as part of a systemic inflammatory response directly following cardiopulmonary bypass. The relation between cell count and circulating VEGF found in the present study may very well reflect the inflammatory response associated with CABG surgery.

The present study shows that CABG surgery in itself induces a transient increase in systemic circulating VEGF, which appears to be related to the inflammatory response following surgery irrespective of global myocardial ischemia. Following surgery and in case of activated leukocyte counts and platelet counts care must be taken in the interpretation of systemic circulating VEGF as a marker for disease activity or treatment response.
REFERENCES


