Correlation of Plasma and Serum Vascular Endothelial Growth Factor Levels with Platelet Count in Colorectal Cancer: Clinical Evidence of Platelet Scavenging?

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ABSTRACT

Most studies measuring circulating vascular endothelial growth factor (VEGF) have sampled serum rather than plasma. There has been much debate whether the collection of sera (which causes the activation of platelets and VEGF release) is a true reflection of tumor angiogenic activity or whether platelets act as scavengers of VEGF. Addressing this issue, we measured serum and plasma VEGF, before and after colorectal resection, with reference to platelet counts. Serum and plasma samples were collected from 116 colorectal cancer (CRC) and 116 control patients. Ninety-five CRC and 32 benign resections were performed. Both plasma and serum VEGF were significantly higher in CRC patients (18.5 and 327 pg/ml, respectively) compared with controls (9.0 and 151.5 pg/ml, respectively; \( P < 0.0001 \)). Paired serum and plasma VEGF measurements correlated in both CRC (\( r = 0.56 \)) and control patients (\( r = 0.73 \); \( P < 0.0001 \)). Serum and plasma VEGF levels correlated with platelet count in CRC patients (\( r = 0.58 \) and 0.44, respectively) but not in controls. Plasma and serum VEGF levels, and VEGF concentration per platelet, increased with advancing disease stage. The correlation of serum and plasma VEGF with platelet counts in CRC but not in benign disease may be attributable to the scavenging of VEGF from the tumor source by platelets, with plasma levels reflecting free circulating VEGF in equilibrium with platelet levels. VEGF levels in citrated plasma are low and lie close to the limits of ELISA sensitivity. We recommend that a standardized measurement of serum VEGF—normalized by the patient’s platelet count to give a value of serum VEGF per platelet—be adopted.

INTRODUCTION

Tumor angiogenesis is an important factor in the progression of tumors because, without the development of new blood vessels, tumors cannot grow beyond 2–3 mm (1). Neovascularization also increases the possibility that tumor cells will enter the circulation and give rise to metastases (2).

There is evidence in some cancers that the degree of angiogenesis (measured by microvessel density or angiogenic cytokine expression levels on biopsies or surgical specimens) may reflect the malignant potential of individual tumors (3) and provide important prognostic information (4). However, if tumor angiogenesis could be reliably measured noninvasively, this would extend the tumor types accessible to study and would possibly provide indications of residual disease after surgery or early indications of responses to therapy. There is currently no consensus on the feasibility of this approach nor standardization of the procedures to be used.

The angiogenic process is mediated by several potent cytokines, one of the most important being VEGF, a specific mitogen for endothelial cells. Most studies that have attempted to assess tumor angiogenesis remotely by measuring angiogenic factors in the peripheral circulation of cancer patients have sampled serum rather than plasma. In CRC, serum VEGF correlates with stage (5), and high levels are associated with rapid tumor progression (6). However, the comparison of serum and plasma VEGF has shown much higher levels in the former (7) because VEGF is stored in the \( \alpha \) granules (8) and is released on platelet activation during clotting (9, 10). Subsequently, serum VEGF levels have been found to correlate with platelet count in a mixed population of metastatic cancers (11) and renal cancer (12), and, hence, it has been suggested that serum VEGF may be an inaccurate indicator of circulating VEGF because of its release during sample collection. Blood taken into citrate tubes avoids platelet activation, and, thus, plasma has been suggested to represent a more accurate assessment of circulating VEGF (13).

One physiological function of platelets may be to act as scavengers of circulating VEGF to restrict angiogenic activity to sites of wound healing. This may also be occurring in the pathological situation of tumor growth (14, 15); however, there is no direct evidence to support this hypothesis at present. This study is the first to measure changes in both plasma and serum VEGF before and after colorectal surgery for both malignant and benign disease in relation to platelet counts. Other studies have been limited in extent and have not included a control group of patients undergoing benign colorectal resection.

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2 The abbreviations used are: VEGF, vascular endothelial growth factor; CRC, colorectal cancer.
PATIENTS AND METHODS

Blood samples were taken from patients with primary CRC presenting to the Mayday University Hospital and Royal Marsden Hospital Gastrointestinal Unit (Sutton, Surrey, United Kingdom). No patients had had previous malignant disease nor had received preoperative radiotherapy. Control blood samples were taken, at the time of colonoscopy, from 84 patients with benign disease (60 patients with normal colons, 16 with benign polyps, and 8 with diverticulosis). Thirty-two patients with benign colorectal disease (9 with nonfistulating diverticular disease, 9 with polyps, 7 with diverticulosis). Thirty-two patients with benign colorectal disease (9 with nonfistulating diverticular disease, 9 with polyps, 7 with nonfistulating inflammatory bowel disease, 4 resection rectopexy, and 3 others) underwent colorectal resection.

The median age of the 116 CRC patients was 68 (range, 19–92) with 59 male and 57 female patients. The 116 patients with benign colorectal disease had a median age of 65.5 (range, 21–95) with 46 male and 70 female patients. Of the 116 CRC patients, 90 underwent surgical resection. The 26 other patients were given either preoperative chemoradiotherapy or palliative care only.

Plasma samples only were taken from 26 CRC patients and 41 control patients. Both plasma and serum samples were taken for comparison on 90 CRC patients and 72 control patients (Table 1). Postoperative samples were taken 4–6 h and 14–20 h after surgery (Table 1). Preoperative and first-day postoperative platelet counts were recorded.

Blood was taken into a citrate tube for plasma analysis and into serum separator tubes or clot-activator tubes for serum analysis. Samples were centrifuged at 1400 g for 10 min within 30 min of collection. The samples were stored at −70°C until assay by ELISA (R&D systems; VEGF ELISA). The limits of sensitivity of the ELISA are 9 and 2000 pg/ml. All of the samples were measured in duplicate.

To correct for variation in platelet counts between patients, the concentration of VEGF per platelet (pg/10^6) was calculated by dividing the serum VEGF concentration (pg/ml) by the platelet count × 10^6/ml. Serum VEGF levels reflect plasma VEGF plus platelet-released VEGF. Therefore, an estimate of “platelet VEGF” can be obtained by subtracting the plasma VEGF level from the serum level and dividing this by the platelet count [(serum – plasma VEGF) ÷ platelets (in pg/ml)] and will be referred to as “plasma-corrected VEGF”/platelet. Statistical analysis was performed with Prism 3.0 software, using nonparametric statistical analysis.

### RESULTS

**Plasma VEGF.** The median plasma VEGF level was significantly higher in CRC patients compared with patients with benign disease. Only 35.3% (41 of 116) of CRC patients had a plasma level of <9 pg/ml (the limit of detection of the ELISA assay) compared with 64.6% (73 of 113) of the control patients. There was a significant increase in plasma VEGF measured both 4–6 h and 14–20 h after surgery in both of the groups (Table 2).

**Serum VEGF.** Serum VEGF was significantly higher in CRC patients compared with benign disease patients (Table 2). In CRC patients, there was a significant increase 4–6 h after surgery, but values had returned to preoperative levels by 14–20 h after surgery. There was no significant difference between curative and palliative resection groups and their preoperative and postoperative serum VEGF levels (data not shown).

Serum VEGF levels were significantly increased 4–6 h after benign resection compared with preoperative levels and remained so (although not significantly) 14–20 h after surgery.

**Correlation between Plasma and Serum VEGF.** In both benign and malignant disease, there was a statistically significant correlation between plasma and serum VEGF levels (r = 0.73 and 0.56, respectively; P < 0.0001). Both serum and plasma VEGF correlated with platelet count in CRC (Figs. 1 and 2) but not in benign disease (r = 0.08 and 0.12, respectively).

**Dukes’ Staging and Plasma/Serum VEGF.** There was an increase in both plasma and serum VEGF levels with advancing stages (Table 3). Correcting each value for variation in platelet count, we found that both the total serum VEGF/platelet

### Table 1 Sample numbers

<table>
<thead>
<tr>
<th></th>
<th>CRC</th>
<th>Benign disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Serum</td>
</tr>
<tr>
<td>All patients</td>
<td>116</td>
<td>90</td>
</tr>
<tr>
<td>Surgical resection</td>
<td>90</td>
<td>67</td>
</tr>
<tr>
<td>4–6 h postsurgery</td>
<td>43</td>
<td>40</td>
</tr>
<tr>
<td>14–20 h postsurgery</td>
<td>68</td>
<td>46</td>
</tr>
</tbody>
</table>

### Table 2 Median plasma and serum VEGF and changes after surgery

<table>
<thead>
<tr>
<th></th>
<th>A (CRC patients)</th>
<th>B (benign disease)</th>
<th>A vs B (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma VEGF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All patients</td>
<td>18.5 (9–36.38)</td>
<td>9.0 (9–10.8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Preoperative</td>
<td>15.8 (9–32.6)</td>
<td>9.0 (9–15.75)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>4–6 h</td>
<td>31.3 (19.7–58.9)</td>
<td>24.7 (14.8–49.4)</td>
<td></td>
</tr>
<tr>
<td>P &lt; 0.0001*</td>
<td>P = 0.0005*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14–20 h</td>
<td>38.1 (22.2–64.2)</td>
<td>35.5 (22.3–50.3)</td>
<td></td>
</tr>
<tr>
<td>P &lt; 0.0001*</td>
<td>P &lt; 0.0001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum VEGF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All patients</td>
<td>327 (212–537.9)</td>
<td>151.5 (95.8–317.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Preoperative</td>
<td>295 (192.9–573.9)</td>
<td>149.4 (99.5–313.1)</td>
<td>0.29</td>
</tr>
<tr>
<td>4–6 h</td>
<td>332.1 (217.1–482.3)</td>
<td>237.9 (145.3–500.1)</td>
<td></td>
</tr>
<tr>
<td>P = 0.036*</td>
<td>P = 0.0001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14–20 h</td>
<td>273.5 (163.4–366.2)</td>
<td>205.2 (144.4–306.2)</td>
<td></td>
</tr>
<tr>
<td>P = 0.34*</td>
<td>P = 0.054*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Paired preoperative and postoperative CRC VEGF levels.
b Paired preoperative and postoperative benign VEGF levels.

Interquartile ranges are in parentheses.
(Fig. 3) and the plasma-corrected VEGF/platelet showed a significant increase with advancing disease stage.

**Serum VEGF, Platelet Counts, and Surgery.** There was no difference in median platelet count between the two surgical groups preoperatively, and both of the groups showed a significant decrease in platelet counts after resection (Table 4). After CRC resection, the increased serum VEGF/platelet reflected an increase of VEGF in the plasma (Fig. 4). This was confirmed when the plasma values were subtracted from the serum values and a comparison of plasma-corrected platelet VEGF levels showed no significant increase 14–20 h after surgery (Table 5).

However, after benign resection, the increase in serum VEGF/platelet was not simply attributable to an increase in plasma VEGF inasmuch as there was a significant increase in plasma-corrected platelet VEGF levels (Fig. 5).

**DISCUSSION**

The close association between VEGF and platelets was first considered when changes in serum VEGF were found to mirror changes in platelet counts during chemotherapy for breast cancer (7). Since then, there has been much debate as to whether plasma or serum should be used to measure circulating VEGF (13, 15, 18, 19). Banks et al. (13) have suggested that the release of VEGF on platelet activation during the clotting process makes serum measurements artifactually high and that plasma, obtained using citrate blood bottles, is a more accurate measurement. In that study, which involved eight normal volunteers, citrated plasma VEGF levels ranged from 9 to 43 pg/ml.

In CRC, plasma VEGF levels have been measured in one study (20), but the numbers were small (n = 26), and EDTA bottles rather than citrate bottles were used, which gave a median value of 105 pg/ml for patients with distant metastatic spread. This is higher than in our study, with a median of 37.3 pg/ml in the authors’ group of patients, and may be attributable to partial platelet or leukocyte activation in the EDTA blood samples (13). This suggests that using EDTA bottles may not give accurate results because the VEGF levels will be affected to an unknown degree by uncontrolled and variable release from platelets.

Surgical resection of CRC resulted in a decrease in serum VEGF after 1 month, but numbers were small (n = 14), and there was no control group of patients undergoing benign colorectal resection (16). Our study compares both serum and plasma VEGF changes after colorectal resection for malignant and benign disease. With a VEGF half-life of 3 min in the circulation, 4–6-h and 14–20-h time points should give ample time for changes to be manifest (21).

In our study, there was a correlation between serum VEGF and platelet count in CRC but not in the control group. Serum VEGF has been shown to correlate with platelet count in malignant disease (11, 15) and, in some studies, in healthy controls (7). However, our results support the study of Salven et al. (22), which demonstrated correlations between serum VEGF and whole blood VEGF and between whole blood VEGF and platelet count in cancer patients but no correlation between whole blood VEGF and platelet count in healthy controls.

Interestingly, citrated plasma levels of VEGF, which should avoid platelet activation, correlated with platelet count in CRC patients but not in controls. It is possible that this reflects the release of VEGF from platelets on centrifugation; however, this is unlikely because spinning samples at different speeds (150, 600, and 1400 × g) did not change the plasma VEGF levels (data not shown). Incomplete inactivation of the clotting cascade by citrate is unlikely inasmuch as 37% of CRC patients and 64% of controls had plasma VEGF levels below the lower limit of the ELISA assay sensitivity and had platelet counts that were similar (237 and 209, respectively). Therefore, plasma VEGF is most likely to reflect free, circulating VEGF, after equilibration with platelet levels.

Both serum and plasma VEGF were significantly raised in CRC patients compared with those with benign colonic disease. There was an increase in serum VEGF with advancing stage,
similar to that reported by Kumar et al. (5). Plasma VEGF also increased with disease stage, with median values ranging from 9.0 to 37.3 pg/ml. It should be noted that many of these values are close to the lower limits of detection of the ELISA assay (9 pg/ml).

Cancer patients have been shown to carry more VEGF per platelet than do normal controls (22). After correcting for platelet count, we have confirmed this finding, and Table 5 shows that the median VEGF/platelet in CRC patients is 56% higher than in benign disease patients. We also showed that there is a stepwise increase in VEGF/platelet with advancing stage. This provides further support to the theory of platelets scavenging circulating, presumptively tumor-derived, VEGF. When the “free” plasma VEGF levels are subtracted from the serum levels to calculate plasma-corrected VEGF/platelet, these values also show an increase with each stage, which suggests that the free plasma VEGF component does not contribute significantly within serum levels.

After colorectal resection for both malignant and benign disease, there was a significant increase in plasma VEGF, probably reflecting release of VEGF in response to surgical trauma. After an initial increase in serum VEGF at 4–6 h in both groups, there was a small decrease in serum VEGF at 14–20 h after CRC resection, but levels remained high after benign resection. When variation in platelet count is excluded by considering serum VEGF/platelet and plasma-corrected VEGF/platelet, the changes after surgery also suggest platelet scavenging. We hypothesize the following: after CRC resection, the increase in plasma VEGF attributable to surgical trauma is counterbalanced by the loss of tumor-derived VEGF, with platelets now scavenging only the VEGF that is released because of surgical trauma. Hence the plasma-corrected VEGF/platelet remains relatively constant compared with preoperative levels. However, after benign resection, there is an increase in plasma VEGF.

### Table 3  Dukes’ stage and VEGF levels: medians

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Benign</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>53</td>
<td>13</td>
<td>25</td>
<td>33</td>
<td>29</td>
</tr>
<tr>
<td>Platelet count</td>
<td>230</td>
<td>235</td>
<td>284</td>
<td>299</td>
<td>268</td>
</tr>
<tr>
<td>Plasma VEGF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9 (9–14.8)</td>
<td>9.0 (9–23.6)</td>
<td>11.93 (9–24.2)</td>
<td>21.4 (9–332.5)</td>
<td>37.3 (13.6–107.8)</td>
</tr>
<tr>
<td>Serum VEGF&lt;sup&gt;b&lt;/sup&gt;</td>
<td>168.3 (95.8–313.2)</td>
<td>122.8 (69.6–364.4)</td>
<td>246.6 (144.5–459.7)</td>
<td>318.9 (263.2–666.4)</td>
<td>515.1 (308–791.8)</td>
</tr>
<tr>
<td>Serum VEGF/platelet&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.72 (0.37–1.61)</td>
<td>0.84 (0.21–1.29)</td>
<td>0.94 (0.64–1.58)</td>
<td>1.31 (0.83–1.79)</td>
<td>1.94 (1.15–2.97)</td>
</tr>
<tr>
<td>Plasma-corrected VEGF/platelet&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.68 (0.38–1.54)</td>
<td>0.75 (0.25–1.19)</td>
<td>0.84 (0.59–1.5)</td>
<td>1.13 (0.74–1.67)</td>
<td>1.43 (0.92–2.39)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Kruskal-Wallis test: P < 0.0001, plasma VEGF and benign, A, B, C, D.
<sup>b</sup> Kruskal-Wallis test: P = 0.0066, serum VEGF and benign, A, B, C, D.
<sup>c</sup> Kruskal-Wallis test: P = 0.022, serum VEGF/platelet and benign, A, B, C, D.
<sup>d</sup> Kruskal-Wallis test: P = 0.018, plasma-corrected VEGF/platelet and benign, A, B, C, D.
Table 5  Median serum VEGF/platelet and plasma-corrected VEGF/platelet (interquartile ranges are in parentheses)

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>CRC Preoperative</th>
<th>Postoperative</th>
<th>Benign Preoperative</th>
<th>Postoperative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum VEGF/platelet (pg/10⁶)</td>
<td>1.08 (0.68–1.7)</td>
<td>1.29 (0.952–1.74)</td>
<td>0.69 (0.38–1.06)</td>
<td>1.03 (0.79–1.34)</td>
</tr>
<tr>
<td>% change</td>
<td>19.4%</td>
<td></td>
<td></td>
<td>14.9%</td>
</tr>
<tr>
<td>Plasma-corrected VEGF/platelet (pg/10⁶)</td>
<td>0.97 (0.61–1.56)</td>
<td>1.02 (0.63–1.4)</td>
<td>0.62 (0.33–1.03)</td>
<td>0.82 (0.53–1.17)</td>
</tr>
<tr>
<td>% change</td>
<td>5.2%</td>
<td></td>
<td></td>
<td>32.3%</td>
</tr>
</tbody>
</table>

This is the largest study of its kind to date and the first to compare serum and plasma levels of VEGF in cancer patients directly, to explore the contribution of platelets to serum VEGF levels, and to examine the effects of surgical removal of both benign and malignant colorectal lesions. Definitive proof of the relationship of circulating VEGF levels to tumor angiogenesis, malignant potential, surgical cure, or responses to therapy has yet to be established in any study. However, by paying attention to methodological details, it is hoped that some of the apparent discrepancies in different studies can be resolved, and standard procedures evolved. Further work is attempting to relate the levels of circulating VEGF to expression of VEGF in resected tumor specimens, because such data are essential to validate the use of indirect estimates of tumor angiogenic capacity.

REFERENCES


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